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14. ABSTRACT (200 words)

In this fourth and final year of DoD funding we have been finalizing a peer-reviewed manuscript entitled "***SRC family kinase FYN promotes the neuroendocrine phenotype and visceral metastasis in advanced prostate cancer***". This manuscript is under review at *Oncotarget*. In this manuscript, we show that the loss of FYN abrogates the invasion of PC3 cells in response to the MET receptor ligand HGF. We also demonstrate that FYN contributes to the metastatic potential of NEPC cells in two mouse models of visceral metastasis. After the first submission of this manuscript, the reviewers expressed strong interest in our findings but they voiced a concern regarding the lack of data from a second prostate cancer cell line. During this past year, we have incorporated data from other prostate cancer lines including ARCaPm and LNCaP cells and we continue to be encouraged that FYN is an attractive therapeutic and diagnostic target in PC. Also, our current data provide further support for ongoing clinical trials of FYN and MET inhibitors in castration-resistant PCa patients. As for future research directions, we have begun to explore the role of FYN in PDL1 expression in metastatic prostate cancer and the manner in which this kinase might be involved in prostate CTC activation.

15. SUBJECT TERMS (key words or phrases identifying major concepts in the report)

Fyn, Src, Met, prostate cancer, metastasis, dissemination, motility

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I. INTRODUCTION

Fyn is a 59-kDa member of the Src family of kinases (SFKs). Src and its family members are dysregulated in prostate cancer (PCa) and other malignancies. Our group identified Fyn as the most upregulated in PCa and led us to investigate the role of this kinase in PCa. Our previous data resulted in the submission of this Idea award pointed toward the role of Fyn in directional cellular motility. The overall goal of this project is to test the underlying hypothesis that the up-regulation of Fyn enhances directional cellular motility thereby, increasing PC metastatic capacity and making it a relevant therapeutic target. These studies continue the work from our original CDMRP PCRP Physician Research Training Award by allowing them to evolve to the next level.

In our fourth and final year of funding, our group has continued to develop and characterize PC cell lines with the goal to understand the role of Fyn in prostate cancer, including a full set of PC3, ARCaPm, and DU145 based constructs. In addition to our data in PC3 cells, we observed that FYN regulates the motility and invasiveness of ARCaPm cells. Also, the activation of pMET in ARCaPm appears to be regulated by FYN. In contrast to PC3 cells, ARCaPm cells do not exhibit NE markers. At this time, a new scientist, Dr. Karen Cavassani, has joined our research group and she is exploring the direct correlation between FYN and NE marker expression. Ongoing experiments will involve the transgenic expression of FYN in an FYN negative PC line (DU145) in order to more directly explore this relationship and finalize our publication.

II. KEYWORDS

Fyn, Prostate Cancer, Metastasis, Neuroendocrine Prostate Cancer, Circulating Tumor Cells, Motility, Invasion

III. OVERALL PROJECT SUMMARY

SPECIFIC AIM 1. Quantify the impact of Fyn modulation on motility, directional velocity, and invasive capacity of prostate cancer cell lines *in vitro*.

A. Measure the impact of Fyn expression on directional motility and invasion in vitro.

B. Quantify the impact of alteration in Fyn expression on its signaling partners in vitro.

Summary: In multiple PCa models, Fyn strongly drives cellular motility. In those lines with higher native Fyn expression, this also drives chemotaxis[1]. Signaling partners identified range from skeletal assembly proteins to microRNAs which are now being studied in collaboration with Dr. Michael Freeman which will continue beyond the completion of this award.

SPECIFIC AIM 2. To test the hypothesis that Fyn expression correlates with dissemination to and colonization of secondary (metastatic) sites in experimental metastasis models.

A. Measure the impact of Fyn expression on tumor cell dissemination from a given tumor volume in vivo.

B. Quantify the change in end organ involvement related to decreased expression of Fyn after fixed tumor cell dissemination via intracardiac injection

As previously reported, the FYN overexpressing models we created did exhibit a greater disposition toward widespread metastasis. Interestingly, we found that the pattern of metastasis favored spread to the lungs, lymph nodes and soft tissues. In discussion with local and national collaborators, our findings distinct from what it have been reported from other studies. In fact, others have been showed that PC3 injection promotes lytic bone lesions, but not visceral lesions. These findings strongly led us to hypothesize that the overexpression of FYN in these cells was promoting behavior that mimicked neuroendocrine prostate cancer (NEPC). These findings prompted us to reconsider FYN in the setting of NEPC (in vivo data has been in the previous report).

Summary: These findings have strongly led us into studying the relationship between Fyn and circulating tumor cells in human prostate cancer. This has led me to form collaboration with Dr. Hsian-Rong Tseng at UCLA who has designed the NanoVelcro Chip that we are using to study live CTCs from patients. As part of our profiling of these CTCs we have designed a DeltaGene assay to quantify Fyn, Met, and other related molecular signals. This collaboration will continue beyond this Idea Award and has gained funding from the NCI through a NanoTechnology U01. We have also applied for an R01 as a collaborative unit that should be reviewed this fall. Finally, we are in the midst of preparing our initial studies for both a physical science in oncology U01 and a DoD PCRP Impact award.

SPECIFIC AIM 3. Determine alterations of Fyn pathway members in human CaP and correlate them with demographic, pathological and clinical outcome parameters.

Our previous studies identified that FYN expression as increased in PC although FYN kinase is typically associated exclusively with neuronal activity. This observation led us to hypothesize that FYN expression might be detectable in a subset of PCa with NE features. Accordingly, Huang and colleagues have reported that the PC3 cell line is a bona fide prostatic small cell carcinoma with NE features[2]. As such, we examined PC3 cells for FYN expression and observed that PC3 cells have greater expression of FYN compared to LNCaP cells (a more acinar or non-NE cell line) consistent with our previous reported observations. FYN expression correlated with the expression of markers of NE differentiation (**Figure 1**).

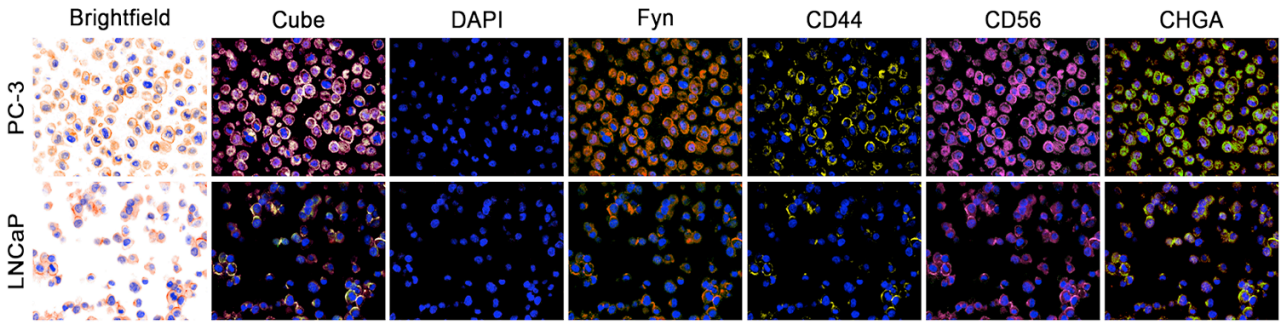

Figure 1. FYN kinase co-expressed with neuroendocrine biomarkers in PC cell lines. Analysis of NE markers (CD44, CD56, and CHGA) and Fyn in metastatic PC3 cells and the indolent LNCaP cells.

Next we studied the impact of FYN on the growth and invasive potential of PCa cells using Matrigel invasion assays. To perform these assays, we chose, ARCaPm cells, a metastatic PC cell line that expresses intermediate levels of FYN. For this set of experiments, ARCaPm cells were transduced with lentivirus with an shRNA targeted against *FYN* (Figure 2A). We first analyzed the role of FYN in the proliferation index of ARCaPm. The lack of FYN impairs the full ability of the cells to proliferate when compared with NT cells (intact FYN control) (Figure 2B), however no significant differences were found. In addition, we observed a decrease in invasive capacity in response to HGF-stimulation for the FYN depleted cells as compared to their corresponding controls (Figure 2C). Thus, we demonstrated that FYN activation regulated PC cell invasion not only in PC3 cells (as shown in the previous report) but in ARCaPm cells as well.

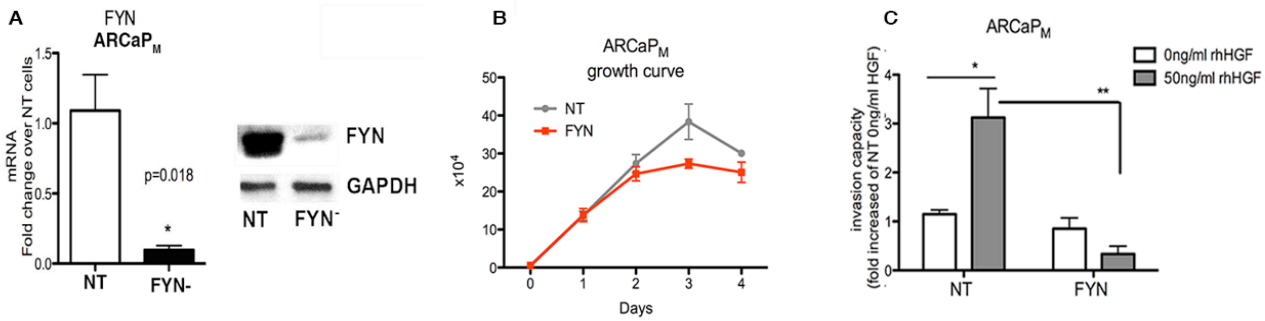

Figure 2. FYN promotes growth and invasion of ARCaPm cells. (A) RT-PCR and Immunoblotting assays of FYN expression in ARCaPm variants confirming decreased expression of this kinase in knockdown line. (B) Proliferation index of NT and FYN- cells were analyzed during four days of culture. (C) Matrigel invasion assays of ARCaPm NT and ARCaPm FYN- at 48 hours post-stimulation with or without 50 ng/ml of rhHGF. *p<0.05 when NT cells stimulated with HGF was compared with no HGF; **p<0.01, when FYN- cells were compared with NT (both groups under HGF stimulation).

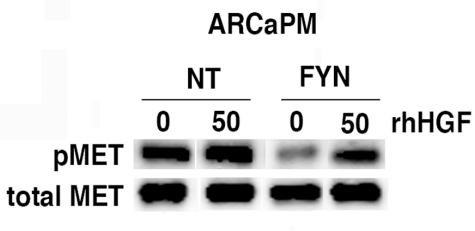

Figure 3. Western blotting analysis shows the phospho-MET profile of ARCaPm NT and FYN- cells after 20 minutes of HGF stimulation.

Our previous studies showed that in the PC3 cell line, the FYN kinase was capable of activating MET- am important pro-oncogenic, pro-metastatic signal in PCa[3-6]. In characterizing the relationship between FYN and MET using FYN-manipulated ARCaPm cells, we found that FYN knockdown suppressed MET activation and the phosphorylation of MET was restored in the presence of HGF (Figure 3A). We propose that FYN has the capacity to regulate MET activation, not exclusively in PC3 cells.

Trying to further characterize the behavior we have seen *in* vivo with this Fyn-associated visceral metastatic behavior, we pursued characterizing a relationship with clinical markers of NEPC. IWe observed an increase in the expression of NE markers including CHGA, CHGB, SYP and SCG3 in PC3 PCa cells

upon stimulation with HGF suggesting possible FYN/MET interaction in regulation of NE markers. On the contrary, results from mRNA and protein levels show that ARCaPm cells did not up-regulate NE markers after HGF stimulation, and the knockdown of FYN did not change the expression of these markers (Figure 4). At this moment, the direct correlation of FYN and NE marker expression is still under investigation by our group, and one experiment will involve the transgenic expression of FYN in an FYN negative PC line (DU145) in order to more directly explore this relationship.

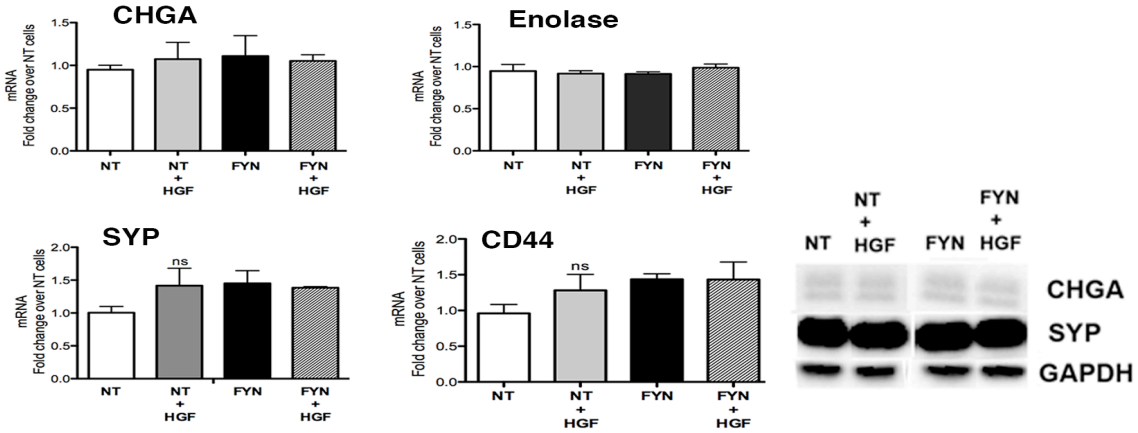

Figure 4. FYN/MET signaling does not regulates NE markers in ARCaPm cells. ARCaPm NT and FYN- were fasted and then stimulated with or without 50ng/ml of rhHGF during 10min. Analysis of the NE expression were performed using RT-PCR and Western blot.

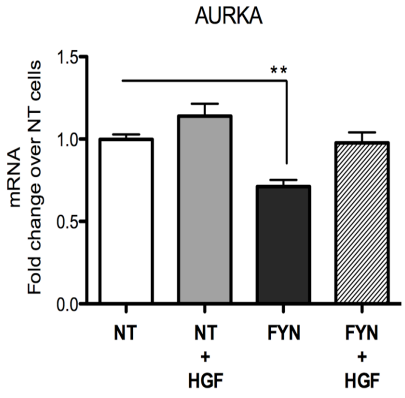

Figure 5. FYN activation regulates the expression of AURKA in ARCaPm cells. RT-PCR analysis of ARCaPm NT and FYN- cells in the presence or absence of 50ng/ml of rhHGF. **p<0.01, when NT cells were compared with FYN- cells.

AURKA has been showed to be amplified in 40% of NEPCs analyzed compared with only 4% of prostate adenocarcinoma[7-10]. We found that the knockdown of FYN expression on ARCaPm significantly reduced the expression of this gene (Figure 5), strongly suggesting that FYN may regulate NE features in ARCaPm but it will be necessary further experiments to compared NE markers in different cell lines constructs.

Summary: These findings have encouraged us to pursue the role of Fyn in NEPC and in patients with visceral metastases. This work will now exceed the scope of the idea award, but have become the core of an R01 application and will be leveraged in a CSMC P01 application on the topic of liver metastasis and the DoD PCRPC Impact Award application for 2015.

IV. KEY RESEARCH ACCOMPLISHMENTS

1. Awarded Grant: NCI U01CA198900-01 (started 8/15/15)- THERMORESPONSIVE NANOVELCRO CTC PURIFICATION SYSTEM FOR PROSTATE CANCER PROFILING (Role: co-PI)
2. Awarded Grant: NCI 2P01CA098912-11 (started 3/15/15)- PROSTATE CANCER BONE METASTASIS BIOLOGY AND TARGETING (Role: co-investigator)
3. Awarded Grant: Steven Spielberg Family Foundation (started 9/1/2013)- THE ECOSYSTEM OF LETHA PROSTATE CANCER (role: co-investigator)
4. Award Grant: Margaret Early Family Trust (started 12/1/14)- UNTANGLING MITOCHONTRIAL MAOA AND NUCLEAR AR COMMUNICATION (Role: co-investigator)

V. CONCLUSION

Fyn remains an interesting and putative target prostate cancer but may be relevant in certain subtypes of prostate cancer. From our work, the most likely clinical subset would be those cases of mCRPC with NE features or those with a disposition to visceral metastases. Profiling of the disease from cell line and xenografts models may not be as relevant to human disease, thus we are continuing to move into profiling of human cancers using circulating tumor cells as tissue source for study. As a clean subset is identified, it may be appropriate to revisit the use of Fyn and other SFK inhibitors in the clinic. Given the existing data with dasatinib[11-13] and our experience with saracatinib (see appendix), we would hesitate to move too quickly into single agent SFK inhibitor studies even using an approach like the randomized discontinuation design used in our phase 2 study of saracatinib. Currently my clinical program is studying the role of cabozantinib in mCRPC with VM[14-16]. The inhibition of MET in these patients may be particularly important as we would speculate that these patients have increased FYN expression driving their VM and hence over activation of MET.

We also believe that it may be important to identify an optimal partner for therapy. From the existing literature, it seems that taxane-based chemotherapy is not an optimal partner[11,17-19]. Rather, we propose that aligned signals such as Met be considered in light of emerging agents and strategies.

VI. PUBLICATIONS, ABSTRACTS AND PRESENTATIONS

Peer-reviewed publications

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Presentations

1. Prostate Cancer Cure: How to do we get there? Healthcare College- Board of Governors, Cedars-Sinai Medical Center- May 3, 2011
2. Targeted Therapy in Prostate Cancer- April 25, 2011
3. Advanced In Renal Cell Carcinoma- June 9, 2011
4. Management of high-risk prostate cancer, ASTRO 2011 August 5, 2011 Miami, FL
5. Updates in Systemic Therapy for Prostate Cancer. Pathology Grand Rounds, Cedars-Sinai Medical Center, August 26, 2011
6. A patient with metastatic-castrate resistant prostate cancer treated with cabozantinib. Expert Forum on MET (CTEP supported). October 21, 2011.
7. Prostate Cancer Therapy in 2011. Medicine Grand Rounds, CSMC 2011
8. Multidisciplinary care in prostate cancer: the medical oncologists perspective. CSMC Multi-disciplinary Cancer Management Program. Spring 2012.
9. Clinical Trials vs. Standard Care: Exploring New Approaches in Cancer Treatment. ACS Doc Talks. September 18, 2012
10. Prostate Cancer Care in the Elderly. First Annual Symposium on the Treatment of Elderly Patients with Cancer. BioMedical Learning Institute. Los Angeles. October 13, 2012.
11. Therapeutic Advances in Prostate, Kidney, and Bladder Cancer. New Therapeutics in Oncology: The Road to Personalized Medicine. CSMC. October 2012.
12. Advances in Systemic Therapy for Prostate and Kidney Cancer. Special Lecture/Heme-Onc Grand Rounds- Siriraj Hopital/Mahidol University, Bangkok, Thailand- Sept 5, 2013
13. Targeting the c-MET pathway: Clinical Applications. New Therapeutics in Oncology: The Road to Personalized Medicine. CSMC November 9, 2013
14. Management of Testicular Cancer in 2013. Hematology Oncology Grand Rounds. SOCCI/CSMC November 18, 2013
15. CTC technology in solid tumors. 10th World Congress on Urologic Research. Society of Basic Urologic Research / European Society for Urologic Research. Nashville, TN. November 21, 2013
16. Advances in Detection and Treatment of Prostate Kidney, and Bladder Cancer. Healthcare Innovations Conference. Guam Medical Association. Guam. October 5, 2013
17. Molecular analysis of circulating tumor cells in prostate cancer: moving toward a liquid biopsy and personalized medicine. UCLA Mathematics Forum. February 10, 2014
18. Targeting metastasis in prostate cancer: Fyn and circulating tumor cells. Grand Rounds, City of Hope. April 8, 2014
19. Clinical perspectives in Translational Research. CSMC-Clinical Translational Research Workshop, July 2, 2014
20. Early Detection and Management of Prostate Cancer. Guam Medical Association. October 18, 2014
21. Advances in Prostate Cancer therapy. XY Congress - Male and Female Cancer Research and Drug. November 6, 2014
22. Prostate Cancer in African-American Men: What You Need to Know. Holman Methodist Church Men's Health Forum. November 8, 2014

23. Targeted therapy in Renal Cell Carcinoma. Kidney Cancer Association. February 7 ,2015
24. Multidisciplinary Care in Prostate Cancer. Huntington Hospital. March 3, 2015
25. Immunotherapy of Genitourinary Malignancies. Advances in Cancer Immunotherapy- Society of Immunologic Therapy for Cancer. June 19, 2015
26. Circulating tumor cell nuclear sizes predict visceral metastasis in prostate cancer patients. Urologic Association of Asia/ Chinese Urologic Association Annual Meeting. Shanghai, China. Sept 5, 2015

VII. INVENTIONS, PATENTS AND LICENSES

Provisional patent application: NanoVelcro vsnCTC assay for visceral metastases (filed August 2015)

VIII. REPORTABLE OUTCOMES

See above

IX. OTHER ACHIEVEMENTS

Posadas Lab Trainees:

- Karen A Cavassani, PhD (2015- current)- Project Scientist
- Rafi Ahmed, MD (2013-2015)- Medical Oncologist, Kaiser Permanente
- Richard Huynh, MD (2015- Current)- Medical Oncology Fellow, Cedars-Sinai Medical Center
- Jeff Chen, MD- Post-doctoral fellow (2014-current)
- Yi-Tsung (John) Lu, MD – Post Doctoral Researcher, Cedars Sinai Medical Center (2012-2014); Residency: internal Medicine- Cook County Hospitals, Chicago, IL
- Shawn Wagner, PhD- (2014-current) Faculty translational mentor
- Elizabeth Kaufman (2014- current)- medical student University of Southern California
- Julie Yang (2012- Current)- PhD candidate- Cancer Biology, CSMC
- Jake Lichterman (2012-2013)- DO candidate- New York Osteopathic Medical College
- Elisabeth Hodara (2014- current)
- Shaleekha Sharma (2012-current)
- Justin Levy - High School Student (Summer 2012)
- Eric Brunner- High school student mentee (2014)- Undergraduate- University of Chicago
- Christian Vazquez- High school student mentee (2014)- Undergraduate- Harvard University

Promotions

- University of California, Los Angeles: Health Sciences Clinical Associate Professor (7/1/14)
- Cedars Sinai Medical Center: Associate Professor (7/1/14)

Appointments

- Medical Staff Leadership Program. Cedars-Sinai Medical Center (2011-2012)
- Co-Medical Director: Urologic Oncology Center of Excellence- Cedars-Sinai Medical Center (2011-2014)
- Cancer Quality Committee Member- Cedars Sinai Medical Center (2011-2014)
- Protocol Review and Monitoring Committee Member (2011-2014)

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XI. APPENDICES

A. Manuscript: Posadas, BJUI 2009- Fyn overexpression in PCa
B. Manuscript: Saito, Cancer 2010- Fyn biology in cancer
C. Manuscript: Jensen, Clin Ca Res 2011- Fyn and PCa cell motility
D. Draft Manuscript: Cavassani, Oncotarget submission- Fyn and the NEPC phenotype
E. Draft Manuscript: Ahmed, Prostate submission- Saractinib as a metastasis inhibitor

BJUI BJU INTERNATIONAL

FYN is overexpressed in human prostate cancer

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Accepted for publication 17 June 2008

Study Type – Aetiology (case control)
Level of Evidence 3b

OBJECTIVE

To test the hypothesis that *FYN*, a member of the SRC family of kinases (SFKs), is up-regulated in prostate cancer, as *FYN* is functionally distinct from other SFKs, and interacts with FAK and paxillin (PXN), regulators of cell morphology and motility.

MATERIALS AND METHODS

Through data-mining in Oncomine (http://www.oncomine.org), cell-line profiling with immunoblotting, quantitative reverse transcription and polymerase chain reaction (RT-PCR) and immunohistochemical analysis, we described *FYN* expression in prostate cancer. The analysis included 32 cases of prostate cancer, nine of prostatic intraepithelial neoplasia (PIN) and 19 normal prostates. Samples were scored for the percentage of stained glands and intensity of staining (from 0 to 3). Each sample was assigned a composite score generated by multiplying percentage and intensity.

RESULTS

Data-mining showed an eight times greater *FYN* expression in prostate cancer than in normal tissue; this was specific to *FYN* and not present for other SFKs. Expression of *FYN* in prostate cancer cell lines (LNCaP, 22Rv1, PC3, DuPro) was detected using quantitative RT-PCR and immunoblotting. Expression of *FYN* and its signalling partners FAK and PXN was detected in human tissue. Comparing normal with cancer samples, there was a 2.1-fold increase in median composite score for FYN ($P < 0.001$) 1.7-fold increase in FAK ($P < 0.001$), and a doubling in PXN ($P < 0.05$). There was a 1.7-fold increase in FYN ($P < 0.05$) and a 1.6-fold increase in FAK ($P < 0.01$) in cancer compared with PIN.

CONCLUSIONS

These studies support the hypothesis that *FYN* and its related signalling partners are up-regulated in prostate cancer, and support further investigation into the role of the FYN as a therapeutic target.

KEYWORDS

FYN, SRC, prostate cancer, paxillin, FAK

INTRODUCTION

Prostate cancer is the most common cancer affecting American men, accounting for >200 000 new cases of cancer diagnosed in 2008 [1]. While many men have disease that is either amenable to local therapy (surgery or radiation), many will develop metastatic disease. It is this population that is at risk of morbidity and death from both the disease and treatment-related side-effects, such as osteoporosis or cardiovascular events. Despite advances in therapy, >30 000 men are expected to die in 2008 from this disease. These figures have driven an aggressive search for promising molecular targets in prostate cancer. Castration is a highly effective and widely used therapy for men with this disease, but most patients will progress to a castration-resistant state. This progression is associated with increased morbidity and mortality rates. At present only docetaxel-based chemotherapy has been shown to extend survival for this population of patients. Thus, many therapeutic targets have been proposed and explored. Tyrosine kinases are known to be dysregulated in prostate cancer, and as clinically useable agents have become available, several of these have been studied in prostate cancer, including the epidermal growth factor (EGF) receptor, vascular endothelial growth factor receptor, and B/C raf-kinase, none of which have yet shown significant clinical efficacy. Gene expression profiling of non-receptor tyrosine kinases in prostate cancer has shown that the SRC family is particularly dysregulated in prostate cancer [2].

The SRC-family of kinases (SFKs) is one of the most studied families of proteins in cancer biology. Since the identification and description of the pp60c-SRC, eight other proteins sharing significant structural homology have been identified. The SFKs have long been recognized as overexpressed in several cancers, including prostate cancer. Each member is distinguished by a unique region that specifies its respective binding partners and hence function.

FYN is a 59-kDa member of this family and was one of the first members to be identified. The gene encoding *FYN* is located on chromosome 6q21. The most abundant transcript encodes a protein composed of 537 amino acids with a structure similar to the other SFKs, except for the unique region. Like

other SFKs, FYN is a non-receptor tyrosine kinase that functions downstream of several cell-surface receptors. Its best characterized functions are in neuronal development and T-cell signalling [3], but FYN also induces morphogenic transformation when overexpressed [4]. FYN is recognized as an important mediator of mitogenic signals and as a regulator of cell cycle entry, growth and proliferation. It is also known to mediate integrin interactions and hence cell-cell adhesion. FYN is known to interact with several molecular signals including FAK and paxillin (PXN) [5,6] which might account for the described morphogenic transformation and possibly lend insight into its role in cancer.

In this report we present the first series of studies showing the specific importance of FYN in prostate cancer. Our approach used a combination of both data-mining and tissue microarray (TMA) immunohistochemical (IHC) analysis, showing overexpression of FYN in human prostate cancer.

MATERIALS AND METHODS

The expression of *FYN* in prostate cancer was queried using the Oncomine database (http://www.oncomine.org) in February 2008. This is a publicly available database summarizing gene-chip experiments across tissue types [7]. Oncomine provides an infrastructure of data-mining tools to query genes and data sets of interest, and to meta-analyse groups of studies. This database was queried for gene expression data for *FYN, SRC, YES, BLK, LCK, FGR, LYN, HCK,* and *YRK.* Studies were included if they compared primary prostate cancers to any of the following: normal or benign epithelium, metastatic prostate cancer, prostatic intraepithelial neoplasia (PIN), BPH or hormone-refractory prostate cancer. The *P* values presented are extracted directly from the Oncomine analysis and have not been repeated manually.

All cell lines used were obtained from the American Type Culture Collection (Manassas, VA, USA). Lines used included standard prostate cancer cell lines: LNCaP, CWR22Rv1, PC3, and DuPro; U87 are malignant astrocytes that were used as a positive control for FYN [8]. Cells were grown according to the supplier's recommendations, in RPMI 1640 with 10% fetal calf serum and penicillin/streptomycin supplement.

All human tissue samples used in the study were obtained from the University of Michigan through an interSPORE collaboration. The use of tissue complied with an institutional review board-approved protocol requiring that all samples were kept anonymous to the primary investigational team.

Tissue was analysed in the form of a TMA, the fabrication of which was described by the University of Michigan group elsewhere [9]. In short, the initial TMA used contained 120 patient specimens planned to have triplicate representation on the TMA; each element was 0.6 mm in diameter. Tissue samples included primary tumour from patients with prostate cancer, with Gleason 6–9 disease, metastatic tumour sites, PIN, proliferative inflammatory atrophy, BPH, prostatic stroma and normal prostate tissue. The identity of patients was withheld from the primary analytical group. Normal glands present on the TMA were taken from patients who had prostatectomy or cystectomy. A patient's sample was only considered useable if represented at least twice on the array.

Commercially available antibodies were used for all immunoblotting and IHC studies. Anti-FYN was obtained from Millipore (Burlington, MA, USA); Anti-FAK was obtained from Invitrogen (Carlsbad, CA, USA); and anti-PXN antibody 5H11 was obtained from Biosource (Invitrogen).

For protein extraction and Western blotting, monolayer cells were grown to 80% confluence then washed in ice-cold PBS. Protein lysates were prepared using lysis buffer (10 mmol/L Tris, pH 7.5, 1 mmol/L β-glycerophosphate, 2 mmol/L DDT, 1 mmol/L EDTA, 150 mmol/L NaCl, 0.5 mmol/L NaF, 2 mmol/L NaVO4, 0.1% NP40, 10 μmol/L phenylmethylsulphonyl fluoride, 1% Triton X-100 w/v, 70 units/mL aprotinin, and one Complete Protease Inhibitor Cocktail tablet, Roche, Basel, Switzerland). Cells were scraped and placed on ice after being passed through a 27-G needle and subsequently centrifuged at 11 000 ***g***. Protein was quantified using the bicinchoninic acid protein assay kit (Pierce, Rockford, IL, USA); 20 μg of protein were subjected to SDS-PAGE and transferred to a HyBond Enhanced Chemiluminescence nitrocellulose membrane (GE Healthcare Biosciences, Piscataway, NJ, USA).

For Western blotting, membranes were blocked at 4 °C overnight in TBS-Tween plus 5% (w/v) non-fat dried milk. After incubation with each antibody diluted in blocking solution for 1 h, the membrane was washed for 10 min in blocking solution and then washed six times for 5 min each in TBS-T. The horseradish peroxidase-conjugated secondary antibody was detected using the Super Signal West Femto Maximum Sensitivity Chemiluminescence Substrate (Pierce) according to the manufacturer's directions. Probed membranes were stripped using Pierce Restore Western Blot Stripping Buffer, washed in TBS-T, and blocked overnight before re-probing. The dilutions of antibodies were: anti-FYN 1:1000, anti FAK 1:1000, anti-PXN 1:500. As a loading control, membranes were probed for actin followed by incubation with a goal antimouse IgM-peroxidase-conjugated secondary antibody (Oncogene Research, Uniondale, NY, USA; 1:20 000 and 1:40 000 dilutions of primary and secondary antibodies, respectively).

RNA from cell lines was extracted using an RNAqueous kit (Ambion, Auton, TX, USA) according to the manufacturer's recommendations. Samples were stored at −80 °C until processed. Customized primers for *FYN* were prepared by Integrated DNA Technologies (Coralville, IA, USA). The left primer was: ATG GAA ACA CAA AAG TAG CCA TAA A; and the right primer: TCT GTG AGT AAG ATT CCA AAA GAC C. Data were calibrated to the expression of glyceraldehyde phosphate dehydrogenase. Quantitative PCR was performed using SYBR Green dye on an ABI 7700 (Applied Biosystems, Foster City, CA, USA).

For IHC, stained TMA sections were analysed by a dedicated urological pathologist (H.A.A.) while unaware of sample origin. Results were reported semiquantitatively on a scale of 0–3 for intensity, where 0 was negative, 1 was weak, 2 was moderate and 3 was strong. The percentage of tumour staining was reported as 0–100% in increments of 10%. A composite score was formed using the product of the intensity and percentage of glands staining. Human breast cancer tissue was used as a positive staining control, as recommended by the manufacturer [10]. Human leiomyomas were used as a negative control. FYN was stained using an antibody concentration of 1:50; FAK at 1:100; and paxillin at 1:100.

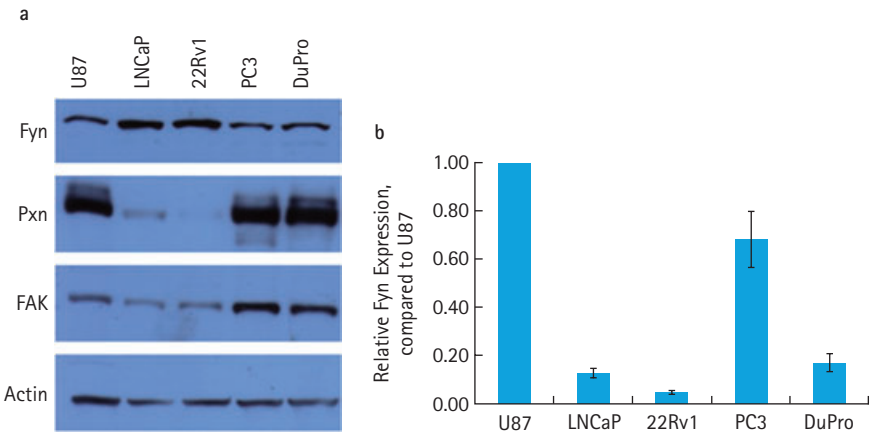
TABLE 1 Primary FYN antibodies tested, with the results

Manufacturer, id#	Results
Cell Signalling, #4023	Several high molecular weight bands seen in addition to FYN
Abcam, ab32022	Single band ≈59 kDa, but on IHC predominantly nuclear staining.
Upstate, 04-353	Single band at 59 kDa. Cytoplasmic staining on IHC.
Chemicon, MAB8900	No bands seen
Santa Cruz, SC-16	Strong band at 59 kDa but several high molecular weight bands, not specific for IHC

TABLE 2 The patient demographics for FYN analysis

Variable	N or median (range)
Total useable patient samples	86
Tumour	32
Gleason 3 + 3	6
Gleason 3 + 4	8
Gleason 4 + 3	3
Gleason 4 + 4	8
Gleason 4 + 5	7
Metastases (all sites)	10
BPH	8
PIN	9
Normal prostate	19
Age, years	64 (43–76)
Race	
Caucasian	50
African descent	2
Other/unknown	34

FIG. 1. Expression of FYN and signalling partners FAK and PXN in prostate cancer cell lines shown by (a) immunoblotting and (b) quantitative RT-PCR. U87 cells (malignant astrocytes) were used as positive control for FYN expression.



To analyse the TMA data, ANOVA was used to compare expression levels (based on the percentage staining or the composite score) across groups. The equal-variance assumption was verified using Bartlett's test [11]. Post-hoc pair-wise comparisons were performed with a Bonferroni adjustment for multiple comparisons. The Kruskal–Wallis test was used to compare the ordinal staining intensity score. Also, a nonparametric trend test [12] was used for further examination of expression levels across the naturally ordered groups. The mean of the duplicate or triplicate samples for each subject was used in the analysis. Statistical significance was indicated at $P < 0.05$.

RESULTS

To identify SFKs for analysis, we reviewed available studies in the Oncomine database. On comparing malignant with normal prostate epithelium, the member of this family that arose as the most consistently and strongly overexpressed was *FYN*, which was eight times greater in cancer ($P < 0.001$) [13]. There was little or no change in the remainder of the SFKs, including *LYN, YES, HCK* and *FGR*. The overexpression of *FYN* further increased by 10 times in the transition from localized to metastatic cancers, while other SFKs were either down-regulated (*HCK, LCK*) or showed no significant changes in expression (*LYN, YES, BLK*, or *SRC*) [14].

FYN was chosen for further investigation as it was identified as the most up-regulated SFK in prostate cancer. Given the homology of the various members of the family, several antibodies were tested and eliminated on the basis of sensitivity and specificity (supplemental data, Table 1). The expression of FYN was evaluated in standard prostate cancer cell lines (Fig. 1a, top). The U87 cell line was used as a positive control, as malignant astrocytes are known to express FYN [8]. Findings were verified by quantitative reverse transcription-PCR (Fig. 1b). There was expression of FYN RNA and protein in all tested cell lines. FYN was not expressed in human leiomyoma samples (immunoblot-verified negative control; data not shown).

We then verified the Oncomine findings in human tissue samples using IHC analysis of a TMA obtained from the University of Michigan, that contained samples of normal prostate, PIN and prostate cancer. There were 86 useable patient samples for the FYN analysis (Table 2). We stained the TMA for total FYN (Fig. 2) and analysed by generating a composite score from the percentage of tumour cells staining and intensity. Several candidate antibodies were tested and discarded (Table 1) if they failed to show sensitivity and specificity to tumour tissues and expected positive control (e.g. lymphocytes) or if the pattern of staining did not correlate with the biology of FYN. For example, an antibody showing predominantly

FIG. 2. Expression of FYN, FAK, and PXN in malignant and non-malignant prostate epithelium. Representative photomicrographs of sections of malignant and non-malignant prostate epithelium.

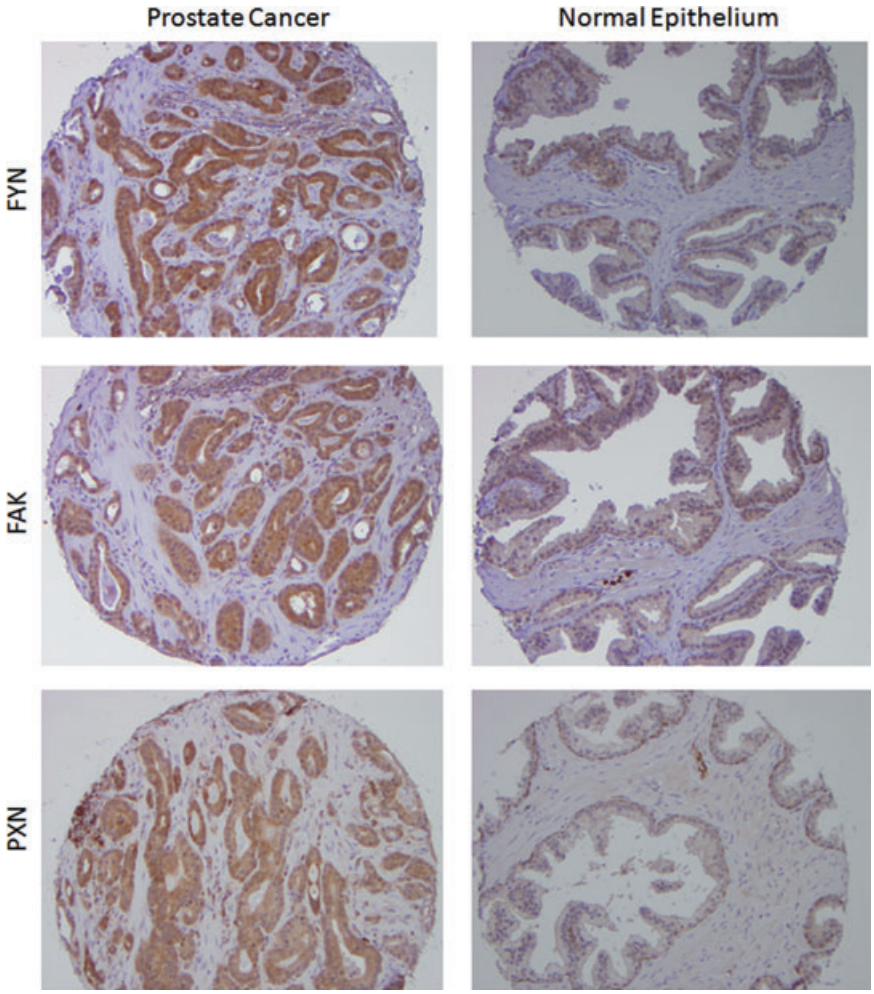
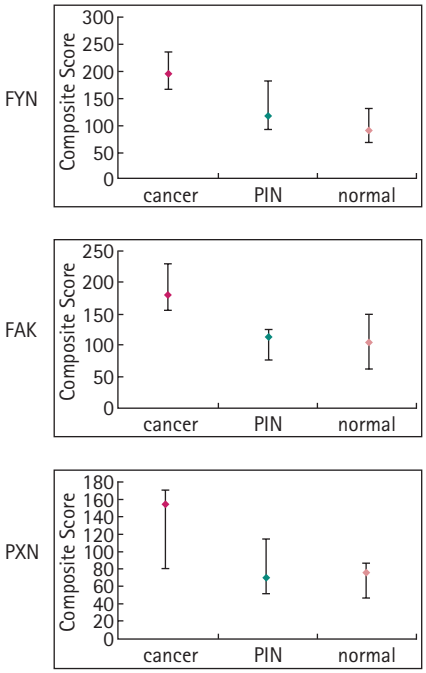


FIG. 3. Plots of (A) FYN (B) FAK and (C) PXN staining in malignant vs non-malignant tissue samples. Composite scores (intensity of staining × percentage of glandular cells staining) are shown on the Y-axis. The median is plotted with the error bars representing the 25th and 75th percentiles.



nuclear staining in all samples was declared to be erroneous.

The median (range) composite score for cancer specimens was 200 (23–300); scores did not correlate significantly with Gleason score (data not shown). Of 32 tumour samples, 19 (59%) had scores of 200–300. For normal epithelium the median (range) score was 93 (7–160) and for PIN, 120 (45–220). Figure 3 shows the distribution of composite scores for FYN. Staining of FYN was strong in primary tumour samples compared with non-neoplastic tissue ($P < 0.001$ for the overall comparison). Differences in expression between normal and cancer and PIN and cancer were both statistically significant, based on the composite score. Specifically, there was a 2.1 times greater median composite score in cancer than normal ($P < 0.001$) and a 1.7 times greater score for FYN for cancer than PIN ($P = 0.03$). Furthermore, there was evidence for increasing expression levels across these three naturally ordered groups ($P < 0.001$ for trend). Ten metastatic tumours were represented from various sites (lymph node, lung, liver), with a median (range) scores of 102 (10–290). With so few samples it was not possible to confirm or deny the absence of a trend in FYN expression, but this merits further study.

As FYN interacts with several regulators of cellular morphology and attachment, cell lines and human tissue samples were re-examined for FAK and PXN. Immunoblotting showed co-expression of FAK and PXN with FYN (Fig. 1a, middle, bottom). Both were most highly expressed in the castrate-resistant cell lines (PC3 and DuPro) consistent with the data-mining presented earlier. Castrate-sensitive lines (LNCaP and 22Rv1) showed expression of both FAK and PXN but at a much lower level.

To extend the studies to clinical material, FAK and PXN expression was evaluated on the TMA. Representative sections stained for FAK and PXN are shown in Fig. 2 (middle and bottom). There were 35 useable tumour samples for FAK and 22 for PXN analysis. Our findings for the TMA population are represented graphically in Fig. 3 (middle and bottom).

The median (range) FAK score was 180 (40–300) in tumour samples. There was a tendency for higher Gleason tumours to have higher FAK scores, but this association was not statistically significant. Twelve of 35 (34%) samples had scores of 200–300. In normal epithelium the FAK score was 107 (53–253) and in PIN it was 113 (35–167). In the final analysis, there was 1.7 times greater FAK expression in cancer than normal tissue ($P < 0.001$) and 1.6 times in cancer than PIN

($P < 0.01$). The score in metastatic lesions was 140 (57–290).

The median (range) PXN score for tumour samples wa 155 (25–300), with no clear relationship with Gleason score. Only two of 22 (9%) useable specimens had PXN scores of 200–300 (285, 300). The score in normal prostate samples was 77 (25–160) and that for PIN 72 (40–150), but only four samples were available for analysis due to poor transfer. There was a doubling of PXN staining score in cancer over that in normal samples ($P < 0.05$). The few PIN specimens precluded any comparisons between PIN and cancer. These data indicate an up-regulation of FAK and PXN in prostate cancer compared with normal epithelium that correlates with FYN overexpression in cancer.

DISCUSSION

Through a combination of data-mining, immunoblotting and IHC we showed up-regulation of FYN, a particular member of the SRC family of kinases, in prostate cancer. The initial Oncomine queries suggested particularly high overexpression of FYN in cancer compared with normal prostate (non-neoplastic, non-hypertrophic) and *in situ* malignancy (PIN). There was expression of FYN in both a panel of prostate cancer cell lines and human tissue samples. This was accompanied by expression of the FYN signalling partners FAK and PXN, factors known to regulate cellular motility and metastasis. There were discrepancies between the magnitude of FYN measured by quantitative RT-PCR and immunoblot, but there are frequent published reports of discrepancies between RNA and protein expression. Specifically, FYN has been shown to undergo post-transcriptional modification which might affect protein expression [15].

The data-mining further suggested that this up-regulation of expression is specific to FYN and not the other members of the SRC family. While the SRC kinases share similarities in sequence and structure they have differences that might be germane to the development of SFK-directed therapies. Most SFK-directed research in cancer has been aimed at the expression of c-SRC. To date, the role of FYN in cancer biology is relatively unexplored. With >2300 citations in Pubmed referencing the role of SRC and SRC kinases in cancer, there are ≈200 studies mentioning FYN expression in various cancer models, only a few of which specifically focus on FYN biology. FYN has been implicated as a mediator of EGF-driven transformation of JB6 cells [16]. In breast cancer, FYN expression was shown to correlate with poorer survival, and correlated with FAK up-regulation [17]. In haematological malignancies, FYN has been identified as a putative target for treating BCR-ABL-expressing adult acute lymphoblastic leukaemia, due to the centrality of its relationship to several important molecular signals suspected to drive the proliferation of malignant leukaemic blasts [18] Compounds active against FYN have shown *in vitro* antiproliferative activity in acute lymphoblastic leukaemia [19]. In other solid tumours such as melanoma, FYN has been implicated as a mediator of integrin signalling, and thus appears to regulate metastatic potential [20].

Interestingly, there is a report of loss of *FYN* expression in prostate cancer [21]. This group recognized an allelic imbalance at 6q14–22 and sought to identify tumour suppressors associated with this region. They identified *FYN* as a potential tumour suppressor, noting that the highest levels of FYN were in BPH, compared with malignant tissues which showed little or no FYN expression. While the results appear to be contradictory, the present study does not specifically address the role of FYN in BPH. Members of the SRC family are known to have several different roles in various cellular contexts, and thus it is entirely possible that in one biochemical context FYN serves as a tumour suppressor, while in the altered biochemical landscape of neoplastic transformation (i.e. in the change from pre-invasive, to invasive, then again to metastatic) that FYN serves another role altogether. Further studies will be needed to show the biological role of *FYN* in these various settings. This type of dynamic signalling behaviour has been seen with other molecular targets (including proposed tumour suppressors) in the setting of prostate cancer [22]. Sørensen *et al.* [21] reported an immunohistochemical analysis similar to that presented here. The present results agree, insofar as there was expression of FYN in normal and hyperplastic epithelium. What requires reconciliation is the absence of FYN staining in tumour tissue as reported by Sørensen *et al.* In the present study, samples from all 32 patients with prostate cancer showed high levels of FYN expression. This might be the result of technical issues, such as the choice of antibody in the IHC results, as we found during our screening. Finally the study of Sørensen *et al.* suggested that by quantitative PCR there was attenuated expression of *FYN* in tumour samples from patients. The approach taken made use of whole-tissue homogenates, making the epithelial cell content difficult to control. This is especially important given the congruent findings of absent FYN expression in the stromal compartment.

FYN is positioned downstream of several important cell-surface receptors and upstream of several cellular signals important for prostate cancer progression. Like other SRC family members, it is known to mediate some cell-shape and migration behaviours. As such, its interactions with mediators of cell shape and motility were important factors to study. Our data also suggest that there is an accompanying up-regulation of FAK and PXN, both of which are important regulators of cell shape and interactions with other cells, and the extracellular matrix. Both FAK [23–26] and PXN [26,27] have been recognized as crucial to motility, and thus invasion, which are cellular processes required for metastatic competence and acquisition of the metastatic phenotype.

The expression of FAK and PXN in prostate cancer have been correlated with disease progression [26,28]. FAK has been shown to play a role in prostate cancer metastasis by disrupting integrin-mediated signalling from the extracellular matrix. The invasive ability of DU145 cells on fibronectin was inhibited by silencing FAK expression via siRNA [29]. SRC kinases have been implicated as potential means of modulating FAK activity in prostate cancer and SRC inhibitors have been shown to down-regulate FAK activation [23]. Overexpression of leupaxin, a member of the PXN family, was shown to cause an increase in cellular motility in PC3 cells [30]. Again, SRC kinase inhibitors have been shown to down-regulate the activation of PXN, which in turn results in decreased cellular motility [5]. Given the overexpression of FYN noted here, and the nonspecific nature of most SFK inhibitors, it is likely that the bulk of this effect is mediated by FYN.

These findings gain translational relevance with the introduction of SRC-family inhibitors into clinical practice. Dasatinib is commercially available for treating chronic myelogenous leukaemia, and is currently

being evaluated as a treatment for castrate-resistant prostate cancer. Other agents such as AZD0530 and bosutinib are currently in clinical development, with a host of others to follow. AZD0530, a potent SRC/ABL inhibitor, has been shown to have a potent effect on cellular motility which is SFK-mediated [31]. While labelled as inhibitors of c-SRC, these drugs are known to have various inhibitor effects on cellular tyrosine kinases, including FYN. Furthermore, several inhibitors of both FAK and PXN are currently under development. This raises the potential for combined approaches with these signal-transduction inhibitors in a vertical fashion, which might have potent effects on cellular motility and invasion. If relatively nontoxic, such an approach might be an effective treatment after definitive local therapy in concert with or after castration.

In conclusion, our findings show a statistically significant up-regulation of FYN and its signalling partners FAK and PXN through data-mining, immunoblotting and IHC. It is hoped that further understanding of the role of FYN in prostate cancer development and progression might provide insights into how FYN-inhibitory agents should be used in the clinic. Given our findings, we think that FYN is a promising molecular target for cancer therapeutics.

CONFLICT OF INTEREST

None declared. Source of funding: investigator-sponsored; charitable donations from private organizations.

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Abbreviations: **SFK**, SRC family kinase; **PXN**, paxillin; **TMA**, tissue microarray; **IHC**, immunohistochemical; **PIN**, prostatic intraepithelial neoplasia.

Review Article

Fyn

A Novel Molecular Target in Cancer

Yoshihito D. Saito, MD, MS[1]; Ana R. Jensen, BS[1]; Ravi Salgia, MD, PhD[1]; and Edwin M. Posadas, MD[1,2]

Fyn is 59-kDa member of the Src family of kinases that is historically associated with T-cell and neuronal signaling in development and normal cellular physiology. Whereas Src has been heavily studied in cancer, less attention has been traditionally awarded to the other Src kinases such as Fyn. Our group has shown that Fyn is particularly upregulated in prostate cancer in contrast to the alternative members of the Src family. This suggests that it may mediate several important processes attributed to Src kinases in prostate cancer and other malignancies. These functions include not only cellular growth and proliferation but also morphogenesis and cellular motility. Together, these suggest a role for Fyn in both progression and metastasis. As several agents in clinical development affect Fyn activation, understanding the role that Fyn plays in cancer is of great importance in oncology. ***Cancer*** **2010;116:1629–37.** *© 2010 American Cancer Society.*

KEYWORDS: Fyn, Src, integrin, FAK, paxillin, AKT, Ras, Erk, Rho, Rac, prostate cancer.

The field of cancer biology has made strides in identifying several molecular events and molecules critical to cancer progression. Tyrosine kinases are an important class of molecules in human biology and particularly relevant to the field of cancer research. Tyrosine kinases (TKs) fall broadly into 2 categories: receptor and nonreceptor TKs. Receptor TKs are membrane bound proteins that receive signals from soluble ligands. These include a variety of molecular targets such as the epidermal growth factor receptor (EGFR), vascular endothelial growth factor receptor (VEGFR), and mesenchymal epithelial transition factor (c-MET). Examples of nonreceptor TKs include members of the following families: Abl, Src, focal adhesion kinase, and the Janus kinase. When activated, these tyrosine kinases activate downstream molecular signals that drive processes crucial to growth and motility of cancer cells. Normally, activation of such molecules is tightly regulated.[1] In cancer, receptor and nonreceptor kinase activation is often dysregulated, leading to altered cellular growth, shape, and function hallmarks of malignancy.[2] Pharmacologic agents that are able to attenuate this uncontrolled signaling have long been pursued as cancer therapies.

Of the Src family kinases (SFKs), Src is the most studied and, hence, the most commonly discussed in cancer. However, there has been growing interest in the other SFKs in both physiological and pathological states. The role of Src in cancer is thoroughly reviewed in several publications and will not be reviewed here.[3] Instead, we will focus upon developments in understanding the role of Fyn in various biological processes such as cellular motility and morphogenesis. In addition, we will discuss the potential role of Fyn and SFK inhibitors in cancer therapy.

THE SRC FAMILY KINASES

Overview and history

The Src family kinases (SFKs) are among those nonreceptor TKs overexpressed in various cancers and have long been proposed as molecular targets for therapy.[4] The prototypical member of this family is c-Src ($pp60^{c\text{-}src}$)—the first discovered oncogene. c-Src was originally described by Rous in the early 1900s. Rous originally described a transforming factor present in tissue of sarcoma bearing chickens that drove the formation of tumors in normal chickens. Injection of a tissue homogenate made from tumor-bearing chickens allowed for transmission of this factor. This tissue factor was later known as the Rous Sarcoma Virus (containing *v-src*). In 1979, J. Michael Bishop and Harold Varmus discovered that normal

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Figure 1. General linear protein structure of Fyn and the Src-family members.

cellular Src (c-Src) had the potential to be altered in a fashion that allowed it to drive a cancerous phenotype. Their work in elucidating the mechanism of malignant transformation won them the Nobel Prize in medicine in 1989 and opened the field of oncogenesis. Subsequent proteomic studies led to the identification of other members of an entire family of proteins related to Src, collectively known as the SFKs. These members include Fyn, Src, Yes, Fgf, Lyn, Hck, Blk, Lck, and Yrk.[1] The features that identify each protein as a member of this family and that define each member's unique identity are discussed below.

SFK Structure and activation

These SFK proteins all share a common structure (Fig. 1) and pattern of activation.[1] The domains of these proteins include SH4, SH3, SH2, and SH1 (kinase) domains followed by a short C-terminal regulatory segment. The SH4 domain is the N-terminal domain and is often myristoylated or palmitoylated to allow for association with the cell membrane. A region known as the unique domain located within the N-terminus specifies the identity of each member of the family. This 60 amino-acid region contains the highest degree of variability and is thought to direct protein-protein binding interactions and, hence, function for each SFK.

The SH2 and SH3 domains are highly conserved regions that further mediate protein interactions: the SH2 domain binds phosphotyrosine residues with a general pYEEI sequence, while the SH3 domain recognizes PXXP-like sequences. Between the SH2 and SH1 domains is the SH2-kinase linker, which is a loop that functions as a pseudo-SH3-binding site. This domain contains a tyrosine residue (Y416) that is activated by autophosphorylation and is required for optimal activity.[5,6] The SH1 domain is the site of kinase activity. Following the SH1 domain is a C-terminal regulatory segment. Dephosphorylation of this tyrosine residue (Y527) leads to activation of SFKs via unmasking of an SH1 tyrosine, which is also regulated by phophorylation.

The genetic information encoding each member can be quite variable depending on the family member. However, the majority of the genetic and proteomic information is well-preserved with the exception of the unique region. The remainder of this review will focus on the structure, function, and role in cancer biology of a specific member of this family, Fyn.

FYN: A BRIEF INTRODUCTION

Gene and protein structure

Fyn (p59-FYN, Slk, Syn, MGC45350, Gene ID 2534) is a 59-kDa protein that comprises 537 amino acids whose genetic information is located on chromosome 6q21. Fyn is a member of the Src family originally identified in 1986 as Syn or Slk through probes derived from *v-yes* and *v-fgr*.[7,8] Fyn is primarily localized to the cytoplasmic leaflet of the plasma membrane, where it phosphorylates tyrosine residues on key targets involved in a variety of different signaling pathways.

There are 3 identified transcript isoforms of Fyn. Isoform 1 (isoform a, Fyn[B]) was the first identified and is the longest of the 3 genomic sequences. Isoform 2 (isoform b, Fyn[T]) tends to be expressed in T-cells and shows a greater ability to mobilize cytoplasmic calcium than isoform 1.[9] These 2 forms differ in the linker region between the SH2 and SH1 domains (exon 7A vs 7B), accounting for some of the differences in regulation between the 2 forms.[7] Isoform 2 differs from 1 by approximately 50 amino acids in the region near the end of the SH2 domain and the beginning of the kinase domain. Although most tissues express a mixture of the 2 isoforms,[9] Fyn(B) is highly expressed in the brain and Fyn(T) is highly expressed in T-cells. Isoform 3 (isoform c) lacking exon 7 (FynΔ7) has been reported. This form has been found in blood cells, but no translated protein has been documented.[10] Additional transcript variations

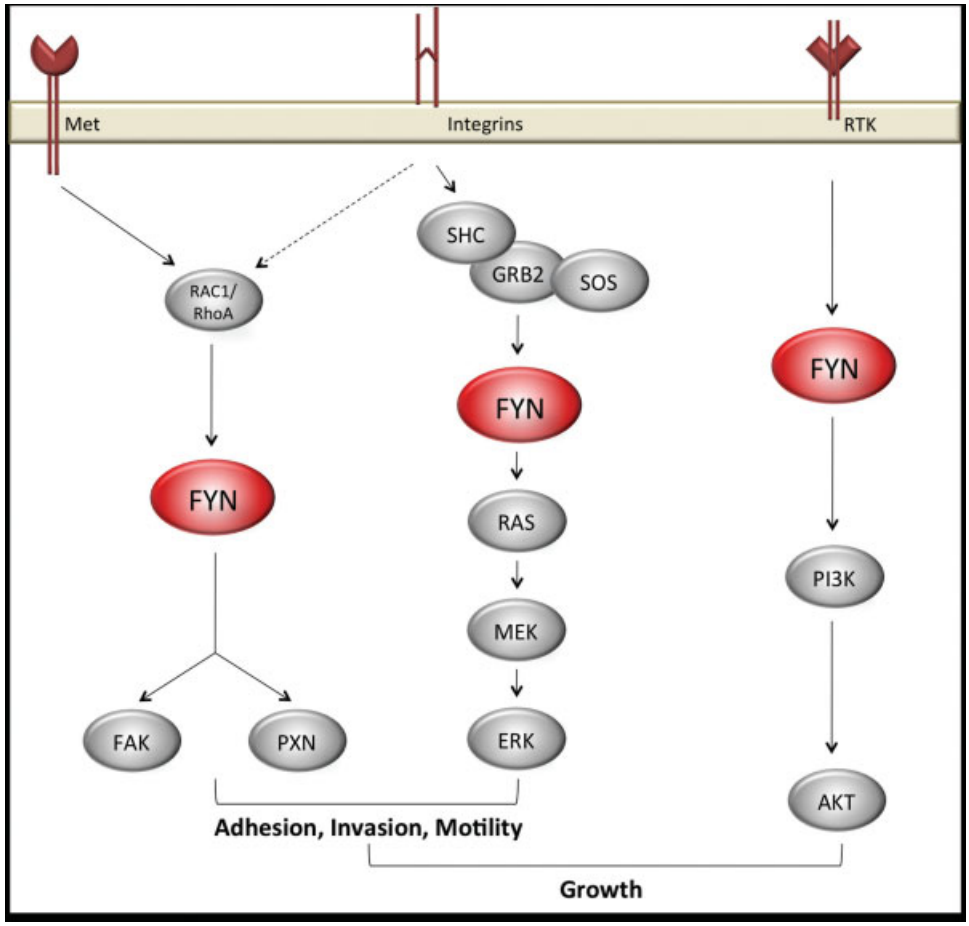

Figure 2. Fyn mediates signals from cell surface receptors to several critical growth and motility pathways.

have also been cataloged at this point but have not been associated with a diseased state.

The biological functions of Fyn are diverse (Table 1). Much of the initial work on Fyn centered on its role in immune and neurological function. However, Fyn has also been recognized as an important mediator of mitogenic signaling and regulator of cell cycle entry, growth and proliferation, integrin-mediated interactions, as well as cell-cell adhesion, as will be discussed below.

Table 1. Biological Functions of Fyn

Function
Growth factor and cytokine receptor signaling
Integrin-mediated signaling
Cell-cell adhesion
Ion channel function
Platelet activation
T-cell and B-cell receptor signaling
Axon guidance
Fertilization
Entry into mitosis
Differentiation of natural killer cells, oligodendrocytes, and keratinocytes

Fyn *in cancer*

Like Src, overexpression of Fyn has been shown to drive a morphologic transformation in normal cells. Overexpression of Fyn in NIH 3T3 fibroblast cells exhibited a cancer-like phenotype with increased anchorage-independent growth and prominent morphologic changes[11] *FYN* is overexpressed in various cancers, including glioblastoma multiformae, squamous cell carcinoma of the head and neck, and melanoma.[12] The role of FYN overexpression in these systems, however, has not been well-defined.

In addition, our group has shown through a combination of datamining, immunobloting, RT-PCR, and immunohistochemistry that FYN expression is

upregulated in the progression to cancer from both normal epithelium and prostate intraepithelial neoplasia (PIN).[13] In the datamining studies, alternative *SRC* kinases were explored. *LYN*, *FGR*, and *HCK* did not show consistent up-regulation in cancer versus normal. There was no significant difference in expression of *SRC* ($P = .056$, $P = .064$). *LCK*, *YES*, and *BLK* showed strong up-regulation in cancer compared with normal epithelium ($P = .00,018$; 0.00,016, 0.019, respectively) but to a lesser degree compared with *FYN*. The studies on human tissue specimens showed a 2.1-fold increase in FYN expression ($P < .001$) in cancer relative to normal prostatic epithelium. Our studies also showed an increase in the signaling partners of Fyn—FAK and paxillin were both upregulated nearly 2-fold.

BIOLOGICAL FUNCTIONS

Cell growth and apoptosis

Inhibition of Fyn, like other SFKs, has been associated with decreased cell growth. Expression of kinase-dead-Fyn (KD-Fyn), a specific competitor of endogenous Fyn, reduced primary tumor weights in a mouse squamous cancer model.[14] The PI3K/Akt/PKB is often implicated in cancer cell growth. Fyn and other SFKs are known mediators of growth factor-induced antiapoptotic activity of Akt/PKB. Knockdown of Fyn, in concert with Src and Yes, resulted in inhibition of Akt activation by EGF.[15] Fyn has been shown to phosphorylate and prevent cleavage of phosphatidylinositol 3-kinase enhancer-activating Akt (PIKE-A), an inhibitor of apoptosis, in HeLa cells.[16] The activation of Fyn has also been shown to be important in prolactin-dependent Akt activation and cell growth.[17] Fyn is thought to relay the antiapoptotic signals of Akt from not only soluble growth factors but also interactions downstream of cell-extracellular matrix interactions. Baillat reported that integrin engagement with SW480 cancer cells during early contact with ECM triggered a subset of focal adhesion kinase (FAK) molecules to be recruited to lipid raft domains within the cellular membrane where it interacted with Fyn. Within the lipid raft, Fyn phosphorylation of FAK at Y861 and Y925 lead to FAK recruitment back out of the lipid raft and simultaneous activation of the PI3K/Akt pathway.[18]

These studies have revealed that the overexpression of Fyn results in promotion of the antiapoptotic activity of Akt. Although activation of Akt has classically been attributed to inactivation of PTEN, it is becoming apparent that SFKs, such as Fyn, also play a role. Expectedly, Akt activation is common in many cancers, including prostate cancer, and particularly in castration-resistant disease. For example, higher levels of Akt immunoreactivity in prostate tissue samples have been shown to correlate with higher Gleason scores in prostate cancer.[19] Further studies need to be conducted to elucidate the precise mechanism of how aberrant Fyn function leads to dysregulated Akt activity. Such studies may reveal further novel molecular targets in the treatment of cancer.

Cell migration

Overview

Understanding how tumor cells interact with and navigate through the extracellular milieu is an important aspect in elucidating how carcinoma in situ progresses to invasive cancers and then to metastatic disease. Metastasis depends on the ability of cancer cells to migrate and adhere to its local microenvironment. Malignancies of different origins have been shown to use various mechanisms to accomplish this and SFKs play an integral role in the mechanism. In particular, SFKs have been shown to mediate extracellular interactions driven by various molecules, including, but not limited to, IL-8, c-Met, EGFR, and integrins.[4] Many of these pathways have been shown to be highly dependent on kinase function and constitutive kinase activity contributes to metastatic transformation of cancer. Less is known about the specific role of Fyn in cell migration and adhesion in cancer, although the growing body of literature as discussed below suggests it may play a prominent role.

Integrins and FAK

Integrins are cell surface receptors that interact with the extracellular matrix (ECM) and mediate various intracellular signals that control cellular shape and motility. FAK is a tyrosine kinase recruited to focal adhesion sites and plays a central role in directed cell movement. FAK-mediated cellular motility requires the participation of SFKs.[20] Fyn and Src have been shown to coimmunoprecipitate with FAK.[21] Typically, FAK is recruited to the β-subunit of integrins and following its association with SFK, the SFK-FAK complex formation leads to autophosphorylation at Y397. This complex formation is further activated by various phosphorylation events and such assembly acts as the centerpiece of the cellular machinery coordinating actin fiber formation, focal adhesion formation, and ultimately cell shape and motility.

Integrins contribute to cellular motility through the recruitment and activation of several SFK complexes, including SFK-FAK and SFK-Shc.[18,22] The SFK-FAK

pathway has been associated with directed chemotaxis, while the SFK-Shc pathway has been associated with random haptotaxis.[23] This difference may be related to the means through which each respective pathway impacts rearrangements of actin and cytoskeletal machinery. Although FAK activation leads to highly organized actin filaments and focal contacts, Shc activation leads to short actin filaments with fewer focal contacts. It is thought that fine regulation between these 2 pathways by SFKs as well as other regulator molecules results in normal physiological cellular movement. Although the exact mechanisms behind this observation have not been completely understood, aberration of Fyn/SFK function presumptively leads to dysfunctional cellular movement. Thus, integrins and FAK appear to play key roles in the mediation of Fyn transmitted cellular events impacting shape and motility.

Rac and the Rho family of GTPases

Downstream of the SFK-FAK activation, a number of molecules affecting cell migration are activated, including JUN, nuclear factor κB (NF-κB), B-raf, GEF, and Akt/PKB. Therefore, dysfunctional Fyn has the potential to interact with multiple motility effectors. A family of major pathways of interest is the Rho family of GTPases, a subfamily of the Ras superfamily. These proteins have been shown to regulate many aspects of intracellular actin dynamics and include Rac1, RhoA, and Cdc42. Interactions between Fyn and the Rho-family GTPases have been shown to control morphologic differentiation of cells such as oligodendrocytes.[24]

RhoA affects stress fiber formation and Cdc42 has been associated with filopodia formation. Rac1 has been shown to control cell motility, affecting actin reorganization at the leading edges of cells. Fyn-deficient (Fyn−/−) mast cells showed a significant defect in cell spreading and lamellipodia formation on fibronectin. In addition, Rac-activation assays showed that Fyn promotes activation of Rac GTPase under stem cell factor (SCF) stimulation.[25] After α_v-integrin stimulation, PTEN has been shown to directly deactivate Fyn, leading to downstream regulation of Rac-GTPase activity as described above.[26] Strong interplay between Fyn and the Rho family of GTPases, such as Rac1, suggests that this may represent another important pathway through which Fyn exerts its effects on cellular shape and motility.

Ras, Erk, MAPKs

Although many integrins couple to FAK through their β-subunits as described above, certain integrins, including α5β1, α1β1, α6β4, and αvβ3, are known to couple through their α subunits to the Ras-extracellular signal-regulated kinase (ERK) signaling pathway *via* Shc and palmitoylated SFKs, such as Fyn and Yes.[27,28] In this pathway, caveolin-1 functions as a transmembrane adaptor to facilitate the recruitment of SFKs.[29] A palmitoylated SFK then binds, via its SH3 domain, to Shc, leading to phosphorylation of Shc at Y317 and forming an activated complex. The activated complex then combines with GRB2-SOS to activate ERK/MAPK signaling via Ras. Activation of this pathway results in increased cell motility and progression through the G1 stage of the cell cycle in response to mitogens driving cellular growth. This process ties cellular adhesion to cell cycle progression in a process known as anchorage-dependent cell growth. Normal cells need to adhere to serum-derived extracellular matrix components for cell growth in vitro, whereas in malignant cells, this requirement is bypassed. Overexpression of Fyn, therefore, can contribute to dysregulated anchorage-dependent cell growth.[29] Evidence for Fyn involvement in this pathway is supported by the finding that PP1, an SFK inhibitor, will inhibit Fyn over Src at lower concentrations, thus preventing the malignant transformation of oncogenic Ras mutants such as v-Ha-*Ras*.[30] The proposed mechanism for this is inhibition of PAK, a serine/threonine kinase required for malignant transformation of v-Ha-*Ras* and a key regulator of anchorage-dependent cell growth.

Cell adhesion, invasion, and EMT

Fyn has also been shown to play a role in sensing and responding to the rigidity of extracellular matrix surfaces. The generation of sheer force on rigid cell-matrix interfaces results in recruitment of various focal adhesion proteins, leading to increased cell adhesion and cell spreading. Receptor-like protein tyrosine phosphatase-α (RPTP-α) and $\alpha_v\beta_3$ integrin form a complex at the leading edge of a migrating cell in an ECM rigidity-dependent manner that results in recruitment and activation of Fyn.[31,32] This recruitment depends on the proper functioning of the palmitoylation site on Fyn and the level of Fyn activation is thought to be force-dependent in which greater forces result in greater reinforcement of integrin-cytoskeleton linkages. Malignancies may, in part, spread aggressively because of overexpression of Fyn causing an exaggerated sensing response to the rigidity of the extracellular matrix.

Table 2. Src Kinase Inhibitors

Name	Reference	Manufacturer	Comments
Clinically studied			
Dasatinib	44	Bristol-Myers-Squibb	FDA approved for imatinib-resistant CML
AZD0530	45	Astra Zeneca	In phase 1/2 clinical studies
Bosutinib (SKI-606)	46	Wyeth	Phase 1
KX2-391	41	Kinex	Phase 1/2 clinical studies
Preclinical only			
PP1	36		Not usable clinically
PP2	36		Not usable clinically
AP23846	47	ARIAD	
Herbimycin A			Benzochinoid antibiotic related to geldanamycin
CGP76030	48	Novartis	
1l (Nbenzyl-1-(2-chloro-2-phenylethyl)-1H-pyrazolo[3,4-d]pyrimidin-4-amine	49		
7-(2,6-dichlorophenyl)-5-methylbenzo [1,2,4]triazin-3-yl]-[4-(2-pyrrolidin-1-ylethoxy)phenyl]-amine	16	TargeGen, WuXi PharmaTech	

Epithelial-mesenchymal transition (EMT) is the process in which cells convert from an epithelial to a mesenchymal phenotype. Key features of EMT include loss of cell adhesion, a switch from E-cadherin to N-cadherin expression, and an increase in cell motility.[33] Furthermore, a characteristic up-regulation of the neural cell adhesion molecule (NCAM) expression is also commonly known. EMT is necessary for several physiologic processes during development but is also seen in progression from localized cancer to metastatic disease.

Fyn has been reported to play a role in EMT. Recently, Lehembre suggested that at low concentrations of NCAM, a series of events occur including a complex formation outside of lipid rafts between NCAM and FGFR, downstream activation of the MAPK pathway, as well as sustained levels of cellular adhesion.[34] However, in EMT, the loss of E-cadherin results in the overexpression of NCAM, leading to its relocalization into lipid rafts. This event, in turn, results in increased motility because of association and activation of Fyn together with the downstream activation of FAK.

Matrix metalloproteinases (MMPs) are regulators of the interface between epithelial cells and their underlying ECM. Dysregulation of MMP function is commonly observed during metastatic progression as they facilitate invasion into metastatic sites by degrading the ECM in pathologic states.[35] β_6 integrin has also been demonstrated to directly phosphorylate and activate Fyn. This results in downstream up-regulation of matrix metalloproteinase-3 (MMP-3) leading to increased cell proliferation and progression to metastatic disease in vivo.[14] Taken together, these findings suggest a role for Fyn as a mediator of metastatic progression of disease apart from local tumor growth.

SFK INHIBITORS IN PRECLINICAL AND CLINICAL MODELS

Tyrosine kinase inhibitors PP1 and PP2 are the earliest reported SFK-selective tyrosine kinase inhibitors. They have been extremely important in elucidating the role of SFK in signal transduction.[36] Since then, several signal transduction inhibitors have been synthesized and are now being brought forward into clinical studies (Table 2).

Agents targeted specifically against Fyn have not been developed clinically at this time. However, SFK inhibitors known to inhibit Fyn activation have been tested in preclinical and clinical models. Dasatinib is a FDA-approved and commercially available SRC/ABL inhibitor[37] that impairs cell migration[38] and inhibits FAK and $p130^{CAS}$ phosphorylation in DU145 and LNCaP prostate cancer cell lines. This may be attributed to the effects of FAK and $p130^{CAS}$ on integrin interaction. Another SFK inhibitor, AZD0530, inhibits growth by inducing G1-arrest in 22Rv1, DU145, LAPC-4, LNCaP, and PC3 prostate cancer cell lines.[39] DU145 cells treated with AZD0530 showed decreased invasion in a Boyden chamber assay and decreased FAK and $p130^{CAS}$ phosphorylation. The investigators in both studies did not specifically determine which SFKs were responsible for the observed phenomenon. In fact, this is a particularly

difficult distinction to establish as the typically used pSRC (Y419) antibody crossreacts with all active SFKs. Both studies suggested a strong correlation with activation of FAK, a known binding partner of Fyn. Given this and the high relative expression of Fyn in prostate cancer models, it is reasonable to hypothesize that Fyn is the major regulator of these processes.

Further work targeted at understanding the role of dasatinib in prostate cancer has been pursued. Park showed that in a murine orthotopic metastasis model, the use of dasatinib was associated with decreased activation of both Src and Lyn and resulted in decreased lymph node metastases from PC3-M cells.[40] This same group showed similar findings with the novel SFK inhibitor, KX2-391.[41] Interestingly, Park's work shows that Lyn and Src function differently in that Lyn regulated metastasis apart from growth, whereas Src regulated growth apart from metastasis. The investigators, however, did not query other SFKs, such as Fyn, to determine what role Fyn may have played in this behavior.

Both dasatinib and AZD0530 have been studied as single-agent therapies for castrate resistant prostate cancer (CRPC). Our group participated in a study reported by Lara,[42] which was a single-agent, phase 2 clinical study of AZD0530 based on the preclinical data showing the inhibition of growth and migration described above. The study was powered to detect a serum prostate-specific antigen (PSA) response rate of 15% or greater. None of the patients treated exhibited such a response by PSA. However, it is important to note that the trial was not designed to look at alternative outcomes such as new metastasis, which may be more relevant to SFK inhibition.

In addition to inhibiting Fyn, dasatinib also inhibits other SFKs such as LCK and SRC. Our group also participated in a single-agent, phase 2 study in chemotherapy naïve patients with CRPC reported by Yu.[43] This study showed that the disease control rate for 15 Response Evaluation Criteria In Solid Tumors - evaluable patients was 67% (10 had stable disease). Of 27 patients with bone scans at 12 weeks, 16 were stable and 1 was improved. Two of 5 patients with greater than 2 bone scans at 24 weeks had stable disease. An improved PSA doubling time was seen in 29 of 36 patients (80.1%). The mode of action of dasatinib in this population is not as clear, but the effects reported are more consistent with what is recognized in the role that SFKs, such as Fyn, may play in prostate cancer. Collectively, these data support ongoing evaluation of SFK inhibitors in prostate cancer.

CONCLUSION

Although Src has long been recognized as an important oncogene, little attention has been given to its family members such as Fyn, which may be more relevant than c-Src in certain cancers. Our initial work shows a particular up-regulation of Fyn in prostate cancer. Given the above-mentioned data showing expression and putative role for Fyn in prostate cancer progression and with the availability of pharmacologic agents to manipulate this target, it is reasonable and timely to test the utility of this molecular target. The additional information, from ongoing studies of Fyn in prostate cancer clarify the role it plays in the disease process to optimize Fyn-directed therapeutics. Regretfully, pharmacologic developments of SFK inhibitors have focused upon inhibition of c-Src rather than other SFKs that may be more relevant to human disease such as Fyn. As more is learned of the specific role that Fyn plays in human disease, we hope that agents specifically targeted at Fyn may be advanced in preclinical and clinical development.

CONFLICT OF INTEREST DISCLOSURES

The authors made no disclosures.

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Human Cancer Biology

Clinical Cancer Research

Fyn Is Downstream of the HGF/MET Signaling Axis and Affects Cellular Shape and Tropism in PC3 Cells

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Abstract

Purpose: Fyn is a member of the Src family of kinases that we have previously shown to be overexpressed in prostate cancer. This study defines the biological impact of Fyn inhibition in cancer using a PC3 prostate cancer model.

Experimental Design: Fyn expression was suppressed in PC3 cells using an shRNA against Fyn (PC3/FYN-). Knockdown cells were characterized using standard growth curves and time-lapse video microscopy of wound assays and Dunn Chamber assays. Tissue microarray analysis was used to verify the physiologic relevance of the HGF/MET axis in human samples. Flank injections of nude mice were performed to assess *in vivo* growth characteristics.

Results: HGF was found to be sufficient to drive Fyn-mediated events. Compared to control transductants (PC3/Ctrl), PC3/FYN- showed a 21% decrease in growth at 4 days ($P = 0.05$). PC3/FYN- cells were 34% longer than control cells ($P = 0.018$) with 50% increase in overall surface area ($P < 0.001$). Furthermore, when placed in a gradient of HGF, PC3/FYN- cells showed impaired directed chemotaxis down an HGF gradient in comparison to PC3/Ctrl ($P = 0.001$) despite a 41% increase in cellular movement speed. *In vivo* studies showed 66% difference of PC3/FYN- cell growth at 8 weeks using bidimensional measurements ($P = 0.002$).

Conclusions: Fyn plays an important role in prostate cancer biology by facilitating cellular growth and by regulating directed chemotaxis—a key component of metastasis. This finding bears particular translational importance when studying the effect of Fyn inhibition in human subjects. *Clin Cancer Res; 17(10); 3112–22. ©2011 AACR.*

Introduction

The Src-family kinases (SFKs) have long been recognized as key players in cancer biology. Currently, the known members of the SFKs include Src, Lck, Fyn, Yes, Fgr, Lyn, Hck, Blk, and Yrk, and all these family members share a common structure and pattern of activation. The existence of a unique domain provides high variability and impacts protein–protein interactions that confer specific physiologic and pathophysiologic function for each member.

Fyn is a ubiquitously expressed SFK that has been previously demonstrated by our group to be overexpressed in prostate cancer (1). Fyn is localized to the inner cytoplasmic leaflet of plasma membrane; a process is driven by posttranslational fatty acid acylation of amino acids in the SH4-domain, typically with myristatic and palmitatic acids, as well as methylation of lysine residues (2, 3). Activation of Fyn results in tyrosine phosphorylation of a variety of target proteins resulting in downstream signaling of a number of pathways. The role of Fyn has been studied in a variety of cellular processes including T-cell and B-cell receptor signaling, oligodendrocyte, keratinocyte and natural killer cell differentiation, platelet activation, integrin and growth factor-mediated signaling, and cell–cell adhesion and cell migration (4–8). These interactions are mediated by signaling partners such as FAK and paxillin that are overexpressed in prostate cancer concurrent with Fyn (1).

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Note: Supplementary data for this article are available at Clinical Cancer Research Online (http://clincancerres.aacrjournals.org/).

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Given our findings, we have made efforts to delineate the causal relationship between Fyn overexpression and prostate cancer progression. Our previous studies demonstrated a concurrent upregulation of FAK and paxillin in prostate cancer tissues suggesting that Fyn may act as a regulator of shape and motility (1). We thus hypothesized that disruption of Fyn activity would result in impaired cellular motility and alternation of cell shape in prostate cancer. Further, upstream of Fyn, there are a number of growth factors and receptors whose downstream signaling may be mediated by the activity of SFKs. Serum concentrations of HGF have been shown to be elevated in men with prostate cancer (9). Furthermore, MET expression has been described in several studies suggesting that this axis may be active in stimulating biochemical events related to disease progression (10); however, the role of the HGF/MET axis has not been well characterized in prostate cancer. In this study, we show that Fyn strongly impacts cellular tropism and shape and that this behavior can be driven by activation of the HGF/MET signaling axis in prostate cancer cell line.

Methods

Cells and *Fyn* knockdown

PC3 cells were a generous gift of Dr. Carrie Rinker-Schaeffer. Cells were propagated and maintained in RPMI 1640 media (Gibco BRL) supplemented with 1% streptomycin/penicillin (Cellgro) and 10% fetal calf serum (Cellgro) at 37°C in humidified air at 5% CO_2, except where noted.

Suppression of *Fyn* expression was achieved using MISSION shRNA Lentiviral transduction particles (Sigma-Aldrich). Transduction conditions were optimized with a GFP containing construct from Sigma using the same lentiviral transduction system. In the presence of hexadimethrine bromide at 8 mcg/mL, PC3 cells were transduced with shRNA against *Fyn* or a nontargeting (control) shRNA named PC3/FYN- and PC3/Ctrl, respectively. Knockdown cell lines were propagated in media containing 0.25 mcg/mL puromycin (Sigma Chemical Co.) as the construct contained a puromycin resistance vector. Immunoblots for Fyn were performed in conjunction with all studies to ensure continued Fyn suppression.

Antibodies

Anti-Fyn antibody for use in immunoblotting, immunohistochemistry (IHC), and immunofluorescence (IF) was purchased from Upstate Biotechnology, Inc. Rhodamine-labeled phalloidin and fluorescein isothiocyanate-conjugated anti-mouse and rhodamine-conjugated anti-rabbit antibodies for use as secondary antibodies for IF were obtained from Molecular Probes. Total MET antibody was obtained from Zymed Laboratories. Two phospho-MET antibodies were utilized for IHC (pY1003 and pY202/3/4, Biosource). HGF antibody was obtained from R&D systems.

Preparation of cell lysates and immunoblotting

Cell lysates were prepared using lysis buffer containing 20 mmol/L Tris, pH 8.0, 150 mmol/L NaCl, 10% glycerol, 1% Nonidet P-40, and 0.42% NaF containing inhibitors (1 mmol/L sodium orthovanadate, 1 mmol/L HALT phosphatase inhibitor cocktail (Thermo Scientific). Cell lysates were separated using a 7.5% Tris-HCl gel with SDS-PAGE under reducing conditions. Protein was transferred to polyvinyl chloride membranes and processed for immnoblotting using established methods with enhanced chemiluminescence techniques (GE Healthcare).

Quantitative PCR for FYN

RNA from cell lines was extracted using an RNAqueous kit (Ambion) according to the manufacturer's recommendations. Samples were stored at −80°C until processed. Customized primers for Fyn were prepared by Integrated DNA Technologies (Coralville). The left primer was: 5′-ATG GAA ACA CAA AAG TAG CCA TAA A-3′; and the right primer: 5′-TCT GTG AGT AAG ATT CCA AAA GAC C-3′. Data were calibrated to the expression of glyceraldehyde phosphate dehydrogenase. Quantitative PCR was performed using SYBR Green dye on an ABI 7700 (Applied Biosystems).

Time-lapse video microscopy and image analysis

All time-lapse experiments were performed using an inverted Olympus IX71 microscope with an attached QImaging Retiga EXi camera. Cells were maintained on a heated stage at 37°C (Omega CN9000A) with a constant flow of 5% CO_2. Image capture was achieved using IPLab version 3.65a (Scanalytics, Inc.). Analysis of still images was performed using the ImageJ software package from the NIH (http://rsb.info.nih.gov/ij/).

Wound-healing assay

Cells were plated onto either 60-mm plates or 6-well plates at a concentration of 1×10^6 cells/cm^2 and allowed to attach overnight. Cells were allowed to grow to approximately 80% confluence by visual inspection prior to scratch assay. At the time of the scratch, cells were washed 3 times with PBS and starved in serum-free RPMI 1640 for 3 hours. A linear wound was then made with a 10 μL plastic pipette tip. After washing 3 times with serum free media, the cells were stimulated with media containing fetal calf serum or HGF. Wound width was measured at 3 randomly chosen sites using ImageJ. Growth factors used included hepatocyte growth factor (HGF), epidermal growth factor (EGF), and basic fibroblast growth factor (bFGF; Cell Signaling). Wound closure was quantified by parallel assessments of wound length at 4 fixed positions over time and expressed as a percentage of baseline wound distance at that point.

Single-cell shape and motility assay

Cells were plated onto 35-mm plates at a concentration of 1.5×10^5 cells/cm^2 and allowed to attach for 48 hours to approximately 20% confluence. The cells were then washed 3 times with PBS and starved for at least 3 hours. Cells were

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then stimulated with 10 to 50 ng/mL HGF and recorded as described earlier using time-lapse video microscopy (TLVM). Cell movements were tracked using the Metamorph 7.6 software package (Molecular Devices). Using this software package, 15 to 25 cells per sample were identified and tracked over a 12-hour period. The tracking data were fed in to the IBIDI cell tracker tool in ImageJ yielding analysis of velocity and path length. Data provided represents an average of the cells tracked. Shape characterizations (area, circularity, and length) were performed by manual measurements using ImageJ using no less than 20 cells. Cellular area refers to a 2-dimensional projection of the cell onto an *XY* plane. For membrane ruffling, cellular perimeter was manually measured to determine the fraction of total membrane perimeter involved in ruffling in ImageJ.

Dunn chamber assay

Cell chemotaxis was studied using a Dunn chamber assay as previously described (11). In brief, a Dunn Chamber is a modified Zigmond chamber in which a diffusion gradient of a chemotactic factor was made by creating a liquid bridge across 2 wells: one containing media with a high concentration of a chemotactic factor and the other well containing media alone. This creates a diffusion gradient across the area where the 2 are connected. Glass coverslips were placed at the bottom of a 35-mm plate and to this was added 1.5×10^5 cells in RPMI supplemented with 10% FCS. Cells were allowed to attach over 24 hours then placed under serum-starved conditions with RPMI for 3 hours. The coverslip was then inverted onto a Dunn chamber (Hawksley) filled with media (no serum). The coverslip was then sealed on the outer edges with hot VALAP mixture (1:1:1 vaseline, beeswax, and paraffin). The outer chamber of the Dunn apparatus was subsequently evacuated and refilled with media supplemented with HGF at a concentration of 10 ng/mL. Cell chemotaxis was then captured by video microscopy over 3 hours. Analysis of motility was completed as described earlier.

Immunofluorescence

PC3 cells were plated onto a glass coverslip in a 6-well plate at a concentration of 1.5×10^5 cells/well and allowed to attach over 48 hours in media supplemented with 10% fetal calf serum. Cells were then fixed with 4% paraformaldehyde, and permeabilized with 0.1% Triton X100-PBS before blocking with 3% bovine serum albumin in TBST. The coverslips were then incubated with primary antibody in TBST at 100:1 dilution for 1 hour. Cells were subsequently washed 3 times in TBST before incubating with secondary antibody and/or Rhodamine-phalloidin in TBST at 50:1 dilution for 1 hour. Cells were washed once again in TBST before mounting onto coverslips using ProLong Gold antifade mounting medium with DAPI (Invitrogen Molecular Probes). Images were analyzed using ImageJ after deconvolution using a Huygen's algorithm. Colocalization was detected using the JACOP plugin for ImageJ.

Human tissue source

All human tissue samples used in this study were obtained from the University of Chicago. Utilization of tissue was performed under an institutional review board approved protocol requiring that all samples were kept anonymous to the primary investigational team.

Tissue was analyzed in the form of 2 tissue microarrays previously fabricated by the Department of Pathology at the University of Chicago. Microarray fabrication has been described elsewhere (12). In short, the arrays used contained specimens from 45 patients planned to have triplicate representation on the array. Each array element was 1.5 mm in diameter. Tissue samples included primary tumor from prostate cancer patients with Gleason scores of 6 to 9. When possible both normal and tumor elements were scored on a section. The identity of patients was kept blinded to the primary analytic group. A patient's sample was only considered usable if represented at least twice on the array.

Immunohistochemistry

For IHC, stained TMA sections were analyzed by a dedicated urological pathologist (H.A.A. or K.D.H.). Results were reported semiquantitatively on a scale of 0 to 3 for intensity, where 0 was negative, 1 was weak, 2 was moderate, and 3 was strong. The percentage of tumor staining was reported as 0% to 100% in increments of 10%. A composite score was formed using the product of the intensity and percentage of glands staining. Staining was performed at the following antibody concentrations: MET at 1:100, MET-Y1003 at 1:20, MET-Y1202/3/4 at 1:25, and HGF at 25 μg/mL. Each TMA contained on-slide controls of lymph node tissue to ensure absence of artifacts contributing to differential staining reported.

Mice

Eight-week-old nude (nu/nu) mice (Strain code: 088, Charles River Laboratories) were kept in a specific pathogen free colony, in microisolator cages, and were fed sterile rodent chow and sterile water *ad libitum*. All protocols were approved by the University of Michigan Animal Care and Use Committee. Tumors were harvested at necropsy and preserved in formalin. Staining was performed using standard hematoxylin and eosin (H&E) as well as CD31 (Abcam, 1:50 dilution) to asses for microvessels. H&E sections were analyzed for mitotic index [per 10 high powered field (HPF) counting up to 30 HPFs], coagulative tumor necrosis (% tumor volume), lymphoid aggregates within the tumor (per 10 HPF), ratio of epithelioid: spindled cells, and neovessel density (CD31+ with luminal formation by pathologists review).

In vivo growth assay

PC3/FYN- and PC3/Ctrl cells were harvested by trypsinization washed twice with PBS and resuspended at a density of 2×10^6 cells in 100 μL PBS for each injection site. Mice were monitored for tumor growth and when detected by palpation, measurement of the tumors began. Tumor

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volumes were calculated by the formula: volume = [(minimum measurement)^2 × (maximum measurement)]/2 as described by Smith (13). Tumors were measured weekly until volume exceeded 1 mm^3. Each mouse was given 2 subcutaneous doses of PC3/FYN- (right flank) and PC3/Ctrl (left flank). Alternatively, a cross product of the longest tumor diameter and one orthogonal to the longest diameter were calculated and compared. At the conclusion of the study, all mice were sacrificed, and tissue samples were collected.

Statistical analyses

All analyses were performed using SPSS version 17.0 for Windows. A general linear model (GLM) was used to compare the effects of FYN expression and HGF stimulation and their interaction on cell morphology parameters from baseline to 12 hours and to compare changes in cell length between baseline and 12 hours. To evaluate the differences of cellular shape and growth between groups (PC3/FYN- and PC3/Ctrl, serum starved and HGF stimulated), the independent-samples *T* test was used for the data based on specific distributional assumptions such as the normal distribution. If the data was of a non-normal distribution, then Mann–Whitney test to assess was appropriately used. The Watson–Williams test was performed for the equality of mean angle of cellular motility using calculating angular movements from a relative origin (14). Comparisons of quantified IHC data were performed using a Wilcoxon signed ranks test. For *in vivo* growth studies, tumor volume and cross product data were logarithmically transformed so that a paired-samples *T* test could be used to assess differences of PC3/FYN- cell growth compared to PC3/Ctrl. A 2-sided $P < 0.05$ was used as a threshold for declaring statistical significance.

Results

Generation of PC3 Fyn knockdown cell line

To characterize the effect of Fyn variation *in vitro*, we generated a knockdown line using PC3 cells and lentiviral transduction particles containing shRNA constructs targeted specifically against *Fyn* (PC3/FYN-). Using a multiplicity of infection of 2, 5 constructs (Supplementary Table S1) were tested. The construct leading to maximal Fyn suppression by immunoblot with minimal effect on non-Fyn SFKs, such as Src, was labeled PC3/FYN- and advanced for further study. A control cell line was developed (PC3/Ctrl) using a nontargeting shRNA construct. Both lines were maintained under continuous selection of puromycin. Fyn mRNA expression was measured with a comparative RT-PCR using PC3/Ctrl as a reference and protein by immunoblot (Fig. 1A and B). Both assays revealed that Fyn expression was decreased by at least 60%. Minimal off target effects were seen as evidenced by the lack of change in Src expression (Fig. 1B). The knockdown effect was stable through serial passage under these conditions.

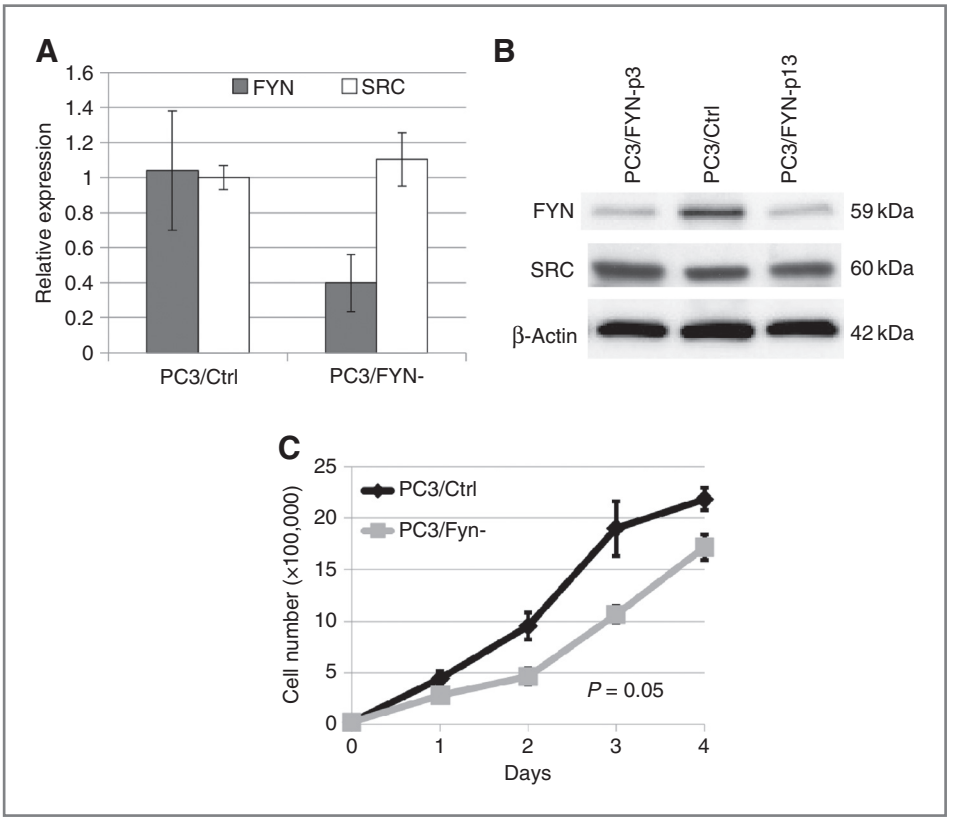

Figure 1. Generation of Fyn knockdown cell lines (PC3/FYN-). A, comparative RT-PCR showing mRNA expression of Fyn and Src in PC3/Ctrl and PC3/FYN- lines. Fyn expression is decreased approximately 60% without significant impact on Src expression. B, immunoblots for Fyn and Src expression showing decreased Fyn expression without significant alteration of Src expression. No significant variation in Fyn expression is seen in the PC3/FYN- line over serial passage. C, 4-day growth curves comparing PC3/FYN- to PC3/Ctrl. Error bars represent standard error of the mean with 3 replicates for each day.

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Fyn knockdown results in impaired *in vitro* growth and motility

Under standard propagation conditions, PC3/FYN- cells showed a small but statistically significant diminishment in growth rate (Fig. 1C). After 4 days under standard conditions, PC3/FYN- cells grew at only 79% the rate of PC3/Ctrl cells ($P = 0.05$).

Wound healing assays in concert with TLVM were performed to characterize motility (Fig. 2A and B and Supplementary movies 1 and 2). Virtually no wound closure was seen in the absence of serum or other mitogens. However, in serum-replete conditions, PC3/Ctrl cells showed near complete closure within 12 hours whereas PC3/FYN- cells failed to show complete closure.

HGF stimulates PC3 motility

Isolated growth factors were utilized to further understand growth and motility. HGF, EGF, and bFGF were selected given overexpression or increased serum concentrations in men with advanced prostate cancer (9, 15, 16). Wound closure was observed using TLVM under conditions of media with a single growth factor at 10 ng/mL. HGF was determined to be the optimal motility stimulus for PC3 cells from this pool as it produced the highest rate of wound closure per unit time (Supplementary Fig. S1).

Fyn knockdown results in altered cell shape and increased cell speed with impaired directional motility

A series of single cell motility experiments with serum or HGF stimulation were performed to measure the impact of Fyn knockdown on morphology and speed of movement.

Cell area. Area was measured as a measure of size and cell spreading. Differential effects of HGF stimulation on cell shape are summarized in Figure 3. At baseline, a small but statistically significant difference was seen between PC3/FYN- and PC3/Ctrl cells. Under serum-starved conditions, the PC3/FYN- cells were 33% larger than the

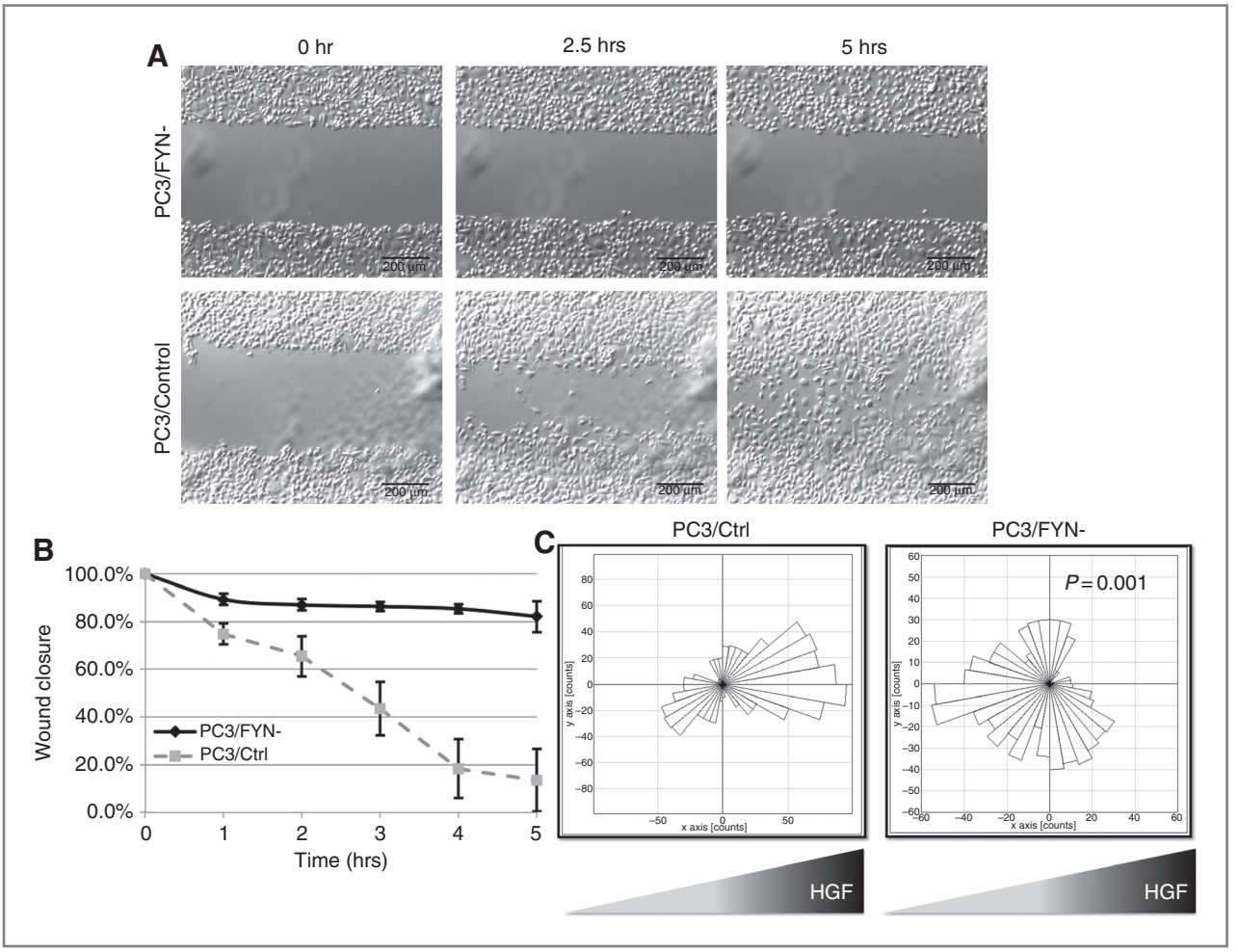

Figure 2. Wound healing assays of PC3/FYN- and PC3/Ctrl cells in serum replete media. Full videos are available in the Supplemental Data. A, representative images from time lapse video. B, graphical representation of wound closure over time. Error bars represent standard deviation of wound closure as a percentage of the original wound distance taken at 5 serial points. C, rose plots (circular histogram) showing collective number of steps in any given direction (in 10° increments) in the presence of an HGF gradient.

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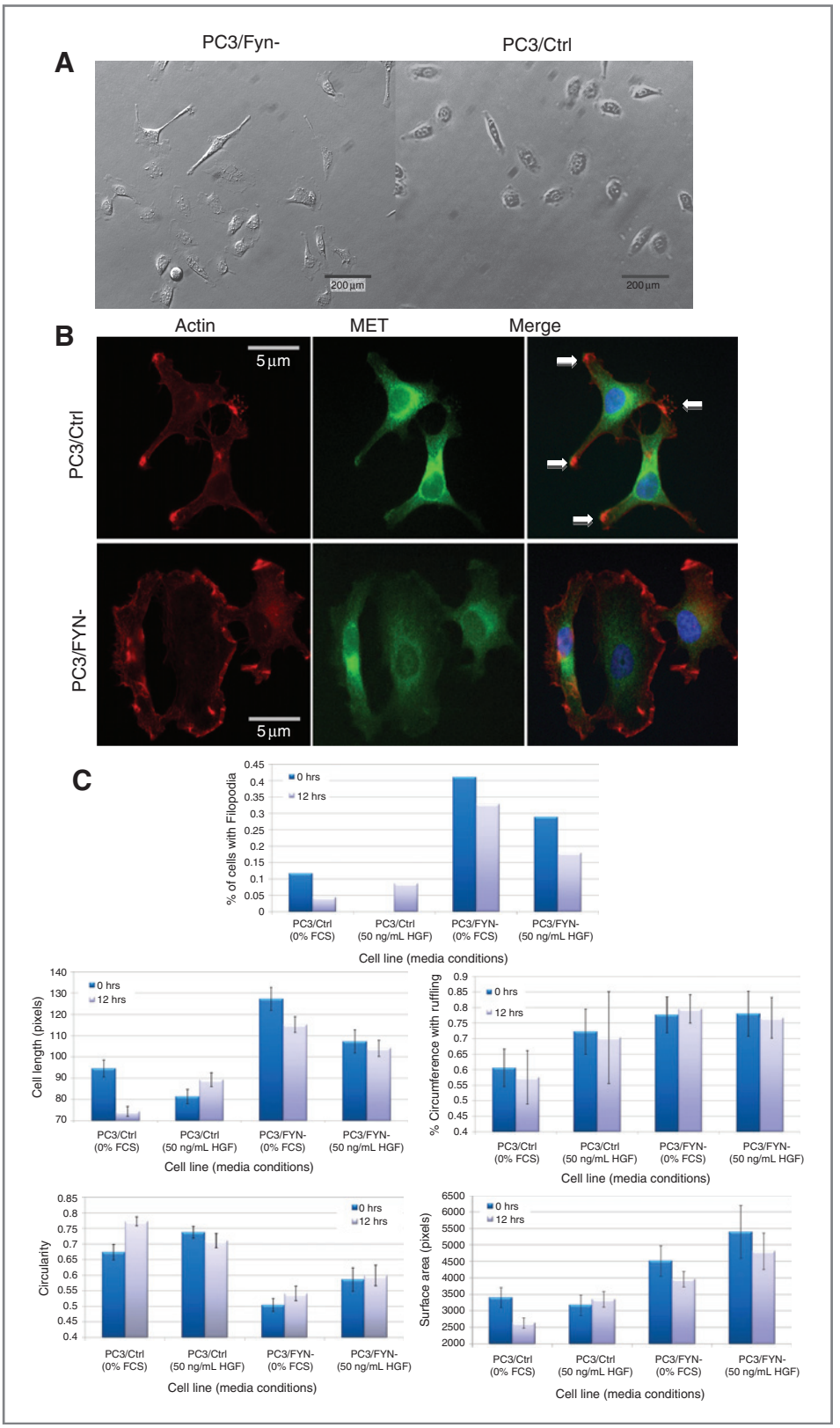

Figure 3. A, representative images of PC3/FYN- and PC3/Ctrl cells on polystyrene plates stimulated by HGF. PC3/FYN- cells were found to have greater cell length, area, and ruffling than PC3/Ctrl cells. B, immunofluoresence of actin and MET in PC3 sublines. Overlap of actin and MET is shown in yellow and demarcated by arrows in the rightmost panels. These areas are most consistent with focal adhesion plaques. As seen in the lower right panel, there is no focal accumulation of both MET and actin to suggest plaque formation. B, rose plots showing direction of movement of PC3 sublines relative to an HGF gradient (source on right). A rose plot is a circular histogram in 10° increments showing cumulative motion in any given direction in 360° without regard to the magnitude of movement. C, graphical representation of quantified morphologic variations between PC3/FYN- and PC3/Ctrl cells.

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PC3/Ctrl cells ($P = 0.018$), and after 12 hours of stimulation with serum there was a 50% increase in surface area ($P < 0.001$) for the PC3/FYN- cells compared to PC3/Ctrl. Comparing Feret's (longest) diameter, the PC3/FYN- cells were 34% longer at baseline ($P < 0.001$) and 56% longer after 12 hours of serum simulation ($P < 0.001$). Membrane ruffling was not significantly different between the 2 cell lines before or after serum stimulation. The effect of HGF was similar on both cell lines; in comparing PC3/FYN- to PC3/Ctrl cells after 12 hours of stimulation we found a 43% increase in area ($P = 0.016$), and 17% increase in Feret's diameter ($P = 0.026$) with no change in ruffling.

Cell length. A similar trend was also observed for cell length as determined by longest cell axis under the same experimental conditions (Fig. 3A and C). After 12 hours of stimulation with HGF, PC3/FYN- cells were 31% longer than PC3/Ctrl cells ($P = 0.003$). In fact, PC3/Ctrl cells showed 17% increase in cell length ($P = 0.010$) after 12 hours in HGF, whereas the PC3/FYN- showed no statistically significant change.

Cell morphology. In response to HGF stimulation, PC3/FYN- acquired a broader distribution of various cell shapes and morphologies (Fig. 3A and C). PC3/FYN- cells also produced a larger number of filopodia compared to PC3/Ctrl- cells. Using representative images of subconfluent cellular monolayers, we manually counted the percentage cells with filopodia. In the absence of HGF supplementation, PC3/Ctrl cells underwent a decrease of the percentage of cells with filopodia from 11% to 4.5%, a 59% decrease over 12 hours. Under the same conditions, the percentage of PC3/FYN- cells with filopodia only decreased 20%, from 40.5% to 32.5%. Under HGF stimulation, PC3/Ctrl cells had a 9% increase in cells with filopodia whereas PC3/FYN- cells had a 12% decrease over 12 hours. There were variations in the percentage of cell perimeter ruffling. PC3/Ctrl cells had a greater degree of ruffle formation in response to HGF supplementation (a 16% increase in percentage of cell circumference with ruffles upon HGF stimulation) whereas PC3/FYN- cells were not observed to have any change in ruffling in the presence of HGF. Cell circularity [as defined by $4\pi(\text{area}/\text{perimeter}^2)$] was calculated as an assessment of cell shape symmetry. There was an increase in circularity under serum-starved conditions over 12 hours for PC3/Ctrl cells of 12%, whereas there was no significant increase observed for PC3/FYN- cells. Under HGF supplementation, no changes in circularity were observed for either cell line.

To test the hypothesis that the variations in shape were related to alterations in the actin cytoskeleton, phalloidin staining with MET immunostaining were performed (Fig. 3B). In response to HGF stimulation, PC3/Ctrl cells showed retraction of small hair-like projections in favor of forming larger cellular extensions. PC3/FYN- cells continued to show these small hair-like projections despite HGF stimulation.

To quantify the effect of Fyn knockdown on focal adhesion formation and MET distribution, colocalization of MET and actin was analyzed by detecting signals above a threshold level to exclude background beyond the cellular membrane—given this colocalization pattern suggests focal adhesion formation. At threshold, signal overlap between actin and MET were expressed as Mander's coefficients expressing degree over overlap. In the setting of HGF stimulation, 28% of the actin signal was associated with MET predominately at focal points along the cell surface and predominately at the tips of filopodia, which would represent focal adhesion plaque formation. Conversely, only 2% of actin was associated with HGF stimulation in PC3/FYN- cells.

Cell speed. Representative films of cellular movement of PC3/FYN- and PC3/Ctrl cells on glass bottom plates are shown in supplemental videos 3 and 4. Although PC3/FYN- cells exhibited decreased ability to migrate over monolayer defects, analysis of single cells showed a relative increase in cellular speed when no gradient was present. Both PC3/Ctrl and PC3/FYN- cells showed decreased cell speed in the absence of HGF stimulation, 24% and 31% from baseline respectively. However, PC3/FYN- cells exhibited increased movement speed compared to PC3/Ctrl with a 41% increase in average cell speed over a 12-hour period of HGF stimulation.

Directed chemotaxis. Given the dichotomy between elevated cell speeds but decreased rates of wound closure, we hypothesized that Fyn regulates vectorial velocity or more specifically, directed chemotaxis. To test this hypothesis we employed a Dunn chamber to allow for the direct observation of cell migration under a chemotactic gradient. Unlike a Boyden Chamber assay, this allows for direct visualization of cells in transit. Individual cells were observed under TLVM for a period of 3 hours. More than 20 cells were tracked to create a composite map of movement represented as a rose plot in 10° increments (Fig. 2). PC3/Ctrl cells moved toward the HGF source whereas PC3/FYN- cells were either insensitive to or moved away from the HGF source. We performed a comparison of direction relative to the vector defined by the initial and final location (Euclidean distance, ED) of the each cell (Table 1 and Supplementary Fig. S2). In brief, this directionality coefficient is a ratio of the shortest linear distance between the

Table 1. Summary of motility parameters from Dunn Chamber assay

	PC3/FYN-	PC3/Ctrl	*P*
Directionality	0.248	0.242	0.822
FMI	−0.042	0.084	0.077
AD	28.5 um	58.8 um	<0.001
ED	6.5 um	14.2 um	0.016
Velocity	0.16 μm/min	0.42 μm/min	<0.000001

Abbreviations: AD, accumulated (total) distance; ED, Euclidean distance; FMI, forward motion index. Supplementary Figure S2 shows a graphical representation of the above parameters.

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ED compared to the total distance traversed by the cell (accumulated distance, AD) such that a value of 1 would represent a perfectly linear path and values below suggest relative degrees of variation from this linear path. The overall directionality did not differ greatly between the 2 conditions ($P = 0.822$). However, when comparing translocation relative to the HGF source using a forward migration index (FMI) quantifying motion only in a fixed vector for each cell (i.e., a vector toward the HGF source), PC3/FYN- cells showed movement away from the gradient; PC3/Ctrl cells showed movement toward. Analysis of membrane edges revealed a greater amount of membrane ruffling in the PC3/FYN- cells relative to the PC3/Ctrl; however, comparing AD and ED, PC3/Ctrl cells were significantly more motile with and AD increase of 200% and a ED increase of 212% resulting in a 263% increase in overall vectorial velocity.

In vivo growth assay

To measure the impact specifically on growth variation in a biologically relevant system, subcutaneous flank injections were performed on 5 nude mice with each mouse receiving 2 injections of PC3/FYN- and 2 injections of PC3/Ctrl. After approximately 8 weeks of growth, mice were sacrificed and tumors measured. Four of the mice grew visible tumors. Figure 4A shows a representative mice bearing PC3/Ctrl (left or top) and PC3/FYN- (right or bottom) tumors as well as the tumors removed at necropsy. In comparing PC3/FYN- to PC3/Ctrl in each mouse (Fig. 4B), an average 33% difference was seen in the size of the PC3/Ctrl and PC3/Fyn- tumors (mean volume day 43: 712 vs. 120 mm^3, $P = 0.014$; day 57: 1299 vs. 449 mm^3; $P = 0.071$). Alternatively, comparing the cross product of the longest diameter and an orthoganol diameter, there was a 66% difference (average cross product day 57: 570 mm^2 vs. 193 mm^2; $P = 0.002$). Histomorphological and immunohistochemical analysis of the tumor samples did not show significant changes in cellular morphology or tumor neovessel formation as evidenced by CD31 staining (Fig. 4C). There was a trend however, to an increase in mitotic index, tumor necrosis, and tumor infiltration by lymphocytes (as evidenced by intratumoral lymphoid aggregates) favoring the PC3/Ctrl tumors without meeting significance criteria. Neovessel density was also not significantly different but favored the PC3/FYN- tumors (Fig. 4D).

HGF and MET expression in prostate cancer tissue samples

The relevance of the HGF/MET signaling axis in human disease was validated by immuhistochemical analysis of a tissue microarray composed of 40 patient samples. Representative sections are shown in Supplementary Figure S3. HGF was up-regulated 1.3-fold in cancer compared to normal (mean composite score 140.74 vs. 179.72, $P = 0.035$). No significant variations in MET, pMET-Y1003, or pMET-Y1349 were found in the sample population between malignant and nonmalignant glands (data not shown).

Discussion

These studies show that focused reduction of Fyn expression apart from other SFKs results in a notable variation in the *in vitro* phenotype. This phenotype is characterized by a change in growth kinetics that is durable over time with a significant impact on cellular shape and motility. The *in vitro* growth studies showed a small but statistically significant separation in growth that became more pronounced during *in vivo* studies due to the significant increase in experimental duration. Further studies of multi-SFK inhibition would potentially be warranted for the future. Analysis of the tumor specimens from the mouse experiment did not show significant differences between the 2 conditions outside of tumor necrosis as this likely reflected the difference in size of the tumors. The lack of differences in mitotic index and neovessel formation could have been affected by tumor size and necrosis as well as loss of antigen retrieval during prolonged formalin fixation.

Our Fyn knockdown cells in general showed an accelerated speed but more importantly, aberration of directional response in PC3 cells. This change in function is accompanied by a change in macroscopic and microscopic cellular structure as shown by the variation in the arrangement of cytoskeletal elements and focal adhesions. In particular, the changes in colocalization of MET with actin points to variation in focal adhesion formation- key step in the metastatic process. This presentation is particular focused on the PC3 model given its extensive use in both *in vitro* and *in vivo* studies. Interestingly, we have found the HGF/MET axis to be a sufficient activator of Fyn-driven events. Elevation of serum HGF is well recognized in prostate cancer. Our finding of increased HGF expression in malignant prostatic glands is certainly consistent with this previous observation (9). Despite this, the expression of the MET in both total and active forms was stable to diminished. The limited sample size restricted our ability to detect more subtle differences in MET expression and activation, but if a difference was present, it would likely only represent a small variation. The role of MET in this system and in prostate cancer in general continues to be an important and exciting area of investigation that exceeds the scope of this particular study.

The SFKs are among the first oncogenes recognized in cancer biology. SFKs have remained of great interest given the central role they play in mediating extracellular stimuli to the nucleus. Although the majority of research in Src biology has focused on the prototypical member of this family, c-Src, emerging data suggests that the various SFKs may affect cancer cells differently. Fyn is known to be ubiquitously expressed under normal physiologic conditions and its functions are typically ascribed to mediating T-cell response and neuronal development (17). Fyn knockout mice have been characterized as having a subtle phenotype in neuropsychological development and T-cell function with little phenotypic characteristics otherwise (18). This has been attributed to the high degree of

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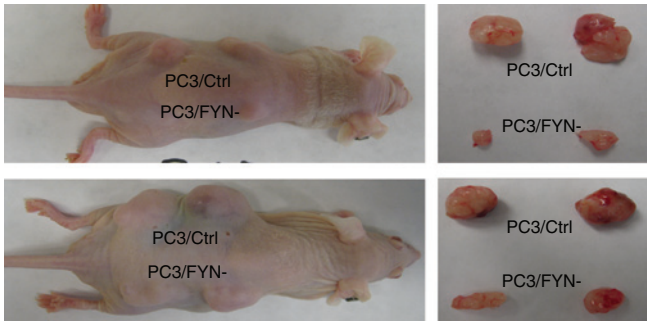
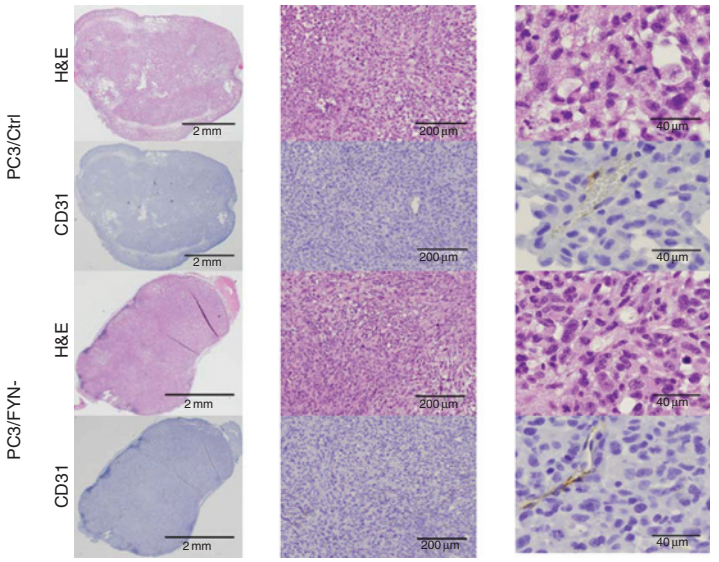
A



B

Mouse ID	Cross product (mm^2) PC3/Ctrl	PC3/FYN-	% Change	Volume (mm^3) PC3/Ctrl	PC3/FYN-	% Change
3170	222	38	**17%**	1374	112	**8%**
3172	161	78	**49%**	878	350	**40%**
3173	196	95	**49%**	1236	443	**36%**
3174	314	181	**58%**	2347	1182	**50%**
3175	26	12	**48%**	63	20	**32%**

C



D

		Mitoses (per 10 HPF)	Tumor necrosis	Lymphoid aggregates (per 10 hpf)	Epithelioid-spindled cells	Neovessels (# per 10 HPF)
PC3/FYN-	3170 R	42	<3%	<1	95:5	25
	3172 R	38	0%	0	90:10	32
	3174 R	46	2%	0	80:20	29
PC3/Ctrl	3170 L	56	10%	2	95:5	19
	3172 L	48	10%	1	90:10	25
	3174 L	51	6%	0	85:15	19

Figure 4. A (left), nude mouse with subcutaneous injection of PC3/Ctrl and PC3/FYN- cells at day 57; (right) tumor recovered at necropsy. B, table of mouse tumor measurements showing both cross products (length × width) and volumetric approximations based on maximal and minimal tumor dimensions. C, immuohistochemical analysis of tumors from *in vivo* growth study. PC3/Ctrl (upper) and PC3/FYN- cells (bottom) were injected into the flanks of nu/nu mice and collected at necropsy. H&E and CD31 staining is shown at 1× (left), 4.2× (middle), and 20× (right). No significant change in cellular morphology or neovessel formation was seen between the 2 conditions. D, table of quantification of mitoses, tumor necrosis, lymphoid aggregate formation, ratio of epithliod to spindled cells, and neovessel density. No significant differences were seen.

homology of the family members that may allow for compensation for the loss or absence of another family member. There is now growing evidence, however, that non-Src SFKs play an important role in tumor progression. In a study by Park and colleagues, inhibition of Lyn expression with RNAi resulted in altered cellular growth apart from migration whereas Src knockdown resulted in impaired metastatic capability (19). Similarly, our group has previously demonstrated that Fyn is up-regulated in prostate cancer (1). Therefore, we have made efforts to define the role that this up-regulated kinase may play in cancer biology.

Our results point toward a role for Fyn in metastatic progression, which are consistent with findings in other models

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(20). This finding holds particular appeal for translation in the availability of SFK inhibitors that have entered clinical studies. It is important to note, however, that these inhibitors display varying activity on the members of the Src family and the majority of their characterization and hence development have relied upon the inhibition of Src itself. Clinical studies with such agents have shown modest benefits (21) but have been strongly dependent on trial design. Several studies with dasatinib have been performed in prostate cancer. Araujo (22–24) has argued that the benefit of such agents may be in altering the microenvironment resulting in a cytostatic effect. A clinical trial of a potent SFK inhibitor, sacratinib (AZD0530), using PSA-driven endpoints showed no significant effect (25), but this would be less expected given the role that Fyn and other SFKs likely play in disease progression. Understanding, however, that the events related to Fyn inhibition are likely not cytotoxic and yet potent demands a more contemporary approach to experimental and clinical trial designs to test Fyn-associated hypotheses.

To optimize Fyn-targeted therapeutic approaches, however, it may be necessary to combine agents rationally to maximize effect. Thus, understanding the upstream and downstream signaling partners of Fyn becomes essential in developing new strategies. Here, we identify HGF and MET as activators of Fyn related downstream events. Identifying and understanding interactions with downstream signaling partners such as FAK and paxillin, which we have also described as up-regulated in prostate cancer (1), may open additional therapeutic strategies that can be tested *in vivo* and in future clinical trials.

Fyn is a member of the SFKs up-regulated and germane to prostate cancer progression. It functions to promote not only growth but more importantly, directed chemotaxis, a finding that makes Fyn a putative target for metastasis-directed therapy in prostate cancer and other malignancies where it is overexpressed.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Clinical Cancer Research

Fyn Is Downstream of the HGF/MET Signaling Axis and Affects Cellular Shape and Tropism in PC3 Cells

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SRC family kinase FYN promotes the neuroendocrine phenotype and visceral metastasis in advanced prostate cancer

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Abstract

FYN is a SRC family kinase (SFK) that has been shown to be upregulated in human prostate cancer (PCa) tissues and cell lines. In this study, we observed that FYN is strongly up-regulated in human neuroendocrine PCa (NEPC) tissues and xenografts, as well as cells derived from a NEPC transgenic mouse model. *In silico* analysis of FYN expression in prostate cancer cell line databases revealed an association with the expression of neuroendocrine (NE) markers such as CHGA, CD44, CD56, and SYP. The loss of FYN abrogated the invasion of NEPC cells in response to MET receptor ligand HGF. FYN also contributed to the metastatic potential of NEPC cells in two mouse models of visceral metastasis with NEPC cell lines. The activation of MET was regulated by FYN and this interaction appeared to regulate neuroendocrine (NE) features as evidenced by increased expression of NE markers in cells treated with HGF and decreased expression of such markers in FYN-depleted cells. Thus, FYN is an attractive therapeutic and diagnostic target in NEPC and provides further support for ongoing clinical trials of SFK and MET inhibitors in castration-resistant PCa patients.

Keywords:
SRC kinase, MET kinase, NEPC, Metastasis, Molecular target, Molecular and Cellular Pathology

Abbreviations

AdCa- adenocarcinoma

AR- Androgen receptor

$ARCaP_M$ – Androgen refractory cancer of the prostate, cell derivative with mesenchymal phenotype

CCLE- Cancer cell line encyclopedia

CHGA- Chromogranin A

CHGB- Chromogranin B

CSMC- Cedars-Sinai Medical Center

DAPI- 4'6-diamidino-2-phenylindole

EGFR – Epidermal growth factor receptor

GEO- Gene expression omnibus

H&E-Hemotoxylin and eosin staining

HGF – Hepatocyte growth factor

IHC- Immunohistochemistry

IRB- Institutional review board

mQDL- Multiplexed quantum dot label

NE – Neuroendocrine

NEPC – Neuroendocrine PCa

NSE- Neuron specific enolase

PCa – Prostate cancer

PDX- patient derived xenograft

PIN- Prostatic intraepithelial neoplasia

PSA- Prostate specific antigen

QD- Quantum dot

SFK- SRC family kinase

SCG3- Secretogranin 3

SYP- Synaptophysin

VEGFR – Vascular endothelial cell growth factor

Introduction

Over 90% of prostate cancers (PCa) occur in the form of adenocarcinomas, which are characterized by dysregulated growth of the epithelial cells that typically secrete prostate specific antigen (PSA). Many of these are tractable when treated with currently available therapies even though nearly every PCa contains a subpopulation of neuroendocrine (NE) cancer cells scattered throughout the tumor that make up 1% or less of the total tumor volume [1, 2].

In some cases of PCa, patients exhibit a clinical phenotype dominated by NE behavior. These NE prostate cancers (NEPCs) do not typically express androgen receptor (AR). Because PSA is a target gene of AR, patients with NEPC typically have very low serum PSA concentrations. Clinically, NEPCs exhibit aggressive metastatic properties leading to disease spread to visceral organs such as the liver and lung. This pattern of clinical behavior has been strongly associated with shortened overall survival [3, 4]. NEPC is distinguished by the expression of markers including chromogranin A (CHGA), chromogranin B (CHGB), synaptophysin (SYP), CD44, and CD56. Since the introduction of next-generation AR-inhibitors, there appears to have been an increase in the incidence of NEPC, which is thought to arise during the development of resistance. NEPCs are typically treated with cytotoxic chemotherapy with platinum-containing regimens, but these therapies are non-curative and relatively toxic. As such, they represent an urgent and unmet clinical and translational problem.

We have determined that the FYN kinase (one of the nine identified SFKs) is overexpressed in PCa [5-7]. Our published studies have shown that FYN plays an important role in cellular motility in cancer [6], particularly when driven by hepatocyte growth factor (HGF), which is found in abundance in the plasma of patients with both acinar prostatic adenocarcinomas and NEPC [6, 8, 9]. Data from our group and others have demonstrated particular importance of FYN and other SFKs in later events in PCa progression. However, these studies did not directly address the role of FYN in NEPC. The role of SFKs, particularly the FYN kinase, in NEPC has not been characterized. *Fyn* knockout mice develop neurological defects such as blunted long-term potentiation (LTP), impaired special learning, and altered hippocampal development, suggesting a neuronal role for Fyn kinase and a potential role in cancers that have NE features [10]. Recent evidence suggests that nerves innervate the prostate microenvironment in

unique fashion. Moreover, there is evidence to show that neuronal cells and endocrine factors promote tumor generation and progression of NEPC [11].

In the present study, Fyn kinase expression was associated with neuroendocrine biomarkers in PC3 cells and PCa liver metastasis derived cells. In vitro and in vivo data demonstrate that FYN promoted the invasion and metastasis of NEPC cells. Together, these data highlight the importance of FYN and its interaction/activation partner MET in the regulation of NE markers, NEPC invasion and metastasis.

Methods and Materials

Cell lines and reagents

PC3 cells were a generous gift from Dr. Carrie Rinker-Schaeffer. TRAMPC2 and LNCaP cells were purchased from ATCC and $ARCaP_M$ obtained from Novicure Biotechnology. PC3 and $ARCaP_m$ cell line variants were generated as previously described [6]. Antibodies used for Western blot analysis or multiplexed quantum dot (QD) labeling (mQDL) were: anti-FYN antibody (Cell Signaling #4023 or Santa Cruz #SC16), phospho-MET and total MET (Cell Signaling #3077, #4560). Anti-CD44, (sc-7297); anti-FYN (MAB8900 clone 1S); anti-CD56 (sc-7326); anti-CHGA (sc-13090), anti-SYP (sc-17750), all obtained from Santa Cruz. Other common reagents for mQDL were used as described previously [12, 13]. Recombinant HGF was purchased from Calbiochem.

Cell culture

TRAMPC2 and $ARCaP_M$ and cells were cultured in T-medium (GibcoBRL) supplemented with 5% heat inactivated fetal bovine serum (FBS; Omega Scientific, Inc). PC3 and LNCaP were cultured in RMPI 1640 with 10% FBS. Each had 50 IU/mL penicillin and 50 µg/mL streptomycin (GibcoBRL) in 5% CO_2 at 37°C. All cells were negative for mycoplasma contamination (MycoAlert Mycoplasma Detection kit from Lonza).

Lentiviral transduction

FYN-altered lines were generated as previously described by our group [6]. In brief, PC3 and $ARCaP_m$ cell lines were transduced with lentivirus with an shRNA targeted against *FYN* (FYN-) or a GC-content matched, non-targeting shRNA control (NT), each containing a puromycin resistance gene. Lentiviral preparation and transduction of cell lines were performed as per the manufacturer's instructions (Sigma Aldrich, St. Louis, MO). Cells were selected in puromycin (for FYN shRNA) before experiments were performed. A rescue/overexpressing PC3 line was created using another lentivirus containing a *FYN* construct with a silent mutation to avoid the shRNA effect from knockdown named PC3 FYN- SIL. The corresponding empty vector control for the PC3 FYN- background was called PC3 FYN- EV. Both constructs contained a blasticidin resistance gene. TRAMPC2 cells were transduced with a retroviral vector with

RANKLgene cloned into the pIRES-GFP-Puromycin vector and cells were selected for puromycin expression.

Growth, invasion and migration assays

$ARCaP_M$ were grown in 12 well plates and counted on day 4. PC3 NT and FYN invasion assay was performed in the BD BioCoat tumor invasion system (24 multi-well plate with 8 µm; BD Biosciences) according to the manufacture's instructions. ARCaPm NT and FYN were starved on T medium 0.1% BSA. $1x10^5$ cells were applied to chamber well 8-micron. The bottom chamber contained medium with or without 50ng/ml of rhHGF. Cells were incubated for 48h and the cells that had invaded and attached to the lower surface of the membrane was fixed and stained with hematoxilin.

Quantitative PCR

Total RNA was isolated from confluent monolayers of cells using the RNeasy Mini Kit (Qiagen). RNA was converted to cDNA using iScript cDNA Synthesis kit (Biorad). Messenger RNA expression levels were determined real time PCR assay and SYBR Green Dye (Applied Biosystems), and mRNA expression was normalized to *Gapdh*. The fold change in transcript expression was calculated over the expression of NT cells. Primers were designed and synthesized at Integrated DNA Technologies.

In vivo *metastasis assay*

The bioluminescent human PC3 and mouse TRAMPC2 prostate carcinoma cell lines were generated by stable retroviral transduction of MSCV-Luc-Hygro vector. PC3 NT or PC3 FYN- cells (0.5×10^6) were injected via intracardiac route into SCID mice (Strain Code: 236, Charles River Laboratories). The TRAMP2 control and TRAMPC2-RANKL cells were injected via intracardiac route into C57BL/6 mice (Jackson laboratories). Briefly, cells were injected into the left ventricle of the heart as an experimental metastasis model. Mice were monitored on a weekly basis by bioluminescence imaging for *in vivo* growth of tumors and metastasis after injection for 4 weeks. For histological analysis, excised lung samples were fixed with 10% buffered formalin and processed using routine histological techniques. Tissue sections were stained with H&E. The Cedars-Sinai Medical Center Institutional Animal Care and Utilization Committee approved all protocols regarding animal procedures and care.

Histopathology and Multiplexed QD Labeling (mQDL)

We performed mQDL procedures, multi-spectral image acquisition, signal unmixing and quantification as described in our published protocol [13]. The cell or tissue specimens were subjected to sequential labeling of CD44, FYN, CD56 and CHGA or SYP as follows: 1) ant-CD44 (1:100), QD655 (1:100), 2 hr at 37˚; 2) anti-FYN (1:50), QD605 (1:100), overnight at 4˚; 3) ant-CD56 (1:50), QD625 (1:150), 2 hr at 37˚; and 4) anti-CHGA or SYP (1:100), QD585 (1:100) overnight at 4˚ and completed with 4'6-diamidino-2-phenylindole (DAPI) mounting (Vector Laboratories) for imaging. Negative controls were performed in parallel by replacing the species- and dilution matched immunoglobulin subtypes applied to an immediately adjacent tissue section. Image acquisition and deconvolution or unmixing, signal quantification, and statistical analyses were performed as described previously [12, 13, 15, 16]. Human tissues analyzed in this study were collected and characterized under CSMC IRB-approved protocols after obtaining informed consent in compliance with the Declaration of Helsinki. Usage of clinical specimens was approved by the Institutional Research Board (IRB# Pro00025216).

Correlation analysis of FYN and NE markers in human PCa

To compute Spearman's correlation coefficients between FYN and NE markers such as Neuron specific enolase (NSE), Chromogranin A (CHGA), Chromogranin B (CHGB), Aurora Kinase A (AURKA), N-Myc (MYCN), and Secretogranin 3 (SCG3). We used global gene expression profiles derived from human PCa tissues. Four independent datasets [15-18] were downloaded from Gene Expression Omnibus (GEO) database. These datasets were selected by the criteria that each dataset contains more than 50 samples of primary PCa. The intensities in each dataset were normalized by quantile normalization method [19]. Given the normalized intensities of the whole genes in the dataset, the intensities of FYN, NSE, CHGA, CHGB, AURKA, MYCN, and SCG3 in primary PCa samples were extracted and utilized to compute Spearman's correlation coefficients.

Statistics

All analyses were performed using SPSS version 17.0 for Windows. Comparisons between control and experimental groups were made using t test. A 2-sided $P < 0.05$

was used as a threshold for declaring statistical significance. For *in vivo* metastasis studies, Kaplan-Meier Survival Curve Analysis was performed using the PRISM software to assess differences in the survival between the two groups of mice.

Results

FYN is overexpressed in NEPC cell lines and tissues

Our previous studies identified that FYN expression is increased in PCa [6] although FYN kinase is typically associated exclusively with neuronal activity. This observation led us to hypothesize that FYN expression might be detectable in a subset of PCa with NE features. Accordingly, Huang and colleagues have reported that the PC3 cell line is a bonafide prostatic small cell carcinoma with NE features [20]. In the present study, we examined PC3 cells for FYN expression and observed that PC3 cells have greater expression of FYN compared to LNCaP cells (a more acinar or non-NE cell line) consistent with our previous published observations [6] (**Figure 1A & B**). FYN expression correlated with the expression of markers of NE differentiation (**Figure 1A & B**) and QD analysis of human PCa patient tissues expressing NE markers including CHGA, CD44, CD56, and SYP confirmed co-expression of FYN (**Figure 1C & D**). In particular, FYN expression was approximately 4-fold higher in NEPC patient tissues compared with a standard adenocarcinoma. Together, these observations suggested that there was a strong correlation between FYN and NEPC.

FYN expression is associated with NE marker expression in PCa

We next examined whether FYN expression was associated with NE tumor marker expression lines cataloged in the Cancer Cell Line Encyclopedia (CCLE, http://www.broadinstitute.org/ccle). Analysis of mRNA expression across the CCLE lines revealed that was expressed at higher levels in the cell lines derived from the tumors such as neuroblastoma, small cell lung cancer, and medulloblastoma. Although the PCa cell lines included in the CCLE were characterized with low expression of FYN when compared to most of the NE cell lines, this was not unexpected as the majority of cell lines used in PCa research are of an acinar adenocarcinoma phenotype. However, NCI-H660 cells (a well-defined NEPC cell line [21, 22]) showed the highest expression of FYN and PC3 showed third highest expression among the 8 PCa cell lines in CCLE (**Figure 2A**). The correlation between FYN and NE markers including NSE, CHGA, CHGB, AURKA, SCG3, and MYCN was next analyzed using gene expression profiles obtained from four public datasets [15-18]. All NE markers showed significant correlation with FYN in at least one of the datasets (**Figure 2B**).

***FYN regulates growth and invasion of NEPC cells* in vitro**

The impact of FYN on the growth and invasive potential of PCa cells was determined using both MTS and standard Matrigel invasion assays. To perform these assays, we generated PCa cell lines in which FYN was depleted via siRNA targeting approaches (**Figure 3A** and **3C**). We first analyzed the role of FYN in the proliferation index of $ARCaP_m$. The lack of FYN impairs the full ability of the cells to proliferate when compared with NT cells (intact FYN control) (**Figure 3B**), however no significant differences were found. In addition, we observed a decrease in invasive capacity in response to HGF-stimulation for the FYN depleted cells as compared to their corresponding controls (**Figure 3B** and **3E**). Thus, we demonstrate that FYN activation regulates PC cell invasion not only in PC3 cells but in $ARCaP_m$ cells as well.

***FYN promotes visceral metastasis of NEPC cells* in vivo**

On the basis of the above *in vitro* work and our previous studies [6], we hypothesized that FYN depletion would reduce the metastatic potential in tumor cells. To address this hypothesis, Luciferase-tagged PC3 NT and PC3 FYN- were introduced into SCID mice via intracardiac injection and tracked periodically by bioluminescent imaging. Mice injected with PC3 FYN- cells developed fewer metastatic lesions and exhibited increased survival when compared to mice injected with PC3 NT cells (**Figure 4A & B**). Mice that were inoculated with PC3 NT cells became extremely moribund and had to be sacrificed at earlier time points as shown in the Kaplan-Meier Survival Curve Analysis (**Figure 4A**). At 3 after inoculation, we observed an increase in tumor growth in the control group but not in the PC3 FYN- group (Figure 4B). Interestingly, non-osseous metastases were detected by imaging, indicating that FYN expression promoted visceral metastasis. This observation was confirmed at necropsy during which tumors were detected within the lung parenchyma (Figure 4C). Histopathological analysis revealed massive tumor cell infiltration in the lungs of mice inoculated with PC3 NT cells with near loss of lung architecture (**Figure 4D**). Conversely, the lung architecture was intact in mice that received PC3 FYN- cells suggesting that FYN expression in NEPC cells was responsible for metastatic colonization (**Figure 4D**).

In a parallel study, we stably expressed RANKL, a bone tropic factor in TRAMPC2 cells [23-27] derived from the TRAMP model of murine PCa (TRAMPC2-RANKL). Protein and mRNA expression data showed that TRAMPC2-RANKL cells overexpress FYN (**Figure**

4E & F), and inoculation of this tumor line via the intracardiac route led to metastasis and lethality in immune intact C57BL/6 mice (**Figure 4G-H**). Histological analysis showed that the tumor load was much greater in the lungs of mice that received TRAMPC2-RANKL compared to the control group. (Figure 4I). Thus, these results demonstrate that FYN is highly expressed in NEPC cells and appears to dictate the propensity to PCa to metastasize to visceral organs including the lung.

FYN activates MET in response to HGF stimulation and regulates expression of NE markers in PCa cells

Recognizing that HGF is a growth factor found in excess in the plasma of patients with NE cancers, and the observed *in vitro* phenotypes of HGF-stimulated PCa cells (see **Figure 3C**), the impact of FYN on MET activation was next examined To characterize the relationship between FYN and MET using FYN-manipulated lines, FYN knockdown was used to suppress MET activation. In fact, PC3 and ARCaP$_m$ FYN- cells have an attenuated ability to phosphorylate MET when stimulated. (**Figure 5A and 5B**). Next, using a PC3 subline with rescued expression of FYN and a control line containing GFP (PC3 FYN- EV), it was apparent that the overexpression of FYN in PC3 cells led to the restoration of MET phosphorylation (**Figure 5C**).

To correlate the FYN/MET signaling axis with the regulation of NEPC markers in PCa cells, we next analyzed the expression of NE markers, CHGA, CHGB, SCG3, SYP, and NSE in PC3 NT and PC3 FYN- cells. In the absence of HGF, FYN regulated the expression of NE markers both *in vivo* and *in vitro*. The addition of HGF to triggers MET phosphorylation (see **Figure 5A**), resulted in a significant increase in all NE markers analyzed. However, this response was suppressed when FYN expression was inhibited in PC3 cells (**Figure 6A-D**). TRAMPC2 cells also express high levels of CHGA and SYP but not NSE (**Figure 6D**). *AURKA* and *MYCN* amplification was identified in the most of NEPCs [28-31]. Interestingly, our findings showed that MYCN and AURKA expression was down-regulated in FYN- PC3 and ARCaPm cells, respectively (**Figure 6E and 6F**). Altogether, these data suggest that the HGF/MET axis activates of FYN-mediated NE expression markers in PCa cells.

Discussion

NEPC is an aggressive subtype of PCa that remains an urgent and growing clinical problem. It is universally recognized as a form of PCa that has a rapidly evolving natural history punctuated by aggressive metastatic features including the appearance of visceral disease. In fact, most patients who develop NEPC survive less than 1 year after diagnosis [30, 32]. The biology of NEPC is a new and growing are of interest. The biological drivers for growth, differentiation, and metastasis have not yet been clearly delineated, though several studies have raised both traditional and non-traditional biomarkers and targets based on the better-studied and more common gastrointestinal forms of neuroendocrine tumors (NETs). These tumors are often identified by the presence of biochemical features indicating neuroendocrine differentiation such as CHGA, CHGB, SYP, and CD56. Non-gastrointestinal forms of neuroendocrine cancer, while bearing these features, do not necessarily have the same biology and hence may not have the same clinical behavior. Understanding the biological drivers of non-gastrointestinal NETs such as NEPC remains an important translational task. Our group has already demonstrated the capacity for FYN to drive metastasis and growth in prostate cancer [6]. The initial and current studies on FYN have focused on its impact on the biology of the PC3 line, which has been characterized as NEPC/SCPC [20]. A better characterization of mechanisms/factors implicated in NE differentiation is likely to lead to the identification of new targets. In the present study, we report the following: (1) FYN is overexpressed in NEPC; (2) knockdown of FYN has reduced the expression of NE markers and metastatic potential of NEPC, and; (3). FYN regulates the activation of the HGF-MET axis via increased expression of NE markers in NEPC.

To date, the standard of care for men with NEPCs remains the use of platinum based chemotherapy. This form of treatment is associated with commonly feared adverse events including nausea, vomiting, alopecia, neuropathy, and cytopenias given its diffuse and potent cytoxic effects. Despite its common use, however, there is little data to support its continued use in this subset of prostate cancers. As such, there continues to be an urgent need for improved therapeutics. At this time, a select few kinase inhibitors have been explored, but to date none have demonstrated clear activity or benefit (such as alisertib). The Src-family kinases (SFKs) have been recognized as promising targets for cancer therapy given their capacity to synergize with other

signaling pathways [6, 7, 33-36]. They have been particularly appealing targets given the availability of well-tolerated agents that can be used to suppress their activation such as dasatinib, saracatinib, and bosutinib. Treatment with SFK inhibitors has been associated with a number of biological effects including reduction of metastatic potential as exhibited in vivo [37]. Early pre-clinical and clinical studies with SFK inhibitors such as dasatinib showed potent biological effects [38, 39]. The subsequent Phase 3 study combining SFK inhibition (using dasatinib) with docetaxel chemotherapy in an unselected pool of men with mCRPC did not yield an improvement in overall survival [23]. This disappointing clinical result is presumably the consequence of two major caveats in the trial design. First, the clinical pairing with docetaxel may not have been optimal since no agent to date has shown the capacity to improve upon the taxane effect [REF- Antonarakis & Eisenberger JCO 2013]. Second, there is tremendous molecular heterogeneity in this patient group. It is likely that were patients with varying degrees of dependence on FYN or other SFKs, thus focusing on a more optimized clinical patient subgroup may lead to a clearer benefit (e.g. providing trastuzumab in a HER-2 negative breast cancer population versus as HER-2 overexpressing population).

Our findings suggest the possibility of a role for FYN inhibition in NEPC growth and metastasis to visceral organs. RNA-sequencing data from NEPC patient tissues and treatment resistant NEPC PDX models reveal specific upregulation of FYN kinase but not c-SRC or LYN [28]. These observations in human clinical samples reveal that FYN could be a potential biomarker and therapeutic target for NEPC. Refining patient selection based on the expression of FYN or related molecular signals (i.e. MET) especially in patients subgroups with NEPC features represents a more refined clinical approach that may have reveal the benefit of FYN inhibition. In addition, combining FYN inhibition with other therapeutics such as cabozantinib might also hold promise in the treatment advanced PCa. According to our results, FYN regulates the expression of MYCN suggesting that a combinatory therapy strategies targeting MYCN, FYN, and/or MET activation. Clearly, future experiments are required performed to elucidate the direct molecular mechanisms that drive NEPC activation and metastasis.

In summary, our studies demonstrate that FYN plays an important role in NEPC metastasis and progression in a xenograft tumor model. Our findings of FYN expression and function in NEPC are timely given and assume great significance due to the

reported frequency of 25% of NEPC in advanced PCa patients who have de novo or emerging resistance to next-generation AR-targeted therapies. Thus, our findings demonstrate that FYN is an important biomarker and therapeutic target worthy of further exploration that may reshape care for men in NEPC.

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Conflict of Interest:

The authors declare no conflict of interest

Figure legends

Figure 1. FYN kinase co-expressed with neuroendocrine biomarkers in primary PCa with neuroendocrine phenotype and in PCa liver metastasis.

Analysis of NE markers (CD44, CD56, and CHGA) and Fyn in cell and tissues were performed by multiplexed quantum dot labeling method. **A)** metastatic human PC-3 cells expressed higher Fyn, and NE markers than the indolent LNCaP cells. **B)** showed the relative fluorescence quantification of FYN and NE biomarkers in PC-3 and LNCaP cells. **C & D)** Biopsy samples from patients with neuroendocrine PCa: (**C**) primary tumor (**D**) pleural biopsy.

Figure 2. Association analysis of FYN expression with NE phenotype. **A**) Bar graphs showing normalized intensities of FYN in 8 PCa cell lines characterized in CCLE. **B**) Heatmap shows Spearman's rho of FYN with expression of NE biomarkers in four independent datasets derived from human PCa tissues.

Figure 3. FYN promotes invasion of PCa cells in vitro in response to HGF stimulation**. A-B**) Immunoblot and RT-PCR assays of FYN protein expression in PC3 and $ARCaP_m$ variants confirming decreased expression of FYN in the knockdown line. **B**) 4-day growth curves comparing $ARCaP_m$/FYN- to $ARCaP_m$/NT. Error bars represent standard error of the mean with 3 replicates for each day. **C-D**) Matrigel invasion assays of PC3 (NT and FYN-) at 16 hours or $ARCaP_m$ (NT and FYN-) at 48h post stimulation with or without 50 ng/ml of HGF. Following incubation, the cells that had invaded and attached to the lower surface of the membrane were fixed and stained with hematoxilin. Cells numbers were counted in 4 different randomly chosen microscope field per membrane and analyzed using Image J software. Duplicates were performed. Data are representative of two independent experiments. *$p<0.05$, when PC3 FYN- was compared with PC3 NT (both groups under HGF stimulation). ***$p<0.0001$ when $ARCaP_m$ NT cells with no HGF was compared with $ARCaP_m$ NT +HGF. #$p<0.0001$ when $ARCaP_m$ NT + HGF were compared with $ARCaP_m$ FYN- under HGF stimulation.

Figure 4. FYN promotes invasion and metastasis of NEPC cells **in vivo.** Luciferase tagged PC cells were introduced into SCID mice via intracardiac injection. A) Kaplan-Meier survival curve of mice (n = 5/group) injected with PC3 NT and PC3 FYN-. B)

Bioluminescent signal of PC3 NT and PC3 FYN- at 4 weeks after intracardiac injection. C) Necropsy analysis and D) Histological analysis of lungs from PC3 NT and PC3 FYN- intracardiac injections in SCID mice. (panels C and D). (E) Western blotting and (F) RT-PCR analysis of FYN expression of TRAMPC2 -Control and TRAMPC2-RANKL cells *p<0.05. G) Kaplan-Meier survival curve of C57BL/6 mice (n = 5/group) injected with TRAMPC2 and TRAMPC2-RANKL cells. I) Bioluminescence signal and (I) H&E sections of lungs from mice injected with TRAMPC2 and TRAMPC2-RANKL cells at day 30 post-injection.

Figure 5. FYN promotes MET activation and phosphorylation in NEPC cells. **A**) Western blotting analysis shows the phospho-MET profile of PC3 NT and PC3 FYN- cells after 20 minutes of HGF stimulation. **B**) phospho-MET profile of PC3 FYN- EV, PC3 FYN- SIL cells without HGF stimulation.

Figure 6. FYN/MET signaling regulates the expression of NE markers in PCa cells. **A-C**) RT-PCR analysis of NE markers (*chga*, *chgb,* and *scg3*) in PC3 NT and PC3 FYN- cells (+/- HGF stimulation). **D**) Protein expression of CHGA, SYP, and NS3 in PC3 NT (+/- HGF), PC3 FYN- (+/-HGF), and TRAMPC2 cells. **E**) RT-PCR analysis of MYCN expression in PC3 NT and PC3 FYN- cells **F**) RT-PCR analysis of AURKA expression in $ARCaP_m$ NT and $ARCaP_m$ FYN- cells with or without HGF. *p<0.05, **p<0.01 when FYN- was compared with NT.

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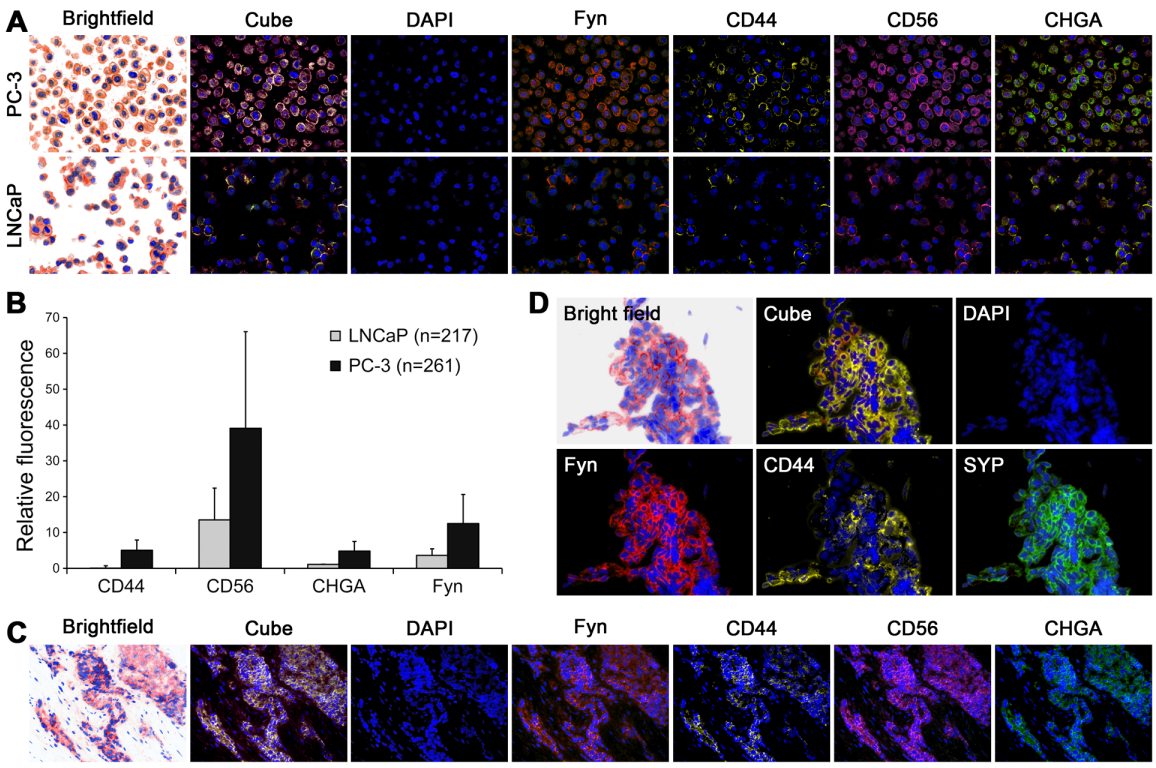
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Cube
DAPI
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CD44
CD56
CHGA
PC-3
LNCaP
B
Relative fluorescence
LNCaP (n=217)
PC-3 (n=261)
CD44
CD56
CHGA
Fyn
C
Brightfield
Cube
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CD56
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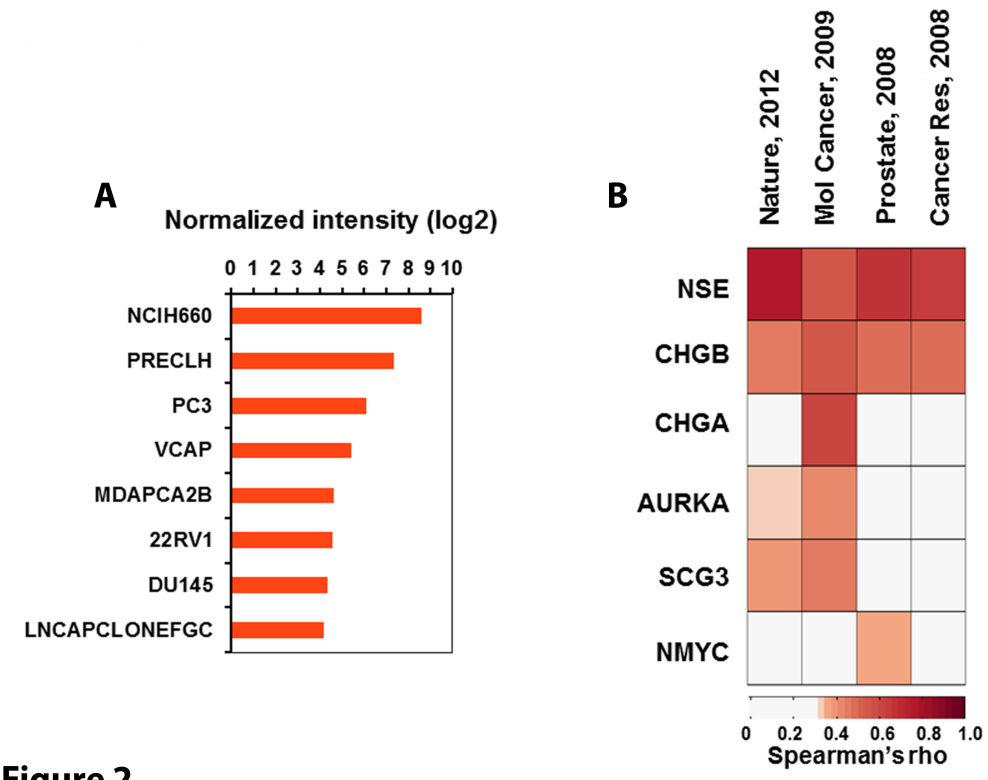
D
Bright field
Cube
DAPI
Fyn
CD44
SYP

Figure 1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Normalized intensity (log2)
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PRECLH
PC3
VCAP
MDAPCA2B
22RV1
DU145
LNCAPCLONEFGC
B
Nature, 2012
Mol Cancer, 2009
Prostate, 2008
Cancer Res, 2008
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CHGB
CHGA
AURKA
SCG3
NMYC
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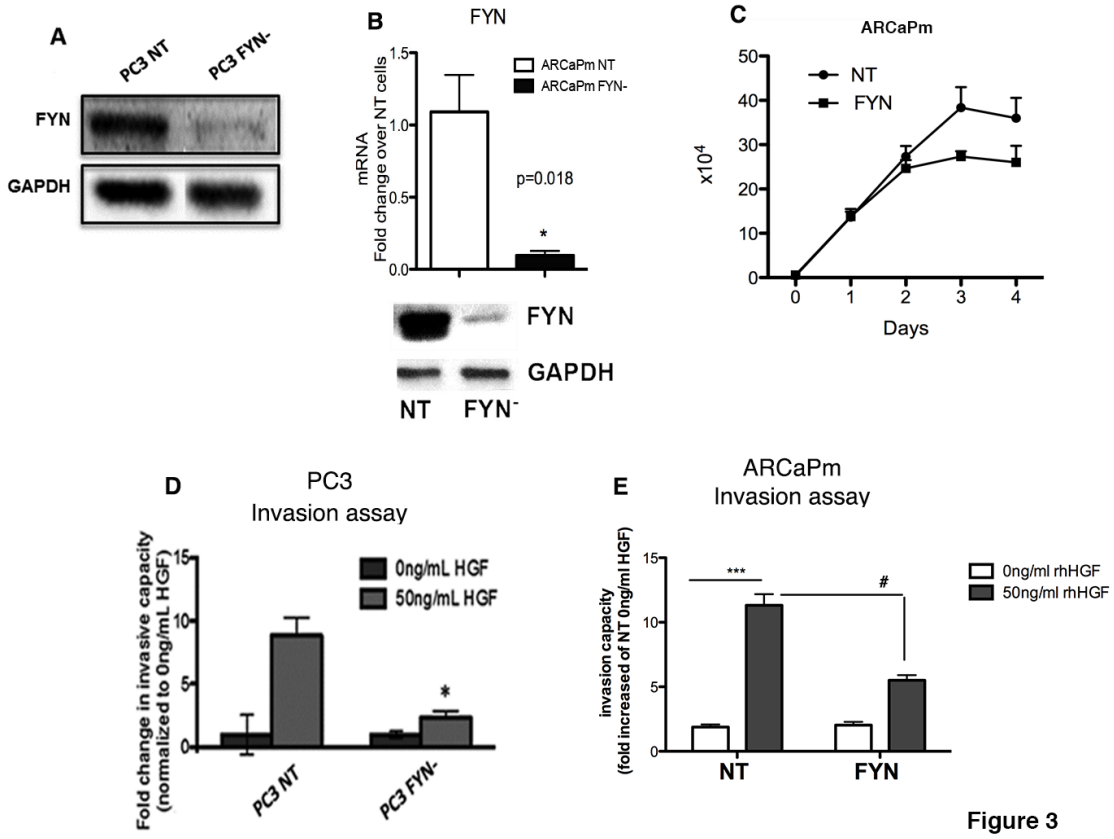
Spearman's rho

Figure 2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PC3 NT
PC3 FYN-
FYN
GAPDH
B
FYN
ARCaPm NT
ARCaPm FYN-
mRNA
Fold change over NT cells
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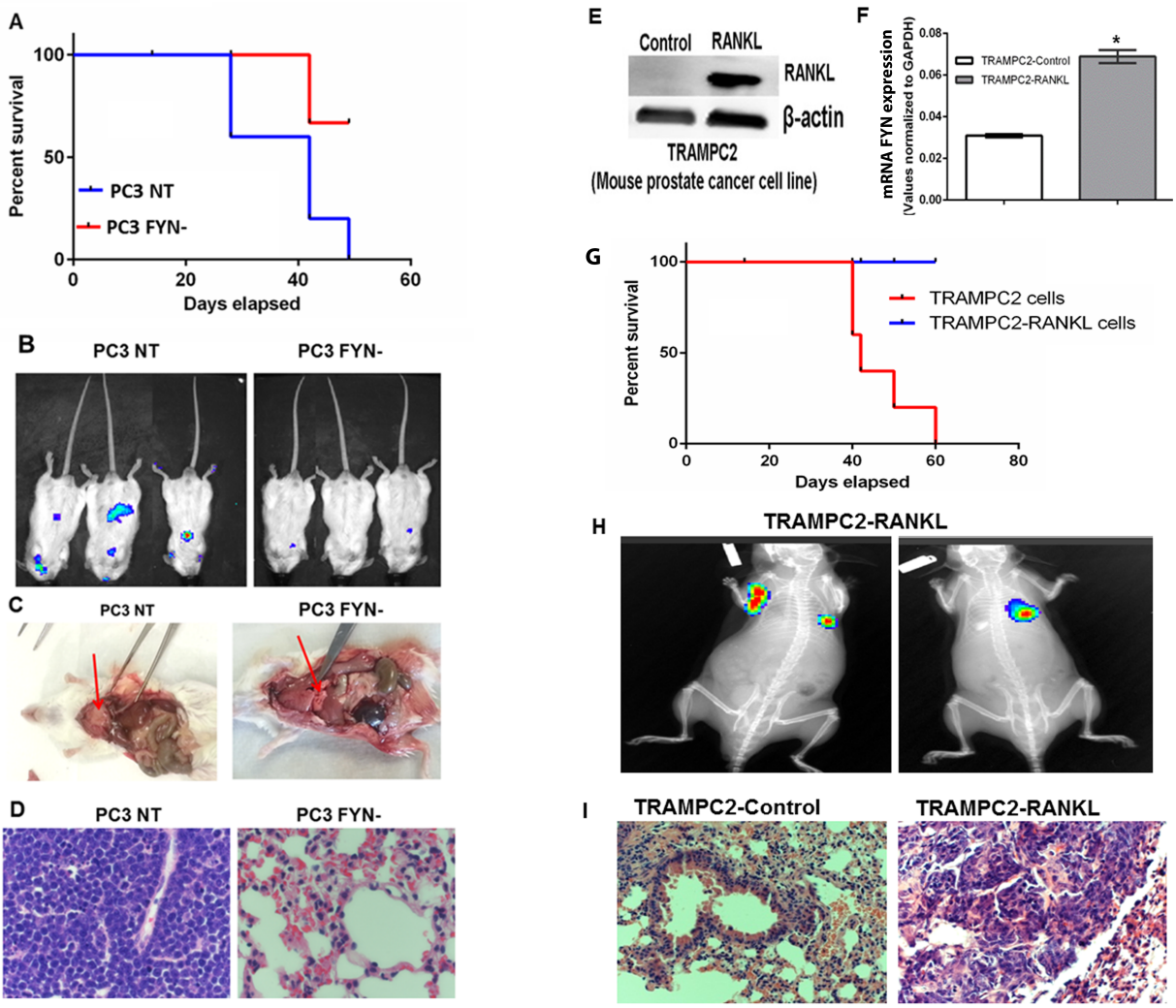
1.0
0.5
0.0
p=0.018
*
FYN
GAPDH
NT
FYN-
C
ARCaPm
NT
FYN
x10^4
50
40
30
20
10
0
0
1
2
3
4
Days
D
PC3
Invasion assay
0ng/mL HGF
50ng/mL HGF
Fold change in invasive capacity
(normalized to 0ng/mL HGF)
15
10
5
0
*
PC3 NT
PC3 FYN-
E
ARCaPm
Invasion assay
0ng/ml rhHGF
50ng/ml rhHGF
invasion capacity
(fold increased of NT 0ng/ml HGF)
15
10
5
0
***
#
NT
FYN

Figure 3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Percent survival
100
50
0
PC3 NT
PC3 FYN-
0
20
40
60
Days elapsed
B
PC3 NT
PC3 FYN-
C
PC3 NT
PC3 FYN-
D
PC3 NT
PC3 FYN-
E
Control
RANKL
RANKL
β-actin
TRAMPC2
(Mouse prostate cancer cell line)
F
mRNA FYN expression
(Values normalized to GAPDH)
0.08
0.06
0.04
0.02
0.00
TRAMPC2-Control
TRAMPC2-RANKL
*
G
Percent survival
100
50
0
TRAMPC2 cells
TRAMPC2-RANKL cells
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60
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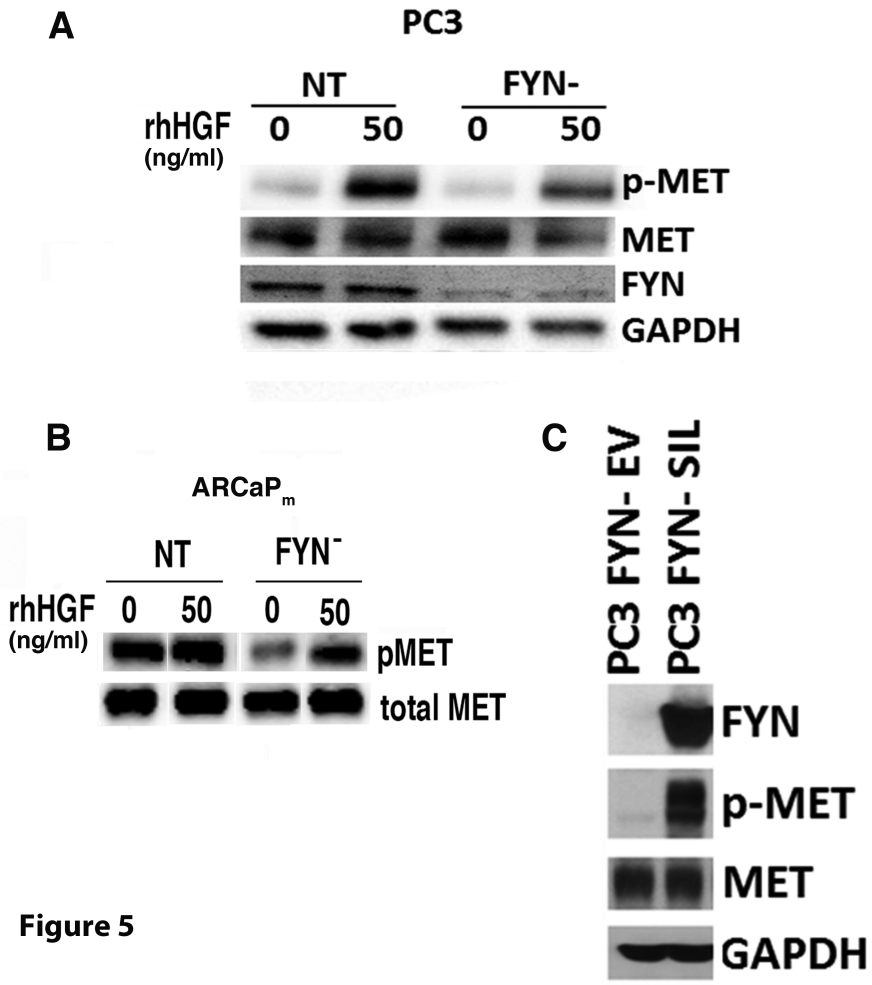
Days elapsed
H
TRAMPC2-RANKL
I
TRAMPC2-Control
TRAMPC2-RANKL

Figure 4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PC3
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FYN-
rhHGF
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50
0
50
p-MET
MET
FYN
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ARCaP$_m$
NT
FYN$^-$
rhHGF
(ng/ml)
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pMET
total MET
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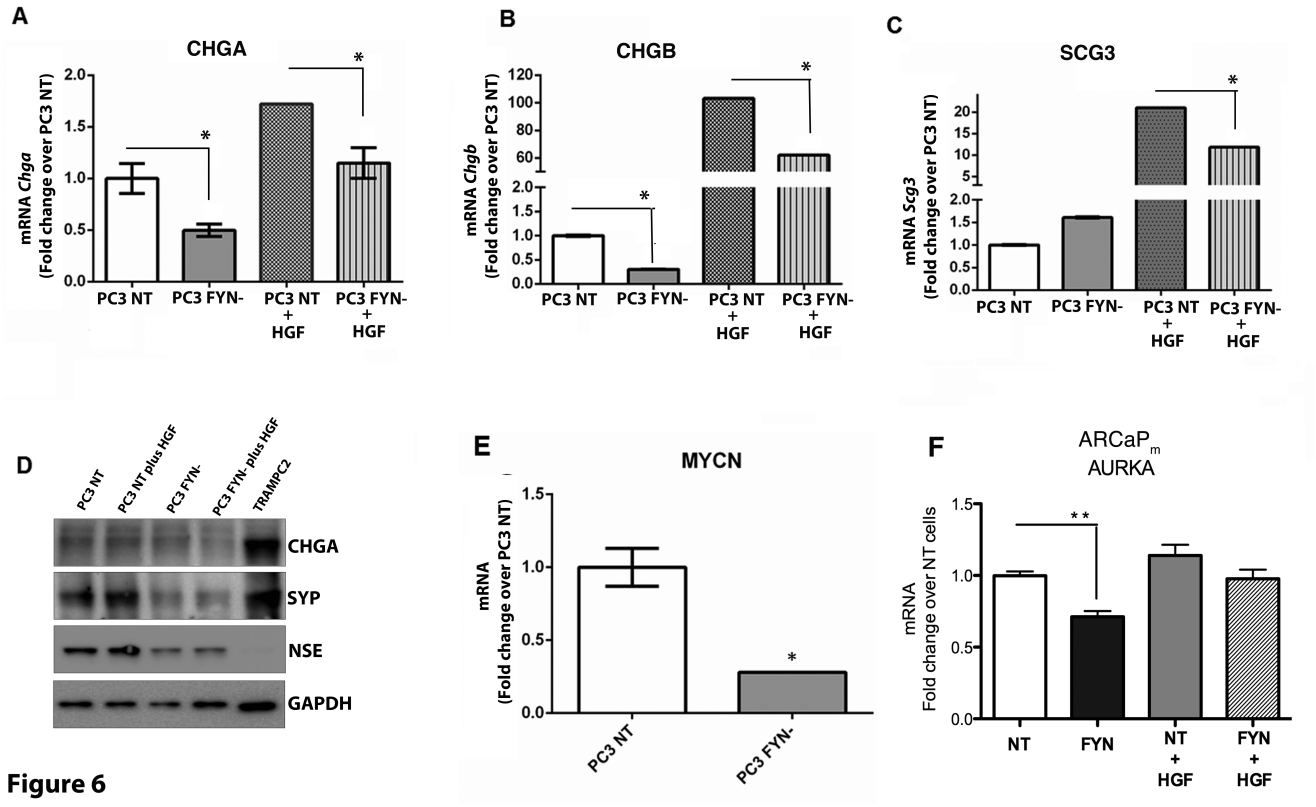
PC3 FYN- EV
PC3 FYN- SIL
FYN
p-MET
MET
GAPDH

Figure 5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CHGA
mRNA Chga (Fold change over PC3 NT)
PC3 NT
PC3 FYN-
PC3 NT + HGF
PC3 FYN- + HGF
B
CHGB
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C
SCG3
mRNA Scg3 (Fold change over PC3 NT)
D
PC3 NT
PC3 NT plus HGF
PC3 FYN-
PC3 FYN- plus HGF
TRAMPC2
CHGA
SYP
NSE
GAPDH
E
MYCN
mRNA (Fold change over PC3 NT)
F
ARCaP$_m$
AURKA
mRNA Fold change over NT cells
NT
FYN
NT + HGF
FYN + HGF

Figure 6

A phase 2 study of saracatinib (AZD0530) as a metastasis inhibitor in advanced metastatic castration-resistant prostate cancer: A University of Chicago Phase 2 Consortium and DOD/PCF Prostate Cancer Clinical Trials Consortium Study.

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Abstract

Background

Fyn, a member of the Src-family kinases, is upregulated in metastatic castration-resistant prostate cancer. Prior studies have noted a relationship between Fyn expression and directional motility. We hypothesized that inhibition of Fyn by Saracatinib would impair successful migration of metastatic tumor cells and thus delay new metastatic lesions.

Methods

Patients with metastatic castration-resistant prostate cancer that had progressed after docetaxel were eligible for enrollment. This study was executed as a randomized discontinuation study. During a lead-in phase, all patients received saracatinib. Afterwards, patients with radiographic stable disease were randomized to either saracatinib or placebo. Patients continued treatment until evidence of new metastasis.

Results

Thirty-one patients were treated on protocol. Only 26% of patients proceeded to randomization. Of the 23 patients that did not proceed to randomization, only 7 patients (30%) had evidence of new metastasis by the end of treatment. The majority of patients who progressed after the lead-in phase exhibited expansion of existing lesions or clinical decompensation.

Conclusions

This study was unable to determine if saracatinib had potential as an inhibitor of metastatic progression. This may have been due to an erroneous hypothesis, inadequate suppression of Fyn and other SFKs, or an inappropriate study population. Use of this study design with careful monitoring of accrual allowed us to terminate this study early. Metastasis inhibition by saracatinib may still be viable in an earlier disease space, but our study highlights particular challenges including patient selection when using new metastasis as an endpoint.

Introduction

The Src-family kinases (SFKs) have been considered one the most important non-tyrosine kinase signaling groups in multiple cancers including prostate cancer. While much of the work in this field has focused on the prototypical member of the family, Src, there are a total of 9 members including Fyn, Lyn, Blk, Fgr, Hck, Lck. Several of these, especially Fyn and Lyn, have been implicated in cancer progression.

Our laboratory has specifically identified a relationship between Fyn and directional motility in response to chemotactic factors such as hepatocyte growth factor (HGF)[1-3]. Our studies also have shown that knockdown of Fyn results in suppression of tumor cell growth. Other groups have also shown anticancer effects related to inhibition of Src[4-7] and Lyn[8,9]. Saracatinib (AZD0530) is a novel anilinoquinazoline that inhibits activation of most SFKs including Fyn, Lyn, and Src[10]. This preclinical data led us to predict that the pharmacologic inhibition of Fyn and other SFKs with saracatinib would impair successful migration of metastatic tumour cells to a secondary site for colonization. As such we hypothesized that treatment with saracatinib would increase the time required to develop new metastatic lesions.

Given the contemporary understanding of metastatic castration-resistant prostate cancer (mCRPC), we identified the population at greatest risk of new metastatic lesions as the population of patients with existing metastatic disease. Our previous studies suggested that these patients should also have higher

expression levels of Fyn and SFKs as compared to those with non-metastatic or castration-sensitive disease[4].

Like other advanced malignant conditions, we anticipated a considerable degree of heterogeneity in the test population. As such, it was advantageous to apply a strategy to further refine the test population so as to optimize the testing of our hypothesis. We selected the use of the randomized discontinuation trial (RDT) design to meet this need.[11,12]

Herein we report our attempt at conducting a clinical trial in the advanced disease population using new metastasis as a primary clinical endpoint.

Materials and Methods

Patients

Eligible patients had histologically confirmed, progressive metastatic prostate cancer despite castration and docetaxel-based chemotherapy. Progressive disease was defined as new clinical or radiographic metastasis or rising PSA of greater than 1.0 μg/L with at least 2 consecutive rises separated by at least 10 days. Prior chemotherapy, surgery, or radiotherapy needed to administered at least 2 weeks prior to start of the trial. At the time of the study, abiraterone, enzulatamide, and radium-223 were not FDA-approved and available for general use. Other inclusion criteria included Eastern Cooperative Oncology Group (ECOG) score of ≤ 1 and adequate organ function.

Exclusion criteria included concurrent usage of non-FDA approved medications or other investigational agents, allergic reactions to compounds or

chemicals similar to saracatinib, or usage of CYP3A4-active agents that may have potential drug interactions with saracatinib. Other exclusion criteria included a history of pneumonitis, cardiac dysrhythmias, prolonged QTc interval (>480 msec), or unresolved toxicity from previous treatments. Use of bisphosphonates was permitted. All patients provided informed consent in compliance with the declaration of Helsinki to participate on this University of Chicago Institutional Review Board approved protocol.

Treatment and design

The primary purpose of this study was to test the hypothesis that treatment with saracatinib would delay the onset of new metastasis in men with mCRPC. As such, the primary endpoint of the study was time to new metastasis by CT and/or bone scan. This study was executed as a RDT (Figure 1). All patients were required to undergo a lead-in phase of saracatinib alone at 175 mg daily for two 28 day cycles. Only patients who were stable by imaging after the lead-in were randomized to saracatinib or placebo. In this manner, patients whose cancers were clearly not sensitive to saracatinib were excluded from further study. We allowed for crossover at post-randomization progression to enhance protocol accrual.

Randomized patients continued treatment until evidence of new metastasis, clinical decompensation, growth of existing lesions by RECIST criteria, or unmanageable drug-related toxicity. Progression by PSA was excluded since prior studies indicated saracatinib had little effect on PSA.[16]

Clinical assessments were performed every 4 weeks and radiographic assessments were required every 8 weeks. Images were evaluated using RECIST 1.1 criteria. Bone scans were evaluated using a modification of PCWG2 criteria that required at least 2 new lesions to be present to declare disease progression. Dose reductions to 125 mg or 100 mg daily were allowed for excess toxicity that recovered to a grade 1 or better. Patients randomized to placebo had the opportunity to crossover to saracatinib if they were fit to continue treatment. Toxicity on trial was captured using the Common Toxicity Criteria for Adverse events (CTCAE) v 4.0.

Statistical considerations

This study was designed with a full accrual of 125 patients. This sample size assumed a randomization rate of 70% (assuming a 30% loss to rapid progression during the lead-in period). With 88 randomized patients, the study was able to detect a hazard ratio of 1.75 in time to new metastases with a one sided α of 0.1 and 80% power based on a log rank test.

Two early termination rules were applied based on the results observed in the lead-in phase: If after the first 40 patients less than 50% (19 or fewer) of the subjects had been randomized, the study would be reviewed for termination. Also, if after the first 40 patients, more than 90% (36 or greater) of the subjects had shown an absence of disease progression, saracatinib would be considered potentially active and appropriate for evaluation in a larger trial and the current

trial would be terminated (95% confidence interval for progression free would be at least 76%).

Results

Patient accrual and demographics

A total of 33 patients from 8 sites were consented from February 2011 to June 2012. Two patients did not proceed to treatment. Thirty-one entered the lead-in phase of this RDT (Figure 2).

Patient characteristics are summarized in Table 1. The patient population enrolled had a median age of 71 years (range 48-87). Approximately 26% of the patients were ≥ 75 years old. Baseline PSA in the study population was high with the average at 360 µg/L (range 2 – 1480 µg/L). One third of the study population had known visceral metastasis prior to enrollment. More than 80% of the study group had received 5 or more therapeutic treatments maneuvers prior to study enrollment. As noted earlier, all patients had prior docetaxel therapy.

Efficacy analysis

Out of the 31 patients who received saracatinib during the lead in, only 8 patients (26%) were considered to have stable disease after 8 weeks of lead-in therapy and were thus eligible for randomization. Given the low rate of patients

randomized, the study was closed. Details of the entire treatment population are shown in Table 2.

Of the 23 patients who did not proceed to randomization, 12 (52%) had radiographic progression, but only 7 patients (30%) had evidence of new metastasis (Table 3). In comparison to patients who were not randomized, 38% of the randomized patients had radiographic progression- all with new metastatic lesions.

Of the 8 patients who proceeded to the randomization phase of the study, 3 were randomized to saracatinib and 5 were randomized to placebo. None of the patients on placebo chose to cross to saracatinib as the protocol allowed at the time of progression. Those who were randomized to saracatinib had a median duration of stable disease of 18 weeks (range 17-19) and those who received placebo had a median duration of 12 weeks (range 9-17) (p=0.05) No significant declines in serum PSA concentration were noted. All patients randomized to saracatinib remained on therapy until objective radiographic progression: Two patients developed new bone lesions, and one patient with a new soft tissue lesion. The patients randomized to placebo all discontinued treatment prior to objective radiographic progression. Three decompensated clinically from growth of existing lesions, one experienced unacceptable toxicity (see below), and one patient withdrew after randomization due to anxiety about the randomization without report of clinically significant toxicity or clinical/radiographic progression

Toxicity

Toxicities are summarized in Table 4. No grade 4 toxicities were encountered on this study as a result of saracatinib. No patients developed pneumonitis during the course of this study by either clinical or radiographic assessments. There were 3 patients who discontinued for toxicity. During the lead-in phase, one patient discontinued due to grade 2 fatigue and diarrhea and another discontinued due to grade 1 vomiting. A patient who was randomized to placebo after lead-in discontinued due to grade 2 transaminitis, nausea, and diarrhea.

Discussion

Prostate cancer continues to be the most common cancer affecting American men and the second leading cause of cancer death[13]. The transition to CRPC represents an important clinical hallmark that indicates an increased risk of death. Since 2004, the number of treatment options for patients has increased[14-18]. Although these therapeutic breakthroughs are encouraging there remains a need for additional strategies offering benefit with acceptable toxicity. Since metastatic progression precedes end organ failure, metastasis inhibition theoretically is a viable form of treatment.

While most studies of SFKs in general and in prostate cancer specifically have focused on Src, our laboratory has demonstrated that Fyn is the most upregulated SFK in advanced prostatic malignancies. Fyn is known to regulate activity of focal adhesion kinase (FAK) and paxillin- key regulators of cell shape

and motility. Studies from our laboratory have confirmed these phenotypic alterations related to the Fyn kinase utilizing a variety of cell lines, xenograft mouse models and clinical samples [24]. These findings have led us to investigate the potential clinical benefit related to SFK inhibition in prostate cancer. More specifically, our laboratory data led us to hypothesize that the clinical benefit of SFK inhibition would manifest as inhibition of metastatic progression and hence prolonged time to new metastasis.

There is one prior study of saracatinib in CRPC by Lara,[29] whose primary endpoint was response based upon modified PSA response criteria (i.e. a 30% decrease from baseline). As was observed in our study, no PSA response was noted. However various kinase inhibitors have been shown to increase PSA secretion from cells independent of an effect on growth[31-33] adding to concerns that PSA measurements may not optimally reflect clinical benefit.

Our trial was unfortunately unable to detect an effect on metastasis, principally due to the paucity of patients who were randomized. As such, this study was unable to fully test the central hypothesis that inhibition of Fyn and other SFKs by saracatinib would delay the development of new metastatic lesions. There are three possible explanations for our clinical observation. It is possible that the underlying hypothesis was erroneous. It is also equally possible that the suppression of Fyn or other SFKs by saracatinib was not sufficient to alter Fyn/SFK-driven metastatic progression. Finally, the population chosen for study was not preselected by biomarker (e.g. Fyn) expression and may not have been optimal for metastasis inhibition. These were generally older, medically-

fragile, heavily-pretreated, late-stage patients with high tumor burdens (reflected by scans and PSA) when compared to other contemporary mCRPC studies (Table 5). Many had developed visceral metastases which have been associated with poorer outcomes[36-38]. This population was apt to discontinue therapy due to overall clinical decompensation from disease progression even without the development of new metastatic lesions. While the growth of pre-existing lesions was not directly relevant to the molecular hypothesis, ethically it prevented investigators from continuing study treatment.

Another noteworthy aspect of our approach was the use of RDT design. Most assays for measuring Fyn or SFK activation in clinical specimens have not been well validated or standardized: It is unclear what level of target expression and/or activation is required to predict sensitivity to saracatinib. This trial design allowed the drug under investigation to select the population as opposed to an assay that has not been validated. A large number of patients in this study were unable to complete the lead-in phase of treatment due to reasons unrelated to the formation of new metastasis. While this led to premature closure of the study, the RDT design allowed us to stop accrual in an efficient manner while still maximizing patient exposure in a way that may not have been accomplished using a traditional randomized phase 2 trial design with 1:1 or even 2:1 allocation.

Our clinical experience during this study points to the need of patient selection and study design, especially when testing novel biological hypotheses in a highly heterogeneous population. In this advanced and highly

heterogeneous population, the use of new metastatic lesions as a primary endpoint is suboptimal. We would speculate that as the other fields mature, such as that of circulating tumor cells (CTCs), an alternative endpoint may be used. In this case, if SFK inhibition resulted in impaired motility, it may be case that this would result in a notable decline in CTC counts which may correlate with reduced metastatic potential. Moreover, our lab and others have been studying the relationship of solid tumors and CTCs hoping to use them as tissue surrogates. These studies are early but show promise and may one day allow for minimally invasive molecular classification of patients.[39,40].

We continue to propose that metastasis inhibition in advanced CRPC remains a novel trial endpoint that is viable and clinically relevant. Given this particular experience, the methodology of testing study hypothesis must further consider patient factors and drug toxicity in future studies. Given emerging trends in the field and an expanding knowledge of biology and biomarkers of disease progression and metastasis, an earlier disease population (post-op and or non-metastatic CRCPC), and study design may be identified in the future.

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Figure Legends:

Figure 1. Trial Design

Figure 2. Trial Profile

Table 1. Patient Characteristics

Patient Characteristics (n=31)		
Age	**Value**	**Range and Percentage**
Median	71	(48-87)
≥ 75 years	8	(26%)
Ethnic Origin	**Number of Patients**	**Percentage**
White	23	(74%)
Black	7	(23%)
Asian	1	(3%)
Baseline Disease	**Number of Patients**	**Percentage**
Visceral	10	(32%)
None	8	(26%)
Unknown	13	(42%)
Baseline PSA	**Value (µg/L)**	**Range**
Mean	360	
Median	230	(2-1480)
Prior Therapy	**Value**	**Range and Percentage**
Median	6 prior therapies	(3-13)
≥ 5	25 patients	(81%)

Table 2. Patients Enrolled in Trial

Patients Enrolled in Trial (n=31)		
Median Time to Progression	**Weeks**	**Range**
	9	(2-19)
Average rise in PSA	**PSA (µg/L)**	**Range**
	349	(0-1984)
Reason for termination of therapy	**Number of Patients**	**Percentage**
New Metastatic Lesions‡	10	(32 %)
Target Lesion Growth	5	(16 %)
Clinical Progression	11	(35 %)
Toxicity	3	(10 %)
Patient Choice	2	(6 %)
ǂ Six patients with new bony metastasis, two patient with new visceral metastasis, two patients with new visceral and bony metastasis (Percentages may not add to 100 due to rounding)		

Table 3. Comparison of Randomized and Non- randomized Patients

Comparison of Randomized and Non-randomized Patients		
	Patients Not Randomized (n=23)	**Patients Randomized (n=8)**
Median Age (years)	72 (range 48-87)	69 (range 50-73)
≥75 years	8 (35%)	0 (0%)
Mean Baseline Serum PSA (µg/L)	370 (range 2-1480)	330 (range 8-1375)
Baseline Visceral Disease	7 (30%)	3 (38%)
≥ 5 Prior Therapies	18 (78%)	7 (88%)
Mean rise in PSA (µg/L)	400 (range 0-1984)	201 (range 1-627)
Median Time to Progression (weeks)	-	16 (range 9-19)
Radiographic Progression	12 (52%)	3 (38%)
New Metastasis	7 (30%)	3 (38%)
Target Lesion Growth	5 (22%)	0 (0%)
Clinical Progression	8 (35%)	3 (38%)
Toxicity	2 (9%)	1 (13%)
Patient Choice	1 (4%)	1 (13%)
(Percentages may not add to 100 due to rounding)		

Table 4. Patient Toxicities

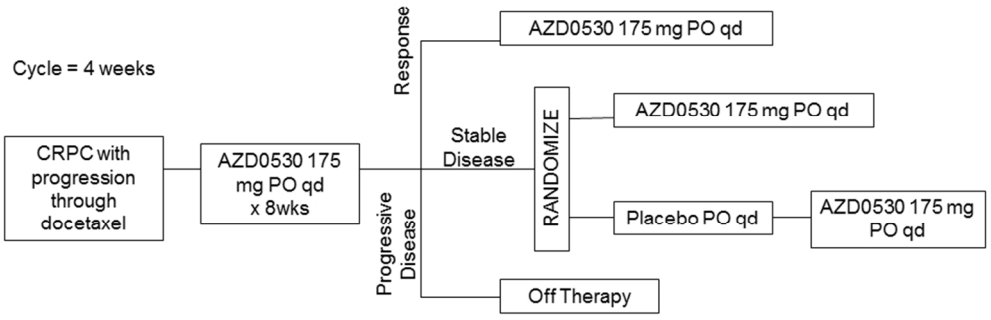
Patient Toxicities				
Adverse Event	**Number of patients with any grade toxicity**	**% of patients**	**Number of patients with grade 3 toxicity**	**% of patients**
Fatigue	10	31 %	2	6 %
CNS Hemorrhage	1	3 %	1	3 %
Dehydration	3	9 %	1	3 %
Thrombocytopenia	3	9 %	1	3 %
Weakness	1	3 %	1	3 %
Anorexia	9	28 %	-	-
Nausea	9	28 %	-	-
Transaminitis	8	25%	-	-
Vomiting	7	22%	-	-
Diarrhea	5	16%	-	-
Anemia	4	13 %	-	-
Constipation	3	9 %	-	-
Renal Dysfunction	3	9 %	-	-
Edema	2	6 %	-	-
Fever	2	6 %	-	-
Flu-like symptoms	2	6 %	-	-
Hematuria	2	6 %	-	-
Leukopenia	2	6 %	-	-
Myalgia	2	6 %	-	-

Table 5. Characteristics of Saracatinib RDT Patients Compared to Previously Studied Patients with mCRPC

Characteristics of Saracatinib RDT Patients Compared to Previously Studied Patients with mCRPC				
Study	**Median Age** (years)	**≥75 years** (average %)	**Baseline PSA** (µg/L)	**Visceral disease** (average %)
Saracatinib RDT	71	26%	360	32%
TAX 327[ǂ]	68	20%	115	23%
Cabazitaxel[Φ]	68	19%	136	25%
Radium - 223[Ψ]	71	29%	160	0%

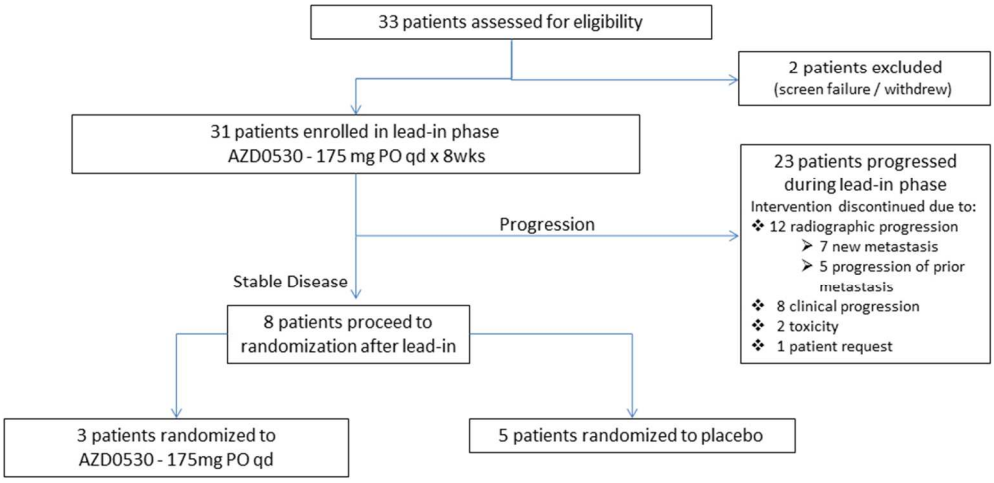
ǂ N Engl J Med 2004; 351:1502-12.
Φ Lancet 2010; 376: 1147–54.
Ψ N Engl J Med 2013; 369:213-23.
Averages were taken for the studies with two treatment arms

Figure 1. Trial Design



Trial Design
254x190mm (96 x 96 DPI)

Figure 2: Trial Profile



Trial Profile
254x190mm (96 x 96 DPI)