The expression and functional role of protein tyrosine phosphatase 1B in breast epithelial cells

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A thesis submitted for the degree of Master of Science

University of Iceland

Faculty of medicine

School of Health Sciences

Tjáning og starfrænt hlutverk
prótein tyrosín fosfatasa 1B in brjóstaþekjufrumum

Ritgerð til meistaragráðu

Bylgja Hilmarsdóttir

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Ágrip

Stofnfrumur brjóstkirtilsins eru nauðsynlegar til að viðhalda greinóttri formgerð kirtilsins. Talið er að ýmsar tegundir brjóstakrabbameina eigi uppruna sinn í þessum stofnfrumum. Skilningur á ferlum sem stjórna stofnfrumu- og krabbameinseiginleikum frumna hafa grundavallargildi til að auka skilning okkar á viðhaldi vefja og krabbameinsmyndun.

D492 er brjóstaþekjufrumulína með stofnfrumueiginleika sem búin var til með innskoti á E6 og E7 genunum með vörtuveiru 16. Frumur D492 mynda greinótta formgerð í þrívíðum ræktunum með innra lag af kirtilþekjufrumum og ytra lag af vöðvaþekjufrumum sem styður undir stofnfrumueiginleika frumulínunnar. Innskotsstaður retróveirunnar í D492 er á litningi 20q13.1, en það svæði er oft magnað upp í brjóstakrabbameini. Það gen sem er næst innskotstað veirunnar er genið sem kóðar fyrir prótein týrósín fosfatasa 1B (PTP1B), og er það staðsett um 95 kílóbasa frá innskotsstaðnum.

Markmið rannsóknarinnar var að kanna hlutverk PTP1B í fjölgun og lifun D492 frumulínunnar. Tjáning PTP1B í þekjufrumum og frumulínum gaf til kynna að próteinið sé yfirtjáð í D492. PTP1B hindri framkallaði stýrðan frumudauða í D492 frumum, en hafði engin áhrif í MCF-7 brjóstakrabbameinsfrumum sem einnig tjá PTP1B í miklu magni. Niðurstöðurnar gefa til kynna að frumudauðinn í D492 sé fyrst og fremst í frumum í skiptingu, hugsanlega í gegnum anoikis, en svo kallast stýrður frumudauði sem verður þegar fruma missir tengsl við millifrumuefnið. Einnig var sýnt fram á að PTP1B getur virkjað Src sem er þekkt æxlisgen og er talið leika hlutverk í anoikis. Þegar PTP1B tjáning í mismunandi frumulínum var metin kom fram að PTP1B er til staðar í tveimur stærðum í sumum frumum. Hugsanlega er minni gerðin af próteininu virkari undireining PTP1B sem klippt er með calpain 2 próteasa. Lyfjahindrun á calpain í D492 framkallaði frumudauða sem gefur til kynna að calpain sinni mikilvægu hlutverki í D492 frumulínunni, hugsanlega í gegnum PTP1B boðferla.

Niðurstöður rannsóknarinnar gefa til kynna að PTP1B gegni mikilvægu hlutverki í D492 og þær varpa ljósi á hlutverk þess í boðferlum frumulínunnar. Ritgerðin byggir góðan grunn að frekari rannsóknum á PTP1B í framtíðinni.

Abstract

Breast epithelial morphogenesis is maintained by tissue specific stem cells. Recent studies indicate that some aggressive forms of breast cancer originate in these stem cells. Therefore, linking the molecular signals and mechanisms that control the fate decisions and branching morphogenesis in breast epithelial stem cells to cancer progression is important.

D492 is a breast epithelial cell line with stem cell properties. It was established by retroviral transduction of the E6 and E7 genes from human papilloma virus 16. When cultured in three dimensional (3D) assays the D492 cell line forms branching structures with an inner layer of luminal epithelial cells and an outer layer of myoepithelial cells clearly showing a bipotential phenotype, correct histoarchitecture and thus stem cell properties. We have recently localized the retroviral insert in the D492 stem cell line 95kb upstream of a gene coding for the protein tyrosine phosphatase 1B (PTP1B) at chromosome 20q13.1, a region that is frequently amplified in breast cancer.

In this work I have studied the role of PTP1B in the proliferation and survival of the D492 cell line as well as other epithelial cells. PTP1B protein expression in various cell lines and primary cells indicated that PTP1B is overexpressed in D492, possibly due to the retroviral insert. Treatment of the D492 cells with a specific PTP1B inhibitor induced apoptosis, but did not induce cell death in MCF-7, a breast cancer cell line which also has high PTP1B expression. My data further indicates that the cell death in D492 is primarily in dividing cells, possibly due to anoikis, i.e. programmed cell death when anchorage dependent cells detach from the surrounding matrix. I also provide evidence that PTP1B can activate Src, a well known oncogene which plays a role in anoikis. Interestingly, I also noticed a smaller form of the PTP1B protein when evaluating PTP1B expression in various cell lines. This smaller form is reported to be a more active subunit of PTP1B cleaved by Calpain2 protease. Pharmacological inhibition on Calpain in D492 resulted in cell death suggesting a role for calpain, possibly through PTP1B.

In summary my data suggest an important role of PTP1B for cell survival in D492 and helps to elucidate its role in signaling pathways. This thesis provides a foundation for further studies on PTP1B during breast morphogenesis and cancer.

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Figures and tables

Abbreviations

BM	Basement membrane
BSA	Bovine serum albumin
cDNA	Complementary DNA
CFSE	Carboxyfluorescein succinimidyl ester
CGH	Comparative genomic hybridization
CISs	Common integration sites
CPT	Camptothecin
ECM	Extracellular matrix
EGFR	Epidermal growth factor receptor
ENDO	Endothelial cells
ER	Estrogen receptor
ESA	Epithelial-specific antigen
FAK	Focal adhesion kinase
FBS	Foetal Bovine Serum
HPV	Human papillomavirus
IGF1R	Insulin-like growth factor-I receptor
LEP	Luminal epithelial cells
MEP	Myoepithelial cells
Mo-MuLV	Moloney murine leukemia virus
MUC	Sialomucin
PBS	Phosphate-buffered saline
PDGFR	Platelet-derived growth factor receptor
PI	Propidium iodid
PTKs	Protein tyrosine kinases
PTP1B	Protein tyrosine phosphatase 1B
PTPs	Protein tyrosine phosphatases
PR	Progesterone receptor
RTKs	Receptor tyrosine kinases
RSV	Rous sarcoma virus
RT-PCR	Reverse transcription polymerase chain reaction
SSC	Squamous cell carcinoma
TDLU	Terminal duct lobular unit
TMA	Tissue microarray

1. The human breast gland

1.1 Structure of the breast gland

The human breast gland is a modified sweat gland, composed of a highly branching epithelial ductal system terminating in structures called terminal duct lobular units (TDLUs) (Ronnov-Jessen et al., 1996). TDLUs are the functional unit of the breast tissue where the milk is produced and pumped to collecting ducts during lactation (figure 1).

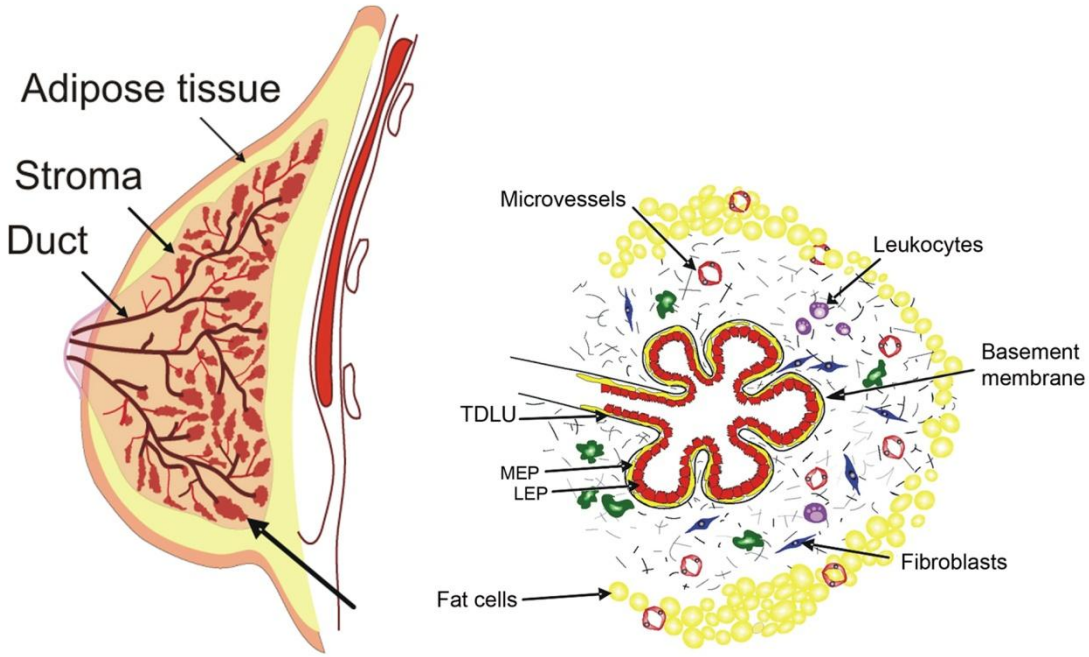

Figure 1. Schematic overview of the adult breast gland and the stromal compartment.
The human breast gland is composed of a highly branching epithelial ductal sytem terminating in the terminal duct lobular units (TDLU). The TDLUs are compoes of bilayerd epithelial structure with an inner layer of luminal epithelial cells (MEP) and an outer layer of myoepithelial cells (LEP). The epithelial compartment of the breast is surrounded by a loose-intralobular stroma surrounded by a more dense-extralobular stroma. The stromal compartment is composed of collagen and growth factors as well as microvessels, fibroblasts, leukocytes and fat cells (Adapted from (Sigurdsson, 2005)).

TDLU are bilayered structures with a hollow central lumen: an inner layer of polarized milk producing luminal epithelial cells (LEP) and an outer layer of contractible myoepithelial cells (MEP) required for milk ejection. Surrounding the mammary ducts and alveoli is a basement membrane (BM) containing laminins, nidogen, proteoglycans and collagen IV. The basement membrane separates the epithelium from the surrounding stroma (Howard & Gusterson, 2000; Nelson & Bissell, 2005).

The stroma constitutes up to 80% of the volume of the breast (Howard & Gusterson, 2000; Ronnov-Jessen et al., 1996) and is composed of cellular components such as microvessels, fibroblasts, macrophages and adipocytes and acellular extracellular matrix (Katz & Streuli, 2007). Interaction between the breast epithelium and the stroma is believed to be important for normal development of the breast as well as tumor progression.

Mammary gland development in the female breast is for the most part postnatal (Howard & Gusterson, 2000; Lanigan et al., 2007). At birth, a rudimentary gland is present and further epithelial growth is hormone-dependent and begins at puberty. During puberty the gland branches and forms ducts and at early pregnancy, lateral buds extend from these main ducts and form lobuloalveolar structures. After lactation the gland goes through a process called involution where lobuloalveolar compartment undergoes massive apoptotic cell death and remodeling. Formation of these branches require invasion of the ductal epithelium through a mature BM and the stroma and is therefore strictly controlled by external signal from the stroma (Lanigan et al., 2007).

1.2 Tissue stem cells in the breast

There is mounting evidence that the two different epithelial cell types that make up the TDLU are derived from breast epithelial stem cells. Shackleton and colleagues demonstrated that a single cell from the mouse mammary gland is capable of generating a functional mammary gland in the cleared fat pad of a syngenic mouse, thereby confirming the existence of a multipotent mammary stem cell (Shackleton et al., 2006). The stem cells are presumed to be responsible for the continuous tissue renewal throughout the reproductive period as well as the massive epithelial expansion and branching during pregnancy. Mammary stem cells are presumed to be important for both organ development and maintaining tissue homeostasis. These cells give rise to mature epithelium of either luminal or myoepithelial lineage via a series of lineage-restricted intermediates. The luminal lineage can be further subdivided into ductal and alveolar luminal cells that line the ducts and constitute the alveolar units that arise during pregnancy. In contrast, myoepithelial cells are specialized, contractile cells located at the basement membrane. In humans, analysis of X chromosome inactivation in human mammary gland suggests that entire lobules could be derived from one cell (Tsai et al., 1996). In human breast, subpopulations of suprabasal epithelial cells have been shown to give rise to both luminal epithelial and myoepithelial cells (Clarke et al., 2005; Gudjonsson et al., 2002).

The vast expansion of mammary epithelium that occurs during puberty and pregnancy and involution after lactation supports the idea of stem cells within the mammary gland that have remarkable regenerative capacity (LaBarge et al., 2007; Visvader, 2009).

2. Breast cancer

Breast cancer is a complex and heterogeneous disease at both the histological and molecular levels. More than one million new breast cancer cases are diognosed worldwide each year (McArthur, 2009). The vast majority of breast cancer arises from the epithelial cells of the breast, predominantly expressing the luminal epithelial phenotype (Sims et al., 2007). New findings suggest that the distinct expression patterns of tumor subtypes and the significant differences in disease outcome are likely to be caused, at least in part, by alterations in specific cellular pathways and/or different cell type origin (Sorlie et al., 2006). A recent development of gene expression microarray and related technologies has great potential to become a method for predicting clinical outcome and relapse (Cleator & Ashworth, 2004).

2.1 Breast cancer subgroups

First reported by the Perou and Sorlie groups (Perou et al., 2000; Sorlie et al., 2001), evidence suggest that breast cancer tumors can be divided into subtypes defined by gene expression patterns which are associated with different prognostic outcome. These subtypes have been termed: basal like, ERBB2, normal-like and luminal types A and B (Kapp et al., 2006; Sims et al., 2007). By far the clearest difference between tumors seems to be the distinction between luminal tumors and basal-like tumors (Cleator & Ashworth, 2004). Luminal tumors generally express the estrogen receptor (ER) with or without coexpression of the progesterone receptor (PR).

Members of the basal-like group are characterized by high expression of basal keratins 5/6 and 17, laminin, and fatty acid binding protein 7 whereas the oncoprotein ErbB2 is overexpressed in the ERBB2 group (Sorlie et al., 2001). Normal-like tumors express genes of normal epithelium. Tumors in the luminal epithelial/ER+ group are ER positive and breast luminal cell markers are relatively overexpressed in these samples. The main difference between Luminal A and B is that luminal B tumors are poorly differentiated and have high proliferative index. The prognosis of patients diagnosed with luminal type cancer is good, although the prognosis is better in patients with luminal A type tumors than luminal B (Normanno et al., 2009). Prognosis for patients bearing tumors from the poorly differentiated ER negative groups are worse, particularly the triple negative basal like subtype (Er-, PR- and ERBB2 negative) .

2.2 Cancer stem cells

The cancer stem cell theory poses that cancer develops from, and is sustained by, a rare subpopulation of tumorigenic cells known as cancer stem cells (CSCs) (Lawson et al., 2009). CSCs are thought to be a distinct subpopulation of cancer-initiating cells that constitute a small percentage of the tumor bulk, while the rest of the tumors cells do not have tumor-initiating capacity. They are defined by a distinct surface expression profile and are capable of asymmetric cell division that renews the CSC population within the tumor while also generating the differentiated cells of the tumor (Lawson

et al., 2009). In breast cancer a small subpopulation has been identified as having self-renewal capacity and being enriched in tumor–initiating cells compared to the majority of carcinoma cells. This proposed cancer stem cell subpopulation is CD44+/CD24- and it is also rich in epithelial-specific antigen (ESA) expression (Garcia Bueno et al., 2008; Mani et al., 2008).

A normal cell, or a normal cell with stem cell properties requires several hits to transform into a cancer stem cell with self-renewal capability. It is unknown if a cancer stem cell is originated from a differentiated cell that acquires self-renewal capacity, or the stem cell/progenitor cell population of the breast (Garcia Bueno et al., 2008).

Evidence that stem cells may play a role in carcinogenesis is the observation that normal stem cells and cancer cells share several important properties. These include: self-renewal, the ability to differentiate, active telomerase, activated anti-iapoptotic pathways, increased membrane transporter activity and the ability to migrate and metastasize (Wicha et al., 2006). Also gene expression studies and marker analyses have suggested that there are similarities between tissue stem cells and CSCs of the breast (Williams et al., 2009). Given the similarities between CSCs and somatic SCs it has been proposed that somatic SCs might be an ideal target of the carcinogenesis process. Numerous studies have been published recently that propose that breast cancers in human and mice may arise from the transformation of either mammary stem cells or early progenitor cells (Dontu et al., 2003; Feinberg et al., 2006; Li et al., 2003; Petersen et al., 2003).

3 Human breast epithelial stem cell line

Studies suggest that human mammary stem cells are contained within the luminal epithelial lineage within the TDLUs (Gudjonsson et al., 2002). As mentioned above, candidate stem cells have been defined as having no luminal contact but are distinctly localized from myopeithelial cells and are referred to as suprabasal. When a normal human breast tissue is double-stained with epithelial-specific antigen (ESA) and sialomucin (MUC), MUC is expressed on the apical surface of luminal epithelial cells and ESA at the basolateral surface. But a subset of MUC^-/ESA^+ not reaching the lumen are referred to as suprabasal and are considered to be candidate stem cells (Gudjonsson et al., 2002). Thorarinn Gudjonsson et al established and characterized a bipotential breast epithelial stem cell-like line referred to as D492 (Figure 2a). D492 was isolated from primary breast tissue with the markers MUC^-/ESA^+ and immortalized using a retroviral transduction protocol containing E6/E7 HPV-16 oncogenes (Gudjonsson et al., 2002). D492 has stable stem cell properties based on in vitro findings where they generate both undifferentiated cells and fully differentiated luminal- and myoepithelial cells. Moreover D492 has a propensity to generate TDLU-like structures with an inner layer of luminal epithelial cells and an outer layer of myoepithelial cells in 3D culture (Figure 2b).

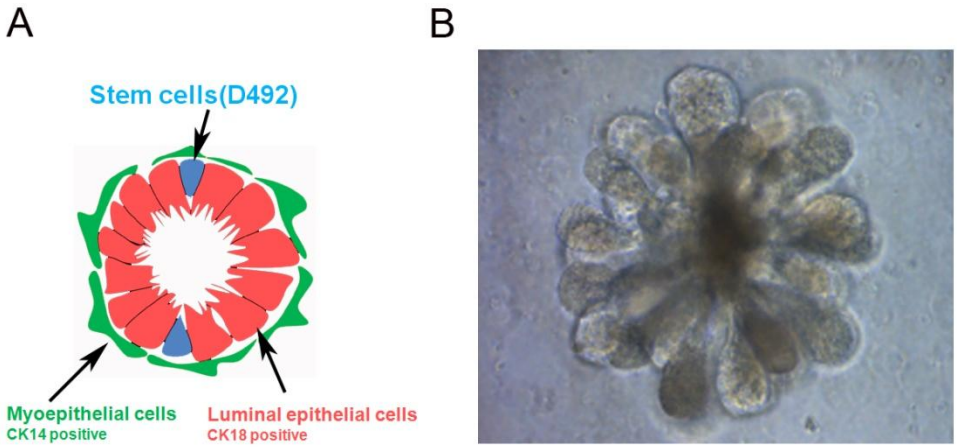

Figure 2. D492 is a bipotential breast epithelial stem cell-like line

A. A scematic picture of a TDLU and a predicted position of the stem cells within the breast gland. **B.** Phase contrast image of D492 cells that form TDLU-like structures in 3D culture (Sigurdsson, 2005).

3.1 insertional mutagenesis

Primary cells can only go through limited number of cell division due to telomere shortening at each cell division before entering replicative senescence. When generating cell lines using primary cells from human tissue it is necessary to immortalize the cells due to this replicative senescence. To achieve this, proteins that control the cell cycle are deactivated, telomerase activated and as a consequence allow the cell to replicate indefinitely (Wazer et al., 1995).

D492 was immortalized using a human papillomavirus (HPV) carrying E6 and E7 oncogenes (Gudjonsson et al., 2002). E6 and E7 deactivate p53 and Rb proteins, respectively, which are two of the principal pathways controlling cell proliferation in mice and humans. We hypothesized that the retroviral insertion could affect genes within its promoter range and this could contribute to the phenotype of the cell line. We therefore decided to clone out the insertion site in D492. Using a reverse PCR technique we were able to localize the retroviral insertion on chromosome 20q13.1. The insert is 95kb upstream of the gene PTPN1 which codes for protein tyrosine phosphatase 1b (PTP1B) (Figure 3) (unpublished results).

Figure 3. Schematic figure of the retroviral insert site in D492.
The D492 cell line was immortalized with a retroviral transfection of E6/E7 oncogenes. The insertion site was located on chromosome 20q13.1 95kb upstream of the PTPN1 gene which codes for PTP1B.

Retroviral transduction is commonly used to transfer genes into cells. In this process, retroviruses integrate into the genome of the host cell where they are expressed through powerful promoters present in the retroviral construct. Insertion within a gene or, up to 300kb distance from a gene can modulate its expression by either enhancing or reducing it. This is called insertional mutagenesis (Kurth & Bannert, 2010). In this manner a retrovirus can cause tumor formation, without harboring any oncogenes. Common integration sites (CISs) are loci where a proviral insertion has been found in multiple tumors. If insertions cloned from multiple independent tumors are found in the same locus, it is not likely due to chance but rather a result of a selective expansion of tumor cells carrying insertion at this locus (Uren et al., 2005). Once a CIS has been found, it can be correlated with other tumor characteristics such as host genotype, background strain, tumor histology and the presence or absence of other CIS within the same tumor.

This insertion site in D492 is known to be a CIS in different tumors mouse models, predominantly in „Cdkn2a-deficient“ mice (Lund et al., 2002). The Cdkn2a locus controls both the p53 and Rb pathway, the same pathways that are impaired in D492 through the E6/E7 oncogenes. This suggests that there might be a connection between PTP1B insertional mutagenesis and oncogenes working through the p53 and Rb pathway. Interestingly, a gain or amplification of the 20q13 chromosomal region, where the insertional site is, has been observed in several cancers and is associated with poor prognosis in breast cancer (Tanner et al., 1996).

4. PTP1B

Protein tyrosine phosphatases (PTPs) and protein tyrosine kinases (PTKs) modulate cellular levels of tyrosine phosphorylation and regulate many cellular events such as differentiation, cell growth, motility and proliferation. Dysregulation of the balance between tyrosine phosphorylation and dephosphorylation events within cells is the bases for many pathophysiological conditions, e.g. cancer, autoimmune disorders and diabetes (Bartholomew et al., 2005).

4.1 PTP1B and its signaling pathways

Protein tyrosine phosphatase 1B (PTP1B) is a 50kDa non receptor phosphatase localized predominantly in the cytoplasmic surface of the endoplasmic reticulum, anchored via its C-terminal region (Hernandez et al., 2006). Cleavage of PTP1B from the endoplasmic reticulum enhances its activity (Cortesio et al., 2008). It is a member of the large family of protein tyrosine phosphatases. Recent studies indicate that PTPs are important for numerous cellular processes and are involved in a number of human diseases (Bartholomew et al., 2005). Substrate trapping and biochemical studies have identified various substrates of PTP1B. Among those are receptor tyrosine kinases, e.g. epidermal growth factor receptor (EGFR), platelet-derived growth factor receptor (PDGFR), insulin receptor and insulin-like growth factor-I receptor (IGF1R). PTP1B also regulates intracellular protein tyrosine kinases like focal adhesion kinase (FAK), c-Src and adaptor proteins like b-catenin and p130Cas (Akagi et al., 2004; Dube et al., 2005; Li et al., 2003; Stuible et al., 2008).

PTP1B has a major role in down-regulating insulin and leptin signaling. PTP1B dephosphorylates the insulin receptor, thus terminating signals from this receptor tyrosine kinase. Therefore PTP1B deficient mice are hypersensitive to insulin and resistant to obesity induced by a calorie-rich diet (Tonks, 2003). For this reason PTP1B has received much attention for the last few years as a novel therapeutic target for the treatment of diabetes and obesity. Consequently the pharmaceutical industry is focusing on PTP1B as a drug target and there are numerous inhibitors against PTP1B at various stages of development.

PTP1B has also a role in other signaling pathways such as growth factor and integrin mediated processes as well as cancer development (Lessard et al., 2009; Stuible et al., 2008). At first it was believed that PTP1B would be a tumor suppressor given it´s activity to dephosphorylate proteins and the fact that PTP1B has been implicated in downregulation of signaling through several receptor tyrosine kinases (RTKs) (Ostman & Bohmer, 2001). This has not been supported in mouse models where PTP1B-deficient mice do not spontaneously develop tumors (Dube & Tremblay, 2004). On the contrary, two papers in 2007 revealed that PTP1B as a positive mediator of the ErbB2-induced signals that trigger breast tumorigenesis (Bentires-Alj & Neel, 2007; Julien et al., 2007). Moreover, in a study of 29 human breast cancer samples, 21 (72%) displayed PTP1B expression greater than the maximum observed in nine samples of normal mammary epithelium (Wiener et al., 1994) . This study also linked the expression of PTP1B to ErbB2 expression, finding that 11 out of 12 (90%) tumors that

overexpressed ErbB2 also overepxressed PTP1B. PTP1B is also known to up-regulate two growth-promoting pathways: It activates Src tyrosine kinase and deactivates $p62^{Dok}$, an inhibitor of the Ras/mitogen-activated protein kinase pathway (Tonks & Muthuswamy, 2007).

PTP1B has been shown to interact with integrin complexes and localize to early cell matrix adhesion sites. This was done with substrate-trapping mutant of PTP1B which formed long-lived complexes with substrates and is crucial for PTP1B detection at adhesion sites (Hernandez et al., 2006). In another study, mouse fibroblasts that expressed dominant negative form of PTP1B had small matrix sites, were impaired in cell spreading and they also had reduced Src activity. This data suggests that PTP1B is an important regulator of integrin signaling pathways making it important for adhesion, spreading and formation of focal adhesion (Arregui et al., 1998). Cells derived from PTP1B KO mice also show defects in cell spreading (Cheng et al., 2001).

4.2 PTP1B and ErbB2

ErbB2 (Her2, Neu) is a protein tyrosine kinase of the EGF receptor family. The gene is amplified leading to protein overexpression in ~25% of breast cancers (Dean-Colomb & Esteva, 2008). ErbB2 amplification is associated with poor prognosis. The main treatment of ErbB2 positive cancer is to employ trastuzumab which is a monoclonal antibody that binds selectively with the ErbB2 receptor. Trastuzumab has had a major impact on the treatment of ErbB2 positive cancer, however patients usually develop resistance to the drug (Penault-Llorca et al., 2009). It is therefore important to understand the signaling events in the ErbB2 pathway to find novel therapeutic targets which could complement existing anti-ErbB2 treatments. Increasing evidence points to PTP1B as a positive mediator of the ErbB2-induced signals that trigger tumorigenesis and metastasis (Bentires-Alj & Neel, 2007; Julien et al., 2007).

In 2007 there were two studies proposing that PTP1B was required for ErbB2-induced breast cancer (Bentires-Alj & Neel, 2007; Julien et al., 2007). Both studies demonstrated that in mice with activated ErbB2, PTP1B deficiency dramatically delays or prevents the onset of tumors. In these mice, Julien et al. also showed significant tumor resistance to lung metastasis and they got the same result using pharmacological inhibition of PTP1B. Furthermore, they showed that overexpression of PTP1B in the mammary gland in mice leads to spontaneous breast cancer development suggesting that PTP1B can act as an oncogene independent of ErbB2 (Julien et al., 2007).

In a recent study Arias-Romero et al confirmed that activation of ErbB2 increased PTP1B expression in the untransformed immortilized cell line MCF10a (Arias-Romero et al., 2009). They also showed that pharmacological or siRNA induced silencing of PTP1B impaired the ability of activated ErbB2 to transform these cells and to activate Src. Furthermore, overexpression of PTP1B in MCF10a lead to distorted acinar morphology, causing unchecked proliferation and loss of polarity. These effects where associated with Src activation.

4.3 PTP1B and Src

c-Src is a membrane-associated non-receptor tyrosine kinase which can phosphorylate a variety of intracellular substrates, affecting cell division, cell differentiation, and cell mobility (Bjorge et al., 2000). The first member of the Src family protein tyrosine kinases was identified as the transforming protein (v-Src) of the Rous sarcoma virus (RSV) in 1977 (Thomas & Brugge, 1997). The discovery of Src family proteins gave an insight into the mechanisms that drive cancer initiation and progression. In 1989 J. Michael Bishop and Harold E. Varmus received the Nopel Prize in Physiology and Medicine for the discovery of " the cellular origin of retroviral oncogenes" for their research on Src family proteins.

Src is a powerful oncogene but it is rarely mutated in human cancer, suggesting that it is involved in later stages of carcinogenesis and plays a supporting, rather than an initiating role (R. Ishizawar & Parsons, 2004). In human breast cancer, Src activity is often increased 4- to 30 fold compared with normal breast tissue and it has also been reported that Src protein level can be elevated in breast cancer (Verbeek et al., 1996). Src activity is upregulated in over of 80% of colon adenocarcinomas relative to normal colonic mucosa and in majority of colon tumor cells, Src is constitutively active (Windham et al., 2002). Numerous studies have shown that elevated catalytic activity of Src is required to confer resistance to cell death, thus identifying Src as a survival factor that protects cancer cells from apoptosis and anoikis, an anchorage dependent cell death (Golubovskaya et al., 2003; Windham et al., 2002).

PTP1B has been shown to activate Src by dephosphorylating the inhibitory tyrosyl phosphorylation site (Y529) on the COOH terminus of the kinase. Studies indicate that PTP1B is the primary protein-tyrosine phosphatase capable of dehosphorylating c-Src in breast cancer cell lines and thus control its kinase activity (Bjorge et al., 2000). Liang et. al showed in 2005 that downregulation of PTP1B in fibroblasts, supressed cell spreading and migration on fibronectin, increased Src Tyr^{529} phosphorylation and decreased phosphorylation of FAK, $p130^{cas}$ and ERK1/2. They concluded that PTP1B promotes integrin mediated responses by dephosphorylating the ihibitory $pTyr^{529}$ and thereby activating the Src kinase (Liang et al., 2005).

4.4 PTP1B and Calpain

Calpains are a ubiquitously expressed family of Ca^{2+}-dependent cysteine proteases that cleave many adhesion-associated and actin regulatory proteins. This includes talin, FAK, paxilin, cortactin and PTP1B (Niapour et al., 2008). Calpain 2 is an intracellular protease that regulates adhesion turnover and disassembly through the targeting of specific substrates. A recent study demonstrated that calpain 2 is upregulated in breast cancer and its expression correlates with increased invasive properties of tumors (Libertini et al., 2005). Furthermore, recent evidence suggests a critical role for calpain in cell migration (Cortesio et al., 2008).

Motile and invasive cells, like cancer cells, often form specialized integrin-mediated adhesive structures known as matrix-degrading invadopodia. Little is known about the regulation of invadopodia in carcinoma cells, but in a recent paper, Cortesio et al. found a novel pathway involving calpain 2, PTP1B and Src that regulates invadopodia dynamics and breast cancer cell invasion (figure 4) (Cortesio et al., 2008). In the study they demonstrated that calpain 2 cleaves full length PTP1B into a more active 41kD form. Earlier it had been established that Src tyrosine kinase and the actin-binding protein cortactin are critical regulators of invadopodia formation and turnover. The Cortesio study demonstrated that calpain 2 functions both upstream and downstream of Src to regulate invadopodia. They treated the breast cancer cells with PTP1B inhibitor or used PTP1B deficient cell lines which impaired the ability of the cells to migrate and invade. Therefore they concluded that PTP1B regulates invadopodia formation and breast cancer invasion.

A recent study showed that in calpain-deficient cells, insulin signaling was diminished just like PTP1B deficient cells, indicating that PTP1B and calpain act in the same pathway (Trumpler et al., 2009).

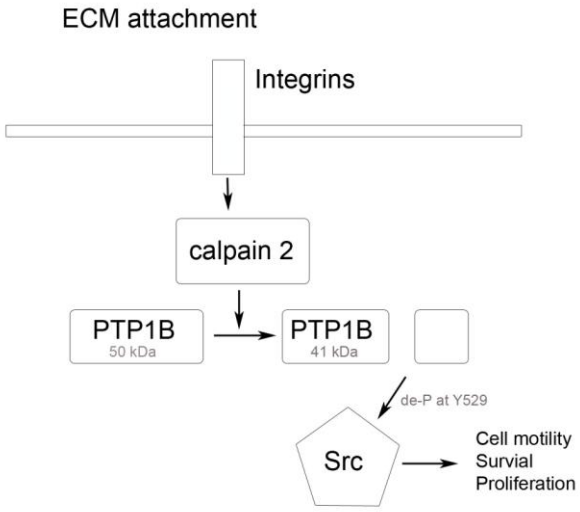

Figure 4. Schematic overview of calpain 2, PTP1B and Src interaction.

Integrin engagement increases calpain 2 activity which in turn cleaves PTP1B and enhances its activity. PTP1B dephosphorylates Src at Y529 thereby activating Src kinase activity inducing cell motility, survival and proliferation (adapted from (Cortesio et al., 2008)).

4.5 PTP1B and Anoikis

The development of the mammary gland is spatillly regulated by the interaction of the mammary epithelium with the extracellular matrix (ECM) (Katz & Streuli, 2007). Epithelial cells require contact with the ECM and this regulation is primarily through a family of adhesion receptor proteins called integrins. A major role of the ECM is to regulate cell-survival in the mammary gland. In adherent cells, cell detachment from the ECM leads to a detachment-induced apoptosis, termed anoikis (Bouchard et al., 2008). Cell adhesion to the ECM triggers tyrosine phosphorylation of several proteins. This is mediated through members of the integrin family and regulation of a subset of PTKs. This integrin-mediated adhesion stimulates activity of FAK and Src family kinases which in turn phosphorylate proteins important for regulating cell migration, proliferation and survival (Bartholomew et al., 2005).

Resistance to anoikis is a common feature of cancer epithelial cells. A variety of cellular mechanisms have been implicated in anoikis resistance, for example activated oncogenes such as Src and Ras, overexpression of FAK and activation of growth factor receptors (Golubovskaya et al., 2003; Windham et al., 2002). Anoikis is a common event in cancer progression and it is therefore important to understand the mechanism and cellular pathways behind resistance to anoikis.

2. Aim of the study

Breast epithelial morphogenesis is maintained by tissue specific stem cells. Cancer cells and stem cells resemble each other in many ways and many genes that were found due to their role in cancer progression also turn out to be important in stem cells. To understand the origin of breast cancer and breast cancer progression we need to understand the function of signaling pathways in breast morphogenesis and to increase our understanding of cancer as a disease where normal cellular signaling has gone awry. D492 is a cell line with stem-cell properties that has a retroviral insert 95kb upstream of PTPN1, a gene that codes for the protein tyrosine phosphatase 1B (PTP1B). This location has been identified as a common integration site in mouse cancer models and thus PTP1B could be important for the stem cell line D492. There is mounting evidence that the PTP1B plays a role in breast tumorigenesis. PTP1B is located on chromosome 20q13.1 which is often amplified in breast cancer. PTP1B activates Src kinase, a known oncogenes, and furthermore its expression correlates with ErbB2 expression in breast cancer tumors. A recent study shows that PTP1B inhibition in ErbB2 overexpressing mice delayed tumorigenesis and PTP1B overexpression induced tumor formation. It is therefore interesting to study the effects of the retroviral insert in D492 as well as general PTP1B expression and function in breast epithelial cells.

The general aim of this study was to study PTP1B expression in D492 stem cell line, MCF-7 breast cancer cell line as well as breast tissue section and primary cells. Furthermore, I wanted to study the role of PTP1B in proliferation and survival of D492.

Specific aims:

1. Examine PTP1B expression in breast epithelial cell lines, primary cells and breast tissue sections.
2. Analyze the effects of pharmaceutical inhibition of PTP1B on breast epithelial cell line with stem cell properties and on breast cancer cell line.
3. Analyze up- and downstream targets in PTP1B signaling pathways.

3. Materials and methods

3.1 Culture of cells

3.1.1 Monolayer

The D492 and D382 cell lines were cultured in the chemically defined medium H14 (Gudjonsson et al., 2002) unless indicated otherwise. H14 consists of DMEM/F12 basal medium (Gibco) supplemented with 50 IU/ml penicillin, 50 µg/ml streptomycin, 10ng/ml EGF, 0.1 nM estradiol, 0.5 µg/ml hydrocortisone, 250 ng/ml insulin, 2.6 ng/ml sodium selenite, 5 µg/ml prolactin and 10 µg/ml transferrin. In certain experiments the D492 cell line was cultured in M10 or CDM4 media. M10 media consists of DMEM/F12 basal medium (Gibco) supplemented with 50 IU/ml penicillin, 50 µg/ml streptomycin, 7.5 µg/ml insulin, 10ng/ml EGF, $1.4x10^{-6}$ M hydrocortisone, 10ng/ml Cholera Toxin and Bovine pituitary extract of unknown concentration. CDM4 media consists of DMEM/F12 basal medium (Gibco) supplemented with 50 IU/ml penicillin, 50 µg/ml streptomycin, 10 µg/ml ascorbic acid, 100 ppm bovine serum albumin (BSA), 10 nM dibutyryl cyclic AMP, 100 ng/ml EGF, 0.1 nM estradiol, 0.1 mM ethanolamine, 20 µg/ml fetuin, 100 ng/ml fibronectin, 0.5 µg/ml hydrocortisone , 3 µg/ml insulin, 2.6 ng/ml sodium selenite, 0.1 nM phosphoethanolamine, 25 µg/ml transferrin and 10nM triiodothyronine and cholera toxin, 10ng/ml (EGF purchased from Peprotech, all other growth factors purchased from SIGMA) .

The MCF-7 cell line was cultured in DMEM/F12 basal medium (Gibco) supplemented with 50 IU/ml penicillin, 50 µg/ml streptomycin and 5% fetal bovine serum (FBS)(Gibco) (Soule et al., 1973), unless indicated otherwise. The MDA-MB-231 cell line was cultured in RPMI1640 basal medium (Gibco) supplemented with 50 IU/ml penicillin, 50 µg/ml streptomycin and 10% FBS(Gibco) (Cailleau et al., 1978). Primary endothelial cells (endo) were cultured in EBM-2 basal medium (Lonza), containing 50IU/ml penicillin, 50 µg/ml streptomycin and ascorbic acid, EGF, bFGF, Heparin, IGF and VEGF of unknown conentrations (Lonza). The EBM medium was supplemented with 30% FBS. Primary luminal epithelial cells (LEP) were cultured in the chemically defined medium CDM3 consisting of all the same factors of CDM4 except CDM3 contains 20ng/ml EGF, 10 µM dibutyryl cyclic AMP and it does not contain Cholera toxin. Primary myoepithelial cells (MEP) where cultured in CDM4. All the cells were cultured on collagen-1 (Nutagon) coated flasks with the exception of MCF-7 and MDA-MB-231.

3.1.2 Three dimensional cell culture

Growth factor reduced matrigel (Becton Dickinson Labware, Bedford, MA) was used in the 3D experiments. Matrigel starts to gel at 4°C to form a rBM gel so cells can be seeded into the gel before that happens. Experiments were carried out in 24 well culture plates. 300µl Matrigel was used for each gel where 10.000 D492 cells were seeded into the gel and incubated at 37°C for 30 minutes allowing gelatinization before adding 1 ml culture medium.

3.2 Reagents and antibodies

The PTP1B inhibitor 3-(3,5-Dibromo-4-hydroxy-benzoyl)-2-ethyl-benzofuran-6-sulfonicacid-(4-(thiazol-2-ylsulfamyl)-phenyl)-amide (Cat.nr.539741, IC_{50}=8µM) was optained from Calbiochem. It is a cell-permeable compound that acts as a selective, reversible and non-competitive allosteric inhibitor of PTP1B. It binds to a novel site away from the catalytic pocket and inhibits PTP1B activity by preventing closure of the WPD loop (Caliper life science 2005, wiesmann 2005, Review:Zhang 2007).

The Calpain inhibitor 3-(4-Iodophenyl)-2-mercapto-(Z)-2-propenoic Acid (Cat.nr. 513022) was optained from Calbiochem. It is a cell permeable, non competitive, selective non peptide calpain inhibitor which is directed towards the calcium binding sites of calpain (citation: Niapor 2008).

The apoptosis inducer camtothecin (Cat.nr.C9911) was optained from Sigma. Camptothecin binds irreversibly to the DNA-topoisomerase I complex, inhibiting the reassociation of DNA after cleavage by topoisomerase I and traps the enzyme in a covalent linkage with DNA thereby blocking the cells in S-phase and inducing apoptosis.

Dimethyl sulfoxide (DMSO) is a commonly used solvent in molecular biology. It dissolves polar and nonpolar compounds. In this study DMSO was used as a control in experiments with inhibitors and camtothecin, always in the amount equivalent to the highest inhibitor concentration. 16µM of PTP1B inhibitor is equivalent to 0.24% DMSO in media. 200µM of calpain inhibitor is equivalent to 0.61% DMSO in media. 10µM of CPT is equivalent to 0,35% DMSO in media.

Table 1. Primary and secondary antibodies used in western blots.

Antibody	Clone	Species	Isotype	Dilution	Company - Order number	Secondary antibody
PTP1B	polyclonal	rabbit	IgG	1:500	RandD systems - AF1366	ECL Rabbit IgG (NA943-Amersham)
Src (phsopho Y529)	polyclonal	rabbit	IgG	1:500	Abcam - ab4817	ECL Rabbit IgG (NA943-Amersham)
Actin	polyclonal	rabbit	IgG	1:5000	Abcam - ab8227	ECL Rabbit IgG (NA943-Amersham)
GAPDH	9484	mouse	IgG2b	1:1000	Abcam - 9484	ECL Mouse IgG (NA931-Amersham)

Table 2. Primary and secondary antibodies used in IF staining.

Antibody	Clone	Species	Isotype	Dilution	Company - Order number	Secondary IF antibody
PTP1B	15	mouse	IgG2a	1:50	BD bioscience - 610139	Alexa Fluor 546 (red) goat anti-mouse IgG2a
PTP1B	polyclonal	rabbit	IgG	1:50	RandD systems - AF1366	Alexa Fluor 546 (red) goat anti-rabbit IgG
CK14	LL002	Mouse	IgG3	1:50	abcam-ab7800	Alexa Fluor 488 (green) goat anti-mouse IgG3
CK18	CD10	Mouse	IgG1	1:50	DAKO-M7010	Alexa Fluor 488 (green) goat anti-mouse IgG1
Vimentin	V9	Mouse	IgG1	1:100	DAKO-M0725	Alexa Fluor 488 (green) goat anti-mouse IgG1
ErbB2	polyclonal	rabbit	IgG	1:50	Abcam - ab2428	Alexa Fluor 488 (green) goat anti-rabbit IgG
β4-integrin	3E1	Mouse	IgG1	1:100	Sigma-Aldrich - A2547	Alexa Fluor 488 (green) goat anti-mouse IgG1
Src (phsopho Y529)	polyclonal	rabbit	IgG	1:50	Abcam - ab4817	Alexa Fluor 488 (green) goat anti-rabbit IgG

F-actin was stained with conjugated Alexa Fluor 488 phalloidin (Invitrogen, Cat.nr. A12379) in dilution 1:40. Phalloidin is a high-affinity probe for F-actin that is made from a mushroom toxin conjugated with bright, photostable, green-fluorescent Alexa Fluor 488 dye.

3.3 RNA extraction

RNA extraction was performed using TriTeagent (Ambion). Media was removed from the cells and the cells were treated with TriReagent (1ml/25cm^2) for 5 minutes. The solution was transferred to a 1.5 ml eppendorf tube and 0.2ml of chlorophorm per ml TriReagent added. Vortexed and left standing at RT for 15 minutes. Spun down at 13.000 rpm for 18 minutes at 4°C. The clear upper liquid phase was transferred to another eppendorf tube but the lower, pink phase discarded. 0.5ml of isopropanol per ml TriReagent was added to the solution, mixed carefully and kept at RT for 5-10 minutes. Then the solution was centrifuged at 13.000 rpm for 12 minutes at 4°C. The liquid was discarded and the precipitate washed in 1ml of 96% EtOH per ml TriReagent and then spun down again at 8000 rpm for 5 minutes. The precipitate was dissolved in 10-30 µl of water and measured for concentration and quality in Nano-drop (Thermo scientific).

3.4 Reverse Transcription

Reverse transcription polymerase chain reaction (RT-PCR) is a variant of polymerase chain reaction (PCR), a technique to generate many copies of a DNA sequence in a process called ”amplification“. In RT-PCR, however, an RNA strand is first reverse transcribed into its complementary DNA (cDNA) using the enzyme reverse transcriptase, and the resulting cDNA is amplified using traditional or real-time PCR. Here I used real-time PCR with sequence-specific DNA primers consisting of oligonucleotides that are labelled with a fluorescent reporter which permits detection only after hybridization of the probe with its complementary DNA target.

3.4.1 cDNA synthesis

Reverse transctiption was carried out with the RevertAid First Strand cDNA Synthesis Kit (Fermentas). First, 1 µl RNA, 1 µl 10x reaction buffer, 7 µl water and 1 µl DNAse was mixed together in an eppendorf tube. The solution was incubated at 37°C for 10 minutes and 1 µl of 25 µM EDTA was added to the mix. After incubation for 10 minutes at 65°C the solution was ready for cDNA synthesis. The RNA solution was mixed with 1 µl of water and 1 µl oligo(dt) (0,5 µg/µl). The solution was incubated at 70°C for 15 minutes and cooled on ice. Then 4 µl of 5x reaction buffer, 1 µl of ribonuclease inhibitor (20U/µl) and 2 µl 10mM dNTP mix were added to the mixture and incubated at 37°C for 5 minutes. Finally 1 µl of reverse transcriptase was added to mixture and the mixture was incubated at 42°C for 60 minutes and followed by inactivation of the enszyme at 70°C for 10 minutes. At this point I had cDNA ready for amplification in the RT-PCR reaction.

3.4.2 RT-PCR

Reagent were mixed as followes: 10 µl master mix (Applied Biosystems), 9 µl sample and 1 µl primers. The reaction was and analyzed by quantification method in 7500 Real time PCR system from Applied Biosystems. The reaction cycle was as follows: 50°C for 2 minutes (1cycle) , 95°C for 10 minutes (1 cycle), 95°C for 15 seconds and 60°C for 1 minute (40 cycles) and 72°C for 10 minutes (1 cycle). To test PTPN1 gene expression I used PTPN1 TaqMan primers (HS00182260_m1) from Applied Biosystems that cover exon boundary 5-6 in the PTPN1 gene. As control I used pre-designed GAPDH TaqMan primers (part no.4326317E) from Applied Biosystems. Quantitative analysis of results was calculated with the 2^-DDCT method, as carried out by the manufacturer (Applied-Biosystems, 1997).

3.5 Protein extraction

Protein extraction was performed using RIPA lysis buffer (20 nM Tris (pH 7.4), 1% Triton-X, 1% sodium deoxycholate, 10% glycerol, 150 mM NaCl, 2.5 mM EDTA 1% aprotinin, 1mM PMSF, 100mM Na_3Vo_4). Briefly, cells were washed with ice-cold phosphate-buffered saline (PBS) and incubated with RIPA lysis buffer on ice for ten minutes. Monolayers were then scraped using a cell scraper and lysates were transferred to microtubes. Lysates were then drawn through a syringe several times to fragment long DNA strands which can interfere with protein electrophoresis and incubated on ice for 10 minutes. Subsequently, lysates were centrifuged at 13.000 rpm for 20 minutes at 4°C and the supernatant transferred to a new microtube for protein quantification, determined by Bradford method in a spectrometer.

3.6 Western blot

Equal amounts of protein was loaded and run on a NuPAGE 10% Bis-Tris gel (Invitrogen, Carlsbad, CA) using MES running buffer (Invitrogen) for 35 min at a constant voltage of 200 volts. Protein were then transferred to a polyvinylidene difluoride membrane (Invitrogen) for 1 hr at 30 volts. The blots were then blocked in 5%-non fat milk for 1 hr, except for GAPDH where 5% BSA was used (table 1). The blots were then washed and incubated with the secondary antibody, horsesadish peroxidase-conjuagetd anti-mouse or rabbit (BD scientfic) at dilution 1:10000 for 1 hr. The protein bands were visualized using enhanced chemiluminescence (ECL) system (Thermo Scientific). The chemiluminescent signal was detected using Hyperfilm (Amersham).

3.7 Deparaffinisation of tissue samples

Tissue sections on microscope slides were dewaxed for 2x5 minutes in xylene, air dried and rehydrated for 2x5 minutes in 96% EtOH. The slides were then rinsed in water for 1 minute and boiled in TE buffer (1 mM Tris, 0.1mM EDTA) for 15 minutes. Slides were cooled in the buffer for 20 minutes and then rinsed in water for 10 minutes and in PBS for 5 minutes.

3.8 Isolation of branching structures from Matrigel

Branching bronchio-alveolar like structures from three dimensional cultures were isolated from the Matrigel. The Matrigel was dissolved by Matrigel lysis buffer (500ml of cold 1x PBS buffer, 500µl 0.5M EDTA, 250µl 0.2M $NaCI_4$, 250µl 0.25M NaF) on ice at mild rotation for 60 minutes. Branching structures were then spun down in pre-cooled rotator at 1000 rpm for 5 minutes at 2°C. The structures were then re-suspended in 30µl PBS and left to dry on glass slides.

3.9 Immunocytochemistry

3.9.1 Immunofluorescence staining of tissue samples

Deparaffinized tissue samples were fixed with methanol for 10 minutes at -20°C and then incubated for 30 minutes in PBS containing 10% FBS. Slides were washed 2x5 minutes and primary antibodies were pre-mixed in PBS containing 10% FBS and incubated for 30 minutes (table 2). The slides were then washed x2 with PBS for 5 minutes and incubated with isotype specific fluorescent antibodies (Invitrogen) mixed in PBS for 30 minutes in the dark. The slides were then washed twice for 5 minutes and incubated with fluorescent nuclear counterstain (TOPRO-3, Invitrogen) for 10 minutes in the dark. Samples were washed 2x5 minutes and then mounted with coverslips using Fluoromount-G (Southern Biothech) for preservation of the fluorescent signal. Immunofluorescence was visualized using a Zeiss LSM 5 Pascal laser scanning confocal microscope.

3.9.2 Immunofluorescence staining of monolayer cells.

D492 cells were fixed with fixed with 3.5% formaldehyde in PBS for 5 minutes followed by 2x7 minutes incubation with 0.1% Trition X-100 in PBS at room temperature and then incubated for 5 minutes in PBS containing 10% FBS. Slides were washed 2x5 minutes and primary antibodies were pre-mixed in PBS containing 10% FBS and incubated for 30 minutes (table 2). The slides were then washed x2 with PBS for 5 minutes and incubated with isotype specific fluorescent antibodies (Invitrogen) mixed in PBS for 30 minutes in the dark. The slides were then washed twice for 5 minutes and incubated with fluorescent nuclear counterstain (TOPRO-3, Invitrogen) for 10 minutes in the dark. Samples were washed 2x5 minutes and then mounted with coverslips using Fluoromount-G. Immunofluorescence was visualized using a Zeiss LSM 5 Pascal laser scanning confocal microscope.

3.9.3 Immunofluorescence of 3D cultures

The branching structures stained for β4-integrin were fixed in methanol for 10 minutes at -20°C but the structures that were stained for ErbB2 and F-actin were fixed with 3.5% formaldehyde in PBS for 5 minutes followed by 2x7 minutes incubation with 0.1% Trition X-100 in PBS at room temperature. Then the slides were rinsed with 100 mM PCS glycin solution (100 mM glycin in PBS) in 3x10 minutes. Next 200 µl of IF blocking solution (10% goat serum, 1% goat anti mouse immunoglobulin G mixed in IF buffer (0.2% Triton X-100, 0.1% BSA. 0.05% Tween 20 all mixed in PBS) was put on the slide for 90 minutes. Next the structures were incubated in primary antibody mixed in IF buffer for 120 minutes (table 2). After that the slides were washed in a coplin jar 3x20 minutes in IF buffer. Then the slides were incubated in the dark with secondary antibody mixed in IF buffer for 45 minutes. The slides were then washed in IF buffer for 20 minutes and 2x10 minutes in PBS. Next the structures were incubated with nuclear stain (TOPRO-3) mixed in PBS for 10 minutes

in the dark, samples were washed 2x5 minutes and then mounted with coverslips using Fluoromount-G. Immunofluorescence was visualized using a Zeiss LSM 5 Pascal laser scanning confocal microscope.

3.10 Apoptosis assay

D492 cell line was treated with DMSO or indicated concentrations of PTP1B inhibitor for 48hrs. Cells were harvested and washed twice with cold PBS and resuspended in 1 x Binding buffer at concentration $1x10^6$ cells/ml. Then 100 µl of the solution ($1x10^5$ cells) were transferred to a 15 ml culture tube. 2.5 µl of FITC Annexin V (BD Pharmingen) were added to the cells and incubated at RT in the dark for 15 minutes. 400 µl of 1 x Binding buffer were added to each tube and 2.5 µl of propidium iodide (PI) just before 10.000 cells from each tube were analyzed in FACSCalibur.

3.11 CFSE-based cell proliferation

D492 cell line was trypsinized and resuspended in 1 ml PBS with 0.1% BSA at 10^6/ml cells. CFSE dye (Invitrogen) was added to the cells in a final concentration of 0.5 µM. The cells where incubated in a water bath at 37°C for 10 minutes. 10ml of FBS was added to stop the reaction. Cells were washed 3 times in PBS and then resuspended in 1 ml of normal growth media and seeded to a 24 well plate. PTP1B inhibitor in a final concentration of 8 µM or equivalent amount of DMSO was added to the cells. Dilution of CFSE was measured by counting 10.000 viable cells with FACSCalibur (BD Bioscience) for 5 consecutive days.

3.12 Cell survival assays

3.12.1 Cell growth curve with PTP1B inhibitor

D492 and MCF-7 cells were plated at 70% confluency per well in a 24-well plate in growth media. One day after plating, the media was replaced with H14 media supplemented with 0.5% FBS containing DMSO or 16 µM concentration of PTP1B inhibitor. Each day after that 3 wells per concentration were washed three times in phosphate-bufferd saline, fixed with 250µl of crystal violet solution (0.25% crystal violet in 20% methanol) for 10 min, washed 6 times in water and dryed. After that the fixed cells were incubated in 500µM lysis solution (33% acetic acid) for 10 minutes. To quantitate the amount of crystal violet in each sample the optical density at 570 nm was determined using the spectrophotometer.

3.12.2 Cell growth curve with calpain inhibitor

D492 cells were plated at 70% confluency cells per well of a 96-well plate in growth media. One day after plating, the media was replaced with growth media containing DMSO, 50 μM or 100 μM concentration of Calpain inhibitor. Each day after that 3 wells per concentration were washed three times in phosphate-bufferd saline, fixed with 50μl of crystal violet solution (0.25% crystal violet in 20% methanol) for 10 min, washed 6 times in water and incubated in 100μM lysis solution (33% acetic acid) for 10 minutes. To quantitate the amount of crystal violet in each sample the optical density at 570 nm was determined using the spectrophotometer.

3.13 Statistical analysis

All growth curves were performed in triplicate for statistical accuracy. Graphs were created in Excel. Error bars represent the standard deviation of the sample (SD).

4. Results

4.1 PTP1B expression

4.1.1 PTP1B expression in primary cells and cell lines

To answer the question if the retroviral insert has an effect on PTP1B expression in D492 I studied expression levels in D492 both at the RNA and protein level and compared it to other cell types (figure 5a). D382 is a Muc1 and ESA positive cell line which represents normal luminal epithelial cells in the breast. D382 was immortalized with HPV carrying E6 and E7 oncogenes like D492, but the insertional site is currently unknown. MCF-7 is widely used and well characterized, spontaneously immortalized breast cancer cell line (Soule 1973). Real-time PCR analysis clearly shows increased expression of PTP1B in D492 compared with D382 and MCF-7 (figure 5a). Western blot confirms increased expression of PTP1B in D492 compared with D382 and primary cells from the breast (Figure 5b), but the difference in mRNA expression between D492 and MCF-7 is not translated to protein level where PTP1B expression is even higher in MCF-7 than D492. Myoepithelial cells seem to express much more PTP1B than luminal epithelial cells and endothelial cells (figure 5b).

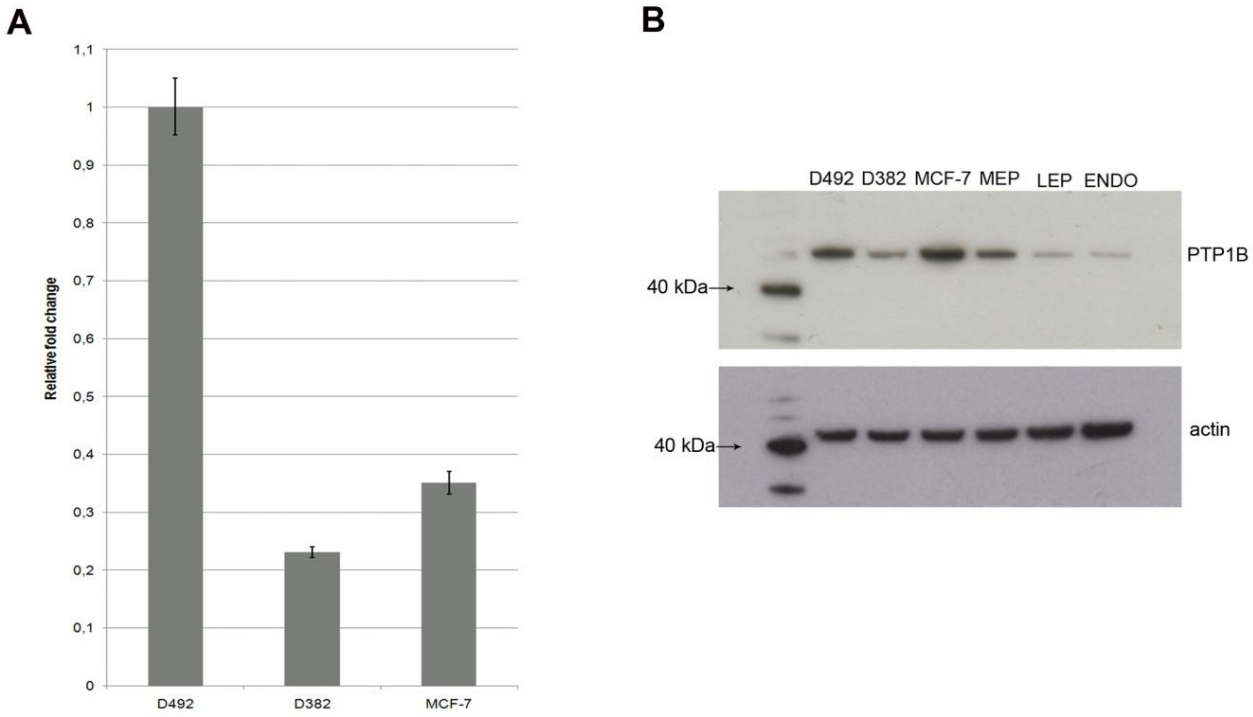

Figure 5. PTP1B expression is much higher in MCF-7 and D492 than primary cells isolated from the breast and the luminal like cell line D382.

A. PTP1B expression assessed with RT-PCR in D492, D382 and MCF-7 cell lines. **B.** PTP1B expression assessed with a western blot in D492, D382 and MCF-7 cell lines and myoepithelial (MEP), luminal epithelial (LEP) and endothelial (ENDO) primary cells isolated from the breast.

4.1.2 PTP1B expression in tissue sections

PTP1B is ubiquitously expressed in epithelial cells in the breast, as well as in some cells in the stroma. Histochemical staining of paraffin wax embedded breast tissue with antibodies against PTP1B, cytokeratine 14, cytokeratine 18 and vimentin revealed that PTP1B is, at least partially, co-stained with each of these markers. CK14 was only expressed in the myoepithelial cells of the breast (figure 6a), whereas CK18 was expressed in the luminal epithelial cells (figure 6b). IF staining of PTP1B suggested that there is a variation in the amount of protein expression within the TDLU structure. The most prominent expression was seen in a linear staining pattern surrounding all normal ducts and lobules, where the myoepithelial cells come in contact with the ECM. The mesenchymal intermediate filament protein vimentin was expressed by the myoepithelial cells of the mammary gland as well as cells in the stroma (figure 6c). It is widely used as a marker for stromal cells. Figure 2c clearly shows that vimentin staining in the stroma is much stronger than PTP1B staining, though PTP1B seems to be partially expressed in stromal cells, for example endothelial cells as confirmed in figure 1b. These histochemical staining confirm the results from the western blot analysis of PTP1B in figure 1b where PTP1B expression was predominantly in the breast epithelial cells.

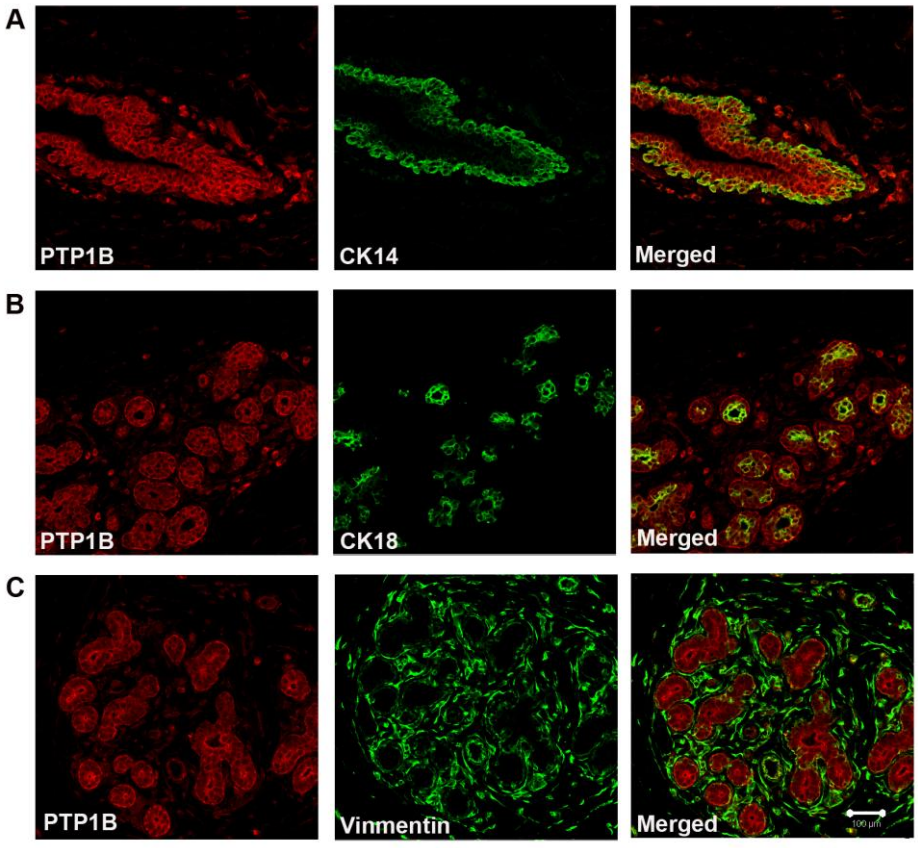

Figure 6. PTP1B is expressed in normal breast tissue, predominantly in epithelial cells.
A,B,C. IF staining on paraffin wax embedded breast tissue of PTP1B (rabbit antibody) (read) and **A.** CK14 (myoepithelial cell marker) **B.** CK18 (luminal epithelial cell marker) **C.** Vimentin (green) (Bar: 100µm).

4.1.3 PTP1B expression 3D structures

PTP1B expression in D492 in three dimensional rBM culture was similar to the expression in normal breast gland (figure 7). Immunofluorence staining revealed that PTP1B is expressed predominantly at the lobular end of the structure. PTP1B appears to colocalize significantly with ErbB2 which is evident on the yellow fluorescent signal in the structure (figure 7a right). F-actin is a cytoskeletal protein that is expressed equally throughout the structure (figure 7b). β4-integrin is localized at the surface of the structure in contact with the basement membrane (figure 7c).

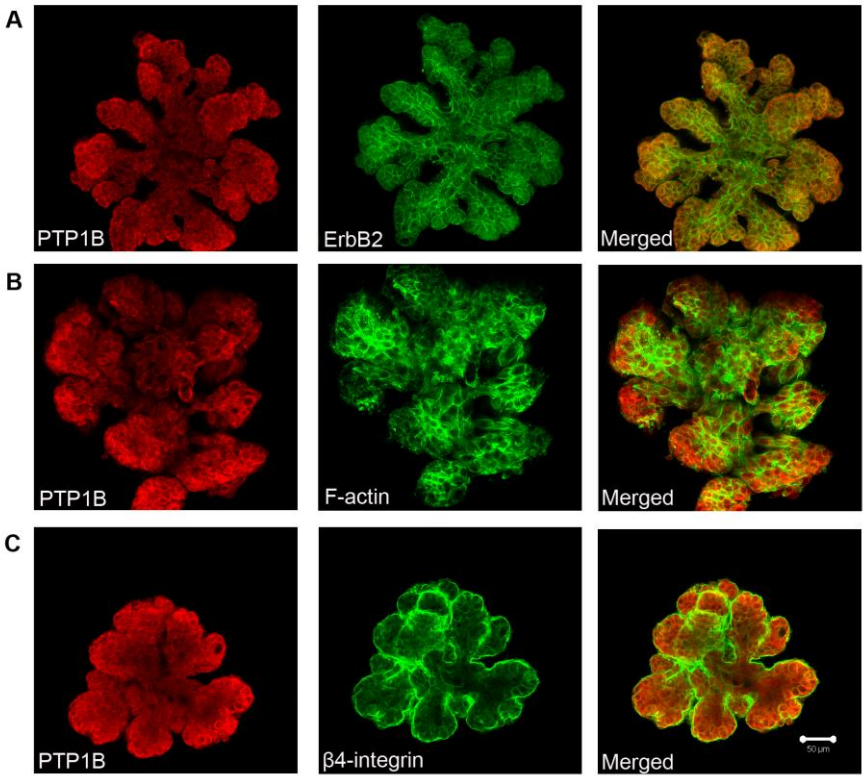

Figure 7. PTP1B is expressed in D492 3D cultures, predominantly at the lobular end of the structure.

IF staining on D492 3D cultures of **A.** ErbB2 (green) and PTP1B (mouse antibody) (read) **B.** F-actin (green) and PTP1B (rabbit antibody) (read) **C.** b4-integrin (green) and PTP1B (rabbit antibody) (read) (Bar: 50µm).

4.2 Functional role of PTP1B in breast epithelial cells

Given the location of the retroviral insert and its close proximity to the PTPN1 gene, I examined if PTP1B might be important for the proliferation and survival of D492. To test this, a specific PTP1B inhibitor was used to inhibit PTP1B catalytic function in the cells.

4.2.1 The effect of PTP1B inhibition on the proliferation of D492 and MCF-7

D492 cell were seeded in H14 media in monolayer culture and the following day treated with 16 μM of PTP1B inhibitor, twice the IC_{50} for the inhibitor. Figure 8 shows how the inhibitor affected the cells by inducing cell death within the first 48 hours. MCF-7 were also treated with the same amount of the inhibitor in DMEM media supplemented with 5% serum, but the treatment did not have any visual effects on the cells.

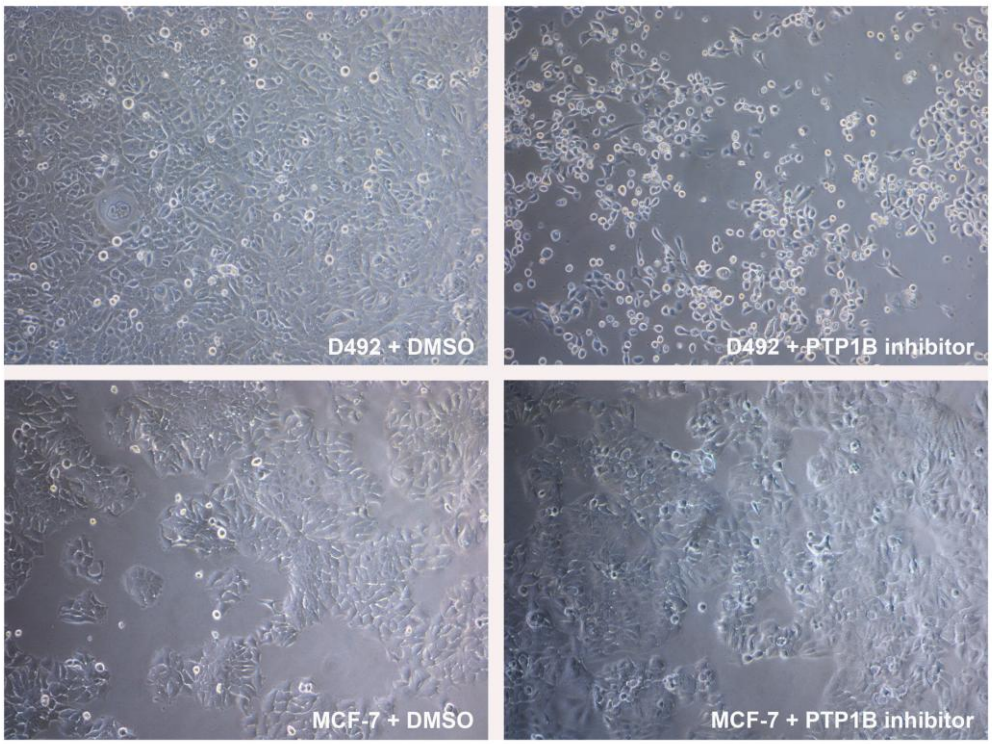

Figure 8. PTP1B inhibitor induces cell death in D492 but does not affect the survival of MCF-7. D492 and MCF-7 were treated with DMSO (control) or 16μM of a specific PTP1B inhibitor (IC_{50} = 8μM) for 48hrs in monolayer (Phase contrast light microscopy, original magnification x100).

When analyzed with regard to cell growth and proliferation PTP1B inhibition had major effect on the survival of D492. As seen in figure 9 growth curve assay shows that cells treated with DMSO proliferate, whereas the majority of D492 cells treated with 16μM of PTP1B inhibitor were dead after 72hrs. In this experiment the cell were cultured in H14 media supplemented with 0.5% FBS. MCF-7

cell were seeded in the same media as D492 and treated with PTP1B inhibitor or DMSO as control. The inhibitor had no effect on the survival or the morphology of MCF-7.

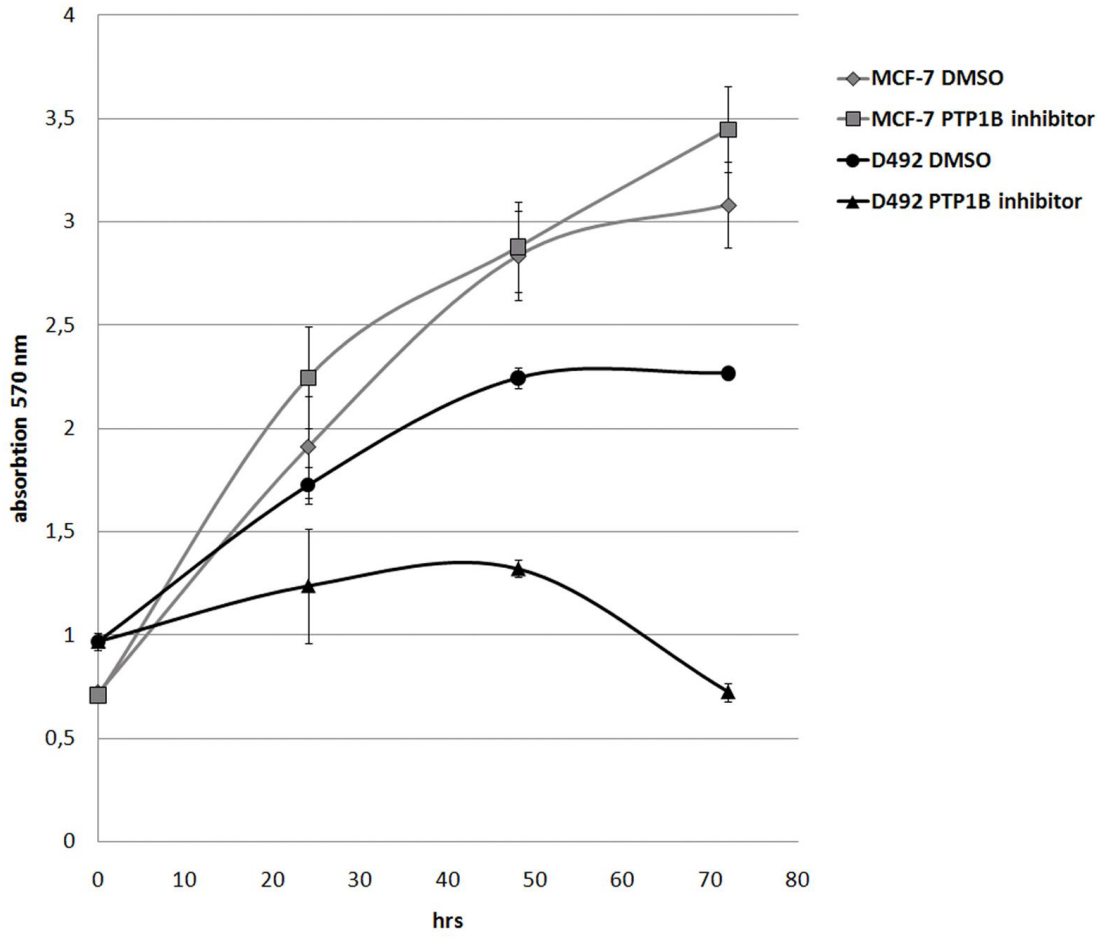

Figure 9. Cell survival assay reveals that PTP1B inhibitor induces cell death in D492 but does not affect the survival of MCF-7.

Growth curve on D492 and MCF-7. Cells were treated with DMSO (control) or 16µM of a specific PTP1B inhibitor for 3 days, stained with crystal violet and the optical density at 570nm determined. All experiments were conducted in triplicate.

4.2.2 PTP1B inhibitor induces apoptosis in D492.

To study the type of cell death induced by the PTP1B inhibitor I used an annexin V apoptosis kit. Apoptosis is the process of programmed cell death that involves a series of biochemical events that lead to variety of morphological changes, including blebbing, changes in the cell membrane such as loss of membrane asymmetry and attachment, cell shrinkage, nuclear fragmentation, chromatin condensation and chromosomal DNA fragmentation.

This apoptosis kit utilizes that annexin V detects cells that express phosphatidylserine (commonly found in the inner leaflet of cell membrane) on the outer cell surface which is a feature found in apoptosis. PI is membrane impermeant fluorescent molecule that stains DNA and is generally excluded from viable cells. It is therefore used to measure necrosis and late apoptosis.

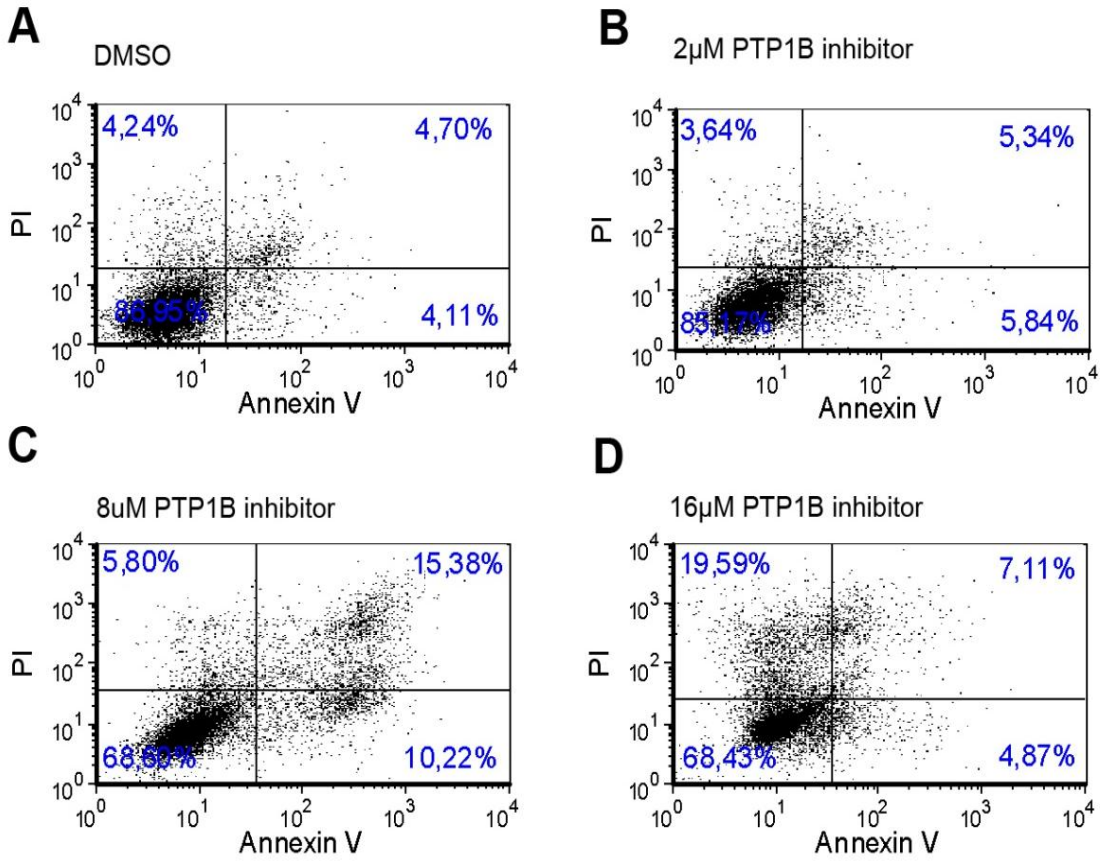

Figure 10. Annexin V and PI staining reveals that PTP1B inhibitor induces apoptopic cell death in D492.

A - D. Annexin V and PI staining of D492 cells treated with DMSO (control) and various concentrations of PTP1B inhibitor.

As seen in figure 10 the Annexin V apoptosis test clearly demonstrates that the PTP1B inhibitor is inducing apoptosis in D492. DMSO treated culture cells were only stained mildly with annexin V and PI (9% and 9%, respectively). When treated with 2µM there is slightly increase in annexin V staining but no increase in PI staining, 11% of cells are stained with annexin V and 9% with PI. When treated with 8µM of the PTP1B inhibitor there is a drastic change where 26% of the cell stain for annexin V and 21% for PI, of which 15% are stained for both markers. This data demonstrates that annexin V/PI staining of cells with increasing PTPB inhibitor concentration shows several stages of programmed cell death, from early to late apoptosis. The highest level of cell death was observed in D492 cells treated with 16µM of the PTP1B inhibitor. Annexin V/PI staining indicates

that the cells are in late apoptosis where 27% of cells stain positive for PI and only 12% are positive for annexin V.

In summary these result indicate that D492 cells are induced to undergo programmed cell death after treatment with PTP1B inhibitor.

4.2.3 PTP1B inhibitor induces cell death in dividing cells

Using the PTP1B inhibitor I performed a Carboxyfluorescein succinimidyl ester (CFSE) experiment to address the effects of PTP1B inhibition on cell division in D492 cells. CFSE is a fluorescent dye that measures cell proliferation. CFSE is retained within non proliferative cells and covalently couples to intracellular molecules. Due to this covalent coupling reaction fluorescent CFSE can be retained within cells for long periods of time (Parish, 1999). Also, due to this stable linkage, once incorporated within cell the dye is not transferred to adjacent cells. CFSE can be used to monitor cell proliferation, both *in vitro* and *in vivo*, due to the progressive halving of CFSE fluorescence within daughter cells following each cell division. High concentrations of the dye can be toxic to cells, but if CFSE labeling is performed optimally, approximately 7-8 cell divisions can be identified before CFSE fluorescence is too low to be detected (Lyons & Parish, 1994). D492 cells were treated with either DMSO as a control or 8μM of the PTP1B inhibitor. As seen in figure 9 (ch.4.2.1), 8μM of the inhibitor induces substantial cell death, but significantly less than at 16μM of the inhibitor. Flow cytometry analysis of the CFSE-stained cells showed that the PTP1B inhibitor affected cell division of D492 while cell proliferation seems not disturbed in the control cells.

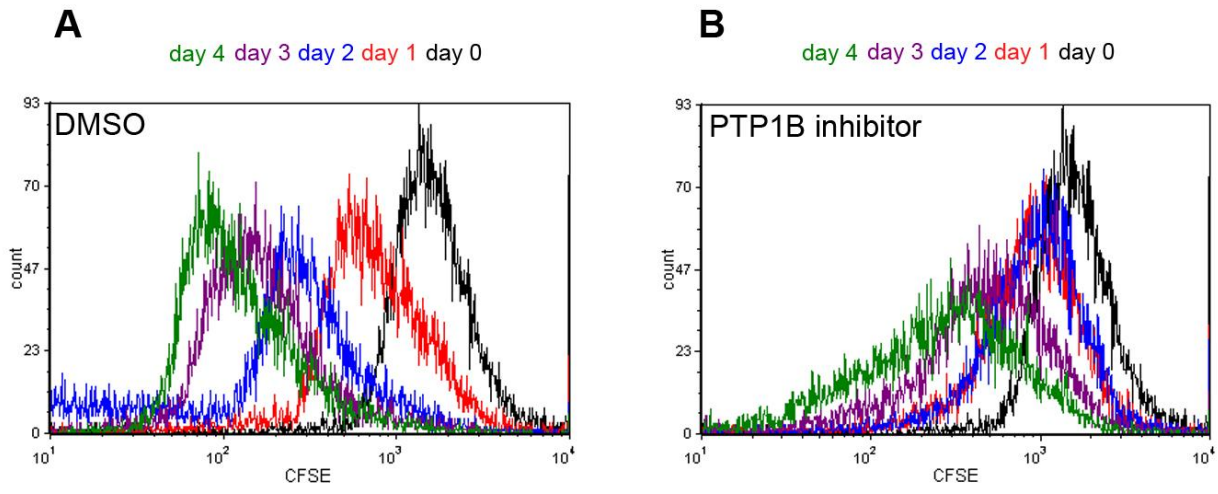

Figure 11. PTP1B inhibitor induces cell death in dividing D492 cells.
D492 cells where stained with CFSE fluorescent dye, treated with **(A)** DMSO and **(B)** 8μM of PTP1B inhibitor and analyzed in flow cytometry over a period of 4 days.

When treated with the PTP1B inhibitor D492 cell proliferation decreased to some extent on the first day and after that there are few cell divisions (Figure 11). The data indicates that the cells that remain in viable culture are the cells that do not divide.

This raises the possibility that the cells are dying through a process called anoikis where loss to the ECM induces apoptotic cell death. Cells undergoing apoptosis go through a process of blebbing, cell shrinkage, nuclear fragmentation and chromatin condensation, whereas cells undergoing anoikis look round shaped with halo around them as they detach from the surface. To compare the morphology of cells undergoing classic apoptosis and cell death induced by PTP1B inhibition, D492 cells were treated with 10μM camptothecin (CPT), an apoptosis inducer or the PTP1B inhibitor. Cells treated with CPT show morphology of classic apoptosis (figure 12b) whereas cells treated with the PTP1B inhibitor look round with characteristics of reduced cell attachment and spreading (Figure 12c). It is evident that the morphology of cells treated with CPT and PTP1B inhibitor is very different and their morphology supports the idea that the PTP1B inhibitor induces anoikis.

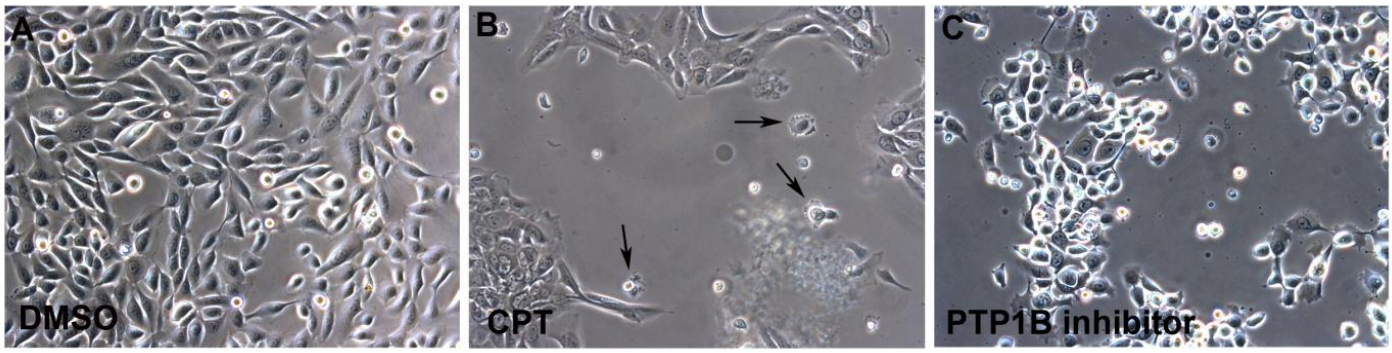

Figure 12. The morphology of D492 cells treated with PTP1B inhibitor is consistent with anoikis.

Cells were treated with **A.** DMSO, **B.** 10 μM Camptothecin (CPT) **C.**16μM of PTP1B inhibitor. Arrows point to cells showing the morphology of apoptosis (Phase contrast light microscopy, original magnification x200).

4.2.4 PTP1B inhibitor decreases Src activity in D492 cells

I assessed the effect of the PTP1B inhibitor on Src phosphorylation as Src activity is regulated by phosphorylation of two distinct tyrosine residues. Autophosphorylation of Tyr^{416} in the kinase domain activates Src, whereas phosphorylation of Tyr^{529} in the C-terminal tail inactivates Src. Consequently, activation of Src requires the removal of the C-terminal phosphate by specific PTPs. D492 cells were treated with various concentrations of the PTP1B inhibitor for 48hrs followed by a protein isolation for western blot analysis. Phosphorylation level at Tyr529 was determined using phosphospecific antibody as shown in figure 13. Treatment of D492 cells with the PTP1B inhibitor caused significant increase in Tyr 529 phosphorylation (figure 13a). Phosphorylation on the inhibitory

tyrosine increased gradually with increased concentration of the inhibitor. To support this data D492 cells were seeded in culture flask and the next day treated with DMSO as control or 16µM of the PTP1B inhibitor for 3 hrs. The cells where then fixed with methanol and stained for PTP1B and p-Src 529 (figure 13b). After 3 hrs there was a drastic change in the phosphorylation status of Src, it increased drastically and appeared to be at the cell membrane in the cell.

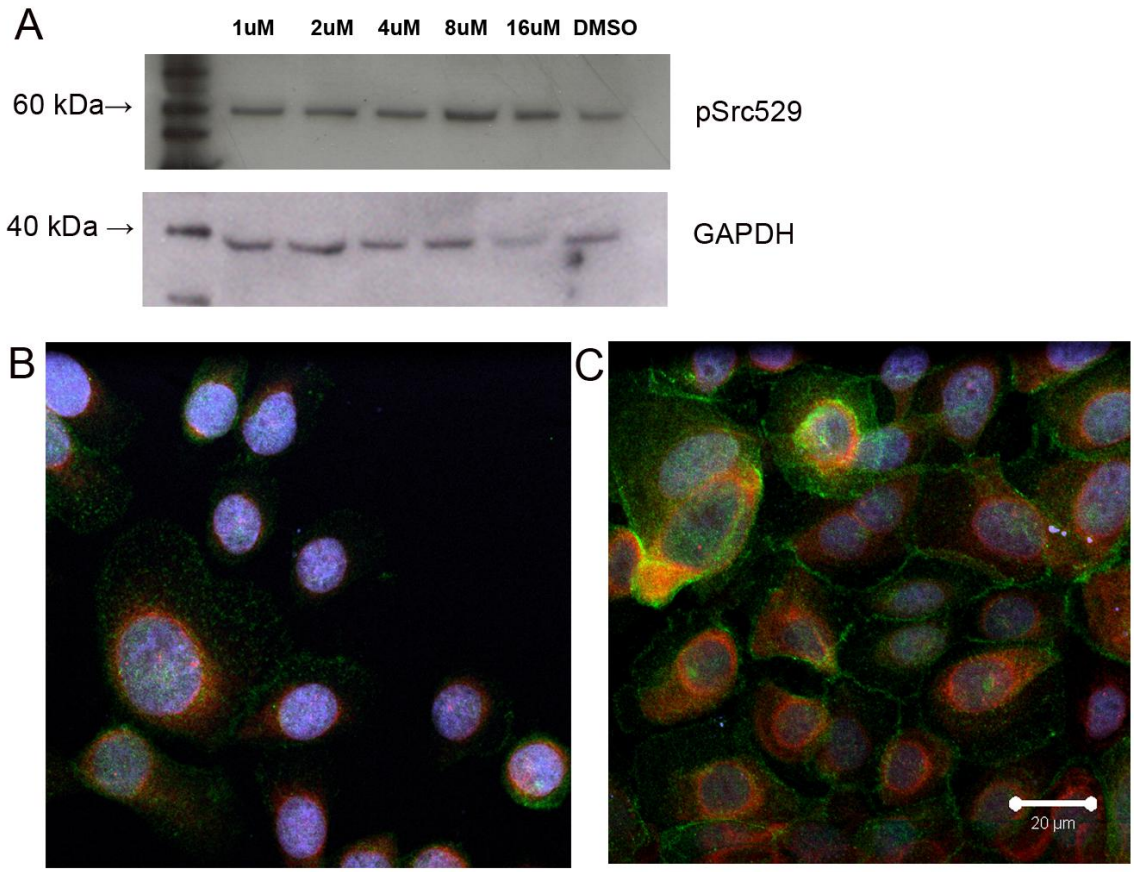

Figure 13. PTP1B inhibitor decreases Src 529 phosphorylation in D492.

A. Western blot analysis of p-Src 529. Protein from D492 cells that were treated with DMSO or various concentrations of PTP1B inhibitor. **B, C.** IF staining of p-Src in D492 cells that were treated with **B.** DMSO **C.** 16µM PTP1B inhibitor for 3hrs; pSrc 529 (green), PTP1B (mouse antibody) (read), TOPRO-3 nuclearstaining (blue) (Bar: 20µm).

4.2.5 D492 cells are sensitive to calpain inhibition

In western blots, after long exposure of PTP1B antibody in protein from selected breast epithelial cell lines, two bands appear. One for the full length protein at 50 kDa and another smaller band at 40 kDa. I hypothesized that this band could represent a proteolytic fragment of PTP1B cleaved by calpain 2 protease (see introduction, figure 4). This band appears in protein from D492 and D382 but not in protein from MDA-231 (breast cancer cell line) or MCF-7 (figure 14a). If this is a

proteolytic fragment of PTP1B and therefore more active form of the phosphatase it could mean that this fragment is significant in the PTP1B pathway and for the survival of the D492 cells.

I therefore did a survival assay after treating the cells with a pharmaceutical inhibitor targeting calpain activity. Treatment of D492 with 100 µM calpain inhibitor induced significant cell death whereas cells treated with DMSO or 50 µM of calpain inhibitor did not display indications of any cell death (figure 14b).

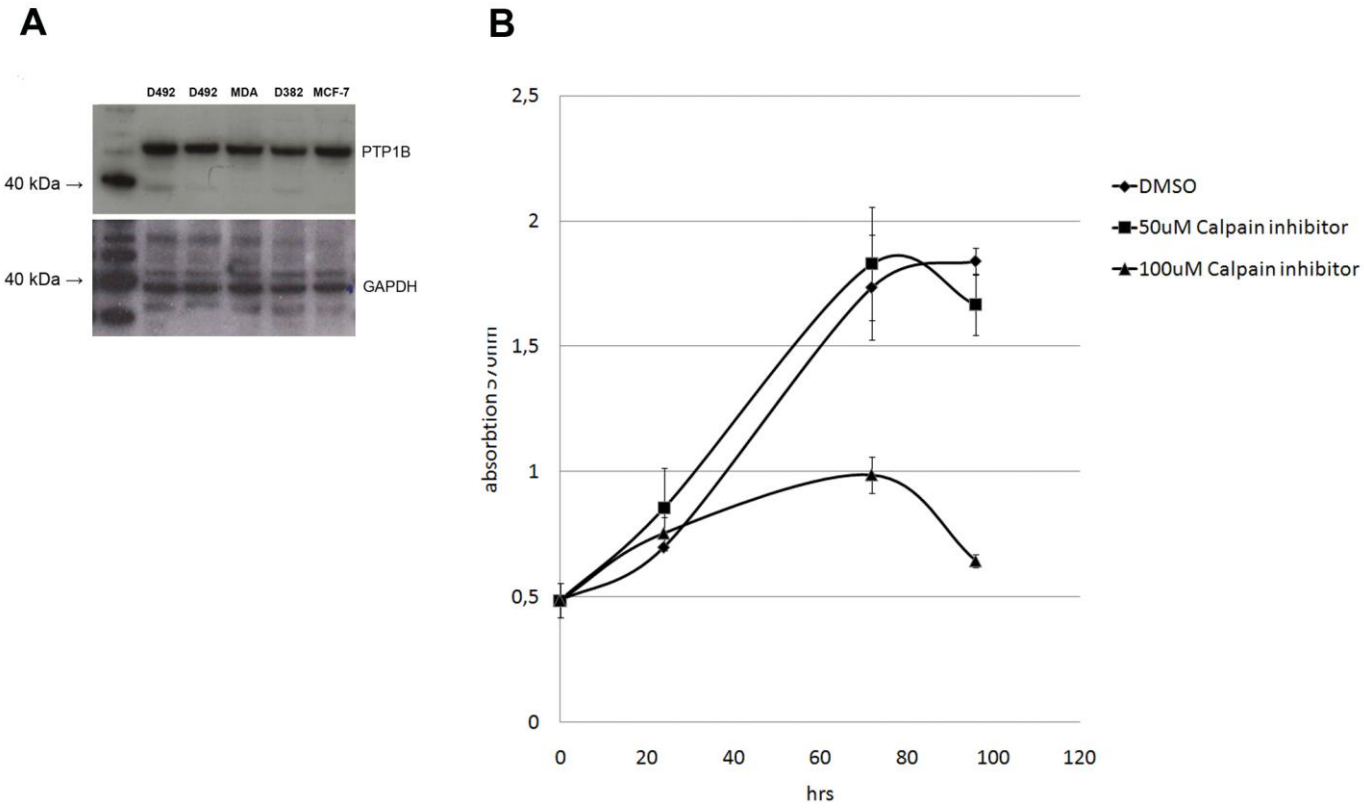

Figure 14. Calpain inhibitor induces cell death in D492.

A. PTP1B expression assessed with a western blot in protein from D492 isolated in 2 independent experiments, MDA-MB-231, D382 and MCF-7 cell lines. When exposure duration reaches 30 minutes a smaller PTP1B protein band appears. **B.** D492 treated with DMSO (control), 50µM or 100 µM of calpain inhibitor for 4 days and stained with crystal violet and the optical density at 570nm determined. All experiments were conducted in triplicate.

Next I examined if the cells were undergoing apoptosis or necrosis using annexin V and PI staining. The number of annexin V positive cells stayed approximately the same until the inhibitor concentration reached 200 µM where the number of annexin V positive cells increased (figure 15). The number of PI positive cells increases from 5% in cells treated with DMSO to 23% in cell treated with 200 µM of the calpain inhibitor. The annexinV apoptosis test clearly shows that the calpain inhibitor is inducing cell death, but it is not as conclusive as the annexin V staining was with the PTP1B inhibitor. Therefore this subject needs further studies to determine if the cells are going through apoptosis or necrosis. The sensitivity of the cells to the calpain inhibitor indicates that calpain protease is important for the survival of D492.

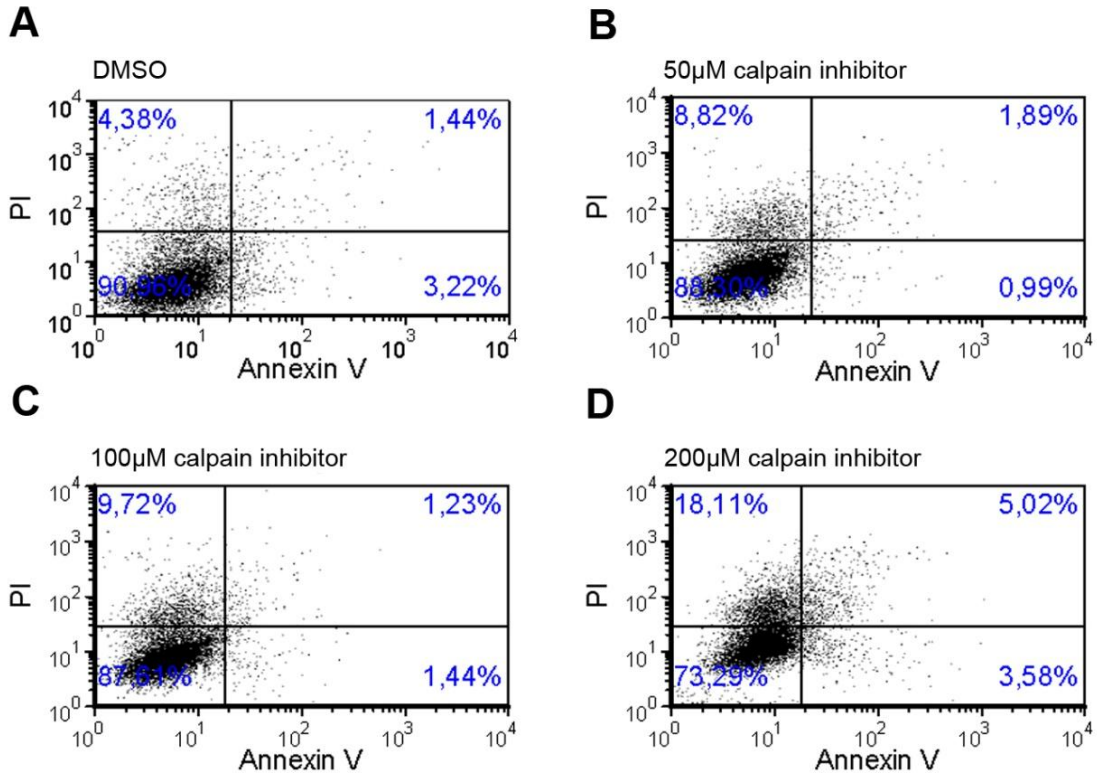

Figure 15. Calpain inhibitor increases annexin V and PI staining D492.
C, D, F, G Annexin and PI staining of D492 cells treated with DMSO (control) and various concentrations of calpain inhibitor.

4.3 Effects of serum on PTP1B induced cell death

Some proteins in serum are known to bind many of its components and in some cases, neutralize drugs in the circulation. In my first experiments with the PTP1B inhibitor D492 cells were cultured in the chemically defined serum-free medium H14 while the MCF-7 cells were cultured in DMEM medium supplemented with 5% serum, raising the possibility that our results comparing the effects of PTP1B inhibition between D492 and MCF-7 could be explained by the presence of serum in the culture media. This possibility was excluded when we showed that MCF-7 remained resistant to PTP1B inhibition in serum free media. (see ch. 4.2.1 figure 9). Figure 8 is an example of an experiment where the effects of PTP1B inhibition were assessed in different kind of media, D492 in defined serum free media while MCF-7 are in serum containing DMEM medium.

4.3.1 Serum protects D492 cells from apoptosis induced by PTP1B inhibitor

The major difference between the culture condition of MCF-7 and D492 is the serum in the MCF-7 medium. I therefore asked whether the apoptopic effects of the PTP1B inhibitor would be altered in the presence of serum. D492 was cultured in H14 with or without 5% serum with various concentrations of the PTP1B inhibitor (figure 16). The serum protected D492 from apoptosis. Cells that were only grown on H14 died quickly but the cells grown in medium supplemented with 5% serum did not show any sensitivity to the PTP1B inhibitor (figure 16).

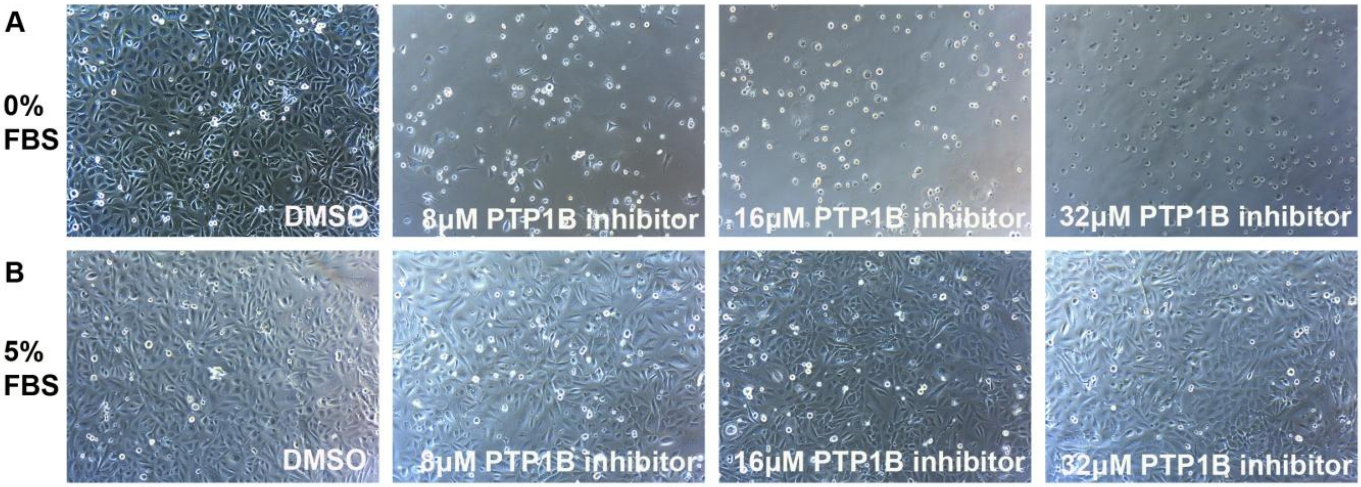

Figure 16. Serum protects D492 from PTP1B inhibitor induced cell death.
A. D492 was grown in H14 medium with different concentration of PTP1B inhibitor for 48 hrs.
B. D492 was grown in H14 medium with 5% serum with different concentration of PTP1B inhibitor for 48hrs (Phase contrast light microscopy, original magnification x100).

These findings suggest that there is something in the serum that either neutralizes the inhibitor or turns on alternative pathway that rescues D492 from cell death. To test which factors in the serum could be rescuing the cells from apoptosis D492 cells were seeded in three different chemically defined medium with DMSO or the PTP1B inhibitor. Interestingly D492 underwent apoptosis in M10 medium and H14 medium but not in CDM4 media (figure 17).

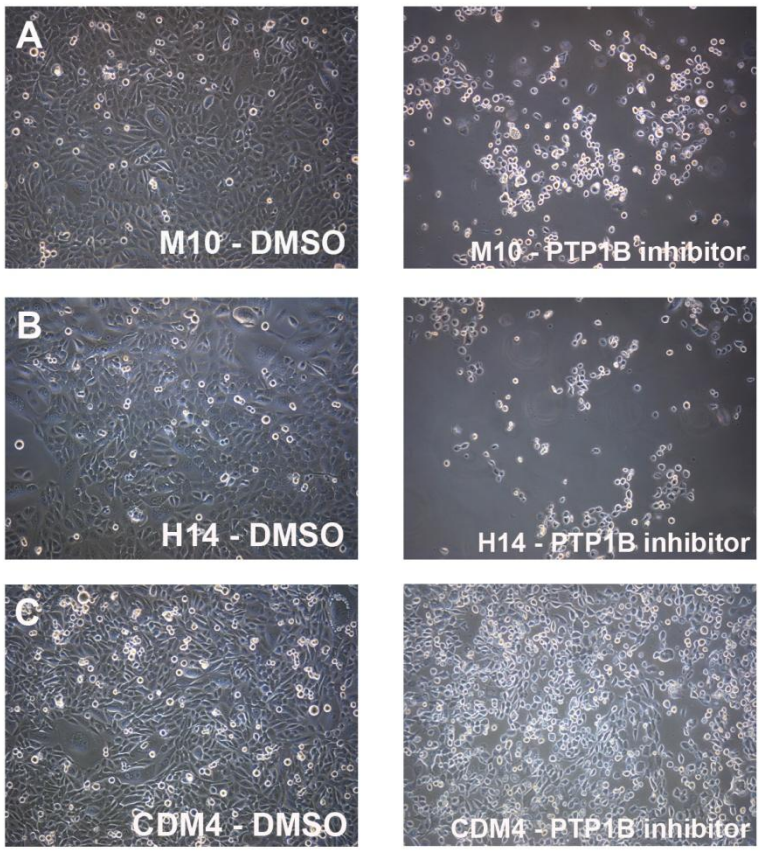

Figure 17. D492 dies when treated with PTP1B inhibitor in M10 and H14 medium, but not in CDM4 medium.

A. D492 grown in M10 medium with DMSO (control) or 16µM PTP1B inhibitor. **B.** D492 grown in H14 medium with DMSO (control) or 16µM PTP1B inhibitor. **C.** D492 grown in CDM4 medium with DMSO (control) or 16µM PTP1B inhibitor (Phase contrast light microscopy, original magnification x100).

These 3 media differ in composition as seen in table 3 where the factors in CDM4 which are not H14 and M10 are found in the rectangle.

Table 3. Components of H14, M10 and CDM4 medium.

CDM4	concentration in media
Insulin (Ins)	3µg/ml
Transferrin (Tf)	25µg/ml
EGF	20ng/ml
Na-Selenite (Na-Sel)	2,6ng/ml
Estradiol (Est)	10^{-10}M
Hydrocortisone (Hydro)	500ng/ml
Cyclic AMP (cAMP)	10^{-5}M
Fibronectin (FN)	100ng/ml
Triiodothyronine (T3)	10^{-8}M
Ethanolamine (Eth)	10^{-4}M
Phosphoethanolamine (Peth)	10^{-4}M
Bovine Serum Albumin (BSA)	0,01%
Ascorbic Acid (C-vít)	10µg/ml
Fetuin (Fet)	20µg/ml
Cholera Toxin	10ng/ml

H14	concentration in media
Insulin (Ins)	250ng/ml
Transferrin (Tf)	10µg/ml
EGF	10ng/ml
Na-Selenite (Na-Sel)	2,6ng/ml
Estradiol (Est)	10-10M
Hydrocortisone (Hydro)	1,4x10-6M
Prolactine	0,15IU

M10	concentration in media
Insulin (Ins)	7,5µg/ml
EGF	10ng/ml
Hydrocortisone (Hydro)	1,4x10-6M
Cholera Toxin	10ng/ml
Bovine pituery extract	unknown

To test which factors in the CDM4 medium were protecting D492 cells from apoptosis, D492 cells were seeded in 9 wells, one contained full medium, each of the other lacked one component of normal CDM4 medium (figure 18). The only well were D492 underwent cell death was the well were bovine serum albumin (BSA) was missing (figure 18G).

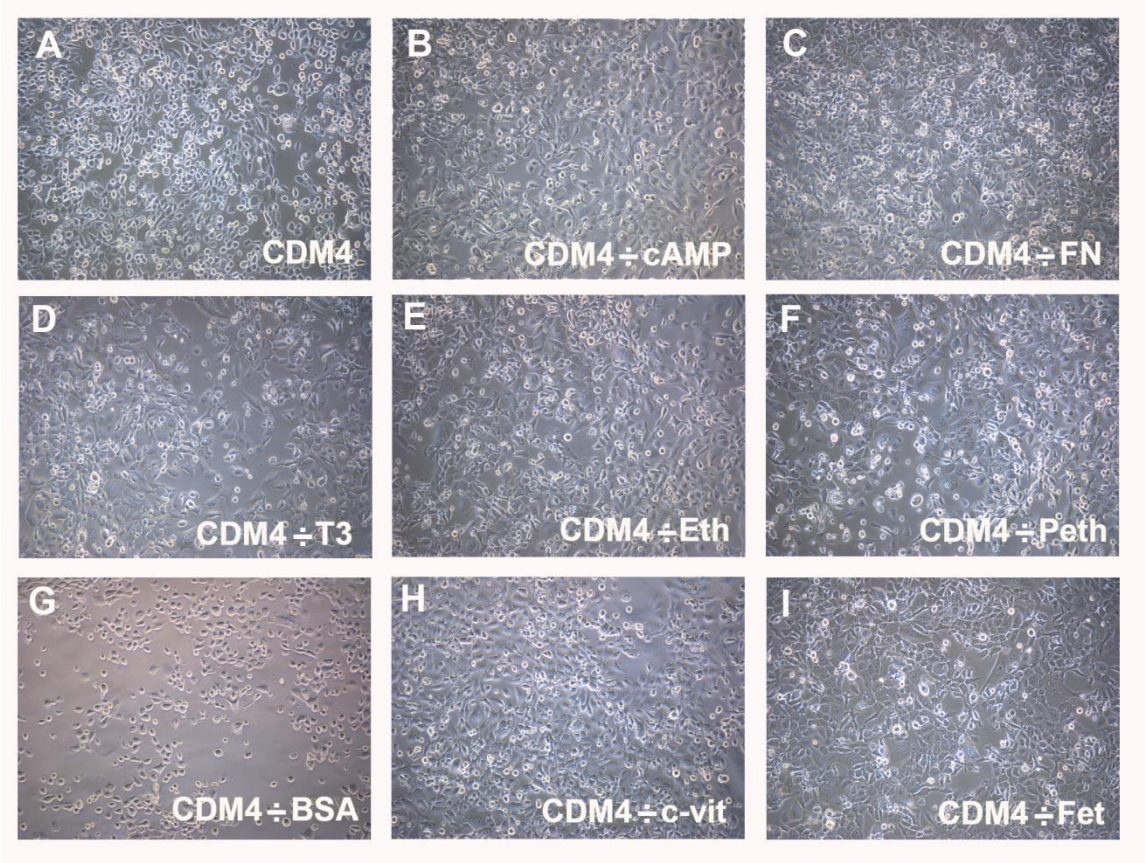

Figure 18. BSA protects D492 from PTP1B inhibitor induced cell death in CDM4 medium.
D492 was seeded in CDM4 with all its ingredients except: **A.** nothing missing, **B.** cAMP **C.** FN **D.** T3 **E.** Eth **F.** Peth **G.** BSA **H.** c-vit **I.** Fet and the following day 16µM of PTP1B inhibitor was added (Phase contrast light microscopy, original magnification x100).

Albumin is the most abundant protein in plasma, present at 50mg/ml, with half life of 19 days in humans (Dennis et al., 2002). It is known to bind many of serum components and among other functions, albumin maintains plasma pH, it functions as carrier of metabolites and fatty acids, and serves as a major drug transport protein in plasma. Noncovalent association with albumin has been shown to extend the half-life of short lived proteins. The diminished ability of the PTP1B inhibitor to induce apoptosis in the presence of serum appeared to be due to its binding affinity to albumin. Many pharmaceutical agents are at least partially protein bound in the circulation and in many cases, only free drug can exert a pharmacological effect. It is therefore most likely the BSA in the serum is neutralizing the PTP1B inhibitor.

5. Discussion

5.1 Summary

In this work I have studied the role of PTP1B in the proliferation and survival of breast epithelial cells including the stem cell line D492. PTP1B gene and protein expression in various cell lines and primary cells indicate that PTP1B is overexpressed in D492, possibly as a result of insertional mutagenesis. PTP1B expression is high in epithelial cells in primary tissue and it is also expressed in D492 3D cultures. Treatment of the D492 cells with a specific PTP1B inhibitor induced apoptosis in the D492 cells, but did not induce cell death in MCF-7 cells treated with the same inhibitor concentration. My data further indicates that the cell death in D492 is primarily in dividing cells, possibly due to anoikis, i.e. programmed cell death when anchorage dependent cells detach from the surrounding matrix. I also provide evidence that PTP1B can activate Src, a well known oncogene which plays a role in anoikis. When evaluating the PTP1B expression in various cell lines a smaller form of the PTP1B protein appeared, consistent with a previously reported active subunit of PTP1B generated through cleavage by Calpain2 protease. Pharmacological inhibition on Calpain in D492 resulted in cell death suggesting a role for this protease, possibly through PTP1B.

5.2 PTP1B expression in D492 and possible effects from the retroviral insert

Retroviral insertional mutagenesis has been successfully utilized in transgenic or gene knockout mice to identify cooperating oncogenes. This approach have been used as a second hit in several studies aimed at determining the genes involved in T-cell lymphomagenesis (Jonkers & Berns, 1996). For example, oncogenes that synergize with a c-*myc* transgene were found after infection with Moloney murine leukemia virus (Mo-MuLV) (Uren et al., 2005). In 2002 Lund et al identified the PTPN1 gene as a common integration site in Cdkn2a deficient mice, which are viable but highly prone to tumor formation (Lund et al., 2002). Mice with Cdkn2a-/- background are deficient in the p53 and Rb pathways, which are two of the principal pathways involved in cell proliferation in human and mouse cells. Insertional mutagenesis at the PTPN1-locus in mice with the Cdkn2a-/- background was much more frequent than in other screens in mice with different genetic backgrounds. Interestingly in my study I used D492 cell line that was generated by retroviral insertion of E6 and E7 oncogenes. The E6 and E7 oncogenes bind and inactivate p53 and Rb making the genomic background of D492 similar to Cdkn2a-/- mice. This indicates that the oncogenicity of PTP1B largely depends on preexisting genetic background. These studies and the interesting location of the retroviral insert in D492 gives us clues about cooperating effects of PTP1B and RB/p53 and that PTP1B could play a role in the unique stem-cell properties of D492.

I assessed PTP1B expression in various cell lines. RT-PCR and western blot confirmed that D492 showed higher expression of PTP1B than the D382 cell line which was immortalized by the same manner as D492. These results indicate that the insert is enhancing PTP1B expression in D492. According to the RT-PCR, PTP1B mRNA expression is very high in D492 compared to MCF-7 but this difference is not translated in western blot where the cancer cell line also expresses high levels of PTP1B protein like D492.

In my study I also analyzed the expression of PTP1B in primary cells as well as PTP1B expression pattern in tissue sections and 3D cell culture. Results indicate that PTP1B is expressed primarily in the epithelial cells of the breast, but also to a much lower degree in stromal cells. PTP1B expression in tissue section correlates with PTP1B expression in western blot. In the 3D culture the most intense expression was at the lobular end of the structure, where cells are proliferating. This high expression of PTP1B indicates that the protein plays an important role in breast epithelium.

5.3 Functional role of PTP1B in D492

The fact that PTP1B expression is high in epithelial cells and D492 provides a unique tool to ask the question if this insertion plays an important role in D492 and if the PTP1B has a functional effect in the breast epithelium.

To test whether PTP1B plays a role in proliferation and survival of D492 cells I employed a commercially available PTP1B inhibitor from Calbiochem (Pnr.539741). The inhibitor is a cell-permeable allosteric inhibitor of PTP1B with an IC_{50}=8μM. It is highly unlikely that the PTP1B inhibitor inhibits other PTPs at this concentration (Sciences, 2005; Wiesmann et al., 2004; Zhang & Zhang, 2007), although off-target effects cannot be excluded. PTP1B inhibition resulted in dramatic effect on the survival of D492 cells by inducing extensive cell death, but no effect on MCF-7 cells was observed.

To demonstrate if D492 cells were dying through apoptosis or necrosis I carried out annexin V and PI staining followed by flow cytometry. The result indicate that the PTP1B inhibitor induces apoptosis in D492 in dose dependent manner. To further investigate the mechanism of D492 cell death I used CFSE staining. The CFSE staining suggests that the cells are undergoing apoptosis during cell division. Furthermore, there were striking changes in the cell morphology during PTP1B inhibitor treatment, the cells gained round-appearance with characteristics of reduced cell attachment and spreading. Collectively from these changes, I hypothesize that PTP1B inhibition in D492 cells could be affecting by anchorage -dependent growth of the D492 cells by inducing anoikis, a cell death where in vivo detachment from the ECM results in apoptopic cell death. Comparison of cell death induced by PTP1B inhibitor and camptothecin, a known apoptosis inhibitor, supports this hypothesis as the morphology of the cells is very different and D492 cell undergoing cell death induced by the PTP1B inhibitor show reported characteristic of anoikis (Hanker et al., 2009; Meredith et al., 1993).

5.4 PTP1B in signaling pathways

Anoikis resistance of carcinoma cells constitutes an essential event in tumor progression to metastases in most cancers of epithelial origin (Frisch & Screaton, 2001). The development of resistance to anchorage-independent growth is an essential event for carcinoma cells to metastasize because they must detach from their ECM, invade into vascular vessels and travel to distant organs. Therefore, studies of anoikis have gained increased attention as the characterization of the mechanisms mediating sensitivity to anoikis can provide important insights into epithelial homeostasis and carcinogenesis. The tyrosine kinase Src is one of the key molecules believed to play a critical role in the development of resistance against anoikis. Numerous studies have shown that Src overexpression significantly contributes to resistance of epithelial cell to anoikis (R. C. Ishizawar et al., 2007; Zhao et al., 2009).

PTP1B has been proposed as a primary regulator of Src activity (Bjorge et al., 2000). My findings suggest that when D492 cells are treated with the PTP1B inhibitor Src activity is diminished, identifying PTP1B as a positive regulator of Src. Assessment of protein expression by western blot showed that increased PTP1B inhibitor concentration enhanced phoshorylation on the tyrosine inhibitory phosphorylation site in Src. This finding was further confirmed by staining cells with a Src-phosphospecific (Y529) antibody where D492 cells treated with PTP1B inhibitor showed increased Y529-phosphorylated Src at the cell membrane as well as in the cytoplasm. This staining correlates with reported Src position in other studies (Arias-Romero et al., 2009).

We also provide evidence that suggest that calpain is important for D492 cell survival which is interesting in light of a recently published study from Cortesio et. al where they identify calpain 2 as an important regulator of PTP1B (Cortesio et al., 2008). Calpains proteases are known to cleave many adhesion associated and actin regulatory proteins, including PTP1B, and calpain 2 could therefore be a crucial factor in regulating PTP1B activity in D492 cells. In the 2008 study Cortesio et al described regulation of invadopodia in carcinoma cells which is frequently formed in metastatic cancer. They provided evidence that calpain 2 mediated protelysis and activation of PTP1B is an important mechanism by which calpain 2 regulates Src activity and invadopodia formation in breast cancer cells. Their findings furthermore indicated that PTP1B regulates invadopodia formation through the regulation of Src activity (Cortesio et al., 2008).

The small 40kDa band in the western blot suggest that the PTP1B proteolytic fragment with enhanced activity is present in the D492 cells. I therefore investigated if D492 cells were sensitive to calpain inhibition in the same manner as PTP1B inhibition. The survival curve clearly shows that D492 cells are sensitive to the calpain inhibitor at 100μM concentration. Annexin/PI assay was inconclusive with regard to determining whether apoptosis was involved.

Collectively, my findings implicate PTP1B, Src and calpain in the same pathway. However, this does not exclude other possible mechanisms through which PTP1B could be working.

5.5 PTP1B in cancer

In this study I evaluated PTP1B expression in primary cells from the breast as well as normal breast tissue. Our findings suggest that PTP1B expression is high in the epithelium which indicates an important functional role in these cells. It is also highly expressed in D492 and could play an important role in its unique properties. PTP1B has been implicated in many cellular processes, and for the last years it is been connected to cancer progression in several different models. Few studied have been done on PTP1B expression in breast cancer, but one was published in 1994 where PTP1B expression was associated with ErbB2 overexpression (Wiener et al., 1994). 29 breast cancer samples were examined of which 21 (72,2%) tumors overexpressed PTP1B. Elevated expression was detected at different developmental stages, indicating that PTP1B overexpression may be an early event in the molecular pathogenesis of breast cancer. They also revealed a possible correlation between PTP1B and ErrB2 expression where nearly all cases (11 out of 12) of ErbB2 overexpression also displayed PTP1B overexpression. Another group studied PTP1B expression pattern in skin squamous cell carcinoma (SSC) (Nanney et al., 1997). Their findings suggested that compared to normal skin, PTP1B expression was markedly increased in the invasive margins of SSCs indicating that PTP1B overexpression could be associated with an invasive cell population. Interestingly PTPN1, the gene encoding for PTP1B, is located within the 20q13 chromosomal region where amplification is frequent in breast cancers and has been associated with poor prognosis (Tanner et al., 1996).

The correlation between PTP1B expression and ErbB2 amplification in mammary carcinomas suggested that they may collaborate in tumorigenesis. Recently, two papers have shown a critical role for PTP1B in ErbB2 induced breast tumorigenesis. In both papers suppression of PTP1B signaling, either through gene knockout or pharmacological inhibition, was associated with markedly suppressed tumorigenesis in transgenic mice expressing activated forms of ErbB2. These mice developed tumors later, the tumors where smaller and less metastatic (Bentires-Alj & Neel, 2007; Julien et al., 2007; Tonks & Muthuswamy, 2007). Furthermore overexpression of PTP1B in mouse mammary gland lead to tumor formation suggesting that PTP1B is truly an oncogene. These two studies suggest that further studies should be carried out to see if patients with ErbB2-positvive breast cancer might benefit from pharmacological inhibition of PTP1B activity in combination with andi-ErbB2 therapies. Together, these results highlight not only the potential of PTP1B inhibitors in cancer, but also emphasize the need for better understanding of the role of PTP1B in breast cancer.

Many studies have shown that overexpression of ErbB2 can induce anoikis resistance in mammary epithelial cells (Reginato et al., 2003; Sheffield, 1998). In a recent study, pharmacological inhibition and Src overexpression was used to show that cellular motility and anchorage-independent growth promoted by the ErbB2/ErbB3 heterocomplex are dependent upon Src (R. C. Ishizawar et al., 2007). It has also been shown that pharmacological inhibition of Src causes reduction in ErbB2-mediated soft agar colony formation and motility (Sheffield, 1998). Most recently Haenssen et al presented results where they identified integrin α5 as a key mediator of Src and ErbB2-survial signaling in low adhesion states, which are necessary to block pro-anoikis mediators (Haenssen et al., 2010).

In this study I show that PTP1B inhibition increases inhibitory phosphorylation on Src, thus inhibiting its activity and furthermore the inhibition induces cell death in D492 cells. My results suggest that the cell death is apoptopic and that it occurs in dividing cells. I propose that the PTP1B inhibitor is inducing anoikis, related to Src activation status in these cells, and possibly ErbB2 activity. These speculations need further investigation to be confirmed.

Recently, some PTP1B inhibitors have been developed as potential therapeutics in the treatment of type 2 diabetes and obesity based on the observation that PTP1B has been identified as a major negative regulator of both insulin and leptin signaling. I have now shown that inhibition of PTP1B can affect the survival of cells with certain properties and others have shown that PTP1B inhibition can delay the onset of certain types of cancer. The link between oncogenic potential and PTP1B may lead to the use of PTP1B inhibition as a cancer therapeutic in certain types of cancer, such as colon and breast cancer.

5.6 Future perspectives

In this study I have demonstrated the effect of PTP1B inhibition on cell survival in the breast epithelial stem cell line D492 cells and explored possible mechanisms that are causing these effects. In the near future my focus will be on performing PTP1B gene-knockdown using siRNA approach to confirm our data obtained with pharmaceutical inhibition of PTP1B. Previously I have attempted to silence GFP and other genes with shRNA retroviral approach with no conclusive results yet. My focus now is to use siRNA oligos to silence PTP1B expression in D492 and in our lab there is ongoing work in setting up a lentiviral system to transfect cells. Lentiviruses have the unique ability to infect non-dividing cells with high transfection efficiency. Using such an alternative approach to suppress PTP1B would confirm that that the effects observed are not off target effects of the pharmacological inhibition. It is difficult to confirm anoikis because the phenomenon is not well defined and there is no one protein that defines cells that are going through anchorage dependent cell death, compared to classical apotposis. In this regard it can though be advantageous to use 3D models and use caspase staining to show apoptosis in cells within the epithelial colonies that are not able to form connection to the extracellular matrix. I also intend to study the effects of PTP1B inhibition/overexpression on colony formation in soft agar and culture cell under non-adherent conditions by seeding them on poly-HEMA-coated dishes. To look further into the molecular effects of calpain inhibition on D492 it would be interesting to look at the effects of the inhibition on PTP1B expression and to study further if the inhibitor is inducing apoptotic cell death. To follow up on the reported correlation between PTP1B and ErbB2 it would be interesting to look at the effects of PTP1B inhibition on ErbB2 activation and vice versa. Currently, ErbB2 overexpressing D492 cells are being generated in the lab. To look at the interactions between PTP1B and E6/E7 an interesting approach would be to express these genes under the same promoter in breast epithelial cell and thus try to replicate the immortalization of D492. I also intend to evaluate PTP1B expression in available breast tumor cancer material that has been

studied using whole genome microarray comparative genomic hybridization (CGH), whole genome microarray gene expression (RNA expression) and tissue microarray protein expression (TMA). The tumor cohort comprises 161 primary breast tumors collected at Landspitali-University Hospital. These studies are already ongoing, in collaboration with Rósa Barkardóttir, Inga Reynisdóttir and Bjarni Agnarsson at the department of cell biology and the department of pathology at Landspitali University Hospital.

6. Concluding remarks

There is mounting evidence that PTP1B plays an important role in breast cancer. D492, a breast epithelial cell line with stem cell properties, was established by transducition of the E6/E7 genes from HPV 16 using a retroviral approach. The insertion has been localized on chromosome 20q13.1, a region that is frequently amplified in breast cancer, 95kb upstream a gene coding for PTP1B. In this study I have shown that PTP1B expression is high in the stem cell line D492 compared to other epithelial cells. To study the role of PTP1B on the survival and proliferation in D492 I used a commercially available PTP1B inhibitor. PTP1B inhibition in D492 induced apoptosis, whereas the inhibitor had no effect on the survival of MCF-7. The cell death in D492 appears to be predominantly in dividing cells, possibly through anchorage-dependent cell death. This was supported by the difference of the morphology of cells dying from PTP1B inhibition or a classic apoptopic cell death. Furthermore, I present data supporting the finding that PTP1B is an activator of Src in D492 and that PTP1B exist in two forms in D492, both at its full length 50kDa and as a smaller 41 kDa more active subunit which is reported to be a proteolytic fragment of calpain 2 protease. Calpain inhibition induced cell death in D492, suggesting a role for calpain in D492, possibly through PTP1B.

In summary my data suggest an important role of PTP1B for cell survival in D492 and elucidates some of its role in signaling pathways. These data call further studies up to elucidate its role in breast morphogenesis and cancer.

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