Relevance of fibroblast growth factor receptor 1 and its isoforms in prostate cancer bone metastases

PhD Candidacy Exam Proposal
Cancer Biology Program
MD Anderson Cancer Center UTHealth Graduate School of Biomedical Science
Fall Semester 2017
Specific Aims

Prostate Cancer (PCa) is one of the most commonly diagnosed malignancies in men. Bone-forming metastases dominate the clinical picture of men with advanced PCa and constitute the main cause of morbidity and mortality of the disease. Androgen deprivation is first line therapy for bone metastases of PCa, but responses to such therapy are short, and eventually, the disease progresses to castration-resistant PCa (CRPC), being bone the primary site of progression. Further development of therapies for bone metastases of PCa requires an understanding of the mechanisms underlying the growth of CRPC in bone.

The fibroblast growth factor (FGF)/FGF receptor (FGFR) complex (FGF axis), a signaling axis that typically mediates epithelial–stromal cell interactions, is central to prostate and bone development, and is commonly altered during PCa. Recent studies have implicated FGF signaling in the pathogenesis of PCa progression in bone, suggested that it mediates a positive feedback loop between PCa cells and bone cells, and identified the FGF axis as a candidate target for therapy. Indeed, blockade of FGFRs with dovitinib (TKI258), a receptor tyrosine kinase inhibitor with potent activity against FGFR and vascular endothelial growth factor receptor (VEGFR) has clinical activity in a subset of men with CRPC and bone metastases. Our preliminary data acquired by large-scale RNA sequencing in human PCa samples indicate that the mean expression of FGFR1 was the highest of all the FGFR family genes studied. Analyses of FGFR1 transcripts identified eight different protein coding transcripts to be the most abundantly expressed, and determined that different human PCa tissue samples express different FGFR1 isoforms. However, whether these isoforms respond differently to FGFR1 blockade and modulate different downstream pathways still needs to be determined.

The overall goal of this proposal is to investigate the molecular and clinical implications of the expression of FGFR1/FGFR1 isoforms in the pathogenesis of PCa bone metastases. Based on the protein lengths of the most abundantly expressed transcripts, we focused on the two best characterized FGFR1 isoforms, alpha and beta, containing 3 and 2 immunoglobulin-like domains, respectively. We hypothesize that FGFR1 alpha and beta confer different phenotypes to PCa cells, and that this may partly explain PCa heterogeneity, pattern of progression, and differences in response to FGFR1 inhibitors (Fig.1). Also, because FGF axis plays an important role in bone biology and we have previously reported the involvement of FGFR1 in PCa cells-bone cell interaction [3], we also propose that FGFR1 mediates PCa cell–bone cell cross talk (Fig.1). We will test these hypotheses in the following Specific Aims:

Aim 1. Analyze FGFR1 isoforms expression in human PCa and its molecular and clinical correlates. Based on our preliminary RNA sequencing studies showing the existence of a repertoire of alternative splice variants in PCa tissue samples, we will test our postulate that PCa tumors are heterogeneous in their expression of alpha and beta isoform levels throughout disease progression. Furthermore, we hypothesize that these two isoforms trigger activation of different associated gene signatures that modulate malignancy. To evaluate molecular and clinical correlates of FGFR1 isoforms, we will (a) mine the TCGA PCa data, and (b) assess the expression of FGFR1 alpha and beta in clinical samples reflecting the progression of the disease (i.e. primary and metastatic PCa). For this last sub-aim, we will develop specific antibodies for each isoform. We will also (c) study the signaling cascade induced by FGFR1 alpha and beta by genetically manipulating FGFR1 isoform expression in PCa cells, and subsequently performing immunoblotting and reverse phase protein array.

Aim 2. Assess the role of FGFR1 (and its isoforms) in the growth of PCa in bone, response to FGFR blockade, and PCa-bone interaction. Given that FGF signaling is a key mediator of bone metabolism and that FGFR1 is involved in PCa progression, we propose that FGFR1 accelerates the bone metastatic phenotype of PCa cells, which is orchestrated by the contribution of both isoforms. We will (a) evaluate the metastatic dissemination of PCa cells after intracardiac injection of these cells in mice mediated by FGFR1 isoforms in vivo, and (b) the induction of PCa growth in bone by direct injection of PCa cells into the femur of mice and treated with a specific Pan-FGFR inhibitor, JNJ-42756493. We will (c) perform co-culture studies in vitro to investigate the role of FGFR1 isoforms in the cross talk between PCa cells and bone cells (osteoblasts).

The experiments described here will provide insight into the interaction between PCa and bone cells and will help us elucidate the pathways involved in the growth of PCa cells in the bone. Dissecting the role of FGFR1 isoforms in PCa bone metastases will significantly contribute to the recognition of FGFR1 blockade responders, to develop new therapies targeting FGFR, and to identify predictive biomarkers of response to this treatment.
Research Strategy

Background and Significance. Prostate cancer (PCa) is the third leading cause of cancer death among American men and is the most commonly diagnosed cancer [4]. Although the survival rate is high for the early stages, the 5-year survival rate drops from nearly 100% to 29% when the disease has disseminated beyond the local site [5].

Patients with advanced metastatic PCa have several therapy options, but none of them are curative. Androgen deprivation is the most effective therapy, but growth of the cancer resumes over time in most cases, and the disease becomes castration-resistant (CRPC). Most CRPC patients are treated with abiraterone and/or enzalutamide, next generation drugs for androgen deprivation therapy. Unfortunately, resistance to these therapies eventually emerges, and because there is no available chemotherapy and/or targeted therapy that are curative, this leads to disease recurrence and death after several different treatment modalities [6]. Identifying new and more effective therapies represent a major clinical challenge in the field.

The FGF axis is a complex signaling pathway, composed of 18 known receptor-binding ligands (FGFs) and 4 tyrosine kinase membrane receptors (FGFRs). The interaction, in the paracrine signaling, is mediated by heparan sulfate (HS) and leads to activation of the FGFR kinases followed by phosphorylation (i.e., activation) of FGFR substrate 2 (FRS2) and recruitment of phospholipase Cγ and downstream cascades and networks (e.g., mitogen-activated protein kinase (MAPK) and protein kinase B (AKT)) [7]. Ultimately, FGF signaling regulates a plethora of biological effects: mitogenesis, differentiation, angiogenesis, survival, and motility/invasiveness, among others [8] (Fig. 2). The FGF pathway plays a central role in various processes that include embryonic and organ development, wound healing, and carcinogenesis [9, 10]. Certainly, all 4 FGFRs have been reported to overexpress several members of the FGF axis including, FGF1, FGF2, FGF6, FGF8, FGF9 and FGF17 as paracrine or autocrine growth factors in PCa [11-14]. FGFR1, in particular, was shown to be implicated in PCa progression in transgenic mice with inducible FGFR1 expression in the prostate (JOCK1), established by the group of Spencer [15]. McKeehan’s lab further demonstrated that specifically FGFR1 stimulates the malignant phenotype of prostate epithelial cells and also highlighted the key function of isoforms in the epithelial-stromal interactions [16, 17]. The FGFs are also important regulators of bone formation at all stages of the osteogenic lineage, as demonstrated by Ornitz and colleagues [18-21].

Studies conducted by our group in mice proposed that PCa cells can alter the bone microenvironment by triggering the expression of FGFs [14]. Indeed, blockade of FGFRs with dovitinib (TKI258, Novartis Pharmaceuticals) has clinical activity in a subset of men with CRPC and bone metastases [3]. Also, dovitinib has antitumor activity in vivo in PCa patient-derived xenografts (PDXs) with PCa cells expressing high FGFR1; while no antitumor activity was observed against PDXs with low FGFR1 expression in the PCa cells. These results provide direction for therapy development of FGFR blockade in PCa. Interestingly, treatment with dovitinib not only targeted tumor cells causing their death, but also blocked the FGF axis in osteoblasts, improving bone quality (increase in bone mass) and interfering with the interaction between the stromal and the epithelial compartments [3]. This raises the motivation to discern the autocrine and paracrine growth mechanisms of FGFR1 expressing tumor cells. In this context, searching for predictive markers will aid to develop the rational bases for beneficial treatment combination in our future studies: drugs that directly affect the tumor cells together with drugs that affect the stromal cells appear as a promising strategy to prolong survival of PCa patients. Additionally, since dovitinib clinically modulates PCa phenotype without decline in serum PSA, it is essential to identify markers to accurately select likely responders and to confirm pathway blockade at the tissue level in order to evaluate responses to treatment.

RNA sequencing of 183 human PCa tissue specimens and PDXs showed that the mean expression level of FGFR1 was the highest of all the FGFR family genes studied [3]. Analyses of FGFR1 transcripts identified various alternative spliced variants, or isoforms, differentially expressed in different samples. Based on the predicted protein lengths of these transcripts, the most abundantly expressed are two well characterized FGFR1 isoforms, alpha and beta, containing 3 and 2 immunoglobulin-like domains, respectively (Fig. 3). The Ig I domain,
or the alpha-exon, deleted by splicing in FGFR1 beta, is flanked by two intronic silencing sequence (ISS) elements. One of the splicing factors associated with this event is the Splicing Factor Proline And Glutamine Rich (SFPQ). Interestingly, a recent study has reported elevated expression levels of SFPQ in advanced PCa [22, 23]. These isoforms allow to modulate responses to a ligand even without overexpression, and depending on the cytoplasmic domains of the receptor, confer growth advantages.

Splicing variants or their downstream targets could serve as potential biomarkers and therapeutic targets. Even the regulated switch to specific splice variants could differentially impact the cell [24].

Since FGFR1 isoforms have been associated with pancreatic cancer, breast cancer and glioblastoma [25-27], it is our interest to address the significance of the presence of these isoforms in PCa, which could have important insights in revealing fundamental mechanisms involved in disease progression. The complexity of the FGF axis, constituted by at least 18 FGFs, 4 highly ubiquitous FGFRs, and their isoforms simultaneously expressed in a tissue, represents an obstacle in unraveling the distinct roles of the different molecules of the FGF axis, and at the same time, constitutes the motivation to decipher the role of each of the different players of the FGF pathway.

Elucidating the involvement of FGFR1 and its isoforms in the dissemination and growth in bone is significant not only to treat PCa that currently use that currently use this pathway to progress under androgen ablation but also because it is expected that some cases progressing on new targeted therapies will use FGFR signaling to grow. For instance, previous reports indicate that progression to cabozantinib, an inhibitor of the tyrosine kinases c-Met and VEGFR2, is associated with FGFR1 upregulation in PDXs suggesting that FGFR1 might be a pathway mediator of treatment resistant [28].

This project is also significant because it addresses an important clinical challenge, the identification of PCa patient candidates for FGFR blockade therapy and the detection of FGFR1 target genes expected to be modulated following FGFR1 blockade.

Innovation. Understanding the molecular heterogeneity of tumors led to the successful application of targeted agents in many cancer types. PCa is a heterogeneous disease yet available therapies continue to be applied homogeneously. The fact that responses to agents with different mechanisms of action were not uniform in PCa patients, showed the biological intra and inter- heterogeneity of these tumors and proves the urgent need to integrate our knowledge of PCa biology into clinical application to address each tumor’s unique behavior. Biomarkers distinguishing the clinically relevant PCa subsets, i.e. selecting the correct patient population, predicting and bypassing mechanisms of acquired resistance, are indispensable for optimal therapeutic improvement.

While initially the vast majority of metastatic PCa rely on the availability of androgens for growth and survival, in their final stages patients with metastatic PCa eventually progress to CRPC. Under the selective pressure of drug treatment, PCa cells are able to acquire molecular changes that allow them to survive in androgen-deprived conditions, gain a selective growth advantage, and finally, cause their host’s death [29, 30]. These molecular changes include the activation of the FGF axis, particularly upregulation of FGFR1 and its isoforms in a subgroup of patients. Therefore, the main innovation of this proposal is our hypothesis that FGFR1 isoforms activate different genes or pathways in PCa cells with implications in disease progression. Although previous studies indicate that FGFR1 isoforms expression predicts progression in breast cancer [31], this is the first study to identify a different signature associated with FGFR1 isoforms in PCa.

Approach.

Specific Aim 1. Analyze FGFR1 isoforms expression in human PCa and its molecular and clinical correlates.

Rationale. There is compelling evidence that clearly suggests that FGFR1 signaling is a major mediator of PCa proliferation and evolution to metastasis [32-34]. Indeed, our studies have shown clinical response to FGF axis blockade in advanced PCa [3], which posted the challenge of better defining which patients will benefit from this therapy, by identifying markers. However, the detailed mechanisms of FGFR1 signaling in PCa cells and the role of this signaling on tumor progression is not yet fully understood. In collaboration with Dr. Arul Chinnaiyan (University of Michigan), we found that tissue samples derived from different PCa express different isoforms of
FGFR1. We hypothesize that the different FGFR1 isoforms are responsible, at least in part, for therapy response to FGFR1 blockade, derived from tumor heterogeneity. Thus, to determine the clinical relevance of FGFR1 isoforms expression in PCa and to sort out the functional differences between the isoforms, we will focus our studies on the two best characterized isoforms of FGFR1, alpha (NM_023110.2), and beta (NM_023105.2), which represent the most abundant predicted protein coding transcripts found in our RNA sequencing study. To accomplish this aim we will (a) use bioinformatics tools. To validate our in silico data, we will (b) develop and use antibodies specific for each isoform. Furthermore, we will (c) engineer PCa cells to overexpress FGFR1 alpha and beta isoforms and we will study the signaling pathways triggered by them. We expect to elucidate details of the significance of the diversity of the FGF axis and its molecular impact. Successful completion of this aim will aid in the knowledge of the biology of action of FGFR1 and its associated signaling pathways in PCa, which will be critical in the development of approaches to control progression of CRPC.

Preliminary data supporting this aim. FGFR1 isoforms alpha and beta are associated with the expression of different genes. Our initial TCGA data mining of molecular correlates of FGFR1 isoforms (sub-aim1a) resulted in two distinct patterns of gene expression associated with each FGFR1 isoform (alpha and beta) (Fig. 4).

Experimental Design. (a) To mine the TCGA PCa data to evaluate molecular and clinical correlates of FGFR1 isoforms. The recent advances in next-generation sequencing has enabled to analyze the fine structure of the human genome; however, simple cataloging genomic mutations and derangements and the transcriptional archive is not enough to unravel the complexity of the biochemical processes. To address this issue, we will collaborate with Dr. Broom (Dept. of Bioinformatics and Comp Biology) to mine the human RNA sequencing data from TCGA for expression of FGFR1 isoforms in PCs and its molecular and clinical correlates. For the search, we will use the specific sequence of each of the FGFR1 isoforms, alpha and beta. To perform the analyses, an FGFR1 splice score will be defined as the ratio of FGFR1 alpha to FGFR1 beta. A high FGFR1 score indicates prevalence of FGFR1 alpha and a low FGFR1 score indicates prevalence of FGFR1 beta. We will assess the expression and clustering of genes and pathways associated with FGFR1 splice score by heatmap analysis. Our search for clinical and molecular correlates of FGFR1 isoforms in silico will also include the analysis of different clinical features/parameters associated with FGFR1 isoforms (i.e. clinical recurrence vs non-recurrence, overall survival, biochemical relapse-free survival, time to progression after hormone treatment). We will complement our analysis mining the Genotype-Tissue Expression (GTEx) project for expression of FGFR1 isoforms in normal prostate tissue. Database mining can be very informative and can provide a molecular framework for the other experimental approaches (described below), enabling the analyses of our findings in the context of clinical data or genomic alterations.

For statistical analysis and data interpretation, we will perform Chi-square tests to study the association between FGFR1 isoforms and clinical features. Univariate and multivariate analyses (considering covariates such as pathologic Gleason Score, serum PSA level at diagnosis, etc.). will be conducted using Cox regression, and Kaplan-Meier plots will be used to evaluate the association between FGFR1 isoforms and survival. The comparison between groups will be done using the log-rank test.

(b) To assess the expression of FGFR1 alpha and beta in clinical samples reflecting the progression of the disease. Since the available commercial antibodies anti-FGFR1 alpha and beta lack specificity for immunohistochemistry (IHC) analysis, we will develop customized antibodies designed to recognize each isoform at the protein level. To recognize FGFR1 alpha isoform, we have designed a peptide (aa 31 to 59) that includes the sequence encoding the Ig-like domain in FGFR1 alpha not present in FGFR1 beta isoform (Ig I) (Fig. 5). To recognize FGFR1 beta isoform, we have designed a peptide (aa 21 to 41) that spans between the signal peptide and Ig II (a sequence that does not include Ig I) (Fig. 5). The sequences were selected based on sequences blast (NCBI) and 3D structure modelling performed by Creative Biolabs (Upton, NY). These peptides were then used by the company to develop FGFR1 isoform specific mouse antibodies using hybridoma technology. Since we have

**Fig. 4.** Snapshot of heatmap of the 2000 most highly correlated genes positively (red) or negatively (green) to FGFR1 isoforms. Samples, in columns, are arranged by the expression ratio of FGFR1 alpha to beta. Each row represents a gene.

**Fig. 5.** Illustration of customized antibodies (red) developed to perform immunohistochemistry.
tested that the expression levels are high and easily detectable by IHC and Western blot analysis, we will test the specificity and sensitivity of these antibodies by IHC and Western blot analyses of PCa cells expressing FGFR1 isoform alpha or beta, or control (empty vector). We will also perform the screening in formalin fixed paraffin embedded tissue samples (i.e. IHC by fixing cell pellets and embedding them in paraffin). Once the experimental conditions are set up, we will study the correlation of FGFR1 isoforms expression with stage of PCa (untreated versus CRPC, primary tumors versus metastases) in clinical samples. We will use formalin fixed, paraffin embedded archived samples from the institution’s tissue bank. We will establish with bioinformaticians the sample size for power calculations. We will inform semi-quantitatively the ratio between the two isoforms. Analysis will be performed in collaboration with Dr. Troncoso, Dept. of Pathology.

(c) To study the signaling cascade induced by FGFR1 alpha and beta in PCa cells. We developed stable C4-2B PCa cell lines expressing FGFR1 and GFP, using vectors containing bicistronic FGFR1 and GFP, or empty vector (GenScript). Stable lines were developed by batch transfection and selection with gentamicin followed by cell sorting of GFP positive cells. We also established PC3 sublines expressing FGFR1 alpha and control empty vector in a stable manner, and are in the process of developing PC3 cell expressing FGFR1 beta.

To assess the signaling pathways activated by FGFR1 alpha and beta, we will stimulate PCa cells expressing FGFR1 isoforms or empty vector with FGF ligands, in particular FGF2 and FGF9, which we found to have the highest expression among FGF ligands in PCa cells and PDXs and because bone cells secrete FGF2 [14, 35]. At the end of treatment, cells will be harvested and we will assess activation of targets downstream of FGFR1 isoforms. Specifically, we will evaluate expression and phosphorylation of known downstream genes regulated by FGFR1 (e.g., FRS2a, p-ERK1/2, p-AKT, PLCγ, Spry 2, sef, MKP3, and FRLT3) as well as genes based on the findings of our in silico studies performed in sub-aim 1a. Activation of genes downstream of FGFR1 isoforms will also be assayed by performing RPPA in the C4-2B stably expressing FGFR1 alpha, beta and empty vector cells treated with vehicle, FGF2 or FGF9. This high-throughput approach, which will be performed in the institution’s RPPA core, will validate and further expand the targets studied. Statistical considerations will be performed in collaboration with Dr. Broom by using software tools. RPPA results will be validated by Western blot analyses of C4-2B stably expressing FGFR1 alpha, beta and empty vector. Western blot experiments will be repeated at least three times and the results will be recorded quantitatively.

As a more relevant preclinical model to represent the biologic complexity of PCa and as a complimentary approach, we will use MDA PCa 118b PDX, that expresses high endogenous levels of FGFR1, prevalently alpha (although low levels of FGFR1 beta isoform can be detected). We will use shRNA lentiviral particles targeting FGFR1, or scramble shRNA as control, that contain GFP driven by the CMV promoter (Sigma) to help confirm delivery of shRNA and to follow the cells expressing it. Since 118b, like most PCa PDXs, cannot be cultured in vitro for a long term, we will use organoids following published protocols [36, 37]. These organoids are currently being developed in the lab.

Altogether, results of these studies will identify signaling pathways and genes activated by FGFR1 and its isoforms in PCa cells.

Expected results, potential pitfalls, and alternative approaches. In sub-aim 1a we expect to find expression of different genes and pathways linked to each FGFR1 isoform. We also expect this approach to be informative regarding clinical features associated with the isoforms. Our pilot preliminary TCGA data mining exploration results provide us with confidence that FGFR1 alpha and beta will trigger different signaling cascades and biological effects in PCa cells. As the samples in TCGA are mainly primary PCa, it is possible that there is not enough information on FGFR1 isoforms for clinical correlates for metastatic disease. To overcome this issue, we will consider The International Cancer Genome Consortium (ICGC) database that might contain more data useful to mine the clinical correlates.

In sub-aim 1b, we expect to elucidate whether there is enrichment of a particular isoform (alpha or beta) during PCa progression. It is possible that the antibodies lack specificity for IHC assay. We will then test the expression of FGFR1 isoforms by RNA in situ hybridization (ISH) in archived samples (formalin fixed, paraffin embedded). For that, we will collaborate with Dr. Palanisamy (Henry Ford Health System, Detroit MI) who has extensive experience in performing RNA-ISH in clinical samples [38-41]. We would use three probes: an alpha specific probe, for the exon-exon junction to detect the alpha-specific exon; a probe for the exon-exon junction to detect the skipping of the alpha-specific exon; and a third probe targeting common sequences between FGFR1 alpha and beta. We would approach this study by dual color assay and take the ratio to decide on the expression level of each variant. Another alternative approach is to perform FGFR1 isoform expression profiling mass
spectrometry. If successful, this method would be the first time that the expression of these isoforms is assessed by detection of the specific peptides using mass spectrometry.

In sub-aim 1c we expect to identify an FGFR1 isoform associated signature, resulting from different molecular outcomes of PCA cells expressing FGFR1 alpha or beta. We expect to identify genes regulated by FGFR1 alpha but not beta and vice versa; these genes will help us to understand the mechanism by which each isoform mediates its action and may have therapeutic implications. This would serve as the bases to determine target inhibition in clinical trials with FGFR blockade. We do not expect major problems with methods as they are well established in the laboratory or by collaborators.

Specific Aim 2. Assess the role of FGFR1 (and its isoforms) in the growth of PCa in bone, response to FGFR blockade, and PCa bone interaction.

Rationale. In spite of the progress achieved in the understanding of the mechanisms mediating bone metastasis, this knowledge has not been yet translated to new, curative treatments for the metastatic disease and thus bone metastasis remains a devastating complication of advanced PCa. The fact that PCa consistently produces bone-forming metastases reveals the existence of a unique communication between PCa and the bone microenvironment. Our previous studies showed how targeting FGF signaling interfered with this interaction [14], consequently altering the bone remodeling process, and thus implicating the FGF axis in the continuous progression of PCa in the bone [3]. However, the specific mechanism by which FGFR1 mediates PCa growth in bone remains poorly understood. The goal of this aim is to assess the effect of FGFR1 isoforms on the ability of PCa cells to spread and grow in bone. We hypothesize that FGFR1 accelerates the bone metastatic phenotype of PCa cells, which is orchestrated by the contribution of both isoforms. We will overexpress FGFR1 alpha and beta in PC3 and C4-2B PCa cell lines (which express very low levels of FGFR1). We will use PCA cells transfected with empty vector as control and will perform the following studies: (a) intracardiac injection to assess the influence of FGFR1 in the metastatic spread of PCa cells; (b) intrabone injection to study the evolution of metastatic lesions impacted by FGFR1 and the response to FGFR1 inhibition; and (c) co-culture studies using Boyden-type chamber system to define the involvement of the FGF axis in the interaction with the bone cells. Achievement of these results will aid elucidate the effects of FGFR1 in metastatic activity to better apply the established axis blockade in therapy.

Preliminary data supporting this aim. Survival of mice was significantly reduced after intracardiac injection of PCa cells expressing FGFR1. In a pilot study, we found that male SCID mice injected intracardially with PC3 cells expressing FGFR1 alpha (sub-aim 2a) exhibited higher death rates compared to mice injected with PC3 control empty vector cells (Fig 6). These results thus suggest that FGFR1 alpha accelerates the aggressive phenotype of PCa cells, suspiciously due to higher bone metastases.

Experimental Design. (a) To evaluate the metastatic dissemination of PCa cells mediated by FGFR1 isoforms in vivo. To assess whether FGFR1 expression favors the metastases of PCa cells to bone in vivo, C4-2B cells expressing luciferase (established by lentiviral transduction) and FGFR1 isoforms or empty vector will be injected intracardially into male immunodeficient SCID mice to assess the effect of FGFR1 isoforms in the generation of PCA bone metastases. Since metastases of C4-2B to bone (which are mixed osteoblastic-osteolytic) are not easily identified by X-ray analyses, bone metastases will be monitored by quantification of bioluminescence using the IVIS imaging system. At the end of the study we will perform X-ray analyses to determine whether there is a change in the PCA induced bone reaction. We will subsequently perform morphological analyses of bone tumors. Findings will be confirmed by using PC3 cells expressing FGFR1 isoforms or empty vector, in this case as PC3 is osteolytic, we would monitor bone metastases as osteolytic lesions by X-ray analyses. We will use 12 mice per cell line.

(b) To evaluate the induction of PCa growth in bone mediated by FGFR1 isoforms and response of FGFR1 isoforms to treatment with a specific Pan-FGFR inhibitor. To assess tumor growth in the bone of PCa cells expressing FGFR1 isoforms and response to FGFR blockade, we will test the effect of C4-2B stably expressing FGFR1 alpha, beta and empty vector growing in the right femur of male SCID mice. Left legs will serve as sham-injected non-tumor bearing controls. In order to evaluate markers of FGF signaling under FGFR1 inhibition, we will use a tyrosine kinase inhibitor with activity against FGFRs (Janssen Pharmaceutical Companies of Johnson&Johnson), JNJ-42756493 (JNJ), which we have identified to have antitumor activity in prostate tumors
expressing FGFR1. JNJ half-maximal inhibitory concentration values are in the low nanomolar range for all members of the FGFR family (FGFR1 to FGFR4), with minimal activity on vascular endothelial growth factor receptor (VEGFR) kinases compared with FGFR kinases (approximately 20-fold potency difference). Following protocols established in our laboratory [3], we will use 6 mice per line (C4-2B stably expressing FGFR1 alpha, beta and empty vector) and per treatment, JNJ or vehicle. We will study the growth of PCa cells in bone by X-ray analyses at different time points and by Magnetic resonance imaging (MRI) at the end of the study to assess tumor volume. After mice are killed, we will dissect tumor bearing bones and study the specimens by high-resolution microCT, bone histomorphometry of undecalcified bone and by immunohistopathology of formalin fixed paraffin embedded tissue. We will assess bone mass, markers of apoptosis and proliferation and expression of FGFR1 downstream factors identified in sub-aim 1c. Regulation of FGFR1 isoform specific downstream target genes by treatment will be tested. When performing IHC, we will also see if FGFR1 is expressed in tumor associated osteoblasts, based on our previous findings [3]. Results will be confirmed using PC3 cells stably expressing FGFR1 alpha, beta and empty vector.

(c) To investigate the role of FGFR1 isoforms in the cross talk between PCa cells and bone cells. Using the mouse osteoblast precursor cell line MC3T3, we will investigate the effect of osteoblasts in PCa cells expressing FGFR1 and its isoforms by co-culturing these two cells types in vitro in a Boyden chamber-type system. We will assess proliferation, apoptosis, migration and signaling pathways activated by osteoblasts in PCa cells under co-culture.

For statistical considerations and analysis plan, all studies will be designed and evaluated in close interaction with our statistical support team (Dept. of Bioinformatics and Comp Biology). Results will be recorded qualitatively and quantitatively and will be expressed as mean ± SD. Two sample t tests will be used for analyses of quantitative data or appropriate tests accordingly. P values less than 0.05 will be considered statistically significant.

Expected results, potential pitfalls, and alternative approaches. We expect that results of studies proposed in sub-aim 2a will indicate whether FGFR1 or a specific FGFR1 isoform mediates the metastatic progression of PCa cells. We expect to find a direct correlation between FGFR1 expression and PCa cell aggressiveness. In sub-aim 2b, we expect to detect differences in the growth or in the effect in the bone mediated by FGFR1 isoforms. Our studies in sub-aim 2b will also help identify men susceptible to respond to FGFR blockade based on isoform expression. In sub-aim 2c we expect that cells expressing the isoforms will be more favored by the interaction with the bone, hence resulting in an increased effect in the parameters assessed when compared to control. Furthermore, we expect to isolate the individual contribution of each of the isoforms in the interaction with bone-forming cells. Altogether, the results from these experiments will help consider isoform activity for a more effective assessment of FGFR1-directed therapy.

Most of the techniques proposed in this specific aim have already been established in the laboratory or in the collaborator laboratories, and major technical difficulties are not anticipated.

As an alternative approach, the use of humanized bone models can be more representative to mimic specific mechanisms of the human disease. It has been tested already that cells injected intracardially disseminate into bone scaffolds implanted subcutaneously [42, 43]. The humanized tissue-engineered bone construct is used by Dr. Dondossola, a collaborator in our Dept. This could serve as an alternative approach to analyze the metastatic dissemination of PCa cells mediated by FGFR1 isoforms.

The use of these scaffolds can also be used as an alternative strategy to our proposed co-cultured studies to study the behavior of the isoforms by other approaches during its interaction with the bone. Other parameters can be assessed by this approach in a more preclinically relevant setting: tumor growth and invasion, reactive remodeling of the stroma and neovessel organization can be monitored by multiphoton microscopy, currently available in our collaborator’s lab in our Dept. (Dr. Dondossola/ Dr. Friedl) [44].

Finally, it is possible that the effect of co-culturing FGFR1-expressing PCa cells with bone cells is on the osteoblasts. We would analyze markers of proliferation and differentiation of osteoblasts, as previously performed in our lab [45, 46].

Conclusive statement. Altogether, an exhaustive analysis of the effects exerted by FGFR1 in PCa and the comprehension of the molecular mechanisms by which FGFR1 and its isoforms act, can contribute to more accurate therapeutic application of an established/developing treatment for this disease, in particular for the aggressive stage.
References


