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Title: NON-CLASSICAL SH3-DOMAIN MEDIATED SIGNALLING THROUGH THE VASCULAR ENDOTHELIAL GROWTH FACTOR RECEPTOR IN BREAST CANCER

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Abstract:

Introduction: In the absence of ligand stimulation, we have shown that a second tier of signalling is initiated by interactions between the SH3 domains of intracellular kinases and the proline rich motifs of receptor tyrosine kinases (RTKs). When this signalling is perturbed it can lead to pathological outcomes. This secondary tier of signalling has been shown to contribute to poorer outcomes with FGFR2 in ovarian cancer but it has not been widely explored for other RTKs or other types of cancers. One RTK of interest is VEGFR2, which is overexpressed in many cancer types including breast cancer (BC). VEGFR2 is a RTK that regulates angiogenesis, proliferation, survival and migration in response to the ligand VEGF-A. Inside a tumour mass, RTKs on the surface of cancer cells will have limited access to extracellular stimuli, including VEGF-A. Here, some of the response of VEGFR2 may be through these non-classical interactions with non-activated RTKs and could possibly be used to aid in cancer progression and survival.

Objective: To determine whether VEGFR2:SH3-domain-containing protein interactions occur under serum starved conditions in breast cancer, and to assess their impact on cancer cell behaviour.

Methods: VEGFR2 expression levels were investigated in BC cell lines (SKBR3 and MCF7 cells) and HEK 293T cells as a control non-cancerous cell line under serum starved conditions for 24 hours.
Protein-protein interactions (PPIs) were determined by endogenous Co-IP and via pull down of VEGFR2 constructs containing mutated SH3-binding sites in HEK 293T cells (low expression of endogenous VEGFR2), SKBR3 and MCF7 (high expression of endogenous VEGFR2) cells grown under serum starved conditions and visualised by immunoblotting.
Protein expression and purification of the C-terminal tail of VEGFR2, containing only the c-terminal proline rich motif, was used in a pull down in cancer cell lysates and PPIs visualised by immunoblotting.

Results: MCF7 and SKBR3 cells expressed high endogenous levels of VEGFR2 and HEK 293T cells had low endogenous expression. After serum starvation, SKBR3 and MCF7 cells had low levels of phosphorylated VEGFR2.
Co-IP and GFP pull down results showed that Src, Nck1 and Plcγ1 were identified to bind to VEGFR2 under serum starved conditions in SKBR3 and MCF7 cells. C-terminal VEGFR2 tail pull down in MCF7 and SKBR3 cell lysates indicate that there are PPIs occurring at the SH3 binding site of VEGFR2.

Conclusions: These data indicate that there are PPIs occurring under serum-starved conditions with VEGFR2 and SH3-domain-containing proteins. The pull down of the VEGFR2 mutated constructs indicate the interactions are occurring through the second SH3-binding site in the C-terminal tail of VEGFR2 in BC cells, but this interaction does not occur in the non-cancerous HEK 293T cells. Further experiments will be to further validate the PPIs through FRET and look at downstream oncogenic signalling outcomes from these interactions.

Keywords: Cancer Biology, Breast Cancer, Cell Signalling, Protein-Protein Interactions

Images/Graphs/Tables:

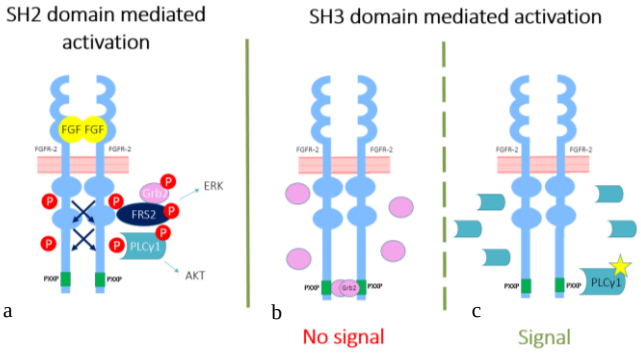


Figure 1 Non-classical SH3-domain binding to FGFR2 induces PLCγ1 activation and AKT signaling a. Canonical binding of adaptor proteins via their SH2 domains to the receptor tyrosine kinase FGFR2 after ligand stimulation with FGF. b. Under non-stimulated conditions FGFR2 is held in a heterotetramer with Grb2 via its SH3 domains to the proline rich motifs (PXXP) in the FGFR2 c-terminal tail. This hold the receptor in an inactive basal state. c. When PLCγ1 is of abundance in the cell cytoplasm it competes with Grb2 to bind to the same binding site on FGFR2 via its SH3 domain under non-stimulated conditions. When PLCγ1 binds to FGFR2 via its SH3 domain it changes the conformation of PLCγ1 such that it acts as if it active. PLCγ1 is then recruited to the membrane and elevates phosphatidylinositol 4,5-bisphosphate turnover which activates the AKT signaling pathway.

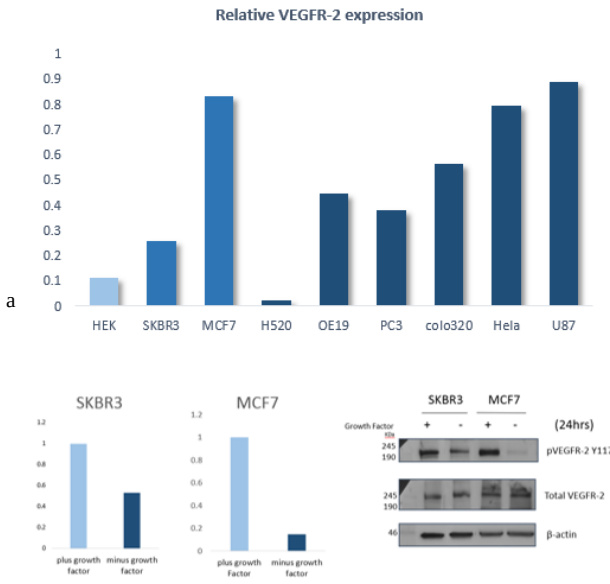


Figure 2 Phosphorylation of VEGFR2 in breast cancer cells is reduced after serum starvation for 24 hours a. The relative protein expression of VEGFR2 in different cancer cell lines. The two BC cell lines showed high relative expression of VEGFR2 compared to the control non-cancerous cell line, HEK 293T. b. The relative phosphorylation of VEGFR2 in the two BC cell lines after serum starvation for 24 hours, showing a reduction of 55% in SKBR3 cells and 85% in MCF7 cells.

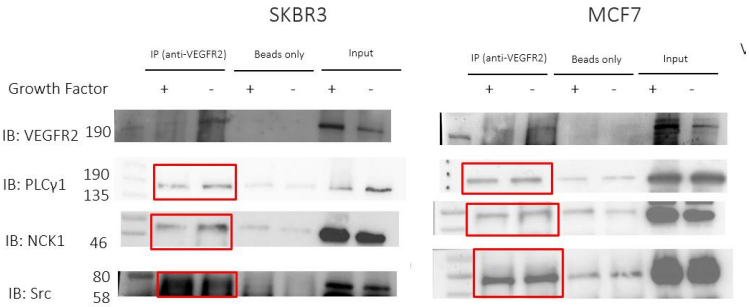


Figure 3 SH3 domain containing adaptor proteins Src, NCK1 and PLCγ1 bind to VEGFR2 under serum starved conditions Endogenous co- immunoprecipitation of SKBR3 and MCF7 BC cells showed binding to Src, NCK1 and PLCγ1 adaptor proteins under both stimulated and serum starved conditions (shown in red boxes).

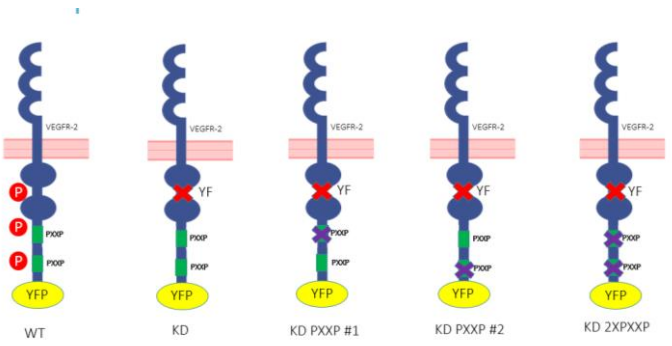


Figure 3 Mutated VEGFR2 constructs to determine SH3-domain binding to proline rich motif of VEGFR2 Four VEGFR2 constructs were made via site-directed mutagenesis from the wildtype (WT). A kinase dead (Y to F) mutation was made in all mutated constructs to diminish SH2 domain mediated binding of adaptor proteins. The two proline rich motifs were mutated in the constructs separately (P to A) and as a double PXXP mutant.

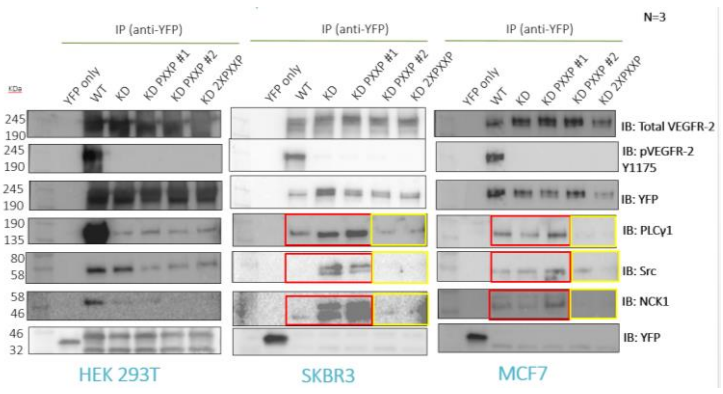


Figure 4 Src, NCK1 and PLCγ1 bind to VEGFR2 via their SH3 domains in BC cells SH3-domain mediated interactions were determined by co-immunoprecipitation of VEGFR2 constructs containing the kinase dead mutation. Binding was visualised in SKBR3 and MCF7 BC cells but not in control HEK 293T cells (shown in red boxes). The introduction of the second proline rich motif mutation seems to abrogate the binding of the adaptor proteins (shown in yellow boxes) suggesting the SH3-domain binding is to the second proline rich motif in the c-terminal tail of VEGFR2.

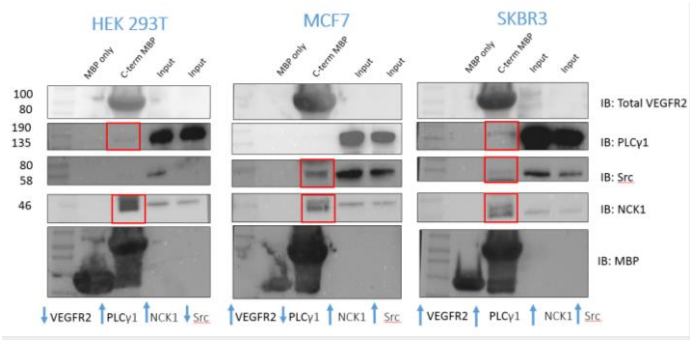


Figure 5 Interactions of adaptor proteins seem to occur based on the proteome of the cell To further validate that the SH3-domain containing adaptor proteins Src, NCK1 and PLCγ1 are binding to the second proline rich motif in the c-terminal tail of VEGFR2, a c-terminal tail pull down of expressed and purified c-terminal VEGFR2 was performed. The c-terminal tail peptide containing only the second proline rich motif was dipped into serum starved cell lysates of HEK 293T, MCF7 and SKBR3 cells. Binding of the adaptor proteins correlated with the relative expression of the proteins in the cell lines (shown in red boxes).

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Biography:

Eleanor Cawthorne undertook her undergraduate degree in Biochemistry with a Research Placement at The University of Huddersfield in 2012. During her time at Huddersfield she was awarded a placement year at the University of Leeds in the Leeds Institute of Cardiovascular and Metabolic Medicine. Here she worked under supervisor Dr. Richard Pease and Dr. Cora Beckers in the area of thrombosis and coagulation. Upon completing her degree she was awarded the Silver Prize for the second highest degree grade in the faculty. After completing her undergraduate degree in 2016 she embarked on a PhD at the University of Leeds in Cancer Biology in Professor John Ladburys research group. Particularly focusing on early signaling events in breast cancer, exploring interactions and cellular outcomes of complex formation at receptors when growth factor deprived. This is set to be complete by November 2020.

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