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Identification of novel targets in prostate cancer progression

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CHAPTER 1

GENERAL INTRODUCTION AND SCOPE OF THE THESIS

PROSTATE CANCER

Prostate cancer is a principal cause of illness and death among men in the United States and Western Europe. Autopsy series have demonstrated small prostatic carcinomas in up to 29 percent of men 30 to 40 years of age and 64 percent of men 60 to 70 years of age. Moreover, the risk of prostate cancer is 1 in 6 and the risk of death due to metastatic prostate cancer is 1 in 30 (1). Curative treatment is usually prostatectomy or radiation to remove or destroy the cancerous cells that are still confined within the prostate capsule (fig. 1). However, many patients are not cured by this form of therapy and their cancer recurs, or they are diagnosed after the cancer has spread to distant sites. Curative treatment of prostate cancer is only possible when tumor cells are predominantly confined to the prostate (1).

MECHANISM OF ANDROGEN ACTION

Why do prostate cancer cells need androgens to grow and survive (2) ? Prostate cancer growth is dependent on the ratio of proliferating cells to those dying. Androgens are considered as the main regulator of this ratio by both stimulating proliferation and inhibiting apoptosis. So it could be concluded that, prostate cancer depends on a crucial level of androgenic stimulation for growth and survival. Androgen blockage causes regression of prostate cancer because without androgen, the rate of cell proliferation is lower and the rate of cell death is increased, leading to extinction of these cells (3). Testosterone - which is the main circulating androgen — is secreted primarily by the testes, but is also synthesized by peripheral conversion of adrenal steroids (2). 90% of the free testosterone entering the prostate cells is converted to dihydrotestosterone (DHT) by the enzyme 5 α -reductase (SRD5A2). DHT is the more biologically active hormone, possessing five-fold higher affinity for the androgen receptor (AR) than does testosterone. DHT binds to the androgen receptors in the cytoplasm, leading to the phosphorylation, dimerization, and subsequent translocation into the nucleus, thereby binding to the androgen-response elements within the DNA, with resultant activation of genes involved in cell growth and survival (2). Almost all metastatic prostate cancers initially require testosterone for growth, and the use of androgen deprivation as a first-line therapy for metastatic prostate cancer has been recognized for more than 60 years (4, 5). Hormone deprivation is achieved by surgical (orchiectomy) or medical (luteinizing hormone-releasing hormone agonists, anti-androgens) castration. Furthermore, simultaneous administration of therapies designed to block adrenal androgen production or the binding of residual androgens to the androgen receptor by synthetic anti-androgens has been recommended by many studies (6, 7). Androgen deprivation therapy leads to remissions lasting 2 to 3 years; however, virtually all patients progress to a clinically androgen-independent state resulting in death in 16 to 18 months (8-12).

MECHANISM OF AIPC DEVELOPMENT

Androgen-independent prostate cancer (AIPC) is a lethal form of prostate cancer that progresses and metastasizes. There are several pathways, which have been shown to be responsible for the development of AIPC. These pathways gives us deep insights into the mechanisms of androgen action and provides us a deeper understanding of the mechanisms by which cancer cells subvert normal growth control and escape attempts to treat the prostate cancer (13). Deeper understanding of these pathways is the first step towards developing new therapies against this lethal form of prostate cancer. These mechanisms are demonstrated in Fig.2 and include:

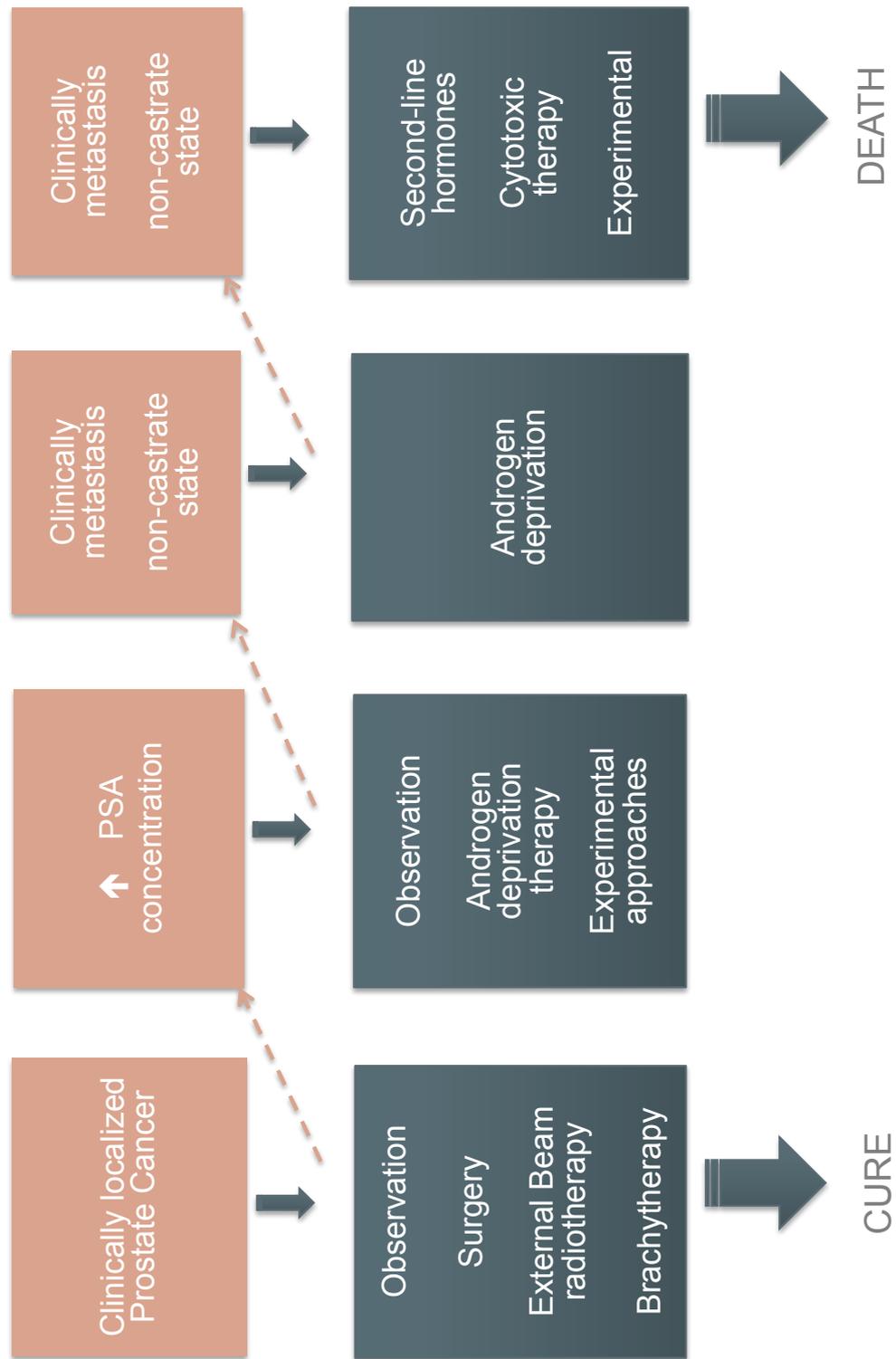


FIGURE 1. Represents clinical states of prostate cancer. Curative therapy is available only for the localized disease when tumor cells are confined to the prostate. "Adapted from Lancet Oncol 4:407-14, 2003"

The hypersensitive pathway

One way in which prostate cancer cells surpass the effects of androgen blockade is by developing the ability to use very low levels of androgens for (14-16), which means that they do not become androgen independent in the classic sense, but rather castration independent (13). There are several mechanisms which could explain response to lower levels of androgens.

One of these responsible mechanism is enhanced expression of the androgen receptor, which eventually allows enhanced binding of ligand. The existence of this pathway is supported by various studies of hormone refractory tumors that show increased expression of androgen receptor as compared to androgen-dependent tumors (14, 17-22). Elevated production of androgen receptor is caused either due to gene amplification or mutations through selective pressure of the androgen-depleted environment, which eventually causes the cells with fewer androgen receptors to die off and leading to clonal expansion of cells expressing more androgen receptor. Chen and colleagues have shown that AR gene expression was up regulated during the progression from androgen-dependent to castration-independent growth (14). Furthermore, they also showed that androgen-independent cells require 80% lower concentrations of androgen than androgen-dependent cells for growth (14).

Increased androgen receptor sensitivity to androgens has been suggested as another important mechanism for castration resistance (15). In support of this, Gregory and colleagues have shown that recurrent prostate tumors possess high level expression of the androgen receptor, increased stability of the androgen receptor, and enhanced nuclear localization of the androgen receptor, which is associated with an increased sensitivity to the growth-enhancing effects of dihydrotestosterone (15).

A third hypersensitive mechanism considered to be important is increased local production of androgens by prostate cancer cells themselves, which most likely occurs due to increased rate of conversion of testosterone to dihydrotestosterone via an increased activity of 5-alpha-reductase (2). There are various evidences that are in support of this mechanism which includes (a) ethnic groups with higher levels of 5-alpha-reductase activity are at a higher chance of development of prostate cancer (23); (b) after the initiation of the androgen ablation therapy, serum testosterone levels decrease by 95%, but concentration of dihydrotestosterone in prostatic tissue reduces by only 60% (24); and (c) genes involved in steroid biosynthesis have been shown to be overexpressed in recurrent human prostate tumors (20).

Promiscuous Receptor

The wild-type androgen receptor is stimulated only by testosterone and dihydrotestosterone, and it has been shown that specificity of androgen receptor is broadened by mutations. The majority of mutations are found to be clustered in the ligand-binding domains leading to binding promiscuity of androgen receptor (25, 26). Due to these mutations the androgen receptor can be activated by nonandrogenic steroid molecules, which are normally present in the circulation as well as antiandrogens (2, 14, 27-32). Such mutations have been demonstrated to be more frequent in androgen-independent prostate cancer cells and it form the basis of antiandrogen withdrawal syndromes in which 10% to 30% of patients treated with long-term antiandrogens will show disease regression with drug withdrawal (33-36).

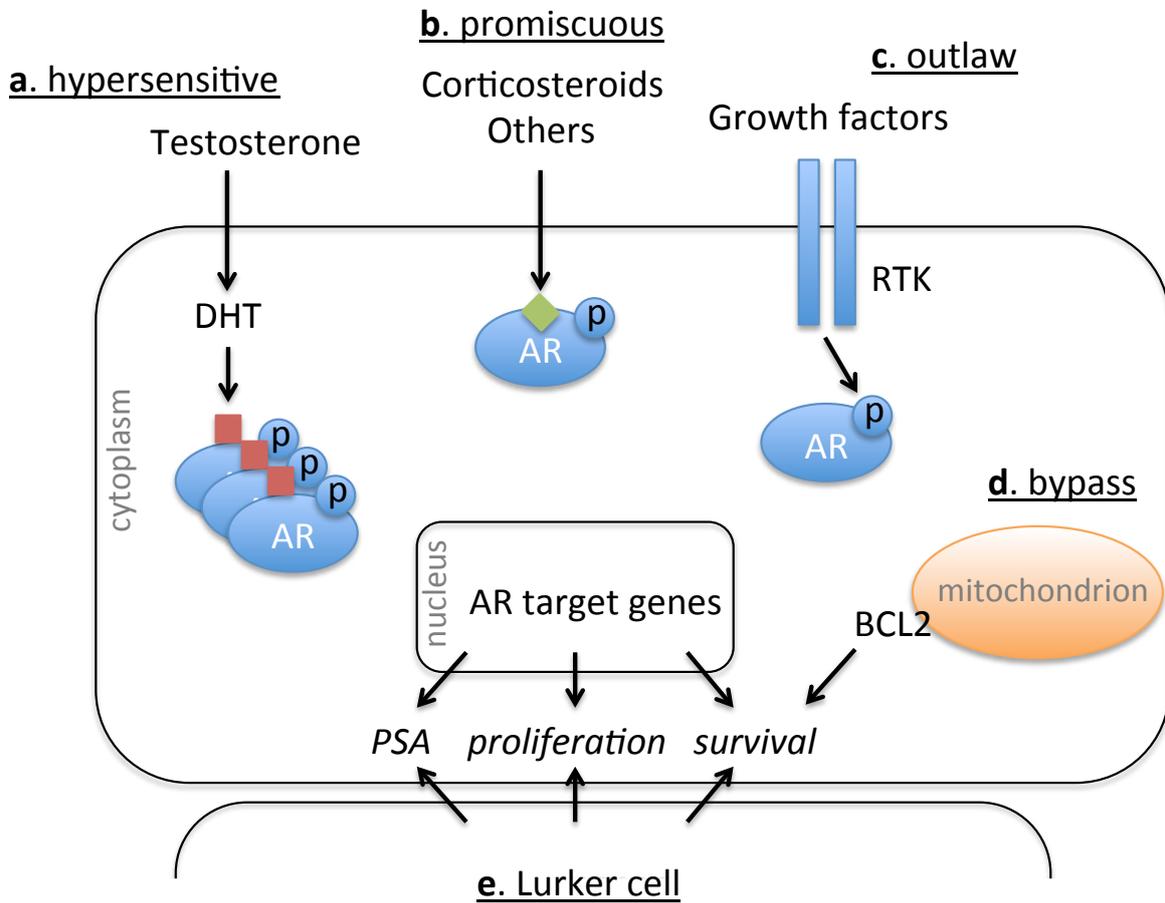


FIGURE 2. Pathways to androgen independence. (a) Hypersensitive pathway : Enhanced expression of the androgen receptor leading to enhanced ligand binding or enhanced conversion of testosterone to more potent dihydrotestosterone (DHT) by 5 α -reductase.(b) Promiscuous pathway : AR(Androgen receptor) activated by non-androgenic molecules present in the circulation leading to the activation of AR.(c) Outlaw pathway : Activation of receptor tyrosine kinases (RTKs) leading to downstream activation of either AKT(protein kinase B) or the mitogen-activated protein kinase (MAPK), which leads to phosphorylation and activation of AR. (d) Bypass pathway : Activation of the parallel survival pathways, thus obviating the need for AR or its ligand.(e) The lurker cell pathway : Presence of prostate cancer stem cells that continually resupply the tumor cell population, despite therapy. Although these cancer stem cells remain a small percentage of the actual percentage of the tumor, they are not affected by androgen-depletion therapy. "Adapted from Nature reviews cancer 1:34-45, 2001"

Outlaw Pathway

It is known that stimulation of androgen receptor can be achieved by ligand dependent binding of nonsteroid molecules or via the activation of downstream signaling of the androgen receptor by ligand independent mechanisms. Such type of activation has been defined as outlaw activation (2, 27, 28, 37). Deregulation of growth factors, for example insulin-like growth factor, keratinocyte growth factor, and epidermal growth factor, and cytokines, including IL-6, have been shown to directly phosphorylate and stimulate the androgen receptor (38, 39). It has been shown that androgen receptor dependent genes can be activated by deregulation of signal transduction pathways (40,41). HER-2/neu receptor overexpression activates androgen receptor-dependent genes in the absence of a ligand (40,41). Furthermore, the overactivity of outlaw pathways demonstrates the potential importance of tumor-microenvironment interactions in the development of castration-independent growth (42,43). Paget in his famous theory hypothesized that a “fertile soil” was required for the successful growth of cancer metastases (44). The interaction of prostate cancer cells with bone stromal cells, osteoblasts, and osteoclasts and bone extracellular matrix, leads to the deregulation of multiple growth factors that the prostate cancer cells can utilize to become androgen independent (2).

Co-activators and co-repressors

A large number of co-activators and co-repressors have been identified that are involved in the regulation of androgen receptor driven transcription (45). Imbalance between co-activators and co-repressors have been shown to influence androgen receptor activation, but the precise mechanisms are unknown (2, 46-49). An increased level of co-activators has been well identified in androgen-independent disease (46, 50-52). Co-activator proteins enhances the activity of the androgen receptor to alternative ligands, thereby sensitizing the receptor to lower levels of native and nonnative ligands, leading to ligand-independent activation(46).

Activation of bypass Pathways

Androgen receptor pathway can be completely bypassed, and prostate cancer cells can develop the ability to survive independent of ligand-mediated or non-ligand-mediated androgen receptor activation. Modulation of apoptosis is the best-known bypass mechanism. In androgen-dependent prostate cancer cells, androgen receptor activation stimulates cell proliferation but depletion of androgen leads to apoptosis of these cells. Androgen-independent prostate cancer cells have been shown to possess higher levels of anti-apoptotic molecules (53-56). Inactivation of the tumor suppressor gene phosphatase and tensin homologue (PTEN) leading to subsequent activation of Akt is one of the best known way in which AIPC cells escape apoptosis in an androgen-depleted environment (28). Another suggested bypass mechanism is the neuroendocrine differentiation of prostate cancer cells (28). Neuroendocrine cells exhibits a low rate of proliferation and are more prevalent in androgen refractory prostate cancers (28). They also secrete neuropeptides, which leads to increase in the proliferation of neighboring cancer cells, ultimately leading to progression in a low-androgen environment (28).

Prostate Cancer Stem Cells

In contrast to the stochastic model of tumorigenesis, the stem cell model of cancer postulates that only a rare subset of tumor cells are tumorigenic (54). Such population of cells comprising 0.1% of prostate tumors,

have been shown to be CD44⁺/alpha2 beta1hi/CD133, which do not express androgen receptors, and is thought to be enriched in prostate cancer stem - or progenitor cells (57). The prostate cancer stem cells in the androgen-depleted environment continually resupply the tumor cell population, and these cancer stem cells are not effected by the androgen-depletion therapy. Differentiation of these cancer stem cells into androgen dependent and independent cells results in the formation of heterogenous androgen receptor phenotype observed in AIPC patients (12).

DEVELOPMENT OF TARGETED APPROACHES AGAINST PROSTATE CANCER METASTASIS

Several molecular abnormalities have been identified that can lead to prostate cancer progression and development of androgen independent state. Many of these targeted therapies are now under various phases of clinical development (13, 58). Although most previous work have been focused on the androgen receptor but it is likely that several other molecular targets leads to the development of prostate cancer progression and androgen independent state (13). Development of these therapies requires the recognition that some of these targets may be relevant to the entire disease spectrum of prostate cancer but others may only be relevant for specific stages of prostate cancer. Many of these hypotheses-driven therapies are undergoing pre-clinical and clinical testing but, still there is a clear need for a better identification and selection of the specific patient population that will experience maximum benefit from specific molecular targeted approach (58). May be in the near future, it will be possible to determine the predominant mechanism of resistance in a tumor as it progresses during treatment and then apply that knowledge for the selection of the appropriate targeted therapy , with the goal of turning prostate cancer into a chronic disease. (58)

MOLECULAR MECHANISMS IN PROSTATE CANCER INVASION

Local invasion is one of the earliest steps in metastasis. To become invasive, the malignant cell should have the ability to downregulate its cell–cell contacts and alter cell–matrix adhesive characteristics, become motile and acquire the ability to degrade the extracellular matrix (ECM) (59). As soon the malignant cell has degraded the basement membrane, then it must enter the vascular or lymphatic circulation by breaching the endothelial barriers. Then the cancer cell should have acquired the ability to migrate via the blood or lymphatic circulation and should have the ability to arrest at a secondary endothelial site before binding to the endothelium, extravasating and then transmigrate through the endothelial layer to reach the interstitium, where it could have various fates as outlined in the figure 3 (60). There are various molecular mediators that have been shown to mediate the local invasion and dissemination of these prostate cancer cells. Some of well-known mechanisms are:

Epithelial to mesenchymal transition (EMT) in prostate cancer

In the normal prostate gland, epithelial cells have restricted migratory capability because the basal cells inside the lumen attach to the basement membrane and to each other forming a layer of interconnected cells with apical-basal polarity. Cell-to-cell adhesion in this epithelial layer is maintained by several types of junctions, such as adherens- and tight junctions. These junctions are composed of protein complexes of cell adhesion molecules (CAMs), such as cadherins. EMT is a cellular process through which epithelial cells lose

their epithelial characteristics like cell polarity and cell-cell junctions. During this epithelial to mesenchymal transition cells undergo changes in cytoskeleton and shape leading to acquirement of mesenchymal characteristics resulting in gain of migratory and invasive properties (61). Disruption of the E-cadherin-catenin adhesion complex is a key step in these processes. In prostate cancer, one of the most important features of cells undergoing EMT is cadherin switching, whereby E-cadherin (expressed in normal epithelial cells) is downregulated and N-cadherin (expressed in mesenchymal cells) is upregulated. Higher gleason grade prostate cancers have lower E-cadherin and higher N-cadherin expression, as compared to prostate cancer in patients with lower gleason grade disease (62-64). Decreased catenin expression has been also shown to be associated with decreased expression of E-cadherin, and with higher grade prostate cancer (65). In prostate cancer, one important signaling axis that can support EMT (in addition to effects on proliferation) is the HGF receptor, MET. Expression of the MET receptor is associated with disease progression and MET inhibitors are actively tested in prostate cancer clinical trials (66)

Prostate cancer invasion: Roles of proteases

Matrix metalloproteinases and serine proteinases (for example urokinase- type plasminogen activator) are the most important families of proteinases that have been shown to be most associated with degradation of extracellular matrix in prostate cancer. It has been shown that primary prostate tumor tissues possess higher levels of MMP-9, and also the ratios of MMP-2/9 to tissue inhibitor of metalloproteinases-1 (TIMP-1) are increased as compared to normal prostate epithelium. Also, the levels of MMP-9 and the ratios of MMP-2/9 are increased in patients with high gleason grade prostate cancer, which is associated with poorer patient survival (67-69). Furthermore, it has been shown that loss of TIMP-1 is correlated with upregulation of MMPs in malignant human prostate cancer tissues(70). Patients with metastatic prostate cancers are observed to have high concentrations of MMP-2 and MMP-9 in their plasma (71). MMP-12 downregulation using RNAi approach reduced the invasiveness of PC3 cells by reducing degradation of Type I collagen in the bone, which supports the fact that MMP-12 also participates in bone-tropic metastasis(72).

Adhesion signaling in prostate cancer migration

Cell migration is a dynamic process mediated by Focal adhesion (FA) signaling. Focal adhesions are the regions where integrins, in large dynamic protein complexes establish a signaling link between the ECM and actin cytoskeleton. Integrin-mediated ECM adhesion leads to activation of downstream pathways involved in gene regulation, proliferation, apoptosis, cell survival, polarity, actin dynamics and cell migration (73, 74). The predominant integrins present in normal prostate epithelial cells are alpha5 beta1, alpha6 beta1, alpha6 beta4, alpha V beta3 and alpha3 beta1 (75). Lee et al., has showed that levels of beta1 integrins and integrin-induced autophosphorylation of FAK are increased in prostate cancer cells in primary prostate cancer and lymph node metastases, which suggests that beta1 integrin activation occurs in metastatic progression of prostate cancer (76). It suggests that alterations in integrin composition and function can thus affect migration of prostate cancer cell through modulation of FAK function. Furthermore, overexpression of FAK has been observed in high-grade and metastatic prostate cancer tissues as compared to normal prostate tissues (77).

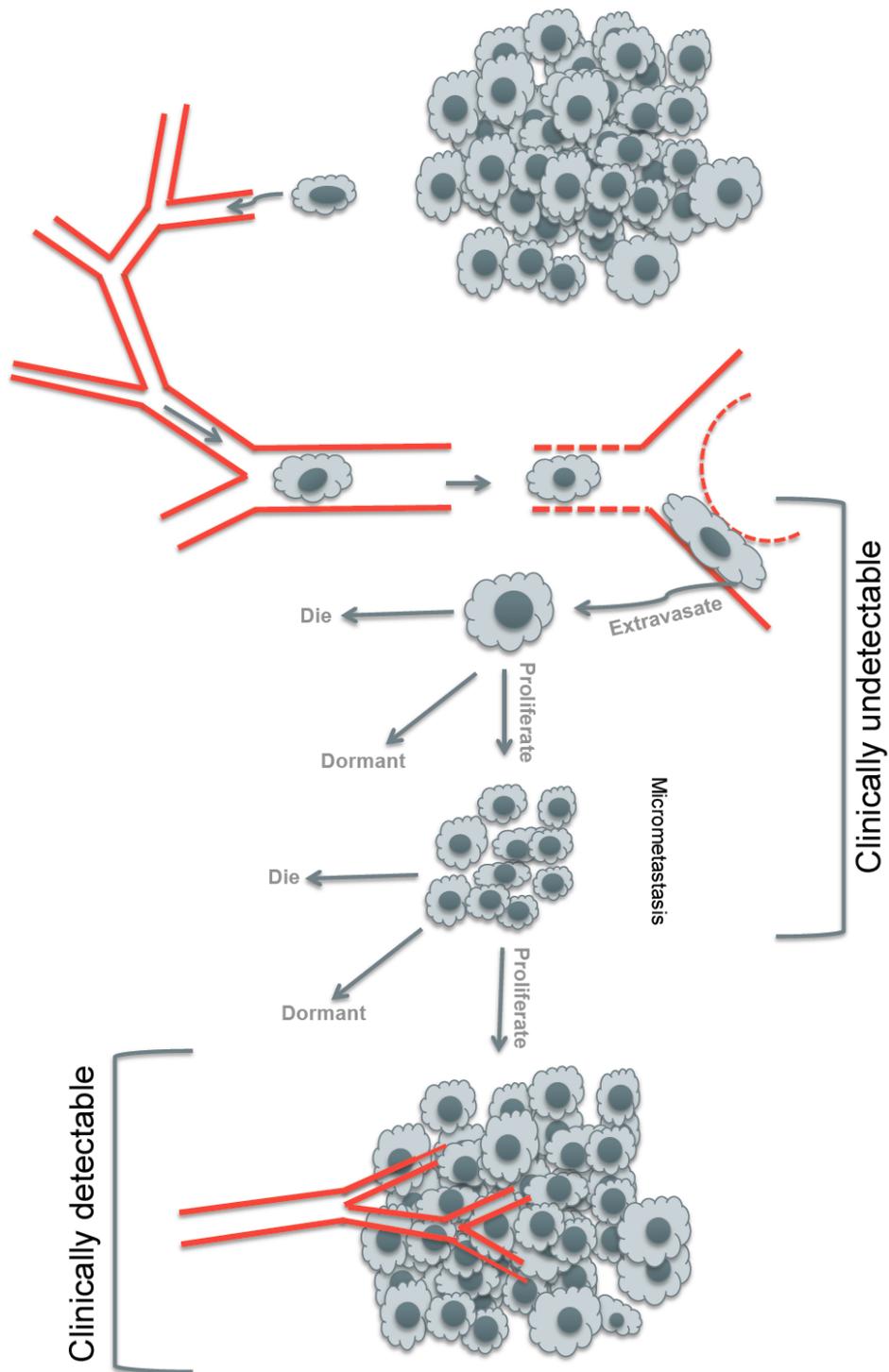


FIGURE 3. *The metastatic cascade* (a) Tumor cells escape from the primary tumor and then arrest at the secondary metastatic sites (b) Cancer cells can have various fates following their arrival at the secondary metastatic sites. At secondary sites, cancer cells can exist in various forms like solitary cells, micrometastasis and vascularized macrometastasis. The balance between the proliferation and survival governs their fate at each step. Cells, which adapt better to the foreign microenvironment (in line with Pagets seed and soil hypothesis) survive, proliferate and progresses to form vascularized macrometastasis. The cells where proliferation is balanced by apoptosis stays dormant, and those cells die where the balance tilts in favour of apoptosis. "Adapted from Nature Rev Cancer 2:563-72, 2002"

MODELS TO STUDY PROSTATE CANCER METASTASIS

As our understanding of prostate cancer becomes more comprehensive, the need for more sophisticated and accurate models increases (78). Experimental therapeutics targeting specific tumor-specific molecules must be validated in well-characterized models that enable the testing of the experimental hypothesis. By establishing models that replicate human prostate cancer progression more closely and by asking more disease specific questions with such models, animal models will continue to play an important role in validating novel cancer targets and evaluating putative cancer therapies (78). Three dimensional extracellular matrix models and animal based models, are being utilized to replicate and model the various steps of metastatic cascade. Figure 3 gives an overview of the steps involved in metastasis cascade. Basic understanding of the dynamics of metastasis cascade is the key to the development of novel models and to the development of novel anti-cancer therapies. In 3D based assays prostate cancer cells are either grown inside the matrix or plated on the top of the matrix to model the complex processes of growth, local invasion and migration. Also these 3D based models provide an ideal platform to study the complex interactions between the cancer cells and the extracellular matrix (79). In animal based models, cancer cells are injected to form a primary tumor orthotopically (in the prostate) or at other, more easily accessible sites (e.g. subcutaneous) and tumor growth as well as metastasis is monitored. Alternatively, tumor cells are injected directly into the circulation to model the later phases of the metastatic process such as extravasation and metastatic colonization. These assays are called, respectively, spontaneous or experimental metastasis assays (80, 81) and the end point of both assays is the formation of visible metastases at a secondary site. These assays have led to the identification of many molecular alterations in cancer cells that can contribute to their ability to metastasize, and selective targeting of these tumor cell specific alterations is the basis of molecular targeted approach (82-87). These models constitute the major preclinical tool required for the further development of new therapeutic strategies. Xenografts are the most commonly used models in drug development and are able to maintain several biological properties of the human tumors they are derived from, particularly when orthotopically transplanted. Osteoblastic bone metastases can also be modeled (78). Non-invasive methods of imaging have been recently developed to significantly reduce the number of mice required and have improved the follow up of tumor growth and response to therapy in these models. Genetic models are very useful for target validation, but have not yet been extensively used for drug evaluation. Finally, some models are more relevant for the study of specific pathways as androgen receptor signaling, or bone metastases development, and others are useful in developing strategies targeting prostate cancer stem cells or mechanisms of resistance to chemotherapy or radiotherapy (78).

The modern drug development technologies increase the number of candidate therapeutics and subsequently increase the costs for preclinical and clinical testing. Mouse models are time, labor, and finance intensive and current limitations have hampered the transition of scientific findings from these models to human clinical trials. Moreover, while the vast contribution of mouse models to advancing our understanding of cancer biology is indisputable, there have been ongoing concerns about the value of mouse models for predicting drug efficacy and usefulness in humans, and the predictive value of tumor xenograft models in cancer research is still a matter of debate. Thus, the development of faster and cheaper animal model systems and improvements in the ability of preclinical models to predict clinical efficacy can have a high impact by lowering the cost of drug development and by helping to prioritize compounds for clinical investigation.

AIM OF THESIS

General purpose

Metastasis is a major cause of death and morbidity in patients with neoplastic disease, but one major obstacle in the progress of prostate cancer studies has been the limited availability of relevant preclinical models (78). Current limitations of these models have hampered and delayed the transition of scientific findings to clinical trials. The ideal prostate cancer model is one that reproduces and replicates the natural history of the prostate cancer, and able to generate long-term spontaneous metastases to lymph nodes, lungs and bones. Such ideal prostate cancer model does not exist (78). However, models for investigating key steps in the metastatic cascade have been developed and can be further improved. The greatest challenge of the post genomic cancer drug discovery is to develop mechanism based agents that act on the key biological tumor specific processes and then sequentially to test the hypothesis that such tumor specific agents are indeed more potent and more specific than the current available generation of anti cancer drugs. There is lot of criticism regarding the value of two-dimensional based in vitro models in anti-cancer drug target discovery (88). Facing this criticism, the cancer researcher is continuously challenged to develop novel models that could enable us to study disease specific question in a setting that nearly recapitulates the natural history of metastatic process. The development of better anti-cancer targets continues, and it is now evident that selection of the appropriate disease models is of utmost importance during the pre-clinical drug discovery process when investigating biological processes like invasion, dissemination and metastatic colonization. One of the most important thing is to use the right models to ask the right questions about the right therapies (78).

Based on this background the **specific aims** were (as schematically outlined in figure 4) :

- To develop novel 3D in vitro and whole organism-based high-throughput models of (prostate) cancer progression that are suitable for both compound as well as RNA-interference screening approaches.
- To Identify/validate novel candidate genes playing a significant role in prostate cancer progression.

OUTLINE OF THESIS

The ultimate aim of this thesis is the identification and validation of novel candidate genes playing a significant role in the prostate cancer metastasis. For that purpose, we established imaging based automated in-vitro and in-vivo models to study growth, migration, invasion and dissemination of (prostate) cancer. First, we established an automated 3D in vitro assay to study growth, local invasion and migration of cancer cell spheroids in an extracellular collagen matrix. The development of this assay is described in **chapter 2**. For prostate cancer, and most other solid cancers, it is necessary to develop whole organism based bio-imaging models to screen for novel candidate target genes which can deepen our understanding of the mechanism underlying metastatic progression. In **chapter 3**, we developed an automated bio-imaging model of cancer progression using xenotransplantation in zebrafish embryos. In **chapter 4**, current state of art in prostate cancer treatment is discussed. Here, we discuss emerging new molecular targets and the potential clinical applications of combining radiotherapy with these molecular targeted agents for the treatment of prostate cancer. It will be important to find more drug targets and this is what we describe in following two chapters. In **chapter 5**, we describe the identification of SYK as a novel candidate prostate cancer target gene derived from a RNA-interference screen exploiting the zebrafish xenotransplantation model. Next to the RNA-interference screening, focused research has been conducted on previously identified novel candidate genes. In **chapter 6**, we describe MST1R as a dominant factor in prostate cancer progression. Finally **chapter 7**, provides a summary and a general discussion on the findings and implications of the work in this thesis.

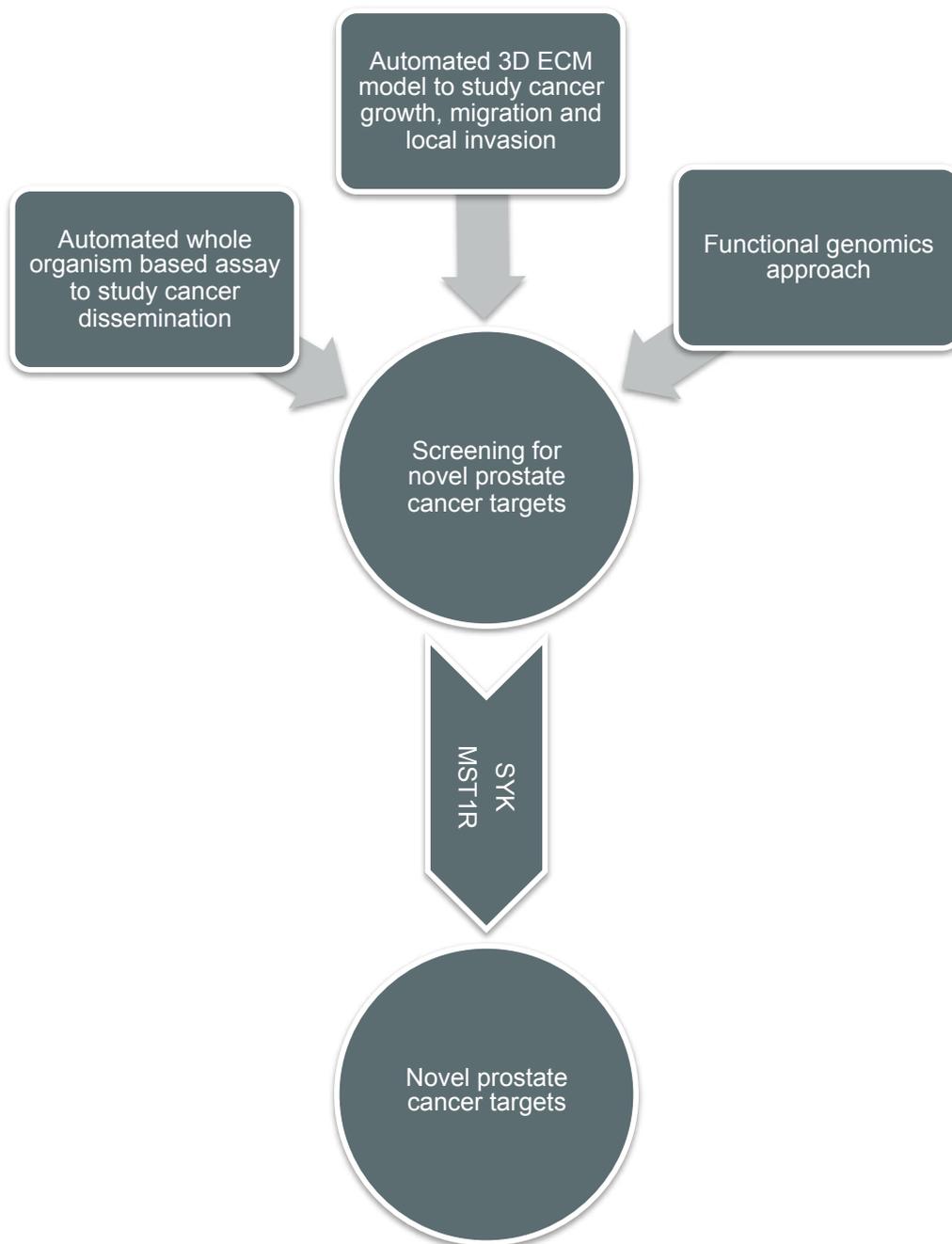


FIGURE 4. Schematic representation of the specific aims/outcomes of the thesis.

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