THE FOURTH CONGRESS ON THE EPH/EPHRIN SYSTEM

Parma, Italy. May 16-17, 2024

UNIVERSITÀ DI PARMA

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CONFERENCE PROGRAMM

MAY 16, 2024		
9:00	**Welcome**	**Magnifico Rettore and Massimiliano Tognolini** *University of Parma, Italy*
	Opening lectures	
9:10	**Quantifying Eph receptor multimerization with time resolved fluorescence spectroscopy**	**Adam Smith** *Texas Tech University, USA*
9:40	**EphA2 multimerization underscores canonical and noncanonical signaling in oncogenesis**	**Bingcheng Wang** *Case Western Reserve University, USA*
	Session: CNS **Adam Smith and Massimiliano Tognolini**	
10:10	**Untangling astrocyte-mediated PV inhibitory synapse development through ephrin-B1/EphB2 signaling**	**Iryna M. Ethell** *University of California Riverside, USA*
10:35	**The roles of ephrin-B2 and EphB2 in memory formation**	**Raphael Lamprecht** *University of Haifa, Israel*
11:00	**EphA1 missense mutations associated with Alzheimer's disease dysregulate receptor signaling function**	**Elena B. Pasquale** *Sanford Burnham Prebys Med Discovery Institute, USA*
11:25	*Coffee break*	
	Session: Cancer I **Bryan Day and Jiangping Wu**	
11:50	**Roles of EphA2-ephrin-A signaling in prostate cancer development and progression**	**Ryan Lingerak** *Case Western Reserve University, USA*
12:15	**EphA4 in head and neck squamous cell carcinoma: dissecting its role in the tumor microenvironment and tumor progression**	**Sophia Corbo** *University of Colorado, USA*
12:40	**Advancing understanding of uveal melanoma progression: integrating Eph/ephrin epigenetics, gene expression, and regulatory networks through comprehensive big data analysis and web resources**	**Georgios Mandrakis** *University of Athens, Greece*
13:05	*Lunch*	
	Session: Molecular Biology **Bingcheng Wang and Maurizio Pellecchia**	
14:30	**Proximity labeling of ephrin-B ligands reveals that EGFR is an EFNB1 interactor**	**Ana Isabel Osornio-Hernandez** *Université Laval, Canada*
14:55	**EphA2 transmembrane helix interactions regulate EphA2 oligomerization**	**Karina Hristova** *Johns Hopkins University, USA*
15:20	**Could the pro-oncogenic role of EphA2 Ser-897 be affected by Eph-ephrin antagonists?**	**Francesca Romana Ferrari** *University of Parma*
15:35	**EphA2 controls EphA1 function via a heterotypic interaction that regulates tumor susceptibility**	**Xiaojun Shi** *Case Western Reserve University, USA*
	Coffee Break	

	Session: Miscellaneous **Mario Cioce and Massimiliano Tognolini**	
16:30	**The relationship between ephrin and NOTCH system in the regulation of tube formation of HUVECs in co-culture with MSCs**	**Irina Beloglazova** (online) *National Medical Research Centre of Cardiology Named after Academician E.I.Chazov, Russia*
16:55	**Ephrin micropatterns exogenously modulate cell organization in organoid-derived intestinal epithelial monolayers**	**Elena Martinez** (online) *Institute for Bioengineering of Catalonia, Spain*
19:30	*Social dinner at Corale Verdi*	

	MAY 17, 2024	
9:00 10:00	*Coffee and Poster session*	
	Session: Drug Discovery **Raphael Lamprecht and Alessio Lodola**	
10:00	**Fifteen years of ephrins in Parma: a PPI-inhibitors story**	**Carmine Giorgio** *University of Parma, Italy*
10:25	**Hunting for original anticancer agents: peptide modulators of EphA2 mediated SAM-SAM interaction**	**Marilisa Leone** *CNR Naples, Italy*
10:50	**Anti-ephrin-B2 neutralizing antibody suppresses fibrosis in preclinical *in vitro* and *in vivo* models**	**Benjamin T. Wilks** *Mediar Therapeutics, USA*
11:15	**Unveiling potential? Targeting Eph-ephrin in glioblastoma stem cells with novel PPI-inhibitors**	**Alfonso Zappia** *University of Parma, Italy*
11:30	**Targeting the Eph-ephrin system in cancer and neurodegeneration**	**Maurizio Pellecchia** *University of California Riverside, USA*
11:55	**EphB2 a viable therapeutic target for the treatment of pediatric medulloblastoma**	**Bryan W. Day** *The University of Queensland, Australia*
12:20 13:50	*Lunch and Poster session*	
	Session: Cancer II **Elena B. Pasquale and Andrew Freywald**	
13:50	**Ephrin's and Eph's are implicated in EGFR-TKI resistance of non-small cell lung cancer and are loaded in extracellular vesicles**	**Albano Cáceres-Verschae** *Karolinska Institute, Sweden*
14:15	**EphA2 phosphorylation sustains the adaptive response of colorectal organoids to oxaliplatin**	**Mario Cioce** *University of Campus-BioMedico, Italy*
14:40	**Harnessing the EphA2 blockade in glioblastoma**	**Andrea Knight** *Masaryk University, Czech Republic*
15:05	**Farewell**	**Massimiliano Tognolini** *University of Parma, Italy*

ABSTRACTS

UNTANGLING ASTROCYTE-MEDIATED PV INHIBITORY SYNAPSE DEVELOPMENT THROUGH EPHRIN-B1/EPHB2 SIGNALING

S.N. Sutley-Koury[1], C. Taitano-Johnson[2], P.W. Hickmott[2], V. Santhakumar[2], P.N. Mimche[3], I.M. Ethell[2]

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Ephs and ephrins are involved in a diverse array of neuronal developmental processes including cell migration, axon guidance, excitatory synaptogenesis, and synaptic plasticity, however their role in inhibitory synapse development and the mechanism by which astrocytes influence it are still unclear. To address these gaps in our knowledge, we present new evidence that astrocytes regulate inhibitory synapse development between parvalbumin (PV)-expressing interneurons and excitatory pyramidal cells (PC) and describe a novel mechanism involving ephrin-B/EphB signaling. In this study we utilize genetic approaches and mouse hippocampus as a model to study the mechanisms of inhibitory circuit development in a combination with whole-cell patch-clamp electrophysiology, optogenetics, immunohistochemical analysis and mouse behaviors. Our findings show that astrocytic ephrin-B1 enhances PV->PC structural and functional connectivity during postnatal day (P)14-P28 developmental period. While inhibitory synapse development is adversely affected by PV-specific expression of EphB2, a strong candidate ASD risk gene, astrocytic ephrin-B1 facilitates PV->PC connectivity by interfering with EphB signaling in PV boutons. In contrast, the loss of astrocytic ephrin-B1 enhances association of EphB receptors with PV boutons and reduces PV->PC inhibitory connectivity, resulting in increased seizure susceptibility and an ASD-like phenotype. Finally, we observe no effects of astrocytic ephrin-B1 overexpression on PV->PC connectivity in PV-specific EphB2 KO mice, suggesting that astrocytes regulate PV->PC connectivity by limiting EphB2 signaling in PV interneurons. The findings underscore the crucial role of astrocytes in regulating inhibitory circuit development and describe a novel mechanism by which astrocytes regulate their development in the hippocampus. Impaired inhibition is suggested to underlie the development of neuronal hyperactivity in several neurodevelopmental disorders (NDDs), including ASD and epilepsy, and EphB2 receptor itself is also implicated in the pathogenesis of ASD. Therefore, this study not only addresses critical gaps in our understanding, but also possesses clinical relevance as ephrin-B/EphB2 signaling in PV interneurons may be a promising therapeutic target to correct inhibitory circuits dysfunction in NDDs.

THE ROLES OF EPHRINB2 AND EPHB2 IN MEMORY FORMATION

K. Agarwal[1] and R. Lamprecht[1]

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Long-term fear memory formation is believed to involve alterations of synaptic efficacy mediated by changes in synaptic transmission and morphology in the basolateral amygdala (BLA). EphrinB2 is involved in regulating synaptic transmission and neuronal morphogenesis. We were therefore interested to study its role in memory formation. Deleting ephrinB2 from excitatory neurons in the basolateral amygdala (BLA) impaired long-term (LTM), but not short-term (STM), fear memory formation. Deleting ephrinB2 from astrocytes in the BLA impaired fear LTM but not STM. Removing ephrinB2 from astrocytes in the BLA reduced the excitatory amino acid transporter 1 (EAAT1) level in these cells. Inhibiting EAAT1 activity in the BLA during fear conditioning, by its specific inhibitor UCPH-101, impaired fear LTM showing that EAAT1 in the BLA is needed for fear LTM formation. The administration of ephrinB2 into the BLA during fear conditioning training enhanced fear LTM. Moreover, ephrinB2 increased the ability of fear conditioning to activate cells in the BLA as detected by c-Fos labeling. EphrinB2 therefore determines the threshold for fear memory formation. In contrast to mature neurons, we show that ephrinB2 in neural stem cells (NSCs) is not needed for fear LTM. However, deleting astrocytic ephrinB2 diminished the DCX immature neuronal marker level in BLA. Increasing the activity of the ephrinB2 receptor EphB2 in NSCs in the BLA increased the number of immature and mature neurons in the BLA and enhanced fear LTM. Our study shows that ephrinB2-mediated molecular functions in excitatory neurons and astrocytes in the BLA are needed for fear memory formation.

EPHA1 MISSENSE MUTATIONS ASSOCIATED WITH ALZHEIMER'S DISEASE DYSREGULATE RECEPTOR SIGNALING FUNCTION

Mike Matsumoto[1*], Maricel Gomez-Soler[1*], Sara Lombardi, Bernhard C. Lechtenberg[2], Elena B. Pasquale[1]

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Missense mutations in the *EPHA1* receptor tyrosine kinase gene have been recently identified in late-onset Alzheimer's patients. We have investigated the effects of four of these mutations on EPHA1 signaling ability in order to gain insight into their potential role in disease pathogenesis. The four mutations, which were chosen based on the prediction that they would affect EPHA1 function, include P460L and R492Q in the second fibronectin type III domain, R791H in the kinase domain, and R926C in the sterile-alpha motif (SAM) domain. EPHA1 is the founding member of the Eph receptor tyrosine kinase family, but there is limited information about its physiological and pathological activities. EPHA1 forward signaling, involving tyrosine phosphorylation and kinase activity, can be induced by ephrinA ligands or by high expression. In addition, EPHA1 S906 in the linker that connects the kinase domain with the SAM domain corresponds to S897 in EPHA2, whose phosphorylation by serine/threonine kinases mediates a non-canonical form of signaling that is independent of ligand binding and kinase activity. We monitored EPHA1 tyrosine phosphorylation in HEK293 cells and generated a phosphospecific antibody to EPHA1 S906 to characterize EPHA1 non-canonical signaling ability. In addition, we examined EPHA1 N-glycosylation and trafficking to the cell surface. We also investigated proteolytic cleavage in the EPHA1 extracellular region, which generates a soluble N-terminal fragment released from the cell surface and a transmembrane C-terminal fragment that can be phosphorylated on both tyrosine residues and S906. Our findings advance understanding of EPHA1 signaling mechanisms. Furthermore, our characterization of the effects of the four Alzheimer's mutations shows that at least two of them severely dysregulate EPHA1 signaling ability, suggesting that abnormal EPHA1 signaling function due to missense mutations contributes to Alzheimer's disease pathogenesis.

ROLES OF EPHA2-EPHRIN-A SIGNALING IN PROSTATE CANCER DEVELOPMENT AND PROGRESSION

R. Lingerak[1,2,3], A. Petty[2], H. Miao[2], H. Guo[2], X. Shi[2], S. Kim[2], H. Lin[2,4], B. Wang[1,2,3]

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Prostate cancer (PCa) is the most common cancer in the US men. While usually indolent or benign, a small fraction (~5%) rapidly progress to malignant disease. PCa is initially responsive to androgen deprivation therapy (ADT) or castration. However, aggressive forms of the disease inevitably become resistant to the therapy, leading progressively to metastatic castration resistant PCa (mCRPC), a fraction of which further progress to neuroendocrine prostate cancer (NEPC) and double negative PCa (DNPC). A major goal of PCa research is to broadly identify molecular and cellular mechanisms aiding nearly inevitable progression to identify vulnerabilities that could be targeted. Multiple receptor tyrosine kinases (RTKs) have been implicated in PCa. A significant body of literature points to an important role of EphA2, a member of the Eph subfamily of RTKs, in PCa. Notably as first reported by Chinnaiyan lab, EphA2 RTK is overexpressed in metastatic CRPC, but not early localized PCa tumors. In tumors where EphA2 is overexpressed, there is loss of the cognate ligand EphrinA1. In fact, Colm Morrissey was the first to discover that EphrinA1 is one of the top three genes whose expression is lost in metastatic PCa, particularly in bone metastases. The Wang lab, a leading group in studying Eph/Ephrin system in cancer biology, discovered that EphA2 has dual opposed roles during tumor development and progression, i.e., a ligand dependent tumor suppressor in the early stage of tumorigenesis and a ligand-independent oncogenic protein in the late-stage tumor progression in several cancer types. Our data indicate that EphA2 is involved in both PCa (1) progression, and (2), initiation. (1) EphA2 expression increases with increasing malignancy, favoring the ligand-independent signaling role. Often this is accompanied by loss of AR which supports the finding of EphA2 overexpression in CRPC. (2) In a spontaneous model of PCa driven by *PTEN* deletion across **our *EphA2-/-*, and *Efna1,3,4-/-* genetically engineered mouse models, we find that deletion of either the EphA2 receptor or its cognate EphrinA ligands has an inhibitory effect on the initiation of PCa.** Our current hypothesis is that EphA2-ephrinA interaction plays a multifaceted regulatory role in prostate cancer (PCa) development and malignant progression toward late stage PCa. The outstanding questions are addressed by examining EphA2 expression across human PCa samples, and modeling PCa progression using *in vitro* and *in vivo* systems.

EPHA4 IN HEAD AND NECK SQUAMOUS CELL CARCINOMA: DISSECTING ITS ROLE IN THE TUMOR MICROENVIRONMENT & TUMOR PROGRESSION

S. Corbo[1], D. Nguyen, B.Van Court, E.B. Pasquale, S.D. Karam

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Head and Neck Squamous Cell Carcinoma (HNSCC) is a deadly cancer with approximately half of locally advanced patients developing disease progression and/or recurrence. We examine the role of the receptor tyrosine kinase EphA4 in HNSCC progression. Previously we developed a model to study HNSCC disease progression where knockout of EphB4 receptor on HNSCC cancer cells results in accelerated tumor growth. In our model of HNSCC disease progression the expression of EphA4 increases within the tumor. Using APY-d3-PEG4, a novel PEGylated peptide that inhibits EphA4 signalling, we have shown that HNSCC progressive tumors treated with APY-d3-PEG4 display decreased tumor growth. To improve our understanding on how EphA4 is mediating tumor growth during HNSCC disease progression we first determined where EphA4 is expressed within the tumor microenvironment (TME). We found that EphA4 is expressed in T regulatory cells (Tregs) and macrophages within the TME. To investigate the role of EphA4 in these cellular populations during HNSCC disease progression we utilized genetically engineered mouse models to knockout EphA4 in Tregs ($EphA4^{fl/fl}FoxP3^{Cre}$) and macrophages ($EphA4^{fl/fl}LysM^{Cre}$). Tumor growth analysis and multicompartmental flow cytometric analysis was performed. To our surprise, we uncovered that EphA4 signalling in Tregs and macrophages have dichotomous effects on tumor growth. EphA4 knockout in Tregs resulted in decreased tumor growth while EphA4 knockout in macrophages resulted in accelerated tumor growth.

ADVANCING UNDERSTANDING OF UVEAL MELANOMA PROGRESSION: INTEGRATING EPH/EPHRIN EPIGENETICS, GENE EXPRESSION, AND REGULATORY NETWORKS THROUGH COMPREHENSIVE BIG DATA ANALYSIS AND WEB RESOURCES

G. Mandrakis[1], I. E. Stergiou[1] and S. Theocharis[1]

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Uveal melanoma (UVM) comprises the most common malignant neoplasm of the eyes, affecting the iris, the ciliary body, and the uveal choroid layer. Unravelling the molecular profile of neoplastic disease, regarding both genetic aberrations and epigenetic modifications with an impact on the expression of molecules implicated in various cellular pathways, drives the development of modern targeted therapies. In this view, research has highlighted multiple associations of the erythropoietin-producing hepatocellular carcinoma receptor (EPH) family, the largest group of receptor tyrosine kinases (RTKs), and their membrane-bound ligands, ephrins, with the pathogenesis of various malignancies. Applying big data analysis tools on the publicly available Gepia2 and The Cancer Genome Atlas (TCGA)-UVM databases, this study aimed to evaluate the potential implications of EPHs/ephrins' epigenetic and post-transcriptional regulation in the development and progression of UVM, focusing on the gene promoter methylation status and the expression of non-coding RNAs (ncRNAs) possibly modulating EPHs/ephrins' biological function. Gepia2 database analysis revealed that lower *EFNA2* promoter methylation levels and the subsequently increased *EFNA2* expression strongly correlated with shorter disease-free survival of UVM patients. Additionally, TCGA-UVM dataset analysis underscored a very strong correlation between long non-coding RNAs (lncRNAs) SNHG14, SNHG15, GAS5 and miR-34b-3p, miR-30a, miR-21, miR-211-3p, miR-510-5p micro-RNAs (miRNAs). Interestingly, the interplay of the above-mentioned ncRNAs has been previously shown to target EPHs or ephrins in the setting of neoplasia modulating biological processes driving tumorigenesis. Overall, our results support the prognostic significance of *EFNA2* expression, regulated by its level of methylation, in UVM, while in terms of functional EPH/ephrin regulation, the possible role of ncRNAs in the pathogenesis and progression of UVM is highlighted, also unveiling these lncRNAs and miRNAs as possible biomarkers. Experimental validation and clinical analysis are necessary to characterize the exact mechanisms of EPH/ephrin regulation and function in UVM, further opening an innovative field for the application of novel therapeutic approaches.

PROXIMITY LABELING OF EPHRIN B LIGANDS REVEALS THAT EGFR IS AN EFNB1 INTERACTOR

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Reverse signaling downstream of transmembrane Ephrins B (EfnBs) can be classified as pTyr-dependent or PDZ-dependent. The pTyr-dependent reverse signaling initiates when EfnBs bind to Eph receptors (EphRs). This event promotes the phosphorylation of Tyr residues within the EfnBs intracellular domain. The resulting pTyr residues are thought to serve as docking platforms for signaling proteins bearing Src Homology (SH) 2 or PhophoTyrosine Binding (PTB) domains, which can then recruit additional signaling proteins in order to control cellular processes such as cell migration and cell adhesion. In addition to pTyr-dependent signaling, EfnBs can initiate downstream signaling pathways through their PDZ binding motif, which mediates protein interactions with PDZ domain proteins. Despite our current knowledge on reverse signaling, the identity of multiple EfnB signaling effectors remains to be elucidated in order to better understand the molecular mechanisms underlying the function of EphR-EfnB signaling in cellular processes. We hypothesized that proximity labeling proteomics of EfnB ligands will allow the identification of new effectors involved in reverse signaling. Hence, we performed experiments with EfnB(1-3) fused to the promiscuous biotin ligase miniTurbo in steady-state and following EphR stimulation. This allowed us to identify 170 novel EfnB proximity partners, from which we could distinguish three main groups: (i) EphR stimulation-dependent candidates; (ii) EphR stimulation-independent candidates and (iii) candidates that are negatively modulated by EphR stimulation. To test whether the TurboID-identified EfnBs proximity partners acted as signaling effectors, we tested 15 candidates in a loss-of-function assay to identify those that contribute to EfnB1-dependent cell adhesion to fibronectin. We highlighted EGFR as an interesting putative effector. We further confirmed the association between EGFR and EfnB1 by co-immunoprecipitation and found out that the interaction is dependent on the PDZ-binding motif of EfnB1. This suggest that the interaction between EGFR and EfnB1 is mediated by a PDZ domain protein, whose identity we are currently investigating. Overall, our work will allow us to better understand how the EfnBs regulate cell adhesion to fibronectin.

EPHA2 TRANSMEMBRANE HELIX INTERACTIONS REGULATE EPHA2 OLIGOMERIZATION

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The transmembrane helices of receptor tyrosine kinases (RTKs) have been proposed to switch between two different dimeric conformations, one associated with the inactive RTK and the other with the active RTK. Furthermore, recent work has demonstrated that some full-length RTKs associate into oligomers that are larger than dimers, raising questions about the roles of the TM helices in the assembly and function of these oligomers. Here we probe the roles of the TM helices in the stability of EphA2 RTK oligomers in the plasma membrane. We employ mutagenesis to evaluate the relevance of a published NMR dimeric structure of the isolated EphA2 TM helix in the context of the full-length EphA2 in the plasma membrane. We use two fluorescence methods, Förster Resonance Energy Transfer and Fluorescence Intensity Fluctuations spectrometry, which yield complementary information about the EphA2 oligomerization process. These studies reveal that the TM helix mutations affect the stability, structure, and size of EphA2 oligomers. However, the effects are multifaceted, and point to a more complex role of the TM helix than the one expected from the “TM dimer switch” model.

COULD THE PRO-ONCOGENIC ROLE OF EPHA2 SER-897 BE AFFECTED BY EPH-EPHRIN ANTAGONISTS?

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Among all Eph receptors, EphA2 is the one with the most profound link to human cancers and correlates with malignancy, poor prognosis, elevated metastatic potential, and reduced survival of patients.
In literature, EphA2 has been characterized as a tumor suppressive and an oncogene. Ephrin-A1-mediated EphA2 canonical activation supports its anti-oncogenic features: the phosphorylation of the intracellular tyrosine kinase domain inhibits Erk and Akt pathways correlating with a reduction of tumor cell growth and survival [1]. On the other hand, in its unbound state, EphA2 becomes a substrate for serine-threonine kinases (i.e., Akt, Erk and PKA) that promote S897 phosphorylation turning EphA2 into an oncogenic protein: the non-canonical signaling enhance cancer progression, invasion, metastasis, angiogenesis, and chemo-resistance [2].
Recently, Shi and colleagues correlated EphA2 multimeric assembly with its pathological features and therapeutic implications. They suggested to attenuate pro-oncogenic EphA2 noncanonical signaling by disrupting Eph-Eph asymmetric interactions between LBD and FN2 of adjacent receptors expressed on the same cell membrane [3].
For all these reasons, EphA2 receptor is an appealing target in cancer therapy, and our research is focused on the identification of new small molecules that act as EphA2-ephrin-A1 antagonists. The compounds are chemical derivatives of UniPR1449, which *in vitro* activity was previously studied [4]. After defining IC_{50} and Ki values in ELISA-like binding assays, compounds were studied in EphA2 phosphorylation and MTT assays to verify respectively their antagonism behavior and cytotoxic or antiproliferative activity on U251 cells. In addition, we investigated possible effects on S897 phosphorylation, and our preliminary results suggest the possibility to modulate pro-oncogenic EphA2 activity using EphA2 antagonists.

References

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EPHA2 CONTROLS EPHA1 FUNCTION VIA A HETEROTYPIC INTERACTION THAT REGULATES TUMOR SUSCEPTIBILITY

X. Shi[1], H. Guo[1], S. Kim[1], H. Lin[1], P. Toth[1], R. Lingerak[1], E. B. Pasquale[1], A. W. Smith[1] and B. Wang[1]

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We observed opposite roles of EphA1 and EphA2 in the control of liver tumor susceptibility. EphA2 suppresses diethylnitrosamine (DEN) induced hepato-carcinogenesis, since deletion of EphA2 in the mouse led to higher tumor burden. In contrast, EphA1 behaves as an oncogene, since deletion of EphA1 in the mouse potently suppressed hepatocarcinogenesis. Using a time-resolved fluorescence spectroscopy known as PIE-FCCS, we discovered distinct homotypic molecular assemblies of EphA1 and EphA2 in live cells which mediate different signaling events. Furthermore, we also observed a strong heterotypic interaction between EphA1 and EphA2, which enables EphA2 to dictate the signaling of EphA1. Our findings challenge the prevailing view that Eph receptors have redundant functions and reveal new opportunities to exploit the unique function of heterotypic interactions to understand the in vivo biology of EphA1 and EphA2 as well as their suitability as targets against human pathologies such as Alzheimer's disease and cancer.

THE RELATIONSHIP BETWEEN EPHRIN AND NOTCH SYSTEMS IN THE REGULATION OF TUBE FORMATION OF HUVECs IN CO-CULTURE WITH MSCs

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VEGF is a soluble factor that initiates angiogenesis, whereas the NOTCH and Ephrin systems mainly regulate this process through intercellular communication. The aim of the work was to study interconnection between Notch and ephrin systems in initiation of angiogenesis. We used in vitro model of tube formation by umbilical vein endothelial cells (HUVECs) in 2D coculture with adipose derived mesenchymal stromal cells (MSCs). We found out that after 19h of coculture in co-cultured HUVECs, EPHA2 and EFNB1 were upregulated and EFNA1, EFNB1, EFNB2, EFNB3, and EPHB4 were downregulated. In the corresponding co-cultured MSCs, EFNA1, EFNA2, EFNB1, EFNB2, EPHB1, EPHB2, and EPHB4 were upregulated and EFNA5, EFNB3, EPHA2, EPHA4, and EPHB6 were downregulated. It is worth noting that in MSCs, EFNB2 ligand is regulated by Notch signaling, which we verified using a Notch inhibitor (compound E). We also analyzed the changes in HUVECs between growing/proliferating cells and cells in the monolayer. We found that the expression of ephrin ligand as well as EPHA4 and EPHB4 was decreased and only EPHA2 was upregulated. EPHB4-EFNB2 is known to be involved in the interaction between endothelial cells and MSCs. Inhibitor analysis revealed that the specific EphB4 inhibitor NVP-BHG712 switches the endothelial cells from an angiogenic phenotype (tubes) to a quiescent phenotype (islets) in a tube formation model in 2D co-culture. A similar effect was observed for the VEGF inhibitor DMH4. The Notch signaling inhibitor (CompoundE), although suppressing tube formation 2-fold, did not change the cell phenotype from tubes to islets. We also found that NVP-BHG712 upregulated DLL1, NOTCH1 and NOTCH4 in HUVEC monoculture. We can conclude that, according to our data, the Ephrin system has a more critical role than Notch in controlling the process of vessel formation in terms of switching the endothelial cell program from an angiogenesis initiation state to a quiescent state and vice versa. Our results may have important implications in the optimization of cell-based strategies to promote angiogenesis during tissue reparation.

EPHRIN MICROPATTERNS EXOGENOUSLY MODULATE CELL ORGANIZATION IN ORGANOID-DERIVED INTESTINAL EPITHELIAL MONOLAYERS

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Eph-ephrin signaling acts as spatial cue to define the tissue boundaries, the axonal growth, or the organization of compartmentalized tissues in vertebrates. By the regulation of tension, adhesion, and repulsion, intermingling of cells expressing the membrane-tethered ligand and cells expressing the membrane-tethered receptor is prevented. Despite being surface-bound, most of the studies addressing Eph-ephrin interactions use soluble ligands, which lack the spatial component needed to study tissue patterning. Here, we demonstrate that spatial patterns of ephrin ligands can influence the organization of different compartments within organoid-derived intestinal epithelial monolayers. We employ a modified microcontact printing technique to create spatial cues of ephrin ligands on basement membrane surrogates. Our findings reveal that both ligand concentration and cellular density can modulate the strength of the repulsive effect induced by Eph-ephrin signaling. Moreover, we show that micropatterned ephrin cues can alter the orientation of intestinal crypts, aligning them according to the pattern. Additionally, through the use of arrays of ephrin-depleted regions, we demonstrate the local ordering of intestinal crypts by the ephrin signal. This approach presents a valuable tool for studying exogenous signals with relevant spatial distributions *in vivo*.

HUNTING FOR ORIGINAL ANTICANCER AGENTS: PEPTIDE MODULATORS OF EPHA2 MEDIATED SAM-SAM INTERACTIONS

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The EphA2 receptor plays a crucial and controversial role in cancer by balancing pro-oncogenic ligand-independent and anti-oncogenic ligand-dependent pathways. Nevertheless, EphA2 is over-expressed in diverse types of tumours and ligand-induced receptor endocytosis and subsequent degradation have been exploited as potential route to reduce tumour malignancy. In this context, the cytosolic Sam (Sterile alpha motif) domain of EphA2 (EphA2-Sam) holds a certain interest, as it represents the engagement site of protein modulators of receptor endocytosis and stability (including the lipid phosphatase Ship2). Ship2 binds EphA2-Sam by forming a hetero-dimeric Sam-Sam complex with canonical Mid Loop/End Helix interaction topology that is highly stabilized by electrostatic contacts. Structural features characterizing the Ship2-Sam/EphA2-Sam association have been deeply described by us and other research groups. Interestingly, Ship2 works as an inhibitor of receptor endocytosis and its interaction with EphA2-Sam should mainly provoke pro-oncogenic effects in cancer cells. With this in mind, in our laboratories, we are employing a variety of structure-based approaches and a multidisciplinary strategy, made up of diverse biophysical techniques coupled to cell-based assays, to design and analyse peptide modulators of Sam-Sam interactions mediated by EphA2 [1]. Our ultimate goal is to develop novel molecular tools provided with anticancer potential. Here, we will present the latest results on positively charged peptide ligands of Ship2-Sam working as weak inhibitors of the EphA2-Sam/Ship2-Sam complex [2].

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ANTI-EPHRINB2 NEUTRALIZING ANTIBODY SUPPRESSES FIBROSIS IN PRECLINICAL *IN VITRO* AND *IN VIVO* MODELS

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Introduction: Fibrotic diseases are characterized by dysregulated synthesis and turnover of extracellular matrix (ECM) leading to the accumulation of scar tissue and loss of organ function. Ephrin signaling, mediated by Eph receptors and their ligands, has emerged as a crucial pathway in the pathogenesis of fibrosis. In particular, the EphrinB2 (EFNB2) signaling axis has been implicated in the progression of numerous fibrotic diseases, making it an attractive target for therapeutic intervention.

Methods: Anti-EphrinB2 antibodies were evaluated in a variety of *in vitro* and *in vivo* models of fibrosis. Primary human umbilical vein endothelial cells (HUVECs) were treated with antiEphrinB2 antibody to characterize inhibition of EphrinB2-induced EphB4 phosphorylation. Similarly, HUVEC migration and chemotaxis were quantified in response to antibody treatment using an Incucyte transwell migration assay. To test *in vivo* efficacy, a bleomycin (bleo) induced lung injury and a bleo induced scleroderma model were evaluated. C57BL/6 mice were administered intratracheal bleo on day 0 in the lung model, or subcutaneous bleo for five days a week for two weeks in the skin model. Anti-EphrinB2 therapeutic treatment was administered from day 7 to day 21. On day 21, fibrotic endpoints in the lung or skin were evaluated including histology, collagen content by hydroxyproline, gene expression, and the levels of proinflammatory cytokines in plasma and bronchoalveolar lavage fluid (BALF). Drug concentrations in plasma and BALF were determined by ELISA and/or MSD.

Results: Our findings demonstrate that anti-EphrinB2 antibody treatment effectively inhibits EphB4 phosphorylation in HUVECs with an IC_{50} of 1.2 nM. Similarly, in vitro migration studies revealed a significant reduction in HUVEC chemotaxis/migration upon treatment with antiEphrinB2 antibody showing dose-dependent inhibition. *In vivo*, anti-EphrinB2 demonstrated significant efficacy in suppressing lung fibrosis, with a reduction in hydroxyproline content by 52.5% compared to vehicle control. Additionally, anti-EphrinB2 significantly attenuated systemic pro-inflammatory cytokine levels including IFNγ, IL-6, KC/GRO, and TNF-a. In addition to showing efficacy in a model of lung fibrosis, anti-EphrinB2 also suppressed skin fibrotic readouts in a bleomycin-induced model of scleroderma. Anti-EphrinB2 antibody led to a 69.9% reduction in hydroxyproline content per mg of skin and histology showed a significant reduction in dermal thickness compared to vehicle group (246.5 ± 8.6 vs 281.7 ± 8.6 µm).

Conclusions: Treatment with anti-EphrinB2 antibody significantly reduced fibrosis in vitro and in preclinical mouse models. These data demonstrate that administration of an anti-EphrinB2 antibody potently suppresses lung and skin fibrosis and supports the potential for this mechanism as a therapeutic intervention for scleroderma or pulmonary fibrosis.

UNVEILING POTENTIAL? TARGETING EPH-EPHRIN IN GLIOBLASTOMA STEM CELLS WITH NOVEL PPI-INHIBITORS

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Background

Eph receptors are the largest family of receptor tyrosine kinases in human and together with ephrin ligands play a critical role in the development of many tumours, included Glioblastoma, the most lethal form of glioma. Research is committed to developing pharmacological strategies to target GBM cells. One of these is to target the interactions within the Eph-ephrin. Here we showed the pharmacological characterization of new small molecules potentially interfering with it.

Methods

Displacement and saturation curves of biotinylated ephrin ligands with recombinant Eph receptors were used to calculate IC_{50} and Ki values in the presence of compounds. Tests were conducted on both non-glioma and glioma stem cell models.

Non-GSCs cells: antiproliferative effects on U251 were assessed via MTT assay at various concentrations over 24h, 48h, and 72h of incubation. Antagonist activity was evaluated by pre-incubating cells with compounds, followed by ephrin stimulation. Eph receptor phosphorylation was quantified via ELISA.

GSCs cells: antiproliferative activity was evaluated using EdU incorporation via immunostaining and flow cytometry. Migration inhibition capability was assessed using the Gap-insert protocol.

Results

For non GSC cell line, the compounds, acting as antagonists, inhibit the phosphorylation of the EphA2 receptor at a concentration of 30 µM. Additionally, they demonstrate antiproliferative activity on U251 cells without inducing non-specific toxicity at concentrations up to 30 µM when incubated for 2 hours.

Regarding the stem cell line compounds showed a decrease in proliferation of cell line after 24h and 48h of incubation. Moreover, there was an increase in the G0 phase with compounds after 48h and 72h. Additionally, there was a reduction in the S phase after 72h. In the presence of natural ligand, migration was reduced at 10µM after 72h. In the presence of Fc, the compounds did not exhibit migration reduction, suggesting true antagonistic activity on the Eph-ephrin system.

TARGETING THE EPH-EPHRIN SYSTEM IN CANCER AND NEURODEGENERATION

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The design of agents that effectively target Eph receptors remains a challenging task. Over the years our laboratory has focused on designing such agents targeting the ligand binding domains of the receptor subtypes EphA2 in oncology and EphA4 in cancer and neurodegeneration. Overexpression of the receptor tyrosine kinase EphA2 is associated with poor prognosis and development of aggressive metastatic cancers. Guided by our recently solved X-ray structure of the complex between an agonistic peptide and the EphA2 ligand binding domain (LBD), we designed and characterized the agent Targefrin, that binds to EphA2-LBD with a 21 nM dissociation constant by isothermal titration calorimetry and presents an IC50 value of 10.8 nM in a biochemical assay. I will present on the design, characterization, and activities of Targefrin in various cellular and animal models of pancreatic and prostate cancers. Similar strategies were deployed to obtain potent agents targeting the EphA4 ligand binding domain. Interestingly, analysis of binding mode of such agents guided by X-ray crystallography and NMR spectroscopy measurements in solution, identified structural features that may direct the activity of designed agents toward agonism versus antagonism. Obtaining both potent agonistic and antagonistic agents targeting EphA4 is particularly useful given that the complex interplay between EphA4 receptor and its ephrin ligands in neurodegeneration has not yet been fully elucidated. For example, we reported that astrocytes from ALS patients induce cell death in co-cultured motor neurons. Furthermore, we found that our synthetic EphA4 agonistic agents effectively protected MNs when co-cultured with reactive astrocytes from ALS patients from multiple subgroups (both sALS and mutant SOD1). Newer generation and more potent EphA4 agonistic agents also provided effective protection at lower therapeutic doses. Combined, the data suggest the pharmacological tools identified provide a significant step towards the development of Eph-based therapeutics in broad therapeutic areas and invites the development of similar strategies for other receptor sub-types.

EPHB2 A VIABLE THERAPEUTIC TARGET FOR THE TREATMENT OF PAEDIATRIC MEDULLOBLASTOMA

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Medulloblastoma (MB) is the most frequent malignant brain cancer to occur in children and remains the leading cause of cancer-related mortality in childhood. Overall survival rates have reached 70%, but the outcome for young children, especially infants, is worse. Those who do survive, suffer from long-term therapy-induced side effects. Thus, more effective tumour-specific targets are urgently needed. Eph family receptors predominantly function during embryonic development and are typically expressed at low levels in healthy tissues. Importantly, Eph receptors have been shown to be re-expressed and functional in many human cancers. The aim of our study was to validate EphB2 as a potential tumour-specific therapeutic target in MB. Analysis of the Medulloblastoma Advanced Genomics International Consortium (MAGIC) dataset show EphB2 is significantly elevated in all MB subgroups when compared to normal cerebellum. EphB2 stimulation, using clustered ephrinB1-Fc and ephrin B2-Fc, resulted in robust kinase activation and functionally resulted in reduced MB tumour cell proliferation and invasion *in vitro*. EphB2 blocking studies, using the SNEW peptide, effectively inhibited kinase phosphorylation and downstream FAK phosphorylation and notably rescued the observed MB anti-proliferative response observed following EphB2 kinase activation. To better understand the contribution of EphB2 to MB tumourigenesis we employed shRNA mediated gene knock down (KD) in Daoy MB cells. EphB2 KD resulted in a significant reduction in proliferation *in vitro* and a significant reduction in tumour formation when engrafted orthotopically into the cerebellum if immunocompromised mice. We have now embarked upon the development of a novel EphB2 targeting therapeutic antibody. This mAb employed as an antibody drug conjugate (ADC) displayed a promising therapeutic killing effect in EphB2 positive brain cancer organoids. Initial *in vivo* safety assessment of the EphB2-ADC has been conducted in NRG mice revealing no significant therapy-related toxicity. In summary, our study has highlighted the functional importance of EphB2 in MB maintenance and progression and paves the way for future EphB2-ADC receptor targeting studies in MB.

EPHRIN'S AND EPH'S ARE IMPLICATED IN EGFR-TKI RESISTANCE OF NON-SMALL CELL LUNG CANCER AND ARE LOADED IN EXTRACELLULAR VESICLES

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Background: Epidermal growth factor receptor Tyrosine kinase inhibitors (EGFR TKIs) has a major impact on treatment of non-small cell lung cancer (NSCLC) patients whose tumor has mutated EGFR (mut. EGFR) as oncogenic driver. Development of resistance is a clinical problem which may be due to mutations in the EGFR kinase domain that block TKI binding but also by-pass signaling alterations in others receptor tyrosine kinases (RTKs). Previous results have shown that EphA2 signaling may be a resistance driver in relation to 1st generation EGFR-TKIs. We earlier reported that knockdown of Ephrin B3 inhibited migration and invasion in NSCLC. Moreover, Ephrin B3 was found to bind to EphA2 and promote EphA2 Ser897 phosphorylation. Here we focus on the role of Ephrin B3 as a driver of EGFR-TKI osimertinib (osi) resistance in NSCLC and in particular Ephrins/Ephs expression in extracellular vesicles (EVs).

Materials and methods: Ephrin B3 was silenced in the EGFR mut. cell line H1975 and its effect on EGFR-TKI erlotinib (erlo) and osi sensitivity was analyzed by clonogenic assays. EVs were isolated from media of t H1975 cells or an osi resistant subline H1975/OR3 in response to osi by size exclusion chromatography (SEC). Nanoparticle Tracking Analysis (NTA) and by western blot was used for EV characterization. Mass spectrometry (MS) was used for EVs protein profiling and data was analyzed by the Qlucore® platform.

Results: The H1975 and H1975/OR NSCLC cells expressed Ephrin B3, Ephrin A1, and EphA2. siRNA-mediated silencing of either Ephrin B3- or EphA2 expression sensitized for erlo as well as osi. Components of the Eprin/Eph pathway i.e. EphA2, EphB2, EphB3, Ephrin B1 and Ephrin B3, were found in EVs isolated from H1975 and H1975/OR cell culture media. Interestingly, EphB2 had a higher expression in the EVs isolated from osi-sensitive H1975 cells while Ephrin B1 expression was increased in EVs obtained from osi-resistant H1975/OR.

Conclusion: Ephrin B3 silencing increases sensitivity to EGFR-TKI osi with reduced colony formation capacity, suggesting that the Ephrin B3/EphA2 pathway could be involved in a by-pass mechanism for osi. Also, we were able to find several Ephrin's and Eph's in EVs. These results should be further analyzed in a context of cellular-to-cellular communication, metastatic signaling and as therapeutic target or biomarkers in NSCLC.

EPHA2 PHOSPHORYLATION SUSTAINS THE ADAPTIVE RESPONSE OF COLORECTAL ORGANOIDS TO OXALIPLATIN

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EphA2 is highly expressed in colorectal cancer (CRC) and patients with colorectal cancer with high EphA2 expression exhibit worse prognosis. Therefore, we explored the dynamics of EpHA2 in a clinically relevant model, namely CRC Patient-derived Organoids (PDOs). PDOs were obtained and validated from four CRC specimens. We evaluated the number of EphA2 positive cells within these CRC PDOs and this revealed that EphA2 positive cells represented a stable cell subpopulation in CRC PDOs. Given the involvement of EphA2 in mediating the response to chemotherapy, we challenged the PDOs with oxaliplatin (OXA) at pharmacologically relevant schedules. This revealed the persistence or increase of EphA2-positive cells both in acute and chronic (21dd) treated cells. To dissect this observation, we analyzed the EphA2 protein levels and its phosphorylation status using zn-Phos-tag gels and indirect ELISA assays. This revealed that the ser897 of EphA2 was an oxaliplatin-sensitive phosphorylation modification in CRC PDOs, correlating with increased EphA2 protein levels. Functionally, we found that RNAi mediated knocking down of EphA2 or attenuated EphA2 ser897 phosphorylation significantly reduced the organoid-forming ability (OFA) of the PDOs after OXA treatment, consistent with a chemosensitizing effect. We speculate that increase of EpHA2 supports the adaptive response of PDOs to OXA and that phosphorylation of EphA2 ser897 may be instrumental to such modulation.

HARNESSING THE EPHA2 BLOCKADE IN GLIOBLASTOMA

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The overexpression of tyrosine kinase EphA2 receptor has been reported in numerous cancers including the most aggressive adult brain tumour; glioblastoma (GBM), with accumulating evidence showing strong association with tumour progression. The EphA2 receptor functions as a powerful oncoprotein and has been listed as the 25. most requested antigen for therapeutic manipulation.
In this study, we determined innate immunity effector lymphocytes Vδ1 a Vδ2 γδ T cell populations in peripheral blood and paired tumour tissue samples in patients following resection and throughout the therapy follow-up. Tumour samples were processed using enzymatic kits and gentleMACSTM Dissociator and tumour-infiltrating γδ T lymphocytes (TILs) were analyzed by flow cytometry.
We found infiltration of intratumoral CD3+ γδ T cell subsets in most tumour samples. Functional studies showed prominent cytotoxicity of magnetically sorted Vδ1 a Vδ2 γδ T cells against GBM cell lines and more importantly against primary tumours.
Next, we identified the EphA2 receptor as one of the targets for tumour-reactive Vδ1 γδ T cells. Specifically, we found that blocking of EphA2 expression by the small molecule inhibitor ALW-II-41-27 and also by knock-outs generated by CRISPR/Cas9 have resulted in significant inhibition of GBM killing mediated by Vδ1 γδ T cells.
Moreover, correlations with clinical data showed a prolonged survival of patients with higher percentages of gamma-delta T cell populations, which can be used as a prognostic parameter determining delay in disease progression. Together, these data further define a role for EphA2 and GBM-reactive Vδ1 γδ T cells.

THE RECEPTOR FOR ADVANCED GLYCATION-END PRODUCTS (RAGE) RAGE INDUCES MIGRATORY AND INVASIVE FEATURES IN BREAST CANCER CELLS VIA EPHRIN TYROSINE KINASE A3 (EPHA3)

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The receptor for advanced glycation-end products (RAGE) is a multiligand recognition receptor belonging to the immunoglobulin superfamily. RAGE and the cognate ligands have been involved in metabolic alterations and inflammatory responses as well as in the progression of diverse malignancies, including breast cancer (BC). In present study, we aimed at providing new insights on the role eexerted by RAGE in BC. To this aim, we stably overexpressed RAGE in MCF7 and T47D estrogen receptor (ER)-positive BC cells. Highthroughput RNA-sequencing studies allowed us to assess the most important transcriptional changes occurring in RAGE-overexpressing BC cells, while Gene Ontology (GO) enrichment analyses have enabled to predict the biological role of the differentially expressed genes (DEGs) obtained. Of note, RNA-sequencing data indicated that RAGE overexpression is associated with a motility-related gene signature in ER-positive BC cells. Accordingly, in vitro assays including migration, invasion, colony formation and scanning electron microscopy highlighted an aggressive and invasive behavior of RAGEoverexpressing BC cells. Notably, *in vivo* experiments using zebrafish xenografts further corroborated the motile phenotype of BC cells driven by RAGE overexpression. Next, flow cytometry, real time-PCR, chromatin immunoprecipitation, immunofluorescence and western blot assays have identified EphA3 as a novel RAGE target gene. Bioinformatics analysis on ER-positive BC patients of the TCGA dataset revealed an association of EphA3 expression with poor outcome, as well as the involvement of EphA3 in invasive events. Of note, a pro-migratory role elicited by EphA3 has been also established in cancer-associated fibroblasts (CAFs), as ascertained by co-culture assays. Collectively, our findings indicate that the overexpression of RAGE, which is a common occurrence in diabetes and obesity, may contribute to the progression of BC triggering EphA3 expression. Therefore, EphA3 should be included among the target genes of RAGE involved in BC invasion and metastasis.