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# Chapter 7

## Summary & discussion

Despite extensive studies to unravel molecular mechanisms underlying breast cancer metastasis, still 3,500 women in the Netherlands die each year. Improving our understanding of metastasis formation remains a challenge for further drug development. The overall aim of the work described in this thesis was to identify novel candidate metastasis genes that could serve as putative drug targets for breast cancer treatment and/or can serve as markers that predict the poor disease outcome in relation to metastasis free survival. One of the earliest steps in cancer cell dissemination and metastasis is tumor cells migrating away from the primary tumor into surrounding tissue. This process is often associated with an epithelial-to-mesenchymal transition (EMT) and requires enhanced cell migration capacity. We therefore focused our studies on candidate genes affecting tumor cell migration and EMT. Below the results from different chapters are discussed in a broader context and future perspectives are lined out.

## 1. Methodologies for in depth tumor cell migration studies

Cell migration is a complex and highly dynamic biological process that is dependent on a series of events that require a high level of spatial and temporal integration. Tight coordination of actin regulation as well as focal adhesions is of major importance. This is mediated through a large set of regulatory components including intracellular signal transduction pathways and genetic programs and numerous external stimuli such as growth factors and chemokines. In the context of cancer progression and metastasis, a deeper understanding of crucial factors in the migratory behavior of cells is necessary in order to develop targeted anti-cancer therapies.

### *1.1 Fluorescent live cell imaging techniques*

Cell migration assays allow the quantification of several motility parameters including cell speed, directionality and persistence. We established a live cell imaging-based random cell migration assay that is suitable for both compound as well as RNA-interference screening in multiple cancer cell lines. The development of this assay is described in **chapter 2**. One of the main advantages of this method over pre-existing methods is the combination of a screening platform with live cell imaging. Other methods, such as the phagokinetic track assay, have proven to be successful in high-throughput screening (see **chapter 4**), but lack information about cell dynamics during the cell migratory process. Acquisition of the dynamic behavior of cells allows the modeling of cell behavior during cell migration, which could be used to improve our understanding of molecular mechanisms underlying different modes of

migration, e.g. single cell versus collective cell migration. In particular the modeling of these events in the context of candidate target genes that were identified in our migration screen (**chapter 4**) would be an interesting follow up strategy to better understand the consequences of our novel tumor cell migration regulating genes including SRPK1, NEK2, ITGB3BP and MAP3K8 in different panels of human cancer cell lines.

*1.2 Relation between cell migration and focal adhesion dynamics*

Cell migration is a highly dynamic process, which is mediated by focal adhesion (FA) signaling. Focal adhesions are large dynamic protein complexes, consisting of over 150 proteins, including signaling proteins as well as structural components and function to establish a signaling link between the extracellular matrix (ECM) and actin cytoskeleton resulting in downstream activation of pathways involved in for instance actin dynamics and cell migration. Throughout this thesis, we have linked cell migration to FA dynamics. FAs that are strongly attached to the ECM are associated with a slow turnover of the structures and consequently, associated with decreased cell migration [1]. This is in agreement with our data in which we show that upon knockdown of SRPK1, FAs are enlarged and stabilized (**chapter 4**). On the contrary, expression of Fra-1 in MCF7 induced FA turnover in association with increased cell migration (**chapter 5**). In addition, we systematically monitored FA organization for the 30 high confidence genes identified in the migration screen. To achieve a more complete view of all individual components that control FA dynamics, a RNA-interference screen was performed in the MCF7 cell line using the same libraries as in the migration screen described in this thesis. The effect of knockdown of a gene on FA disassembly induced by nocodazole treatment and FA assembly following wash out was determined (Le Dévédec et al., manuscript in preparation). Although the screens are performed in two different human cancer cell lines with large differences in migratory behavior, some overlap in the identified hits has been found. For example, knockdown of PTEN, MYO9B, LCK and PVRL3 in MCF7 resulted in increased FAs, which is in line with the observed decrease in cell migration in the migration screen. No direct interaction between the proteins encoded by these genes is known, but based on sequence alignment they all interact with the well studied FA protein focal adhesion kinase (FAK), either directly (PTEN [2] and LCK [3]) or indirectly (MYO9B and PVRL3). This observation emphasizes the central role for FAK signaling in regulating FAs and cell migration and possibly adds new partners to this signaling route. Combining cell migration assays with FA dynamics studies

using model cell lines provides new insight into the critical genes involved in these processes and contributes to an increased understanding of molecular mechanisms underlying tumor cell migration. Repeating this combination for multiple cancer cell lines following by identification of common denominators increases the chance of defining a FA dynamics and cell migration relation that is cell type independent and more generally applicable.

### *1.3 Modes of cell migration and cancer progression*

Pre-specified patterns of cell migration are classified into single cell migration, including amoeboid and mesenchymal, or collective cell migration, including cell sheets, strands, tubes and clusters. Cell migration patterns are often cell type specific and dependent on extracellular guidance cues as well as intrinsic molecular programs [4; 5]. For example, high Rac activity results in cell elongation and mesenchymal migration, whereas active Rho in combination with little or no Rac activity supports cell rounding associated with amoeboid migration [6; 7]. In **chapter 6**, we showed that knockdown of paxillin in 4T1 alters Rac1 signaling resulting in loss of cell-cell adhesion and decreased directional cell migration. 4T1 cells switched from collective sheet migration in control cells towards single cell migration upon paxillin depletion. Possibly, 4T1 cells switched to amoeboid migration upon paxillin knockdown, since this is associated with a loss of directionality compared to other modes of migration [8]. Our current models do not allow to distinguish between different modes of single cell migration but the previously discussed modeling of dynamic cell behavior during migration would additionally contribute to this. Since amoeboid migration is characterized by blebbing and small pseudopodia and mesenchymal migration by polarized cells with a clear lamellipodium at the leading edge of the cell, modeling of cell shape during cell migration would help to distinguish these two types of single cell migration. Collective migration in strands and tubes is generally seen in three dimensional cancer cell invasion assays. This assay will be discussed in the next section.

### *1.4 Translation of 2D tumor cell migration to the* in vivo *situation*

Tumor cell migration is typically studied in two dimensional (2D) assays and has provided valuable understanding of molecular mechanisms underlying cell migration. However, 2D cell culture conditions differ strongly from the *in vivo* situation and have been shown to affect cell behavior, including cell survival, proliferation, differentiation and cell shape [9-14]. In addition, cell migration is possibly altered due to the large

difference in stiffness of the substrate and cancer cell invasion can not be studied in 2D [15]. To improve translation to the in vivo situation, three dimensional (3D) invasion assays have been developed. This includes the Boyden chamber assay in which cells migrate through an ECM-coated membrane containing pores towards a chemotactic stimulus. However, this method does not properly mimic tumor cells migrating away from a solid tumor. Major improvements in this respect have been made with the tumor cell spheroid assays. The microenvironment of solid cancers is mimicked by compact cell spheroids, which contain an oxygen- and nutrient-depleted core [16; 17]. Importantly, these cell spheroids are surrounded by ECM that ideally mimics both chemical as well as physical properties of organ tissue. The development of an automated 3D cell spheroid culture system allows for quantitative high-throughput assessment of compound libraries in the context of tumor cell invasion [18]. We applied this method in **chapter 6**, in which we showed that paxillin depletion in 4T1 drastically inhibited spheroid outgrowth, indicating decreased cell invasion. This was in agreement with our *in vivo* data, in which we showed that paxillin is required for efficient metastasis to the lung in an orthotopic breast cancer model. In 2D, paxillin knockdown had only a mild effect compared to control cell migration. Since *in vivo* paxillin depletion almost completely inhibited metastasis formation, the 3D cell spheroid invasion models have improved predictive value compared to 2D culture methods.

## 2. Improved *in vivo* methodologies to study breast cancer progression and metastasis

For breast cancer it is necessary to develop animal models to ultimately evaluate treatments for metastatic cancer and to deepen our understanding of the mechanism underlying metastatic progression. In **chapter 3**, we described an improved mouse breast cancer model that is suitable to study cell autonomous processes of metastasis formation. Although in this case rat (MTLn3) and mouse (4T1) breast cancer cell lines were used, we further explored this system and it is now also possible to use this immunodeficient mouse model with clinically more relevant human breast cancer cell lines. We successfully exploited the MCF7 cell line to demonstrate a crucial role for Fra-1 in tumor progression as is described in **chapter 5**.

*2.1 The role of the immune system in cancer metastasis*

The animal experiments described in this thesis are performed in immunodeficient mice, and, likewise exclude the possible role of the immune system in cancer progression towards metastasis. Consequently this model present only a part of the complex puzzle of cancer progression. Indeed, an important role for the immune system in metastasis formation is demonstrated. Monocytes, neutrophils, regulatory T cells and myeloid-derived suppressor cells (MDSCs) are attracted to tumors where they secrete various cytokines that can activate tumor growth [19]. Increased MDSCs levels are found in breast cancer patients and mouse models including a 4T1 transplantation model and correlate with enhanced capacity to form lung metastasis when co-inoculated with 4T1 cells in naive mice [20; 21]. Tumor-associated macrophages (TAMs) are another example of tumor promoting immune cells. For example, Fra-1 expression in TAMs is upregulated upon interaction with breast cancer cells. Fra-1 in turn initiates the activation of the IL-6/JAK/Stat3 signaling pathway, which results in a malignant switch. Knockdown of Fra-1 in TAMs reduced tumor cell invasion, angiogenesis and metastasis in a mouse breast cancer model [22].

Recently, the tumor self seeding theory has been published, proposing a novel mechanism of tumor progression. Instead of an unidirectional process of dissemination tumor cells from a primary tumor resulting in a secondary tumor at distant organs, it considers circulating tumor cells to be able to also colonize their tumors of origin. Circulating tumor cells are attracted by tumor-derived cytokines IL-6 and -8 and accelerate stromal recruitment through seed-derived factors including the chemokine CXCL1. Tumor self seeding presumes the involvement of the microenvironment to establish a pre-metastatic niche. To study the role of our identified novel metastasis candidate genes in tumor self seeding, syngeneic immunocompetent mouse models are required [23].

*2.2 Intravital imaging of cell migration in the primary tumor*

In many mouse breast cancer models, organs are isolated at the end of the experiment and end-point metastasis is being quantified. However, fluorescent and bioluminescent whole animal imaging techniques allow the evaluation of tumor growth as well as the formation of secondary tumors at distant organs within the same animal over time [24; 25]. An important drawback of this technique is the relative low resolution, which makes it impossible to follow metastatic events at the single cell level. The development of intravital imaging techniques allows the

visualization of tumor cells within the primary tumor in an intact living animal, which provides critical insight into the migratory behavior of tumor cells *in vivo* [26; 27]. This, in combination with inducible gene expression, allows us study the role of individual candidate metastasis genes in tumor cell dissemination within the primary tumor [28; 29]. Also the development of a mammary imaging window in combination with photo-convertible fluorophores demonstrated the possibility to mark a region within the primary tumor and follow migratory events within the same region for days [30; 31]. We showed that knockdown of SRPK1 (**chapter 4**), Fra-1 (**chapter 5**) and paxillin (**chapter 6**) inhibit tumor cell migration in human breast cancer cell lines and block metastasis formation in an *in vivo* breast cancer model. Although we can not say this for sure, it is likely that metastasis formation was blocked upon SRPK1, Fra-1 or paxillin depletion through a reduced migratory capacity of the tumor cells *in vivo*. Intravital imaging of migratory events within the primary tumor would be a powerful method to test this hypothesis.

*2.3 Zebrafish model to study tumor cell invasion and metastasis*

Recently, zebrafish embryos have been used in tumor development, invasion and metastasis studies. Zebrafish are inexpensive to maintain, breed in large numbers and develop *ex vivo* in a short time. Importantly, most cancer genes and tumor suppressor genes are highly conserved between zebrafish and humans and zebrafish develop spontaneous tumors with similar histopathological and gene expression profiles as human tumors [32-34]. Moreover, zebrafish are suitable for xeno-transplantation of human tumor cells [35] and since they are transparent, enable visualization of fluorescently labeled tumor cells. A semi-automated quantitative bio-imaging assay has been developed that allows analysis of cancer cell dissemination in zebrafish embryos in a high-throughput manner [36]. This model offers the possibility for testing genes identified in large scale RNA-interference screens for their *in vivo* relevance, prior to more time- and money-consuming studies in mouse models. It would be interesting to use this model for testing the genes identified in our RNA-interference migration screen described in **chapter 4**. Since our initial screen was performed in a lung cancer cell line, it was surprising to see a strong correlation for eight genes with respect to clinical metastasis free survival in breast cancer. This however does not exclude that other identified genes still have a role in invasion and progression cancer cell types, in particular lung cancer. To gain more insight into these genes, it would be useful to test those genes in the zebrafish model, as well as the 3D collagen invasion assay.

## 3. Signaling networks in breast cancer metastasis

Various genes involved in cell migration are associated with tumor cell motility and cancer metastasis. Increased signaling through EGF or HGF receptor family members promotes actin reorganization and cancer cell invasiveness and metastasis and is associated with poor disease outcome [37-39]. Alternative, activation of TGFβ receptor induces an epithelial-to-mesenchymal transition of otherwise non-motile epithelial cancer cells [40]. Downstream effectors such as the Rho-GTPase RhoC stimulates invasiveness and cancer cell dissemination [41]. And also focal adhesion signaling molecules such as β1 integrin, focal adhesion kinase, Src and p130Cas have been associated with metastatic potential [42-45]. We performed a multiparametric quantitative high content imaging-based RNA-interference screen to identify novel regulators of tumor cell migration. Since tumor cell migration is likely controlled by enhanced oncogenic (receptor-mediated) signaling that drives the dynamic rearrangement of the actin cytoskeletal network and cell matrix adhesions we focused on kinases, phosphatases and all adhesion-related genes (**chapter 4**).

### *3.1 How do our identified migration genes fit in established metastasis signaling networks?*

The RNA-interference screen exploiting the phagokinetic track assay, in which we identified a set of genes as novel regulators of cell migration is described in **chapter 4**. Although we focused our follow up studies at the splicing related kinase SRPK1 (discussed in the next section), seven more clinically relevant genes are identified. Amongst them is the well known migration and metastasis regulator SHC1. SHC-transforming protein 1 couples activated receptor tyrosine kinases, including EGF and HGF receptor family members, to Ras via GRB2/SOS complex and this is implicated in propagation of mitogenic signals [46]. In addition, the MAPK family members MAP2K2 and MAP3K8 are identified. MAPK signaling regulates a number of cellular processes linked to metastasis, including proliferation, differentiation, cell survival and apoptosis. Also a role for MAPK signaling in cell migration has been described [47]. The receptor tyrosine kinase ROS1 may downstream activate MAPK signaling, but is also related to the PI3 kinase and mTOR signaling pathways [48]. It was recently described that TGFβ-induced activation of mTOR complex 2 drives EMT and cell invasion [49]. ROS1 could potentially be part of this EMT program. The non-receptor kinase NEK2 is involved in the control of centrosome separation in mitotic cells, but potentially also contributes to centrosome orientation during directional cell migration [50]. A role for NEK2 in directionality of cell migration has not been

demonstrated before, but would be interesting to investigate. LCK is a member of the Src family kinases and has been shown to activate LIM domain only 2 (Lmo2) promotor through direct interaction [51]. Lmo2 plays a role in prostate cancer cell motility and invasion, possibly via repression of E-cadherin expression [52]. This could (indirectly) link LCK to EMT. ITGB3BP can interact with the NFKB1 NF-kappa-B subunit and thereby interferes with its transactivation domain. NF-kappa-B is essential for EMT and metastasis in a breast cancer model and ITGB3BP possibly plays a role in metastasis via this signaling route [53].

*3.2 SRPK1-related splicing events in the control of breast cancer progression*

We showed that SRPK1 is required for tumor cell migration and is a clinical relevant breast cancer metastasis promoter. SRPK1 is reported to be part of the so-called spliceosomes. Within these organelles, genes are regulated through the splicing of pre-mRNA into mRNA. While the spliceosome consists of 141 core components, it co-purified with over 200 proteins, which includes the catalytic snRNPs and members of the SRSF protein family [54]. SRPK1 is one of these noncore proteins, which are the presumed link between the spliceosome and other cellular machineries such as transcription factors [55]. Given the molecular diversity of the spliceosomes, it is likely that additional core as well as noncore proteins play a role in tumor cell migration and breast cancer progression. We started to explore this with a targeted RNA-interference screen affecting all SRSF family members in the H1299 as well as the MDA-MB-231 cell line. Preliminary data showed that knockdown of SRSF7 and 8 significantly inhibited cell migration in both cell lines, but these genes did not show clinical relevance in our breast cancer patient cohort. On the other hand, knockdown of SRSF5 resulted in an increase in cell speed, and high expression levels are associated with better patient prognosis, suggesting a tumor suppressing function for this splicing factor.

SRPK1 has a role in both constitutive as well as alternative splicing by regulating intracellular location of splicing factors [56]. Alternative splicing events in tumor cell migration have been reported. For example, downregulation of SRPK1 resulted in altered splicing of MAP2K2 leading to imbalanced mitogen-activated protein kinase pathway signaling in various tumor cell lines [57]. Interestingly, we also identified MAP2K2 as a migratory regulator in our screen. Knockdown of both SRPK1 as well as MAP2K2 resulted in inhibition of cell migration. Additionally, SRPK1 seems involved in alternative splicing of MST1R (Ron) via phosphorylation of SF2/ASF. SF2/ASF-mediated splicing activity results in ΔRon induced EMT and increased

cell migration and ΔRon is accumulated in breast and colon cancers [58; 59]. We also identified MST1R as a migration regulating gene in our RNA-interference screen. Expression of this gene did not show clinical relevance in our breast cancer patient cohort but specific splice isoforms of the gene were not taken in to account.

Splicing events are not only involved in tumor cell migration but also in additional aspects of metastasis formation. For example, SRPK1 was shown to regulate VEGF splicing in podocytes and thereby induces angiogenesis [60]. Additionally, an EMT-driven alternative splicing program was recently shown to be involved in human breast cancer and modulation of cellular phenotype [61]. We speculate that SRPK1 is possibly involved in EMT. We observed an increase in cell-cell contacts in the H1299 cell line upon depleting of SRPK1 with increased ZO-1 localization and E-cadherin expression (data not shown). This could however be an indirect result of impaired cell migration caused by SRPK1 knockdown. The establishment of a role for SRPK1 in EMT requires additional research. Focused studies to unravel splicing events underlying tumor cell migration and metastasis could make a significant contribution to a deeper understanding of these biological complex processes and open opportunities for therapeutic intervention. In particular, detailed next generation RNA-sequencing is required to identify the gene networks that are modulated by SRPK1 through splicing.

### *3.3 Targeting breast cancer metastasis by inhibition of Fra-1 signaling*

In **chapter 5**, we demonstrated that ectopic expression of Fra-1 is sufficient to induce a full EMT genetic program in the luminal breast cancer cell line MCF7. This resulted in cell scattering, anchorage independent growth and increased cell migration with enhanced focal adhesion turnover. Importantly, Fra-1 expression induced tumorigenesis as well as metastasis formation in an *in vivo* orthotopic breast cancer mouse model. Gene expression profiling followed by unsupervised hierarchical clustering demonstrated a switch towards basal B-like breast cancer cells upon introduction of Fra-1 in the luminal MCF7 cell line. Finally, knockdown of Fra-1 in basal B cell lines reduced their migratory capacity and metastatic potential. Together the results indicate that Fra-1 is a dominant factor in breast cancer progression and metastasis and pin-point Fra-1-based signaling as a valid target for development of breast cancer therapies.

Of main interest is the role for Fra-1 in the induction of EMT. Different transcriptional repressors of E-cadherin were affected by Fra-1 expression, including upregulation of SNAI2 and ZEB1/2. The Fra-1-ZEB1/2 axis has previously been

described to induce EMT in normal breast cancer cells. Possibly, Fra-1 directly drives the expression of the different E-cadherin repressors leading to a full EMT program. Interestingly, miR-221/222 was recently demonstrated as a target of Fra-1, which suppresses the expression of the TRPS1, a repressor of ZEB2. Next to E-cadherin suppression, our transcriptomics data analysis revealed additional receptor signaling routes that could contribute to Fra-1-induced EMT including Wnt-, HGF-mediated signaling. Possibly Fra-1 induces the expression of these ligands with the subsequent activation of their receptors and initiation of the EMT program. A link between Fra-1 and HGF signaling in the context of EMT has not been established before. HGF signaling is known to have crosstalk with other signaling routes, including Wnt signaling via induction of β-catenin [62]. Our data indicates that Fra-1 expression promotes a Wnt signaling program. Canonical Wnt signaling is linked to EMT via hypoxia-inducible factor 1 alpha (HIF1α) in human prostate cancer cells [63; 64]. Activation of canonical Wnt signaling in periodontal ligament fibroblasts resulted in increased Fra-1 mRNA levels [65]. Our data suggests that Fra-1 also acts upstream of Wnt. Further work is required to decipher the role of these different signaling programs in Fra-1-mediated EMT and cancer metastasis. This is likely to lead to the identification of additional relevant and novel anti-metastasis target genes.

Another interesting observation is the possible role for Fra-1 in breast cancer cell stemness, as is suggested by the observed dramatic decreased expression of the putative BC stem cell marker, CD24, in MCF7-Fra-1 cells. Cancer stem cells are also referred to as tumor initiating cells and have increased expression levels of proinvasive genes and enhanced tumorigenicity. Therefore, targeting the cancer stem cells, possibly by inhibition of Fra-1, has potential to be highly effective as a treatment in breast cancer patients.

*3.4 Targeting paxillin-mediated signaling to prevent breast cancer metastasis formation*

In **chapter 6**, we aimed to investigate the role of the focal adhesion protein paxillin in breast cancer progression. We showed that upon paxillin depletion in the 4T1 cell line, 2D migration as well as 3D invasion is decreased. This is in line with our *in vivo* data, in which we showed that paxillin is required for efficient metastasis to the lung in a breast cancer model. This is possibly explained by the observation that paxillin is required for directional cell migration in the 4T1 through Rac1 mediated signaling. Paxillin depletion resulted in altered membrane dynamics through actin-cytoskeleton reorganization, which could explain the defect in directional migration. However,

more details are required to determine the exact molecular mechanism underlying these observations. Given the critical role of individual domains and Tyr and Ser phosphorylation sites in paxillin in the control of different downstream events, it will be interesting to define which domains are critical for the downregulation of actin dynamics and focal adhesion activity. Moreover, we need to determine whether Rho GTPase activity is altered in paxillin depleted cells, and whether this is related to local perturbations of individual Rho GTPase family members including Rac1, RhoA and Cdc42 activity. This could be achieved using specific fluorescence resonance energy transfer (FRET) sensors [66].

Paxillin strongly inhibited metastasis formation and it is likely that this is related to inhibition of tumor cell motility and invasion in the primary tumor, preventing intravasation of tumor cells followed by their dissemination to distant organs. Alternatively, paxillin may affect the expression of candidate genes that control the local tumor microenvironment for example by influencing the immune system. Indeed our gene expression data indicate that the top twenty affected pathways after paxillin knockdown include pathways related to immune responses, in particular antigen presentation, pathways involved in bacterial infections and CD40 signaling (unpublished data). CD40 is a member of the TNF receptor superfamily that can interact with several TRAF family members and is expressed in antigen presenting cells, including B cells and macrophages, as well as endothelial and epithelial cells. Interestingly, paxillin and CD40 have previously been linked in the context of endothelial barrier function and B cell activation. Upon B cell adhesion to endothelial cells via CD40 and β1 integrin, paxillin, together with FAK and ERK2, is dephosphorylated resulting in endothelial cell activation followed by chemokine production and lymphocyte infiltration [67]. Therefore, paxillin knockdown possibly decreases metastasis through impaired chemokine production and lymphocyte infiltration. Although paxillin itself does not contain domains with enzymatic activity that could be targeted by small molecule inhibitors, searching for molecules that could block interaction between paxillin and crucial downstream effectors or target paxillin controlled gene expression would be a feasible approach to inhibit cell migration and prevent metastasis formation.

## 4. Towards full understanding of cancer metastasis – future perspectives

The complexity and the large number of signaling pathways underlying cancer metastasis is a major challenge for researchers as well as pharmaceutical companies. The development of preclinical models and the identification of relevant signaling

pathways and genetic regulators of cancer metastasis have greatly contributed to improved cancer treatment. However, parts of the complex metastasis puzzle are still missing and unfortunately current cures are insufficient to treat all breast cancer patients, in particular with respect to recurrence and metastasis. Future research and improved data processing and integration is needed to improve our understanding of cancer metastasis.

Nowadays, the goal of most RNA-interference screens is to improve our knowledge of complex biological processes, like the migration screen described in this thesis. However, large parts of these datasets, especially for image-based screens, are not included as part of scientific publications, risking valuable information to be lost or overlooked. To improve this, investments are being made to make all images and associated numerical data available in online databases. This enables the scientific community to search for genes, biological phenotypes and gene ontology (GO) terms across a variety of RNA-interference screens. Likewise, many research groups have invested in translational research. For example those that used gene expression profiling to establish a breast cancer stem cell signature in a study with cells derived from breast cancer lung metastases [68]. Databases like Oncomine, a compendium of cancer transcriptome profiles integrated with an analysis engine, allows researchers to get information about their genes of interest in the context of the latest and cutting-edge cancer studies. The challenge ahead lies in combining various screening data, including functional genomics as well as proteomics and transcriptomics, with translational clinical research. This will help researchers to focus on those genes that are critical in a certain biological process as well as show clinically relevance, thereby increasing its chances to enter further drug development. Alternatively, one could start by determining genes associated with metastasis free survival for specific cancer patient cohorts and test the effect of gene knockdown in high-throughput cell migration assays for functional validation. Verification of relevance in human disease, for example by tissue micro arrays, is however still required.

The unraveling of the whole human genome has resulted in commercial available RNA-interference libraries but recently also pushed the field of genetic research forward. The development of next generation sequencing allows the unprecedented generation of very large sequencing datasets on a short time scale and at affordable cost. For example, the targeted next generation sequencing of a cancer transcriptome enhances detection of sequence variants and novel fusion transcripts [69]. Of particular interest would be to define mutations within migration

associated genes, in particular genes in the adhesome network. This will contribute to our understanding of focal adhesion hierarchy and regulation of its dynamics in the context of tumor cell migration and metastasis. Next generation sequencing is also applicable for clinical molecular diagnosis. In context of the research described in this thesis, breast cancer patients could for example be screened for SRPK-1 mediated alternative splicing patterns and receive personalized treatment.

## 5. Conclusions

In conclusion, we established a live cell imaging-based random cell migration assay that is suitable for both compound as well as RNA-interference screening in multiple cancer cell lines. In addition, we successfully established a suitable *in vivo* breast cancer model, which provides insight into cell autonomous processes that are required for tumor cells to metastasize. Furthermore, a RNA-interference screen exploiting the phagokinetic track assay allowed us to identify a novel set of candidate genes that act as regulators of cell migration. In particular, this has led to the identification of SRPK1 as a clinical relevant breast cancer metastasis promoter. Next to the RNA-interference screening, focused research has been conducted on previously identified novel candidate genes. We described Fra-1 as a dominant factor in breast cancer progression and metastasis through induction of EMT and pin-point Fra-1-based signaling as a valid target for development of breast cancer therapies. Finally, we investigated the role of the focal adhesion protein paxillin in breast cancer using an established mouse breast cancer model. Here we showed that paxillin is controlling directional tumor cell migration possibly through modulating Rac1-mediated signaling. Future research to elucidate underlying molecular mechanisms for in particular SRPK1-, Fra-1- and paxillin-mediated metastasis in more detail will help us understand the key pathways that control breast cancer metastasis and open opportunities for therapeutic intervention.

## References

1. Palecek, S. P., Loftus, J. C., Ginsberg, M. H., Lauffenburger, D. A., & Horwitz, A. F. (1997). Integrin-ligand binding properties govern cell migration speed through cell-substratum adhesiveness. Nature, 385(6616), 537-540.

2. Hlobilkova, A., Knillova, J., Bartek, J., Lukas, J., & Kolar, Z. (2003). The mechanism of action of the tumour suppressor gene PTEN. Biomed Pap Med Fac Univ Palacky Olomouc Czech Repub, 147(1), 19-25.

3. Goldmann, W. H. (2002). p56(lck) Controls phosphorylation of filamin (ABP-280) and regulates focal adhesion kinase (pp125(FAK)). Cell Biol Int, 26(6), 567-571.

4. Friedl, P. (2004). Prespecification and plasticity: shifting mechanisms of cell migration. Curr Opin Cell Biol, 16(1), 14-23.

5. Friedl, P., & Wolf, K. Plasticity of cell migration: a multiscale tuning model. J Cell Biol, 188(1), 11-19.

6. Sahai, E., & Marshall, C. J. (2003). Differing modes of tumour cell invasion have distinct requirements for Rho/ROCK signalling and extracellular proteolysis. Nat Cell Biol, 5(8), 711-719.

7. Sanz-Moreno, V., Gadea, G., Ahn, J., Paterson, H., Marra, P., Pinner, S., Sahai, E., & Marshall, C. J. (2008). Rac activation and inactivation control plasticity of tumor cell movement. Cell, 135(3), 510-523.

8. Huttenlocher, A., & Horwitz, A. R. Integrins in cell migration. Cold Spring Harb Perspect Biol, 3(9), a005074.

9. Beliveau, A., Mott, J. D., Lo, A., Chen, E. I., Koller, A. A., Yaswen, P., Muschler, J., & Bissell, M. J. Raf-induced MMP9 disrupts tissue architecture of human breast cells in three-dimensional culture and is necessary for tumor growth in vivo. Genes Dev, 24(24), 2800-2811.

10. Bissell, M. J. (1981). The differentiated state of normal and malignant cells or how to define a “normal” cell in culture. Int Rev Cytol, 70, 27-100.

11. Bjerkvig, R., Tonnesen, A., Laerum, O. D., & Backlund, E. O. (1990). Multicellular tumor spheroids from human gliomas maintained in organ culture. J Neurosurg, 72(3), 463-475.

12. Corcoran, A., De Ridder, L. I., Del Duca, D., Kalala, O. J., Lah, T., Pilkington, G. J., & Del Maestro, R. F. (2003). Evolution of the brain tumour spheroid model: transcending current model limitations. Acta Neurochir (Wien), 145(9), 819-824.

13. Kenny, P. A., Lee, G. Y., Myers, C. A., Neve, R. M., Semeiks, J. R., Spellman, P. T., Lorenz, K., Lee, E. H., Barcellos-Hoff, M. H., Petersen, O. W., Gray, J. W., & Bissell, M. J. (2007). The morphologies of breast cancer cell lines in three-dimensional assays correlate with their profiles of gene expression. Mol Oncol, 1(1), 84-96.

14. Walpita, D., & Hay, E. (2002). Studying actin-dependent processes in tissue culture. Nat Rev Mol Cell Biol, 3(2), 137-141.

15. Kraning-Rush, C. M., & Reinhart-King, C. A. (2012). Controlling matrix stiffness and topography for the study of tumor cell migration. Cell Adh Migr, 6(3).

16. Mueller-Klieser, W. (1987). Multicellular spheroids. A review on cellular aggregates in cancer research. J Cancer Res Clin Oncol, 113(2), 101-122.

17. Sutherland, R. M. (1988). Cell and environment interactions in tumor microregions: the multicell spheroid model. Science, 240(4849), 177-184.

18. Truong, H. H., de Sonneville, J., Ghotra, V. P., Xiong, J., Price, L., Hogendoorn, P. C., Spaink, H. H., van de Water, B., & Danen, E. H. Automated microinjection of cell-polymer suspensions in 3D ECM scaffolds for high-throughput quantitative cancer invasion screens. Biomaterials, 33(1), 181-188.

19. Coussens, L. M., & Werb, Z. (2002). Inflammation and cancer. Nature, 420(6917), 860-867.

20. DuPre, S. A., & Hunter, K. W., Jr. (2007). Murine mammary carcinoma 4T1 induces a leukemoid reaction with splenomegaly: association with tumor-derived growth factors. Exp Mol Pathol, 82(1), 12-24.

21. Yang, L., Huang, J., Ren, X., Gorska, A. E., Chytil, A., Aakre, M., Carbone, D. P., Matrisian, L. M., Richmond, A., Lin, P. C., & Moses, H. L. (2008). Abrogation of TGF beta signaling in mammary carcinomas

recruits Gr-1+CD11b+ myeloid cells that promote metastasis. Cancer Cell, 13(1), 23-35.
22. Luo, Y. P., Zhou, H., Krueger, J., Kaplan, C., Liao, D., Markowitz, D., Liu, C., Chen, T., Chuang, T. H., Xiang, R., & Reisfeld, R. A. The role of proto-oncogene Fra-1 in remodeling the tumor microenvironment in support of breast tumor cell invasion and progression. Oncogene, 29(5), 662-673.
23. Kim, M. Y., Oskarsson, T., Acharyya, S., Nguyen, D. X., Zhang, X. H., Norton, L., & Massague, J. (2009). Tumor self-seeding by circulating cancer cells. Cell, 139(7), 1315-1326.
24. Henriquez, N. V., van Overveld, P. G., Que, I., Buijs, J. T., Bachelier, R., Kaijzel, E. L., Lowik, C. W., Clezardin, P., & van der Pluijm, G. (2007). Advances in optical imaging and novel model systems for cancer metastasis research. Clin Exp Metastasis, 24(8), 699-705.
25. Kaijzel, E. L., van der Pluijm, G., & Lowik, C. W. (2007). Whole-body optical imaging in animal models to assess cancer development and progression. Clin Cancer Res, 13(12), 3490-3497.
26. Condeelis, J., & Segall, J. E. (2003). Intravital imaging of cell movement in tumours. Nat Rev Cancer, 3(12), 921-930.
27. Kedrin, D., Wyckoff, J., Sahai, E., Condeelis, J., & Segall, J. E. (2007). Imaging tumor cell movement in vivo. Curr Protoc Cell Biol, Chapter 19, Unit 19 17.
28. Le Devedec, S. E., Lalai, R., Pont, C., de Bont, H., & van de Water, B. Two-photon intravital multicolor imaging combined with inducible gene expression to distinguish metastatic behavior of breast cancer cells in vivo. Mol Imaging Biol, 13(1), 67-77.
29. Le Devedec, S. E., van Roosmalen, W., Pont, C., Lalai, R., de Bont, H., & van de Water, B. Two-photon intravital multicolour imaging to study metastatic behaviour of cancer cells in vivo. Methods Mol Biol, 769, 331-349.
30. Gligorijevic, B., Kedrin, D., Segall, J. E., Condeelis, J., & van Rheenen, J. (2009). Dendra2 photoswitching through the Mammary Imaging Window. J Vis Exp(28).
31. Kedrin, D., Gligorijevic, B., Wyckoff, J., Verkhusha, V. V., Condeelis, J., Segall, J. E., & van Rheenen, J. (2008). Intravital imaging of metastatic behavior through a mammary imaging window. Nat Methods, 5(12), 1019-1021.
32. Amatruda, J. F., Shepard, J. L., Stern, H. M., & Zon, L. I. (2002). Zebrafish as a cancer model system. Cancer Cell, 1(3), 229-231.
33. Berghmans, S., Jette, C., Langenau, D., Hsu, K., Stewart, R., Look, T., & Kanki, J. P. (2005). Making waves in cancer research: new models in the zebrafish. Biotechniques, 39(2), 227-237.
34. Lam, S. H., & Gong, Z. (2006). Modeling liver cancer using zebrafish: a comparative oncogenomics approach. Cell Cycle, 5(6), 573-577.
35. Haldi, M., Ton, C., Seng, W. L., & McGrath, P. (2006). Human melanoma cells transplanted into zebrafish proliferate, migrate, produce melanin, form masses and stimulate angiogenesis in zebrafish. Angiogenesis, 9(3), 139-151.
36. Ghotra, V. P., He, S., de Bont, H., van der Ent, W., Spaink, H. P., van de Water, B., Snaar-Jagalska, B. E., & Danen, E. H. Automated whole animal bio-imaging assay for human cancer dissemination. PLoS One, 7(2), e31281.
37. Hynes, N. E., & Lane, H. A. (2005). ERBB receptors and cancer: the complexity of targeted inhibitors. Nat Rev Cancer, 5(5), 341-354.
38. Rijken, P. J., Hage, W. J., van Bergen en Henegouwen, P. M., Verkleij, A. J., & Boonstra, J. (1991). Epidermal growth factor induces rapid reorganization of the actin microfilament system in human A431 cells. J Cell Sci, 100 ( Pt 3), 491-499.
39. Xue, C., Wyckoff, J., Liang, F., Sidani, M., Violini, S., Tsai, K. L., Zhang, Z. Y., Sahai, E., Condeelis, J., & Segall, J. E. (2006). Epidermal growth factor receptor overexpression results in increased tumor cell motility in vivo coordinately with enhanced intravasation and metastasis. Cancer Res, 66(1), 192-197.
40. Zhang, H. J., Wang, H. Y., Zhang, H. T., Su, J. M., Zhu, J., Wang, H. B., Zhou, W. Y., Zhang, H., Zhao, M. C., Zhang, L., & Chen, X. F. (2011). Transforming growth factor-beta1 promotes lung adenocarcinoma

invasion and metastasis by epithelial-to-mesenchymal transition. Mol Cell Biochem, 355(1-2), 309-314.
41. Rosenthal, D. T., Iyer, H., Escudero, S., Bao, L., Wu, Z., Ventura, A. C., Kleer, C. G., Arruda, E. M., Garikipati, K., & Merajver, S. D. (2011). p38gamma promotes breast cancer cell motility and metastasis through regulation of RhoC GTPase, cytoskeletal architecture, and a novel leading edge behavior. Cancer Res, 71(20), 6338-6349.
42. Danen, E. H. (2005). Integrins: regulators of tissue function and cancer progression. Curr Pharm Des, 11(7), 881-891.
43. Di Stefano, P., Leal, M. P., Tornillo, G., Bisaro, B., Repetto, D., Pincini, A., Santopietro, E., Sharma, N., Turco, E., Cabodi, S., & Defilippi, P. (2011). The adaptor proteins p140CAP and p130CAS as molecular hubs in cell migration and invasion of cancer cells. Am J Cancer Res, 1(5), 663-673.
44. van Nimwegen, M. J., Verkoeijen, S., van Buren, L., Burg, D., & van de Water, B. (2005). Requirement for focal adhesion kinase in the early phase of mammary adenocarcinoma lung metastasis formation. Cancer Res, 65(11), 4698-4706.
45. Zhang, X. H., Wang, Q., Gerald, W., Hudis, C. A., Norton, L., Smid, M., Foekens, J. A., & Massague, J. (2009). Latent bone metastasis in breast cancer tied to Src-dependent survival signals. Cancer Cell, 16(1), 67-78.
46. Patrussi, L., Savino, M. T., Pellegrini, M., Paccani, S. R., Migliaccio, E., Plyte, S., Lanfrancone, L., Pelicci, P. G., & Baldari, C. T. (2005). Cooperation and selectivity of the two Grb2 binding sites of p52Shc in T-cell antigen receptor signaling to Ras family GTPases and Myc-dependent survival. Oncogene, 24(13), 2218-2228.
47. Kim, E. K., & Choi, E. J. (2010). Pathological roles of MAPK signaling pathways in human diseases. Biochim Biophys Acta, 1802(4), 396-405.
48. Charest, A., Wilker, E. W., McLaughlin, M. E., Lane, K., Gowda, R., Coven, S., McMahon, K., Kovach, S., Feng, Y., Yaffe, M. B., Jacks, T., & Housman, D. (2006). ROS fusion tyrosine kinase activates a SH2 domain-containing phosphatase-2/phosphatidylinositol 3-kinase/mammalian target of rapamycin signaling axis to form glioblastoma in mice. Cancer Res, 66(15), 7473-7481.
49. Lamouille, S., Connolly, E., Smyth, J. W., Akhurst, R. J., & Derynck, R. (2012). TGF-beta-induced activation of mTOR complex 2 drives epithelial-mesenchymal transition and cell invasion. J Cell Sci, 125(Pt 5), 1259-1273.
50. Mardin, B. R., Lange, C., Baxter, J. E., Hardy, T., Scholz, S. R., Fry, A. M., & Schiebel, E. (2010). Components of the Hippo pathway cooperate with Nek2 kinase to regulate centrosome disjunction. Nat Cell Biol, 12(12), 1166-1176.
51. Venkitachalam, S., Chueh, F. Y., & Yu, C. L. (2012). Nuclear localization of lymphocyte-specific protein tyrosine kinase (Lck) and its role in regulating LIM domain only 2 (Lmo2) gene. Biochem Biophys Res Commun, 417(3), 1058-1062.
52. Ma, S., Guan, X. Y., Beh, P. S., Wong, K. Y., Chan, Y. P., Yuen, H. F., Vielkind, J., & Chan, K. W. (2007). The significance of LMO2 expression in the progression of prostate cancer. J Pathol, 211(3), 278-285.
53. Huber, M. A., Beug, H., & Wirth, T. (2004). Epithelial-mesenchymal transition: NF-kappaB takes center stage. Cell Cycle, 3(12), 1477-1480.
54. Hegele, A., Kamburov, A., Grossmann, A., Sourlis, C., Wowro, S., Weimann, M., Will, C. L., Pena, V., Luhrmann, R., & Stelzl, U. (2012). Dynamic protein-protein interaction wiring of the human spliceosome. Mol Cell, 45(4), 567-580.
55. Agafonov, D. E., Deckert, J., Wolf, E., Odenwalder, P., Bessonov, S., Will, C. L., Urlaub, H., & Luhrmann, R. (2011). Semiquantitative proteomic analysis of the human spliceosome via a novel two-dimensional gel electrophoresis method. Mol Cell Biol, 31(13), 2667-2682.
56. Ghosh, G., & Adams, J. A. (2011). Phosphorylation mechanism and structure of serine-arginine protein kinases. FEBS J, 278(4), 587-597.
57. Hayes, G. M., Carrigan, P. E., & Miller, L. J. (2007). Serine-arginine protein kinase 1 overexpression is

associated with tumorigenic imbalance in mitogen-activated protein kinase pathways in breast, colonic, and pancreatic carcinomas. Cancer Res, 67(5), 2072-2080.
58. Collesi, C., Santoro, M. M., Gaudino, G., & Comoglio, P. M. (1996). A splicing variant of the RON transcript induces constitutive tyrosine kinase activity and an invasive phenotype. Mol Cell Biol, 16(10), 5518-5526.
59. Ghigna, C., Giordano, S., Shen, H., Benvenuto, F., Castiglioni, F., Comoglio, P. M., Green, M. R., Riva, S., & Biamonti, G. (2005). Cell motility is controlled by SF2/ASF through alternative splicing of the Ron protooncogene. Mol Cell, 20(6), 881-890.
60. Amin, E. M., Oltean, S., Hua, J., Gammons, M. V., Hamdollah-Zadeh, M., Welsh, G. I., Cheung, M. K., Ni, L., Kase, S., Rennel, E. S., Symonds, K. E., Nowak, D. G., Royer-Pokora, B., Saleem, M. A., Hagiwara, M., Schumacher, V. A., Harper, S. J., Hinton, D. R., Bates, D. O., & Ladomery, M. R. (2011). WT1 mutants reveal SRPK1 to be a downstream angiogenesis target by altering VEGF splicing. Cancer Cell, 20(6), 768-780.
61. Shapiro, I. M., Cheng, A. W., Flytzanis, N. C., Balsamo, M., Condeelis, J. S., Oktay, M. H., Burge, C. B., & Gertler, F. B. (2011). An EMT-driven alternative splicing program occurs in human breast cancer and modulates cellular phenotype. PLoS Genet, 7(8), e1002218.
62. Muller, T., Bain, G., Wang, X., & Papkoff, J. (2002). Regulation of epithelial cell migration and tumor formation by beta-catenin signaling. Exp Cell Res, 280(1), 119-133.
63. Heuberger, J., & Birchmeier, W. (2010). Interplay of cadherin-mediated cell adhesion and canonical Wnt signaling. Cold Spring Harb Perspect Biol, 2(2), a002915.
64. Jiang, Y. G., Luo, Y., He, D. L., Li, X., Zhang, L. L., Peng, T., Li, M. C., & Lin, Y. H. (2007). Role of Wnt/beta-catenin signaling pathway in epithelial-mesenchymal transition of human prostate cancer induced by hypoxia-inducible factor-1alpha. Int J Urol, 14(11), 1034-1039.
65. Heo, J. S., Lee, S. Y., & Lee, J. C. (2010). Wnt/beta-catenin signaling enhances osteoblastogenic differentiation from human periodontal ligament fibroblasts. Mol Cells, 30(5), 449-454.
66. Seth, A., Otomo, T., Yin, H. L., & Rosen, M. K. (2003). Rational design of genetically encoded fluorescence resonance energy transfer-based sensors of cellular Cdc42 signaling. Biochemistry, 42(14), 3997-4008.
67. Reyes, L. I., Escobar, P., Bono, M. R., & Rosemblatt, M. (2002). Adhesion of B cell lines to endothelial cells from human lymphoid tissue modulates tyrosine phosphorylation and endothelial cell activation. J Immunol, 169(10), 5881-5888.
68. Shipitsin, M., Campbell, L. L., Argani, P., Weremowicz, S., Bloushtain-Qimron, N., Yao, J., Nikolskaya, T., Serebryiskaya, T., Beroukhim, R., Hu, M., Halushka, M. K., Sukumar, S., Parker, L. M., Anderson, K. S., Harris, L. N., Garber, J. E., Richardson, A. L., Schnitt, S. J., Nikolsky, Y., Gelman, R. S., & Polyak, K. (2007). Molecular definition of breast tumor heterogeneity. Cancer Cell, 11(3), 259-273.
69. Levin, J. Z., Berger, M. F., Adiconis, X., Rogov, P., Melnikov, A., Fennell, T., Nusbaum, C., Garraway, L. A., & Gnirke, A. (2009). Targeted next-generation sequencing of a cancer transcriptome enhances detection of sequence variants and novel fusion transcripts. Genome Biol, 10(10), R115.