Novel regulators of prostate cancer stem cells and tumor aggressiveness

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General discussion and future perspectives

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Prostate cancer consist of heterogeneous epithelial cell subpopulations of which prostate cancer cells with stem/progenitor-like characteristics (CSCs) have been increasingly recognized as the “driver” cancer cell subpopulation in tumor initiation, local and distant relapse, hormone refractory disease, castration, metastasis and chemotherapy resistance (1-4). Therefore, unraveling the molecular properties of malignant subpopulation of CSCs may represent a promising strategy to identify new attractive targets for therapeutic intervention.

The work presented in this thesis covers two aspects of the molecular characteristics of CSCs: in the first part the identification of miRs as novel regulators of gene expression in CSCs are described; in the second part two studies are presented that focus on the identification of new potential markers and functional factors that are involved in prostate cancer pathogenesis, progression and bone metastasis. A schematic representation and graphic summary of our observations is depicted in **Fig. 1**.

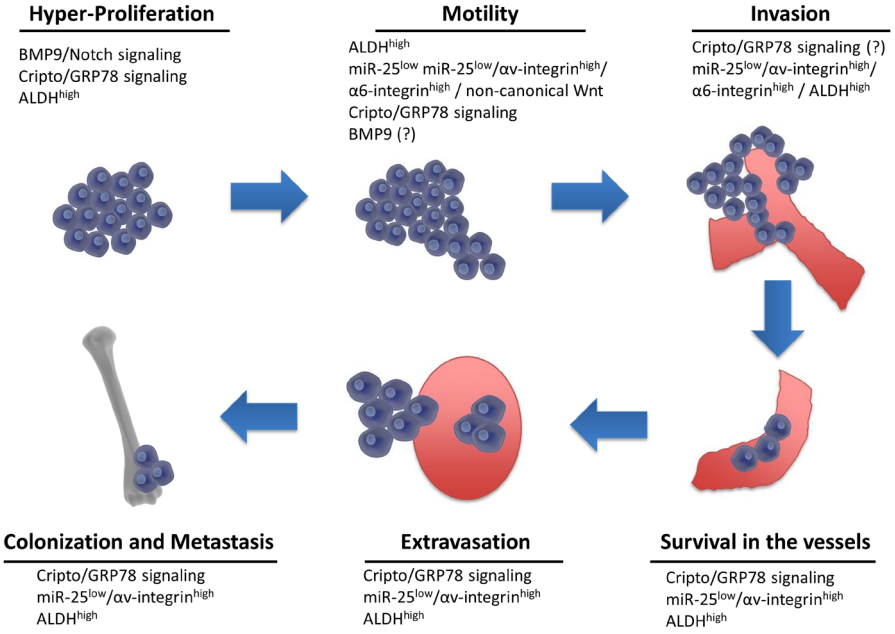

Figure 1. Schematic representation of the metastatic cascade. The involvement of miR-25, α_v, α_6 integrins, non-canonical Wnt signaling, ALDH, BMP9 and Cripto signaling pathways are highlighted in the different steps of the metastatic process.

Molecular characteristics of highly aggressive prostate cancer stem-like cells

Cellular heterogeneity is an important characteristic of many epithelial cancers, including prostate cancer. The major aim of this thesis was to identify the molecular properties of selected subpopulation of highly metastatic cancer stem/progenitor-like cells in human prostate cancer. In the first part of this thesis, we characterized the miR expression of two subpopulation of cells: the tumor- and metastasis-initiating $ALDH^{high}$ cancer stem/progenitor-like cells and the more differentiated, poorly tumorigenic/metastatic $ALDH^{low}$ cells (5). In the past 10 years, high aldehyde dehydrogenase activity has been progressively established as a maker to identify highly aggressive and metastatic prostate cancer stem cells (5-8) also in clinical studies (5,9). However, to the best of our knowledge, none of the previous studies have systematically investigated the molecular characteristics (e.g. miR expression) of $ALDH^{high}$ vs. $ALDH^{low}$ cells in human prostate cancer.

In **Chapter 3**, microRNA expression profiling of cultured $ALDH^{high}$ and $ALDH^{low}$ prostate cancer cells revealed a number of differentially expressed miRs (10). Our results are strengthened by clinical profiling data of a comparison of three subpopulations of transformed epithelial cells isolated from primary prostate tumors, namely: the stem-cell subpopulation, the $\alpha2\beta1^{hi}$ /$CD133^{-}$ transient-amplifying cells and the $\alpha2\beta1^{low}$ cells committed for terminal differentiation (11). Our study shows that miR-25 is low/absent in the $ALDH^{high}$ subpopulation isolated from prostate cancer cell lines and in the $\alpha2\beta1^{hi}$ /$CD133^{+}$ basal stem-cell subpopulation isolated from patients and steadily increases during differentiation to $\alpha2\beta1^{hi}$/$CD133^{-}$ transit amplifying cells and $\alpha2\beta1^{low}$ committed basal cells. miR-25 is part of the miR-106b-25 cluster (12). Consistent with our findings, the expression of the miR-106b-25 cluster appears to mediate neuronal differentiation of adult neural stem/progenitor cells and, interestingly, induction of miR-106b-25 in hypoxic conditions has been linked to increased expression of neuronal markers in prostate cancer cell lines (13,14). Moreover miR-25 has recently been identified in PC12 cells (15,16) as regulator of neuronal differentiation, supporting the involvement of this microRNA in differentiation processes (17).

The data of our study, highlight the limitations of molecular profiling approaches in cell cultures, heterogeneous cell lines and heterogeneous bulk clinical tissues. For example, it was found that the miR-106b-25 cluster was up-regulated in primary tumors and distant metastases from multiple solid cancers, including those of the human prostate (12,18-21). Importantly, none of this studies focus specifically on miR-25 expression but solely on the expression of the miR-106b-25 cluster. Additionally,

microRNAs within one cluster might be regulated differently by different transcription factors and miR-25 has been shown to be uncoupled from the MCM7 host gene (22,23). A likely explanation for these apparent contradictory observations is that cancer cell lines and bulk tumor tissues are not homogeneous and consist of a mixture of heterogeneous subpopulations of cells (24).

Therefore, we speculated that the increase in absolute expression levels of miR-25 in bulk tissues during prostate cancer progression may be indicative of an increase in the proportion of more differentiated, less invasive, "miR-25^{high}" more differentiated, "luminal" epithelial cells. This is reinforced by the fact that, forced miR-25 overexpression led to a decrease in α2-integrin and β1-integrin expression (**Chapter 3**), markers of epithelial basal stem cell (25). Our bioinformatic analysis revealed that αv-integrin and α6-integrin are target genes of miR-25. The identification of these genes confirmed the results of previous studies showing a significant higher expression αv-integrin and α6-integrin selectively in the ALDH^{high} subpopulation compared to ALDH^{low} in prostate cancer and in high-risk prostate cancer patients (26,27). Such studies also demonstrated that knockdown (26) or targeting of αv-integrin (28) significantly diminished the acquisition of a metastatic stem/progenitor cell phenotype and reduced the formation of prostate cancer bone metastasis in preclinical *in vivo* models. Consistent with these data, miR-25 overexpression significantly reduced the migratory potential of both bulk cell lines and selected ALDH^{high} subpopulation.

The induction of dramatic morphological changes after miR-25 overexpression prompted us to study the underlying mechanism(s) of these phenotypic alterations. Our analysis showed that overexpression of miR-25 dramatically impaired F-actin polymerization, thus reducing focal adhesion sites. Integrins provide a structural link between F-actin and the extracellular matrix and contribute to formation of these focal adhesion points (29). Additionally, integrins (αv-integrin in particular), are also involved in the activation of latent TGF-β which represents one of the major driver during EMT (30-32). As already discussed in **Chapter 3**, organization of F-actin is linked to activation of integrin-transmembrane receptors which regulates the activation of Rho-GTPases, RAC1 and CDC42 (33). Given the ability of miR-25 to target αv-integrin and α6-integrin it seems likely that regulation of these integrins by miR-25 has a major impact of the cellular phenotype. Interestingly, cells with a stellate, mesenchymal morphology like PC-3M-Pro4Luc2, often require activated RAC1 for migration (34). These observations are in line our findings that show reduced RAC1 mRNA upon forced miR-25 expression (10). Taken together, our functional and molecular profiling data highlight a pivotal role

of miR-25 as a non-coding RNA for the regulation of tumor aggressiveness by the regulation of cytoskeletal organization and motility.

In **Chapter 4** we describe a potential, other role for miR-25 in the modulation of the invasive program in human prostate cancer through modulation of canonical and non-canonical WNT signaling of which RAC1 is also a component (35,36). The WNT/PCP pathway is considered the β-catenin independent branch of WNT signaling. Beside the involvement of WNT signaling in the pathogenesis of prostate cancer and bone metastasis (reviewed in (37-41)), accumulating evidence revealed the role for non-canonical WNT/PCP signaling in prostate cancer progression, invasion and metastasis (36). Interestingly, canonical WNT signaling and non-canonical WNT/PCP signaling are part of a negative feedback-loop in which WNT/PCP negatively regulates canonical WNT signaling and *vice versa* (42). This led us to hypothesize that a possible differential basal level of canonical and non-canonical WNT signaling could maintain the mesenchymal and motile phenotype in PC-3M-Pro4Luc2 human prostate cancer cell line. Therefore, we speculated that the highly migratory phenotype in this cell line is due to a imbalance between canonical WNT and non-canonical WNT/PCP pathway. Indeed, in **Chapter 4** we describe that the non-Canonical WNT/PCP pathway is approximately 10-fold more active that the canonical counterpart in our model. Moreover, administration of TGF-β, a known inducer of EMT in prostate cancer, strongly increased non-canonical WNT/PCP signaling with a concomitant decrease in canonical WNT signaling. Using the Smad-3 dependent TGF-β reporter (CAGA-luciferase) we demonstrated that miR-25 can attenuate the activation of TGF-β signaling in human prostate cancer and is capable of blocking TGF-β-driven invasiveness. Overexpression of miR-25 also produced a significant increase in canonical WNT signaling, suggesting a modulation of the crosstalk between canonical and non-canonical WNT signaling pathway. Although additional experiments are warranted to confirm a specific and direct effect of miR-25 on non-canonical WNT/PCP signaling, we found that DACT1 knockdown recapitulated the induction of canonical WNT signaling on a bioluminescent reporter that we also detected upon miR-25 overexpression.

Taken together, the data described in this thesis support a key role of miR-25 in the regulation of motility invasiveness and epithelial differentiation in human prostate cancer. Furthermore, our data are in line with the literature concerning an intriguing contribution of non-canonical WNT signaling in prostate cancer progression and emphasize that targeting of this pathway might represent an interesting strategy to restrain EMT, invasion and metastasis.

In **Chapter 2**, we reviewed the established miR-gene interactions among TGF-β, Notch and Wnt signaling pathway and identified a miR signature that highlighted the crosstalk between these pathways. Beside the relevance of CREBBP and EP300 in EMT, which has already been addressed in **Chapter 2**, we wanted to test whether the list of genes identified could be linked to our findings about the role of miR-25 in aggressiveness of prostate cancer stem cells.
Interestingly, we found that CREB1 is identified as predicted target gene of miR-25 in two independent bioinformatics online available tools: TargetScan and microT-CDS. Although direct evidence is (still) lacking one can speculate that miR-25 may be involved in CREB1 downregulation and, as a result, would have a functional impact also on CREBBP (CREB-binding protein) and on the interaction with its signaling partner EP300.

The value of the signature identified in **Chapter 2**, is supported by multiple connections with pathways analyzed in other chapters of this thesis (e.g. Cripto and Notch signaling). In **Chapter 6** we have discussed the role of Cripto as emerging gene whose expression turns out to be involved in the formation of bone metastasis in prostate cancer. Interestingly, two miRs identified in our signature, (miR-15 and the miR-16) have been previously shown to directly interact with Cripto (43). Given the documented role of Cripto in EMT in prostate cancer (44), and its interaction with multiple TGF-β, Wnt and Notch signaling networks (45), this observation supports the involvement of the miRs signature during EMT. Moreover, in **Chapter 5** we show that the soluble chimeric protein ALK1Fc (ACE-041) (46) reduces BMP9 signaling and decreases proliferation of highly metastatic and tumor initiating human prostate cancer cells *in vitro* and *in vivo*. Interestingly miR-34a and miR-24, that were also identified in our miR signature, are shown to target BMP9 (GFD2) by TargetScan online predictive tool. Taken together, our observations emphasize the functional value of the miR signature and further strengthen the role of TGF-β family members and the Notch pathway in human prostate cancer.

As already discussed, EMT represents a crucial process that characterize the early phase of tumor invasion and generate the basis for the metastatic spread of the tumor. The role of CRIPTO during EMT in prostate cancer has already been described (44), however, to the best of our knowledge, the involvement of CRIPTO in the formation of bone metastasis by prostate cancer has not been reported.

Given the clinical problem of bone metastasis and the fact that Grp78 (i.e. Cripto signaling partner) has been associated with the development of castration resistance (47), in **Chapter 6** we tested whether we could register an involvement of Cripto in the onset of bone metastasis in prostate cancer.

Immunohistochemic analysis of clinical bone metastases collected shows that Cripto is strongly expressed and co-localize with cytokeratin-18 positive prostate epithelial cells. This support our hypothesis and suggest a functional involvement of Cripto in the formation of bone metastasis. Our *in vitro* experiments support the role of Cripto and Grp78 in the maintenance of aggressive characteristics in prostate cancer cell lines. The knock-down of Cripto and Grp78 induced a decrease in invasiveness and self-renewal properties in prostate cancer cell lines. Moreover, inoculation of Cripto and Grp78 knock-down cells in the circulation of zebrafish embryos via the duct of Cuvier (10,48), resulted in formation of significantly lower number of experimental metastasis compared to control cells. Finally, we demonstrated that Cripto knockdown significantly reduced the metastatic outgrowth in a preclinical mouse model of prostate cancer bone metastasis.

Our results suggest that Cripto and Grp78 might represent novel markers that could predict the formation of bone metastasis and be indicative of the initiation of invasiveness. This could therefore be of great value for the identification of new therapeutic targets.

miR-25 and Cripto in the landscape of new markers for prostate cancer monitoring and prediction

Although the progress in the molecular diagnostic research for prostate cancer in the last years, there is still a urgent need for the identification of novel additional predictive markers for prostate cancer monitoring and progression.
In this context, the AR has represented the major target for studies focused on prostate cancer treatment. Such studies have addressed the status of the AR and its modification in response to drugs (e.g. abiraterone and enzalutamide both resulted in increased expression of certain AR variants (49,50)). Given the fact that the distant relapse represents the lethal phase of the cancer progression, other approaches have focused on the identification of novel molecules involved in the interaction between the supportive stroma and the tumor cells (51). However, the identification of reliable, predictive markers for the castration resistant phase is still challenging.

In the landscape of the emerging molecular markers for CRPC, recent studies have highlighted the role of small non-coding RNA miR-1247-5p and of its target gene myc-binding protein 2 (MYCBP2) (52). Other recent work has revealed that the tri- and tetra-antennary N-glycan might be associated with the castration resistant status, therefore representing a potential predictive biomarker for castration-resistant prostate cancer (53). Moreover, the TMPRSS2-ERG fusion gene is currently in clinical testing for its

diagnostic and prognostic value, however ERG fusions have been reported to be positive or negative for clinical outcome (54). The data described in **Chapter 3** and **Chapter 6** of this thesis suggest that new approaches and new molecules respectively might contribute to the identification of new markers of interest in CRPC. In **Chapter 3**, we have identified miR-25 as novel microRNA downregulated in the stem-cell compartments isolated from CRPC patients (10) and our results presented in **Chapter 6** show that high expression of Cripto is associated with poor survival in prostate cancer and that Cripto is highly expressed in prostate cancer bone metastasis collected from CRPC patients.

Many studies have already successfully measured proteins and noncoding RNAs in blood or urine collected from prostate cancer patients (reviewed in (55)). However, the findings presented in this thesis highlight the difference of analyzing miRs and gene expression in bulk tissues compared to selected subpopulation of cells. The manipulation of selected targets in specific subpopulation of cells introduce additional layers of complexity linked to the development of targeting strategies that can selectively hit those highly metastatic cluster of cells dispersed within the tumor.

In other words, we prove here that miR-25 is crucial for prostate cancer cell migration and invasion, but we show that miR-25 is downregulated in aggressive prostate cancer subpopulation of cells which makes it a “negative marker”, thus difficult to apply in the clinical practice. However, our molecular studies could contribute to the identification of novel target genes, to be employed as therapeutic targets. This notion is supported by the fact that αv-integrin, that we prove here to be targeted by miR-25, has already been shown as interesting therapeutic target involved in the formation of bone metastasis in human prostate cancer (28).

We believe that the discovery of specific molecules, selectively expressed on highly malignant clones might help in the development of new targeting strategies, especially if those malignant clones and cells are spread and under-represented in the bulk tumor mass (only few aggressive cancer stem/progenitor-like cells are detected (11,56)). From this perspective, the membrane-bound nature of Cripto makes it an interesting target for the development of new molecules capable of targeting highly metastatic cells for diagnostic and therapy purposes. Additionally, this strategy could also be employed in the development of probes capable of revealing the localization of Cripto expressing cells during surgical intervention. Moreover, the soluble nature of Cripto, makes it an interesting molecule for the development of diagnostic and prognostic test to monitor the progression of prostate cancer in patients. Soluble Cripto could be detected in the blood collected from prostate cancer patients by ELISA kits

already available for research purposes. Interestingly, manipulation of Cripto signaling has already been shown to be successful in the modulation of the maintenance of mammary stem cells in breast cancer (57).

Clinical relevance, possible therapeutic opportunities and future perspectives

The study presented in **Chapter 5**, supports the role of BMP9 as a tumor-promoting factor in human prostate cancer cells *in vitro* and *in vivo,* acting on the Notch signaling pathway. To our knowledge, our study represents the first functional evidence for a role of BMP9 and the functional evidence of its targeting in human prostate cancer. Several approaches have been described for therapeutic targeting of the Notch signaling pathway in various diseases, but the majority of these clinical studies failed due to significant adverse effects (58,59). Interestingly, current options to interfere with Notch signaling originate from Alzheimer's disease research where γ-secretase inhibitors (GSI) are employed to prevent the accumulation of amyloid-β peptides (60). The γ-secretases enzymes contribute to the cleavage of the transmembrane portion of the Notch receptor and represent crucial players in the activation of the Notch signaling pathway. Unfortunately, animal and human safety trials revealed a significant toxicity involving gastrointestinal bleeding and immunosuppression following the administration of GSI applied to T-cell acute lymphoblastic leukemia (T-ALL) (58,59). Interestingly, promising results have been achieved in preclinical studies by combining GSIs with conventional chemotherapeutic agents (61) with minimal toxicity. In prostate cancer, administration of GSIs blocks tumor angiogenesis and enhances the docetaxel-mediated antitumor response, indicating a causal role of Notch signaling in mediating therapy resistance in human prostate cancer (61).

Our data in **Chapter 5** suggest that ALK1Fc might impact on cellular proliferation via an indirect effect on Notch signaling pathway. Therefore, this indicates that ALK1Fc might represent an interesting molecule to contain prostate tumor growth. Given the combinatorial effect of GSIs and current therapies, these results support the development of studies to investigate whether employment of ALK1Fc could contribute to sensitize prostate cancer cells to current treatment. Given the fact that ALK1Fc has recently been shown to be well tolerated by patients with advanced refractory cancer (62), showing promising antitumor activity, this might represent a promising and alternative strategy to circumvent the toxic side effects produced by GSIs. Additionally, ALK1Fc has been shown to reduce the vascular density in various solid tumors (63). Considering that angiogenesis is a crucial process coupled to osteogenesis (51,64) in osteoblastic bone metastasis (such those originated from prostate cancer), the testing

of ALK1Fc in preclinical bone metastatic models might be promising. Moreover, the recent experimental evidence that endothelial Notch activity promotes angiogenesis and osteogenesis in bone (65), reinforces the application of ALK1Fc in a metastatic setting (ALK1Fc interferes with Notch signaling see **Chapter 5**). However, a limitation of the animal models employed in the metastatic setting, is that osteoblast progenitors and hematopoietic stem cells (HSCs), responsible for the bone remodeling are tightly associated with "type H", $CD31^{high}$ vessels (64). These vessels are at their highest peak in young 4-week-old animals compared to old 11-week-old mice (64). Thus, a possible strategy to evaluate the efficacy of ALK1Fc in a metastatic setting in murine models, could combine the treatment with ALK1Fc together with the stimulation of the bone growth.

The results presented in **Chapter 6** suggest that Cripto and its signaling partner Grp78 could be employed as novel target genes to identify selectively metastatic prostate cancer cells. Interestingly, Grp78 has been shown to be involved in therapy resistance (66) and Grp78-targeted nanotherapy has already been tested in human prostate cancer (67). Additionally, the development of new molecules capable of targeting Cripto has already been shown to be effective in breast cancer (57). Moreover, the availability of monoclonal antibody specifically targeting Grp78 support the developing of strategies targeting the Cripto/grp78 pathway (68,69). These together support the development of preclinical studies to investigate the application and the relevance of such molecules in prostate cancer treatment.

The results in **Chapter 3**, support that the identification of new putative (up-regulated) miR-25 predicted target genes, could help in the identification of new factors involved in the maintenance of the aggressiveness in prostate cancer. Such research could then be exploited to identify novel small molecules capable to target these factors.

A direct translation of our direct findings would consist of an overexpression of miR-25 selectively in those highly migratory and invasive aggressive clones. Obviously this imply the application of selected targeting of specific cells which, up to date, is still under testing and development (2). Such targeting would require knowledge of the differences between normal and cancer stem cells. The latest developments in targeted therapy comprise the design of novel siRNA, miRNA, and antisense nucleotide therapy against CSCs. In this context, miR-25 could represent an interesting target for this type of new therapies and for nanotherapeutic approaches that have already investigated the application of such strategy to target both genes active in CSCs and the CSCs niche (2,70).

The targeting of CSCs might be achieved by four approaches: targeting component of the CSCs niche, targeting resistance mechanisms, inhibition of self-renewal signaling pathways and elimination therapy (2). The last one involve the eradication of CSCs based on specific characteristics of these cells for example the expression of specific molecules/antigens. In this context, the identification of new specific markers for these cells would be of great value and help.

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