# Identification of novel targets in prostate cancer progression

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# CHAPTER 7

## SUMMARY AND DISCUSSION

The complexity of a biological process such as metastasis necessitates that with our current technology discrete steps of the problem are analyzed. We decided to focus our studies on androgen independent prostate cancer (AIPC), which is generally highly aggressive and metastatic. Reliable and well-characterized model systems of prostate cancer progression are of utmost importance to achieve the challenge of finding a cure especially for the AI stage of prostate cancer. The dream of molecular targeted therapies is that some day a patient's own cells will be rapidly tested in vitro with a palette of therapeutic agents to find the one best suited to the patient's specific needs (1). An obvious goal of tissue engineers and cancer modelers is to progress from good models of tissue complexity and function into models that can be standardized for drug screening purposes. In order to effectively hunt for targets in preclinical testing it is necessary to scale the reliable models for medium or high throughput screening (HTS) (1). Because of the technical challenges involved, there are few accomplished metastasis models that have the ability to support HTS (1).

In order to meet this challenge, we worked in the direction of developing new models that recapitulate key steps in the metastatic cascade. We developed a 3D culture and a xenograft model to study growth, invasion and dissemination. First, we developed a three-dimensional collagen matrix based spheroid assay of growth and invasion (*chapter 2*). This assay recapitulates the steps of growth and invasion of tumors at primary or metastatic sites. Collagen type 1 is one of the predominant matrix proteins in the bone, and hence the model provides an ideal environment to study the growth and invasion characteristics of the androgen independent metastatic prostate tumors. Furthermore, we adapted this model in 96 well plates and demonstrated the applicability of this model in high throughput screening platforms. We also applied this method to cell suspensions derived from resection specimens, which opens the possibility to test therapeutic strategies on freshly isolated material from individual patients. So far, sarcoma biopsies were used; it remains to be established whether this will also be successful for carcinomas, including prostate cancer. For the future, we would also like to adapt/expand this assay to study the complex interactions between tumor cells and stromal cells including for instance endothelial cells, which could be achieved by injecting these spheroids together in one matrix. This could provide us novel information regarding the process of tumor induced neoangiogenesis or vasculogenesis, which could be further useful for the development of novel anti-angiogenic therapies against cancer. In addition, we have used PC3 cells to study prostate cancer invasion in this in vitro model. A major challenge will be to modify the culture conditions such that also less aggressive prostate cancer cell lines could be studied in the same system for instance by modification of the ECM environment or inclusion of supportive stromal cells. Ultimately this could provide a 3D in vitro model that mimics more closely prostate cancer in patients.

Secondly, we turned our attention to the zebrafish, which is proving to be an excellent whole animal based model to study tumor development, invasion, and the dynamics of the metastasis cascade. Several research groups have demonstrated successful xenotransplantation of human cancer cells of different origin in zebrafish, and have carried out in vivo assessment of invasiveness, tumor induced angiogenesis (2-6), metastatic behavior (7-10) and response to anticancer therapies (2, 11-14). The small size, transparency and availability of various transgenic fluorescent zebrafish lines prompted us to couple this model to automated confocal imaging and image analysis (*chapter 3*). To the best of our knowledge, this assay represents the first ever semi-automated, quantitative whole organism based bio-imaging assay in a vertebrate that allows

analysis of dissemination of cancer cells in a high throughput manner. Further automation that is developed for the injection of embryos will further develop this pipeline into a fully automated system. With the continued development in the field of imaging technology and availability of novel transgenic zebrafish lines, It will also be extremely interesting to use our assay to xenotransplant cell lines expressing combinations of fluorescent markers allowing quantification of apoptosis, proliferation and dissemination. Furthermore, incorporation of specific transgenic zebrafish lines expressing fluorescently labeled immune cells or tissues into our automated assay will provide us valuable information regarding the tumor cell and microenvironmental interactions. There are also some outstanding questions on the use of zebrafish; what is the predictability of the zebrafish xenograft model for other (rodent) modes; to what extent do effects observed correlate with the effects of compounds seen in man; what is the predictability of the zebrafish with respect to the PK-PD (Pharmacokinetics-Pharmacodynamics) relationships(15)? These questions need to be urgently addressed to warrant use of this model in drug discovery/development pipelines.

In *chapter 5*, we expanded this assay further and xenotransplanted a panel of aggressive (AI) and non-aggressive (AD) prostate cancer cell lines. Prostate cancer cell lines, which are known to be androgen-independent and/or metastatic in mice (LNCaP-derived C4-2 and C4-2B; DU145 and PC3) showed enhanced dissemination in zebrafish embryos as compared to androgen-dependent non-metastatic cells (LNCaP). Our findings show that this assay closely reflects the results obtained in more expensive and much lower throughput assays such as rodent xenografts. Based on these data, zebrafish embryos provide a microenvironment that allows successful engraftment, proliferation and dissemination of AIPC cell lines. Therefore, this assay represents an ideal platform to screen for targets mediating invasion and dissemination of hormone refractory prostate cancer using RNA interference or compound screens.

Current treatment options for prostate cancer are often unsuccessful, and in *chapter 4* we discuss some strategies for improvement. However, there is clear need for new drug targets to widen the possibilities for treatment. Discovery of such new targets has been the major goal of our work. Therefore, we used our experimental models to address the following questions:

- Can we use them to identify critical new targets involved in aspects of prostate cancer metastasis?
- Are these targets validated in other models?
- Are these targets relevant for the human situation?

To answer these questions we devised an adenoviral based RNAi screen for mediators of prostate cancer dissemination using our automated whole animal based zebrafish model of xenotransplantation (*chapter 5*). Based on this screen, we identify SYK as a kinase supporting human prostate cancer dissemination. In line with zebrafish and 3D models, depletion of SYK also led to a strong reduction in metastatic colonization of androgen independent prostate carcinoma cells in a preclinical mouse model for prostate cancer bone metastasis. In the present xenotransplantation models, the study of human material grafted into the mouse has various limitations, including a compromised immune system, differences in species specificity of proteins and an inability to capture early events in tumorigenesis (16). The use of genetically engineered models (GEM) that mimic prostate cancer progression provides a possibility for further validation of candidate targets identified in our screen (17). Thus, a key next step in studying the role of SYK in prostate cancer pro-

gression would be to silence or delete the SYK gene in the TRAMP (transgenic adenocarcinoma mouse prostate) model that recapitulates the pathophysiological features of the prostate cancer progression in humans (18). SYK has been implicated in lymphoid malignancies and appears to play opposing roles in different solid tumors but has not been previously associated with prostate cancer (19). SYK is believed to act as an oncogene because of its ability to increase phosphorylated AKT, which may imply the involvement of PI3K (20, 21), which is a key molecule implicated in migration and chemotaxis of both tumor and endothelial cells (22). Phosphorylation of SYK at Tyr323 forms a docking site for the p85 subunit of PI3K (23). The effect of SYK depletion on PI3K or AKT activity has to be determined but our findings indicate that it is indeed the SYK kinase activity that supports prostate cancer invasion and dissemination: the effect of SYK knockdown on metastatic colonization is reversed by wild type but not kinase dead SYK expression; pharmacological inactivation of SYK using R-406 and BAY-61- 3606 recapitulated the effect of silencing the SYK gene in vitro and in zebrafish model of xenotransplantation.

It will be important to study the effect of SYK inhibition also in the mouse model used by us and other preclinical mouse models mentioned above. We found that SYK regulates surface expression of adhesion receptors at the posttranslational level. This is a potential underlying mechanism for the support of prostate cancer dissemination by SYK: decreased expression of two of these candidate targets of SYK, integrin alpha-2 and CD44 leads to similar defects as depletion of SYK. It has been previously shown that SYK can regulate turnover or transport of adhesion receptors in other cell types (23,24) through a mechanism requiring ITAM-mediated SYK and AKT activation and inducing membrane translocation or stabilization of adhesion receptors. Future experiments should further unravel the mechanism of action of SYK in this respect. SYK acts as an oncogene in lymphomas, chronic leukemias, gastric cancers, and – based on our findings – in prostate cancer. Paradoxically it appears to be a tumor suppressor in breast cancer : downregulation of SYK levels correlate with growth and invasion in the latter case. Importantly, the SYK gene undergoes alternative splicing and the apparent opposite roles of SYK in different cancers may be related to this (25). Most studies have not distinguished between global and splice isoform–specific increases in SYK expression. It is possible that differential expression of SYK (L) and SYK (S) may contribute to these disparate observations in different cancers. It will be important to extend our own studies to identifying which isoforms are expressed and upregulated with progression (25).

Another gene that was identified in the RNAi screen was MST1R. This MET-related receptor tyrosine kinase has been previously implicated in prostate cancer growth and angiogenesis. Its function in prostate cancer growth at least in part can be explained by a need for MST1R in cancer-associated macrophages (26). Nevertheless, two studies indicate that this receptor also plays a role on prostate cancer cells themselves (27, 28). In *chapter 6*, we show that downregulation of MST1R leads to a phenotype that is also observed when MET (the receptor for HGF) is downregulated in different cancer cell types: cells appear less scattered and form epithelial islands. We show for the first time that MET and MST1R both regulate prostate cancer cell invasion/migration in 3D collagen, and MST1R downregulation also inhibits dissemination in the zebrafish model. An increasing number of basic, translational and clinical studies have shown the importance of MET in prostate cancer progression. MET is overexpressed in primary prostate cancers, and its expression is further increased in bone metastases and is associated with the development of castrate-resistant disease. Be-

cause of its importance as a therapeutic target, MET inhibitors have reached clinical trials for advanced, castrate-resistant prostate cancer (29). A robust pipeline of high-quality inhibitors targeting different aspects of MET activation is already being explored in phase II, III clinical trials across multiple tumor types. The best-known example is the ARQ-197, which has already reached the late phase of clinical development in various tumors including prostate cancer (30,31).

In the screen that was the basis for *chapter 5*, two MET shRNAs did not significantly affect PC3 dissemination. Since levels of knockdown were not analyzed in this screen, this does not exclude a role for MET in PC3 dissemination in this model. In fact, based on the literature, effective stable knockdown of MET may well interfere with dissemination similar to silencing of MST1R. Notably, there is also evidence for extensive cross talk between MET and MST1R (32). To test if the attenuated invasion caused by MST1R downregulation was due to inactivation of MET signaling, PC3shMST1R were treated with HGF. The fact that HGF treatment restored invasion of PC3shMST1R cells shows that MET signaling was intact.

In agreement, MST1R silencing did not prevent MET phosphorylation in response to HGF. Whether MST1R and MET signaling act in parallel and HGF-stimulated MET activity compensates for the loss of MST1R, or whether HGF-induced MET activation resulted in transphosphorylation of MST1R in our experiments as reported earlier (33, 34) remains to be established. Importantly, as observed for SYK, MST1R is confirmed in the mouse model as an important player in the later stages of prostate cancer metastasis perhaps playing a role in extravasation or outgrowth in the bone metastatic niche. Moreover, MST1R expression correlates with androgen-independence / metastatic potential in a panel of cell lines indicating that it may serve as another potential target in prostate cancer progression. As discussed above for SYK, further studies in the different prostate cancer mouse models will be required to further implicate MST1R in prostate cancer progression and metastasis. Our current study, in combination with work by others on its role in tumor growth, warrants investment in this direction.

In conclusion, the results from this thesis have contributed small pieces of knowledge to our overall understanding of the problem of prostate cancer metastasis. We have developed novel fluorescence bio-imaging based automated models to screen for novel candidate targets involved in prostate cancer metastasis. Utilizing these models and adopting a functional genomics based approach; we identified SYK as a novel regulator of prostate cancer progression. We also identified functional involvement of MST1R in regulating the progression of prostate cancer. For both of these targets, there is supporting human clinical data to validate our results in prostate cancer. Although much further insight is required in the mechanism of action of these kinases as well as in their RNA expression levels, splicing, protein levels, and activity in human prostate cancers, this is reassuring: it shows that results obtained in these models may be applicable to humans.

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