Role of DARPP-32 in Breast Cancer Cell Signalling and Migration

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Abstract

Breast cancer is the most common form of cancer in women. While the prognosis for breast cancer patients without local or distal dissemination is relatively favorable, the prognosis is considerably worse once distal metastasis has been established. It is therefore imperative to identify molecular targets and develop novel anti-metastatic therapies that will stop, reduce or delay the spread and growth of breast cancer metastasis.Low expression of Wnt-5a in a primary breast tumor is associated with shorter recurrence free survival, suggesting that Wnt-5a acts to restrict breast cancer metastasis. Moreover, Wnt-5a is known to inhibit migration of breast epithelial cells in culture and expression of Wnt-5a potentiates activation of the receptor tyrosine kinase DDR1, a collagen receptor implicated in cell adhesion and migration.

This thesis describes the identification of DARPP-32 as a novel interaction partner to DDR1. We demonstrate that DARPP-32 inhibits MCF-7 cell migration and that this effect requires phosphorylation of threonine-34, an event catalyzed by protein kinase A (PKA) and strongly induced by detachment of cells from the culture substrate. DARPP-32 mediated inhibition of migration proved to be dependent on DDR1 expression, consolidating a functional relevance of the interaction between DARPP-32 and DDR1. In addition, we found that Wnt-5a could directly trigger a cAMP response that resulted in phosphorylation of DARPP-32 and stimulation with Wnt-5a was necessary for DARPP-32 mediated inhibition of cell migration in wound healing assay.

The anti-migratory effects of Wnt-5a and DARPP-32 were reduced by dominant negative-CREB, which suggests that CREB plays a functional role in this signalling mechanism. Finally, we found that phospho-DARPP-32 inhibited the activity of the focal adhesion kinase (FAK) in MCF-7 breast cancer cells, and that MCF-7 cells expressing phospho-DARPP-32 displayed less filopodia formation.
These results suggest that DARPP-32 restricts the migration of breast epithelial cells via both a transcription dependent mechanism that involves CREB and a transcription independent mechanism affecting FAK. Pharmacological activation of this pathway may constitute a novel way of limiting breast cancer metastasis.

Key words: DARPP-32, Wnt-5a, DDR1, Migration, Breast Cancer, Protein kinase A

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Here is Edward Bear, coming downstairs now,
bump, bump, bump, on the back of his head,
behind Christopher Robin.

It is, as far as he knows, the only way of coming downstairs,
but sometimes he feels that there really is another way, if only
he could stop bumping for a moment and think of it.

And then he feels that perhaps there isn't.

from “Winnie the Pooh” by AA Milne

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Cover: MCF-7 cells transfected with T34A-DARPP-32 expression plasmid and stained for F-actin (green), T34A-DARPP-32 (red) and nucleus (blue).

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II: **Christian Hansen**, Anders Tengholm, Oleg Dyachok, Wolfgang F. Vogel, Angus C. Nairn, Paul Greengard and Tommy Andersson: Wnt-5a-induced phosphorylation of DARPP-32 inhibits breast cancer cell migration in a CREB dependent manner. *Manuscript submitted.*

III: **Christian Hansen** and Tommy Andersson:DARPP-32 phosphorylation on residue Thr34 leads to suppression of focal adhesion kinase activity in breast cancer cells. *Manuscript.*

Abbreviations

aa	Aminoacid residue
AC	Adenylyl cyclase
AKAP	A kinase anchoring protein
ATP	Adenosine tri phosphate
APC	Adenomatous polyposis coli
Bcl-2	B-cell Lymphoma 2
BRCA	Breast cancer susceptibility gene
cAMP	cyclic adenosine monophosphate
CaMKII	Calmodulin-dependent protein kinase II
CaMKIV	Calmodulin-dependent protein kinase IV
Caspase	Cysteine aspartyl specific protease
CBP	CREB binding protein
CDK:	Cyclin dependent kinase
CRE	cAMP responsive element
CREB	CRE binding protein
DARPP-32	Dopamine and cyclic AMP dependent phospho-protein of 32 kDa
D1	Dopamine receptor type-1
D2	Dopamine receptor type-2
DDR1	Discoidin domain receptor 1
DDR2	Discoidin domain receptor 2
DNCREB	Dominant negative CREB
ECM	Extra cellular matrix
EMT	Epithelial-mesenchymal transition
ER	Estrogen receptor
FADD	Fas associated death domian
FAK	Focal adhesion kinase
GEF	Guanine nucleotide exchange factor
GSK-3β	Glycogen synthase kinase 3β

Abbreviations

GTP	Guanosine tri phosphate
HER2	Human epidermal growth factor 2
I-1	Protein phosphatase inhibitor 1
I-2	Protein phosphatase inhibitor 2
KO	Knock out
LEF	Lymphocyte-enhancing factor
LRP	Low density lipoprotein receptor related protein
MAPK:	Mitogen activated protein kinase
MMP	Matrix metalloproteinase
Na/K	Sodium/potassium,
NIPP-1	Nuclear inhibitor of protein phosphatase 1
PBS	Phosphate buffered saline
PDE	Phosphodiesterase
PDGF	Platelet derived growth factor
PI3 kinase	Phosphatidyl inositol 3 kinase
PKA	Protein kinase A
PKR	Protein kinase R
PP1	Protein phosphatase 1
PP2A	Protein phosphatase 2A
PTB domain	Phopho tyrosine binding domain
RB	Retinoblastoma protein
Ror2	Receptor tyrosine kinase like orphan receptor
Ryk	Receptor related to tyrosine kinase
Ser	Serine
SH2 domian	Src homology 2 domain
TCF	T cell factor
Thr	Threonine
Tyr	Tyrosine
VEGF	Vascular endothelial growth factor
Wt	Wild type

Introduction

One in ten women develop breast cancer during their lifetime, and hence the disease represents the most common form of cancer in women. Approximately 30% of all women diagnosed with breast cancer succumb to the malignancy, and, as in most cancers, death is not caused by growth of the primary tumor, but is due to metastasis.

The majority of breast cancers arise from the epithelial cells that line the ducts and the lobules of the breast. In order for transformed breast epithelial cells to metastasize from the primary tumour, they need to change their migratory behaviour. Accordingly, learning more about the signalling pathways that restrict the migration of breast epithelial cells, could prove to be an important tool for development of new approaches to anti-metastatic treatment of breast cancer.

The extracellular protein Wnt-5a is known to inhibit migration of breast epithelial cells and absence of Wnt-5a expression in primary breast tumours is associated with increased probability of dying from the cancer. Expression of Wnt-5a potentiates activation of the receptor tyrosine kinase DDR1, a collagen receptor implicated in cell adhesion and migration, and in a screening for DDR1 interaction partners expressed in the human breast, I isolated the phospho-protein DARPP-32.

In the present study I have explored the role of the phospho-protein DARPP-32 in restricting breast cancer cell migration both downstream and independent of Wnt-5a.

General background

The breast

Development

The human breast is a dynamic organ that does not go through all its developmental stages until the occurrence of pregnancy and childbirth. Up to the onset of puberty, the structure of the breast is much the same in the males and females and their internal structure is similar – a collection of ducts emptying into the nipple. During puberty, the female breast responds to release of the female sex hormones estrogen and progesterone, which stimulate increased deposition of adipose tissue within the gland and elongation and branching of the ducts into a more extensive network. However, the breast does not become fully developed until the events of pregnancy and lactation. When lactation ceases, the breast involutes to a state resembling that seen in a nulliparous woman. At menopause the secretion of female hormones diminishes, which leads to replacement of connective tissue with adipose tissue and an accompanying decrease in the size of the lobules.

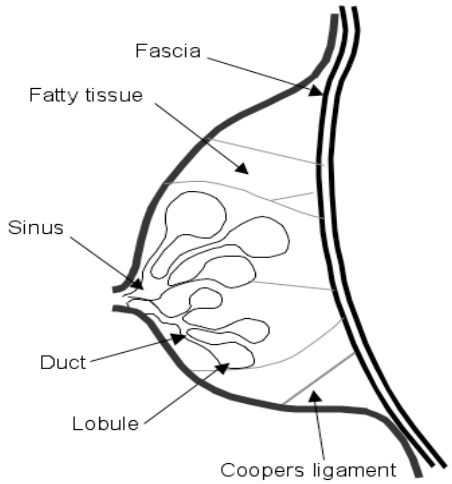

Figure 1: Anatomy of the human breast

Anatomy

The breast is made up of secretory glandular tissue surrounded by adipose tissue (Figure 1). The glandular tissue consists of 15 to 20 lobes with varying numbers of ducts and lobules surrounded by connective tissue. Each lobule is connected to a duct, several of which converge to form a lactiferous sinus or milk chamber. These sinuses

empty into the nipple, which has a number of duct openings. Beneath the tissues of the breast lie the muscles of the chest wall, and between the two is a layer of connective tissue. Fibrous (Cooper's) ligaments support the mammary gland and hold it against the chest.

Breast Cancer:

Breast cancer is the most frequent cancer form found in women. 1 in 8 women in the United States and 1 in 10 in Sweden are diagnosed with breast cancer during their lifetime, and an estimated 30% of those women today die of the disease.

Classification & prognostic factors:

The prognosis of patients with newly diagnosed breast cancer is largely determined by the size of the primary tumor and whether there are metastases in the lymph nodes. Tumor staging is employed as a tool to evaluate the progression of a patient's cancer. In 1992, use of the TNM classification system for that purpose, was accepted by American Joint Committee on Cancer (AJCC) and the International Union Against Cancer (UICC). This classification takes into account the size of the primary tumor (T), the presence of lymph node metastasis (N) and distant metastases (M). According to these parameters the tumors are assigned to stage I, II, III and IV. Stage I tumors have no metastasis and a primary tumor of less than 2 cm in diameter whereas stage IV tumors have widespread metastasis (1).

Histological grading of the primary breast tumor is another valuable tool used to assess the aggressiveness of the cancer. This method is based on the morphology of the primary tumor, and the most widely used system was first established by Bloom and Richardson and was later modified by Elston and Ellis (2,3). This classification system takes into accout the three morphological features of tubule formation, nuclear polymorphism, and mitotic count, each of which is givn a score of 1-3. The overall tumor grade is determined by the sum of the three scores: grade I: Well differentiated (3-5), grade II moderately differentiated (6-7) and grade III poorly differentiated (8-9). In general, less differentiated tumors are associated with a smaller chance of survival. Important prognostic factors in breast cancer include the estrogen receptor (ER), progesterone receptor and human epidermal growth factor receptor 2 (HER-2). Furthermore, evaluation of expression of these markers in breast cancer is important for the choice of treatment for individual patients (see section headed "Treatment").

Risk factors

Most cases of breast cancer (approx. 90%) arise sporadically and the disease almost exclusively hits females, although a small proportion of males also do contract the disease, and the risk of getting breast cancer increases with age. Ethnicity is also a contributing factor, as illustrated by the observation that white American women are at greater risk of breast cancer compared to Afro-American women, whereas Afro-American women are more likely to die of the disease if they get it.

Obesity, high alcohol consumption, lack of exercise, having no children or a first child after the age of 30, and postmenopausal hormone therapy are other factors that increase the risk of getting breast cancer (American Cancer Society, www.cancer.org).

The remaining proportion of the cases (10%) represents hereditary breast cancer. Genetic factors that increase the risk of the disease are in chiefly due to mutations in the BRCA1 and BRCA2 genes, although malignancy-inducing aberrations can also occur in the genes encoding p53, Atm, CHEK2 and PTEN are also found (4,5).

Treatment

The obvious first choice of treatment is surgical removal of the primary breast tumor in conjunction with postoperative radiotherapy to reduce the risk of recurrence.

An additional and important option in treatment of breast cancer is hormonal manipulation. This was first reported more than 100 years ago, in 1882, when the importance of ovarian function in the growth of breast tumors was recognized by Thomas William Nunn, who described the regression of breast cancer in a woman who had gone through menopause (6). Fourteen years later, Dr. George Beatson was the first to successfully treat advanced breast cancer, by removing the ovaries of a patient (7). Importantly, Beatson also concluded that removal of the ovaries might induce involution similar to that seen in normal breast tissue and thereby decrease proliferation of breast cancer cells (7). However, when oophorectomy (surgical removal of one or both ovaries) set up as a treatment method, a tumor response was seen in less than half of the patients who underwent such surgery and the use of the procedure was further discouraged by the high mortality it led to at that time (8). However, today treatment of breast cancer by ablation of the ovaries, is sometimes selected, in particular for post-menopausal women (9).

In 1967, the estrogen receptor was isolated and characterized, which facilitated prediction of what patients would be most responsive to hormonal manipulation (10). A hallmark in hormone treatment of breast cancer arose with the discovery of tamoxifen, a compound that was first marketed in the United Kingdom as a fertility drug. Tamoxifen binds to the ER and prevents the receptor from forming an active complex with its natural ligand estrogen (11). It is now known that tamoxifen has both agonistic and antagonistic effects on ER function in different tissues. In breast cancer cells, tamoxifen antagonizes the actions of estrogen which leads to G1 cell cycle arrest and apoptosis (12). A side effect of tamoxifen is its agonistic influence on the uterus which results in endometrial thickening and an increased risk of endometrial cancer (13). Clinical trials with tamoxifen on breast cancer patients revealed a 49 percent reduction of fatal outcome in one of the largest clinical trials ever made (14), establishing tamoxifen as one of the most successful anti- breast cancer drugs to date. Unfortunately, tamoxifen has a major drawback that is logically related to its function: It is effective only in women with ER positive breast cancer, which represent 70 % of all cases of the disease. The Herceptin antibody (trastuzumab), which blocks the activity of the HER-2 receptor, is another success story in hormonal treatment of breast cancer, and it is often used in combination with chemotherapy (14). However, this

treatment is only effective in the 20-30 % of breast cancer patients that overexpress the HER2 receptor (15).

Tumorigenesis

Several events must transpire to allow transformation of a normal cell into a cancer cell. Upregulation of proliferative pathways is crucial in this process, as is blocking of the limitation on the number of cell cycles that can occur, which is normally regulated by telomere shortening. In addition to these alterations, which enable uncontrolled grow, the cell must be able to evade the apoptotic system, by upregulating the activity of survival proteins and/or by deactivating of pro-apoptotic proteins.

Proliferation

The eukaryotic cell cycle consists of the following phases (Figure 2): G1 phase is the phase between the "birth" of a cell after mitosis and the initiation of DNA synthesis. At this stage the cell can either leave the cell cycle and enter a non-proliferative G0 phase or continue into the S phase, during which the genome is duplicated. The period between the S phase and the mitosis marks the G2 phase. The last step in the cell cycle is the M-phase entailing mitosis, where the parental cell and its double set of chromosomes segregates into two daughter cells in G1 phase. Entry into and exit from these phases are controlled by a number of proteins, particularly the cyclin dependent kinases (CDKs), which are serine/threonine kinases that have to be in complex with their activating partners, the cyclins, in order to exert their effects on cell proliferation. For example, the activity of CDK1 is required for the G2/M phase transition, and influence of CDK2 is needed for the G1/S phase transition. In addition, RB-induced regulation of the E2F transcription factor is particular important for resricting cells in G1phase from entering S phase, and Rb is frequently inactivated in breast cancer (16). Signalling pathways that lead to activation of cell cycle effector proteins, such as the CDKs, promote proliferation and in many cases they also mediate cell survival. One such pathway involves the oncogene Ras, which is frequently mutated in cancer. Activation of the Ras/Raf/MAPK kinase pathway by proteins such as growth factor activated receptor tyrosine kinases, leads to upregulation of cyclin D, which in turn stimulates cell cycle progression (17,18). Other proteins that are know to induce proliferation such as Src and beta-catenin, also stimulate expression of cyclin D (17,19). Cyclin D1 is overexpressed in a substantial amount of breast cancers (20), demonstrating the importance of finetuning expression of this protein in order to avoid cell transformation resulting in malignant disease.

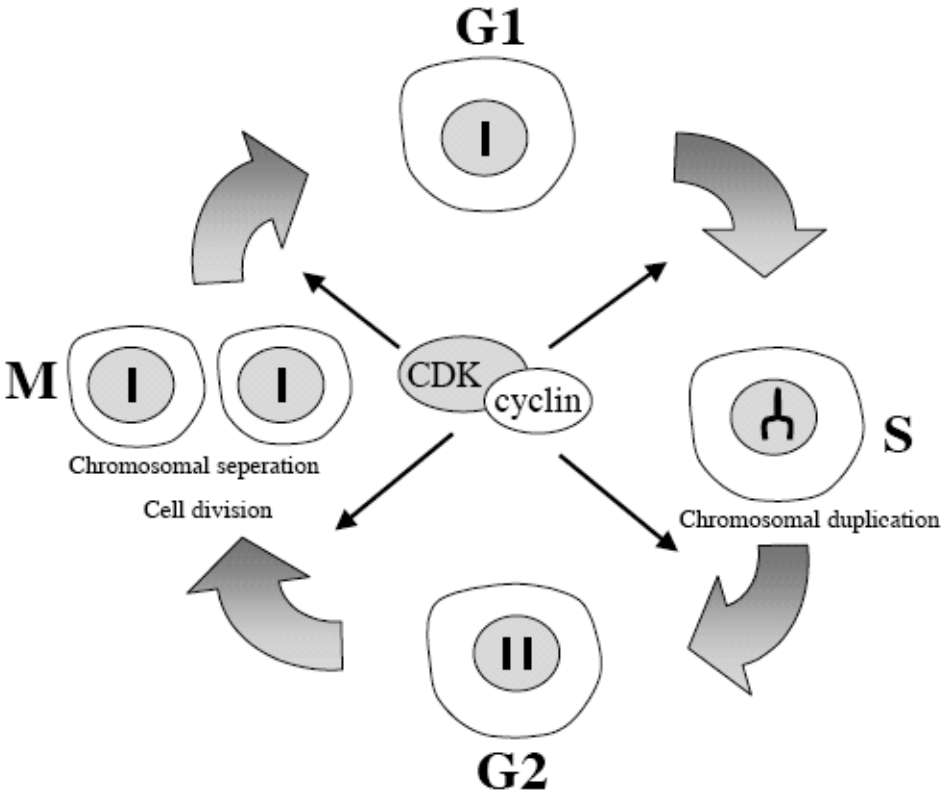

Figure 2: The cell cycle.

Apoptosis

Apoptosis is a genetically controlled mode of cell death that is evolutionarily conserved and beneficial for multi-cellular organisms. The word "apoptosis" comes from the Greek word meaning the "dropping off" of petals or leafs from plants or trees. This is a quite fitting term because when the leaf dies and fall of a tree or plant it leaves the rest of the organism unharmed. Analogously, in the human body, an apoptotic cell leaves the surrounding cells unharmed. In contrast, cells that succumb to the more spontaneous cell death necrosis, release substantial amounts of cytotoxic agents that damage the surrounding cells and tissue.

The mechanism of apoptosis is required during development and for the integrity of the immune system throughout the lifespan of a human being. In addition, apoptosis serves as a protection system that induces "suicide" of cells that no longer carry out their intended function, for one reason or another. This effect is highly relevant as a protection against potential cancer cells, which must evade that mode of death in order to undergo malignant transformation. Therefore, an effective apoptotic system is the body's own system for protection against cancer. If cells that are about to transform

were not removed through apoptotic clearance, the rate of cancer would be many fold higher, because the genes in all cells frequently undergo spontaneously and environmentally induced mutations.

Even though the first publication on the subject apoptosis dates back to 1972 (21), this mechanism was not widely accepted before many years later. A breakthrough came from the finding of Bcl-2 as an anti-apoptotic gene (22). In addition, significant progress on the understanding of apoptosis was obtained from the genetic studies of nematode *Caenorhabditis elegans*. Of the 1090 somatic cells that are formed during development, 131 die through apoptosis and the simplicity of the organism facilitated the discovery of 3 genes named CED-3, -4 and -9, centrally involved in apoptosis (23). The mammalian orthologues of these 3 proteins were found to be caspase-3, apaf-1 and bcl-2 respectively.

The family of proteases called caspases plays a central role in the execution of apoptosis. The caspases can be divided into two groups: The initiator caspases and the effector caspases. Caspases are expressed as inactive pro-forms, which are activated by clevage. A prominent example of an initiator caspase is caspase-8. This caspase trimerizes upon association with the Fas-associated death domain (FADD) in response to stimulation with Fas-ligand (24). This leads to autocleavage and activation of Caspase-8. Caspase-induced cleavage of downstream proteins causes release of cytochrome c from the mitochondria and formation of the apoptosome, which consists of apaf-1, cytochrome c and caspase 9. That event triggers activation of the effector caspases 3,6 and 7, which subsequently results in cleavage of a range of proteins and ultimately cell death that is characterized by nuclear condensation, DNA laddering and membrane blebbing. Apoptosis can be induced by both extrinsic and intrinsic stimuli. Examples of the former are Fas and TNF, and examples of the latter are oncogenic stress, the unfolded protein response and UV irradiation (24). A key molecule in intrinsically stimulated apoptosis is the tumor suppressor gene p53, which is very frequently mutated in cancer cells (5). This gene has been referred to as the “guardian” of the cell, because it can induce cell cycle arrest and apoptosis in response to oncogenic stress (25). One way a transformed cell can evade apoptosis is through inactivation of important pro-apoptotic genes, and another route involves upregulation of survival genes. Prominent examples of such proteins are Bcl-2, which was originally found to be overexpressed in B cell lymphomas, and the IAP’s, inhibitors of the effector caspases (26). Survivin is a member of the IAP family that is particularly often overexpressed in cancer. This protein is produced ubiquitously during development, whereas there is virtually no expression in adult tissues, and hence it has attracted significant attention as a promising target for drug-based treatment of cancer (27).

Metastasis

In general, cancer cell metastasis can be divided into the following major events: 1. Detachment of cells from the primary tumor and breakdown of the ECM, 2. intravasation into the bloodstream, 3. transportation in the circulation, 4. extravasation and 5. establishment of a new tumor. For a cancer cell to achieve these steps, it has to adapt its adhesion and migration capacities, and must be able to induce angiogenesis

once it invades a new tissue. The basic aspects of these cellular mechanisms are discussed here.

Adhesion:

Normal breast epithelial are situated on a basement membrane that consists of a number of different ECM proteins. The main constituents of the basement membrane are fibronectin, collagen type IV, laminin, entactin and heparan sulphate. Of special interest in relation to the present study is collagen, which is the most abundant protein in the human body and also serves as the ligand for the receptor tyrosine kinase DDR1. Collagens form triple helixes but vary with regard to their overall structure. Collagen I forms long fibrils, and is the most abundant type of collagen in the connective tissue, whereas collagen IV forms more sheet like structures in the basement membrane.

The integrins constitute a family of proteins that are of major importance for the cell in sensing the ECM, and they play an essential role in cell adhesion and migration. These molecules exist as heterodimers of an α and a β subunit, both of which subunits have a large glycosylated extracellular domain and a short cytoplasmic tail. In mammals there are 18 different α and 8 different β subunits that gives rise to at least 24 different heterodimers, each of which can bind a specific set of ECM ligands (28). Once integrins are activated by ECM molecules, their cytoplasmic tails sequester a range of proteins to form focal adhesion complexes, at the ECM/integrin interaction junctions. The establishment of these complexes is highly important for both cell adhesion and migration. The actin cytoskeleton-binding proteins Tensin and Talin bind the cytoplasmic tail of the integrins and thereby link the cytoskeleton to the focal adhesions (28). The discoidin domain receptors DDR1 and DDR2, also play critical roles in cell adhesion and migration. However, relatively little is known about how they actually participate (see section headed “DDR1”).

Other groups of cell surface receptors are involved in for cell-cell adhesion and some of the most important proteins in that context are the Cadherins (designated E-, N- and H-) and the selectins (denoted E-, L- and P-). These molecules are important, not only for cell-cell adhesion, but also for intravasation and extravasation, features that are important for tumor cell metastasis. In addition, during epithelial-mesenchymal transition (EMT), which is an essential event in some developmental processes, there is loss of cell adhesion, and expression of E-cadherin is repressed resulting in increased cell motility. These phenomena are also characteristics of the initiation of cancer metastasis. (29)

Migration:

Migration is a cyclical process in which a cell extends protrusions at its front and retracts its trailing end. Upon encountering chemotactic molecules the cell organizes, into a “front” and a “back”, also called the leading edge and the rear of the cell, respectively. Moving forward requires remodelling of the cytoskeleton. In order for the cell to migrate, protrusions are formed, in which there is a constant polymerization and remodelling of actin fibers. The two main forms of protrusion are the broad, flat

sheetlike lamellipodia and the thin, cylindrical needle like filopodia. The actin filaments are grouped into bundles in filopodia, whereas they are more cross-linked into a lattice-like meshwork in lammelipodia. Both types of protrusions need proteins such as arp 2/3, WAVE, and WASP in the actin remodelling process. However, filopodia and lamelliodia differ markedly in that they require different RhoGTPases in their formation: Cdc42 in the former and RAC in the latter (30).

Whereas the formation of protrusions that are needed to form contacts in front of the cell body are orchestrated by one set of proteins, other proteins participate in the relocating the cell body forward. In non-muscle cells, a major important protein in this process is Myosin II. Myosins have the unique ability to move along the actin filaments to couple hydrolysis of ATP to conformational changes, and hence they function a mechano-chemical enzymes that converts chemical energy into mechanical energy (31). The formation of new focal contacts is a fundamental part of cell migration that occurs primarily at the leading edge of a cell, whereas disassembly of these protein complexes mainly takes place at the rear of the cell. Stabilization of focal contacts increases adhesion and impairs the rate of migration, whereas a reduction in adhesion, to a certain extent, propels migration (32). FAK is a key protein in the regulation of focal adhesion turnover and thereby also in migration (see section headed "Focal adhesion kinase"). At the rear of a moving cell, the calcium regulated protein calpain plays an important role in disassembly of focal adhesion by cleaving focal adhesion-associated proteins and attachments to the cytoskeleton, important for the retraction of the trailing edge (33). In addition, Rho-mediated activation of ROCK and subsequent phosphorylation of myosin light chain leading to activation of myosin II is of major importance for retraction of the trailing edge (30).

Angiogenesis:

Like most solid tumors, breast tumors that are larger than a few millimeters in diameter require the formation of new blood vessels (angiogenesis) in order to grow. Besides helping the tumor fulfil its metabolic requirements, angiogenesis also provides new routes for metastasis. One the most important factors for angiogenesis is the vascular endothelial growth factor (VEGF). This protein is highly upregulated in some breast tumors (34). The upregulation of VEGF can be mediated by a number of different factors such as HIF-1α (35) and estrogen (36). In addition, HER2 overexpression in breast cancer cells lead to upregulation of VEGF, whereas a neutralizing antibody directed against HER2 lowers the level of VEGF in these cells (37). Inhibitors of VEGF or the VEGF receptor are obviously interesting targets for treatment of breast cancer patients, but so far no suitable drug has been made. The monoclonal antibody avastin directed against VEGF was used for clinical trial, but without any improved diagnosis for the patients.

Specific background

Cyclic AMP

A major function of second messenger cyclic AMP (cAMP) is to trigger Protein Kinase A (PKA)-activity (38), although cAMP can also stimulate activity of the GEF-protein EPAC (39) and the cAMP gated ionchannel CNG (40). Production of cAMP is in generally triggered by binding of a hormone or a neurotransmitter to a surface receptor leading to activation adenylyl cyclase (AC), via the surface receptor-coupled heteromeric G-proteins.

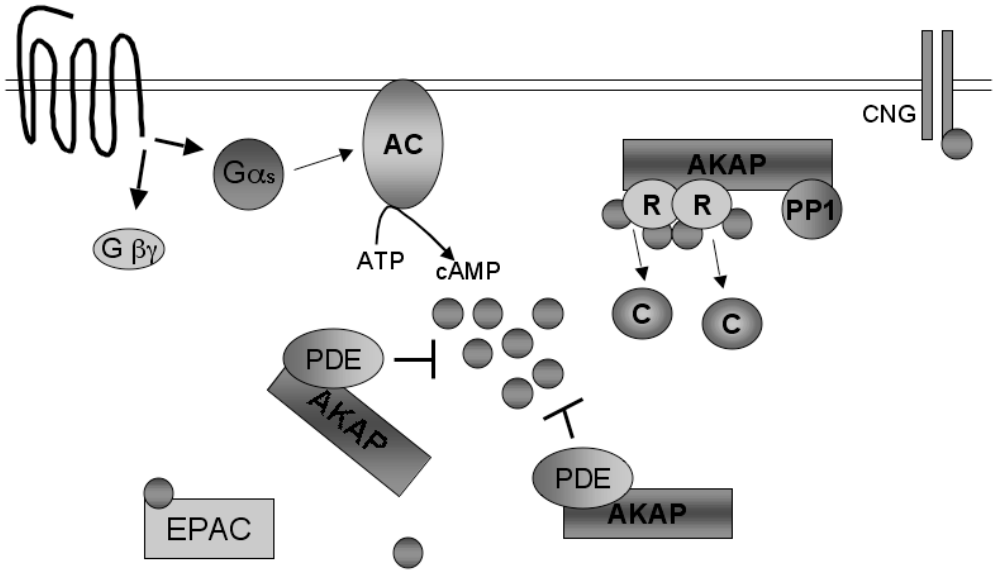

Figure 3. Essentials of cAMP signaling. Adapted from (41). R and C denote the regulatory and catalytic subunits of PKA, respectively. Binding of cAMP to the R-subunits leads to the release and catalytic activity of the C-subunits. Inhibitory arrows indicate how the localization of PDE and processing of cAMP lead to cAMP concentration gradients in the cell.

There are at least 9 different membrane bound isoforms of AC and one soluble form in humans. Interestingly, AC isoform 1, 3 and 8 can be triggered by a rise in cytosolic calcium concentration via calmodulin activity, whereas isoform 5 and 6 are inhibited by calcium (42). A subgroup of the Phosphodiesterases (PDE)s, in particular the PDE4 isoforms, are responsible for inducing the hydrolysis that converts cAMP into 5' AMP (43). These enzymes are important for both the downregulation and the specific subcellular localization of cAMP signalling. The subcellular localization of PDE's is frequently regulated by binding to A kinase anchoring proteins (AKAPs see next

section), which thereby keeps PKA activity and the deactivating PDE enzymes, in close proximity to each other (43).

Protein kinase A

Protein kinase A (PKA) is a serine/threonine kinase, with a central role in the first signalling cascade ever described (38). This heterotetrameric enzyme consists of two catalytic and two regulatory subunits. Upon binding of cAMP, the catalytic subunits are released form the regulatory subunits and kinase activity is exerted. PKA has targets in almost every part of the cell including the plasmamembrane, cytoplasm, nucleus, mitochondria and the cytoskeletal network (42)

Members of the AKAP family are structurally very diverse, but they all have a PKA regulatory subunit binding domain (41), and they bind to PKA to regulate the subcellular concentrations the kinase (41). Several AKAPs such as WAVE-1, -2, -3 and AKAP-Lbc are involved in regulation of the actin cytoskeleton (42), which could be important for the action of PKA in cell migration. Furthermore, it is intriguing that other AKAPs, such as Yotiao (44), AKAP220 (45) and AKAP350 (46) can bind to both PKA and PP1. Since PKA and PP1 play important roles for the function of DARPP-32 (see section headed "DARPP-32") it is tempting to believe that some of these AKAPs could participate in regulation of DARPP-32 activity, by bringing together PKA and PP1.

PKA phosphorylates a large number of different targets (42), which in some cases leads to an anti-migratory response and in other situations to a pro-migratory reaction. Therefore, a possible negative or positive effect on cell migration induced by inhibition of PKA, is very specific to the type of cell in question and depends on the balance of expression levels and activities of pro- and anti- migratory targets.

PKA has been shown by Howe and Juliano to be activated by cell detachment in NIH3T3 fibroblasts (47). Notably, the authors found that PKA phosphorylated PAK *in vitro* and *in vivo*, which resulted in attenuation of cdc42 activity, and they observed that inhibition of PKA activity increased activity of FAK (47). In addition, the same research group showed that PKA activity inhibits migration of NIH3T3 fibroblasts (48). Negative effects of PKA on cell migration have also been reported for endothelial cells (49). On the other hand PKA activity is required for the activation of Rac and Cdc42 that leads to lamellipodia and filopodia formation, in several other cell systems (50,51), which is in contrast to the impact of PKA on Cdc42 activity in NIH3T3 cells.

The transcription factor CREB is a downstream target of PKA (see section headed "CREB"), and both of these molecules have important roles in embryogenesis (52). Recently, an interesting connection was found between PKA/CREB and Wnt signalling as PKA was shown to be required for Wnt mediated myogenesis, and downstream activation of CREB (53).

Protein Phosphatase-1

While the humane genome encodes approximately equal number number of tyrosine kinases and tyrosine phosphatases (approximately a 100 of each), there is a huge

difference in the number of Ser/Thr kinases (approximately 400) and Ser/Thr phosphatases (approximately 25). Protein phosphatase 1 (PP1) is a ubiquitously expressed Ser/Thr phosphatase, that can dephosphorylate a wide range of cellular targets (54). Mammals have 3 different PP1 genes, which encode the isoforms PP1α, PP1γ and PP1β/δ. In order to finetune the activity of PP1, it is regulated by a number of different inhibitors (see next sections) and other regulatory subunits, like the AKAPs, regulate subcellular localization of PP1. Moreover, overexpression of the nuclear inhibitor of PP1, NIPP1, induces nuclear translocation of PP1, which demonstrates that this PP1 inhibitor also regulates PP1 localization (55). Indeed, it is believed that PP1 never exists in the cell as a monomer in the cell, but that it is always associated with some sort of regulatory subunit. PP1 is involved in the regulation of a wide range of processes, including migration, cell cycle, apoptosis, gene transcription, translation, ionchannel activity, glycogen and lipid metabolism (54). It would be too comprehensive and outside the scope of this thesis to thoroughly describe the role of PP1 in all these processes, so I have chosen to focus on studies that I find most relevant to my work, namely cell migration, gene transcription and translation.

PP1 in cell migration:

Modulation of the actin-cytoskeleton is of major importance for cell motility and migration. The neurabins, Neurabin-I and Spinophilin (also named Neurabin-II) are proteins with an N-terminal F-actin cross-linking domain (56). Neurabin-I is highly enriched in the brain (57) whereas Spinophilin is expressed more ubiquitous (58). The F-actin cross-linking activity of both proteins is regulated by PP1-mediated dephosphorylation. PKA phosphorylates spinophilin at Ser94 (59), which can in turn be reversed by PP1mediated dephosphorylation (60). Thus, phosphorylation of DARPP-32 at Thr34 enhances Ser94 phosphorylation by PKA (60). Overexpression of Neurabin-I in kidney cells induced filopodia formation (61). In contrast, neurons from spinophilin knock out mice exhibit a large increase in filopodia formation (62). Doublecortin is a protein involved in neuron migration (63), and it interacts with spinophilin, whereupon spinophilin recruits PP1 to the spinophilin/doublecortin complex, which leads to dephosphorylation of doublecortin by PP1 (64). However, it is not known whether spinophilin and doublecortin, or some other proteins with similar functions are expressed in breast epithelial cells to allow PP1 regulation of cell migration in the same manner.

Another role of PP1 in cell migration is to localize and regulate of phospho-activity at the focal adhesions. PP1α interacts with Tensin (65), a protein that is found in focal adhesions where its task is to anchor the actin filaments to the focal adhesions (66). Moreover, tensin serves as a positive regulator of cell migration (67), suggesting that PP1α mediated dephosphorylation of tensin could play a role in cell migration, although this remains to be demonstrated. Furthermore, PP1-mediated dephosphorylation of Ser722 in FAK, leads to increase in FAK activity resulting in increased cell migration (68).

PP1 in gene transcription and translation:

Transcriptional activation by RNA polymerase II relies on phosphorylation of the C-terminal domain of the largest subunits of the polymerase by Cdk7 and Cdk9. Although dephosphorylation of these sites has been ascribed to the phosphatase FCP1, it has recently been shown that PP1 is also involved in that process (69), which suggests a broader role for PP1 in gene transcription. Another role of PP1 is to suppress gene transcription mediated by the transcription factor CREB, which is achieved through dephosphorylation of Ser133 (70). This is described in greater detail in the "CREB" section.

eIF2α is an initiation factor for translation and act as a general repressor of the translation of stress responsive genes (71). eIF2α is activated by phosphorylation mediated by protein kinase R (PKR). PP1 can both dephosphorylate eIF2α directly (72) or it can dephosphorylate PKR, leading to attenuation of PKR activity (73), suggesting that PP1 can block eIF2α activity to upregulate translation of stress responsive genes. Interestingly, in this context, it has been shown that PKA phosphorylation of the PP1 inhibitor I-1, and subsequent inhibition of PP1 in complex with GADD34 mediated by I-1, is responsible for PKA-induced inhibition of translation (74).

Protein phosphatase-1 inhibitors

Inhibitor-1, Inhibitor-2 and DARPP-32 exhibit a high degree of sequence similarity. In addition, Inhibitor-1 and DARPP-32 in particular have several functions in common, which will be described in more detail in the next sections. In light of this and since very little is known about the actions of DARPP-32 outside the neuronal system, it would no doubt be highly valuable to gain an understanding of how these other PP1-inhibitors work. The list of PP1 inhibitors is long and also includes proteins such as NIPP-1, RIPP-1, KEPI, CPI-17 and GBPI, but these are not discussed here.

Inhibitor-1

Inhibitor-I (I-1) was originally identified in rabbit skeletal muscle (75). It is expressed in a wide array of tissues (76), and is converted into a potent and specific inhibitor of PP1 when it is phosphorylated on Thr35 by PKA (77). The protein shares the following functional similarities with DARPP-32: They share high sequence similarity, especially in the N-terminus (78), are phosphorylated by PKA at similar positions and are both PP1 inhibitors. Since I-1 was identified before DARPP-32, it most definitely facilitated the process of understanding how DARPP-32 functions. I-1 is more widely expressed than DARPP-32, and it is plausible that a lot of functions are similar for the two proteins. In line with this assumption, I-1 knock out mice displayed impaired locomotor activity in response to cocaine similar to DARPP-32 knockout mice (79). However, the impaired response to cocaine was not more pronounced in mice lacking both genes (79). Furthermore, conditional overexpression of I-1 in neurons of transgenic mice promoted CREB phosphorylation and gene transcription (80) and resulted in accelerated learning, by preventing PP1 mediated dephosphorylation of

CREB (80). This establishes I-1 as a modulator of CREB activity, a function that has also been subscribed to DARPP-32. As a connection to cell migration, I-1 has been linked to regulation of cell motility in neurons as I-1 regulates growth cone guidance via inhibition of PP1 activity (81).

I-1 also has several important functions outside the neuronal system where it is phosphorylated in response to stimuli such as the β-adrenergic agonist isoproterenol (82). That event regulates cardiac contractility by inhibiting PP1 and thereby preventing the dephosphorylation of proteins such as Na/K-ATPase troponin I and voltage gated calcium channels, which are involved in regulation of the contractile state of heart muscle (83). I-1 and DARPP-32 are co-expressed in the beta-cells of the pancreas which opens up for a role of these two proteins in PP1-regulated glycogen metabolism (84).

Inhibitor-2:

Inhibitor-2 (I-2) was isolated along with I-1 from rabbit skeletal muscle extracts (75). Unlike I-1 and DARPP-32, I-2 does not have to be phosphorylated by PKA in order to inhibit PP1. Instead, I-2 can be phosphorylated on Thr72 by GSK-3, which does not release it from binding to PP1 but restores the phosphatase activity of PP1 (85). An additional interesting characteristic of I-2 is that its expression fluctuates during the cell cycle peaking at S-phase and mitosis (86). I-2 has also been shown to regulate sperm motility by associating with PP1. Upon phosphorylation of I-2, mediated by GSK-3, PP1 is re-activated and acts to restrict sperm motility (87). These data suggest that I-2 acts to facilitate sperm motility.

DARPP-32

DARPP-32 was originally isolated from homogenates of rat neostiatum and was found to incorporate ^{32}P-labeled phosphate upon stimulation with cAMP or dopamine (88), thereof the name Dopamine and cAMP regulated phospho-protein of 32 kDa, abbreviated DARPP-32. Furthermore, it was established that the catalytic subunit of PKA could catalyze this incorporation of phosphate (88). Due to sequence similarity with the previously identified PP1 inhibitor I-1, it was tested whether phosphorylated DARPP-32 could inhibit PP1 activity. The results showed that this was indeed the case, and lower nanomolar concentrations were sufficient to prevent the catalytic activity of PP1 (89). The amino acid phosphorylated by PKA was identified as Threonine-34 (90). The N-terminus of DARPP-32 is involved in the binding to PP1, and the phospho-group on Thr34 binds to block the catalytic site of PP1 (91)(figure 4). DARPP-32 is expressed primarily in the brain, particularly in the dopaminoceptive neurons (88), where the concentration of DARPP-32 can be as high as 50 μM (92). Dopamine receptors can be classified into two groups: Those that stimulate adenylyl cyclase (the D1 subtypes) and those that inhibit adenylyl cyclase (the D2 subtypes). Consequently, binding of dopamine to the D1 subtype stimulates Thr34 phosphorylation, whereas that effect is antagonized by dopamine binding to the D2 subtype dopamine receptor. Thr34 phosphorylation can also be triggered by other neurotransmitters such as serotonin, adenosine, GABA and neurotensin (93). Another

site in DARPP-32, Thr75, is phosphorylated by the kinase Cdk5 (94) which inhibits PKA activity, leading to a decrease of Thr34 phosphorylation (94). Conversely, activation of PKA stimulates activity of PP2A, a phosphatase that dephosphorylates Thr75 (95), and hence increased phosphorylation at one of those two sites generally leads to dephosphorylation of the other (Fig. 5). Accordingly, phosphorylation of Thr75 in DARPP-32 adds another level to the fine tuning of PKA activity, since the effect of some neurotransmitters, (e.g. dopamine signaling through a D2-type receptor) inhibit PKA activity through such phosphorylation in DARPP-32 (96).

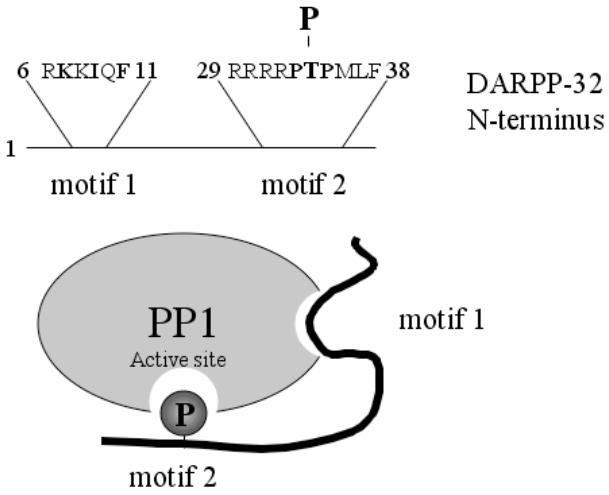

Figure 4. Mechanism of DARPP-32 binding to and inhibition of PP1. Adapted from (91). The docking site at the N-terminus of DARPP-32 binds to PP1 and the phospho-Thr34 binds in the catalytic cleft. Only the N-terminus of DARPP-32 is represented here. Full length human DARPP-32 is 204 aa long. Residues important for binding to PP1 are marked in bold.

PP2B is the enzyme responsible for dephosphorylation of Thr34 (97). Like PP2A, PP2B is activated by calcium. Therefore, stimulation with glutamate via an NMDA or AMPA receptor, which triggers elevation of the cytosolic calcium level, cause a decrease in phosphorylation at both sites (98). Two additional phosphorylation sites on DARPP-32 have been identified, Ser102 and Ser137, which are phosphorylated by the Casein Kinases 1 and 2, respectively (99,100). As of yet, not much is known about the functional consequences of phosphorylation at those sites, except that phosphorylation of Ser137 decreases the rate of dephosphorylation at Thr34 (101). A list of neurotransmitters that induce phosphorylation or dephosphorylation of the mentioned four DARPP-32 phosphorylation sites is shown in Table 1.

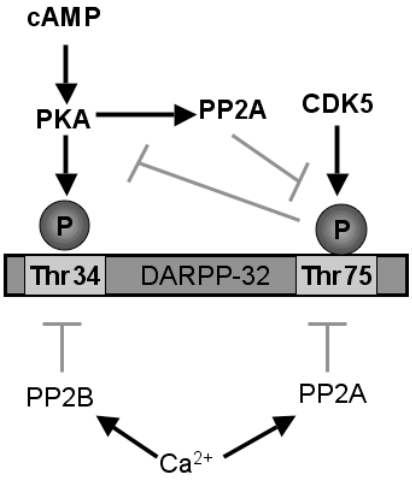

Figure 5. Signalling mechanisms by which phospho-Thr34 and phospho-Thr75 antagonizes each other.

Targeted disruption of the DARPP-32 gene produces mice that have greatly diminished response dopamine as well as other neurotransmitters, which results in effects such as attenuation of activity of the transcription factor CREB and altered activity of ionchannels (102). Svenningsson and colleagues found that a reduction in CREB-mediated transcription, led todiminished dopamine-induced expression of targets such as c-fos and prodynomorphin in DARPP-32 knock out-mice, whereas no effect on transcription of these genes was seen by knocking out the I-1 gene, which demonstrates that the function of DARPP-32 and I-1 is not always similar (103).

PKA phosphorylation sites on proteins such as CREB and spinophilin are also susceptible to dephosphorylation catalyzed by PP1. Therefore, phosphorylation of DARPP-32 at Thr34, leading to inhibition of PP1, is often regarded as a mechanism that enhances the amplitude and duration of the PKA activity. Consequently, it has been speculated that the most prominent function of DARPP-32 is to work as an enhancer of PKA activity.

Many behavioral defects have been observed in DARPP-32 knock out mice, including impaired learning and memory and impaired locomotor activity upon stimulation with caffeine or amphetamine (104-106). This seems to be a logic consequence of the importance of DARPP-32 in neurotransmitter signalling. In addition, numerous drugs of abuse such as caffeine, nicotine, amphetamine, cocaine (107) as well as several therapeutic agents (93) have been found to trigger phosphorylation of DARPP-32. In addition to this, DARPP-32 is involved in (P)-facilitated sexual receptivity in female mice, since DARPP-32 knock out mice exhibited minimal levels of P-facilitated sexual receptivity when compared to their wild-type littermates (108). A list of the

neurotransmitters and some drugs that trigger DARPP-32 phospho-activity is shown in Table 1.

Table 1: Effects of neurotransmitters and drugs on phosphorylation of the indicated four sites in DARPP-32. Adapted and modified from (93). * denotes that the stimulus inhibits acitivty of the receptor, whereas it stimulates activity of the receptor in all other cases.

Stimulus, receptor	Thr34	Thr75	Ser102	Ser137
Dopamine, D1	↑	↓		
Dopamine, D2	↓	↑		
Serotonin, $5HT_2$				↑
Serotonin, $5HT_{4/6}$	↑	↓		
Glutamate, AMPA/NMDA	↓	↓		
Glutamate, $mGlu_{1/5}$		↑		↑
GABA, $GABA_A$	↑			
Adenosine, A_{2A}	↑	↓		
NO	↑	↑		
Opioids, **μ/δ**	↓			
Neurotensin, $NTR_{1/2}$	↑			
CCK, CCK_B	↓			
Nicotine, nAChR	↑	↓	↑	↑
Amphetamine, D1	↑	↓		
Cocaine, D1	↑	↓		
Caffeine, A_1^* A_{2A}^*	↓	↑		
Raclopride, D2*	↑	↓		

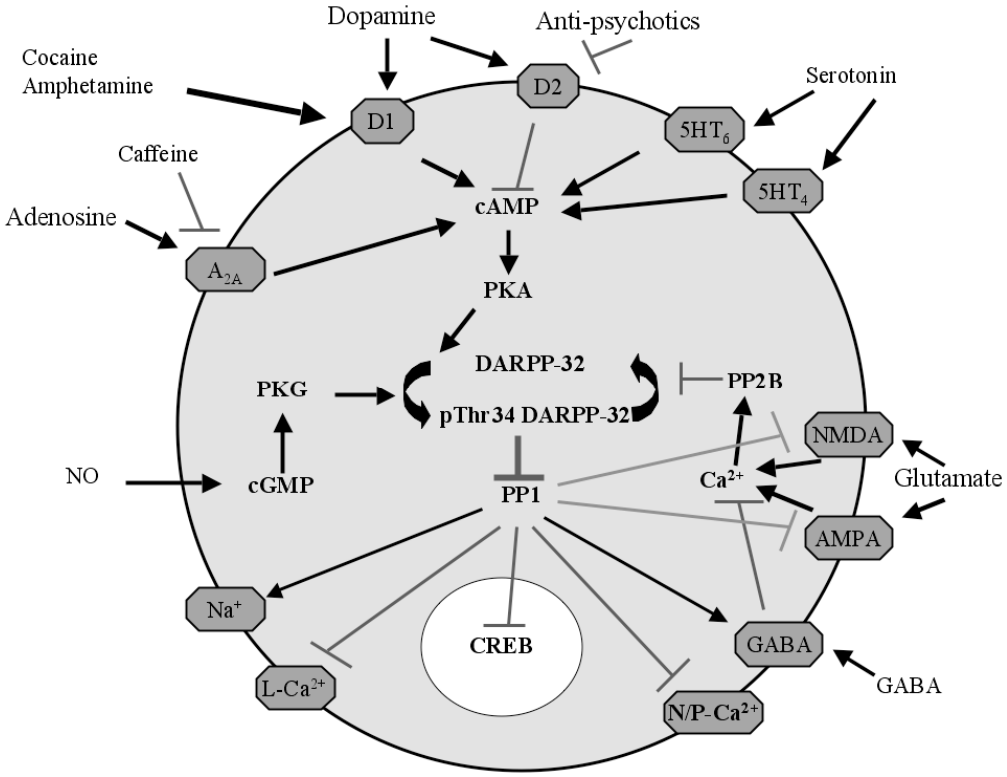

Figure 6. Simplified model of the consequences of the signalling involving DARPP-32 mediated inhibition of PP1 in neuronal cells. Adapted from (109).

DARPP-32 in non-neuronal cell signaling and cancer

The functions DARPP-32 have been extensively studied in neuronal cells, but very little research has focused on the activity of this protein in other tissues. However, recent work has established that DARPP-32 does indeed play important roles outside the neuronal system as well. Besides the brain, DARPP-32 expression has so far been found in breast (110), esophagus (111), pancreas (84), thyroid (112), kidney (113), eye(114), adipose tissue (115), ovary (116) and colon (117).

An interesting study of DARPP-32 was performed in thyroid cells. The authors could show that DARPP-32 expression is strongly induced by FRTL-5 mediated differentiation of thyroid cells (112). This effect was reversed by cell transformation with K- or HA-RAS (112). In line with those findings, Brady and colleagues have observed that DARPP-32 is induced by adipocyte differentiation of 3T3-L1 cells (115). These findings seem to suggest that DARPP-32 might be expressed specifically in differentiated cells. Other studies of kidney cells have revealed that DARPP-32 is also a critical component of the system that regulates salt balance, because it by restricts Na+/K+ ion pump activity (113).

Table 2: Sequence alignment of DARPP-32 from different species. Name of species is followed by an accession number for the specific sequence. 1: mouse (NM_144828), 2: rat (NM_138521), 3: human t-DARPP (NM_181505), 4: human DARPP-32 (NM_032192), 5: rhesus monkey t-DARPP (XM_001089386), 6: c.e.m. monkey (DQ535487), 7: zebrafish (NM_001002538), 8: frog (NM_001089226). The phosphorylation sites Thr34 and Thr75 are conserved between species, whereas Ser102 and Ser137 seems to be less conserved.

```
                                34                                      75
 1 MDPKDRKKIQFSVPAPPSQLDPRQVEMIRRRRPTPAMLFRVSEHSSP-EE EASPHQRTSGEGHHPKSKRPNPCAYTPPSLKAVQ-----HLQTISNLSEN
 2 MDPKDRKKIQFSVPAPPSQLDPRQVEMIRRRRPTPALLFRVSEHSSP-EE ESSPHQRTSGEGHHPKSKRPNPCAYTPPSLKAVQRIAESHLQTISNLSEN
 3 -----------------------------------MLFRLSEHSSP-EE EASPHQRASGEGHHLKSKRPNPCAYTPPSLKAVQRIAESHLQSISNLNEN
 4 MDPKDRKKIQFSVPAPPSQLDPRQVEMIRRRRPTPAMLFRLSEHSSP-EE EASPHQRASGEGHHLKSKRPNPCAYTPPSLKAVQRIAESHLQSISNLNEN
 5 -----------------------------------MLFRLSEHSSP-EE EASPHQRASGEGHHLKSKRPNPCAYTPPSLKAVQRIAESHLQSISNLNEN
 6 MDPKDRKKIQFSVPAPPSQLDPRQVEMIRRRRPTPAMLFRLSEHTSP-EE EASPHQRASGEGHHLKSKRANPCAYTPPSLKAVQRIAESHLQSISNLNEN
 7 MEPNSPKKIQFAVPLFQSQLDPQAAEHIRKRRPTPATLVIYNEPSASGDD KQSTGHQTEAQNAQLSPAQRKQSVYTPPTMRELQLVVEQHFQRQ------
 8 MEANSPRKIQFTVPFLEPHLDPEAAEQIRRRRPTPATLVLSSDQSSP-EI DEERVPNPLQKSLSMSPHQRKKMSRITPTMKELQLLAEHHLCKQ------

   102                             137
 1 QASEEEDELGELRELGYPQEDDEEDEDE--EEDEEEDSQAEVLKGSRGTV GQKPTC--GRGLEGPWERPPPLDEPQRDGNSEDQVEGRATLSEPGEEPQH
 2 QASEEEDELGELRELGYPQEDDEEDEDEDEEEDEEEDSQAEVLKGSRGTA GQKLTS--GQGLEGPWERPPPLDEPQRDGNSEDQGEGRATQSEPGEEPRH
 3 QASEEEDELGELRELGYPREEDEEEEEDDEEEEEEEDSQAEVLKVIRQSA GQKTTC--GQGLEGPWERPPPLDESERDGGSEDQVED-PALSEPGEEPQR
 4 QASEEEDELGELRELGYPREEDEEEEEDDEEEEEEEDSQAEVLKVIRQSA GQKTTC--GQGLEGPWERPPPLDESERDGGSEDQVED-PALSEPGEEPQR
 5 QASEEEDELGELRELGYPREEDEEEEEDDEEEEEEEDSQAEVLKVIRQSA GQKTTC--GQGLEGPWERPPPLDEPERDGSSEDQVED-PALSEPGEEPQR
 6 QASEEEDELGELRELGYPREEDEEEEEDEEEEEEEDSQAEVLKVIRQSA GQKTTC--GQGLEGPWERPPPLDEPERDGSSEN-----PALSEPGEEPQH
 7 ----EQQEAGLSDSPDTPSPITTQHFATGAQWANHNSSEPNGNQSYVSTE GQPGSS--GAGGN------------------------TAESSGSEQKNL
 8 ----GSEEEKIPHLQNNLDDRPDLGCCCHGNTASTQASQSHIPCSCFTQD GQHDTNSLGSR-----------HSSKEDSLDSHVSDGNMQICEPKKQNSH

 1 PSPP----
 2 PTPPESGT
 3 PSPSEPGT
 4 PSPSEPGT
 5 PSPSEPGT
 6 PSPSEPGT
 7 SSPSSVSR
 8 ISFIEDK-
```

Most studies of DARPP-32 expression in non-neuronal cells have been performed in relation to a potential role in cancer. El-Rifai and co-workers identified DARPP-32 as a protein frequently overexpressed in gastric cancer by a microarray specific for genes expressed on chromosome 17 (118). Later data have raised questions concerning whether DARPP-32 promotes gastric cancer, or if it is simply co-amplified with the oncogene Erbb2, since the genomic loci of these two genes are located very close to each other (119). In another paper, El-Rifai and colleagues claimed that they had detected overexpression of DARPP-32 in breast, colon, prostate and stomach (117). Yet, they did not address this with calculations of statistical significance in any of the studies. Although the lack of statistical analysis makes it difficult to evaluate the

overall importance of the study, it is interesting to note that the authors reported that epithelial cells express high levels of DARPP-32 in normal breast tissue, whereas myoepithelial cells do not express DARPP-32 (117). El-Rifai and co-workers found that DARPP-32 is also expressed as a shorter splice form that lacks the first 36 amino acid residues, and thus should be incapable of inhibiting PP1 (118). The existence this shorter isoform has been confirmed by the fact that this isoform has been cloned in several independent studies in human and in Rhesus monkey as well (see Table 2). In addition, the size of DARPP-32 expressed in MDA-MB-468 cells in our own studies (110) could very well correspond to the truncated DARPP-32, although this remains to be verified.

Furthermore, group of El-rifai showed that stable overexpression of DARPP-32 and in particular t-DARPP-32, leads to overexpression of Bcl-2 in gastric cancer cells, (120). Moreover, DARPP-32 inhibited apoptosis induced by camptothecin in the same studies (120).

Other studies of DARPP-32 expression in cancer have concluded that survival rate is lower for patients with oesophageal tumors that are DARPP-32 negative compared to those with DARPP-32 positive tumors (111,121), and that expression of DARPP-32 is decreased in oral cancer (122).

CREB

CREB is a transcription factor that binds as a dimer to the cAMP esponsive element (CRE) of a CREB target gene. The kinases Rsk, CaMKIV and PKA can all phosphorylate CREB at Ser133, which leads to association with CREB binding Protein (CBP), and subsequent recruitment of RNA polymerase II, resulting in gene transcription. The major phosphatases responsible for dephosphorylation at Ser133 are PP1 and PP2A, which leads to de-activation of CREB (123). As CREB can be phosphorylated and dephosphorylated by PKA and PP1 respectively at the same site, it represents a prominent example of a protein, in which PKA-induced phosphorylation is enhanced by DARPP-32. CREB is a downstream effector of dopamine and plays a central role in processes such as memory, addiction, depression and many other psychological phenomena (123), effects that concur with several important functions of DARPP-32. Another major function of CREB activity is to mediate cell survival by inducing transcriptional expression of pro-survival genes (124). However, ablation of the CREB gene in sympathetic neurons has been shown to protect these cells against apoptosis (125), which demonstrates that CREB does not always act as a mediator of survival. The role of CREB in cell migration is still poorly understood. However, studies by Reusch and co-workers have provided evidence for that CREB expression and activity leads to inhibition of smooth muscle cell migration (126). On the other hand CREB has also been shown to have the opposite effect on smooth muscle cells (127). CREB has been suggested to have more than a thousand target genes (128). Still, identification of those that are specifically involved in cell migration have not been identified.

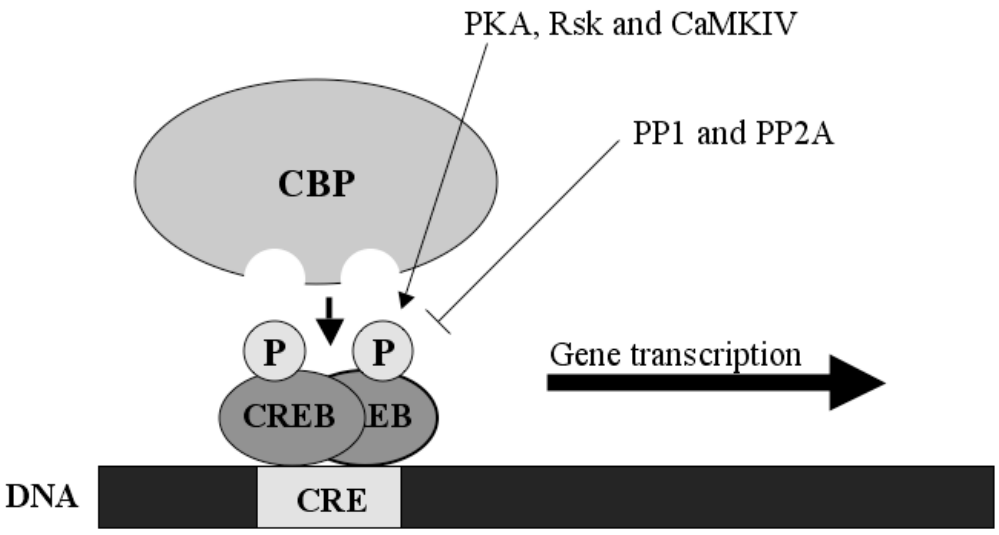

Figure 7: Regulation of CREB phosphorylation and CREB-mediated transcription. CREB binds to the CRE elements as a dimer. Upon phosphorylation of CREB, CBP is recruited, which ultimately leads to gene transcription.

DDR1

Discoidin Domain Receptor 1 (DDR1) is a receptor tyrosine kinase, which is activated by extracellular matrix collagens. Rutter and colleagues isolated the receptor from human placenta library in the early 1990s and found that it is expressed in breast carcinoma cells (129). They also noticed that an extracellular domain of the receptor resembles the discoidin domain originally characterized in the slime mold *Dictyostelium discoideum*, and hence the name discoidin domain receptor, abbreviated DDR. Later, a second mammalian tyrosine kinase receptor with an extracellular discoidin domain was cloned (130), and thus the original receptor was given the designation DDR1 and the second receptor was denoted DDR2. Interestingly, the discoidin protein of the *Dictyostelium discoideum* functions as a lectin, and is involved in the aggregation process of the slime mold (131). It is not yet known whether the mammalian DDR's also function as lectins.

Expression of DDR1 and DDR2 is generally mutually exclusive, in that DDR1 is expressed in the epithelial cells of the tissues and DDR2 in the stromal cells (132). A breakthrough in the understanding of the discoidin domain receptors came when it was discovered that these proteins bind and are activated by extracellular matrix collagens (133,134). DDR1 bind to all triplehelixed collagens tested so far, whereas it seems that DDR2 bind only to fibrillar collagens(133) (134).

It is therefore not surprising that many studies have linked DDR1 to regulation of cell adhesion and migration. DDR1 is important for cell adhesion in several cell types, as primary cells from DDR1 knock out mice adhered less strongly to collagen than wt mouse cells did (135,136). Furthermore, DDR1 promotes migration of smooth muscle cells (136,137)and leukocytes (138) in 3D collagen gels and supposedly upregulation of the matrix metalloproteinases MMP-2 and 9 are important for this process (137). In contrast, DDR1 inhibits cell migration in Madin-Darby canine kidney cells (139,140). In breast epithelial cells expression of Wnt-5a leads to increased activation of DDR1 upon cell plating onto collagen coated surfaces and decreased cell migration (141). Furthermore, we have found that DDR1 expression is required for DARPP-32-mediated inhibition of migration in breast cancer cells (110). Of special interest in this regard is that DDR1 is essential for mammary gland development as DDR1 knock out mice display hyperproliferation and abnormal branching of the mammary ducts, and moreover the DDR1 female knock out mice are unable to lactate (142).

Activation of the DDR1 receptor by collagen (measured as overall tyrosine phosphorylation status) can take up to 18 hours to reach a maximum level (133), which represents unusually slow kinetics compared to ligand-dependent stimulation of other receptor tyrosine kinases. It is believed that DDR1 dimerizes and autophosphorylates upon ligand binding, a classical activation mechanism of receptor tyrosine kinases. The juxtamembrane region, (i. e. the region between the transmembrane domain and the tyrosine kinase catalytic domain), is much longer in DDR1 than the corresponding region in other receptor tyrosine kinases (Fig. 8), and it is moreover exceptionally rich in proline and glycine amino acid residues. This part of DDR1 is responsible for phospho-tyrosine independent binding to DARPP-32, by a so far unknown mechanism (110). Far most characterized intracellular interaction partners of receptor tyrosine kinases bind phospho-tyrosine dependently via SH2 domains or PTB-domains (143). Several such interaction partners have also been identified for DDR1, including ShcA (133), Nck2 (144), Shp-2 (144) and PI3 kinase (145). Interestingly, ShcA binds via a PTB domain to a motif that is present only in the DDR1b isoform, which carries a 37 amino acid insert in the juxtamembrane region, that is not present in the DDR1a isoform (132). This suggests that DDR1 isoform expression adds an additional level to regulation of DDR1 activity. Besides DDR1a and DDR1b three other isoforms has been shown to exist, at least on the RNA level (146). The isoform DDR1d is often used as a tool for dominant negative disruption of DDR1 signalling, since it has an intact extracellular domain but no intracellular catalytic domain (140). Primary kidney cells isolated from DDR1 knock out mice grow slower than the corresponding wt cells (135), which suggests that DDR1 also in this sense is an atypical receptor tyrosine kinase, as expression and activation of the majority of other receptor tyrosine kinases induces proliferation. DDR1 is overexpressed in ovarian cancer (147) but not in breast cancer (148).

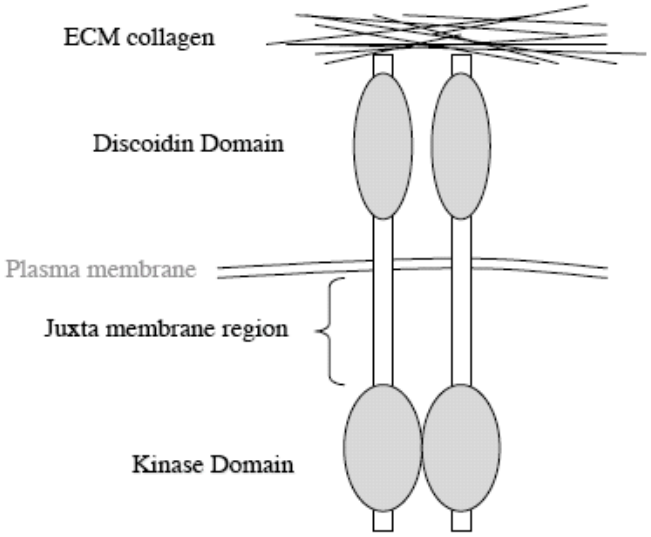

Figure 8: The structure of DDR1.

The Wnt-family of proteins

The Wnt family of proteins constitutes a 19 member large family of secreted glycoproteins in humans. They can roughly be divided into two main groups: The canonical and the non-canonical Wnts. Canonical Wnts, such as Wnt-1, -3a, -8 and –8b, have the ability to induce transformation C57mg mammary epithelial cells and stabilization of β-catenin(149), whereas the non-canonical Wnts such as Wnt-4, -5a and -7b cannot transform C57mg cells and they are generally incapable of inducing β-catenin stabilization (149).

For many years, the 7 transmembrane Frizzled receptors G-protein coupled receptors (150) were the only identified receptors for Wnt proteins. However, recent identification of the Ror2 (151) and Ryk (152) receptor tyrosine kinases as receptors for Wnts in mammalians has opened up for other ways of Wnt-triggered intracellular signaling. In addition, Lrp5 and Lrp6 have been found to serve as important co-receptors for Wnt mediated signaling via frizzled receptors, although it remains to be determined whether this is a general mechanism or if it applies specifically to canonical Wnts (153,154).

Canonical Wnt signalling

The most characteristic feature of canonical Wnt signaling is the ability to induce β-catenin stabilization. This on/off switch of β-catenin stabilization is a very energy wasteful process since cytosolic β-catenin is constantly being targeted for proteosomal degradation in the absence of stimulation with a canonical Wnt protein. In short,

association of β-catenin with the APC/axin/Gsk-3β complex results in phosphorylation of β-catenin by Gsk-3β, which in turn leads to ubiquitination and proteosomal degradation of β-catenin (155). Binding of a canonical Wnt-protein to a frizzled receptor and Lrp 5/6 induces phosphorylation of the adapter protein dishevelled, which then translocates to disrupt the APC/axin/Gsk-3β/β-catenin complex, and thereby hinders Gsk-3β from phosphorylating β-catenin, resulting in β-catenin accumulation (155). β-catenin then translocates to the nucleus where it induces transcription of target genes by binding to the Tcf/Lef transcription factors. One important target gene of β-catenin mediated transcription is cyclinD1, a strong inducer of proliferation, Overexpression of which is correlated with overexpression of β-catenin in more than 50 % of breast cancers (156).

Wnt-5a

Wnt-5a is a non-canonical Wnt and unlike canonical Wnts, it does not have the capacity to transform C57 mammary epithelial cells (149). Furthermore, some studies have shown that Wnt-5a can antagonize the accumulation of β-catenin or cell transformation induced by canonical Wnts (157,158). Wnt-5a induces intracellular signaling by associating with frizzled receptor types 2, 3, 4 or 5 (158-161). Furthermore, Wnt-5a also activates the receptor tyrosine kinase Ror2 (151). In addition, Wnt-5a is essential for embryogenesis, and a noteworthy finding is that Wnt-5a and Ror2 KO mice display some of the same embryonic defects (162,163).

It is now well established that stimulation of cells with Wnt-5a induces upregulation of the cytosolic calcium concentration as tested in Zebrafish (164), mouse (165) and human cells (166,167). This effect can then lead to activation of the transcription factor NFAT via dephosphorylation mediated by the phosphatase calcineurin (166). Moreover, Wnt-5a stimulation has been reported to cause calcium-dependent activation of CamKinase II(168).

The role of Wnt-5a in cell migration is more controversial, since Wnt-5a stimulation can either increase (169-171) or reduce (141,167) cell migration in different cell types. A possible explanation for these opposite findings is that Wnt-5a augments adhesion (141) and thereby decreases migration in cell that are already strongly adherent such as breast epithelial cells (141), whereas it increases migration in cells that are less adherent prior to the stimulation, such as melanoma cells (169). However, additional experiments are needed to substantiate this hypothesis. It is believed that the collagen receptor DDR1 plays an important role in Wnt-5a-mediated increase of cell adhesion, since Wnt-5a expression increases DDR1 activation upon plating on collagen (141).

Wnt-5a expression is altered in different forms of cancer: Wnt-5a expression is downregulated in higher histological grade breast cancer and lack of Wnt-5a expression in breast cancer correlates with poorer prognosis (172). Other studies of Wnt-5a mRNA levels in breast cancer have shown that the mRNA level for Wnt-5a is upregulated in breast cancer (173,174). A plausible explanation for this finding is that Wnt-5a expression is negatively regulated on translational level by HuR, a protein that is known to be upregulated in breast cancer (175). In gastric cancer, Wnt-5a expression

is upregulated on protein level and in gastrointestinal cells it stimulates FAK and RAC activity (170). Theese observations indicate that Wnt-5a plays opposite roles in different types of cancer.

Focal Adhesion Kinase

Focal Adhesion Kinase (FAK) is a ubiquitously expressed tyrosine kinase that plays a central role in regulation of cell migration. It was independently identified in 1992, by different research groups, as a substrate of the Src kinase and as a highly tyrosine-phosphorylated protein localized to integrin enriched cell adhesion sites (176-178). Upon activation of the integrins by extracellular matrix (ECM) molecules such as collagen or fibronectin, focal contacts are formed at the ECM-integrin junctions leading to the assembly of intracellular focal adhesion protein complexes. Both cytoskeletal and signalling proteins are recruited to the focal adhesions, which thereby link the focal adhesion complexes to the actin cytoskeleton (179)

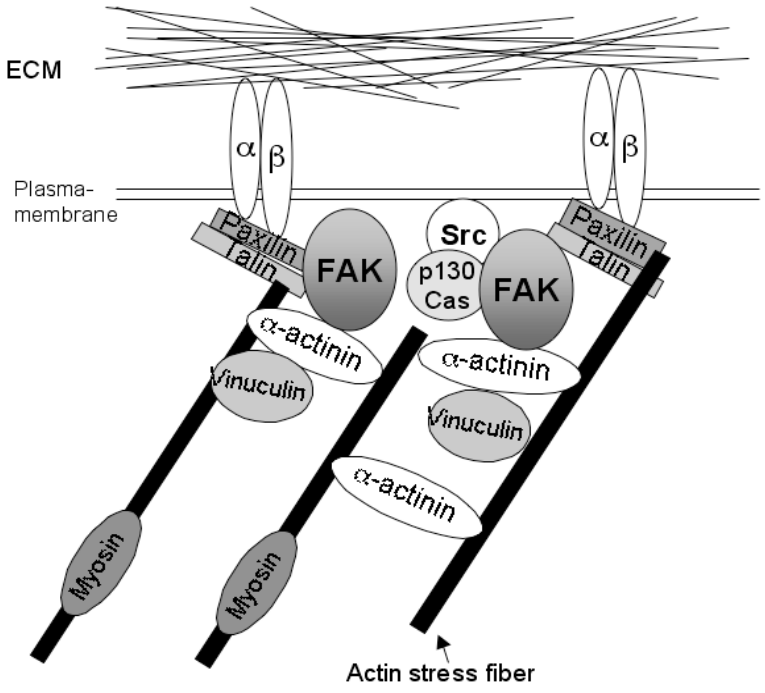

Figure 9. Focal adhesion complex. Adapted from (180).

FAK is not essential for generation of focal adhesion complexes, as demonstrated by a study in which FAK-null fibroblasts where found to exhibit an excess rather than a decrease in the number of focal contacts (181). However, FAK-null cells display

defective motility, and it is believed that FAK is important for both the maturation and the turnover of focal adhesions (182). Interaction with p130Cas and Src seems to be particular important for the role of FAK in cell migration as wt FAK can rescue migration of FAK null fibroblasts, while a mutant FAK, that was unable to recruit Src and p130Cas, could not. Another important function of FAK in cell migration is its involvement in activation of the GTP'ases RAC and Cdc42, which are necessary for formation of lamellipodia and filopodia formation, respectively (183,184).

FAK phosphorylates numerous proteins that participate in cell migration such as α-actin, p130Cas and paxilin (185). However, FAK has an equally important function as an adapter/scaffolding protein (see figure 9). A prominent modification of FAK is tyrosine phosphorylation at residue Tyr397 and phosphorylation at this site is generally assumed to indicate FAK-activity (179). Phosphorylation of this residue enables recruitment of SH2 domain containing proteins such SHC (186), p85 subunit of PI3 kinase (187) and Src (188). FAK-activity can trigger proliferation by recruiting the adapter protein Grb2, which leads to activation of the Ras MAPK kinase pathway (189).

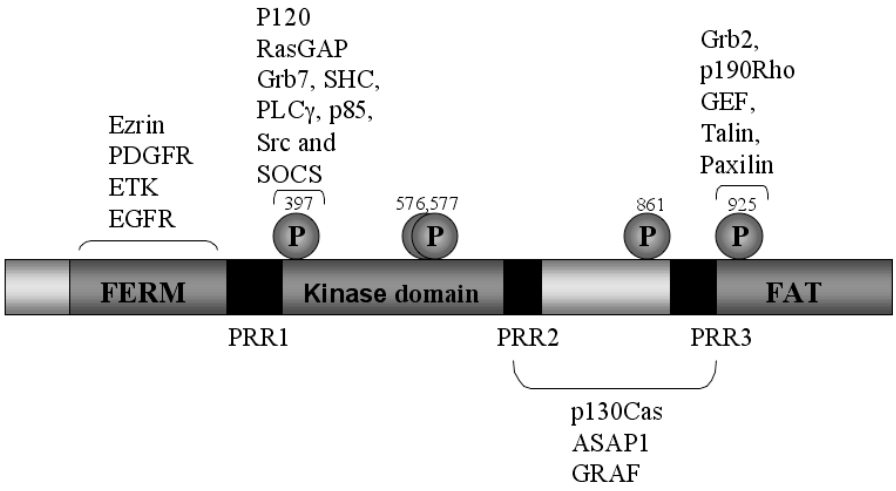

Figure 10: Structure, phosphorylation sites and interaction partners of FAK. Adapted from (180). PPR stands for proline rich regions. These regions are important for binding to the SH3 domain of interaction partners. Phosphorylation of tyrosine residues 397 and 925 leads to recruitment of SH2 domain containing proteins.

In addition, FAK can trigger proliferation via recruitment of PI3 kinase (187). Due to its role a an inducer of proliferation and cell migration it is perhaps not surprising that expression of this protein is often upregulated in different types of cancer (190), including breast cancer (191).

The present investigation

The goal of the present investigation was to explore signalling mediated through Wnt-5a and its downstream target DDR1, and the impact of this signalling on cell migration. By the identification of DARPP-32 as a binding partner to DDR1, the following goal has been to evaluate the role of this phospho-protein in breast cancer cell signalling and migration, which could potentially lead to a novel way of inhibiting breast cancer metastasis.

Paper I

Our research group had previously found that DDR1, a receptor tyrosine kinase activated by collagen, displayed enhanced tyrosine phosphorylation when plated on collagen in the presence of Wnt-5a expression. We believed this could be important for the role of Wnt-5a in cell migration and thus the goal of this study was to identify DDR1binding partners, in order to further shed light on the role of DDR1 in cell migration downstream of Wnt-5a.

To identify such targets we conducted a yeast two hybrid screening of a human mammary library using the juxta membrane region of DDR1 as bait. This approach resulted in the identification of DARPP-32, a phospho-protein that until recently was presumed to be expressed solely in neuronal cells. The interaction of DDR1 and DARPP-32 was confirmed by co-immunoprecipitation (co-IP) of the endogenous proteins in HB2 cells and by co-IP of DDR1 with overexpressed DARPP-32 in MCF-7 cells. Moreover, we found that DARPP-32 was phosphorylated on residue Thr34, but not on Thr75 when the cells where detached. Since both Wnt-5a and DDR1 affect cell migration we tested whether DARPP-32 too would be able to modulate to migratory behaviour of MCF-7 breast cancer cells. The results showed that DARPP-32 did indeed inhibit migration, and we also found that phosphorylation of the Thr34 site was required for this function of DARPP-32. As PKA activity was required for phosphorylation of this site, the PKA inhibitory peptide PKI, abolished the effect of DARPP-32 on cell migration, in line with the importance of this PKA target site for DARPP-32 mediated inhibition of cell migration. A functional connection between DDR1 and DARPP-32 in cell migration was found as DARPP-32 did not inhibit cell migration in the DDR1 deficient cell line MDA-MB-231 unless DDR1 was reintroduced. Interestingly, we also noted that DARPP-32 was expressed much more strongly in two non-cancer cell lines than in the four cancer cell lines tested.

The main findings of this paper, was the identification of DARPP-32 as a novel binding partner to the collagen receptor DDR1 and that DARPP-32 inhibited cell migration phospho-Thr34 dependently.

Paper II

In order to elucidate whether Wnt-5a and DARPP-32 would be coupled in cell migration we investigated the possibility that Wnt-5a could induce Thr34-phosphorylation of DARPP-32 expressed in MCF-7 breast cancer cells. Wnt-5a

triggered an increase in DARPP-32 phosphorylation that peaked after five minutes at a level approx. 3 fold above the basal. We also found that this mechanism was dependent on activation of PKA, since the PKA inhibitors H89 and RPcAMP abolished the induced phosphorylation. Furthermore, we observed that Wnt-5a alone could trigger elevation of cAMP beneath the plasma membrane.

In search of functional relevance of the signalling mechanism, we found that DARPP-32 suppressed of MCF-7 cell migration in wound healing, Wnt-5a dependently. DARPP-32 interacted with PP1 in transfected MCF-7 cells, with increased amounts binding to PP1 when DARPP-32 was phosphorylated. This result indicated that DARPP-32 could inhibit PP1 in breast epithelial cells in a similar mechanism to what has been observed in neurons. Therefore, we proceeded to test whether CREB, which is a downstream target of PP1, would be altered by DARPP-32 phosphorylation. We found that Wnt-5a alone could trigger CREB phosphorylation, which was further enhanced by expression and phosphorylation of DARPP-32. Moreover, dominant negative CREB (DNCREB) partially diminished the inhibitory effect of Wnt-5a and DARPP-32 on cell migration.

Since PKA is responsible for phosphorylation of CREB, we speculate that DARPP-32 acts to enhance the effect of PKA by inhibiting PP1-mediated dephosphorylation. Therefore, Wnt-5a triggering of DARPP-32 represents a mechanism whereby DARPP-32 is activated to enhance activity of the transcription factor CREB, important for inhibition of breast cancer cell migration.

In summary, we found that Wnt-5a could induce cAMP elevation leading to activation of DARPP-32 and that this signalling mechanism is important for restricting cell migration via activation of PKA and CREB.

Paper III

Focal Adhesion Kinase plays a key role in cell migration. We asked the question whether DARPP-32 phosphorylation might affect FAK-activity since other investigators had reported that PP1 activity can increase FAK activity, and thus DARPP-32 might block the effects of FAK via inhibiting PP1.

Detachment induced phosphorylation of DARPP-32 in transfected MCF-7 cells and replating on collagen coated plates lead to less FAK-activity than empty vector transfected cells treated in the same manner. We found that this mechanism was dependent on phosphorylation of Thr34, as T34A mutant DARPP-32 could not mediate downregulation of FAK-activity in the same type of experiment.

The Src inhibitor SU6656 inhibited FAK-activity in MCF-7 cells. To explore whether the actions of Src and FAK were involved in DARPP-32 mediated inhibition of cell migration we tested this hypothesis using a boyden chamber migration assay. SU6656 alone inhibited MCF-7 cell migration as did detachment-activated DARPP-32. However, DARPP-32 did not further suppress migration in MCF-7 cells incubated with SU6656, which suggests the kinase activities of Src and FAK are required for DARPP-32-mediated cell migration.

We also observed that phosphorylated DARPP-32 co-localized with PP1 in the cytoplasm. Incubation of MCF-7 cells with the PP1 inhibitor okadaic acid also caused inhibition of FAK activity, which confirmed that PP1 activity is important for FAK-activity. Surprisingly, okadaic acid did not alter phosphorylation of the PP1 target site Ser722 in FAK in MCF-7 cells, which had previously been reported to be the mechanism by which PP1 affects FAK-activity.

When MCF-7 cells transfected with DARPP-32 expression plasmid were detached and then allowed to adhere to collagen coated glass slides over night we found that phospho-DARPP-32 stained cells showed less staining for active FAK, at the focal adhesions, than did untransfected cells. This again was Thr34 phosphorylation specific as mutant T34A-DARPP-32 did not induce the same phenotype. Moreover, when we stained DARPP-32 transfected cells for F-actin we observed that DARPP-32 transfected cells had less filopodia staining than untransfected cells. This phenotype was again not seen in T34A-DARPP-32 transfected cells.

In conclusion we found that FAK activity is suppressed by phospho-Thr34-DARPP-32, and possibly as a consequence hereof cells expressing phospho-DARPP-32 also displayed less filopodia formation. Src activity is important for DARPP-32-mediated suppression of cell migration, quite possibly because it regulates the activity of FAK. DARPP-32 effected inhibition of FAK is mediated via inhibition of PP1 by a mechanism that remains to be identified.

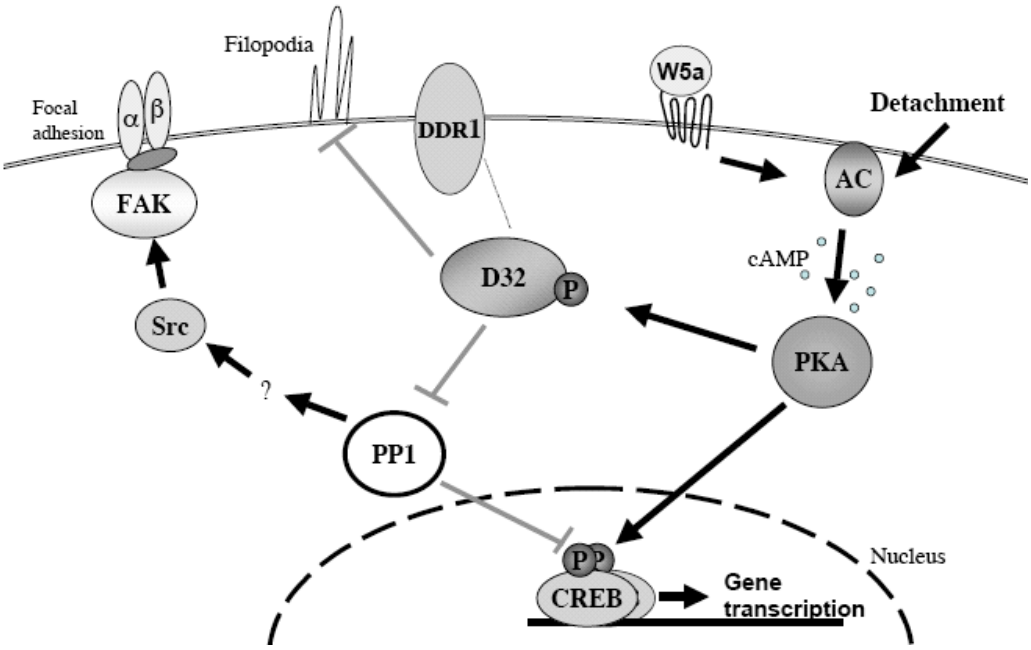

Figure11: DARPP-32 in breast epithelial cell signalling.

Discussion

The finding of the DDR1/DARPP-32 protein-protein interaction in paper I provide new insight into the function of both molecules. The interaction is unusual, since it does not depend on phosphorylation of DDR1. Instead, it seems that DARPP-32 preferentially binds to non-phosphorylated DDR1. It is possible that less DARPP-32 co-immunoprecipates with tyrosine phosphorylated DDR1 because other molecules, like SHC and Nck-2, interact with the juxtamembrane region of DDR1, and thereby compete with DARPP-32 for binding to phosphorylated DDR1. In such a scenario, DARPP-32 might participate in regulation of the adapter molecules that bind to this region of DDR1, and thereby modulate signalling downstream of DDR1. However, at present the downstream consequences of SHC and Nck-2 binding to DDR1 are not known.

The interaction between DDR1 and DARPP-32 is relatively weak but has been detected both by co-IP of the endogenous proteins in HB2 cells and by co-IP of overexpressed Myc- DARPP-32 and endogenous DDR1 in MCF-7 cells. Moreover, it is interesting to note that the proteins interact several fold stronger immediately after cell detachment. However, our unpublished observations from co-IP experiments of DDR1 with either wt DARPP-32 or T34A mutant DARPP-32 immediately after detachment did not reveal any differences, suggesting that the increase in interaction between DDR1 and DARPP-32 following detachment is not phospho-Thr34 dependent (Paper I). This observation was somewhat surprising, since cell detachment resulted in a strong induction of Thr34-DARPP-32 phosphorylation. We believe that the specificity of the interaction with DDR1 may be due to other modifications of DARPP-32 in response to cell detachment. This could for example be phosphorylation at sites such as Ser102 and Ser137 or not yet identified phospho-sites of DARPP-32. Alternatively, the interaction between DDR1 and DARPP-32 could be phosphorylation independent and induced by other protein modifications or more DARPP-32 and DDR1 could be co-localized in the cell upon cell detachment.

In neurones, PKA-mediated phosphorylation of Thr34 in DARPP-32 converts it into a potent inhibitor of PP1, and Cdk5-induced Phosphorylation of Thr75 in DARPP-32, converts DARPP-32 into a potent inhibitor of PKA, which antagonizes Thr34 phosphorylation. We have not been able to detect any changes in phosphorylation status of the Thr75 site with either detachment (Paper I) or Wnt-5a (Paper II), which could be explained by the fact that Cdk5 is specifically expressed in neurons. Nevertheless, there is a detectable Thr75 phosphorylation when DARPP-32 is overexpressed in MCF-7 cells, and this was enhanced by mutation of the Thr-34 in DARPP-32 to alanine (Paper I). Therefore, it seems likely that either CDK5 is weakly expressed in MCF-7 cells or that another kinase can phosphorylate Thr75 in these cells.

We found that DARPP-32 inhibits MCF-7 cell migration. Notably, we also found that the choice of migration assay is important for the impact of DARPP-32 on cell migration, as we in the beginning unknowingly, triggered Thr34 phosphorylation of DARPP-32, at the start of the boyden chamber experiment by cell detachment. We

found that a mutant T34A DARPP-32 could not inhibit MCF-7 cell migration in the same type of experiment whereas the T75A mutant inhibited cell migration with an impact similar to wtDARPP-32 (Paper I). In contrast, when we performed wound healing assays we observed that DARPP-32 expression in MCF-7 cells did not inhibit migration by itself, since the phospho-Thr34 of DARPP-32 started at basal level in this type of assay (Paper II).

Albeit we do not know much about the interaction mechanism between DARPP-32 and DDR1, we found that DDR1 has importance for the anti-migratory function of DARPP-32, as DARPP-32 could not inhibit MDA-MB-231 cell migration unless DDR1 was co-expressed in this normally DDR1 and DARPP-32 deficient cell line.

In order to proceed with the study of DDR1/DARPP-32 interdependence in cell migration it will be important to map the interaction site of DARPP-32 on DDR1 and vice versa. Such information would also help us determine why DARPP-32 interacts more strongly with DDR1 following cell detachment.

Prior to my work, our research group had found that expression of Wnt-5a can enhance DDR1 phosphorylation upon plating of breast epithelial cells onto collagen. In addition, we had found in paper I that Wnt-5a and DARPP-32 share the ability to inhibit cell migration. Therefore, we addressed the question of whether Wnt-5a stimulation of DARPP-32 expressing MCF-7 cells could induce phosphorylation of DARPP-32. Wnt-5a did indeed induce phosphorylation of DARPP-32 at Thr34, but not at Thr75 (paper II). We confirmed that this mechanism was PKA dependent as both PKA inhibitors RPcAMP and H89 abolished Wnt-5a-induced Thr34 phosphorylation.

Since research had not yet shown whether Wnt-5a could induce a cAMP response, we further substantiated our finding by showing that Wnt-5a alone could trigger elevation of cAMP concentration beneath the plasma membrane, in a novel sensitive detection system of cAMP, by means of evanescent fluorescence microscopy (Paper II).

These results altogether establishes the novel finding that Wnt-5a can induce a cAMP response, although it remains to by explored which receptor that mediates this response. Wnt-5a has been shown to act through the frizzled receptors 2, 3, 4 and 5 and also the untypical Wnt-receptors Ror2 and Ryk. None of these have previously been linked to cAMP signalling, although structural analysis of the frizzled receptors seems to suggest that frizzled-3 would be the most likely frizzled receptor to couple to $G\alpha_s$ and triggering of the adenylyl cyclase (192).

Downstream of DARPP-32 phosphorylation we found that Thr34 phosphorylated DARPP-32, as induced by forskolin stimulation, interacted stronger with PP1 than did unstimulated DARPP-32. Phospho-Thr34-DARPP-32 inhibition of PP1 has been well characterized by *in vitro* studies and studies in neurons. Therefor it seems reasonable to assume that phopho-Thr34-DARPP-32 inhibits PP1 acitivity in breast epithelial cells as well (Paper II).

CREB is a downstream target of PP1, since it is inactivated when it undergoes PP1-mediated dephosphorylation. Therefore, we investigated whether DARPP-32-mediated

inhibition of PP1 could induce CREB phosphorylation. We found that DARPP-32 alone did not induce CREB phosphorylation, whereas it did enhance CREB-phosphorylation in combination with Wnt-5a stimulation. Moreover, stimulation with Wnt-5a alone was enough to trigger CREB phosphorylation (Paper II).

DARPP-32 has previously been shown to enhance CREB phosphorylation in neurons, but our finding that Wnt-5a alone induced CREB phosphorylation is new and interesting it itself. Wnt-5a can inhibit cell migration in MCF-7 cells regardless of DARPP-32 expression (paper II) and have also been shown to inhibit cell migration of MCD-MB-468 cells (193), which contain very little of a truncated version of DARPP-32. Therefore, DARPP-32 is not essential for Wnt-5a mediated inhibition of breast epithelial cell migration. More likely, DARPP-32 enhances Wnt-mediated inhibition of cell migration, as it functions as an enhancer of PKA signalling. Wnt signalling, but not Wnt-5a specifically, has previously been linked to CREB in mouse myogenesis. The finding that dominant negative CREB diminished the anti-migratory signal of Wnt-5a and DARPP-32 suggests that a signalling pathway of Wnt-5a, DARPP-32 and CREB pathway can act to restrict breast epithelial cell migration (Paper II). The next step will be to identify transcriptional targets downstream of CREB that are involved in controlling this process.

The fact that detachment could trigger DARPP-32 phosphorylation and migration also demonstrates that DARPP-32 mediated inhibition of cell migration is not nessecarily dependent on Wnt-5a, but can be induced by other factors that trigger a cAMP response as well (Paper I). However, the specific molecular mechanism that causes DARPP-32 phosphorylation upon cell detachment remains to be clarified.

The basis of paper III is the discovery that detachment of MCF-7 cells induced Thr34 phosphorylation of DARPP-32, which in turn inhibited Tyr397 phosphorylation of FAK. Tyr397 phosphorylation of FAK it widely accepted as an indicator of FAK-activity, as phosphorylation of this residue induces recruitment of a long range of adapter proteins important for its function in cell migration and proliferation. However, it should be emphasized that Tyr397 phosphorylation is does not necessarily always correspond to activity of FAK, since FAK can recruit interaction partners to sites, that are not dependent on Tyr397 phosphorylation (Figure 10).

We found that Src activity was crucial for FAK activitity, which has been shown in other cell types before (194). In continuation of this we found, that the DARPP-32 was not capable of inhibiting cell migration of breast epithelial cells incubated with the Src-inhibitor SU6656. This suggests that Src and FAK are important for the anti-migratory function of DARPP-32 (Paper III).

Moreover, the finding that phospho-Thr34-DARPP-32 stained MCF-7 cells have less active FAK and less filopodia formation seems to suggest that DARPP-32 downregulates FAK activity resulting in some defectiveness in actin polymerization or remodelling, possibly through inhibition of cdc42, as cdc42 is important for filopodia formation. However, we can not exclude that the effect seen on filopodia formation could be independent of FAK activity (Paper III).

Staining of MCF-7 cells revealed that DARPP-32, and phospho-DARPP-32 are uniformly distributed in the whole cytoplasm, both with or without stimulation with forskolin, to burst the cAMP response (Paper III).

It would be interesting to evaluate the localization of endogenously expressed DARPP-32 from HB2 or T47D cells, since it is possible that a specific membrane localization of a fraction of DARPP-32 is masked by the strong expression of DARPP-32 in transfected MCF-7 cells. Additionally, it would also be interesting to elucidate whether siRNA mediated downregulation of DARPP-32 expression in HB2 or T47D cells could increase cell migration, to consolidate the findings from experiments with DARPP-32 in MCF-7 cells.

Although many aspects of the role of DARPP-32 in cell migration still remains to be explored, we believe that DARPP-32 may act to keep breast epithelial cells from detaching from the basement membrane thereby preserving their functional integrity. Moreover, this could be a mechanism that blocks initiation of cancer metastasis, by inhibiting the step of cell detachment from the primary tumour. This could explain why we have detected less or no DARPP-32 expression in the breast cancer cell lines compared to non-cancer breast epithelial cell lines.

Summary

The thesis describes these following novel findings:

- DARPP-32 is expressed in breast epithelial cell lines, preferentially in non-cancer cell lines
- DDR1 interacts with DARPP-32
- DARPP-32 inhibits breast cancer cell migration
- DARPP-32 Thr34 phosphorylation is induced by cell detachment
- DARPP-32 mediated inhibition of cell migration is phospho-Thr34 dependent
- DARPP-32 inhibition of MB-MDA-231 cell migration depends on introduction of DDR1 expression into the otherwise DDR1 depleted cell line
- Wnt-5a induces a cAMP response
- Wnt-5a induces cAMP/PKA dependent phosphorylation of Thr34 in DARPP-32
- Wnt-5a induces DARPP-32 mediated suppression of MCF-7 cell migration
- Wnt-5a induces activation of the transcription factor CREB
- DARPP-32 enhances Wnt-5a mediated activation of CREB
- DN-CREB diminishes Wnt-5a/DARPP-32 mediated inhibition of cell migration
- Phosphorylation of DARPP-32 leads to suppression of FAK-activity
- DARPP-32-effected inhibition of MCF-7 cell migration is dependent on Src and FAK activity
- Phospho- DARPP-32 inhibits filopodia formation

Popularized summary

Breast cancer is the most common cancer form in women today. One in ten women in Sweden contract the disease during their lifetime and about 30% of this group succumb to the cancer. It is not the growth of the primary tumour that is lethal, but the spreading (metastasis) to other tissues, which is the cause of death.

It is believed that most cases of breast cancer originate from the epithelial cells that line the lobules and the ducts in the breast. In order to spread from a primary tumour, transformed breast epithelial cells must alter their capacities for adhesion and migration.

The work described in this thesis involved studies of the processes that regulate the migration of breast epithelial cells. It is particularly important to understand these processes in order to learn how to prevent, stop or at least delay breast cancer metastasis.

The protein Wnt-5a is downregulated in breast malignancies, and a low Wnt-5a expression in the primary tumor is correlated with a poor prognosis of recurrence free survival. Wnt-5a increases adhesion and decreases migration of breast epithelial cells. Signalling by Wnt-5a leads to enhanced activation of the cell surface receptor DDR1 when the cells are plated on collagen. Collagen is expressed in the basement membrane, which the breast epithelial cells normally attach to.

The first article in this thesis describes the finding of a named DARPP-32, identified in a systematic search for binding partners to DDR1, expressed in human breast. We found that expression of DARPP-32 inhibited breast epithelial cell migration, and that the anti-migratory function of DARPP-32 required the addition of a phosphate group (i.e. phosphorylation) to a single amino acid residue, designated Threonine-34 (Thr34). The anti-migratory function of DARPP-32 was also dependent on association with DDR1, since DARPP-32 did not inhibit cell migration in the DDR1 deficient breast cancer cell line MDA-MB-231 unless DDR1 was co-expressed

In the second study, we found that Wnt-5a could directly trigger phosphorylation of the Thr-34 in DARPP-32 via activation by a protein that catalyzes the addition of phosphate groups to proteins (such a protein is called a kinase), the protein kinase A. We also found that Wnt-5a could induce DARPP-32 dependent inhibition of cell migration in MCF-7 breast cancer cells.

In addition to this, we observed that Wnt-5a could trigger phosphorylation of the transcription factor CREB, a protein that plays an important role in regulating the expression of other proteins via binding to DNA. This effect was enhanced by expression of DARPP-32. A dominant negative version of the CREB protein diminished the anti-migratory effects of Wnt-5a and DARPP-32, which suggests that Wnt-5a-induced activation of CREB and DARPP-32, mediated by protein kinase A, represents a pathway that restricts breast epithelial cell migration.

Popularized summary

In the third study we found that cell detachment induced phosphorylation of Thr34 in DARPP-32 lead to suppression of Focal adhesion kinase (FAK) activity. FAK is vital for cell migration, because it regulates the formation and degradation of focal adhesions. The focal adhesions are interaction points between the cell and the surface which are needed in order for the cell to adhere to a surface or to move on a surface. A migration assay revealed that the activity of FAK as well as the FAK interacting protein Src kinase, is nessecary for the anti-migratory function of DARPP-32.

DARPP-32-mediated suppression of FAK was effected through inhibition of PP1, a protein that removes phosphate groups from proteins (i.e. a phosphatase). Furthermore, staining MCF-7 cells with antibodies specific for active FAK and phospho-DARPP-32 revealed that cells expressing the latter protein showed less staining for the active form of FAK. Finally, we observed that phospho-DARPP-32 reduced the formation of filopodia, which are the needle-shaped protrusions in the front edge of a cell that are used for locomotion.

In short, we believe that DARPP-32 might serve to keep breast epithelial cells from leaving the surface on which they are normally situated, called the basement membrane. This supports the functional integrity of the cells, because they must adhere to the basement membrane in order to exert their proper function. We believe that this mechanism might block the initiation of cancer metastasis, by inhibiting detachment of cells from the primary tumor.

Populärvetenskaplig sammanfattning

DARPP-32s roll i bröst cancer cellsignalering och migration

Bröstcancer är vanligaste cancerformen hos kvinnor idag. En av tio kvinnor i Sverige drabbas av sjukdomen under sin livstid och 30% av denna grupp dör av bröstcancer. Det är inte tillväxten av den primära tumören som är dödlig i sig utan spridningen till andra vävnader (metastaseringen) som orsakar döden.

Idag anser man att de flesta fall av bröstcancer har sitt ursprung i epitelceller som omger mjölkkörtlarna (lobulär cancer) eller mjölkgångarna (ductal cancer). För att kunna sprida sig måste de transformerade bröstcancer cellerna ändra sin kapacitet för adhesion och migration.

Det utförda arbetet i denna avhandling innefattar studier av de processer som reglerar migration av bröstepitelceller. Det är speciellt viktigt att förstå dessa processer för att komma underfull med hur man kan förhindra, stoppa eller åtminstone fördröja bröstcancer metastasering.

Proteinet Wnt-5a är nedreglerat i elakartade brösttumörer, detta korrelerar med en sämre återfallsfri överlevnads prognos. Signalen via Wnt-5a leder till ökad aktivering av ytcellreceptorn DDR1 när cellerna sätts på collagen. Collagen uttrycks på basal membranet som bröstepitel cellerna normalt binder till.

Den första artikeln in denna avhandling beskriver fyndet av DARPP-32, som identifierades i en systematisk sökning för potentiella bindningspartner för DDR1. DDDR1 uttrycks i human bröstcancer. Vi visar att DARPP-32 förhindrar bröstcancer cellmigration och att den anti-migratoriska funktionen av DARPP-32 kräver addition av en fosfatgrupp på Threonin-34 (Thr-34). DARPP-32s anti-migratoriska funktion är också beroende av association med DDR1, eftersom DARPP-32 inte kan inhibera cellmigration i bröstcancer cellinjen MDA-MB-231 som saknar DDR1 uttryck, om inte DDR1 överuttrycks.

I den andra studien, visar vi att Wnt-5a kan fosforylera Thr-34 i DARPP-32 direkt. Detta kräver dock att Wnt-5a aktiveras av ett protein som katalyserar additionen av fosfatgrupper till proteiner, (dessa proteiner kallas för kinaser) nämligen protein kinas A.

Vi visar också att Wnt-5a can inducera DARPP-32 beroende inhibering av cellmigration i bröstcancer cellinjen MCF-7.

Utöver detta kan observerade vi också att Wnt-5a kan inducera fosforyleringen av transkriptionsfaktorn CREB, ett protein som spelar en viktig roll i uttrycket av andra proteiner genom att binda till DNA. Denna fosforylering ökar då DARPP-32 uttrycket. En dominant negativ version av CREB minskade den anti-migratoriska effekten av Wnt-5a och DARPP-32. Detta antyder att Wnt-5a inducerad aktivering av CREB och

DARPP-32 som medieras av PKA representerar ett sätt att begränsa bröstcancer cellmigration.

I den tredje studien visar vi att då celler frigörs från ytan induceras Thr34 av DARPP-32 vilket leder till en dämpning av Fokal adhesions kompleks (FAK) aktivitet. FAK är viktigt för cellmigration eftersom det bildar och degraderar fokal adhesions. Fokal adhesions är interaktions punkter mellan cellerna och ytan som behövs för att cellerna ska kunna binda till ytan eller röra sig längs ytan. En migrationsstudie visade att FAK aktivitet samt Fak interaktionsproteinet Src kinas, är viktiga för den anti-migratoriska funktionen av DARPP-32.

DARPP-32 medierad nedregulering av FAK påverkades av inhibering av PP1, ett protein som tar bort fosfatgrupper från proteiner (dvs fosfataser). Infärgning av MCF-7 celler med antikroppar specifika för FAK aktivitet samt phospho-DARPP-32 avslöjade att celler som uttrycker det sistnämnda proteinet visade mindre infärgning av den aktiva formen av FAK. Slutligen observerade vi att phospho-DARPP-32 reducerar bildningen av filopodia, som är nålformade utsträckningar i cellens framdel och som används för cellrörelse.

Sammanfattningsvis tror vi att DARPP-32 kan hindra bröstepitelceller från att lämna ytan som de normalt sitter på, dvs basal membranet. Detta stödjer cellernas integritet, eftersom de måste binda till basal membranet för att utöva deras korrekta funktion. Vi tror att denna mekanism kan blockera initieringen av metastasering genom att förhindra celler från primärtumören att lossna.

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