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PRINCIPAL INVESTIGATOR: Bingcheng Wang, Ph.D.

CONTRACTING ORGANIZATION: Case Western Reserve University
Cleveland, Ohio 44109

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Bingcheng Wang, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Case Western Reserve University
Cleveland, Ohio 44109
email resadm@po.cwru.edu

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13. ABSTRACT *(Maximum 200 Words)*

Cancer metastasis involves tumor cell dissemination to and uncontrolled growth at distal organs. Research in this lab shows that activation of endogenous EphA2 kinase in prostate cancer cell lines suppressed cell proliferation by targeting Ras/MAPK pathway and inhibited integrin-mediated cell migration. The goal of the proposal is to develop novel EphA2 agonists and to test their effects on prostate cancer progression. Toward this goal, we proposed to solve the crystal structure of EphA2 kinase in complex with the native ligand as well as a small peptide that we have isolated from phage display library. In addition, we planned to investigate the molecular mechanisms behind the suppressive effects of EphA2 on Ras/MAPK pathway. Much progress has been made during the past year. A new method has been developed to purify EphA2 ligand binding domain and ephrin-A1 ectodomain, which are being used in structural studies. New in vivo model systems have adapted for in vivo testing of new EphA2 agonists. The potential involvement of candidate signaling and adaptor proteins in the suppression of Ras/MAPK pathway has been examined. The results complement those from domain swapping and mutagenesis studies, and suggest that novel signaling pathways are initiated from the activated EphA2 kinase.

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Table of Contents

Cover.....1

SF 298.....2

Table of Contents.....3

Introduction.....4

Body.....4

Key Research Accomplishments.....9

Reportable Outcomes.....9

Conclusions..... 11

References.....11

Appendices..... ...12

A. INTRODUCTION

The central goal of this proposal is to test the feasibility of inhibiting prostate cancer metastasis using EphA2 agonists. For a prostate cancer patient, the difference between life and dead is whether the tumor cells have metastasized or not. There are two key determinants in successful tumor metastasis. One is tumor cell dissemination to a distal organ; the other is metastatic tumor cell growth at the new site. Cell motility plays a critical role in tumor cell dissemination, both during invasion of surrounding tissues at the original tumor site, and during extravasation and migration to favorable growth sites at the distal organ. Indeed, prostate cancer cell motility in vitro has been directly correlated with metastatic potential of prostate cancer in vivo (1;2). Metastatic tumor cell growth at distal sites is regulated by paracrine and autocrine factors, many of which converge on Ras/Raf/MEK/ERK1/2 MAP kinase (MAPK) growth stimulatory signaling pathway. Recent studies have shown that MAPK activity is elevated in prostate cancer specimens compared with normal tissue (3;4), the degree of which is correlated with tumor progression (3;5). Therefore, agents that can suppress either prostate cancer cell motility or MAPK activity can potentially be exploited to prevent metastasis in vivo. Agents that can accomplish both feats will be even more desirable. We have found that agonists of EphA2 kinase possess this unique combination of properties, i.e. they were capable of suppressing both cell motility (6) and Ras/MAPK signaling cascade (Appendix 1) (7). EphA2 activation by its natural ligand or an agonistic peptide isolated from peptide phage display libraries inhibited cell adhesion, spreading and migration by targeting integrins and focal adhesion kinase (FAK). Unexpectedly, activated EphA2 also potently suppressed the ERK1/2 MAPK activity. In keeping with the growth regulatory role of MAPK, activation of EphA2 kinase inhibited cell proliferation. The central hypothesis of the original proposal is that EphA2 agonists can prevent prostate cancer progression by inhibiting both cell migration and proliferation.

Over the past year, we have made progress on a number of projects in the proposed studies, the details of which will be outlined below. One important development for this proposal during the past year is the awarding of two NCI grants to this PI. Most importantly, both grants are a direct result of the Phase I and Phase II New Investigator Award from the USAMRMC. The Prostate Cancer Research Program at USAMRMC deserves much congratulations and gratitude for introducing this new investigator into prostate cancer research for a long time to come. Also importantly, because one of the grants (CA92259) has overlap with the current USAMRMC funding, this PI has suspended the expenditure from the current DOD grant since April 2002. We are currently collecting new data in order to request an extension of existing funding to new and exciting areas of research that are closely related to the original USAMRMC funding, but are not funded by any other sources. As a result, although this report will cover the activities from whole year last year, please bear in mind that recent progresses since April 2002 are funded by NCI.

B. BODY

B1. List of original SPECIFIC AIMS

1). **To conduct structure-function studies on EP1 peptide**
   a) In collaboration with Dr. D. Nikolov, we will solve the X-ray crystallographic structure of EphA2 ligand-binding domain bound with the EP1 agonistic peptide.
   b) The structural information will be used to guide the design of new libraries for affinity selection of a new generation of peptides that exhibit higher affinity and potency than EP1.
   c) The new peptides will be tested directly in vivo for activities against prostate cancer growth and metastasis. They can also serve as lead molecules for synthesis of small organic compounds targeting EphA2 kinase.

2). **To delineate the molecular mechanisms of EphA2 activation-induced inhibition of cell proliferation and Ras/MAPK signaling pathway**

a) Since both FAK and integrins have been implicated in MAPK activation, the role of FAK and integrin inactivation by EphA2 will be investigated.
b) We will map the domain(s) in EphA2 that mediate inhibition of Ras/MAPK signaling. The responsible domain will be used in GST-pull down or yeast two hybrid system to identify EphA2 effectors for Ras/MAPK suppression.

B2. PROGRESSES (Itemized according to statement of work)

B2.1 TASK 1 To investigate the structure-function relationship of EphA2 agonistic peptides, and to isolate high potency peptides based on the structural studies.

1). To clone EphA2 ligand binding domain to human IgG1 heavy chain (EphA2LBD-Fc), containing a thrombin cleavage site between EphA2 and Fc (Months 1-3).
2). To purify EphA2LB-Fc on Protein A-Sepharose affinity column, and to cleave off EphA2LB (Months 1-3).

Both of these tasks have been completed. We have generated over 55 mg of EphA2-LBD-Fc. The recombinant protein was cleaved at the junction between the EphA2-LBD and Fc moieties (Fig. 1), leading to the purification of over 14 mg of EphA2-LBD.

Moreover, encouraged by the success of the system, we have expanded on the original proposal, and produced 16 mg of ephrin-A1 in the same fashion. We are now trying to co-crystallize EphA2/ephrin-A1 complex.

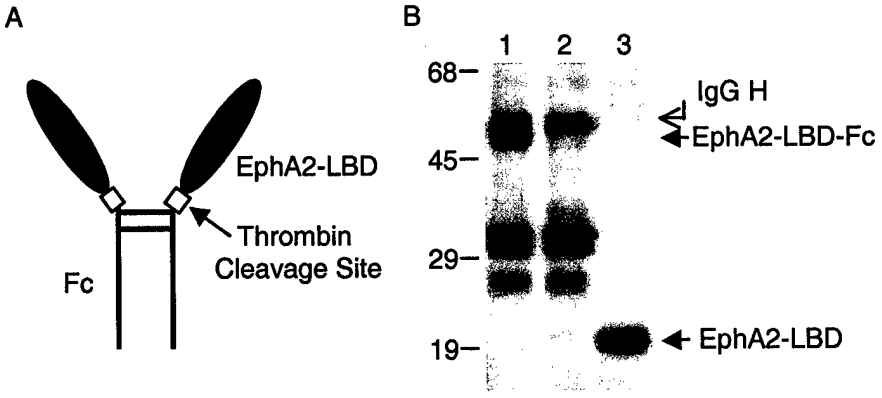

Fig. 1. A) Schematic illustration of EphA2-LBD-Fc with a thrombin cleavage site engineered in. **B)** Production of EphA2-LBD for X-ray crystallography. EpA2-LBD-Fc was cloned into pcDNA3 expression vector and chimeric protein was produced in stably transfected 293 cells. The fusion protein was purified from culture medium on a Protein A Sepharose column. After washing the column with cleavage buffer (50 mM Tris pH 7.5, 150 mM NaCl, 2.5 mM $CaCl_2$) , thrombin (0.02% of the total fusion protein on the column) was added to the column and incubated for 5 hours at RT. Shown is an SDS-PAGE gel stained with Coomassie Blue. Lane 1, Column-bound materials before thrombin cleavage; lane 2, column-bound materials after thrombin cleavage; lane 3, thrombin cleavage product eluted from the column. The indicated IgG heavy chain was from residual bovine IgG from low IgG serum used to culture cells. Similar strategies have been used to produce mammalian ephrin-A1.

3). To co-crystallize EphA2LBD with EP1 (Months 4-6).
4). To solve the X-ray crystallographic structure of EphA2LBD bound to EP1 (7-12).

Using the purified EphA2LBD protein, our collaborators at the Memorial Sloan Kettering Cancer Center, Drs. Dimitar Nikolov and Juha Himanen, are testing crystal growth conditions. Preliminary results show that the EphA2-LBD recombinant proteins can indeed be crystallized, which is quite encouraging. However, so far we have not been able to obtain big crystals that diffract well. They are improving the crystallization conditions. With the new ephrin-A1 recombinant protein, they are also trying to co-crystallize EphA2LBD with ephrin-A1, which may help stabilize the structure of both proteins. In addition, the small size of the EphA2-LBD and ephrin-A1 ectodomain also makes it possible to perform NMR structural studies. In case that either protein does not diffract well, we will adapt NMR strategy. Dr. Jun Qin, a well-established NMR expert at the Cleveland Clinic Foundation, has agreed to assist us should we decide to use this approach.

5). To design new custom Phage Peptide Display Libraries based on the structural information (Months 13-18).
6). To affinity-select high potency EphA2 agonistic peptides using the new libraries (Months 13-18).

We have expanded on these SOWs and incorporated molecular modeling in these studies. A new postdoc, Eugene Myshkin, has recently joined the PI's laboratory to do structural analyses using molecular modeling. We have already modeled the structure of the EphA2 based on the published structure of EphB2, and docked EP1 peptide into the binding pocket (Fig. 2). With this approach, we have identified potential contact residues in EP1 peptide that will be used to guide new library design.

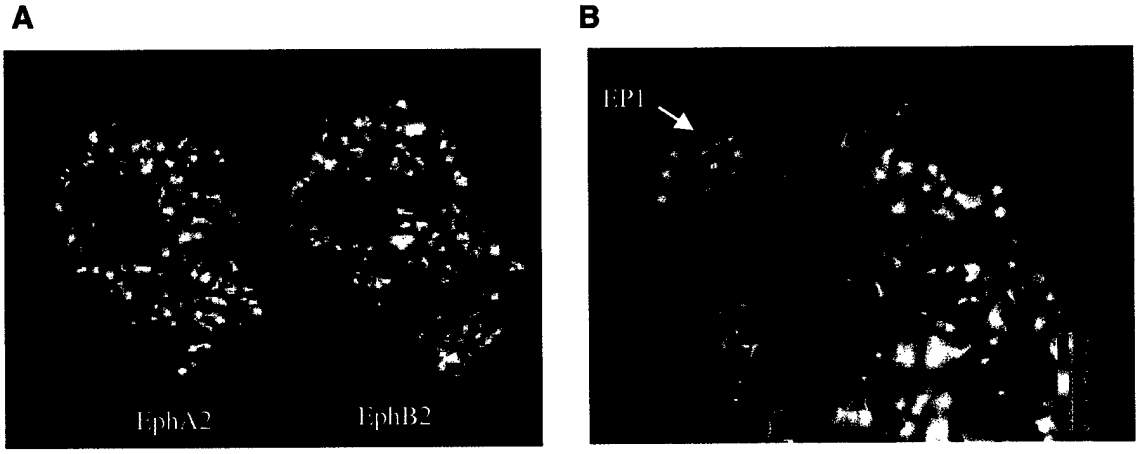

Fig. 2. A) Predicted structure of EphA2 using molecular modeling based on the published structure of EphB2. B) Docking of EP1 peptide into the predicted binding pocket of EphA2 using molecular modeling.

7). To characterize the peptides in vitro inhibit prostate cancer cell growth and motility (Months 18-24).
8). To test the efficacy of the best peptides identified above in inhibiting prostate cancer progression in xenograft model (Months 18-24).

A central objective of these studies is how Epha2 agonists will impact prostate cancer metastasis. To this end, a good model system is required. We have recently adapted green fluorescence labeled cells to facilitate detection of tumor metastases as small as a single cell. An example from GFP-labeled Dunning Rat Met-LyLu cells is shown in Fig. 3.

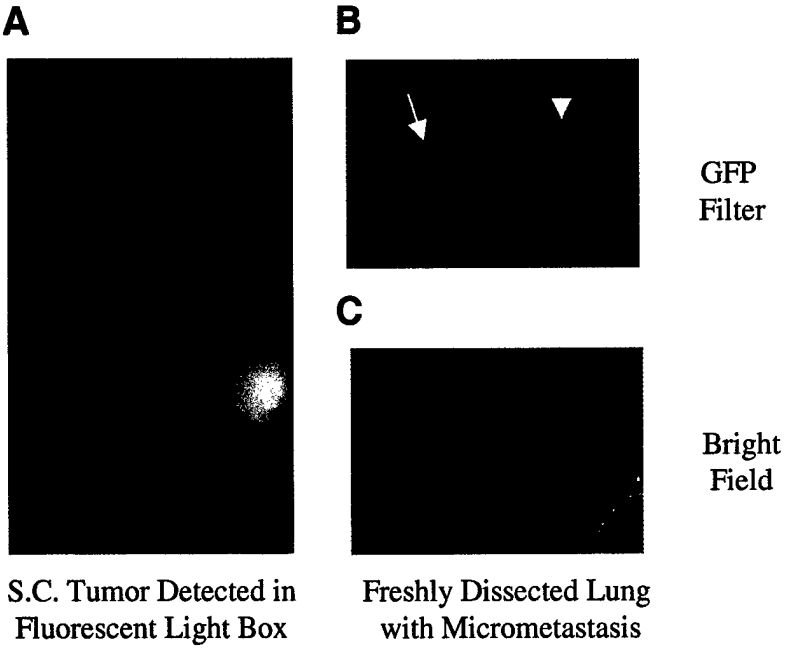

Fig. 3. A) Growth of GFP-tagged MatLyLu cells two weeks after s.c. implantation. The mouse was placed in a fluorescence light box to visualize GFP. B) A dissected lung from the mouse in (A) was placed under a fluorescent microscope fitted with a GFP filter. Arrow and arrow head point to single and multiple cell metastases, respectively. C) Bright field image of the same field as in (B).

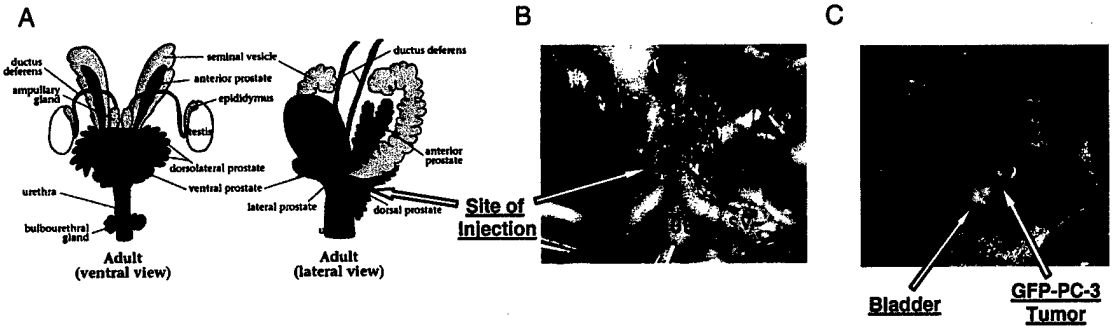

Fig. 4. A) Diagram of the male urogenital system in adult mice (adapted from Cunha et al. 1987). **B)** A ventral view of prostate gland after a mouse was opened up. DL: dorsolateral gland; UB: urinary bladder; SV: seminal vesicle. **C)** Fluorescence imaging of intraprostatic GFP-PC-3 tumor growth four weeks after orthotopic implantation of $1x10^5$ cells.

As prostate cancer metastasis originates from the prostate gland, one model that is believed to better mimic this pathophysiological process is orthotopic implantation (8), where prostate cancer metastasis is monitored following injection of tumor cells directly into prostate glands. Recently, we have successfully adapted this model in conjunction with GFP-tagging as is shown in Fig. 4. With

this powerful model, we are in an ideal position to investigate how EphA2 agonist may impact prostate cancer metastasis.

B2.2 TASK 2 To characterize the molecular mechanisms of EphA2 activation-induced inhibition of Ras/MAPK signaling pathway.

1). To investigate the involvement of candidate signaling and adaptor proteins, including Shc, Grb2, and RasGAP. Since we have shown that both integrins and FAK were inactivated by EphA2, their role will also be examined (Months 1-3).

Our studies so far have ruled out the involvement of FAK, as $FAK^{-/-}$ cells still respond to ephrin-A1 stimulation and show the same degree of MAPK inhibition. The phosphorylation status of Shc and Grb2 recruitment to Shc did not change significantly either (Fig. 3), suggesting that other novel mechanisms are involved. With the ongoing effort to identify regions in EphA2 responsible for the inhibition of MAPK, we will be in better position to determine the potential mechanisms involved.

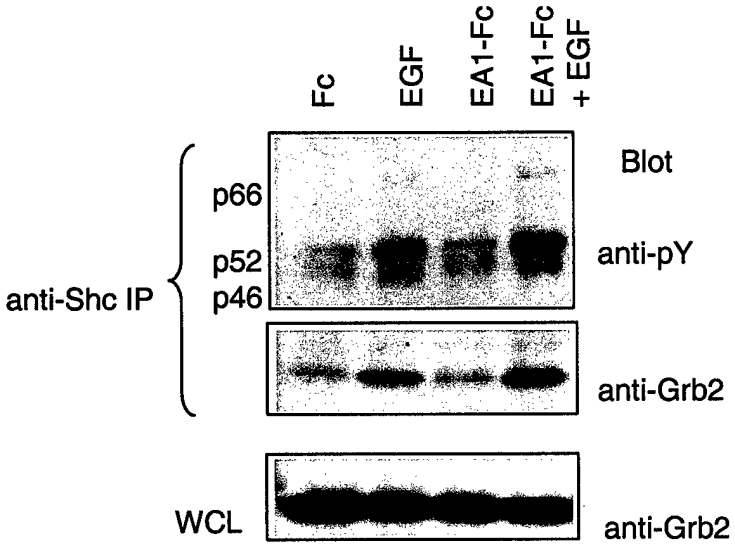

Fig. 5. EphA2 activation does not alter the phosphorylation status of Shc and recruitment of Grb2 to to Shc. PC-3 cells were stimulated with 1 μg/ml ephrin-A1-Fc for 10 min. Cell were lysed in buffer containing 1% TX-100. Shc was immunoprecipitated and blotted for phosphorylation status and the associated Grb2.

2). To identify regions in EphA2 responsible for suppressing Ras/MAPK by carrying out domain-swapping between EphA2 and EphB3 kinases, as we have found that EphB3 activation increased MAPK activity (Months 4-9).

3). To create EphB3/A2 domain swapping mammalian expression constructs and to establish stable 293 transfectants (Months 4-9).

4). To test the effects of chimeric receptors on MAPK activation and cell proliferation.

New results using the domain-swapping mutant outlined in the original proposal show that the PDZ domain binding site residing in the very carboxyl terminal 6 amino acids is not required. Neither is

the 70 amino acid SAM domain next to it (not shown). We are currently testing other domain-swapping mutants.

5). To generate deletion and site-directed mutants of EphA2 to confirm findings from above (Months 10-14).

6). To use the domain identified above in yeast two-hybrid screening to identify mediator of Ras/APK inhibition of EphA2 kinase (Months 10-24).

These studies are in progress.

C. KEY RESEARCH ACCOMPLISHMENTS

- Designed a novel strategy to purify recombinant EphA2 ligand binding domain and ephrin-A1 ectodomain produced in mammalian cells that preserve the natural folding of the proteins. EphA2LBD crystals have been obtained, and crystallization conditions for EphA2/ephrin-A1 complex are being tested.
- Purified sufficient quantities of fusion proteins for crystallographic studies.
- Performed molecular modeling of EphA2 based on the published data, and docked EP1 peptide into the predicted binding pocket on EphA2.
- Completed screening of key candidate proteins that were suspected in mediating EphA2 activation-induced inhibition of Ras/MAPK pathway.
- Developed new strategies for testing sensitive detection of single cell prostate cancer metastasis.

D. REPORTABLE OUTCOMES

1) Funding obtained based on the studies funded by DOD:

NCI 1 R01 CA96533. Eph kinase signaling in prostate cancer. 9/30/01-8/31/06.

NCI 1 R01 CA92256. EphA2 agonists as novel inhibitors of tumor progression, 4/1/02-3/31/07.

CaP CURE award, 2001.

2) Publications:

Khan, S., Koepke, A., Jarad, G., Schlessman, K., Cleveland, R. P., Wang, B., Konieczkowski, M., and Schelling , J. R. (2001). Apoptosis and JNK activation are differentially regulated by Fas expression level in renal tubular epithelial cells. **Kidney International** 60:65-76.

Miao, H., Wei, B.-R., Peehl, D. M., Li, Q., Burnett, E., Alexandrou, T., Sedor, J. R., Schelling, J. R., and Wang, B. (2001). EphA kinase activation inhibits Ras/MAPK pathway. **Nature Cell Biology** 3:527-530.

Zantek, N. D., Walker-Daniels, J., Stewart, J., Hansen R. K., Robinson D., Miao, H., Wang, B., Kung, H.J., Bissell, M. J., Kinch, M. S. (2001). MCF-10A-NeoST: A New Cell System for Studying Cell-ECM and Cell-Cell Interactions in Breast Cancer. **Clinical Cancer Research** 7:3640-3648.

Jarad G, Wang B, Khan S, DeVore J, Miao H, Wu K, Nishimura SL, Wible BA, Konieczkowski M, Sedor JR, Schelling JR (2002). Fas activation induces renal tubular epithelial cell b8 integrin expression and function in the absence of apoptosis. **Journal of Biological Chemistry** (*in press, published online 9-02*).

Han, D. C., Shen, T. L., Miao, H., and Wang, B. Guan, J.-L. (2002). EphB1 associate with Grb7 and regulate cell migration. **Journal of Biological Chemistry** (*in press, published online 9-02*).

3) Manuscript:

Wei, B.-R. and Wang, B. (2002). Systemic administration of ephrin-A1 suppressed growth of androgen-independent prostate cancer in *nu/nu* mice. **Oncogene** (*Submitted*).

Miao, H., Strebhardt, K., Paquale, E. B., and Wang, B. (2002). Inhibition of integrin-mediated cell adhesion and spreading, but not migration requires the catalytic activity of EphB3 kinase. **Journal of Biological Chemistry** (*submitted*).

4) Invited Oral Presentations:

Phage display, NIH Workshop on "Genomics and Proteomics in Kidney and Urologic Diseases" July 2001.

Eph kinase in prostate cancer growth and metastasis. Department of Cancer Research and Genomics and Gene Therapy, Berlex Biosciences, San Francisco, July 2002.

Eph kinase signaling in development and disease. Department of Anatomy and Massey Cancer Center, Virginia Commonwealth University, Sep., 2002.

FAK and MAPK as downstream effectors of Eph kinases: Implications in prostate cancer therapy. Ireland Cancer Center, Case Western Reserve University, Sep. 2002.

EphA2 Receptor Tyrosine Kinase Regulates Renal Epithelial Cell-Cell Adhesion through Rho GTPases. 38th Annual Meeting of America Society of Nephrology, Philadelphia, PA, Nov. 2002.

Eph kinase signaling and tumor progression. 10th SCBA International Symposium, August, 2003.

Regulation of cell growth and proliferation in prostate cancer. Department of Pharmacology, Emory University, Atlanta, GA, Jan. 2003.

5) Reagents

1) A number of expression plasmids were created that express different mutant form of EphA2 and EphB3 kinases.
2) Several new fusion proteins were designed and created.

6) New model system.

1) A new model system was developed to study prostate cancer metastasis, involving tagging tumor cells with GFP in conjunction with orthotopic implantation.

2) A novel strategy was adapted to produce recombinant proteins in mammalian cells in large quantities for crystallographic and NMR studies. This strategy should make it possible to solve structure of proteins that are previously difficult or impossible to solve because aggregation in bacteria and insect cells.

E. CONCLUSION

In sum, over the past year significant progress has been made in virtually all the approved SOWs. In fact, in several areas we have expanded on the original SOWs and ventured into new directions and new systems that strengthened the power to test our original hypothesis. These include the adaptation of molecular modeling approach and novel systems for detecting single cell metastases. While molecular modeling will enable us to rationally design new generation of EphA2 peptides based the structure, the sensitive GFP-tagging/orthotopic implantation model system will greatly facilitate the testing of novel EphA2 agonists in vivo. Finally, the strongest evidence of accomplishment of these DOD funded projects is the fact that it has led to significant additional federal funding to the PI from NCI. This will ensure continued long-term success of these studies.

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Activation of EphA receptor tyrosine kinase inhibits the Ras/MAPK pathway

Hui Miao*†, Bih-Rong Wei*†, Donna M. Peehl‡, Qing Li*, Terry Alexandrou*, Jeffrey R. Schelling*, Johng S. Rhim§, John R. Sedor*, Elisabeth Burnett* and Bingcheng Wang*†¶

*Rammelkamp Center for Research, MetroHealth Campus, and †Ireland Cancer Center and Department of Pharmacology, Case Western Reserve University School of Medicine, 2500 MetroHealth Drive, Cleveland, Ohio 44109 USA

‡Department of Urology, Stanford University, Stanford, California 94305, USA

§Department of Surgery, Uniformed Services University of the Health Sciences, Bethesda, Maryland 20814, USA

¶e-mail: bxw14@po.cwru.edu

Interactions between Eph receptor tyrosine kinases (RTKs) and membrane-anchored ephrin ligands critically regulate axon pathfinding and development of the cardiovascular system, as well as migration of neural cells. Similar to other RTKs, ligand-activated Eph kinases recruit multiple signalling and adaptor proteins, several of which are involved in growth regulation[1,2]. However, in contrast to other RTKs, activation of Eph receptors fails to promote cell proliferation[3,4] or to transform rodent fibroblasts[5], indicating that Eph kinases may initiate signalling pathways that are distinct from those transmitted by other RTKs. Here we show that stimulation of endogenous EphA kinases with ephrin-A1 potently inhibits the Ras/MAPK cascade in a range of cell types, and attenuates activation of mitogen-activated protein kinase (MAPK) by receptors for platelet-derived growth factor (PDGF), epidermal growth factor (EGF) and vascular endothelial growth factor (VEGF). In prostatic epithelial cells and endothelial cells, but not fibroblasts, treatment with ephrin-A1 inhibits cell proliferation. Our results identify EphA kinases as negative regulators of the Ras/MAPK pathway that exert anti-mitogenic functions in a cell-type-specific manner.

To investigate how activation of EphA2 kinase affects the MAPK signalling pathway, we used recombinant ephrin-A1 dimerized by fusion to human immunoglobulin G (IgG; the construct is termed ephrin-A1–Fc) to stimulate pRNS-1-1 and PC-3 human prostatic epithelial cells, mouse embryonic fibroblasts (MEFs), and primary bovine aortic endothelial cells (BAECs). Stimulation of all four cell types with ephrin-A1–Fc caused potent and sustained inhibition of $p42^{MAPK}$ and $p44^{MAPK}$ (extracellular-signal-regulated kinase 2 (ERK2) and ERK1; Fig. 1a). Similar results were obtained using NIH 3T3 and human embryonic kidney 293 cells (data not shown). The inhibition occurred 2–5 min after addition of ligand and persisted for at least 2 h. The four cell types primarily expressed EphA2 kinase (PC-3 > MEF = pRNS-1-1 > BAEC), as well as low levels of other EphA-subfamily kinases including EphA1 (PC-3 and pRNS-1-1 cells) and EphA3 (MEFs). As Eph–ephrin interactions are promiscuous[6], ephrin-A1 was likely to bind to and activate most EphA kinases in these cells. Thus, activation of endogenous EphA2, and possibly of other EphA kinases, by ephrin-A1 suppressed MAPK activity. The inhibitory effects seemed to be specific to ERK1/2 MAP kinases, as JNK activity was not affected (data not shown).

An important function of MAPK activation is regulation of cell proliferation[7]. To test the effects of ephrin-A1 on cell growth, we subjected pRNS-1-1 cells to a clonal-growth assay (see Methods)[8]. Treatment with soluble ephrin-A1–Fc significantly reduced clonal growth compared with the Fc control (Fig. 1b). Cell counting revealed a 68% reduction in total cell number. Although there were similar numbers of colonies, they were significantly smaller in wells treated with ephrin-A1–Fc (Fig. 1b), indicating a slower growth rate. We confirmed this by assaying incorporation of 5-bromodeoxyuridine (BrdU), and observed a 35% inhibition in BrdU incorporation ($P = 0.01$) in cells treated with ephrin-A1–Fc (Fig. 1c). Fluorescence-activated cell sorting (FACS) analyses for cells with subdiploid DNA content, and terminal deoxynucleotidyl transferase-mediated nick end labelling (TUNEL) staining for apoptotic cells showed that there was no increase in apoptosis in cells treated with ephrin-A1–Fc compared with control cells (data not shown). Together, these results indicate that reduced cell proliferation, rather than increased apoptosis, was responsible for the reduction in cell number. Growth inhibition was also observed in PC-3 cells (data not shown) and in BAECs (Fig. 1d). *In vivo*, interactions between Eph kinase and ephrin ligand occur upon cell–cell contact. To mimic cell-contact-induced activation of EphA2 kinase *in vivo*, we immobilized ephrin-A1–Fc on nitrocellulose-coated wells. Immobilized ephrin-A1–Fc inhibited cell growth to the same extent as soluble ephrin-A1–Fc (Fig. 1b). MAPK activity remained lower in cells treated with soluble or immobilized ephrin-A1–Fc, compared with control cells, for the duration of the experiments (data not shown). However, not all cell types were growth-inhibited by ephrin-A1. For example, MEFs were resistant to growth inhibition by ephrin-A1 (data not shown), despite the fact that MAPK activation was similarly suppressed in these cells (Fig. 1a). These results indicate that the anti-proliferative effects of activated EphA kinases may be cell-type-specific.

Inhibition of MAPK can be mediated by suppression of the Ras/Raf/MEK/MAPK signalling cascade, or by activation of MAPK phosphatases (MKPs). MKPs are dual-specificity phosphatases that are regulated at the level of transcription and protein degradation[9,10]. However, both of these regulatory mechanisms exhibit slower kinetics with a delay of ~30 min, whereas inhibition of MAPK by ephrin-A1 was evident within 2–5 min, indicating that MKP activation was unlikely to contribute significantly to MAPK inactivation.

To investigate the involvement of the Ras/Raf/MEK/MAPK signalling cascade, we first determined the effects of activation of EphA kinases on MEK, an upstream kinase of MAPK. In pRNS1-1 cells, stimulation with ephrin-A1–Fc inhibited MEK activation in a dose-dependent manner (Fig. 2a, left panel); the degree of inhibition was correlated with inhibition of MAPK (Fig. 2b, left panel). We then compared the activation status of MAPK and MEK in parental pRNS-1-1 cells and in pRNS-1-1 cells that were transformed with constitutively activated Ras (pRNS-1-1-Ras). Transformation with constitutively activated Ras completely blocked inhibition of MEK and MAPK by the ligated EphA kinases (Fig. 2a, b). Moreover, Ras also rendered cells refractory to growth inhibition by ephrin-A1–Fc (Fig. 2c). EphA kinases were expressed at similar levels, exhibited a similar distribution (Fig. 2d,) and were

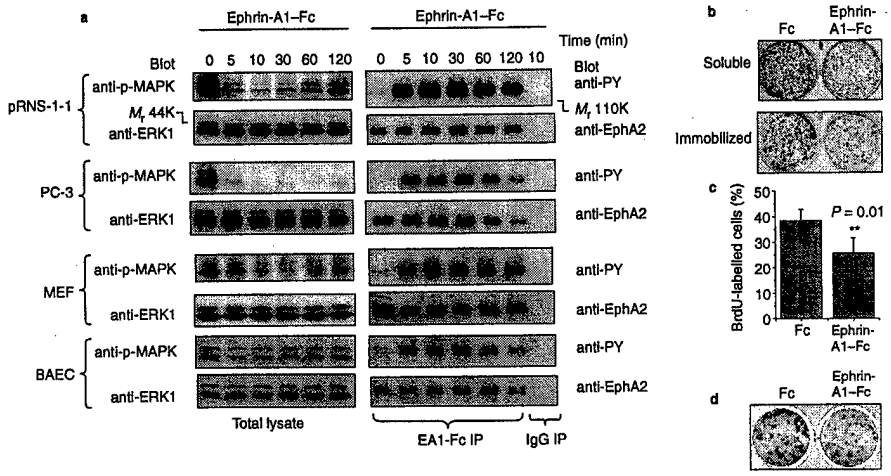

Figure 1 **Ephrin-A1–Fc stimulation inhibits p42^{MAPK} and p44^{MAPK} and suppresses proliferation of pRNS-1-1 cells. a**, Suppression of MAPK by stimulation with ephrin-A1–Fc. Exponentially growing cells in six-well dishes were stimulated with ephrin-A1–Fc for the indicated times. Left panel, total cell lysates were immunoblotted with a specific antibody against phospho-MAPK (p-MAPK) to determine the activation status of MAPK, or with anti-ERK1 antibody as a loading control. Right panel, EphA kinases were immunoprecipitated (IP) with ephrin-A1–Fc, and were immunoblotted for phosphotyrosine (PY) to monitor their activation status. In PC-3 cells, EphA2 was internalized and degraded after stimulation for 60 min. **b**, Clonal-growth assay (see Methods). Upper panel, cells were plated on uncoated six-well dishes in the presence of 0.3 μg ml^{-1} Fc or ephrin-A1–Fc. Lower panel, 0.5 μg cm^{-2} Fc or ephrin-A1–Fc was immobilized on nitrocellulose-coated six-well dishes before plating. Cells were stained with crystal violet 10 days later. Representative results from three experiments are shown. **c**, BrdU-incorporation assay. pRNS-1-1 cells were plated on coverslips in 24-well dishes to allow cell attachment. Fc or ephrin-A1–Fc was added 24 h later and incubated for a further 24 h. BrdU was added to wells for 1 h, and BrdU incorporation was determined by immunofluorescence staining. Seven randomly selected images were obtained using a microscope equipped with a Spot-RT digital camera. Labelled and unlabelled nuclei were counted; values are mean percentages ± s.d. of cells that were labelled. **d**, Inhibition of clonal growth of BAECs by soluble ephrin-A1. BAECs were plated onto 12-well dishes (100 cells per well) in the presence of Fc or ephrin-A1–Fc, cultured for 8 days and stained as in **b**.

activated to a similar degree (Fig. 2a) in pRNS-1-1 and pRNS-1-1-Ras cells, indicating that the loss of responsiveness to ephrin-A1 stimulation was not due to altered expression and/or activation of EphA.

To obtain direct evidence for suppression of Ras activity, we used a construct consisting of the Ras-binding domain of Raf fused to glutathione-*S*-transferase (GST–Raf-RBD) to pull down GTP-bound active Ras[11]. Stimulation with ephrin-A1–Fc caused a significant reduction in the amount of GTP-bound Ras compared with Fc-treated controls in pRNS-1-1 cells, MEFs and BAECs (Fig. 2e). Inhibition was evident ~2 min after stimulation, which is consistent with the kinetics of MAPK inactivation. Together, these results show that activated EphA kinases are potent upstream negative regulators of the Ras/MAPK signalling pathway.

The Ras/MAPK signalling cascade is activated by a variety of extracellular stimuli, including peptide growth factors, cytokines and hormones[7]. We therefore determined whether ephrin-A1 could counteract the stimulatory effects on MAPK of growth-factor receptors (GFR). As expected, both EGF and PDGF activated MAPK, whereas ephrin-A1–Fc inhibited MAPK in MEFs (Fig. 2f). Co-stimulation with ephrin-A1–Fc and EGF or PDGF substantially reduced growth-factor activation of MAPK in these cells. Similarly, VEGF-induced activation of MAPK in BAECs was inhibited in the presence of ephrin-A1–Fc (Fig. 2g). Thus, the GFR-mediated activation of the Ras/MAPK signalling pathway can be attenuated by EphA kinases.

A recent study has shown that overexpression of EphA2 kinases is associated with malignant progression of prostate cancer[12]. We found that primary epithelial cells isolated from six different prostatic cancer specimens all expressed high levels of EphA2. Ephrin-A1 stimulation significantly inhibited MAPK activity in all strains (Fig. 3a). In the clonal-growth assay, 5 out of 6 strains showed 31–72% growth reduction as a result of ephrin-A1 treatment (Fig. 3b). Cells from one specimen, E-CA-44, were similarly responsive to MAPK inhibition compared to other strains, but were not growth-inhibited, indicating that they have developed mechanisms to escape the growth-inhibitory effects of EphA2 activation.

EphB2 and EphB3 kinases have been reported to recruit RasGAP, a negative regulator and positive effector of Ras[13,14]. Interestingly, it has recently been reported that transient transfection of EphB2 into 293 cells leads to MAPK activation[15]. We have also found that EphB3 stably transfected into 293 cells activates MAPK activity in a ligand-dependent manner (H.M. and B.W., unpublished observations). Earlier studies have shown that EGF and PDGF receptors recruit RasGAP upon ligand stimulation[16], but are prototypic activators of the Ras/MAPK pathway[7]. Therefore, if the recruited RasGAP suppresses the Ras/MAPK pathway, it may exhibit slower kinetics than the Ras-activation events. This is in contrast to suppression of Ras by EphA kinases, which occurs within 2 min of ligand stimulation. More importantly, no association between RasGAP and EphA kinase was detected in any of cell types we examined, whereas an interaction between RasGAP and EphB3 was observed under identical conditions (H.M. and B.W., unpublished observations). Another study also failed to detect an association between EphB1 and RasGAP[17], indicating that distinct signalling pathways may be used by even the most closely related Eph kinases. Together, these results indicate that inhibition of the Ras/MAPK pathway induced by activation of EphA kinases is unlikely to be due to recruitment of RasGAP, and that other mechanisms are probably involved.

The rapid and potent suppression of the Ras/MAPK cascade by EphA kinases is in direct contrast to other RTKs, most if not all of which activate the Ras/MAPK cascade[7,18]. We found that EphA activation attenuates activation of MAPK by EGF, PDGF and VEGF

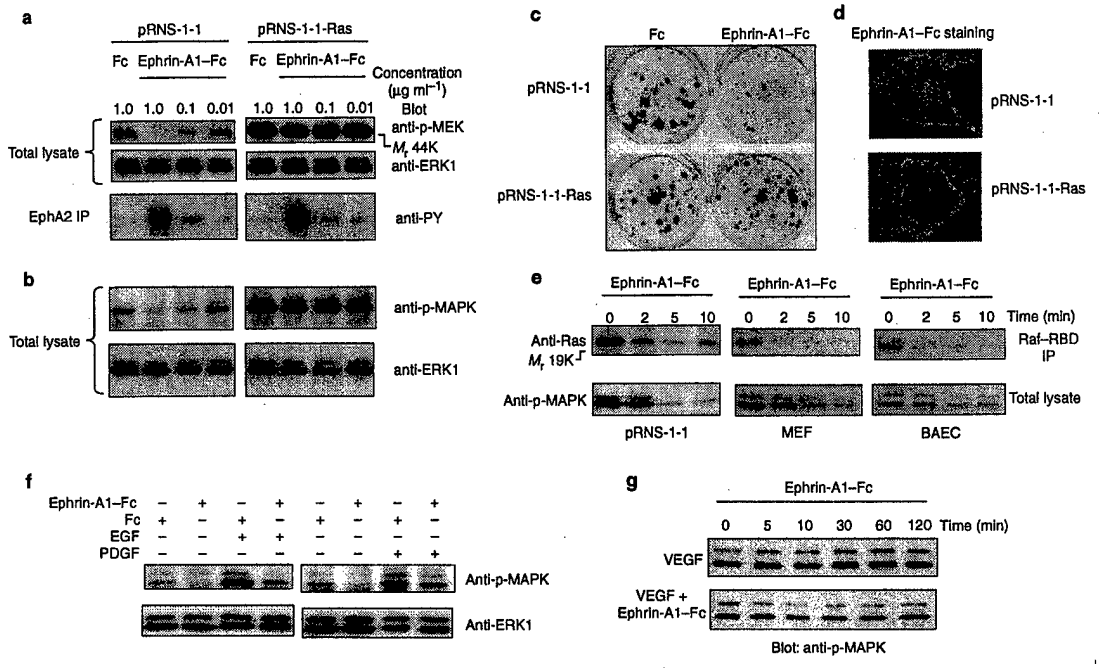

Figure 2 **Activated EphA kinases target Ras.** Transformation by constitutively activated Ras abolishes ephrin-A1–Fc-induced inhibition of MEK (**a**) and MAPK (**b**). Samples were analysed as described in Fig. 1a. **c**, pRNS-1-1-Ras cells are refractory to growth inhibition by ephrin-A1–Fc. A clonal-growth assay using soluble ephrin-A1–Fc was carried out as described in Fig. 1, except that 50 cells were seeded on 12-well dishes. **d**, Ras transformation does not alter the expression or subcellular distribution of EphA kinases. Cells cultured on coverslips were incubated with ephrin-A1–Fc for 60 min on ice. Bound ephrin-A1–Fc was detected using a Cy2-conjugated antibody against human Fc. **e**, Ephrin-A1–Fc stimulation inhibits GTP loading of Ras. Cells were stimulated with 1 μg ml^{-1} Fc or ephrin-A1–Fc for the indicated times. GST–RBD was used to pull down GTP-bound Ras (see Methods), and precipitated materials were blotted with anti-pan-Ras antibody. **f**, Ephrin-A1–Fc treatment attenuates the stimulatory effects of EGF and PDGF on MAPK. MEF cells were treated with Fc or ephrin-A1–Fc, either alone or together with 10 ng ml^{-1} EGF or 20 ng ml^{-1} PDGF, for 10 min and the activation status of MAPK was analysed in total cell lysates. Representative results from at least three different experiments are shown. **g**, BAECs were starved overnight in low-serum (0.5%) medium, and stimulated with VEGF for the indicated times in the presence of Fc or ephrin-A1–Fc.

receptors. EphA2 was initially termed epithelial cell kinase (Eck) because of its wide expression in epithelial cell types *in vitro* and *in vivo*[19]. We have identified the Ras/MAPK cascade as a point of convergence between EphA and other RTKs; the resulting counterbalance may help to maintain homeostasis in normal epithelium as well as endothelium. Moreover, the negative regulation of Ras/MAPK is likely to contribute to other functions of Eph kinases. For example, Eph–ephrin interactions repulse migrating axons and cells[20] partly by inactivating integrins and focal-adhesion kinase[21]. As Ras/MAPK activation is known to stimulate cell motility by phosphorylating and activating myosin light chain kinase[22], inhibition of the Ras/MAPK cascade may contribute to the repulsive guidance of axon and cell motility. □

Methods

Cell culture.

pRNS-1-1 (ref. 23) and pRNS-1-1-Ras (ref. 8) cells were maintained in keratinocyte serum-free medium (KSFM) supplemented with 50 μg ml^{-1} bovine pituitary extract and 5 ng ml^{-1} EGF (GibcoBRL). MEFs were cultured in DMEM supplemented with 10% FBS, 100 U ml^{-1} penicillin and 100 μg ml^{-1} streptomycin. RPMI 1640 medium supplemented in the same manner was used to maintain PC-3 cells. Primary cell cultures derived from prostatic adenocarcinomas were initiated and maintained as described[24]. Primary BAECs were obtained by scraping endothelial cells from bovine aorta, and were cultured in DMEM/F12 medium supplemented with 10% FBS, 100 U ml^{-1} penicillin and 100 μg ml^{-1} streptomycin. Cells from passage 5–8 were used for experiments.

Cell stimulation, immunoblotting and immunoprecipitation.

Cells in logarithmic growth phase were stimulated with 1 μg ml^{-1} Fc or ephrin-A1–Fc for the indicated times and then processed for immunoblotting and immunoprecipitation as described[21]. Briefly, cells

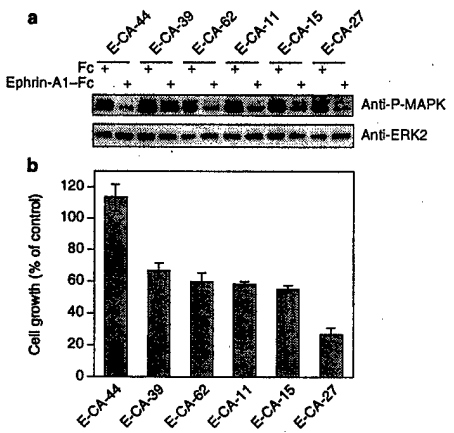

Figure 3 **Ephrin-A1–Fc treatment reduces MAPK activity and inhibits proliferation of primary human prostatic cancer cells.** Fresh specimens of human prostate cancer were used to isolate epithelial cells as described[24]. **a**, Cell strains at ~70% confluency were stimulated with 1 μg ml^{-1} ephrin-A1–Fc or Fc for 10 min. Cell lysates were analysed for MAPK activity. **b**, Clonal-growth assay of the cell strains as described[8]. Results represent growth relative to the Fc-treated control and are expressed as mean ± s.e.m.

were washed with ice-cold-PBS and lysed in modified RIPA buffer. Clarified lysates were immunoprecipitated either with ephrin-A1–Fc, which precipitates all EphA kinases, or with an EphA2-specific antibody (Santa Cruz). Total cell lysates or immunoprecipitates were boiled in SDS–PAGE loading buffer and separated on 4–20% gradient gels (Novex, Carlsbad, CA, USA). Other antibodies were against the following proteins: phospho-MAPK, ERK1 and ERK2 (Santa Cruz).

Clonal-growth assay.

For studies using soluble ephrin-A1–Fc, ~200 cells were plated in triplicate directly onto 6- or 12-well cell culture dishes in the presence of Fc or ephrin-A1–Fc; media were changed every three days. After 7–10 days in culture, cells growing as small colonies were fixed and stained with crystal violet as described[8], or were trypsinized and counted. For studies using immobilized ephrin-A1–Fc, 6- or 12-well cell culture dishes were first coated with nitrocellulose as described[21]. Ephrin-A1–Fc was coated at 0.5 μg cm^{-2} overnight at 4 °C. Cells were plated and monitored as described for soluble ephrin-A1–Fc above.

RasGTP-loading assay.

The GTP-loading status of Ras was analysed essentially as described[11]. Cells were stimulated with Fc or ephrin-A1–Fc as described above, and were lysed in buffer containing 25 mM HEPES pH 7.5, 150 mM NaCl, 1 mM EDTA, 10% glycerol, 1% Nonidet P-40, 1 mM sodium vanadate and protease inhibitors. GST–Raf-RBD was immobilized on glutathione–sepharose 4B beads (20 μg per reaction) and incubated with cell lysates from 1 to 3 subconfluent 100-mm plates. GTP-bound Ras was separated by SDS–PAGE and blotted with anti-pan-Ras antibody (Transduction Laboratories, Lexington, KY, USA).

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Correspondence and requests for materials should be addressed to B.W.