Focal adhesion kinase and paxillin : mediators of breast cancer cell migration

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CHAPTER 1

Introduction:

Breast cancer biology and adhesion signaling

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1. Breast cancer

Breast cancer is one of the most common cancers in women world-wide. Despite major advances in diagnostics and treatment over the years, breast cancer is still the leading cause of cancer-related death today (1). Extensive research on various aspects of the disease has been performed over the past decades, but many unresolved tumor cell biological and clinical problems remain. Breast cancer is not a single disease but is composed of different subtypes that are associated with different clinical outcomes (2). Two major breast cancer subtypes can be distinguished: the lobular and the ductal subtype, with the ductal subtype constituting the majority of all diagnosed cases of breast cancer.

1.1 Breast cancer development and classification

The exact etiology of breast cancer is ill-defined, but family history is one of the strongest determinants of risk. Germline mutations in high-penetrance cancer susceptibility genes (like the tumor suppressor genes BRCA1 and BRCA2) account for less than 25% of excess risk, implying that variations in other genes must explain the majority of cases (3). Other risk factors are: sex, age, lack of childbearing or breastfeeding and higher hormone levels.

To explain breast carcinogenesis two models exist: the sporadic clonal evolution model, proposing that any breast epithelial cell can be the target of random mutations, and the cancer stem cell model, which states that only stem and progenitor cells can initiate and maintain tumor progression (bombonati). The multiple transforming events occurring in the breast cancer (stem) cells may be genetically predisposed changes or mutations induced by environmental factors such as UV radiation or exposure to chemicals or by somatic factors such as hormonal fluctuations. Cancer progression occurs by the accumulation of more genetic changes as well as clonal expansion and selection (2). Epidemiological and morphological observations have led to the development of several models of breast cancer progression. For the ductal subtype, the classic model proposes that neoplastic evolution starts in normal epithelium that develops into flat epithelial atypia (FEA), progresses to atypical ductal hyperplasia (ADH), advances to ductal carcinoma in situ (DCIS) and finally culminates as invasive ductal carcinoma (IDC) (4,5).

Breast cancers can be classified in different ways and usually diagnosis and prognosis includes multiple classification aspects. The stage or TNM

classification of breast cancer is defined by three tumor characteristics: the size of the tumor (T), whether or not the tumor has spread to the lymph nodes draining the breast (N), and whether or not the tumor has metastasized (M). TNM staging can range from stage 0 (pre-malignant disease) through stages 1-3 (early cancer) to stage 4 (advanced or metastatic disease). Furthermore, the biopsies taken from the breast and the sentinel lymph nodes are analyzed to classify the tumor according to its histopathological features. Another way to classify breast cancer is by grading the tumor according to its differentiation: well-differentiated or low grade tumors have a better prognosis than poorly-differentiated or high stage tumors. Finally, tumors can be analyzed for certain molecular factors or prognostic markers: hormone receptors and HER2/neu. Nowadays, breast tumors are routinely checked for the expression of the hormone receptors for estrogen and progesterone (ER and PR respectively); these markers can predict prognosis and response to treatment. In about 70% of breast cancer patients ER is overexpressed and these women have a better prognosis than those that have ER-negative tumors (6). Another important prognostic factor is the expression of epidermal growth factor receptor 2 (EGFR2 or HER2/neu); upregulation of this receptor renders tumor cells more aggressive and it worsens the clinical outcome (6). The development of a small molecule inhibitor of HER2, herceptin, has led to significantly prolonged survival for these patients (7). Approximately 15% of breast cancers do not express ER, PR or HER2, this subtype is called triple-negative breast cancer (8).

1.2 Treatment of breast cancer
After the diagnosis of breast cancer is confirmed, treatment depends on the disease stage and pathological features such as receptor status and tumor grade. Improvements in diagnosing breast cancer have made earlier discovery possible and therefore, most new cancer cases are discovered at the ductal carcinoma in situ stage. Most carcinomas in situ are relatively harmless, unless they develop into invasive breast cancer. Stopping carcinomas in situ progression is one of the main aims of treatment. Likewise, current research is often focused on the better understanding of breast cancer progression.

Breast cancer is usually treated first with surgery and then with molecular or chemotherapy and/or radiation. The aggressiveness of the treatment usually mirrors that of the cancer according to the prognosis and the risk of recurrence.

1.2.1 Surgery

Treatment almost always includes surgical removal of the tumor. This is done by removing the part of the breast that contains the tumor or by removing the whole breast (segmental or complete mastectomy, respectively). In cases where the tumor cells have spread to the lymph nodes, these nodes will also be removed.

One of the major challenges of breast cancer surgery is the removal of every last cancer (stem) cell. One sole cancer (stem) cell can potentially form a new tumor (local recurrence) and this can eventually lead to the development of invasive cancer, or distant metastatic disease. Therefore, surgery is usually not the only treatment of breast cancer.

1.2.2 Induction of apoptosis

Tumor cells are rapidly dividing cells in which proliferation signaling is constitutively activated by mechanisms that are highly complex. All cells of the body are constantly monitoring their health and responding to environmental influences. Upon cellular damage, cells can respond according to the type and amount of damage done. They can go into cell cycle arrest, allowing time to repair this (reversible) damage. If the injury is very severe, cells can directly initiate a programmed form of cell death called apoptosis (9). Dividing cells are especially sensitive to the induction of damage as they are constantly multiplying their DNA to form daughter cells. The therapies described below are aimed at causing damage to rapidly dividing tumor cells.

1.2.2.1 Endocrine therapy

During the female menstrual cycle, breast cells are continuously exposed to changes in concentrations of the hormones estrogen and progesterone. The binding of these hormones to their receptors can provide the cells with proliferation signals and can contribute to the development of breast cancer. Indeed, a large portion of breast cancer cases is positive for the presence of ER (10). Hormonal treatment of ER-positive breast cancer is aimed at inhibition of estrogen-induced signaling. The most common endocrine therapy is the use of anti-estrogen agents such as tamoxifen which inhibits the association of estrogen with the ER ultimately leading to cell cycle arrest. Another way to interfere with ER function is to inhibit the synthesis of estrogen by so-called aromatase inhibitors (eg. anastrazole, letrozole) (11). Endocrine therapy is frequently limited by the development of tumor cell

resistance and therefore endocrine therapy is often combined with or followed by radiation and/or chemotherapy. These are also the treatments that are used for tumors that are not positive for ER.

1.2.2.2 Radiation therapy

Radiation therapy involves the application of ionizing radiation directly to the site of the tumor, leading to the induction of cellular damage, cell cycle arrest and ultimately apoptosis. It may also have a detrimental effect on the tumor microenvironment by killing the endothelial lining of tumor microvasculature (12). In order to protect the surrounding tissue, this type of therapy usually consists of many sessions of low-dose irradiation (13). Still, radiation therapy often causes damage to healthy cells, leading to side-effects like skin irritation and fatigue.

1.2.2.3 Chemotherapy

Another way to cause damage to tumor cells is by the use of anticancer chemicals, or chemotherapy. Because these types of drugs are aimed at stopping cell division, they are also called cytostatics. In contrast to radiation therapy, which is applied directly to the site of the tumor, chemotherapy is administered systemically. Because chemotherapeutics act primarily on dividing cells, they not only affect cancer cells, but also rapidly dividing (stem) cells in hair follicles, the digestive tract and the immune system, leading to side-effects like hair loss, nausea and increased vulnerability towards pathogens.

Cytostatics can be classified by the type of damage they cause (14). Alkylating agents like cyclophosphamide add an alkyl group to DNA, thereby preventing cell division. Platinum-based drugs such as cisplatin and carboplatin damage DNA directly by binding to it. Anthracyclines, such as doxorubicin and epirubicin, exert their action by inhibiting topoisomerase II, an enzyme that plays a crucial role in maintaining DNA structure during replication, transcription, recombination and condensation/decondensation of chromosomes. Anti-metabolites interfere with metabolism, examples are: methotrexate acting to inhibit the metabolism of folic acid and the pyrimidine analog fluorouracil (5-FU) which works through noncompetitive inhibition of thymidylate synthase. Taxanes, such as pacitaxel and docetaxel, bind reversibly to β-tubulin and induce tubulin polymerization, thereby disrupting the balance between polymerization and depolymerization and blocking mitosis. Vinca alkaloids, such as vincristine and

vinorelbine, also interfere with microtubule polymerization and arrest cells in the G2/M phase of the cell cycle, blocking cell cycle progression. Combinations of different classes of cytostatics are used routinely in the clinic. One of the most common treatments is cyclophosphamide plus doxorubicin (Adriamycin), known as AC; sometimes a taxane, such as docetaxel, is added, and the regime is then known as CAT. Another common treatment is comprised of cyclophosphamide, methotrexate, and fluorouracil (CMF). The development of new anticancer drugs and the improvement of existing cytostatics are areas of extensive research (14,15). Moreover, understanding the mechanisms of drug resistance is critical to increase the efficacy of anticancer therapy.

1.2.2.4 Molecular therapy
Over the last years, new anticancer agents have been developed and investigated that are completely different from the classical chemotherapeutics. These drugs have their effects on specific molecules in signal transduction pathways that are most often involved in tumor cell survival and proliferation signaling. In general, in these pathways, receptor tyrosine kinases transmit extracellular signals that influence cancer cell growth, survival and differentiation. These receptors are located within the cell membrane and contain an extracellular domain that is the site for ligand binding, a transmembrane domain that anchors the receptor into the cell membrane and an intracellular kinase domain that harbors ATP-binding sites necessary for protein phosphorylation and activation. The receptor tyrosine kinases activate multiple downstream target proteins, and both the receptor tyrosine kinases and their downstream targets are areas of extensive study and search for anticancer drugs (16,17).

The first molecular-based anti-receptor tyrosine kinase therapeutic approved for breast cancer therapy was herceptin (also called Trastuzumab. Herceptin is a humanized murine monoclonal antibody developed against the human epidermal growth factor receptor 2 (EGFR2 or Her2) that has increased expression in approximately 25% of human breast cancer patients and therein correlates with poor prognosis. In tumor cells, herceptin inhibits tumor cell proliferation and induces cell death through multiple mechanisms of action, one of which is the increase of the cyclin-dependent kinase inhibitor p27Kip1, a protein that inhibits cell proliferation (7,18). Herceptin is used specifically to treat patients

with Her2-overexpressing metastatic breast cancer, usually in combination with conventional chemotherapeutics like paclitaxel (19).

Another tyrosine kinase that is targeted by novel molecular therapies is Src kinase, a transducer of diverse cellular signals including survival, migration and promotion of EMT, all involved in various stages of tumor cell progression or invasion. A number of small molecule inhibitors of Src kinase, such as saracatinib (AZB0530), have been developed and preclinical studies show that they can suppress cancer growth and invasion of tumor cells *in vitro* and inhibit disease spread *in vivo* (20). Interestingly, it has been proposed that dual inhibition of Src together with the crucial mediator of growth factor and integrin signaling and the main subject of this thesis, focal adhesion kinase (FAK) could represent a promising new anticancer strategy (21). Indeed, several small molecule FAK inhibitors, such as PF-562,271 are also being evaluated for their antitumor potential (22,23).

1.3 Resistance to treatment

One of the largest limitations of the therapies described above is the occurrence of a resistant phenotype (24). During the course of treatment, cancer cells can become insensitive towards the anticancer agent used until they finally acquire complete resistance. This can result in even more aggressive and invasive cancer cells (25). Resistance can also be an inherent trait of a subpopulation of cancer cells. Resistance to chemotherapy is an intensively studied subject, though it is still not completely understood. Tumor cells can acquire resistance through many different mechanisms, including enhanced DNA repair, altered membrane transport, genetic responses, alterations in target molecules or growth factors and metabolic effects (26).

It has been proposed that in ER-positive breast cancer, cross-talk between the ER and growth factor receptor signaling pathways can occur, leading to sustained ligand-independent activation of the ER, despite the use of anti-hormones such as tamoxifen (27). In some breast cancer cells, chemoresistance to anthracyclines, alkylating agents and taxanes is mediated by mitogen-activated protein kinase (MAPK) phosphatase-1 (MKP-1) (28). Altered cell signaling can be mediated by MAPK pathways and tight regulation of these pathways is of vital importance for cell fate. Activation of MAPKs is facilitated by MAPK kinases (MKK) while MAPKs can be negatively regulated by MKPs. In BT-474 and

MDA-MB-231 cells overexpression of MKP-1 counteracts chemotherapy-induced apoptotic signaling through the MAPK c-jun N-terminal kinase (JNK) (29). Another mechanism by which resistance can be acquired is the upregulation of drug transporters or drug-metabolizing enzymes that rid the tumor cells of chemotherapeutic drugs before they can exert their action. In particular, the overexpression of glutathione S-transferases (GST) and efflux pumps in tumors may reduce the reactivity of various anticancer drugs (30). Interestingly, GSTs are also involved in the control of apoptosis by inhibition of the JNK pathway (31,32). Reduced protein expression or gene mutations in the tumor suppressor p53 can also be the cause of chemoresistance. The p53 protein is an important regulator of the cell cycle and is sensitive to any DNA damage caused during replication. Normally p53 will cause cell cycle arrest or apoptosis upon genotoxic stress to prevent the production of defective cells, but in cancer cells p53 is frequently mutated or lost leading to defects in p53-mediated apoptosis and resistance to DNA-damaging agents (33). Recently, it has been recognized that the microenvironment of tumors consisting of fibroblasts, vascular network, inflammatory cells and extracellular matrix (ECM) plays a critical role in responses to anticancer treatments. For example, some regions of the tumor microenvironment (TME) are hypoxic due to high proliferation rates of cancer cells. The delivery and uptake of drugs in hypoxic areas is decreased due to limited blood flow and drugs that require oxygen to generate free radicals cannot exert their action. Furthermore, hypoxia can lead to a decrease in cell proliferation rates while anticancer drugs specifically target fast-proliferating cells. Conversely, hypoxia can induce adaptation increasing cell survival signaling. All these consequences of hypoxia can contribute to the development of tumor cell chemoresistance (34).

Many (pre)clinical drug efficacy studies focus on the use of different treatment regimens including the combination or sequential use of endocrine therapy, chemotherapy and/or molecular therapy. When tumor cells have acquired resistance towards one agent, treatment will be continued with another drug. Also, the order of different treatments can be varied: for example treating the patient with chemotherapy before surgery (neoadjuvant) or after (adjuvant). Treatment of breast cancer is becoming increasingly multidisciplinary and patient-tailored.

2. Metastasis formation

As mentioned before, primary breast tumors can progress to invasive breast cancer, and this is related to a poor prognosis. Metastasis formation involves several distinct steps (35), which will be described below (figure 1).

First of all, primary tumor cells must migrate through and invade into the surrounding tissue, to find their way to neighboring tissue or a blood or lymphatic vessel. The change in phenotype that is necessary for tumor cells to be able to invade their surroundings is typically called epithelial to mesenchymal transition or EMT (36,37). The tumor cells lose their epithelial characteristics including their polarity and specialized E-cadherin-based cell-cell contacts and they acquire a migratory phenotype (38). The cells also secrete enzymes called matrix metalloproteinases (MMPs) that can degrade the surrounding ECM (39). In this way, the tumor cells can escape the primary tumor. Once these mesenchymal cells have invaded the surrounding tissue and intravasated into blood or lymphatic vessels after degrading their vessel walls, they must be able to survive inside these vessels without the environmental cues they normally receive from the primary tumor environment. In contrast to normal cells, in which the loss of these cell adhesion cues results in a specific type of cell death called anoikis, invasive tumor cells have acquired a phenotype that enables them to resist the induction of anoikis (40). When tumor cells inside the vessel have reached the target organ site, they will adhere to the vessel wall and extravasate into the target tissue or they will get stuck in the microvasculature. Next, the tumor cells will start proliferating to form new tumors called secondary tumors or metastases. At these sites, tumor cells can also enter a dormant state and in this case, they will start proliferating only after they have received the right local molecular triggers.

For each type of cancer, the clinical course of the events described above occurs with distinct temporal kinetics and to unique distant organ sites (41,42). The sites most frequently colonized by primary breast tumor cells are bone and lung, and to a lower extent liver and brain (43). Each organ places different demands on circulating cancer cells for the establishment of metastases. For example, brain metastasis formation requires tumor cells to travel across the blood brain barrier and to interact with glial cells, while colonization of the lungs demands extravasation through lung capillary walls and survival and proliferation in the lung-specific chemokine and cytokine microenvironment.
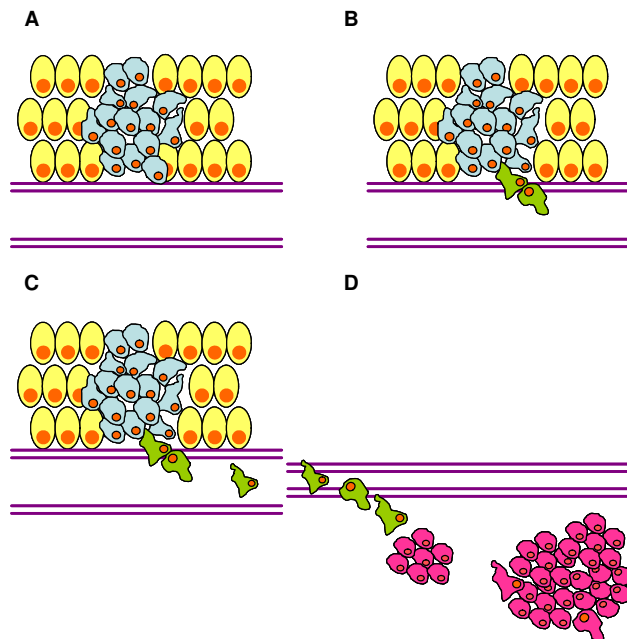
Figure 1. Key steps in metastasis formation. For metastasis formation to occur, cells must go through the metastatic cascade, consisting of multiple steps. (A) By mutation(s) or other stimuli cells from the primary tumor can acquire a metastatic phenotype and start to undergo epithelial-to-mesenchymal transition. (B) These cells can then degrade the ECM and intravasate into the bloodstream or lymphatic vessels. (C) When these cells survive without their normal adherence cues inside these vessels, they can finally arrest in distant organs and adhere to the vessel wall. (D) There, the cells extravasate, migrate through the target tissue and start to proliferate to form a micrometastasis. Dormancy of tumor cells may occur, rendering them inactive until, upon stimulation by proliferative cues, they will start to proliferate once more to form a macrometastasis.

Finally, bone-marrow homing and interaction with osteoclasts is necessary for tumor cells to be able to form bone metastases (44). All these different processes require the transcription of specific genes, and these genes can be identified by molecular profiling of cell lines, (tumor) samples from animal models, or human (tumor) samples. Genome-wide expression profiling has unraveled sets of genes that are associated with organ-specific metastasis. This has resulted in the

definition of metastasis gene expression signatures with a predictive value for the development of secondary tumors in a specific organ, like lung and bone (45,46,41). The lung metastasis signature identified by Minn *et al* includes genes encoding for the collagen-protease MMP-1, involved in degradation of the extracellular matrix, the EGFR ligand epiregulin, promoting growth and survival signaling, chemokines like CXCL1 and the cyclooxygenase COX2 (41). EGFR ligands and COX2 were also identified in a brain metastasis signature, suggesting that these mediators are shared by brain and lung metastasis formation. In contrast, the alpha2,6-sialyltransferase ST6GALNAC5 specifically mediates the formation of brain metastases. The expression of ST6GALNAC5 is normally restricted to the brain, and breast cancer cells enable their adhesion to brain endothelial cells and their passage through the blood-brain barrier by expressing this sialyltransferase (45).

2.1 Models to study metastasis formation

Inhibiting the invasiveness of tumor cells would result in a large improvement of breast cancer prognosis, and therefore the (molecular) mechanisms of metastasis formation are studied extensively (40,47). Different models to study these processes exist, ranging from simple models studying breast cancer cell migration *in vitro* to very complex models studying metastasis formation in an *in vivo* setting. A short outline of the available models and the models used to generate the data discussed in this thesis will be given here.

2.1.1 *In vitro* models

To study breast cancer cell behavior *in vitro*, breast cancer cell lines are available that differ in their biological characterisitics (eg source, tumor type, ER/Her2/p53 status, basal-like versus luminal-like). Some examples of frequently used human breast cancer cell lines are MCF-7, MDA-MB-231, BT-474 and SK-BR-3. Rat or mouse-derived breast cancer cell lines are also available, and an advantage of their use is their compatibility with syngeneic animal models, as described below. In this thesis, all studies were performed using MTLn3 mammary adenocarcinoma cells. These are invasive breast cancer cells that have a high migratory and metastatic capacity.

Often, cell lines are genetically manipulated by transient or stable transfections or RNA interference-based approaches. *In vitro* assays to study

processes involved in metastasis formation include: measurement of random cell migration, wound healing assays, transwell migration, 3D invasion assay or soft agar colony formation, measurement of focal adhesion turnover using total internal reflection fluorescence (TIRF) microscopy and measurement of focal adhesion dynamics using fluorescence recovery after photobleaching (FRAP). In this thesis, almost all of these assays were used to study the role of the focal adhesion-asscoiated proteins FAK and paxillin in breast cancer cell migration.

2.1.2 *In vivo* models

Animal models remain essential to understand the fundamental mechanisms underlying cancer progression and metastasis formation and these models are indispensable in the discovery of new methods to diagnose, treat and prevent cancer. Because of its complexity, no single model can mimic all aspects of the disease. Many different experimental animal model are available, each studying different features of cancer. Transgenic mice that either have a (tissue-specific) ectopic expression of oncogenes (e.g Her2) or a (conditional) knockout of tumor suppressors (e.g p53) are often used to study the development and progression of primary tumors. The metastatic process is more often studied in orthotopic xenograft models in which (genetically manipulated) tumor cell lines are injected into immunodeficient mice. A third model that can be used is the so called syngeneic model, in which a tumor cell line isolated from a spontaneous or chemically-induced tumor is injected back into animals with the same genetic background. In this way, tumorigenesis and (experimental) metastasis formation can be studied, including the contribution of the immune system. In this thesis, a syngeneic rat model (chapters 2, 3 and 4) and an immunodeficient mouse model (chapter 6) have been employed.

The syngeneic rat model we used involves Fisher 344 rats and the rat mammary adenocarcinoma cell line MTLn3. The MTLn3 cell line originates from a mammary tumor cell line called MTC, these were derived from a chemically-induced mammary gland tumor of a female Fisher 344 rat (48). MTLn3 cells can easily be cultured and manipulated *in vitro* to study certain cell biological processes (49,50,51,52), but the cells can also be injected back into Fisher 344 rats to study processes occurring *in vivo*. For chapter 2, 3 and 4 of this thesis, genetically modified MTLn3 cells were injected either into the mammary fat pad to study primary tumor growth or into the lateral tail vein to study experimental lung

metastasis formation. Although the MTLn3 cells do not give rise to immune cell activation due to their genetic compatibility, we show that MTLn3 cells injected into Fisher rats are rapidly killed by natural killer (NK) cells of the innate immune system. As a consequence, MTLn3 cells are not able to efficiently form metastases unless the female Fisher 344 rats are depleted of NK cells by prior treatment with a NK-depleting antibody. In chapters 3 and 4, we made use of the same syngeneic model, including NK cell depletion.

The involvement of immune cells can also be avoided by making use of immunodeficient animals, lacking certain cells of the immune system. The *in vivo* mouse model described in chapter 6 makes use of transgenic immunodeficient Rag2$^{-/-}$γc$^{-/-}$ mice. In contrast to the most widely used immunodeficient mice (nude and severe combined immunodeficient (SCID) mice) that still harbor cells of the innate immune system including natural killer (NK) cells, Rag2$^{-/-}$γc$^{-/-}$ mice are completely void of NK cells. We showed that NK cells are capable of killing circulating MTLn3 tumor cells, thereby preventing metastasis formation (chapter 2). By making use of animal models that lack NK cells, tumor cell autonomous metastasis programs can be studied independently of NK cell interference (53). In chapter 6 we describe a model in which Rag2$^{-/-}$γc$^{-/-}$ mammary fat pads are injected with rat mammary adenocarcinoma cells (MTLn3) expressing GFP-tagged wildtype or mutant paxillin (for details on paxillin, see below). During the experiment, the size of primary tumors is measured using calipers and at the end of the experiment, mice are anesthetized and primary tumors are removed and weighed. In addition, the lungs are removed and GFP-tagged protein-containing secondary tumors are visualized and counted.

3. Cellular responses to anticancer drugs

There are many different ways to treat breast cancer, as outlined above. The responsiveness of individual patients to their drug treatment depends on the response of single tumor cells to the anticancer drugs that are used. Cellular responses to anticancer drugs were also studied in this thesis: two anticancer agents were used, doxorubicin and vincristine. Below, their mechanisms of action and effects on cellular signaling will be discussed.

Doxorubicin is an anthracycline antibiotic that induces apoptosis by blocking DNA synthesis in two ways. Firstly, it inserts itself into DNA and thereby blocks DNA synthesis in the interphase of cell division. Secondly, doxorubicin

inhibits topoisomerase II and eventually, this leads to strand breaks in the DNA. Doxorubicin is also responsible for the formation of reactive oxygen species (ROS) through its reduction to a free radical intermediate. Doxorubicin is very common in the treatment of metastatic breast cancer (54,55) and its mechanisms of action have been studied in great detail. Interestingly, in MTLn3 cells, doxorubicin causes activation of the survival protein kinase B (PKB) pathway dependent on FAK localization and function at focal adhesions. Inhibition of FAK sensitized MTLn3 cells to doxorubicin-induced apoptosis (52). In chapter 4, our knowledge on the involvement of FAK in the doxorubicin response was utilized to study a possible synergy between chemotherapy (doxorubicin) and molecular therapy (inhibition of FAK) *in vivo*.

Vincristine, a member of the vinca alkaloids derived from the plant *Catharanthus roseus*, is a microtubule-interfering agent. Microtubules are a major component of the cell's cytoskeleton and are very important in the process of mitosis by facilitating the separation of the duplicated chromosomes to form two daughter cells. By directly targeting microtubules, microtubule-interfering agents have antimitotic (56) and apoptosis-inducing (57,58) effects. Vincristine, like other microtubule-interfering agents, causes a drastic activation of the c-jun N-terminal kinase (JNK) signaling cascade in human breast and ovarian cancer cell lines (59,58) or other MAPKs like p38 or extracellular signal-regulated kinase (ERK). Subsequent substrate activation may differ between different microtubule-interfering agents, indicating that JNK activation does not always result in activation of the same downstream signaling routes: in cervical carcinoma cells vinblastine acts through a transcription factor activator protein-1 (AP-1)-dependent mechanism, while taxol works AP-1 independently (60). In MTLn3 cells, exposure to vincristine leads to JNK activation and subsequently to phosphorylation of the AP-1 family member c-jun (chapter 5).

Because microtubules make up an important part of the cell's cytoskeleton, interfering with their structure also has profound effects on cell morphology and motility. Although vincristine is not used to treat breast cancer, it is an excellent model compound to study cytoskeletal reorganization and the signaling networks it employs (61,62,59). Microtubule-interfering agents induce an increase in F-actin stress fibers and focal adhesions, related to increased contractility. This change in the adhesion phenotype of tumor cells is very interesting in the light of the changes that occur in tumor cells when they acquire a metastatic phenotype. The signaling

networks influenced by vinca alkaloids are most likely also involved in cell migration (and metastasis formation), which is a highly complex process involving the constant remodeling of the cytoskeleton. In chapter 5, the response of MTLn3 cells to vincristine is studied.

3.1 Signal transduction pathways

Eukaryotic cells can respond to changes in the physical and chemical properties of environment by managing signal transduction pathways inside the cell. Many signal transduction pathways participate and cooperate in these responses and because of their complexity, it is not feasible to discuss them all. In this introduction the following signaling pathways involved in cellular responses to stress and relevant for this thesis are discussed: MAPK pathways and the downstream AP-1 transcription factors (in particular Fos-related antigen-1 (Fra-1)).

3.1.1 MAPK signaling

Mitogen-activated protein kinase (MAPK) signal transduction pathways are among the most widespread mechanisms of cell regulation (reviewed in (63)). Depending upon the stimulus, a distinct signaling pathway can be activated, allowing cells to respond correctly to a wide variety of stimuli, including hormones, growth factors, inflammatory cytokines, chemokines and environmental stresses like radiation, osmotic shock, ischemic injury, heat shock, oxidative stress and chemicals. These stimuli may act through different types of receptor families coupled to MAPK pathways, such as G protein-coupled receptors (GPCRs), cytokine receptors, receptor tyrosine kinases (RTKs) and Ser/Thr kinase receptors. Activation of MAPK pathways coordinates diverse cellular activities like gene expression, differentiation, cell cycle progression, metabolism, motility, survival and apoptosis.

MAPKs are regulated through a three-kinase module: each MAPK is activated by dual phosphorylation mediated by a MAPK kinase (MAPKK or MAP2K) that is activated through phosphorylation by a MAPKK kinase (MAPKKK or MAP3K). Three major MAPK families have been described in mammalian systems: extracellular signal-regulated kinase (ERK), p38 and c-Jun N-terminal kinase or stress-activated protein kinase (JNK/SAPK). The ERK family of MAPKs is activated primarily by mitogenic stimuli, while p38 and JNK respond mainly to environmental stresses like genotoxic stress, withdrawal of growth factors, heat shock, metabolic stress, UV irradiation, osmotic shock and

inflammatory cytokines such as tumor necrosis factor (TNF) and interleukin 1-β (64).

Two isoforms of ERK are known (ERK1 and ERK2). ERKs are generally activated by growth factors and cytokines but they can also be activated by osmotic stress, microtubule disorganization, oxidative stress, for example through growth factor receptors such as EGFR and platelet-derived growth factor receptor (PDGFR) (65). ERK generally functions in a cascade initiated by activation of cell surface receptors (eg RTKs or GPCRs). The signal is then transduced to small G proteins, such as Ras, which transmit the signal by recruiting a MAP3K molecule like c-Raf kinase. Activated c-Raf (or other MAP3K) binds to and phosphorylates the MAP2Ks MEK1 and MEK2, which in turn phosphorylate and activate ERK1/2. Active ERKs can consequently phosphorylate numerous substrates in different cellular compartments, including cytoskeletal proteins (for example paxillin (66,67,68)), other kinases (for example FAK (69,70)), phosphatases and transcription factors (like c-jun and c-fos). These diverse targets downstream of ERK have key roles in cell proliferation and angiogenesis and interestingly also in cell migration (71), invasion and metastasis (72,73,74).

The mammalian JNKs are encoded by three genes (JNK1, JNK2 and JNK3). JNKs respond strongly to cytokines, UV irradiation, growth factor deprivation and DNA damage. JNKs are activated by phosphorylation on Thr and Tyr residues within a conserved Thr-Pro-Tyr motif in their activation loop by upstream activators MKK4 and MKK7; MKK4 is primarily activated by environmental stress, while cytokines are the activators of MKK7. Upstream activators of MKK4 and MKK7 include several MAP3Ks: MEKK1-4, MLK2/3 and ASK1/2 (75) with different MAP3Ks being specific for different stimuli. JNK is named after its most extensively studied substrate c-jun, which induces AP-1 transcriptional activity upon phosphorylation. But JNK has numerous substrates located in multiple cellular regions. Besides c-jun, nuclear substrates include: other proteins of the jun family, proteins of the fos family and activating transcription factor (ATF) family, the tumor suppressor p53 and nuclear hormone receptors (like PPARγ). Non-nuclear substrates of JNKs are scaffold proteins JIP1 and JIP3, cytoskeletal proteins such as paxillin and microtubule-associated protein-2 (MAP-2) and other kinases such as Akt (63). The diversity of JNK substrates mirrors the diverse array of biological responses mediated by JNK: apoptosis (76), cell survival, development and differentiation and cell migration (77,78,79). The

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contribution of JNK signaling to both apoptosis and survival may seem contradictory. However, the duration and the magnitude of JNK activation is crucial in the determination of biological outcomes regarding cell survival or apoptosis, but also processes like EMT and invasion (80,81).

Four p38 isoforms exist: p38-alpha, p38-beta, p38-gamma and p38-delta. Cytokines, environmental stress stimuli and ischemia-reperfusion can activate p38 signaling pathways. The activators directly upstream of p38 are MKK3 and MKK6. Some studies show that p38 can also be activated by JNK activators MKK4 and MKK7 (82,83), suggesting that to some extent cross-talk may exist between JNK and p38 signaling pathways. Upstream activators of MKK3 and 6 are many and include: MTK1, MLK2/3, ASK1 and TAK1. G proteins Rac and Cdc42 have also been identified as p38 activators, through the downstream mediator p21-activated kinase (PAK) (84,85), linking p38 to cytoskeletal organization. Activated p38 has been shown to phosphorylate several cellular targets including transcription factors (ATF1, 2 and 6, p53), kinases (MAPK-activated protein kinase 2 (MK2), MK3 and MAPK-interacting kinase (MNK1)) and (cytoskeletal) scaffold proteins (paxillin (86)). The activity of p38 is required for the induction of apoptosis by chemotherapeutic agents (87), and several other biological processes such as cell cycle arrest, inflammation, development and differentiation can be mediated by p38 activity.

As outlined above, the signaling capacity of MAPK families is high and diverse. How can one cell, containing all these different molecules, coordinate the signaling events to ensure the correct biological outcome? The specificity of the different signals transmitted by the MAPK cascades is regulated by several mechanisms: the duration and magnitude of signals, the interaction with diverse scaffold/adaptor proteins, subcellular localization, the presence of isoforms at each level of the MAPK cascades, cross-talk between different MAPK pathways as well as between MAPK pathways and other pathways and finally, other post-translational modifications in addition to phosphorylation. All or some of these mechanisms are applied whenever cells encounter stress, like also the exposure to cytostatic drugs. Elucidating the multiple signaling pathways activated by different cytostatics may be helpful in the pinpointing of important players that may serve as targets for the improvement of existing therapies.

3.1.2 Fra-1 signaling

Besides post-translational modifications of proteins, mediated by kinases that phosphorylate their targets, changes in gene expression are an important way of altering cellular responses. AP-1 transcription factors include Jun and Fos families. The Jun family consists of c-jun, JunB and JunD and the Fos family includes c-Fos, FosB, fos-like antigen-1 (Fra-1) and Fra-2. The AP-1 transcription factor complex is a dimer consisting of jun-jun homodimers or jun-fos heterodimers (88). AP-1 regulates a variety of cellular processes, including survival, apoptosis, proliferation, differentiation, migration, invasion (89,90,91,92) and contributes to both basal and external stimulus-induced gene expression (e.g. growth factors, chemokines and ECM). Activation of AP-1 occurs at transcriptional and post-translational levels, and is signaled mainly through the MAPK cascade (93,94,95). (Abnormal) induction and/or activation of AP-1 proteins by chemical exposure or mitogens can contribute to the development and progression of various diseases, including cancer (96).

Elevated Fra-1 mRNA and protein levels have been detected in multiple human carcinomas and tumor cell lines, including ER-negative breast tumor cells (97,98,99). Fra-1 may also play an active role in motility and invasion of mammary tumors: Fra-1 expression correlates with the mesenchymal characteristics of epithelial tumors and overexpression of Fra-1 in epithelioid carcinoma cells greatly influences cell morphology, motility, and invasiveness (100,101). One interesting mechanism by which Fra-1 regulates motility and invasiveness, is through the induction of MMP-2 and MMP-9 expression as well as EGFR-activated signaling (89). Furthermore, in chapter 4 of this thesis, we found a link between adhesion signaling and Fra-1 activity. FAK inhibition induced Fra-1 expression in MTLn3 cells, and loss of Fra-1 in these cells reduced cell adhesion and migration in association with increased stable focal adhesion formation. Knockdown of Fra-1 sensitized MTLn3 cells towards doxorubicin, in agreement with its role in cell survival.

3.2 Adhesion and survival signaling

One very important aspect in a cell's decision to live or die is the adhesion of the cell to its surrounding ECM. The adhesion in itself gives rise to survival signaling, which counteracts possible apoptotic signals. On the other hand, a special type of apoptosis, called anoikis, is induced when cells are deprived of their cell-ECM

contacts for too long. Adhesion to ECM is mediated by heterodimeric transmembrane receptors, called integrins. Upon ECM ligand binding, integrin signaling is directed in two ways: organization of the cytoskeleton and regulation of cellular behavior through survival, differentiation or growth (figure 2). Also, there is extensive cross-talk between integrins, growth factor receptors and cytokine receptors (102). Pathways that are activated by adhesion include MAPK, FAK/Src and phosphatidylinositol-3-kinase (PI3K)/Akt pathways. An interesting concept is that the pathways involved in adhesion-mediated survival signaling not only mediate migration and thereby metastasis formation, but also play key roles in the protection against cell killing by cytostatics (103). To facilitate these essential survival triggers, cells are equipped with specialized structures that mediate cell adhesion-based survival signaling pathways: the focal adhesions.
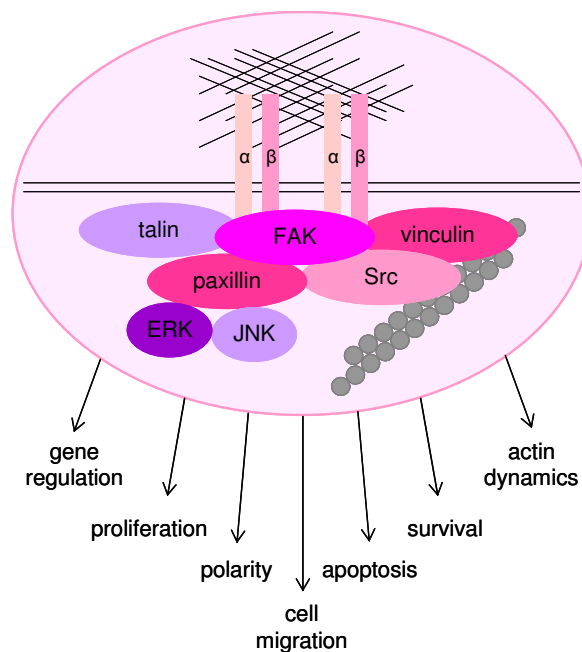
Figure. 2. General model of cell–matrix adhesions and their downstream regulation. Cell-ECM adhesions containing clusters of integrins recruit cytoplasmic proteins, which in cooperation with other cell surface receptors control diverse cellular processes, functions, and phenotypes.

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4. Focal adhesions

As mentioned above, a cell's connection to the ECM is of vital importance for its survival and proliferation. Cells attach to the ECM through specialized receptors called integrins, which link the ECM to the intracellular actin cytoskeleton (104). Upon binding to ECM components, integrins cluster and numerous cellular proteins are recruited into large aggregates called focal adhesions. Focal adhesions are the closest contacts between cells and ECM. In addition to a structural role, these events also initiate signaling pathways involved in cell growth, survival and migration. One of the first proteins to assemble on clustered integrins is FAK. Binding to integrins causes activation of FAK, and this recruits other signaling, adaptor and structural proteins (105). Depending on the stimulus or ECM component, the focal adhesion composition can vary.

4.1 Focal adhesions and cell migration

The attachment of cells to the substratum is not only crucial to survival cues; it is also a major factor in cell migration. To migrate, cells must be able to detach and attach in a controlled manor. Focal adhesions must be disassembled at detachment sites, and new adhesions need to be formed at areas of attachment. These processes require a tightly regulated and highly dynamic organization of focal adhesions. In this thesis, we have investigated the role of two focal adhesion components, the non-receptor tyrosine kinase FAK and the adaptor protein paxillin, in survival signaling and cell migration of breast cancer cells. Their significance and importance in these processes will be explained in a short introduction on both proteins below.

5. Focal adhesion kinase

FAK is a 125 kD non-receptor protein-tyrosine kinase that localizes to focal adhesions. It was identified in 1992 as a substrate of the viral Src oncogene. FAK is expressed in almost all tissues and cell types and plays an important role in signal transduction initiated either at sites of cell attachment or at growth factor, chemokine or G protein-coupled receptors. Activation of FAK leads to a variety of biological processes including survival, attachment, migration and proliferation. It is also crucial during the early stages of embryogenesis. FAK mRNA and protein levels increase from embryonic day 7.5 of mouse development and FAK-null embryos die at embryonic day 8.5 (106).

5.1 FAK structure and activity

FAK is composed of an N-terminal FERM (band four point one, ezrin, radixin and moesin homology) domain, followed by a proline-rich region, a central kinase domain, two more proline-rich regions and a C-terminal FAT (focal adhesion-targeting) domain (107). See figure 3 for a representation of FAK structure. The FERM domain facilitates signal integration from growth factor receptors such as the Eph family (108,109), HGF (107), EGF, and PDGF (110), and the non-receptor tyrosine kinase Etk (111). This domain also interacts with JSAP1, a scaffold for the JNK pathway (112), the cytoskeletal-associated protein ezrin (113), SUMO proteins (114) and the cytoplasmic tail of beta-integrins (115,110).

The first proline-rich region has been identified as a SH3 binding site for Trio (116) and Src family members (117). The second and third proline-rich regions bind other SH3-containing proteins such as p130Cas (Crk-associated substrate) (118,119), the Graf (GTPase regulator associated with FAK) Rho-GTPase activating protein GAP (120) and ASAP1 (Arf-GAP containing SH3 domain, Ankyrin repeats and PH domain) (121).

The kinase domain contains tyrosine 397, phosphorylation of which is considered to be crucial for integrin-mediated FAK activation. After integrin clustering, FAK is recruited and undergoes a conformational change resulting in a release of the interaction of the FERM domain with the kinase domain. Autophosphorylation of tyrosine 397 is the result and phosphorylation of this residue induces the formation of a SH2 binding site for Src, leading to increased Src activity and subsequent maximal activation of FAK by phosphorylation of tyrosines 576 and 577 within the kinase domain activation loop. Src activity in complex with active FAK promotes the phosphorylation of tyrosines 861 and 925, the latter becoming a binding site for the adaptor protein Grb2 (growth factor receptor binding protein 2). Adaptor proteins such as Shc, paxillin and p130Cas as well as cytoskeletal proteins like alpha-actinin can be tyrosine phosphorylated by the active FAK-Src complex (122,117).

FAK is localized to sites of integrin clustering at focal adhesions through interactions of the FAT domain with adaptor and cytoskeletal proteins such as paxillin and talin (123,124,125). There are also reports that FAT can directly interact with integrins (126,127).

The ability of FAK to integrate incoming and outgoing signals controlling the processes of survival, proliferation, attachment and motility are regulated by the complex interactions with and phosphorylation by other proteins.
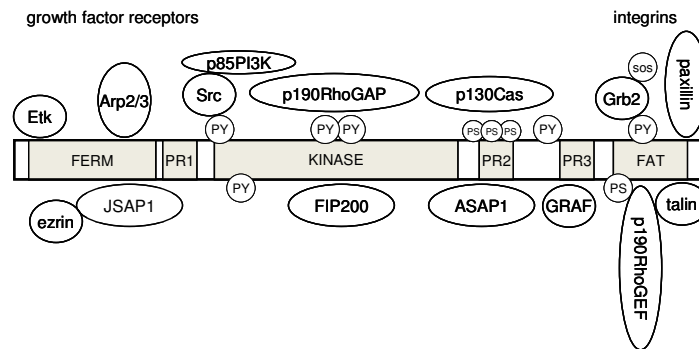
Figure 3. FAK structure and binding partners. FAK is comprised of the amino-terminal FERM domain, the central kinase domain and the carboxy-terminal FAT domain. Furthermore, FAK contains three proline-rich regions (PR1-PR3) and many putative phosphorylation sites (PY, PS). Many FAK-binding proteins have been identified that are involved in cell proliferation, survival signaling and cell motility.

5.2 FAK signaling

FAK activation is a point of convergence for different signaling pathways to affect changes in cell behavior. For example, several pathways downstream of FAK contribute to cell survival. Phosphorylated tyrosine 397 can bind to the p85 subunit of PI3K (128). Phospholipid phosphorylation mediated by PI3K can lead to activation of Akt which in turn, can inhibit apoptosis by regulating several components of the cell death machinery (129,130). FAK has also been shown to suppress apoptosis through other mechanisms, such as activation of JNK or inhibition of the protein kinase RIP with the death receptor complex (131). Interaction of the FAK FERM domain with p53 suppresses transcriptional activation of a number of p53 target genes that inhibit apoptosis. A FAK/p53 complex can also be detected in the nucleus, where FAK promotes ubiquitin proteasome-mediated p53 degradation, contributing even more to inhibition of apoptosis and thus to cell survival (132). Thus, the pathways through which FAK promotes cell survival include both kinase-dependent and kinase-independent mechanisms.

Several FAK signaling pathways have been shown to stimulate cell proliferation. The phosphorylation of tyrosine 925 by Src mediates the interaction

of FAK with Grb2 leading to activation of the Ras-ERK pathway, while the interaction of FAK with adaptor protein Shc also contributes to proliferation through this pathway (133,134). Furthermore, FAK activity can stimulate cell cycle progression by increasing the expression of cyclin D1 and decreasing the expression of p21 (135).

In addition to a positive role in cell survival and proliferation, FAK signaling contributes to efficient cell motility. FAK-null mouse embryo cells have motility defects (106) which can be rescued by reconstitution with wildtype FAK, but not mutants lacking tyrosine 397 (136,137). Cell motility events are largely controlled by the Rho family GTPases that regulate actin cytoskeleton dynamics. FAK signaling has been implicated in regulating the activities of Rac1 and RhoA, which promote the formation of lamellipodia and focal adhesions respectively (138).

Rac1 activity is regulated by FAK through two downstream substrates: the adaptor proteins p130Cas and paxillin. The SH3 domain of p130Cas binds to FAK proline-rich regions, allowing FAK to recruit Src to mediate p130Cas phosphorylation. Next, a signaling complex is formed, consisting of Crk, DOCK180 and ELMO. The Crk/DOCK180/ELMO complex recruited by p130Cas in adhesions has GEF activity towards Rac1, leading to actin polymerization and subsequent plasma membrane protrusions (139).

Paxillin can bind to the FAT domain of FAK and can be directly phosphorylated by FAK (and Src) at tyrosines 31 and 118, promoting Crk binding and some of the same downstream pathways described above for p130Cas (140). Also, these phosphorylated tyrosines can bind to p120RasGAP, which induces the release of an inhibitory interaction between p120RasGAP and p190RhoGAP. Once released, p190RhoGAP suppresses the activity of RhoA, thereby stimulating Rac1 activity (141).

Alternatively, FAK signaling has been implicated in the downregulation of Rac1 activity. Knockdown of FAK or paxillin by RNA interference in HeLa cells resulted in an increase of peripheral Rac1 activity (142), and in another study, phosphorylation of the ArfGAP PKL (paxillin kinase linker) by FAK or Src suppressed Rac-induced lamellipodia formation (143). In addition, FAK was found to interact with p190RhoGEF to promote RhoA activity in neuronal cells (144).

Taken together, these findings show that FAK signaling contributes to the coordination of Rac1 and RhoA activities, critical for the maintenance of cell

polarity and directional movement. Another process that involves FAK signaling and is, like cell motility, indispensable for metastasis formation, is cell invasion. Through its interaction with p130Cas, FAK can promote JNK-mediated transcriptional activation of MMP-2 and MMP-9, leading to matrix-degradation which could facilitate invasion of cells into the surrounding tissue (145).

The processes described above all implicate that FAK could be an important mediator of tumor cell progression (increased cell survival, resistance to apoptosis), invasion and/or motility. Below, the evidence of FAK's involvement in the pathogenesis of cancer is discussed in more detail.

5.3 FAK and cancer

Although FAK itself has not been demonstrated to be an oncogene, overexpression and/or increased activation of FAK is widely observed in a large number of human malignancies, both at early and advanced stages of tumorigenesis. Theses cancers include melanoma, sarcoma, cervical carcinoma, prostate, colon, breast and ovary. Furthermore, amplification of the region of chromosome 8q24, that encodes the FAK gene, has been found in human tumors and tumor-derived cell lines of squamous cell carcinomas, lung, breast and colon. Using paired normal and neoplastic tissue samples, increased levels of FAK mRNA and protein were found in a vast majority of invasive and metastatic tumors compared to normal tissues or non-invasive benign tumors (107).

The increased expression of FAK in cancer may be the result of deregulation at several levels. First of all, FAK mRNA can be elevated, as was found in colon, breast, prostate and hepatocellular carcinomas (146,147,148). FAK overexpression can also be the result of and increase in FAK gene dosage, as is the case in squamous cell carcinomas of the head and neck. Increased gene copy number and FAK protein levels are not strictly correlated, suggesting additional levels of regulation, such as gene transcription, RNA processing, translation and protein stability (149,150).

A very important question is whether FAK overexpression has predictive value for disease prognosis. Studies addressing this question have found conflicting results. While FAK expression was of no prognostic significance in a study of node-negative breast cancer (151), FAK overexpression was found to be a good indicator of patient survival in hepatocellular carcinoma (152) and in colorectal cancer, high levels of FAK and Src were predictive for tumor recurrence (153).

Because FAK overexpression does not necessarily lead to more FAK signaling, it may be more informative to focus on the analysis of levels of FAK activity.

FAK has been proposed as a potential target for anticancer therapy, based on its common overexpression in cancer and its role in the promotion of cell survival and migration/invasion (154,23). Several small-molecule inhibitors for FAK have recently been described, and some of them have already moved to phase I clinical trial (107).

6. Paxillin

Another major player at focal adhesions is paxillin, a 68 kD adaptor protein. Paxillin was first identified as a tyrosine-phosphorylated protein in cells transformed by the Src oncogene (155). Next, paxillin was purified from chicken gizzard smooth muscle tissue and characterized as a direct binding partner for the focal adhesion and actin binding protein vinculin (156). Since then, it has been established that paxillin is a multi-domain adaptor protein that recruits both structural and signaling molecules to focal adhesions, where it coordinates the transduction of growth factor and adhesion signals. In this way, paxillin is essential for changes in cell migration, adhesion dynamics and cell survival. Paxillin is expressed in almost all adult human tissues, except for brain (157,158). In a mouse paxillin knockout model, critical developmental defects were found and this, together with the fact that the paxillin-null embryos are not viable past embryonic day 9.5, confirms the critical importance of paxillin during development (159).

In higher eukaryotes, three paxillin alternative splice isoforms have been identified: paxillin alpha is the most ubiquitously expressed isoform; paxillin beta and gamma show more restricted expression (157). All three isoforms seem to also be expressed in mouse (160). A fourth isoform called paxillin delta is the product of alternative translation initiation, it has not been extensively described (140,161). Two other members make up the paxillin family: hydrogen peroxide inducible clone-5 (Hic-5), which demonstrates widespread expression (162), but is absent from leukocyte-rich tissues like spleen and thymus (163), and leupaxin whose expression is limited to leukocyte populations (164).

6.1 Paxillin structure

Paxillin serves as a molecular scaffold facilitating, integrating and coordinating efficient cell signaling, and its structure of multiple protein-binding domains

reflects its function (figure 4). The N-terminal part of paxillin contains five leucine- and aspartate-rich LD domains (LD1-LD5) with the consensus sequence LDxLLxxL (165). LD domains were first characterized as binding regions for vinculin and FAK (166,167), since then, many other binding partners have been identified (168). Although the LD domains share a large amount of sequence homology, they are capable of mediating protein interactions that are both specific and overlapping. The paxillin N-terminus also contains a proline-rich region, originally identified as a binding site for the SH3 domain of Src (169).

Within the carboxy terminus are four LIM (Lin-11, Isl-1, Mec-3) domains (LIM1-LIM4), double zinc-finger motifs that regulate protein-protein interactions, as well as paxillin's localization to focal adhesions (170,171,172,173). Several other structural and regulatory proteins, including tubulin and PTP-PEST, bind to paxillin LIM domains, and these interactions have important roles in controlling focal adhesion dynamics (174,175).
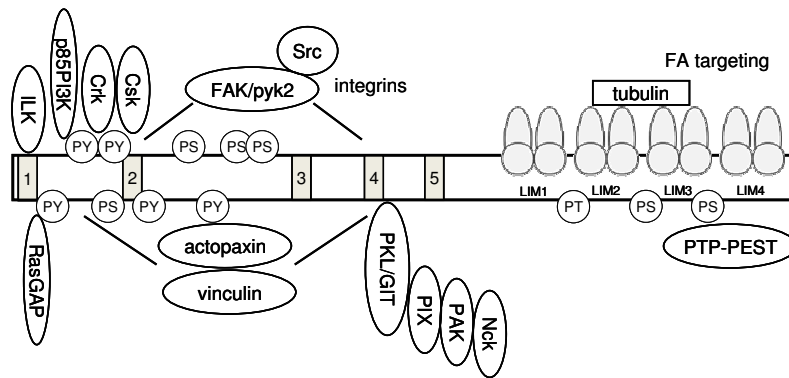
Figure 4. Paxillin structure and binding partners. Paxillin is comprised of multiple protein-binding motifs, including the amino-terminal LD motifs (1-5), the carboxy-terminal LIM domains (LIM1-LIM4), and several tyrosine, serine and threonine phosphorylation sites (PY, PS and PT respectively). Numerous paxillin binding partners have been identified: structural proteins including actopaxin, vinculin and tubulin, kinases such as FAK, Src and ILK, phosphatases such as PTP-PEST and regulators and effectors of the Rho family of small GTPases (eg Crk, PKL/GIT).

Dispersed throughout the paxillin molecule are multiple tyrosine, threonine and serine phosphorylation sites targeted by an array of kinases in response to various adhesion and growth factor stimuli. These kinases include FAK, Src (176), PAK (177), JNK (77,78), ERK (66,68), p38 (86,78), cyclin-dependent kinase 5

(CDK5) ((178) and Abl (179). The phosphorylation of paxillin helps to regulate the recruitment of numerous regulatory and structural proteins. Together these proteins can coordinate responses to the stimuli provided by the environment.

6.2 Paxillin signaling

Through its interaction with other proteins, paxillin is involved in many cellular processes. Below, paxillin's role in the signaling for cell survival and apoptosis, adhesion dynamics and cell migration will be discussed in more detail.

The interaction of vinculin with paxillin LD1 and LD2 motifs has been shown to regulate cell survival. The tail domain of vinculin competes with FAK for paxillin binding and in this way promotes ERK signaling through FAK or paxillin to prevent apoptosis (180). Paxillin is also a substrate for cleavage by caspase-3, through paxillin degradation, integrin-mediated cell survival signals can be inhibited (181) and apoptosis and anoikis are promoted. In cardiomyocytes, overexpression of the FAK homolog Pyk2 induces apoptosis, and this can be inhibited by the co-expression of paxillin (182).

Cell adhesions are highly dynamic structures. Paxillin is one of the earliest proteins that can be detected in new adhesions, and it is rapidly organized there. This suggests that paxillin plays an important role in the assembly of adhesions, and in coordinating their molecular composition (183). Paxillin is also important in the disassembly of focal adhesions, as is indicated by the stabilization of focal adhesions in paxillin-deficient fibroblasts (175). Paxillin-mediated disassembly may be accomplished in several ways. Paxillin phosphorylated at tyrosine residues 31 and 118 localizes to dynamic adhesions (184,185). Mutation of these residues to non-phosphorylatable amino acids impairs the disassembly of focal adhesions at the leading edge of migrating cells and expression of a paxillin mutant lacking the LD4 domain has a similar effect (175). The mechanism by which paxillin regulates adhesion disassembly seems to involve interactions with ERK (66) and FAK-Src to regulate myosin light chain kinase- (MLCK) dependent contractility (175). Also, the proteolysis of paxillin by calpain 2, a calcium-dependent cysteine protease, has been shown to induce disassembly of adhesions in smooth muscle cells (186), and to stimulate protrusive activity in fibroblasts (187). Serine phosphorylation of paxillin at residues 188 and 190 stimulates adhesion turnover by preventing polyubiquitylation and subsequent degradation of paxillin (188,189). From the

above, it is clear that paxillin can regulate the dynamics of adhesions in a number of ways.

As explained before, cell migration is a highly complex process, fundamental for wound healing, immune responses, angiogenesis, embryogenesis, but also for tumor cell invasion and metastasis formation. During cell migration, key proteins that regulate the necessary dynamics of cell adhesions and the actin cytoskeleton are members of the Rho family of small GTPases, Cdc42, RhoA and Rac1 (190). Rho-GTPases function as molecular switches which cycle between an active (GTP-bound) and inactive (GDP-bound) state. The activity of Rho-GTPases is regulated by guanine-nucleotide exchange factors (GEFs), which catalyze the exchange of GDP for GTP, GTPase-activating proteins (GAPs), which promote the hydrolysis of GTP to GDP and various effector proteins (191). Paxillin contributes to the regulation of the Rho-GTPases by recruiting several GEFs, GAPs and effector proteins to sites of cell-ECM contact, thereby coordinating downstream signaling to the cytoskeleton. As described before, upon binding of integrins to fibronectin or collagen, paxillin can be phosphorylated by FAK-Src at tyrosines 31 and 118 (189,192,193,194), enabling signal transduction through the CrkII/DOCK180/ELMO complex. This complex can bind to phosphorylated paxillin through the SH2 domain of CrkII (195,193,196) and can regulate Rac1 activity to promote cell migration through GEF activity of DOCK180 (197,198). Phosphorylation of tyrosines 31 and 118 has also been shown to inhibit RhoA through RhoGAP activity (141). So paxillin that is phosphorylated at tyrosines 31 and 118 can indirectly activate Rac1 and inhibit RhoA, and both of these activities are important for efficient cell migration.

The LD4 region of paxillin is of particular importance in the regulation of Rho-GTPase signaling and cell migration. The domain recruits a protein complex that consists of G-protein-coupled receptor interacting proteins GIT1 or GIT2/PKL (paxillin kinase linker), PAK-interacting exchange factor (PIX), PAK, and Nck to the leading edge of migrating cells (199,200,201). GIT1 and GIT2 can bind directly to LD4; they are negative regulators of Arfs, small GTPases that control membrane trafficking and can indirectly stimulate Rac1 activity and modulate actin dynamics (202,201). PIX displays Rac1 and Cdc42 GEF activity and PAK is a Rac1-Cdc42 effector protein (203,204,205,206,199,200,201). In fibroblasts that express a LD4 deletion mutant, the GIT-PIX-PAK-Nck complex cannot be recruited to focal adhesions, resulting in sustained Rac1 activity and abnormal

membrane-protrusions. These cells are also defective in polarized cell migration and focal adhesion turnover (200,175).

Serine phosphorylation of paxillin also plays a role in cell migration. JNK phosphorylates paxillin at serine 178 and regulates the migration of fish keratocytes and rat bladder tumor epithelial NBT-II cells (77,78). JNK phosphorylation of paxillin is also important in neurite extension of mouse N1E-115 neuroblastoma cells, a process that requires cytoskeletal remodeling regulated by Rac1/Cdc42 signaling (207). Furthermore, recent studies show that phosphorylation of paxillin serine 178 by JNK is a requirement for the association of paxillin with FAK and subsequent phosphorylation of paxillin at tyrosines 31 and 118, leading to the processes described above (79).

The action of phosphatases is essential for adhesion turnover and efficient cell migration. The tyrosine phosphatase PTP-PEST is recruited to focal adhesions by binding to LIM3 and LIM4 domains of paxillin and decreases Rac1 activity, thereby regulating cell spreading, cell migration and protrusion (208,209). PTP-PEST interacts directly with paxillin, and inhibits signaling cascades that are mediated by LD4 binding partners and/or phosphorylation of tyrosine residues 31 and 118 (210).

It is becoming clear that the regulation of cell survival, adhesion dynamics and cell migration by paxillin is tightly regulated and highly complex and may be cell-type and/or context-specific. State-of-the-art techniques and the use of more *in vivo*-relevant 3D ECM models in combination with whole-animal models will be important to elucidate the exact role of paxillin and its binding partners these processes *in vivo*.

6.3 Paxillin and cancer

Amplification of chromosome 12q24, the location of the paxillin gene, was found in many bladder tumors (211). A role for paxillin in papillomavirus-mediated cell transformation associated with cervical cancer has been suggested to be mediated partly by an association of paxillin with the viral protein E6 (212). Another link to cancer is the fact that association of paxillin with the tumor suppressor NF2 may mediate pathogenesis of neurofibromatosis type 2 in humans (213). Heregulin treatment of breast cancer cells transcriptionally upregulates paxillin and the increased expression of paxillin directly correlates with Her2/3 receptor expression in both aggressive breast cancer lines and grade III human breast tumors (214).

However, a decrease in paxillin levels has been associated with metastatic breast cancer in feline and canine models (215). This is consistent with results found in normal murine mammary gland epithelial cells transfected with eGFP-paxillin or a paxillin tyrosine phosphorylation null-mutant, where a decrease in cell migratory capacity was found (216). In small cell lung cancer cell lines and tissues, decreased paxillin levels were found (217) and metastatic Lewis lung carcinoma cells are more migratory and have reduced paxillin tyrosine phosphorylation compared to nonmetastatic cells (218). Similarly, in non-small cell lung cancer, no somatic genetic changes in the paxillin gene were found (219). In proliferative prostate epithelium however, upregulation of paxillin was observed and correlated to increased metastatic potential (147). Paxillin upregulation has also been reported in metastatic renal carcinoma (220).

Recently a number of studies have tried to identify the prognostic value of paxillin expression for cancer patients, but the data are in contradiction. Paxillin is overexpressed in human oesophageal squamous cell carcinoma, but no correlation was observed between expression of paxillin and overall patient survival (221). In feline and canine breast cancer, low levels of paxillin together with high levels of p130Cas may be useful prognosticators for malignancy (215). FAK expression in combination with Src, but not paxillin, was found to be predictive for recurrence in colorectal cancer patients, but in corresponding distant metastases, the levels of FAK, Src and paxillin were maintained (153). The conflicting findings regarding a direct correlation between paxillin upregulation or phosphorylation and cancer aggressiveness or a predictive value for disease progression probably result from context-specific and tissue-specific roles for paxillin in cellular function.

7. Aim and outline of this thesis

Despite major advances in breast cancer diagnostics and treatment over the years, the disease is still a leading cause of death in women worldwide. Primary breast tumors can be treated relatively well with radiation, surgery, chemotherapy or a combination of these treatments. The occurrence of distant metastases derived from the primary tumor however, results in a considerable decrease in disease prognosis. Metastasis formation occurs through a series of distinct cell biological steps (outlined above). Understanding the molecular mechanisms that underlie each of these steps will help in the development of more successful anti-metastasis treatments. In this thesis, both *in vitro* and *in vivo* studies are described that aim at

unraveling some of the processes involved in metastasis formation: signaling by components of the focal adhesions and cell migration.

The results shown in **chapter 2**, allowed us to optimize a syngeneic MTLn3-Fischer 344 experimental metastasis model. Depletion of NK cells in Fischer 344 rats prior to injection of MTLn3 tumor cells resulted in the formation of lung metastases, whereas MTLn3 cells injected into rats that were not depleted of NK cells, failed to induce secondary tumors in the lungs. In **chapter 3**, this model is employed to study the role of focal adhesion kinase in the early phase of lung metastasis formation. MTLn3 cells that are inducible for the expression of FRNK, an inhibitor of FAK localization to focal adhesions, are injected into mammary fat pads and the early expression of FRNK in those cells results in a decrease in the amount of lung metastases. This indicates that FAK is required in the process of breast tumor cell invasion and migration. In **chapter 4**, the syngeneic model was used to show that inhibition of FAK function improves the sensitivity of MTLn3 tumor cells to the anticancer drug doxorubicin. In this chapter, a model is proposed where FAK-mediated signaling is linked to expression of the transcription factor Fra-1. Another anticancer drug with a completely different mechanism of action, vincristine, is studied in **chapter 5**. Vincristine is a microtubule-interfering agent that induces cytoskeletal changes in MTLn3 cells. We exposed cells to this drug to study the role of the focal adhesion adaptor protein paxillin in cytoskeletal remodeling, which is an important part of the migratory process in metastasis formation. Paxillin proved to be a substrate for the stress kinase JNK, and the paxillin-JNK signaling axis was studied in more detail. The focal adhesion-associated adaptor protein paxillin and its association with JNK were also the subjects of study in **chapter 6**. Here, we mutated the JNK phosphorylation site on paxillin to study the importance of this phosphorylation in growth factor-induced tumor cell migration and metastasis formation. Finally, the results are summarized and discussed in **chapter 7**.

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