The Role of EphA2 in Vascular Smooth Muscle Cell Proliferation, Migration, and Mitogenic Signaling

Matthew L. Scott[2], Alexandra C. Finney[1], and A. Wayne Orr[1,2]

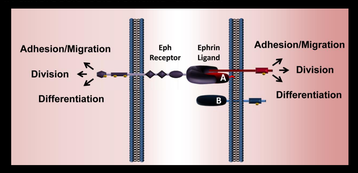
Departments of [1]Cellular Biology and Anatomy and [2]Pathology and Translational Pathobiology, Louisiana State University Health Science Center-Shreveport, Shreveport, LA 71103

ABSTRACT

Vascular smooth muscle cells (VSMCs) are known to be involved in atherogenesis, during which VSMCs undergo phenotypic modulation from a contractile, quiescent phenotype to a more proliferative and synthetic phenotype. Within plaques, SMCs can deposit extracellular matrix and form a protective cap that protects the vessel lumen from plaque contents. EphA2 is a receptor tyrosine kinase that exacerbates many cancers, although our lab has discovered a role for EphA2 in atherogenesis. EphA2 is found to be expressed only by synthetic SMCs within the plaque. We determined that EphA2 signaling is involved in SMC phenotypic modulation and SMC proliferation both in vitro and in vivo. EphA2 signaling is complex, in that the receptor is phosphorylated at both ligand-dependent (Y588/Y772) and ligand-independent (S897) sites that direct distinct signaling outcomes. To determine the role of EphA2 in VSMC proliferation and mitogenic signaling, we use primary aortic or carotid artery VSMCs and mouse aortic EphA2-KO VSMCs. Interestingly, our data show that EphA2 is simultaneously required for maximal SMC proliferation and migration and detrimental to both cellular processes, dependent upon the context of EphA2 activation. siRNA-mediated EphA2 knockdown in primary VSMCs reduces sustained Erk1/2 and Akt activation and significantly impairs maximal VSMC proliferation and migration; however, EphA2 ligation with Fc-EphrinA1 also diminished Erk1/2 and Akt activation, proliferation, and migration. Further, serum treatment of VSMCs promotes robust S897 (ligand-independent) activation, which is associated with increased proliferation and migration/invasion of many cancers. The S897 phosphorylating kinase differs between different cells and different stimuli; therefore, to determine which mediator is mediating S897 phosphorylation of EphA2 in VSMCs, we used various signaling mediator inhibitors and observed that Erk1/2 and RSK inhibition prevented S897 phosphorylation. RSK activation is Erk-dependent, so we used siRNA-knockdown of RSK1 and RSK2 and observed that loss of RSK2, not RSK1, significantly reduces S897 activation at baseline and in response to serum. Interestingly, RSK2-KD abrogation of S897 phosphorylation did not affect SMC proliferation, but does prevent EphA2 localization to lamellipodia structures, implicating S897 phosphorylation in VSMC migration. We confirmed this with cells expressing the S897A-EphA2 that cannot be phosphorylated via single cell tracking migration. Understanding how EphA2 modulates SMC behavior in terms of phenotypic modulation, migration, and proliferation will provide a basis to target this receptor to stabilize plaques, preventing deadly thrombotic events from manifesting.

INTRODUCTION

Eph receptors are the largest family of receptor tyrosine kinases and are traditionally known for their roles in various biological functions such as angiogenesis, neuronal guidance, and carcinogenesis. Ephs are becoming increasingly recognized for their role in inflammation and immune response. Recently, our group demonstrated a novel pro-inflammatory role for EphA2 during atherogenesis in which there was enhanced expression of EphA2 and its corresponding ligand, ephrinA1 in both human and murine atherosclerotic lesions. Despite these findings, a specific role for EphA2 in atherosclerosis remains unclear.



Vascular smooth muscle cells (SMCs) are classically recognized for their role in vascular hemodynamics. During atherosclerosis, smooth muscle cells will undergo a phenotypic alteration characterized by a decrease in contractile markers and an increase in proliferation and migration, as well as enhanced collagen deposition. These “synthetic” vascular smooth muscle cells ultimately promote plaque progression and formation of the fibrous cap. To date there are no studies that have examined EphA2 expression and activity in vascular smooth muscle cells during atherogenesis. EphA2 downstream signaling differs dependent upon context of activation; ephrinA1 (ligand) binding induces Tyr588/Tyr772 phosphorylation, resulting in reduced mitogenic signaling and proliferation, while activation in response to FBS/growth factors induces the ligand-independent phosphorylation of the Ser897 site that promotes mitogenic signaling. Ser897 phosphorylated is known to promote proliferation, migration, and invasion in cancer models. The goal of this project is to characterize the roles of both ligand-dependent and independent EphA2 signaling in smooth muscle incorporation into the plaque, and the subsequent formation of the fibrous cap.

EphA2 expression regulates the expression of SMC contractile markers and genes involved in SMC proliferation and survival

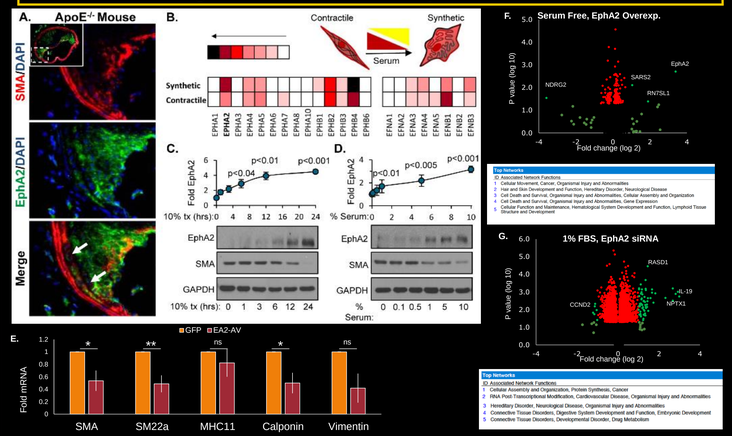


Figure 1: A) EphA2 expression in atherosclerotic SMCs correlates with areas of reduced SMA expression. n=6-7. B) RNA Seq analysis of SMCs under serum free (contractile) or serum treated (synthetic) conditions. C/D) SMCs were cultured in serum-free media for 3 days to induce quiescence and then treated with (C) 10% complete media the indicated time points or (D) increasing serum concentrations for 24 hours. n=3-4. E) SMCs were transduced with Adenovirus expressing GFP or EphA2, and serum starved for 72h, followed by RNA isolation and RT-qPCR. n=4-5. F/G) RNA-seq analysis of (F) quiescent SMCs expressing exogenous EphA2 or (G) synthetic SMCs with EphA2 siRNA. n=3.

Model of EphA2 ligand-dependent, independent, and matrix signaling

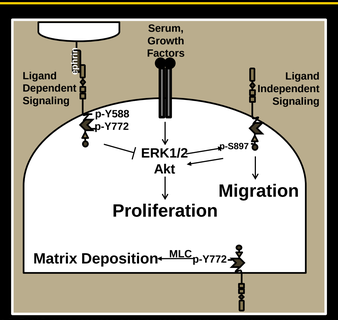


Figure 2: EphA2 ligand-dependent signaling has been previously shown to inhibit proliferation in cancer models, while ligand-independent S897 phosphorylation has been associated with increased proliferation and migration in cancer models. Our lab recently published findings showing that ligand-independent Y772 EphA2 phosphorylation is present within matrix adhesions and facilitates deposition.

EphA2 simultaneously promotes and inhibits proliferative signaling depending on context

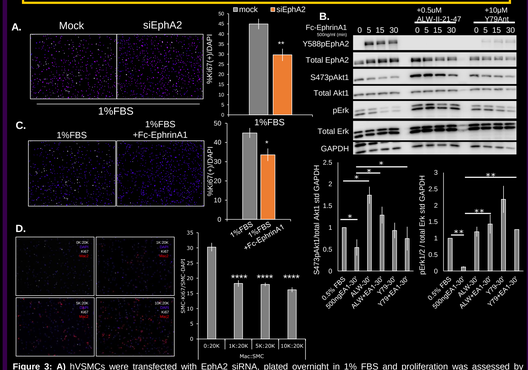


Figure 3: A) hVSMCs were transfected with EphA2 siRNA, plated overnight in 1% FBS and proliferation was assessed by nuclear Ki67 stain. ** p<0.01; by Student’s T-test. n=4. B/C) EphrinA1 reduces (B) mitogenic signaling and (C) Ki67 staining, which is blocked by AUW-II-41-27 and the (B) Y79 peptide antagonist. (D) RAW264.7 Macrophages were plated at varying densities and hVSMCs were co-cultured at consistent density with proliferation being measured by SMC+ Ki67 staining. **** p<0.0001 by Students T-test. n=3.

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EphA2 KO and fc-ephrinA1 treatment both significantly reduce wound healing

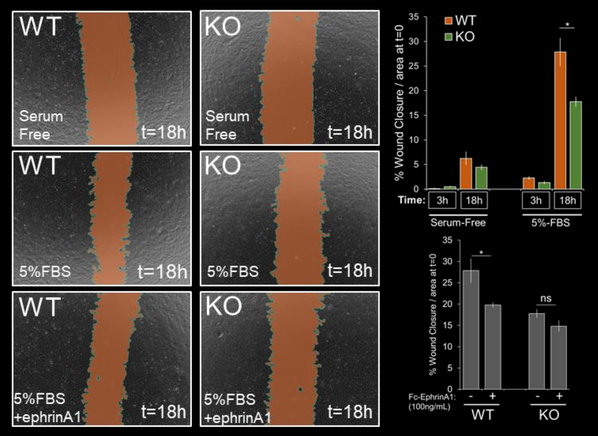


Figure 4. EphA2 wildtype and knockout mouse aortic SMCs were plated to confluence in serum-free media for 5-6 hours, followed by scratching to create wound. Media was supplemented with 5%FBS +/- 100ng/mL fc-ephrinA1. Wound closure was tracked for 18 hours. *=p<0.05; Student’s T-test. n=4-5.

RSK2-dependent EphA2 Ser897 phosphorylation colocalizes with actin-rich lamellipodium

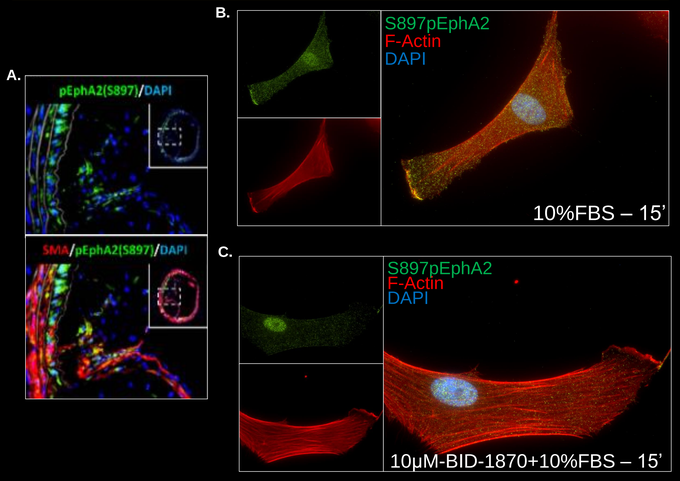


Figure 6. A) p-EphA2 (S897) in mouse atherosclerosis. B/C) Ser897 phosphorylation (green) in the actin-rich lamellipodium is reduced by RSK inhibition (10μM BID-1870). Phalloidin (red) and DAPI (blue)

RSK2-mediated S897 EphA2 activation does not contribute to SMC proliferation

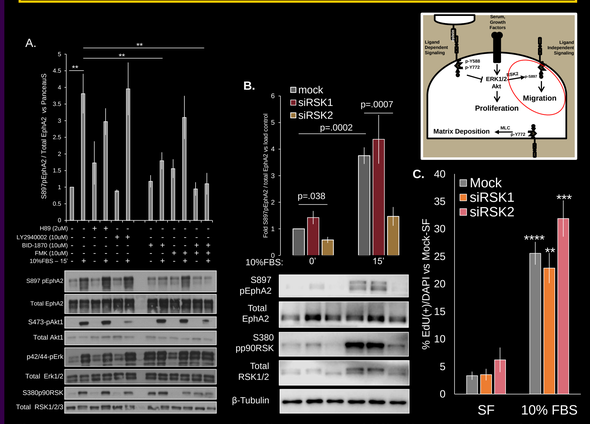


Figure 5: A) hVSMCs were serum starved for 3h, then pretreated (60’) with various inhibitors, followed by stimulation with FBS for 15’. B/C) Knockdown of RSK2, but not RSK1, in VSMCs shows (B) reduced serum-induced EphA2 Ser897 phos., but (C) no change in proliferation. ** p<0.01; *** p<0.001; **** p<0.0001 by Student’s T-test. n=3-5.

EphA2 S897A mutation impairs cell migration

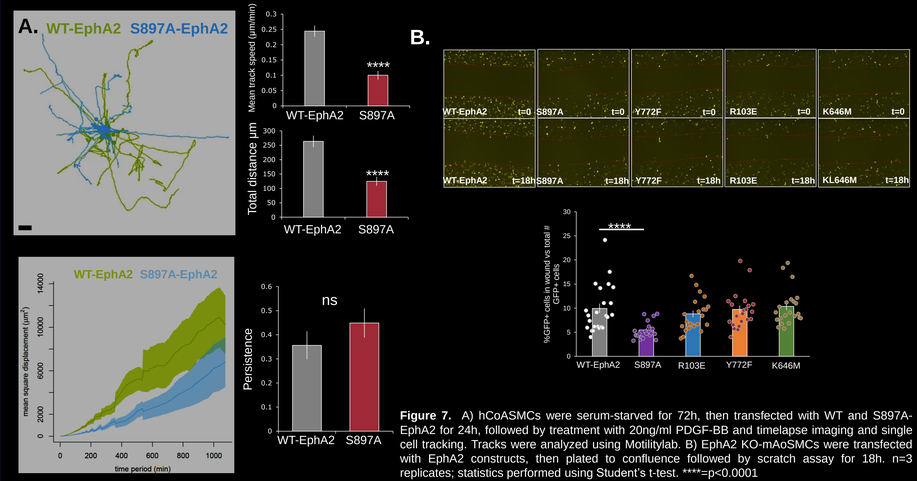
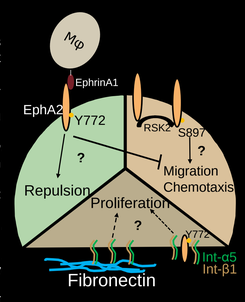


Figure 7. A) hCoASMCs were serum-starved for 72h, then transfected with WT and S897A-EphA2 for 24h, followed by treatment with 20ng/ml PDGF-BB and timelapse imaging and single cell tracking. Tracks were analyzed using Motilitylab. B) EphA2 KO-mAoSMCs were transfected with EphA2 constructs, then plated to confluence followed by scratch assay for 18h. n=3 replicates; statistics performed using Student’s t-test. ****=p<0.0001

Future Directions

- We recently published evidence that, contrary to canon, EphA2 Y772 phosphorylation occurs within SMC focal adhesions, where it is necessary for fibroproliferative remodeling of the matrix and fibronectin deposition
  - Fibronectin has been shown to contribute to SMC proliferation, indicating a possible role for matrix-associated Y772 phosphorylated EphA2 in mediating this effect
  - Assessing whether this effect is direct (mediated by matrix-Y772) or indirect (facilitated through FN deposition) is one focus of future studies
- Fc-EphrinA1 mediated suppression of migration and proliferation is established; however, *in vivo* SMCs will encounter fc-ephrinA1 that is attached to another cell, whereas we treat with soluble Fc-conjugated ephrinA1
  - The response (dead stop, repulsion, etc…) of migrating SMCs to cell-cell mediated ligation of EphA2 has not been characterized and will be the focus of future studies
- Ligand-independent S897 phosphorylation does not appear to contribute to proliferation, suggesting a role in other cell processes, such as migration
  - The contribution of S897 phosphorylation to SMC migratory responses, whether chemotactic (i.e. towards PDGF-BB concentrations) or exploratory (sensing ECM to assess environment), is currently unknown, and will be the focus of future studies
- To these ends, we have successfully generated mice that express WT or mutant (Y773F or S898A) EphA2 after addition of Cre
  - The primary cells harvested from the mice will be used in each of these experiments, and the mice will be used to address these questions *in vivo*.



Summary

- EphA2 overexpression significantly blunts the expression of SMC contractile markers in quiescent SMCs
  - RNA-seq analysis implicates EphA2 in regulating expression of migration, proliferation, and survival associated genes
- Ligand-dependent (fc-EphrinA1) EphA2 activation blunts mitogenic signaling (Erk/Akt), SMC proliferation, and migration
- Ligand-independent EphA2 Ser897 phosphorylation is mediated by RSK2
  - Proliferation was not reduced in siRSK2-treated cells, suggesting S897 phosphorylation does not facilitate EphA2-dependent proliferation
- Ser897 phosphorylation is detected in actin-rich lamellipodia, indicating a potential role in SMC migration, consistent with what is observed in cancer models
  - Blocking RSK2-mediated S897 phosphorylation prevents lamellipodia colocalization
  - Re-expression of S897A mutant impairs migration speed, distance traveled, and persistence as quantified by single-cell tracking and wound healing assay