Image-based phenotypic screening for breast cancer metastasis drug target discovery

Fokkelman, M.

Chapter 6

General discussion and summary

Breast cancer is the most common type of malignancy in women. In the Netherlands, approximately 14,500 cases of breast cancer are reported each year[1]. Despite extensive studies to understand the genetic makeup and molecular mechanisms that drive breast cancer progression and metastasis, nearly 3,100 patients in the Netherlands pass away each year. The majority of these patients suffer from metastatic cancer, an advanced stage of the disease where tumor cells have migrated away from the primary tumor into the surrounding tissues and distant organs. The metastatic spreading of tumor cells relies on the acquisition and enhancement of cell migration, a fundamental cell biological program. Therefore, improved understanding of the molecular determinants of tumor cell migration will help to lay the fundamental ground-work for future advances in targeted therapeutic development and personalized health care. The work in this thesis was aimed at elucidating the signaling and regulatory networks that drive tumor cell migration in context of breast cancer progression and metastasis. Below the results of the different chapters are discussed in a broader context and future perspectives are lined out.

1. Methodologies to study cell adhesion and cell migration

From the movement of unicellular organisms to embryonic development and immunity in multicellular organisms, cell migration is a fundamental process in biology[2]. Cell migration is a highly complex and asymmetrical process that demands a high level of spatial and temporal control of key events, including adhesion dynamics, Rho signaling and actin contractility[3–6]. Investigation of these biological phenomena and the spatiotemporal dynamics of their molecular machineries requires high-resolution time-lapse microscopy, making it difficult to study these in high-throughput fashion. However, such high-throughput studies provide new levels of precision in molecular understanding and are fundamental in broadening our knowledge of basic cell biological programs in order to advance anti-cancer therapies[7]. Throughout this thesis, different imaging-based assays are set up to study cell adhesion and cell migration without the need for time-lapse microscopy, thereby enabling high-throughput imaging-based (high-content) screening of adhesion dynamics and cell migration. In addition to developing assays, extensive image and data analysis was required. For this purpose, custom scripts were established to recognize nuclei, cells, and cell-matrix adhesions, and to calculate different aspects such as size, shape, and speed. Together these advances made it possible to perform this research and identify novel regulators of cell adhesion and tumor cell migration.

1.1 PhagoKinetic Track assay for high-throughput screening of single cell migration

In **Chapter 2**, the development of a single cell migration assay, based on the PhagoKinetic Track (PKT) procedure[8], is described. This imaging-based assay of single cell migration is suitable for RNAi and compound screening, and has previously been used to screen for regulators of cell migration[8,9]. Here, we further optimized the experimental procedures, thereby reducing the

costs of materials with approximately 75%. We also tested numerous breast cancer cell lines, showing that this assay can be applied to other cell lines that display single cell migration. One of the main advantages of this assay is that it is a fixed end-point assay, allowing automated microscopy of multiple plates using a robotic plate handler. Another advantage is the straight forward image analysis, which can be performed with open source software and does not require complex algorithms for tracking objects over multiple frames. These advantages have been fully exploited to examine the role of adhesion G-protein coupled receptors (GPCRs) in cell adhesion and migration (**chapters 3**) and to perform a broad RNAi screen to systematically map the signaling landscape that defines cell migration in breast cancer (**chapter 4**). Other fixed high-throughput compatible cell migration assays, based on the principle of wound-healing or an exclusion zone, are available, however these are directed at collective cell migration and are less suitable for studying single cell migration. Nevertheless, the PKT assay requires cells to be trypsinized, to rapidly adhere, and to phagocytose small particles as they migrate. To confirm screening results and assess changes in cell migration dynamics, live microscopy assays are recommended, and allow a more detailed analysis of migratory behavior (speed, directionality, persistence, and cell morphology). This will be discussed in the next section.

1.2 Fluorescent live cell imaging

Live microscopy cell migration assays allow the quantification of multiple motility parameters such as speed, directionality, and persistence, as well as analysis of cell morphological features and their dynamics. To perform such analysis, a live cell imaging-based random cell migration assay in 96-well format was established using GFP-expressing Hs578T and MDA-MB-231 breast cancer cells (**chapter 4**). Compared to classical migration assays based on Differential Interference Contrast and nuclei imaging, the use of fluorescent marked cells poses several benefits. First, it allows faster image acquisition, as only one channel (GFP) has to be acquired, compared to two channels (DIC and Hoechst). Secondly, DIC images are notoriously difficult to analyze, hampering fully automated analysis of large datasets, whereas fluorescence microscopy allows tracking of the GFP-positive cell body compared to only nucleus, and provides an accurate measurement of cell position. Lastly, given the faster acquisition interval, a larger number of images (and thus cells) can be acquired in a shorter time frame, thereby providing more data on single cell behavior at the population level after perturbation (e.g. RNAi knockdown). This fast and improved assay set up allowed us to rapidly validate candidate metastasis genes derived from our cell migration screens (**chapter 4**) using live cell microscopy, as well as confirm the role of ADGRG2 (Adhesion GPCR G2, or GPR64) in tumor cell migration (**chapter 3**). In general, the overall quality of images greatly impacts the analysis results, indicating the importance of improving both assay and image acquisition set up.

1.3 Image and data analysis of single cell migration

Live cell migration studies produce vast amounts of imaging data and require robust image analysis tools to reliably extract quantitative data from time-lapse images[7]. Furthermore, in these studies an automated yet accurate tracking method is essential to quantitatively assess the temporal behavior of individual cells. Here, we developed a complete image and data analysis pipeline for single cell migration using open source tools (ImageJ, CellProfiler, and R) (**Chapter 4**). This novel analysis method provided a means to study temporal dynamics of individual cells as well as single cell migratory behavior at the cell population level, while retaining the natural heterogeneity that arises in such described in **chapters 3, 4** and **5**. Furthermore, this analysis method could also be applied to track nuclei of migrating epithelial cells (in **chapter 5**), thereby highlighting its adaptability, and proved to be more accurate than existing methods (data not shown). Proper image analysis is critical to derive biological meaning from microscopy images. The analysis tools described here provide a robust method for tracking of objects in image data, generating reliable quantitative data on the dynamic behavior of cells.

1.4 Advances in automated microscopy

Over the past decade, the field of confocal light scanning microscopy (and other microscopy techniques) has made tremendous progress, in terms of speed, sensitivity and possibilities. However, microscopes do not function fully automatically, and require a user to find and focus on the object of interest (e.g. cell, organelle, adhesion). The most recent software versions on a state-of-the-art microscopy set up with an automated xy-stage do provide user-friendly tools, such as predefined plates, stitching and multi-positional imaging. Nevertheless, these tools have only been available recently and are insufficient for fully automated imaging, i.e. a system that searches for cells and defines the optimal focus by itself. In **chapter 5**, we present such a set up for automated high-resolution confocal microscopy of cell-matrix adhesion structures. A set of macro's was custom developed to execute several tasks: generate multiple positions per well for a 96-well plate, correct for xyz-offset, randomly search for cells, and ultimately define the optimal focus (up to 0.05 µm sensitivity in z). This platform was subsequently used to perform a high-throughput and high-content RNAi screen on the dynamic organization of cell-matrix adhesions (**chapter 5**). These advances in automated microscopy will help to accelerate and standardize imaging-based research, and have either been implemented by Nikon in their latest microscopy software (NIS Elements) or by our lab as modular macro's to supplement such software. Nevertheless, automated imaging is becoming more standard with the arrival of High Content Analyzers, and has been used by others to perform a screen on cell-matrix adhesions in HeLa cells[10]. Here, we used a confocal microscope with a unique combination of laser- and image-based auto-focus, instead of epifluorescence, to obtain high-resolution images with minimal background signal. The obvious benefit of confocal-derived images improved the quality of our segmentation and thus assessment of adhesion size under different conditions.

2. Importance of inhibiting cell migration

Enhanced cell migration and invasion is one of the hallmarks of cancer and required for the dissemination of cancer cells leading to metastases[11,12]. The metastatic cascade comprises of several distinct steps, which heavily rely on cell migration, and include local invasion, intravasation, extravasation, and dissemination in distant tissues[13–15]. The rapidly advancing field of intravital microscopy has provided important insights in these processes, by visualizing the dynamic interactions between tumor cells and their local and distant environment *in vivo*[16–20]. These approaches have revealed tumor heterogeneity at single cell level, as well as mechanistic understanding of intravasation coordinated by tumor cells and their local microenvironment. Nevertheless, high-throughput screens are still necessary to systematically unravel the signaling and regulatory networks that drive different aspects of tumor progression, such as proliferation, therapy resistance and cell migration. These studies allow us to understand how tumor cells function, how basic cell biological programs (e.g. sustained proliferation) are affected, and how to exploit vulnerabilities in tumor cells to combat the disease. In this thesis, we performed two high-throughput screens, one focused on tumor cell migration (**chapter 4**) and one on cell adhesion dynamics and migration (**chapter 5**). In **chapter 4**, we aimed to uncover the landscape of signaling molecules that functionally drive tumor cell migration in triple negative breast cancer (TNBC). Therefore, we screened ~4200 target genes in two highly motile TNBC cell lines for defects in single cell migration, thereby covering the majority of signaling components. We identified 215 candidate metastasis genes in total that impaired cell migration in Hs578T and/or MDA-MB-231 (133 for Hs578T, 113 for MDA-MB-231, 31 overlap). More importantly, high expression of 26 of these candidate genes is associated with decreased metastasis-free survival in breast cancer patients, suggesting that inhibition of these targets may impair dissemination of breast cancer thus improving survival. For several candidate genes we have created breast cancer cell lines with stable knockdown using lentiviral shRNA approaches; these are currently tested in *in vivo* orthotopic mouse model for breast cancer metastasis to determine the role of candidate genes in metastasis formation. Additional studies using intravital imaging could provide mechanistic insights and pinpoint which part of the metastatic cascade is most affected. Thus, a pipeline from screening to hit validation, in vivo mouse models to breast cancer patients is in place to identify and evaluate the validity of candidate drug targets to combat breast cancer metastasis.

Performing such a large screen as described in **chapter 4** produces a vast amount of results, i.e. genes that affect cell migration, some mildly and others very strong. We focused our follow up studies on the screen hits that showed the largest effects on cell migration, and only selected the top 160 for each cell line and 145 that were effective in both cell lines. As a consequence, there are still many potential hits left that may affect cell migration and cancer metastasis and are of interest for further investigations. Indeed, our data showed that downregulation of p85β

(PIK3R2), a regulatory subunit of PI3K, inhibited cell migration, confirming earlier findings that increased p85β levels regulated breast cancer progression through PI3K pathway activation and enhanced cell invasion[21]. Similarly, silencing of the adhesion GPCR GPR116 impaired cell migration in MDA-MB-231 cells, inhibited metastasis formation, and correlated with disease progression[22]. Knockdown of GPR116 showed similar results in our cell migration screen, however was not among the strongest hits and not studied further. Another adhesion GPCR, GPR64 or ADGRG2, was shown to be involved in cell adhesion and migration through Gαq and non-canonical NFκB signaling (**chapter 3**), substantiating the role of adhesion GPCRs in cell adhesion and migration. Besides adhesion GPCRs, an additional 16 G-protein coupled receptors were validated to inhibit cell migration using live microscopy assays (**chapter 4**). Further investigation of the primary screen data could reveal more GPCRs and their downstream signaling components to play a role in cell migration, and could be used to improve our understanding of GPCR-signaling in context of TNBC progression. Conversely, some of our top candidates have been previously identified by others to play a role in breast cancer metastasis through regulation of cell migration. For instance, downregulation of TRPM7 and SRPK1 impaired cell migration via adhesion dynamics and alternative splicing, respectively, and reduced metastasis formation *in vivo*[9,23]. These two candidates were among our top hits (**chapter 4**) and we were able to reproduce and validate these findings, thereby confirming our approach to candidate drug target discovery while also highlighting the clinical relevance of blocking cell migration in cancer cells.

Tumor cell migration is a highly heterogeneous biological process and cells can display different modes of migration. Single cell motility occurs via two distinct modes: mesenchymal and amoeboid migration, whose differential mechanisms have necessitated their independent analysis[24,25]. Adaptive switching between these modes of migration is often referred to as migratory plasticity and this capacity to change migration strategy aggravates the metastatic process. Indeed, multiple studies have reported tumor cells switching to another migratory mode in response to genetic alterations, the tumor microenvironment, and molecular targeting[26–30]. Such inverse responses to putative therapies, where amoeboid migration was unintentionally increased while mesenchymal migration was inhibited (or vice versa), harbor dangerous side-effects. These examples highlight the importance of understanding each migration mode and its spatiotemporal behavior independently. In **chapter 4**, we purposely used the two cell lines Hs578T and MDA-MB-231 cells, which display differences in lamellipodial organization and protrusive activity, to unravel commonalities in the migration machinery. In a 3D environment, these differences are retained and each cell line displays similar morphology as in their 2D migration mode (data not shown). Nevertheless, 3D assays are required to exclude potential migratory plasticity, i.e. cells switching to another migration mode, as a next step of validation with our candidate metastasis genes. We have started to develop a fully automated 3D RNAi-

screen set up, however the low temporal resolution as a consequence of Z-stack imaging has restrained the throughput. Ultimately, high-resolution 3D migration assays will provide critical information on the invasive and migratory behavior upon silencing of candidate metastasis genes.

3. Signaling and transcriptional networks in breast cancer metastasis

Cancer dissemination is the main cause of death in patients. Cells that have metastasized are less sensitive to chemotherapy, may stay dormant for years, and have the capacity to become highly proliferative. Each step of the metastatic cascade requires integration of signaling from multiple networks. In **chapter 4**, we took a systematic approach to unravel the signaling landscape that drives tumor cell migration. We identified two sets of genes (of 133 and 113 genes) responsible for cell migration in two highly motile triple negative breast cancer cell lines with different migratory behavior. Some of these genes have previously been shown to regulate cell migration during dissemination of breast cancer, such as SRPK1 and TRPM7 (discussed above)[9,23]. Other have been implicated in breast cancer progression as well, affecting different stages and/or different cell biological programs. These include (but are not limited to) BUD31, which was shown to be required for the survival of MYC-hyperactivated triple negative breast cancer cells *in vivo*[31]; BCL10, which modulated NFκB signaling in tumor initiating cells[32,33]; and PSMC3, a component of the proteasome required for survival of triple negative breast cancer cells[34]. Interestingly, only BCL10 was incorporated in our zero-order interaction network, suggesting that these other three are most likely connected through downstream signaling (e.g. via unidentified partners). Indeed, downstream signaling hubs connect our cell migration genes to protein-protein interaction (PPI) networks of other cell migration and invasion gene signatures. Our comparative network analysis shows that different gene signatures drive cell migration through a common set of regulators, which could function as a main signaling hub in control of multiple migration modes and could be exploited therapeutically to inhibit dissemination. Interestingly, gene signatures for breast cancer progression and metastasis are shown to affect the same signaling pathways and regulatory networks, indicating that our candidate genes impair cell migration through clinically relevant pathways.

The majority of large RNAi screens in the field of cancer research focus on a relatively simple (single value) readout, such as proliferation and viability of cancer cells under specific conditions (e.g. therapy resistance or synthetic lethality). Extensive pathway analyses are subsequently performed to identify and highlight a specific biological process or cell compartment, which becomes the main focus of follow-up studies. This way, Petrocca *et al.* showed that inhibition of the proteasome results in cell death in basal-like breast epithelial cells and that proteasome inhibition could be exploited *in vivo* as a vulnerability in basal-like triple negative breast cancer[34]. Interestingly, we identified several proteasome components in **chapter 4**, which reduced cell

migration upon knockdown. However, a drastic reduction in cell number was also observed, suggesting that inhibition of cell migration was an effect of impaired viability. Proteasome inhibition by RNAi or bortezomib in 3D cell cultures of Hs578T cells confirmed this effect on viability (data not shown) and suggests a therapeutic opportunity in mesenchymal TNBC. In a similar way, Hsu *et al*. used data from an RNAi screen for synthetic lethality with MYC hyperactivation (reference [35]), to reveal that the spliceosome is essential for survival of MYC-driven cancer[31]. They identified BUD31 to play a critical role in alternative splicing, leading to intron retention upon knockdown. Interestingly, we identified BUD31 to inhibit cell migration after silencing, and observed only a small decrease in proliferation in contrast to reported before. These findings suggest that splicing is critically involved in the correct regulation of cell biological programs as division and migration, or alternatively, that reduced motility is a downstream effect of impaired proliferation. A myriad of studies have shown that splicing and splice variants are involved in either cell migration or cancer progression and metastasis[9,36–41]. Nevertheless, either hypothesis requires further studies to be confirmed.

Often, a number of cell lines within the specific subclass of cancer (e.g. within TNBC) are employed to validate and confirm findings from large RNAi screens. Using multiple cell lines often strengthens the results and suggest that results are genuinely *true* and *specific* for that type of cancer. In our cell migration screen, **chapter 4**, we started with two cell lines to reduce off-target effects and focus on candidates that inhibit cell motility independent of migration mode. This way, we aimed to reduce the chance of unintentional migratory plasticity in 3D and *in vivo* follow-up studies. Interestingly, our Hs578T cells displayed extravascular migration (angiotropism) when injected in zebrafish embryos (Claudia Tullotta, data not shown). This type of *in vivo* migration was recently discovered and reported by Bentolila *et al*, providing a novel route for tumor cells to invade tissues next to classical intravasation[42]. It remains to be discovered whether this extravascular migration along vessels is capable of producing metastases.

4. Towards full understanding of cancer metastasis

Cancer metastasis is a highly complex process, consisting of different steps which are all regulated by a large number of signaling pathways. This high level of complexity constitutes a major challenge for researchers, clinicians, and pharmaceutical companies. The rapid development of research technologies in the past decade have greatly contributed to understanding individual signaling pathways, genetic regulators, and fundamental cell biological programs. Nevertheless, a complete and comprehensive systems-level understanding of cancer metastasis is still needed.

In order to get to a full understanding of cancer metastasis, advances in *in vitro* research on fundamental cell biological programs are required. More specifically, approaches such as 3D cell

culture, systems microscopy techniques, and integrative analysis of multi-omics data are essential to study the highly plastic and dynamic behavior of tumor cells. Systems microscopy approaches resolve spatial and temporal dynamics of organizational and behavioral features of single cells, thereby providing a systems-level view of biological processes at the molecular to cellular scale[6,43]. This highly quantitative research strategy enables us to perform detailed and sophisticated analyses of cellular systems, thereby elucidating how dynamic cellular processes occur in space and time. Similarly, integration of multi-omics data, such as functional genomics, proteomics and transcriptomics, will aid researchers to find and focus on the most important candidate genes in screening projects. These examples rely on advances in data analysis, ranging from data mining to multivariate statistics and computational modeling. Fortunately, these advances are in progress, with the latest analytical tools in systems microscopy and multi-omics integration being made accessible to the research community through open access and open source platforms[44,45].

In addition to advances in *in vitro* research, which aims to elucidate how the fundamental processes in cell biology work, investments in translational research are needed to bridge the gap between *in vitro* findings and clinical studies. Recent research has shown that there is a profound heterogeneity within tumors and this heterogeneity negatively impacts diagnostics and therapeutic interventions[46,47]. Furthermore, it has been shown that the genomic profiles of metastases are quite distinct from their matched primary tumors[48–50]. These results indicate that biopsies of primary tumors may miss a number of opportunities for targeted therapies, and highlight the importance of studying the steps in the metastatic cascade. Indeed, current studies explore the potential of circulating tumor cells for diagnostic purposes and therapeutic intervention. Gene expression profiles of circulating tumor cells could be compared to matched primary and metastatic tumors, which may provide essential information on the signaling pathways that regulate metastatic dissemination. Combined with the cell migration gene signatures presented in this thesis, this could lead to the identification of pivotal gene networks behind cancer metastasis. Alternatively, to bridge the gap between fundamental and translation/clinical research, the field of intravital imaging is rapidly advancing, allowing us to study tumor heterogeneity at single cell level in translational (*in vivo*) models[16,18]. As a next step, intravital microscopy with cells that have stable knockdown of candidate metastasis genes may be very helpful to visualize the dynamic interactions between the tumor cells and their local environment. Ultimately, future research on fundamental cell biology and clinical translation is needed to develop new methods of data integration across all types of platforms, which will help us understand the complex signaling landscape that drives tumor cell migration and metastasis formation.

5. Conclusions

Overall, the main aim of this thesis was to unravel the signaling and regulatory networks that drive tumor cell migration during breast cancer metastasis. Understanding how tumor cells migrate, how this process is differentially regulated, and how this highly heterogeneous and plastic behavior is coordinated during metastatic dissemination, will ultimately provide novel insights in therapeutic opportunities. The work presented in this thesis includes different microscopy techniques and analytical tools to study migratory behavior (**Chapters 2, 3, 4, and 5**), reveals an important role for adhesion GPCRs in cell adhesion and migration (**Chapter 3**), provides a compendium of genes that drive tumor cell migration in breast cancer (**Chapter 4**), and investigates the intricate connection between adhesion dynamics, cell migration and contractility (**Chapter 5**). Collectively, this work provides novel insights in the molecular determinants of tumor cell migration and will help us better understand the signaling landscape that drives the formation of breast cancer metastases.

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1. Source: Nederlandse Kankerregistratie, beheerd door IKNL © July 2016.
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