Focal adhesion kinase signaling in metastasis and breast cancer treatment

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8

General Discussion and Conclusions

1 Inhibition of FAK by inducible expression of FRNK.

The non-receptor tyrosine kinase Focal adhesion kinase (FAK) is overexpressed in a variety of human cancers [1, 2, 3, 4] and implicated in multiple aspects of tumor cell biology. Therefore the involvement of FAK in the various processes regarding tumorigenesis and metastasis are being extensively studied. Several approaches are used to study FAK in (cancer) cells, such as the complete knock-down by means of antisense methods, siRNA and FAK knock-out techniques. Another, more delicate way of interfering with FAK can be achieved by selectively blocking its function either by preventing FAK's kinase activity or by preventing its binding to (signalling) proteins, i.e. interfering with its scaffolding function. Importantly, FAK is involved in a large number of signalling pathways, which are not all initiated at the focal adhesions. However, there are strong indications that FAK-mediated survival and migration signalling, processes important in tumorigenesis, progress from the focal adhesions. Therefore, we decided to inhibit FAK by preventing its localization at the focal adhesions. To do so, a negative acting splice variant of FAK, FAK-related non-kinase (FRNK) [5] was expressed. This splice variant lacks the kinase domain but does localize at the focal adhesions, resulting in the selective inhibition of FAK function at the focal adhesions in the presence of functional FAK-signalling in, for instance, the nucleus. To be able to study the effect of disabling FAK-signalling in distinct processes at different time-points, an inducible HA-tagged FRNK expressing rat mammary adenocarcinoma cell line was created: MTLn3-tetFRNK.

The created MTLn3-tetFRNK cell line expressed HA-FRNK in a time and doxycycline-concentration dependent manner (Chapter 5). Under control conditions, expression of HA-FRNK did not alter cell morphology, apoptosis or endogenous tyrosine 397 FAK phosphorylation, indicative for the kinase activity of FAK [6]. The interference of HA-FRNK with the localization of endogenous FAK was confirmed using fluorescence microscopy: HA-FRNK was located at the focal adhesions and as a result less FAK was present at these sites of cell-extracellular matrix contacts (Chapter 5). Although FAK has been shown to promote survival signals downstream of the integrins [7, 8], inducible expression of HA-FRNK in MTLn3 cells did not cause apoptosis (Chapter 3, 5). Adenoviral gene transduction of FRNK in breast cancer cells, resulting in high expression levels of FRNK, caused loss of adhesion and did induce apoptosis [9]. In accordance to this, high expression levels of FRNK resulted in loss of FAK and paxillin from the focal adhesions in myocytes and subsequent detachment and apoptosis [10]. Transient transfection of MTLn3 cells with eGFP-FRNK, resulting in much higher expression levels than inducible expression of HA-FRNK, also resulted in apoptosis (Chapter 3). This indicates that high expression levels of FRNK are required to induce apoptosis. In contrast to the above mentioned studies using adenoviral

transduction of FRNK [9, 10], but in accordance to results obtained using inducible expression of FRNK in a transformed kidney cell line [11], inducible expression of HA-FRNK in MTLn3 cells did not affect anoikis (Chapter 5). This again confirmed that the expression levels of HA-FRNK do not directly interfere with cell survival signalling of the MTLn3 cells.

Inducible expression of HA-FRNK did alter cell motility of the MTLn3 cells: adhesion and migration processes were delayed (Chapter 5) under low serum conditions. Although FAK plays a functional role in integrin signalling leading to cell adhesion [12] and migration [13], spread FAK-null cells displayed an increased number of focal adhesion [14]. Likely, FAK is important in breaking down the focal adhesions in MTLn3 cells. Indeed, inhibition or downregulation of FAK impairs focal adhesion turnover in other cells [14]. During migration, focal adhesions are also very dynamic structures: they have to be formed at the front of the cell, while they have to be broken down at the rear of the cell. Similar to the FRNK-expressing MTLn3 cells, FAK-null cells showed an impaired migratory phenotype [14]. Live-cell imaging revealed that tyrosine 397 phosphorylation is crucial in the disassembly of focal adhesions [15]. Expression of FRNK in spreading MTLn3 cells under low serum conditions delayed tyrosine 397 phosphorylation of FAK. This is indicative for delayed focal adhesion formation. These combined data lead us to speculate that the changes in migration and adhesion behaviour can most likely be attributed to a FRNK-induced decrease in focal adhesion turnover. To confirm this, live-cell imaging of FAK in the MTLn3 cells should be performed. For example, by FRAP analysis [15] of a stable transfected fluorescently tagged focal adhesion protein, i.e. paxillin or vinculin, in the absence and presence of HA-FRNK in the MTLn3tetFRNK cells. In complete medium, in which many growth factors are present, adhesion and migration differences caused by expression of HA-FRNK were no longer significant in MTLn3 cells. Migration studies using fibroblasts showed that EGF induced migration also required FAK [16]. Thus, growth factors might be able, even in the presence of HA-FRNK, to trigger activation of FAK, thereby localize FAK at the focal adhesion and stimulate adhesion and migration processes.

In conclusion, by creating an inducible HA-FRNK expressing breast cancer cell line, we obtained a model in which FAK is delicately modulated. FRNK expression does not result in apoptosis but only affects more physiological functions of FAK, like adhesion and migration.

2 FAK AND TUMOR FORMATION.

Multiple *in vitro* studies have provided evidence for an important role of FAK in processes involved in tumorigenesis and metastasis, like proliferation, migration,

survival and invasion of cells. In addition, *in vivo* experiments point out the importance of FAK in chemical induction of skin cancer [17], whereas inducible deletion of fak after formation of papillomas revealed that FAK was directly required for the malignant conversion *in vivo* [18]. Overexpression of FAK in kidney cells [19] and in astrocytoma cells [20] resulted in increased tumor cell proliferation in xenograft models. In these studies, expression of FAK was decreased or upregulated, so both the phosphorylation and scaffolding functions of FAK were, albeit at decreased or increased levels, still present. Thus, the question remains, which domains of FAK are crucial in tumorigenesis.

Constitutive expression of FRNK in v-Src transformed fibroblasts [21] did not alter tumor growth. However, in most cell lines the constitutive expression of FRNK induced apoptosis. Thus in this cell line (other) survival signalling pathways have to be upregulated and therefore this cell line differs from the founder cell line. By using our inducible HA-FRNK expressing cell line, we showed that continuous expression of FRNK did reduce primary tumor growth (Chapter 5). However, expression of FRNK not starting until day 9 after injection of tumor cells, did not affect primary tumor growth (Chapter 6). Thus, proper FAK signalling is very important at the start of the tumor formation. In contrast to *in vitro* data that show that expression of FRNK reduces proliferation (Chapter 5), *in vivo* expression of FRNK is not able to reduce proliferation of already existing primary tumors. Since the absence of growth factors can be limiting for tumor growth, an explanation for the decreased tumor growth when FRNK is continuously expressed could be a reduced angiogenesis. This would be in line with Mitra *et al.* who showed that expression of FRNK, via inhibition of ERK2 activity and VEGF expression, resulted in the formation of tumors that were hardly vascularized [22]. However, in our model, no differences in tumor vasculature was observed by studying H&E stained tumor sections and no reduced VEGF levels could be detected using microarray analysis of our MTLn3-tetFRNK cells.

Immuno-histochemical analysis showed that primary tumors that continuously expressed FRNK consisted of a lower percentage of HA-FRNK positive cells compared to tumors in which FRNK was only expressed starting three days before isolation of the tumors. Since expression of HA-FRNK after 9 days did not decrease tumor size (Chapter 6), it is not likely that HA-FRNK expressing cells are more sensitive towards apoptosis. This was confirmed by staining primary tumors that continuously expressed HA-FRNK for active caspase-3, 28 days after tumor cell injection: no differences in caspase-3 activity in the HA-FRNK expressing tumors compared with the control tumors could be observed (Chapter 5). A possible explanation for the decreased percentage of HA-FRNK expressing cells in the tumors is that during the first couple of days after injection into the fat pad, selection of

HA-FRNK negative cells (<10% of the injected cell population) occurs, either because of an increased apoptosis in HA-FRNK positive cells during the first few days or by an increased proliferation of HA-FRNK negative cells. To test this, the number of proliferating and the number of apoptotic cells shortly after injection, for example after 1 or 2 days, should still be determined.

3 FAK and experimental metastasis.

The decrease in primary tumor growth in response to continuous expression of HA-FRNK, strongly suggests that proper FAK functioning is especially important in the process of tumor cell homing. Likewise, inhibition of FAK could interfere with metastasis, since in this process cancer cells have to home at a distant target site. To verify this hypothesis, first the syngeneic MTLn3-Fischer 344 experimental metastasis model was optimized. To discriminate between the molecular biological pathways that have to be activated in tumor cells and the anti-metastasis role of the immune system, a role for the immune system had to be excluded. *In vitro* cytotoxicity studies showed that in the syngeneic model that in theory should not show any immune reactivity towards MTLn3 cells, the NK cells efficiently induced apoptosis of MTLn3 cells via the perforin/granzyme B pathway (Chapter 4). NK cell activity is strictly regulated by a balance between inhibitory and activating signals [23]. The cause of the NK cell reactivity towards MTLn3 cells was not found. However, loss of MHC-I which results in strong NK cell activity, could be excluded since OX-18 staining revealed a clear expression of MHC-I (Chapter 4). Most likely, prolonged culturing of the MTLn3 cells resulted either in an altered expression of adhesion molecules, providing a strong NK-MTLn3 cell interaction, or in the expression of ligands for activating NK receptors, both resulting in NK cell mediated killing. Depletion of NK cells from the Fischer 344 rats strongly increased the number of experimental metastases, thereby allowing the dissection of the distinct molecular processes involved in metastasis. This improved syngeneic model in combination with the inducible HA-FRNK expressing MTLn3 cells provides an excellent model to study the role of FAK in the different steps of metastasis.

Others already provided some evidence for a role of FAK in metastasis: stable expression of FRNK in v-Src transformed 3T3 cells reduced metastasis [21] without altering migration or proliferation and constitutive expression of FRNK in B16-F10 melanoma cells reduced the number of lung metastases by 50% [24]. However, using these stable expression approaches, it cannot be excluded that this reduction was caused by increased apoptosis since FRNK continuously inhibits a major survival signalling pathway. Moreover, these studies do not provide any clues about the exact process of metastasis, i.e. migration, invasion, survival or proliferation, in which FAK is crucial. Selective expression of HA-FRNK only prior and during the first days after

injection of the tumor cells into the tail vein, revealed the requirement for FAK in the early phase of experimental metastasis (Chapter 5). In this study HA-FRNK was only expressed for a short period of time, and therefore clearly pinpoints the importance of FAK signalling to the early phase in experimental metastasis. The exact process(es) in which FAK is required could still not be determined. Thus, during the first couple of days the tumor cells have to survive in the circulation, must adhere to the vasculature, invade the lung tissue and attach to either lung matrix or lung cells to provide itself with survival signals. Differential survival rates of control and HA-FRNK expressing cells in the circulation can likely be excluded since *in vitro* studies showed no effect of the expression of HA-FRNK on anoikis (Chapter 5). *In vivo* expression of HA-FRNK did delay attachment and did decrease migration of MTLn3 cells. Therefore the reduced metastatic phenotype of HA-FRNK expressing MTLn3 cells can most likely be attributed to a reduced invasive capacity of the cells. In agreement with this, no dormant cells could be found in the lungs of animals that were continuously exposed to doxycycline; thus the reduced number of metastases was not caused by a reduced outgrowth of the HA-FRNK expressing cells that invaded the lungs, but was a direct consequence of a lower number of HA-FRNK expressing cells that managed to enter the lungs. An important study to verify the reduced invasive capacity HA-FRNK expressing cells is the *in vitro* invasion assay. Interestingly, the tumor suppressor NF2/merlin inhibits invasiveness by attenuation of FAK tyrosine 397 phosphorylation [25], whereas downregulation of FAK hampered invasion of ErbB2/3 transformed fibroblasts [26]. The invasion assay should be a critical component of any further studies using the inducible HA-FRNK expressing MTLn3 cells on the involvement of FAK in tumorigenesis.

Interestingly, the size of the experimental metastases was significantly reduced when FRNK was expressed. Although HA-FRNK reduced proliferation *in vitro*, this reduction was not observed during primary tumor growth, suggesting that under *in vivo* conditions other signalling pathways leading to proliferation are activated. To verify this, *in vivo* proliferation studies using BrdU might be very informative. Again, no HA-FRNK-induced increase in apoptosis was observed. Therefore, it is more likely that the HA-FRNK expressing cells that do succeed in invasion of the lungs, required more time to attach to the matrix and/or to the lung cells prior to the onset of proliferation, possibly as a result of a decreased focal adhesion turnover. In line with this, a role for FAK in the establishment of stabilized adhesive interactions required for the formation of distant metastases was shown in EGFP-FRNK transfected Hep-G2 cells [27]. Since the tumor size increases exponentially, a delayed onset of proliferation of only couple of days can turn out into a significant reduced tumor size in 4 weeks. To verify this possible explanation, it would be interesting to isolate the lungs for example three days after tumor cell injection. According to our hypothesis,

control MTLn3 cells will then already have formed small groups of cells, whereas the HA-FRNK expressing cells might still be found as single cells.

Obviously, the best way to study the entire process of metastasis is the spontaneous metastasis model. Since continuous expression of HA-FRNK reduced primary tumor growth, studying differences in spontaneous metastasis starting from the control tumor and the significant smaller HA-FRNK expressing tumor was not an option. Therefore, the experimental metastasis model was introduced and optimized. However, subsequent studies using the primary tumor model showed that expression of HA-FRNK starting at day 9 did not reduce primary tumor growth. With this in mind, the spontaneous metastasis model can now be employed using our inducible MTLn3-tetFRNK cell line in Fischer 344 rats: nine days after tumor cell injection HA-FRNK should be expressed, whereas three days later (up until the end of the experiment), NK cells should be depleted in all animals to prevent NK cell mediated killing of metastasizing cells. Two weeks later, when the maximum allowed primary tumor size is reached, the lungs should be isolated and the number of micrometastases in the lungs will reveal the importance of FAK in this spontaneous metastasis model.

Although our data do still not exactly indicate how interference of FAK results in both a reduction in primary tumor growth and a strong reduction in the number of lung metastases, our studies demonstrate FAK seems to be a very promising target in the development of new anti-cancer drugs.

4 DOXORUBICIN: EFFECT ON MTLN3 CELLS.

In addition to the development of new therapies for the treatment of cancer, improvement of the currently used anti-cancer therapies might be a successful way to help cancer patients on the short term. Patients who suffer from breast cancer metastases are often treated with chemotherapeutic agents. The balance between survival signals (mediated by for example growth factors, cell-cell or cell-matrix contacts) and apoptotic signals (activation of cellular pro-apoptotic pathways) determines whether a cell dies (either through apoptosis, necrosis or autophagy), goes into growth arrest or survives in response to a cytotoxic stimulus. As shown previously [28], doxorubicin induced apoptosis of MTLn3 cells in a time and concentration dependent manner (Chapter 3) and attachment of the cells to the extracellular matrix protects against cytostatic-induced apoptosis (Chapter 3, [29, 30]). Prior to apoptosis, which was not observed until 12 hours after exposure, doxorubicin caused the reorganization of the cytoskeletal network in combination with increased focal adhesion formation. Since focal adhesions mediate survival signalling, this initial response to doxorubicin could be regarded as a possible reaction of the cells

to increase the survival signalling and, thereby, keeping the balance towards survival instead of apoptosis. In agreement with this, when focal adhesion formation increased, no caspase-3 activity could be found. However, after the initial increase in focal adhesions, the number and especially the size of the focal adhesions decreased and subsequently the cells started to round up and caspase-3 became active. Since these morphological changes in response to doxorubicin were independent of the activation of the apoptotic cascade, as shown by overexpression of Bcl-2 and co-incubation with the pan caspase-inhibitor zVAD-fmk (Chapter 3), these changes occur prior to the onset of apoptosis rather than being a result of this. FAK, a major component of the focal adhesions, was dephosphorylated and cleaved in response to doxorubicin. Importantly, abrogation of the apoptotic signalling cascade either at the level of the caspases (by addition of a pan-caspase inhibitor zVAD-fmk) or at the level of the mitochondria (by overexpression of the anti-apoptotic protein Bcl-2) prevented cleavage of FAK but did not prevent FAK dephosphorylation. Thus, FAK is dephosphorylated in response to doxorubicin exposure, prior to activation of the apoptotic machinery. These data indicate that phosphorylated FAK plays a survival stimulating role in cells exposed to doxorubicin and upon loss of FAK activity the balance is tipped towards apoptosis.

When the sensitivity towards cytostatic-induced apoptosis of cancer cells is studied, most of the time high concentrations that directly induce apoptosis within 24 hours are used. Although this is very useful to provide insight into the molecular mechanisms of cytostatic-induced apoptosis *in vitro*, it is questionable whether, due to severe side affects, these high concentrations can be reached in patients. By using lower concentrations of doxorubicin in a soft agar colony assay, in which the cells form groups of cells instead of monolayers, better insight in the clinical implications of doxorubicin could be provided. Doxorubicin dramatically decreased the colony formation ability of MLTn3 cells in a concentration dependent manner (Chapter 6). Thus, although MTLn3 cells initially survive exposure to low concentrations of doxorubicin, long-term colony growth is severely inhibited. In addition to differences in the concentrations of chemotherapeutic agents used *in vitro* and *in vivo*, it is very hard to compare the effects of chemotherapeutic agents on cancer cells under strictly regulated *in vitro* conditions with the effects on tumor cells in patients, in which a complex mixture of growth factors and cell-cell/matrix interactions provide survival signalling to the tumor cells. Exposure of rats bearing either primary tumors or experimental lung metastases to sub-lethal concentrations of doxorubicin failed to reduce tumor growth or lung tumor burden. This is in line with the observations by Toyota *et al.* [31] who showed the insensitivity of MTLn3 cells towards doxorubicin. In our model, three days after exposure of the rats, no difference in the levels of active caspase-3 in doxorubicin treated rats compared to control rats was found

(Chapter 6). Thus, although doxorubicin is able to induce apoptosis of MTLn3 cells under *in vitro* conditions, *in vivo* treatment was very ineffective (Chapter 7).

5 Combination therapy: inhibition of FAK and doxorubicin treatment.

Since exposure of MTLn3 cells to doxorubicin reduced the phosphorylation of FAK prior to activation of the apoptotic machinery, FAK could play a role in the activation of survival signalling, thereby preventing doxorubicin-induced activation of apoptotic pathways. Exposure of inducible HA-FRNK expressing cells to doxorubicin confirmed these speculations: doxorubicin induced higher levels of apoptosis when FAK signalling was inhibited (Chapter 3, 6). In contrast to some other studies [32], overexpression of FAK was not able to inhibit doxorubicin-induced apoptosis (data not shown), indicating that FAK mediated survival signalling in these MTLn3 cells is already optimal. Either overexpressed FAK is not activated due to competition with endogenous FAK at the focal adhesions, or FAK is not able to further stimulate the downstream survival pathways. These possibilities should be tested by introducing a constitutively activated FAK mutant: when this result in increased survival, the activation of FAK, and not the downstream signalling, in MTLn3 cells is limited.

Several studies showed that inhibition of FAK either by antisense or siRNA approaches, or by expression of negative acting deletion mutants, resulted in cell detachment and apoptosis [33, 34, 7]. Transient transfection of eGFP-FRNK alone (data not shown) also induced apoptosis of MTLn3 cells that could be counteracted by overexpression of Bcl-2. Since FRNK itself induced apoptosis and FRNK increased doxorubicin-induced apoptosis, these two stress response pathways may converge. FAK is able to stimulate several survival signalling pathways: activation of the PI-3 kinase/PKB pathway [35, 36], inhibition of the tumor suppressor p53 [7, 37] and activation of the p130Cas, MAPK pathway [8], of which only the p130Cas / MAPK survival route can be activated in the absence of growth factors. Exposure of MTLn3 cells to doxorubicin, resulted in phosphorylated PKB, supporting the data on an initial upregulation of the survival pathways to prevent apoptosis. (Chapter 3). Expression of HA-FRNK prevented this PKB phosphorylation, whereas either pharmacological inhibition of PKB using wortmannin or molecular biological interference by PTEN increased doxorubicin-induced apoptosis. Thus, FAK-mediated survival signalling seems to be mediated via activation of the PI-3 kinase/PKB pathway. A model in which PKB is constitutively activated could be used to verify that activation of the PI3-kinase/PKB pathway provides sufficient survival signalling to counteract doxorubicin-induced apoptosis. By creating an MTLn3 cell line that inducible expresses constitutively activated PKB, the protective role of the PKB survival signalling pathway in the prevention of doxorubicin-induced apoptosis can be determined.

Although the exact mechanisms are still not fully understood, expression of HA-FRNK acts synergistically with doxorubicin exposure under these *in vitro* conditions and inhibition of FAK in combination with chemotherapeutic agents might therefore be worthwhile to improve cancer therapy. Expression of HA-FRNK slightly reduced colony formation of cells exposed to low concentrations of doxorubicin. Although not significantly, this reduced colony formation capacity in response to HA-FRNK again provides evidence for a synergistic effect of inhibition of FAK in combination with doxorubicin exposure. Whereas neither HA-FRNK was able to inhibit proliferation of already existing primary tumors, nor doxorubicin itself reduced primary tumor size, combination therapy significantly reduced primary tumor size. More importantly, expression of HA-FRNK drastically reduced experimental lung tumor burden after doxorubicin treatment. Since most cancer deaths are related to the occurrence of metastases, this latter result is very promising for the improvement of the treatment of metastasized cancer. Understanding the molecular mechanisms of this combination therapy is very important to optimize this treatment for cancer patients.

No direct effect of combined inhibition of FAK and doxorubicin treatment on apoptosis was observed, as analysis of dissected primary tumor tissue three days after doxorubicin exposure did not reveal increased levels of active caspase-3. In agreement with this, no reduction in the percentage of HA-FRNK positive cells was shown, nor a decrease in experimental metastasis size. Of course, the activation of caspases is time dependent, thus it cannot be excluded that at earlier or later time periods after doxorubicin treatment an increase in caspase activity was present. Time-course analysis of HA-FRNK expressing, doxorubicin-exposed primary tumors is needed to verify that combination therapy does not reduce primary tumor growth via apoptosis of the tumor cells. However, since apoptosis is a very fast process and under *in vivo* conditions apoptotic bodies are rapidly removed by cells of the immune system, it might be very hard to detect increased apoptotic levels. A decrease in tumor cell proliferation or a (temporal) tumor growth arrest could also account for the decreased primary tumor size after combination therapy. Since tumors grow exponentially, a growth arrest of a couple of days can already result in a significant decrease in primary tumor size. Yet, this can not explain the reduced number of experimental lung metastases: analysis of lung sections ruled out the possibility of small, dormant lung metastases. Although HA-FRNK only slightly further decreased the doxorubicin-induced decrease in colony formation capacity, this is most likely the cause of the effective combination therapy *in vivo*.

Thus, although doxorubicin induced apoptosis of MTLn3 cells *in vitro*, doxorubicin alone was not able to induce apoptosis *in vivo*, whereas a combination of HA-FRNK and doxorubicin did reduce tumor and metastasis burden, but most likely

not via the induction of apoptosis. Thus, these *in vivo* results differ from results obtained *in vitro*, emphasizing the importance of good *in vivo* models, not only to unravel the molecular biological processes involved in tumorigenesis and metastasis, but also to understand the molecular mechanisms of anti-cancer therapies.

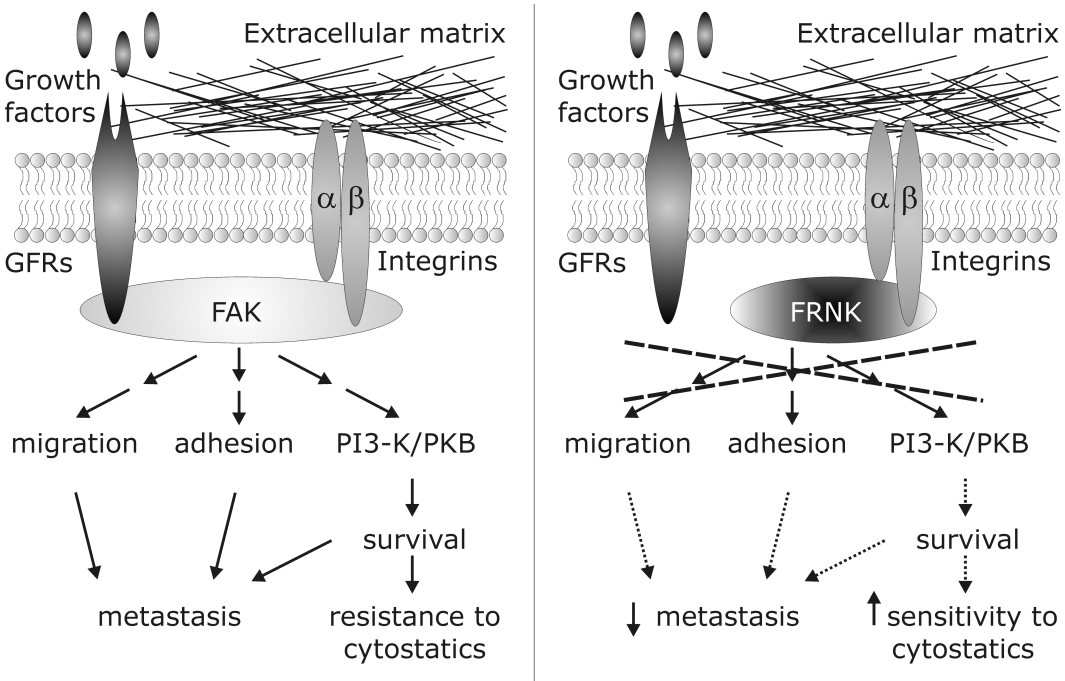
6 Anti-cancer therapy by inhibition of FAK?

Data presented in this thesis combined with a large number of other studies provide evidence for an important role for FAK in both tumorigenesis and metastasis. Therefore, FAK is a very promising target in the development of new anti-cancer drugs. Moreover, the doxorubicin-treatment sensitizing effect of inhibition of FAK offers alternative approaches for the currently difficult to treat metastases.

The main question is how to inhibit the function of FAK. FAK consists of multiple domains, all being important in the activation of specific and overlapping signalling pathways. Inhibition of its kinase domain would prevent phosphorylation and thereby activation of a number of downstream signalling pathways. Since the catalytic domains of a range of kinases show a high degree of amino acid sequence similarity, specificity of the FAK kinase inhibitor would be hard to achieve. This does not necessarily have to be a problem: other kinases are shown to be involved in tumorigenesis and metastasis as well and therefore inhibition of multiple kinases in addition to FAK might improve anti-cancer treatment. However, some selective inhibitors of tyrosine kinases have already been developed such as inhibitors of the EGF-receptor (Iressa), HER2 (Trastuzamab, Lapatinib) and c-Kit (Imatinib) [38]. Another possibility for the manipulation of FAK is blocking one (or multiple) of its adapter functions, by preventing binding of proteins to FAK's tyrosines, proline rich domains, or the focal adhesion targeting site. Blocking of the latter prevents the localization and thereby the activation of FAK at the focal adhesions. By using this approach, specific downstream signalling pathways will be blocked due to the lack of association of FAK with certain binding partners. Since almost all signalling cascades can be activated by multiple triggers, other kinases might take over some of the functions of FAK and thereby some of the intended signalling pathways might still be activated. Yet, a functional peptide that prevents the interaction of FAK to a binding partner was recently developed. Using a phage display library a peptide was identified that prevents the interaction of FAK with VEGFR-3. Expression of this peptide resulted in cell detachment and apoptosis in breast cancer cells, but not in normal breast cells [39]. These results are very promising for the development of peptides that prevent binding of FAK to the focal adhesions. One must not forget that FAK is expressed in virtually all tissues and that one of its major functions is to provide survival signalling. Therefore, systemic manipulation of FAK might result in severe side effects. Interestingly, systemic administration of FAK siRNA in mice did not

result in severe side effects [40]. One of the main characteristics of tumor cells is the disturbed survival signalling pathways.Therefore, these cells probably rely to a greater extent on FAK-mediated survival signalling and thereby inhibition of FAK affects these cells more than non-cancer cells. In addition to this, the expression pattern of FAK increases during the initial stages of embryogenesis, but decreases during ageing [41]. This impliess that in normal cells FAK is especially important during embryonic development, thus temporal pharmacological inhibition of FAK during cancer treatment in adults might not cause too severe side effects. Of course the potential toxicity of the inhibition of FAK is a very important issue and extensive research is required to explore these safety issues.

One interesting new anti-cancer treatment approach is the manipulation of FAK in primary tumors, thereby reducing the metastatic capacity of the cancer cells. Since we, and others, showed the importance of FAK in a number of processes involved in the formation of metastases, reducing FAK activity seems to be a promising method to reduce metastasis. The effect of inhibition of FAK is highly dependent on the type of cancer cell, thus in addition to the studied mouse and rat models it is of great importance to test the effect of inhibition of FAK on the metastatic capacity of several human tumor cell lines. This approach would be most successful in cancer cells that exhibit an increased expression or activity of FAK.



Overall figure: Inhibition of FAK by expression of FRNK inhibits migration, adhesion and PKB-mediated survival signalling and thereby reduced metastasis and increases sensitivity to cytostatics.

Another promising approach is the manipulation of FAK prior to treatment of the tumors/metastases with chemotherapeutic agents. The sensitizing effect of inhibition of FAK towards doxorubicin might enable the use of lower concentrations of this chemotherapeutic agent. Thereby combination therapy might not only enhance the efficacy but also reduce the severe side effects of this treatment. It would be very interesting to study the inhibition of FAK in combination with other chemotherapeutic agents but also in combination with radiation therapy.

Although we and others have provided strong evidence for an important role of FAK in tumorigenesis/metastasis and in the response of cancer cells to treatment with chemotherapeutic agents, the precise mechanisms by which FAK mediates these processes are still not fully understood. Understanding the underlying mechanisms is important to apply manipulation of FAK into the development of new anti-cancer drug therapies. For example, maybe one specific signalling pathway downstream of FAK is highly involved in the increased sensitivity towards chemotherapy and therefore might turn out to be a better target to manipulate than FAK. Also, by understanding the underlying mechanisms, the safety issues might be better understood and studied, providing the possibility to counteract or minimize some side affects.

7 CONCLUSIONS (SEE OVERALL FIGURE).

Inhibition of FAK by inducible expression of HA-FRNK delayed cell spreading and attachment, reduced migration but did not induce apoptosis or sensitized MTLn3 cells towards anoikis *in vitro*. Under *in vivo* conditions continuous expression of HA-FRNK decreased primary tumor growth, but expression of HA-FRNK in already existing primary tumors did not affect tumor cell proliferation. Using an NK cell depleted MTLn3-Fischer 344 rat syngeneic model, temporal expression of HA-FRNK during the first days after injection of the MTLn3 cells into the tail vein of Fischer 344 rats revealed the requirement for FAK in the initial steps of the experimental metastasis process. Although all evidence points into the direction of an effect of HA-FRNK on the adherence to, and invasion of the tumor cells into the lungs, the exact processes that are disturbed by the expression of HA-FRNK have not yet been identified. Combination therapy of the chemotherapeutic agent doxorubicin and inhibition of FAK by expression of HA-FRNK resulted in an additive effect on doxorubicin-induced apoptosis *in vitro*. Treatment of primary tumor bearing or experimental lung metastases bearing rats with doxorubicin did not reduce tumor burden. Combination of doxorubicin and expression of HA-FRNK significantly reduced both primary tumor size and lung metastasis burden.

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