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TITLE: **"Epigenetic Control of Prostate Cancer Metastasis: Role of Runx2 Phosphorylation"**

PRINCIPAL INVESTIGATOR: Renny T. Franceschi

CONTRACTING ORGANIZATION: University of Michigan
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6. AUTHOR(S)
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7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
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8. PERFORMING ORGANIZATION REPORT

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13. SUPPLEMENTARY NOTES

14. ABSTRACT

The goal of this project is to determine the role of ERK/MAP kinase phosphorylation of the RUNX2 transcription factor in the metastasis of prostate cancer cells. Major accomplishments were:
a. Demonstration that Runx2 must be phosphorylated at Ser 301 and Ser 319 to stimulate prostate cancer cell gene expression, in vitro cell migration and invasion as well as in vivo tumor growth.
b. Demonstration that anti-Runx2-S319-P antibody is able to specifically stain human prostate tumor samples on tissue microarrays without staining normal prostate tissue or non-cancerous prostate sample (e.g. benign prostate hyperplasia). In addition, this antibody was able to discriminate between invasive, high Gleason score tumors and non-metastatic tumors

These results support our overall hypothesis that RUNX2 phosphorylation is a critical determinant of prostate tumor formation/growth and metastasis and that P-Runx2 is a cancer biomarker and potential therapeutic target.

15. SUBJECT TERMS
prostate cancer, metastasis, epigenetics, phosphorylation, kinase, Runx2, transcription

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Epigenetic Control of Prostate Cancer Metastasis: Role of Runx2 Phosphorylation

Study Site Information:
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1. INTRODUCTION

Bone metastasis leading to the formation of mixed osteoblastic and osteolytic lesions is seen in ~80% of men with metastatic prostate cancer (PCa)(1). Little is known concerning the cellular signals controlling the metastatic behavior of the primary tumor. The presence of the RUNX2 transcription factor in prostate cancer cells has been related to invasive/metastatic behavior(2-5). This project is based on preliminary studies suggesting that metastatic behavior of prostate tumors requires activation of the ERK/MAP kinase pathway, which phosphorylates the RUNX2 transcription factor. Previous work from our laboratory had shown that S301, S319 phosphorylation of RUNX2 is critical for its transcriptional activity in bone(6), and we hypothesized that these sites are also important for RUNX2-dependent metastatic activity. During the first year of this grant, efforts were focused on determining if RUNX2 phosphorylation is related to metastasis-associated cell properties. In addition, preliminary studies were conducted with prostate cancer tissue microarrays to determine if there is a relationship between RUNX phosphorylation levels and tumor outcome. In year 2, we initiated studies related to RUNX2 phosphorylation and epigenic changes in metastasis-associated genes, expanded studies on the role of RUNX2 phosphorylation in stimulating in vitro cell migration and invasion using non-tumorigenic cells, began in vivo metastasis experiments and completed a large tumor tissue microarray study to confirm the association of P-RUNX2 with prostate cancer in human samples. In year 3, we completed a study showing that adenovirus transduction of PC3 cells with wild type RUNX2 stimulated in vivo tumor formation and angiogenesis in immunodeficient mice while phosphorylation-deficient RUNX2 was inactive. During a no cost extension year 4, we completed this work and described our detailed findings in a major paper published in the journal, Oncogene. These studies together with additional preliminary experiments are the basis for several newly submitted grant applications that will further explore the importance of Runx2 and Runx2 phosphorylation in prostate cancer stem cell formation and de novo tumor formation in animal models.

2. KEY WORDS: prostate cancer, phosphorylation, transcription,

3. ACCOMPLISHMENTS

What were the major goals of the project?
What was accomplished under these goals?

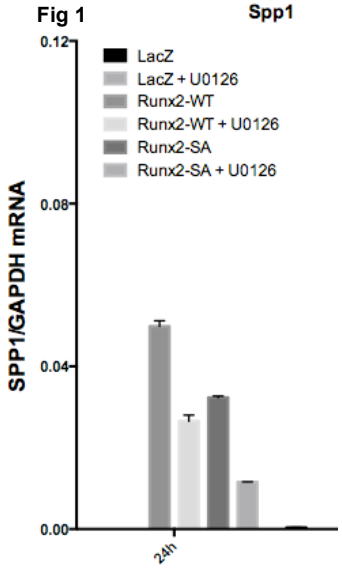
Project Tasks: Accomplishments are listed according to original tasks described in the Statement of Work. Most accomplishments were reported in a major publication: Ge C, Zhao G, Li Y, Li H, Zhao X, Pannone G, Bufo P, Santoro A, Sanguedolce F, Tortorella S, Mattoni M, Papagerakis S, Keller ET and **Franceschi RT**. Role of Runx2 Phosphorylation in Prostate Cancer and Association with Metastatic Disease. Oncogene, 2015- in press http://www.nature.com/doifinder/10.1038/onc.2015.91 (Publication 1 in appendix). This progress report will refer to relevant figures in these papers or additional unpublished data as necessary.

Task 1: Establish the relationship between MAP kinase signaling, Runx2 phosphorylation and transcriptional activity in normal prostate epithelial and PCa cells and determine how Runx2 phosphorylation controls VEGF gene expression. Months 1-36

a. Levels of total and P-ERK, total and P-Runx2 and Runx2-dependent activation of metastasis-associated genes will be compared in normal human prostate epithelial cells, K-ras-transformed prostate epithelial cells, non-metastatic LNCaP cells and metastatic cell lines (PC-3, C4-2B).

Levels of total and phosphorylated Runx2 were compared between highly metastatic (PC3, CB-2B) and non-metastatic prostate cancer cell lines (LnCaP). We found that Runx2 is preferentially expressed in the more metastatic cell lines (PC-3> C4-2B > LNCaP). Furthermore, the ratio of phospho-Runx2/total Runx2 as well as P-ERK/total ERK is greater in PC-3 and C4-2B versus LNCaP cells (Publ. 1 Fig 1)). For this analysis, P-Runx2 was measured using an antibody generated by the project laboratory that specifically detects Runx2-S319-P. As we previously showed, phosphorylation at this site is closely correlated with increased Runx2 transcriptional activity(6,7).

b. Specific inhibitors of ERK/MAPK, Src and PI3K/AKT pathways as well as siRNA inhibition and overexpression of key pathway intermediates will be used to assess the relative importance of each signal transduction pathway in expression of metastatic genes.



The MAP kinase inhibitor, U0126, was shown to inhibit Runx2-dependent gene expression. An example of this is shown in Figure 1. Here, U0126 inhibited Runx2-dependent induction of the metastasis-associated gene, Spp1, by 50%. Partial inhibition was also seen for gene expression induced by a Runx2 mutant containing Ser to Ala mutations in two previously characterized phosphorylation sites at S301 and S319 (Runx2-SA). This indicates that additional MAPK phosphorylation sites may be involved. In addition, we showed that U0126 could block Runx2-induced cell migration (see Task 2c). Other workers showed that the PI3K/AKT pathway is also important for metastatic gene expression(8). For this reason, we did not pursue studies with other pathway inhibitors.

c. Runx2-responsive regions of the proximal Vegf promoter will be identified using deletion/mutation analysis, functional assays and chromatin immunoprecipitation (ChIP).

In separate studies with bone cells not supported by this project, we identified Runx2 binding regions of the Vegf gene at -855, -606 and -266bp upstream of the translation start site using chromatin immunoprecipitation analysis (9).

d. ChIP assays will be used to resolve whether binding of P-ERK to Vegf chromatin requires bound Runx2.

Because we previously established that Runx2 binding sites are present in Vegf(9) and that Runx2 is required for P-ERK binding to chromatin of other genes such as Bglap2 (osteocalcin) and Ibsp (bone sialoprotein)(10), we decided to not repeat this work with Vegf.

e. Use wild type Runx2, S301A/S319A (non-phosphorylated) or S301E/S310E (constitutively active) Runx2 mutants to determine whether Runx2 phosphorylation is necessary for histone phosphorylation, acetylation and activation of Vegf transcription.

In separate studies, the role of Runx2 phosphorylation in control of chromatin epigenetic changes was examined during osteoblast differentiation. Because of time constraints, we were not able to repeat these studies in PCa cells. However, the bone work which is being submitted for publication clearly showed that MAP kinase-dependent Runx2 phosphorylation was necessary for transcription-related epigenetic modifications in chromatin including histone methylation and acetylation.

Task 2: Determine if Runx2 phosphorylation is necessary for *in vitro* and *in vivo* proliferative, invasive and metastatic behavior of PCa cells stably transfected with wildtype Runx2 or Runx2 phosphorylation site mutants. Months 13-36

a. MLV-based retrovirus vectors will be developed that express β-galactosidase (negative control), wild type Runx2, S301A/S319A (non-phosphorylated) or S301E/S310E (constitutively active) Runx2 mutants. Retrovirus were constructed to stably introduce wild type and mutant RUNX2 into cells. Because of poor

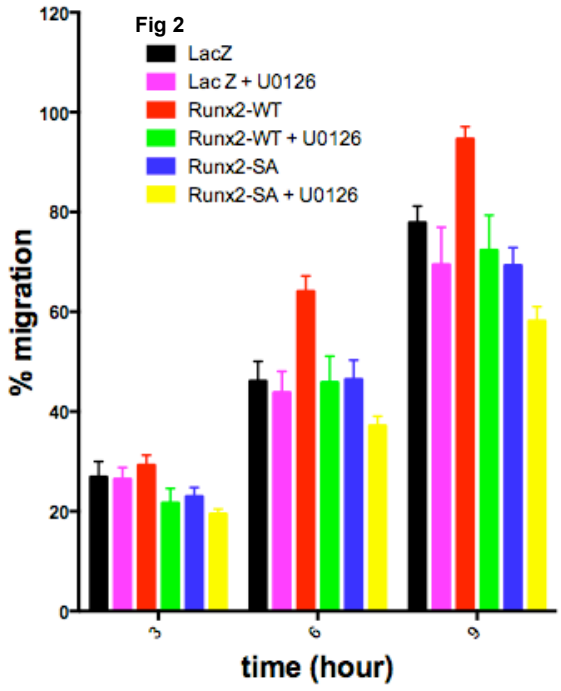
transduction efficiency seen with retroviral vectors, we recently constructed lentivirus vectors, which are able to transduce cells with higher efficiency. These vectors are being used to develop stable cell lines using LNCaP and PC3 cells that are in the process of being characterized. Once this step is completed, these cells will be used to examine the effect of Runx2 phosphorylation on long-term tumor cell survival and metastasis to bone and other tissues in vivo. Although it was not possible to complete these studies with support from the present grant, we still intend to complete them in the coming year using other support mechanisms.

As an alternative approach, we also developed adenovirus vectors to express wild type and S301A/S319A mutant RUNX2. These vectors were used for analysis of metastasis-associated cellular activities in tissue culture and tumor growth after in vivo cell implantation (see below).

b. LnCaP cells with low intrinsic invasive/metastatic activity and Runx2 levels will be transfected with retrovirus vectors and stable lines will be isolated expressing β-Gal, wild type Runx2, S301A/S319A or S301E/S310E Runx2 mutants.

Initial attempts to develop stable lines of PC3 and LnCaP cells expressing β-galactosidase (negative control), wild type Runx2, S301A/S319A (non-phosphorylated) or S301E/S310E (constitutively active) Runx2 were not successful. As described above, we are now pursuing the approach of using lentivirus vectors, which can be grown to higher titers and have higher transduction efficiencies. These vector properties will allow us to make stable luciferase-expressing RUNX2 lines of PCa cells having low (LnCaP) and high (PC3) basal metastatic activity as well as non-tumorigenic prostate cells (RWPE1 cells). These cells will then be used to measure the role of RUNX2 phosphorylation on in vivo metastasis, which requires long-term stable expression of the RUNX2 protein.

As an alternative approach to determine if RUNX2 phosphorylation is important for in vitro cell activity and in vivo tumor cell xenograft growth, adenovirus vectors encoding wild type and S310A/S319A RUNX2 were developed and used to show that wild type, but not phosphorylation-deficient RUNX2 could stimulate metastasis-associated gene expression (MMP9, VEGF and osteopontin-OPN), angiogenesis, cell migration and invasion in vitro. Two approaches were used to measure the requirement for MAPK-dependent Runx2 phosphorylation in metastasis-related gene expression (Publ. 1 Fig3ab). First, Meksp, a constitutively- active MEK1 mutant, stimulated Runx2-P and activation of an Spp1-luciferase reporter in PC3 cells while dominant-negative Mekdn was inhibitory. In contrast, Runx2 containing mutations in 2 MAPK phosphorylation sites (S301A,S319A; Runx2 SA) was refractory to MAPK. Second, expression of wild type Runx2 increased endogenous *Mmp9*, *Vegfa* and *Spp-1* mRNAs while Runx2 SA was less active (Publ. P1 Fig3c-j).



c. In vitro migratory activity of stable LNCaP lines will be compared. PC3 and RWPE1 cells were transduced with AdLacZ, AdRUNX2wt or AdRUNX2 S301A/S319A mutant and plated onto glass slides. A central line of cells was removed using a Pasteur pipette and we measured the ability of cells to migrate into the cell-free area. WT RUNX2 clearly stimulated migration of both cell types, while migration of cells transduced with the phosphorylation-deficient mutant RUNX2 was similar to that of control cultures (Publ 1, Fig 4). As shown in Fig 2, the ability of Runx2 to stimulate cell migration also requires MAP kinase activity since the MAPK inhibitor, UU0126, blocked Wildtype Runx2-dependent migration (compare red vs green bars), but not migration stimulated by the phosphorylation-deficient Runx2 mutant (Runx2-SA, blue vs. yellow bars).

d. The in vitro invasive activity of LNCaP lines will be compared using a matrigel invasion assay. Wild type RUNX2 stimulated migration of PC3 and RWPE1 cells across a Matrigel$_{TM}$ membrane, a cell culture model of tumor invasion. In contrast, a phosphorylation-deficient RUNX2 mutant had no activity in this assay (Publ 1, Fig 5).

f. In vivo proliferative activity of LNCaP cell lines will be measured after subcutaneous and intrafemoral implantation into immunodeficient mice. We examined the acute effects of RUNX2 on *in vivo* growth of tumors from implanted PC3 cells. PC3 cells stably expressing a firefly luciferase reporter (PC-luc cells) were transduced with LacZ, Runx2-WT or Runx2-SA adenovirus vectors to give equivalent amounts of Runx2 protein, suspended in Matrigel$_{TM}$ and subcutaneously implanted into immunodeficient mice (Publ P1 Fig 6). Tumor development was monitored over a 16-day period by measuring whole-body luciferase activity. At day 17, mice were sacrificed and tumors were weighed and used for RNA analysis and immunohistochemistry. Wild type Runx2 significantly increased tumor development as measured by *in vivo* luminescence (Publ P1 Fig 6a,c) and tumor size/weight at sacrifice (Fig. 6 d,e). In contrast, tumors formed by cells transduced with the Runx2-SA virus were not significantly different from LacZ controls. Similar to cell culture results, tumors form Runx2-WT-treated cells expressed higher levels of VEGF, MMP9 and SPP1 mRNAs relative to LacZ controls while levels were generally lower with Runx2-SA transduction (Fig 6 f-h).

These studies support our hypothesis that Runx2 phosphorylation is necessary for in vivo tumor formation.

g. In vivo metastatic activity of LNCaP cell lines will be measured using an orthotopic (intraprostate implantation) model.
We were unable to complete these studies due to delays in making stable cell lines expressing wild type and phosphorylation-deficient/phosphorylation-mimetic Runx2 (see 2b above). Now that lentivirus vectors have been successfully constructed, we hope to conduct these studies using other grant funds.

h. In vivo metastatic activity of LNCaP cell lines will be measured using an intracardiac injection model.
We were unable to complete these studies due to delays in making stable cell lines expressing wild type and phosphorylation-deficient/phosphorylation-mimetic Runx2 (see 2b above). Now that lentivirus vectors have been successfully constructed, we hope to carry out these studies using other grant funds.

Task 3: Correlate phosphorylated (S319-P) Runx2, total Runx2 and P-ERK immunoreactivity with tumor outcome using a panel of human PCa tissue microarrays (TMAs) composed of normal prostate tissue, benign prostatic hyperplasia, prostatic intraepithelial neoplasia and prostate cancer. Months 1-15

a. TMAs provided by the Michigan Prostate Center SPORE will be immunostained using total and P-Runx2-specific antibodies as well as total and P-ERK antibodies.
b. Patterns of immunoreactivity will be correlated with tumor history; statistical models will be used to evaluate the predictive values and relationship to clinical parameters for each marker.

To examine the relationship between Runx2 phosphorylation and PCa, we screened a human prostate cancer TMA from 129 patients using our phospho-Runx2 (S319-P)-specific antibody(7). Strong P-Runx2 staining was detected in all stages of prostate cancer including prostate intraepithelial neoplasia (PIN) with little or no staining in normal prostate, prostatitis or BPH. Publ P1 Fig 7 shows representative histology from TMAs. Overall, high P-Runx2 staining was strongly correlated with primary prostate cancer (PPC) when compared with BPH or prostatitis ($p < 0.001$), with very low staining observed in the latter two conditions (Publ. 1 Table 1). Within the PPC cohort, significant correlations were also noted between percent P-Runx2 staining and high Gleason score ($p = 0.047$), primary prostate cancers with lymph node metastases at the time of initial clinical staging ($p = 0.033$) and primary prostate cancer with prostatic intraepithelial neoplasia (PIN, $p = 0.041$). The detection of phospho-Runx2 in PIN suggests this may be an early event in tumor development and an excellent biomarker for early disease (Fig7c). Notably, staining in PIN samples was in both basal and luminal areas (arrow), both of which may contain prostate stem cells(11). P-Runx2 staining is exclusively nuclear indicating that transcriptionally active Runx2 is being selectively detected.

These studies indicated that P-RUNX2 is an excellent biomarker for early and late stages of prostate cancer.

What opportunities for training and professional development has the project provided?
Nothing to Report

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4. IMPACT

What was the impact on the development of the principal discipline(s) of the project?

This project clearly established the following:

1. Phosphorylation of the Runx2 transcription factor is an important regulatory event controlling gene expression, migration, invasion and in vivo growth of prostate cancer cells.

2. Runx2 phosphorylation, measured using a specific antibody to Runx2-S-319-P, can be used to discriminate human prostate cancers from benign prostate hyperplasia and prostatitis. In addition to staining all prostate cancer samples, this antibody shows strongest staining with the most aggressive cancers (High Gleason Score) and cancers that have metastasized to peripheral tissues.

This project has 2 important impacts of our understanding of prostate cancer:

1) In showing that Runx2 and Runx2 phosphorylation control tumor formation, it has identified Runx2 as an important potential target for therapy. Recent progress has been made developing inhibitors of the Runx family of transcription factors that act by preventing dimer formation between Runx proteins and the common partner protein, Cbfβ(12). We are currently evaluating this class of compounds to determine if they can inhibit Runx2-dependent transcription.

2) Our studies showed that P-Runx2, measured with our anti-Runx2-S319-P antibody, is an important prostate cancer biomarker that can discriminate cancer from benign prostate hyperplasia and prostatitis and may be able to detect the most aggressive cancers. We are hoping to do additional studies to determine if P-Runx2 levels in primary tumor biopsies can predict disease progression.

What was the impact on other disciplines?

Nothing to Report

What was the impact on technology transfer?

Several requests were recently made from "for profit" pharmaceutical companies for the anti-Runx2-S319-P antibody we developed. We are currently beginning negotiations to make this reagent available to them.

In addition, we are entering into collaborations with several other university laboratories studying Runx2-related cancers to determine if Runx2 phosphorylation is also important in the etiology of these cancers.

What was the impact on society beyond science and technology?

Nothing to Report

5. CHANGES/PROBLEMS

In general, we were able to closely follow the Statement of Work submitted with this proposal. Studies in Task 1e, which were designed to determine P-Runx2-dependent epigenetic changes in PCa, were not carried out because we had just conducted a similar analysis in bone cells as part of another project.

6. PRODUCTS
Publications, conference papers and presentations:

Meeting Abstracts:
Abstract 1. 11th International Conference of Cancer-Induced Bone Disease, Chicago, IL, November 30-December 3, 2011.
Zhao Z, Ge C, Pannone G, Bufo P, Santoro A, Sanguedolce F, Tororella S, Mattoni M, Papagerakis P, Papagerakis S and **Franceschi RT**. RUNX2 phosphorylation as a prognostic marker of metastatic disease in prostate cancer. 11th International Conference of Cancer-Induced Bone Disease Proceedings (2011) Abst P145. (copy in appendix)

Abstract 2. American Society for Bone and Mineral Research Annual Meeting, September 12-14, 2014. Ge C, Zhao G, Zhao X, Li Y, Li H, Li B, Pannone G, Buro P, Santoro A, Sanguedolce F, Tortorella S, Mattoni M, Papagerakis S, Keller ET, Franceschi RT. Runx2 Phosphorylation Increases Migration and Invasive Activity of Prostate Cancer Cells and is Associated with Metastatic Disease. J Bone Miner Res. 2014 Abstract 1036 http://www.asbmr.org/ItineraryBuilder/PresentationDetail.aspx?pid=426a5f83-6a69-4cfa-b364-5d6600827898&ptag=AuthorDetail&aid=00000000-0000-0000-0000-000000000000

Publications:

Publication 1. Ge C, Zhao G, Li Y, Li H, Zhao X, Pannone G, Bufo P, Santoro A, Sanguedolce F, Tortorella S, Mattoni M, Papagerakis S, Keller ET and **Franceschi RT**. Role of Runx2 Phosphorylation in Prostate Cancer and Association with Metastatic Disease. Oncogene, 2015-in press http://www.nature.com/doifinder/10.1038/onc.2015.91 (copy in appendix).

Websites: After acceptance of the above referenced paper in Oncogene, our work was featured on the University of Michigan website and a press release was issues.

Reagents Developed:

The following RUNX2 expression vectors were generated :
AdRUNX2 WT- adenovirus expresses wildtype murine RUNX2 at high levels in prostate cancer cells.
AdRUNX2 Mut- adenovirus expresses S301A, S319A phosphorylation-deficient RUNX2 at high levels in PCa

cells.
Lenti-RUNX2 WT- Lentivirus expresses wildtype murine RUNX2 at high levels in prostate cancer cells.
Lenti-RUNX2 SA Mut- Lentivirus expresses S301A, S319A phosphorylation-deficient RUNX2 at high levels in PCa cells.
Lenti-RUNX2 SE Mut- Lentivirus expresses S301A, S319E phosphorylation-mimetic RUNX2 at high levels in PCa cells.

Funding applied for based on work supported by this award:

Title:	In Vivo Role of Runx2 and Runx2 Phosphorylation in Prostate Cancer Progression
Role in Project:	PI
Percent Effort:	20
Source of Funds:	DOD PC131001 Dept of the Army USAMRAA (submitted 10/13)
Support Period:	04/01/14-03/31/17
Total Direct Cost:	
Status:	Not funded

Title:	In Vivo Role of Runx2 and Runx2 Phosphorylation in Prostate Cancer Progression
Role in Project:	PI
Percent Effort:	20
Source of Funds:	DOD PC131001 Dept of the Army USAMRAA (submitted 10/14)
Support Period:	04/01/15-03/31/18
Total Direct Cost:	
Status:	Not funded

Title:	In Vivo Role of Runx2 and Runx2 Phosphorylation in Prostate Cancer Progression
Role in Project:	PI
Percent Effort:	20
Source of Funds:	NCI
Support Period:	04/01/16-03/31/20 ,
Total Direct Cost:	
Status:	Pending

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on project?

Name	Renny Franceschi
Project Role	PI, Professor of Periodontics and Oral Medicine
Research Identifier	
Nearest Person Month Worked	9 Calendar Months
Contribution to Project	Directed all research and planned experiments in collaboration with Co-Is. Wrote all manuscripts and abstracts.
Funding Support	N/A
Name	Silvana Papagerakis
Project Role	Co-I, Research Assistant Professor
Research Identifier	

Nearest Person Month Worked	8 Calendar Months
Contribution to Project	Assisted in immunohistochemical analysis of prostate cancer samples
Funding Support	N/A
Name	Chunxi Ge
Project Role	Co-I , Assistant Research Scientist
Research Identifier	
Nearest Person Month Worked	4 Calendar Months
Contribution to Project	Conducted experiments on control of prostate cancer cell gene expression by P-Runx2. Assisted with analysis of in vivo tumor results.
Funding Support	N/A
Name	Yan Li
Project Role	Research Investigator
Research Identifier	
Nearest Person Month Worked	5 Calendar Months
Contribution to Project	Conducted gene expression analysis using Spp1 promoter-luc reporter assay. Assisted with in vivo tumor analysis.
Funding Support	N/A
Name	Hanshi Sun
Project Role	Research Lab Specialist
Research Identifier	
Nearest Person Month Worked	1 Calendar Month
Contribution to Project	Developed lentivirus expression vectors for wild type and mutant Runx2
Funding Support	N/A
Name	Guisheng Zhao
Project Role	Research Lab Specialist
Research Identifier	
Nearest Person Month Worked	1 Calendar Month
Contribution to Project	Conducted in vitro cell migration and invasion assays and analysis of in vivo tumor growth.
Funding Support	N/A
Name	Christopher Wilson
Project Role	Research Fellow
Research Identifier	
Nearest Person Month Worked	2 Calendar Months
Contribution to Project	Assisted with image analysis of prostate tumors
Funding Support	N/A

What other organizations were involved as partners?

Organization Name: Department of Clinical and Experimental Medicine, Section of Anatomic Pathology, University of Foggia
Location of Organization: Foggia, Italy
Partner's contribution to the project: Collaborators Drs. G. Pannone, P. Bufo and coworkers conducted immunohistochemical analysis of human prostate cancer tissue microarrays using anti-Runx2-S-319-P antibody as well as statistical analysis of results. This portion of work was supported by I.R.C.C.S Centro di Riferimento Oncologico Della Basilicata Rionero in Vulture grant to Dr Bufo.

8. SPECIAL REPORTING REQUIREMENTS- None

9. APPENDICES

Abstracts:

Abstract 1. 11th International Conference of Cancer-Induced Bone Disease, Chicago, IL, November 30-December 3, 2011.
Zhao Z, Ge C, Pannone G, Bufo P, Santoro A, Sanguedolce F, Tororella S, Mattoni M, Papagerakis P, Papagerakis S and **Franceschi RT**. RUNX2 phosphorylation as a prognostic marker of metastatic disease in prostate cancer. 11th International Conference of Cancer-Induced Bone Disease Proceedings (2011) Abst P145.

RUNX-2 Phosphorylation as a Prognostic Marker of Metastatic Disease in Prostate Cancer

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Background: The three mammalian Runt homology domain transcription factors (Runx1, Runx2, Runx3) are important regulators of tissue differentiation. Runx proteins also function as cell context-dependent oncogenes or tumor suppressors. The transcriptional activity of RUNX-2 is stimulated by signals known to be hyperactive in tumors including ERK1/2 and p38 MAP kinase and BMP/TGFbeta/Smad pathways. RUNX2 regulates a number of metastasis-associated genes including vascular endothelial growth factor (VEGF) and matrix metalloproteinase-13 (MMP-13) and stimulates angiogenic and invasive activity of prostate and breast tumors. During normal bone development, RUNX2-dependent transcription is activated by ERK1/2-mediated phosphorylation at Ser 301, Ser 319. This study examines the role of RUNX2 phosphorylation in angiogenic and invasive activity of prostate tumor cells in vitro as well as the relationship between RUNX2-S319 phosphorylation and tumor stage in vivo.
Methods: Total and RUNX2-S319-P were measured in LnCaP, C4-2B and PC-3 prostate adenocarcinoma cell lines using Western blotting and immunofluorescence. A TMA of prostate cancers containing 72 primary and 13 metastatic prostate adenocarcinomas was analyzed for immunoreactivity with total and RUNX2-S-319-P antibodies using standard linked streptavidin-biotin staining. LnCaP cell migration and angiogenesis activities were measured using Boyden chambers and endothelial cell tube formation assays, respectively. VEGF was measured by ELISA. Adenovirus vectors were used to express wild type and non-phosphorylated RUNX2 mutants (S301A, S319A).
Results: RUNX2 was preferentially expressed in the more invasive PCa cell lines (PC-3> C4-2B > LnCaP). Furthermore, the ratio of RUNX2-S319-P/total RUNX2 as well as P-ERK/total ERK was greater in PC-3 and C4-2B vs LnCaP cells. Adenovirus-mediated overexpression of wild type RUNX2 in LnCaP cells (low endogenous RUNX2) increased in vitro migration, angiogenic activity and VEGF secretion while the RUNX2-S301A,S319A mutant was inactive. Analysis of TMAs revealed that P-Runx2 is strongly over-expressed in the nuclei of prostate cancer cells, especially in cases with high Gleason score. Furthermore, P-RUNX2 was consistently elevated in metastatic prostate cancer cases.
Conclusions: The phosphorylation state of RUNX2 is related to its tumorigenic activity with RUNX2-S319-P being positively correlated with negative tumor outcome and in vitro invasive/angiogenic activity.

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Runx2 Phosphorylation Increases Migration and Invasive Activity of Prostate Cancer Cells and is Associated with Metastatic Disease

Category: Bone Tumors and Metastasis

Oral Presentations, Presentation Number: 1036 Session: Concurrent Orals: Greg Mundy Memorial Session: Bone and Cancer Saturday, September 13, 2014 3:15 PM - 3:30 PM, George R. Brown Convention Center, Grand Ballroom A

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The osteogenic transcription factor, Runx2, is abnormally expressed in prostate cancer where it stimulates expression of several metastasis-associated genes. During normal bone development, Runx2 transcriptional activity is activated by signals known to be hyperactive in prostate cancer including the ERK/MAP kinase pathway, which phosphorylates Runx2 at Ser 301 and Ser 319. This study examines the role of these phosphorylation sites in Runx2-dependent activation of metastasis-associated gene expression, *in vitro* migration and invasive activity of prostate cell lines as well as the relationship between Runx2 phosphorylation and tumor stage. Runx2 was preferentially expressed in more invasive prostate cancer cell lines (PC-3> C4-2B > LNCaP). Furthermore, the ratio of Runx2-S319-P/total Runx2 as well as P-ERK/total ERK was greater in PC-3 and C4-2B versus LNCaP cells. Phosphorylated Runx2 had an exclusively nuclear localization and was enriched in PC3 cultures while total Runx2 had a cytoplasmic and nuclear distribution. Overexpression of wild type Runx2 increased *Mmp9*, *Vegfa* and *Spp-1* mRNA and increased *in vitro* migratory and invasive activity in PC-3 cells as well as the non-tumorigenic prostate cell line, RWPE1. In contrast, a phosphorylation-deficient Runx2 mutant (Runx2-S301A,S319A) had greatly reduced activity. Analysis of tissue microarrays from 129 patients revealed strong nuclear staining with a Runx2-S319-P-specific antibody in primary prostate cancers and metastases. P-Runx2 staining was positively correlated with Gleason score and occurrence of lymph node metastases. Little or no Runx2 phosphorylation was detected in normal prostate tissue, benign prostate hyperplasia or prostatitis indicating that Runx2 phosphorylation is closely associated with prostate cancer induction and progression towards an aggressive phenotype.

Publications:

Publication 1. Ge C, Zhao G, Li Y, Li H, Zhao X, Pannone G, Bufo P, Santoro A, Sanguedolce F, Tortorella S, Mattoni M, Papagerakis S, Keller ET and **Franceschi RT**. Role of Runx2 Phosphorylation in Prostate Cancer and Association with Metastatic Disease. Oncogene, 2015-in press

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ORIGINAL ARTICLE

Role of Runx2 phosphorylation in prostate cancer and association with metastatic disease

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The osteogenic transcription factor, Runx2, is abnormally expressed in prostate cancer (PCa) and associated with metastatic disease. During bone development, Runx2 is activated by signals known to be hyperactive in PCa including the RAS/MAP kinase pathway, which phosphorylates Runx2 on multiple serine residues including S301 and S319 (equivalent to S294 and S312 in human Runx2). This study examines the role of these phosphorylation sites in PCa. Runx2 was preferentially expressed in more invasive PCa cell lines (PC3 > C4-2B > LNCaP). Furthermore, analysis using a P-S319-Runx2-specific antibody revealed that the ratio of P-S319-Runx2/total Runx2 as well as P-ERK/total ERK was highest in PC3 followed by C4-2B and LNCaP cells. These results were confirmed by immunofluorescence confocal microscopy, which showed a higher percentage of PC3 cells staining positive for P-S319-Runx2 relative to C4-2B and LNCaP cells. Phosphorylated Runx2 had an exclusively nuclear localization. When expressed in prostate cell lines, wild-type Runx2 increased metastasis-associated gene expression, *in vitro* migratory and invasive activity as well as *in vivo* growth of tumor cell xenografts. In contrast, S301A/S319A phosphorylation site mutations greatly attenuated these Runx2 responses. Analysis of tissue microarrays from 129 patients revealed strong nuclear staining with the P-S319-Runx2 antibody in primary PCas and metastases. P-S319-Runx2 staining was positively correlated with Gleason score and occurrence of lymph node metastases while little or no Runx2 phosphorylation was seen in normal prostate, benign prostate hyperplasia or prostatitis indicating that Runx2 S319 phosphorylation is closely associated with PCa induction and progression towards an aggressive phenotype. These studies establish the importance of Runx2 phosphorylation in prostate tumor growth and highlight its value as a potential diagnostic marker and therapeutic target.

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INTRODUCTION

Prostate cancer (PCa), the most common male malignancy, is frequently associated with bone metastases.[1] The metastatic process begins in the primary tumor with activation of genes that promote angiogenesis, tumor invasion and migration leading to colonization of peripheral tissues including bone.[2] A major challenge for PCa treatment is to identify factors controlling tumor growth and metastasis.

Runx2, a transcription factor required for bone development, is abnormally elevated in PCa.[3,4] The presence of Runx2 in PCa is positively correlated with increased Gleason score and metastasis.[5–7] Furthermore, Runx2 stimulates growth, migration and osteolytic activity of prostate and breast tumors.[6,8,9] Runx2 also directly induces genes associated with angiogenesis, invasiveness and metastasis including *Vegf*, *Spp1*, *Mmp9* and *Mmp13* and stimulates epithelial to mesenchymal transition of primary tumors.[5,8,10] Lastly, transgenic overexpression of Runx2 predisposes mice to T-cell lymphomas, suggesting an oncogene function.[11,12] Other runt domain transcription factors are also associated with cancers; Runx1 chromosomal translocations/mutations are commonly found in myeloid leukemias while Runx3 may function as a tumor suppressor in gastric cancers (for reviews, see Blyth *et al.*[12,13]).

MAP kinase (MAPK), PI3K/AKT and non-receptor tyrosine kinase signaling pathways are also elevated in PCa. Increased MAPK signaling due to RAS-RAF mutations is seen in 43% of primary tumors and 63% of metastases.[14,15] Furthermore, RAS/MAPK activation positively correlates with disease progression.[16] Significantly, transgenic overexpression of RAS stimulates EMT and PCa formation in genetic models of PCa.[17] Similarly, targeted expression of mutant BRAF in prostate epithelium induces invasive carcinomas in mice.[18] PI3K/AKT and non-receptor kinases have also been related to PCa initiation and progression.[19,20] However, there is currently no clear explanation for why kinase activation in PCa is associated with an invasive phenotype.

On the basis of previous work in bone, we propose that Runx2 and the RAS/MAPK pathway cooperatively interact in PCa to regulate metastasis-related gene expression. During osteoblast differentiation, ERK1/2 and p38 MAPKs phosphorylate Runx2 on several serine and threonine residues.[21–24] Of these, Ser 301 and Ser 319 (murine type I Runx2 sequence) are particularly important for Runx2-dependent transcriptional activity.[21] ERK phosphorylates Runx2 directly on the chromatin of target genes.[21,25,26]

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Phosphorylated Runx2 then stimulates epigenetic changes including histone acetylation and transcription leading to induction of gene expression.[25,27]

In the present study, we show that Runx2 is phosphorylated in PCa cells and that the same phosphorylation sites previously demonstrated to be important for osteoblast gene expression are also required for Runx2-dependent stimulation of metastasis-associated gene expression, *in vitro* cell migration, invasion and *in vivo* tumor growth. Furthermore, the presence of P-Runx2 as measured with a P-S319-Runx2-specific antibody is correlated with PCa onset and severity in a patient population.

RESULTS

Runx2 is preferentially phosphorylated in metastatic PCa cell lines

Runx2 expression was previously compared between different human PCa cell lines.[6,7] PC3 cells have high metastatic potential whereas LNCaP cells have little or no activity. C4-2B cells are a metastatic subclone derived from LNCaP cells.[28,29] These cell lines were compared to determine whether there is a correlation between MAPK activity, Runx2 phosphorylation and metastatic potential (Figure 1). Phosphorylated Runx2 was detected using an anti-S319-phospho-Runx2-specific antibody.[26] Although MAPK phosphorylates Runx2 at additional sites including S301, phosphorylation at S319 is closely correlated with Runx2 transcriptional activity in osteoblasts and is likely to reflect phosphorylation at other MAPK sites for which immune reagents are not currently available.[21,24,26] Runx2 mRNA and protein were highest in PC3 cells followed by C4-2B and LNCaP (Figures 1a and b). Panels 1c and 1d compare the S319-phospho-Runx2/total Runx2 ratio with the level of MAPK activation (P-ERK/total ERK) in each cell line. For P-Runx2 analysis, protein loading was adjusted to give the same amount of total Runx2 in each lane. C4-2B and PC3 cells had high MAPK activity and Runx2 phosphorylation. S319-phospho-Runx2/total Runx2 and P-ERK/total ERK ratios in the metastatic cells were more than twice the ratios seen in LNCaP cells.

Differences between cell lines were also noted when total and S319-P-Runx2 staining was examined by confocal microscopy (Figure 2, Supplementary Figure S1). Most cells were positively stained with total Runx2 antibody (Supplementary Figure S1). However, clear differences in cellular distribution were noted; Runx2 was mainly nuclear in PC3 and C4-2B cells, whereas a diffuse cytoplasmic and nuclear distribution was observed in LNCaP cultures (Figure 2). These differences could not be

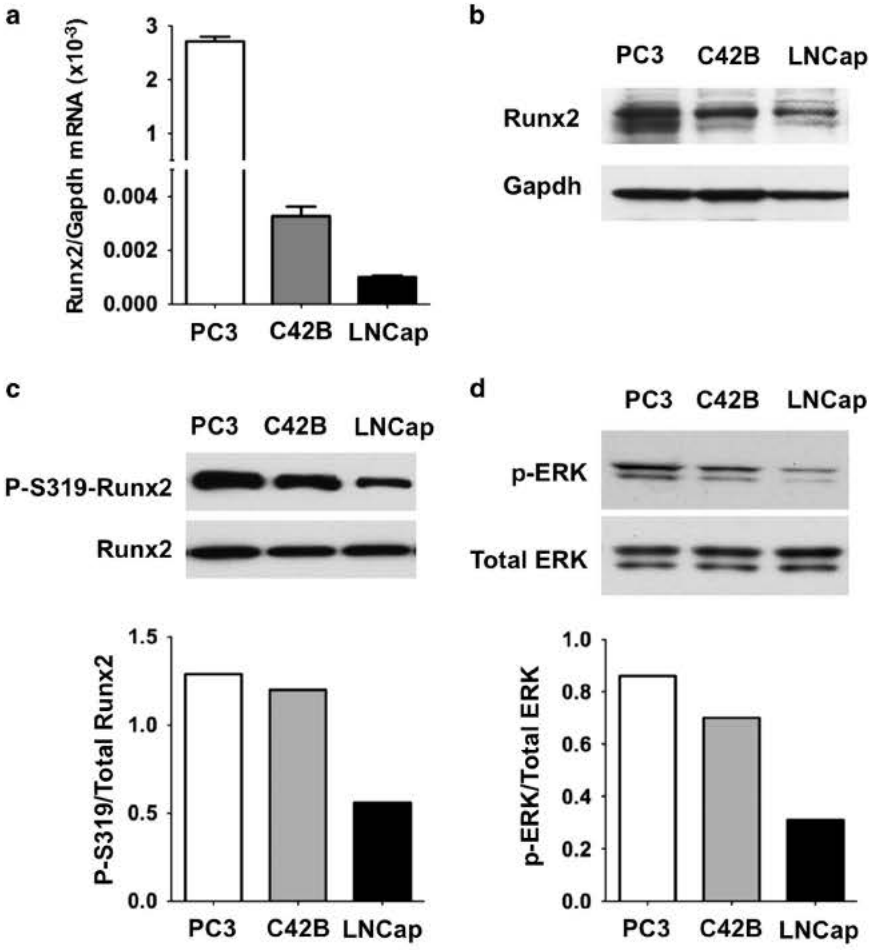

Figure 1. Comparison of total Runx2, phospho-S319-Runx2 and MAPK activity in human PCa cell lines with high (PC3, C4-2B) and low (LNCaP) metastatic potential. (**a**, **b**) Runx2 mRNA and protein levels. Runx2 mRNA was measured by real-time RT/PCR. Total Runx2 protein was measured by western blotting (10 μg protein/lane) using an anti-Runx2 antibody (MBL monoclonal antibody). Loading efficiency was assessed by reprobing each blot with a Gapdh antibody. (**c**) Comparison of P-S319-Runx2/total Runx2 ratio. On the basis of the results shown in panel **b**, sample loading from each cell line was adjusted to give an equivalent amount of total Runx2 protein (note increased GAPDH in C4-2B and LNCaP lanes). Blots were then probed with a P-S319-Runx2-specific antibody or anti-Runx2 antibody and the ratio of P-S319-Runx2/total Runx2 was determined by densitometry (lower panel). (**d**) MAPK activity. Cell extracts (10 μg protein/lane) were probed with P-ERK and total ERK antibody and the P-ERK/total ERK ratio was determined by densitometry (lower panel).

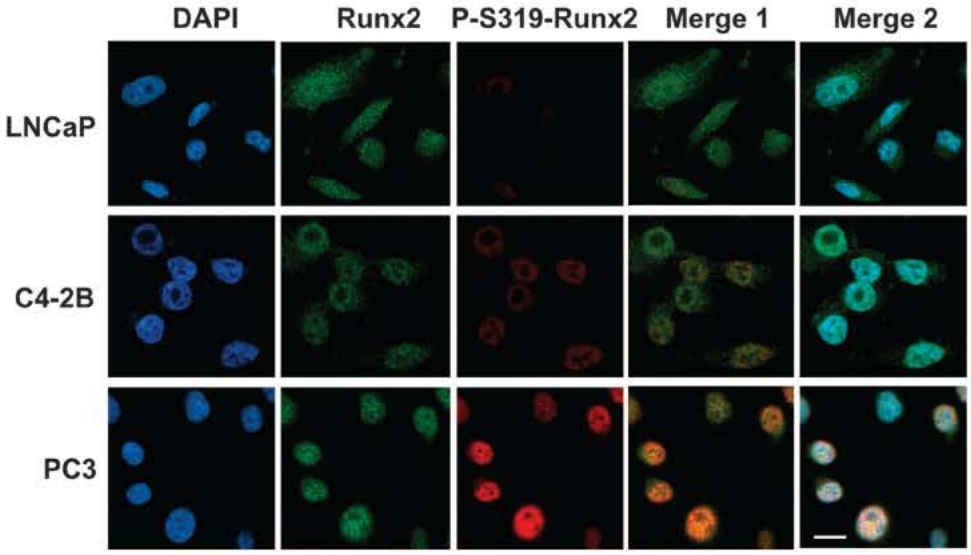

Figure 2. Cellular distribution of total Runx2 and phospho-S319-Runx2 immunofluorescence in PCa cell lines. LNCaP, C4-2B and PC3 cells were plated on glass cover slips, fixed, permeablized and stained for DNA (DAPI), total Runx2 (Abcam primary Mab, secondary antibody Alexa Fluor 488 green) and P-S319-Runx2 (rabbit anti-P-S319-Runx2 primary polyclonal antibody, secondary antibody Alexa Fluor 555 red) as indicated in Methods. High magnification (×100 oil objective) Z stack confocal images are shown. Merge 1 contains total Runx2 and P-S319-Runx2 images. Merge 2 combines total and P-S319-Runx2 with the DAPI image to facilitate visualization of the cytoplasmic Runx2 staining in LNCaP cells. Scale bar = 5 μm. Lower power images and IgG controls are shown in Supplementary Figures S1 and S2.

explained by non-specific staining of LNCaP cells with the total Runx2 antibody (Supplementary Figure S2). In contrast, S319-P-Runx2 staining was exclusively nuclear in all three lines with strongest staining observed in PC3 followed by C4-2B and LNCaP cells. The percentage of Runx2-positive cells also staining for P-Runx2 (yellow/orange staining in merged images, Supplementary Figure S1) was as follows: PC3, 72.5 ± 0.8; C4-2B, 39.5 ± 0.2; LNCaP, 16.5 ± 0.5.

These results indicate that nuclear P-S319-Runx2 staining is increased in more metastatic PCa cell lines.

Requirement for Runx2 S301, S319 phosphorylation in metastasis-related gene expression

As previously shown, MAPK activation of Runx2 transcriptional activity requires phosphorylation at Ser 301 and Ser 319.[21] Two approaches were used to determine whether these same two phosphorylation sites are also required for induction of metastasis-associated gene expression in prostate cells (Figure 3). First, PC3 cells were transfected with a pOPN-luc reporter plasmid containing the proximal promoter of *Spp1*, a gene previously associated with metastatic activity,[30] and Runx2 phosphorylation and transcriptional activity were examined with or without MAPK activation (Figures 3a and b). *Spp1* contains a previously characterized, functional Runx2 binding site between −136 to −130 bp of the proximal promoter.[31] Wild-type Runx2 strongly stimulated promoter activity and this response was further increased with Meksp, a constitutively active MEK1 mutant.[32] Meksp also increased Runx2 phosphorylation as measured with anti-S319-P-Runx2 antibody (Figure 3b). In contrast, dominant-negative MEK1 strongly inhibited Runx2 phosphorylation and activity. To assess whether increased Runx2 phosphorylation was necessary for MAPK-dependent transcriptional activity, cells were transfected with Runx2 containing S301A, S319A mutations (Runx2 SA). Although basal stimulation with Runx2 SA was similar to wild type, mutant Runx2 was resistant to activation by Meksp, as would be expected if the two phosphorylation sites were necessary for MAPK activation. These results cannot be explained by differences in levels of wild type versus Runx2 SA. Also, immunoreactivity of the anti-Runx2 antibody was not affected by Runx2 phosphorylation as similar band intensities were obtained with or without Meksp or phosphorylation site mutation (Figure 3b).

In a second approach, we examined the effect of phosphorylation site mutation on the ability of Runx2 to stimulate endogenous expression of three metastasis-associated genes; *Vegf*, *Mmp9* and *Spp-1*.[6,33] PC3 cells were transduced with adenovirus-expressing LacZ (control), wild-type Runx2 or phosphorylation-deficient Runx2 (Figures 3c and f). To assess whether Runx2 could also stimulate gene expression in non-tumorigenic cells, we examined RWPE1 cells, a non-transformed, immortalized cell line derived from normal human prostate epithelium (Figures 3g and j).[34] Adenovirus vectors were toxic to LNCaP cells, preventing them from being included in this study. In these experiments, Runx2 was activated by endogenous MAPK rather than by added Meksp. Viral titers were adjusted to produce equivalent amounts of wild type or mutant Runx2 protein as measured on western blots (panels 3c and 3g). Note that owing to the high level of Runx2 expression, western blots were exposed for a shorter time than in Figure 1 so endogenous Runx2 in PC3 cells was not visible in the LacZ group, but was detected when blots were deliberately overexposed (Supplementary Figure S3). We were not able to detect Runx2 protein in RWPE1 cells (result not shown). Wild-type Runx2 strongly induced *Vegf*, *Mmp9* and *Spp1* mRNAs in both PC3 and RWPE1 cells. In all cases, the S301A, S319A Runx2 mutant had considerably less activity than wild-type Runx2.

Role of Runx2 phosphorylation in cell migration and invasion

Runx2 is required for *in vitro* PCa cell migration and invasion.[5,33] The following experiments assessed the requirement for Runx2 phosphorylation in these two tumor-related processes using LacZ-, Runx2-WT- or Runx2-SA-transduced PC3 and RWPE1 cells.

Migration assays measured the movement of cells into an initially cell-free area (Figure 4). Panels 4a and 4b show representative images of cells at the beginning of the experiment and after a specific migration period while panels 4c and 4d show the percent occupancy of the initially cell-free area over time.

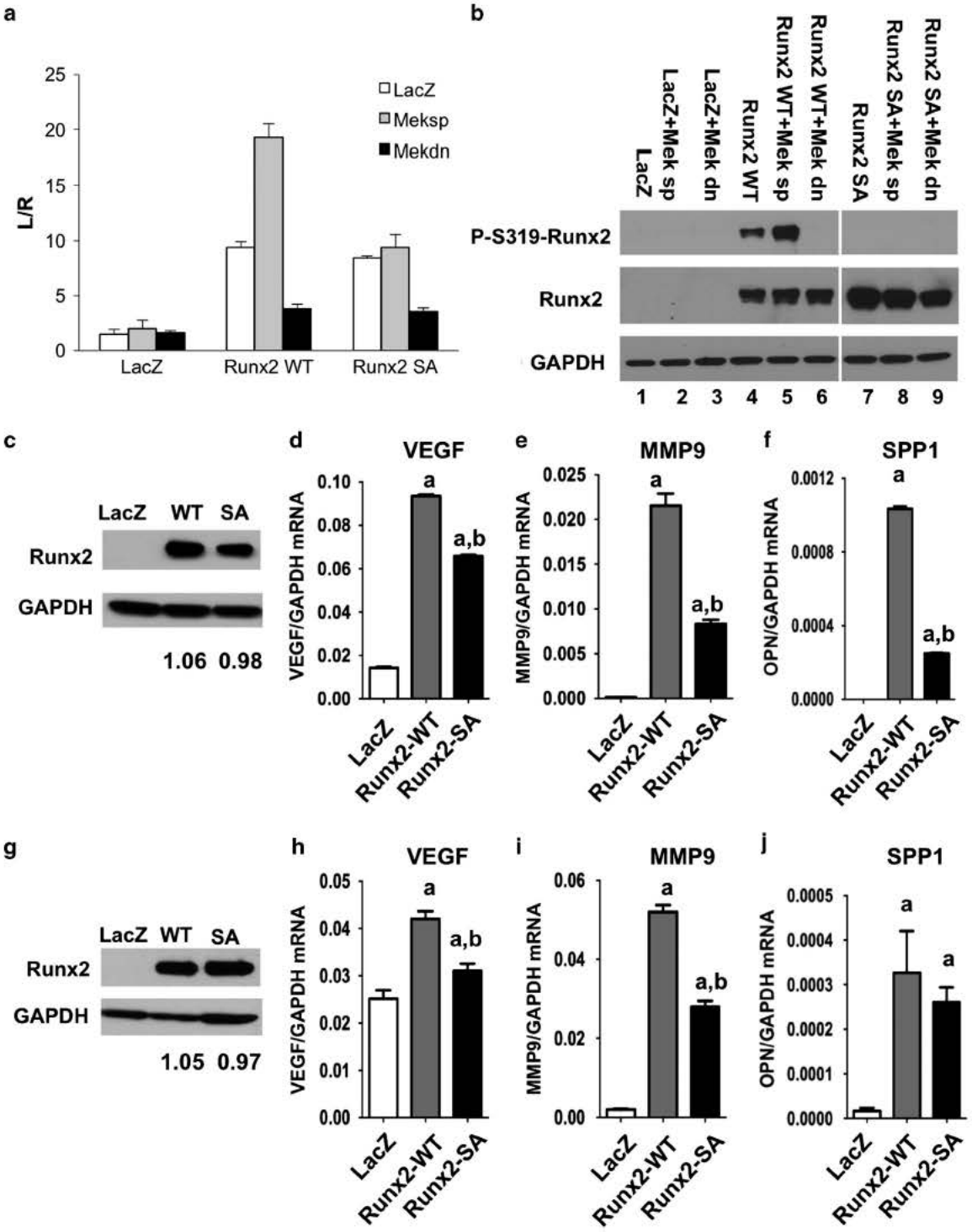

Figure 3. Requirement for Runx2-S301, S319 phosphorylation for induction of metastasis-associated genes. (**a**, **b**) Spp-1 promoter activity. PC3 cells were transfected with pOPN-Luc reporter, pRL-SV40 *renilla* luciferase normalization plasmid, lacZ (control), wild-type Runx2 (Runx2WT) or Runx2-S301A, S319A mutant (Runx2 SA) and either constitutively active (Meksp) or dominant-negative (Mekdn) MEK1 expression vectors as indicated. Firefly luciferase activity was normalized to *renilla* luciferase. Levels of P-S319-Runx2 and total Runx2 protein are shown in panel **b**. Samples in lanes 7 9 were run on a separate gel from those in lanes 1 6, but were blotted and exposed under identical conditions. (**c** **j**) mRNA induction. PC3 (**c** **f**) or RWPE1 cells (**g** **j**) were transduced with LacZ, Runx2-WT or Runx2-SA adenovirus expression vectors, and total protein and RNA was isolated after 48 h. Viral titers were adjusted to give equivalent levels of Runx2 protein as measured on western blots; values below each lane indicate the Runx2/Gapdh protein ratio as determined by densitometry (**c**, **g**). *VegfA* (**d**, **h**), *Mmp9* (**e**, **i**) and *Spp-1* (**f**, **j**) mRNAs were measured by real-time RT/PCR. Statistics (**d** **f**, **h** **j**): [a]Significantly different from LacZ control ($P < 0.01$), [b]Significantly different from Runx2-WT ($P < 0.01$); $N = 3$ per group.

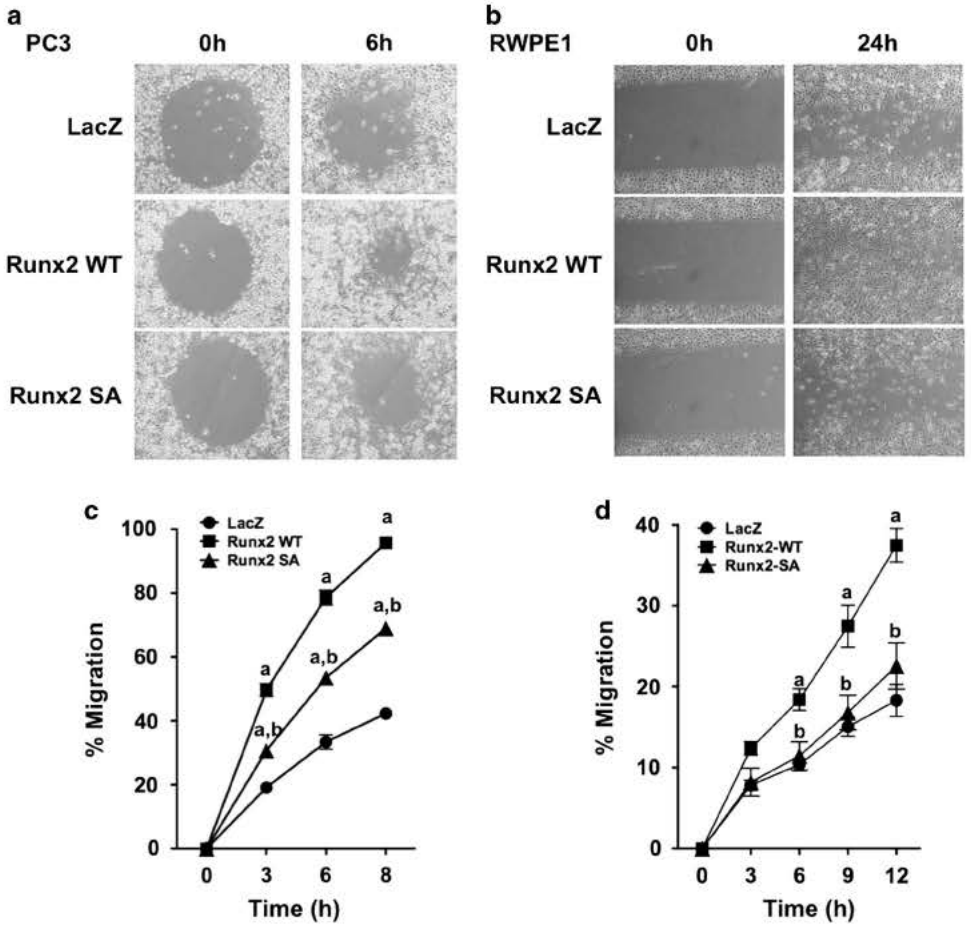

Figure 4. Requirement for Runx2-S301, S319 phosphorylation in stimulation of prostate cell migration. PC3 and RWPE1 cells were transduced with LacZ, Runx2-WT or Runx2-SA adenovirus vectors. After 48 h, cells were trypsinized and cell migration was measured as described in Methods. (**a**, **c**) PC3 cell migration. Cell images after 0 and 6 h (**a**). Percent occupancy of the initially cell-free area was determined after the indicated times (**c**). (**b**, **d**) RWPE1 cell migration. Images of cells after 0 or 24 h (**b**). Percent occupancy of the initially cell-free area was determined after the indicated times (**d**). Statistics: **c**, [a]Significantly different from LacZ control ($P < 0.001$), [b]Significantly different from Runx2-WT ($P < 0.001$); **d**, [a]Significantly different from LacZ control ($P < 0.05$), [b]Significantly different from Runx2-WT ($P < 0.01$), $N = 4$ per group.

Consistent with their metastatic phenotype, PC3 cells migrated faster than the non-transformed RWPE1 cell line. For example, after 6 h, control PC3 cells filled approximately 30% of the initially cell-free area versus only a 10% fill for RWPE1 cells. However, wild-type Runx2 stimulated migration of both cell types with PC3 cells filling 80% of the cell-free area after 6 h and nearly the entire area after 8 h and RWPE1 cells filling 18% of the cell-free area after 6 h and 38% after 12 h. In contrast, equivalent levels of phosphorylation-deficient Runx2 had approximately half the activity of wild-type Runx2 in stimulating migration of PC3 cells and no significant stimulatory activity on RWPE1 cells.

To measure cell invasion, PC3 or RWPE1 cells were plated in the upper well of a Transwell apparatus precoated with Matrigel. The lower chamber contained 10% fetal bovine serum and SDF-1, a chemoattractant. Migration of cells through the Matrigel and Millipore membrane was scored after 24 h (Figure 5). Representative images are shown in panels 5a and 5b while invasion is quantified in panels 5c and 5d. Wild-type Runx2 increased the invasion activity of PC3 cells fivefold, whereas phosphorylation-deficient Runx2 only increased the activity twofold. Similarly, wild-type Runx2 stimulated activity of RWPE1 cells 2.3-fold, whereas the phosphorylation-deficient mutant was inactive. These results cannot be explained by differences in proliferation as cell numbers in each group were identical over the time frame of these experiments (result not shown).

Role of Runx2 phosphorylation in tumor growth *in vivo*

In previous studies, Runx2 stimulated *in vivo* tumor formation by PC3 cell xenografts.[9] To determine whether this response requires Runx2 phosphorylation, PC3 cells stably expressing a firefly luciferase reporter (PC-luc cells) were transduced with LacZ, Runx2-WT or Runx2-SA adenovirus and subcutaneously implanted into immunodeficient mice. Tumor development was monitored over a 16- day period by measuring whole-body luciferase activity. Mice were then killed at day 17 and tumors were weighed and used for RNA analysis or immunohistochemistry. Wild-type Runx2 significantly increased tumor growth as measured by *in vivo* luminescence (Figures 6a and c) and tumor size/weight at the time of killing (Figures 6d and e). The increased growth of Runx2-transduced cells was first detected at day 4 with the most dramatic increase observed between day 9 and day 16 (Supplementary Figure S4). In contrast, tumors formed by the Runx2-SA group were not significantly different from LacZ controls. Similar to cell culture results, tumors from Runx2-WT-treated cells expressed higher levels of VEGF, MMP9 and SPP1 mRNAs relative to LacZ controls, whereas levels were lower with Runx2-SA transduction (Panel 6f).

To further explore the basis for differences in tumor growth, tumors were stained for human Ki67, a cell proliferation marker, and Caspase 3, a marker of apoptosis. The Ki67 antibody uniformly stained control, Runx2-WT and Runx2-SA tumors, indicating that

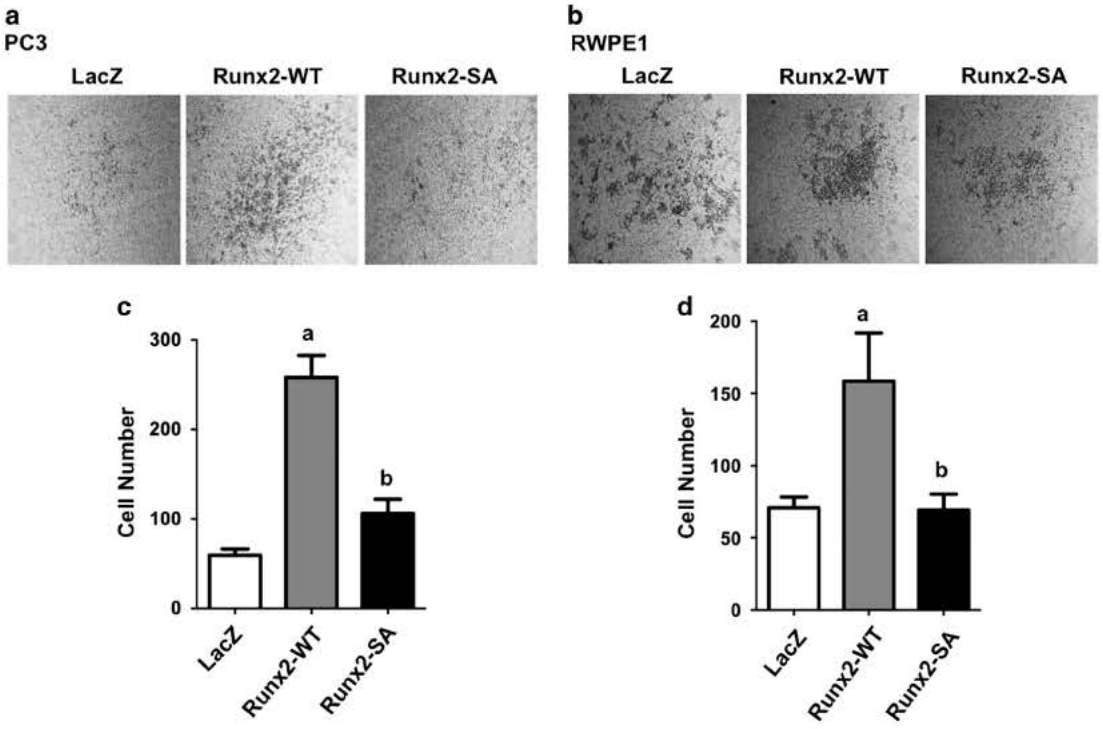

Figure 5. Requirement for Runx2-S301, S319 phosphorylation in stimulation of prostate cell invasion. PC3 (**a**, **c**) and RWPE1 cells (**b**, **d**) were transduced with LacZ, Runx2-WT or Runx2-SA adenovirus vectors, trypsinized and cells were assayed for invasive activity using Matrigel containing transwells as described in Methods. (**a**, **b**) Representative fields of stained cells migrating to bottom side of transwell membrane are shown. Cells were counted in 5 fields per insert and averaged, 3 inserts per group (**c**, **d**). Statistics: Panel **c**, [a]Significantly different from LacZ control ($P < 0.0001$), [b]Significantly different from Runx2-WT ($P < 0.0001$); Panel **d**, [a]Significantly different from LacZ control ($P < 0.05$), [b]Significantly different from Runx2-WT ($P < 0.05$), $N = 4$ per group.

Runx2 did not significantly affect PC3 cell proliferation (Supplementary Figure S5). In contrast, Caspase 3 staining was reduced by approximately 60% in wild-type Runx2 tumors. Values for the Runx2-SA group were highly variable and not significantly different from controls (Supplementary Figure S6). These results suggest that Runx2 phosphorylation increases tumor growth by preferentially suppressing apoptosis.

Association of Runx2 phosphorylation with PCa in a patient population

A tissue microarray (TMA) of normal and malignant prostate tissue and metastases was prepared from biopsies from 129 patients and examined for the presence of P-S319-Runx2 by immunohistochemistry. Results were correlated with clinical tumor parameters as described in Materials and Methods. Figure 7 shows representative immunohistochemistry of normal prostate, benign prostate hyperplasia (BPH), prostate intraepithelial neoplasia (PIN), low and high Gleason score primary PCa (PPC), and a lymph node metastasis (LNM). Little or no staining was seen in normal tissue. Light nuclear staining was observed in a small number of cells in the BPH sample while intense, localized nuclear staining was seen in PIN, particularly in basal cells of crypts and luminal cells. Nuclear staining was more dispersed in advanced cancer and further elevated in high Gleason score tumors and LNM. Table 1 provides quantitation of P-Runx2 staining for all 129 biopsies, expressed as the percentage of cells showing positive staining in each sample. Overall, high P-Runx2 staining was strongly correlated with PPC when compared with BPH or prostatitis ($P < 0.001$), with very low staining observed in the latter two conditions. Within the PPC cohort, significant correlations were also noted between percent P-Runx2 staining and high Gleason score ($P = 0.047$), PPCs with LNM at the time of initial clinical staging ($P = 0.033$) and PPC with prostatic intraepithelial neoplasia (PIN, $P = 0.041$). Although strong P-Runx2 staining was observed in LNM, levels were not significantly different from than those observed in the primary tumors. On the basis of these results, we conclude that Runx2 S319 phosphorylation is a common event in PCa that can be used to distinguish between BPH, prostatitis and neoplastic disease and is also correlated with disease severity and metastases.

DISCUSSION

Here, we establish the importance of Runx2 S301, S319 phosphorylation sites in PCa gene expression, migration, invasion and *in vivo* tumor growth, and identify P-S319-Runx2 as a marker for more aggressive metastatic disease in a patient population. On the basis of these findings, we propose that the elevated MAPK activity in PCa cells phosphorylates Runx2 and stimulates transcription of genes necessary for increased tumor growth and metastasis. These studies provide a potential link between previously reported increases in MAPK signaling and Runx2 in PCa as well as a plausible mechanism to explain how Runx2 and MAPK cooperatively interact to regulate the expression of metastasis-related genes.

Effects of MAPK on Runx2 transcriptional activity in PCa cells are similar to those previously reported in osteoblasts.[21,22,26] Specifically, transfection of PC3 cells with constitutively active MEK1 strongly stimulated Runx2 phosphorylation and activation of an *Spp1* promoter-luciferase reporter. Furthermore, this response required intact S301 and S319 Runx2 phosphorylation sites. Similarly, endogenous metastasis-related genes such as *Vegf*, *Mmp9* and *Spp1*, which all contain functional Runx2-specific enhancer sequences in their proximal promoter, were strongly induced by wild-type Runx2, whereas a phosphorylation-resistant S301A/S319A Runx2 mutant was uniformly less active (Figure 3).[8,10,31,35]

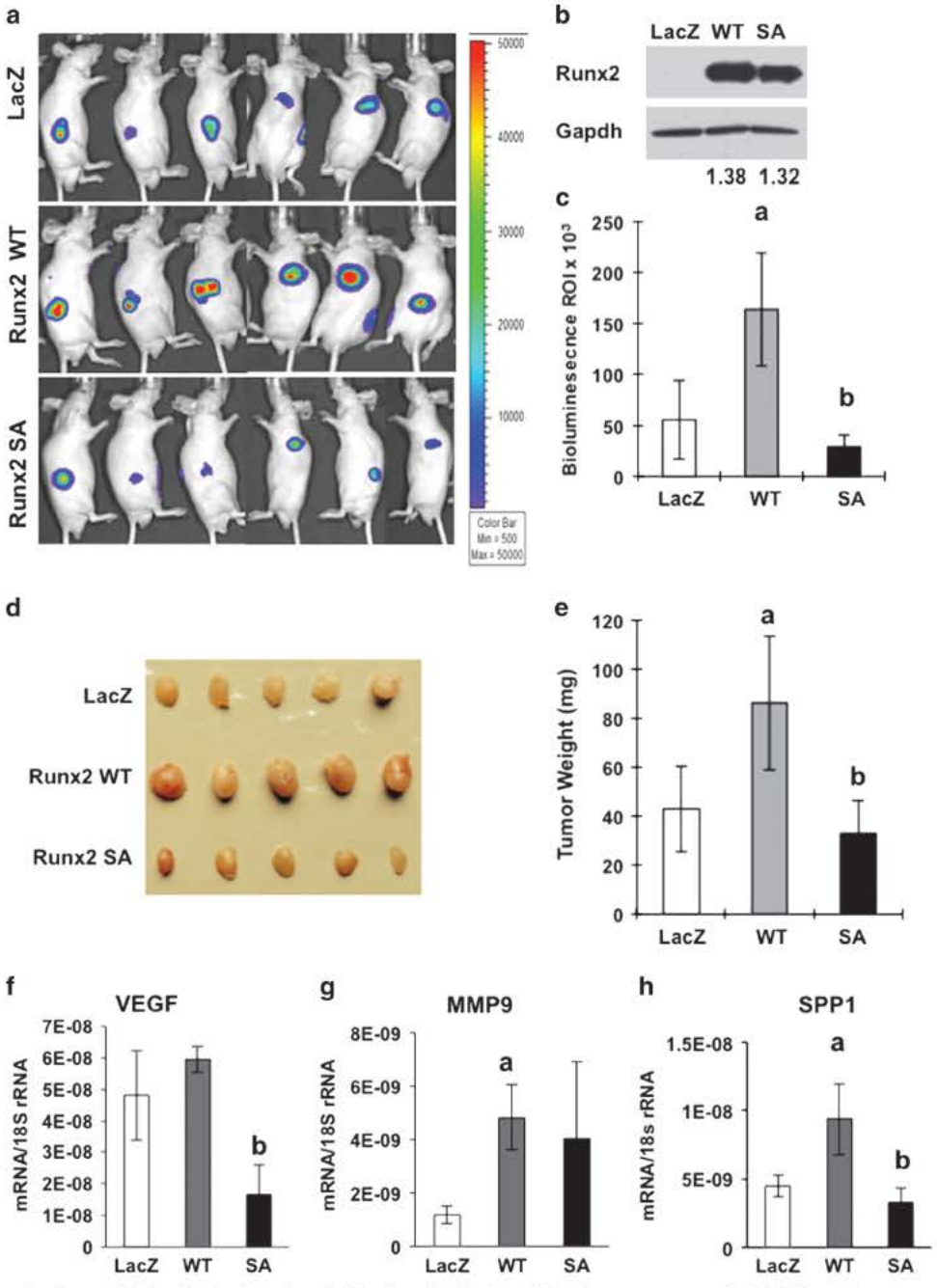

Figure 6. Requirement for Runx2-S301, S319 phosphorylation in stimulation of *in vivo* tumor growth. PC3-luc cells were transduced with LacZ, Runx2-WT or Runx2-SA adenovirus vectors, trypsinized, suspended in Matrigel and subcutaneously implanted into immunodeficient mice. Equal levels of wild-type and mutant Runx2 protein expression were verified on western blots (**b**). Values below each lane indicate the Runx2/Gapdh protein ratio as determined by densitometry. PC3-luc cells were detected after 16 days by *in vivo* bioluminescence imaging (**a**, **c**). Mice were killed at day 17. Tumors were photographed (**d**), weighed (**e**) and analyzed for *Vegf* (**f**), *Mmp9* (**g**) or Spp-1 (**h**) mRNA normalized to 18 S rRNA. Statistics: Panels **c** and **e**, [a]Significantly different from LacZ control ($P < 0.05$), [b]Significantly different from Runx2-WT ($P < 0.01$); Panels **f** **h**, [a]Significantly different from LacZ control ($P < 0.05$), [b]Significantly different from Runx2-WT ($P < 0.01$), $N = 6$ per group.

Intact S301 and S319 phosphorylation sites were also shown to be required for two previously demonstrated Runx2 actions in PCa, stimulation of *in vitro* cell migration and invasion (Figures 4, 5).[6,8,9] More importantly, this same requirement was seen when growth of PCa cell xenografts was measured *in vivo* (Figure 6). However, in this experiment wild-type and phosphorylation-deficient Runx2 were transiently expressed using adenovirus vectors, which remain active for only 7 to 10 days.[36] For this reason, effects of Runx2 on longer-term responses including metastasis to peripheral tissues could not be measured. The basis for the stimulatory effects of Runx2 on tumor mass appears to be mainly through suppression of apoptosis as Caspase 3 immunostaining was reduced in tumors expressing wild-type Runx2 (Supplementary Figures S5 and S6). This is consistent with a previous report showing that Runx2 can suppress apoptosis via direct upregulation of Bcl-2.[37] The *in vivo* finding of reduced tumor growth with expression of the less active, phosphorylation site mutant Runx2 is consistent with previous studies that

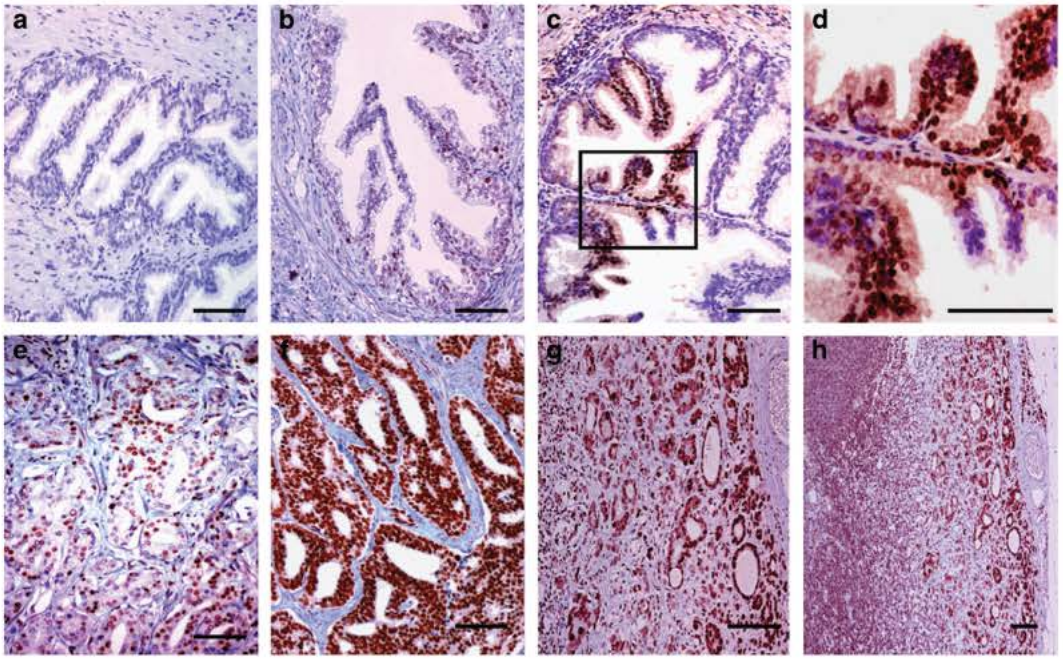

Figure 7. Immunohistochemical staining of prostate tissue and metastases using anti-P-S319-Runx2 antibody. Representative samples from TMAs are shown. (**a**) Normal prostate tissue, (**b**) BPH, (**c**) PIN, (**d**) higher power view of boxed region in **c** showing strong nuclear staining of basal cells, (**e**) moderate staining in low Gleason score PCa, (**f**) very strong staining in high Gleason score PCa, (**g**) LNM. (**h**) Lower power view of LNM showing strong staining in extra-capsular spread (ECS). Bars = 100 μm.

Table 1. TMA analysis of P S319 Runx2 in prostatic diseases, primary cancers and metastases

Groups	n	*Mean P S319 Runx 2+ (percent)*	*Standard deviation*	*Standard error*	*Comparisons*	*ANOVA (P)*	*Student Newman Keuls P < 0.05*
PPC	93	57.48	30.75	3.19	PPC vs BPH	< 0.001	Yes
BPH	15	1.87	2.07	0.53			
PPC	93	57.48	30.75	3.19	PPC vs LNM	0.464	No
LNM	13	64.23	33.16	9.20			
PPC	93	57.48	30.75	3.19	PPC vs P	< 0.001	Yes
P	8	4	0	0			
LG PPC	68	53.65	29.20	3.54	LG PPC vs HG PPC	0.047	Yes
HG PPC	25	67.92	33.01	6.60			
PNP PPC	75	58.43	30.39	3.51	PNP PPC vs NoPNP PPC	0.549	No
NoPNP PPC	18	53.56	32.79	7.73			
VP PPC	1				VP PPC vs NVP PPC	N/A	
NVP PPC	92	57.5	30.8	3.18			
N+PPC	13	74.23	29.29	8.12	N+PPC vs N0 PPC	0.033	Yes
N0 PPC	80	54.76	30.29	3.39			
PIN PPC	16	71.75	25.51	6.38	PIN PPC vs NoPIN PPC	0.041	Yes
NoPIN PPC	77	54.52	31.05	3.54			
ISM PPC	56	58.36	30.81	4.12	ISM PPC vs FSM PPC	0.738	No
FSM PPC	37	56.16	31.03	5.10			
EPE PPC	13	64.62	27.50	7.63	EPS PPC vs I PPC	0.370	No
I PPC	80	56.33	31.25	3.49			

Abbreviations: ANOVA, analysis of variance; BPH, benign prostatic hyperplasia; EPE, extra prostatic extension; FSM, free surgical margins; HG, high Gleason; I, intraprostatic; ISM, involved surgical margins; LG, low Gleason; LNM, lymph node metastases; N/A, not applicable; N+PPC, PPC with lymph node metastasis; N0 PPC, PPC without lymph node metastasis; NoPNP; No perineural permeation; NVP, no vascular permeation; P, prostatitis; PIN, prostate intraepithelial neoplasia; PNP, perineural permeation; PPC, primary prostate cancer; VP, vascular permeation.

demonstrated orthotopic injection of metastatic breast or prostate cell lines modified by nearly complete knockdown of Runx2 resulted in inhibited tumor growth and osteolytic bone disease.[6,8]

In osteoblasts, differentiation is initiated by synthesis of a collagenous extracellular matrix that activates MAPK signaling via β1 integrins. This activation is accompanied by translocation of P-ERK from the cytoplasm to nucleus where it binds and phosphorylates Runx2 already associated with regulatory regions of target genes; MAPK activation does not affect Runx2 distribution, which remains exclusively nuclear in undifferentiated and differentiated cells.[25,27] Nuclear Runx2 localization was also seen in the more highly metastatic PCa cell lines, PC3 and C4-2B. However, in non-metastatic LNCaP cells, considerable cytoplasmic staining was also observed (Figure 2). Although Runx2 is generally considered to be a nuclear protein, cytosolic localization has previously been reported.[7,38,39] For example, one study proposed

that Runx2 can shuttle between nucleus and cytoplasm where it binds to microtubules.[38] Also, cytoplasmic Runx2 staining was previously reported in BPH samples.[39] Thus, the concept that Runx2 can exist in the cytoplasm under certain conditions remains an intriguing subject for further study.

Cellular distribution studies using anti-Runx2 antibody are to be contrasted with results obtained with antibody against S319-P-Runx2, which in all cases showed an exclusively nuclear localization. While most nuclei in the three PCa cell lines were positively stained with anti-Runx2 antibody, the percentage of nuclei staining positive with anti-S319-P-Runx2 antibody progressively increased when LNCaP, C4-2B and PC3 cells were compared and this correlated with the level of MAPK activation and metastatic potential (Figure 1).

Phosphorylation of Runx2 by the RAS/MAPK pathway was previously analyzed using a combination of *in vivo* and *in vitro* kinase assays, mass spectroscopy and mutational analysis.[21] In addition to the serine 301 and 319 sites discussed here, phosphorylation sites were identified at serine 43, 282 and 510. The role of these additional sites in osteoblast differentiation was considered to be minor because S301A/S319A mutations eliminated most MAPK-dependent transcriptional activity.[21] However, S43, S282 and S510 may have greater roles in other cell types including PCa cells. Notably, in the present study, S301A, S319A mutant Runx2, although always being considerably less active than wild-type protein, still had appreciable ability to induce gene expression and stimulate migration/invasion. In this regard, studies on Runx1, a runt domain transcription factor required for hematopoiesis and associated with acute myelogenous leukemia, may be relevant. Runx1 is also phosphorylated by the RAS/MAPK pathway on serine residues homologous to S301, 319 and 510 in Runx2 as well as additional sites homologous to T326 and S329. Although mutation of the first two sites reduced the transforming activity of Runx1, it did not block hematopoietic activity, which required mutation of three additional sites.[40,41]

Runt domain transcription factors are also substrates for other kinases including Src, Pim-1, Cdc2, GSK3β, PKA and AKT.[42–47] Of these, the AKT pathway is of particular interest because of its role in PCa.[19] Inactivation of PTEN, a negative regulator of AKT, is frequently observed in metastatic PCa.[48] In fact, prostate-selective knockout of PTEN is a widely used mouse model for PCa.[49] Activation of the RAS/MAPK pathway in PTEN-null mice dramatically accelerates PCa progression suggesting possible cooperativity between RAS/MAPK and PI3K/AKT pathways.[17,50] Interestingly, Runx2 is elevated in the prostates of PTEN-null mice.[51] Consistent with these findings, AKT phosphorylation of Runx2 stimulates transcriptional activity and *in vitro* invasion by breast carcinoma cells.[47] The putative AKT phosphorylation sites (S203, T205, T207) are all in the runt domain of Runx2 and distinct from MAPK sites located mainly in the C-terminal region. Like results obtained with MAPK sites, AKT site mutation reduced, but did not abolish, all Runx2 activity. It is, therefore, possible that both RAS/MAPK and PI3K/AKT pathways phosphorylate Runx2 in cancer cells and that both types of phosphorylation contribute to overall Runx2 activity.

An anti-Runx2-S319-P antibody previously developed in the project laboratory was found to be an excellent reagent for discriminating between PCa and benign prostatic disease.[26] This antibody strongly stains nuclei of primary prostate tumors at both early (PIN) and later stages of disease progression, but minimally stains BPH or prostatitis samples. Furthermore, the amount of staining in primary tumors correlated with Gleason score and presence of LNM. Previous studies using commercially available anti-Runx2 antibodies gave variable results with noncancerus and early-stage PCa tissues. Some studies reported diffuse cytoplasmic and intense nuclear staining for total Runx2 in normal and cancerous prostate tissue while others reported staining only in more advanced high Gleason score cancers and not in early PIN.[6,7] In contrast, anti-Runx2-S319-P antibody strongly stained basal and luminal cell nuclei in PIN (Figure 7 and Table 1). This may indicate both that this antibody is more sensitive and that early Runx2 phosphorylation is a hallmark of PCa that becomes more intense with disease progression. As noted above, anti-Runx2-S319-P antibody staining is exclusively nuclear and associated with transcriptional activation.[25,27] Thus, the detection of P-S319-Runx2 in patient samples may indicate that Runx2 is in an active form capable of stimulating the genetic program necessary for invasion and metastasis. Detection of P-S319-Runx2 in primary tumors may also have predictive value in discriminating between tumors that will remain localized and those that will progress to metastasis.

MATERIALS AND METHODS

Antibodies

Antibodies used in this study were obtained from the following sources: Phospho ERK and total ERK antibodies from Cell Signaling (Beverly, MA, USA), total Runx2 antibodies from MBL (cat. no. D130 3, mouse monoclonal antibody; Woods Hole, MA, USA) or Abcam (cat. no. ab76956, mouse monoclonal antibody; Cambridge, MA, USA), anti human Ki67 monoclonal antibody from Dako North America, Inc. (cat. no. F7268; Carpinteria, CA, USA) and Caspase 3 polyclonal antibody from Cell Signaling (cat. no. 9661). A rabbit polyclonal antibody specifically detecting Runx2 S319 P was previously described.[26]

Cell culture and measurement of metastasis-associated mRNAs

LNCaP, C4 2B, PC3 and RWPE1 cells were obtained from the ATCC (Manassas, VA, USA). LNCaP, PC3 and C42B cells were maintained in RPMI 1640, and 10% fetal bovine serum while RWPE1 cells were cultured in Keratinocyte SFM (Gibco Life Technologies, Grand Island, NY, USA). RNA was isolated from cells and tumors using TriZol reagent (Invitrogen, Grand Island, NY, USA) and mRNAs were measured by quantitative real time RT/PCR as described previously.[25]

Western blot analysis and immunofluorescence

Western blot analysis of cell and tissue extracts was conducted as previously described.[26] All primary antibodies were used at a 1:500 dilution. Sheep anti mouse IgG or donkey anti rabbit IgG secondary antibody conjugated with horseradish peroxidase was used at a 1:10000 dilution and HRP activity was detected by ECL (Amersham, PIttsburgh, PA, USA). Films were scanned for densitometric analysis using ImageJ 1.46 image analysis software (open source, http://en.softonic.com/s/imagej 1. 46r). For immunofluorescence, cells were grown on glass cover slips, fixed and permeabilized with 0.25% Triton X 100 as previously described.[25] Total Runx2 (Abcam antibody) and S319 P Runx2 antibodies were used at a 1:500 dilution. Fluorescence was detected using an Olympus FluoView 500 Laser Scanning Confocal Microscope (Olympus Inc., Waltham, MA, USA).

Transient transfections, luciferase reporter assays and virus transduction

Transient transfection of PC3 cells was accomplished using lipofectamine reagent (Invitrogen), 0.5 μg pOPN Luc reporter plasmid containing a 1454/+5 bp region of the proximal murine *Spp1* promoter, 0.05 μg of pRL SV40 plasmid encoding *Renilla reformis* luciferase with or without 0.5 μg Runx2 and/or MEK1 expression plasmids.[32,52,53] Cells were harvested 48 h after transfection and assayed using a dual luciferase kit (Promega, Madison, WI, USA). Adenovirus expressing β galactosidase (AdLacZ), wild type Runx2 (AdRunx2 WT) or phosphorylation deficient S301A, S319A mutant Runx2 (AdRunx2 SA) were previously described.[21] Viral titers were adjusted to give equivalent expression of Runx2 protein.

Cell migration assay

For PC3 cells, migration was measured using a Radius 24 Well Cell Migration Assay Kit according to the manufacturer's instructions (Cell Biolabs Inc., San Diego, CA, USA). Cells were transduced with adenovirus vectors and, after 48 h, were trypsinized and plated in migration wells at a density of 50 000 cells/cm^2. After 24 h, the Radius biocompatible gel layer was removed and cells were allowed to migrate to the cell free area. For RWPE1 cells, migration was measured by plating cells in 12 well tissue

culture plates and, after 24 h, removing a consistent area of cells by scraping with a Pasteur pipette. In both cases, pre migration and post migration images were captured with an inverted microscope at various time points and migration of cells into the initially cell free area was analyzed using ImageJ 1.46 image analysis software.

Cell invasion assay

Cell invasion was measured using a Cytoselect 24 Well Cell Invasion Assay Kit according to the manufacturer's instructions (Cell Biolabs Inc.). Cells were trypsinized, suspended in serum free medium and plated at 3×10^5 cells per well in the upper well containing Matrigel and allowed to invade toward the lower chamber, which contained 10% fetal bovine serum and 100 ηg/ml SDF 1. After 24 h, invasion of cells to the bottom side of the insert membrane was measured by staining with Coomasie Blue and counting cells in five separate fields per insert.

Subcutaneous tumor growth and immunohistochemistry

All procedures were approved by the University Committee on the Use and Care of Animals. PC3 cells stably expressing firefly luciferase[54] were transduced with AdLacZ, AdRunx2 WT or AdRunx SA vectors. After 24 h, cells were trypsinized and 1×10^6 cells were mixed with Matrigel and subcutaneously injected into 16 week old immunodeficient mice (Foxn1nu/Foxn1nu; 2 injection sites per mouse). Tumor growth was monitored over a 16 day period using bioluminescence imaging. For analysis, 50 ul of 40 mg/ml D luciferin substrate in sterile phosphate buffered saline was injected into each mouse and the luminescence signal was captured on an IVIS Spectrum instrument (Perkin Elmer, Waltham, MA, USA) in the University of Michigan Small Animal Imaging Core. Image analysis was performed using Living Image software version 4.0 (Perkin Elmer). Mice were then killed and tumors were dissected free of connective tissue before analysis.

For immunohistochemistry, tumors were fixed in 4% paraformaldehyde before paraffin embedding, sectioning and immunohistochemical staining. Sections were stained with anti human Ki67 or Caspase 3 primary antibody followed by HRP anti mouse or rabbit IgG. Samples were counterstained with hematoxylin and eosin.

TMA construction and analysis

Tissue samples from a total of 129 caucasian patients with prostate disease were used to construct TMAs. Among these were 106 tumor cases represented by 93 PPCs treated by total prostatectomy with curative intent and 13 LNM from biopsies of patients with inoperable primary prostate adenocarcinomas, 15 cases of BPH and 8 cases of prostatitis (P) (4 prostatic granulomatosa and 4 prostatic xantogranulomatosa). Paraffin blocks were retrieved from the archives of University of Foggia, Italy. All tissues were obtained with patient informed consent. Tissue procurement and analysis was approved by the University of Foggia Ethical Committee. For TMA construction, areas rich in non necrotic tumor cells were identified on corresponding hematoxylin and eosin stained sections and marked on the source paraffin block. Cores (3 mm) were obtained from the source block and transferred to the recipient master block. Hematoxylin and eosin staining of a 4 μm TMA section was used to verify all samples. Immunohistochemical analysis was performed using Ventana Benchmark XT autostainer (Ventana Medical Systems, Tucson, AZ, USA) and/or manual standard linked streptavidin biotin horseradish peroxidase technique. Primary anti Runx2 S319 P antibody was diluted 1:200 with phosphate buffered saline and incubated with TMA sections for 30 min. Negative control slides without primary antibody were included for each staining. Two pathologists (PB, GP) independently evaluated immunostaining results. The study was double blinded and investigators did not have real time access to the clinical data. For quantitative analysis, a score based on the percentage of P Runx2 stained cells was calculated by evaluating 10 high power representative fields (×400) on complete tumor sections. P Runx2 staining in PPC samples was compared with benign prostatic hyperplasia (BPH) and prostatitis as well as LNM. Within the PPC group, P Runx2 staining was related to Gleason score (Low Gleason 3+3, 3+4, 4+3; High Gleason 3+5, 4+4, 4+5, 5+3, 5+4), perineural permeation, presence or absence of LNM at initial clinical staging, presence or absence of PIN; positive or negative surgical margins; and intraprostatic or extraprostatic extension.

Statistical analysis

For cell culture experiments, statistical analyses were performed using SPSS 16.0 Software (IBM Software, Armonk, NY, USA). Unless indicated otherwise, each reported value is the mean ± s.d. of triplicate independent samples. Statistical significance was assessed using a one way analysis of variance. For TMAs, analysis was conducted using Stanton Glantz statistical software 3 (MS DOS McGraw Hill Medical, New York, NY, USA) and GraphPad Prism software version 4.00 for Windows (Graph Pad Software, San Diego, CA, USA; www.graphpad.com). P values < 0.05 were considered significant. The one way analysis of variance and the Student Newman Keuls tests were used to study the differences between groups.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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