UNIVERSITÀ DEGLI STUDI DI PARMA

First congress on the Eph/ephrin system

Parma, Italy

May 5-6, 2016

State of the art, challenges and opportunities

Scientific committee:

M. Tognolini, University of Parma, Italy

E. Pasquale, SBP Medical Discovery Institute, La Jolla, USA

A. Lodola, University of Parma, Italy

Organizing committee

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I. Zanotti, University of Parma, Italy

C. Giorgio, University of Parma, Italy

S. Russo, University of Parma, Italy

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Thursday 5 May 2016	
8.30-13.00	*Registration*
9.00-9.20	*Welcome and introductory remarks*

Biology and structural biology *Chairs: R.Klein (Germany) - M.Tognolini (Italy)*	
9.20-9.50	Y. Maru *Tokyo Women's Medical University, Tokyo, Japan* **Eph receptors a historical overview**
9.50-10.10	D. Wilkinson *The Francis Crick Institute, Mill Hill Laboratory, London, UK* **Role of homotypic and heterotypic cell responses in Eph-ephrin mediated border formation**
10.10-10.40	Y. Jones *University of Oxford, UK* **A structural perspective on ephrin and Eph cell surface signalling assemblies**
10.40-11.00	C. Hogan *Cardiff University, UK* **Differential EphA2 signalling induces the segregation of Ras-transformed epithelial cells from normal neighbours**

11.00 -11.30	*Coffee break*

Eph and cancer *Chairs: D. Wilkinson (UK) – B. Wang (USA)*	
11.30-11.50	B.W. Stringer *QIMR Berghofer Medical Research Institute, Brisbane, Australia* **The transforming ability of EphA3 is independent of its tyrosine kinase activity in a Cdkn2a null neural stem cell model of glioma**
11.50-12.10	S. Parrinello *Imperial College London, London, UK* **EphrinB2 drives perivascular invasion and proliferation of glioblastoma stem-like cells**
12.10-12.30	A. Freywald *University of Saskatchewan, Canada* **The EPHB6 receptor both augments growth and suppresses drug resistance in triple negative breast tumours**

12.30-13.00	B. Wang *Case Western Reserve University, Cleveland, USA* **USA It takes two to tango: both EphA2 receptor and ephrin-A ligands are required for tumor suppression**
13.00-14.30	*Lunch*
Eph system in other pathologies *Chairs: E.Pasquale (USA) – E. Barocelli (Italy)*	
14.30-15.00	A. Zapata *Universidad Complutense de Madrid, Spain* **Eph and Ephrins affect thymus biology by governing thymocyte-thymic epithelial cell interactions**
15.00-15.20	M. Cauquil *INSERM/Université Paul Sabatier Toulouse. France* **Ephrin-B1 blocks adult cardiomyocyte's proliferation: impact on cardiac regeneration**
15.20-15.50	F. Neipel *Universitätsklinikum Erlangen, Germany* **The role of Eph-receptors in viral infections**
15.50-16.20	*Coffee break*
16.20-16.50	R. Klein *Max-Planck Institute, Munich, Germany* **Novel mechanisms of Eph/ephrin repulsion via extracellular and intracellular vesicles**
16.50-17.10	B. Di Benedetto *University of Regensburg, Germany* **The glia-neuron ephrinA/EphA system in major depressive disorder - a novel target for alternative therapeutic approaches?**
17.10-17.40	W. Robberecht *University of Leuven, Belgium* **Ephrins in the pathogenesis of axonal degeneration**
Evening	***SOCIAL EVENT***

Friday 6 May 2016	
9.00-10.20	*Breakfast at the poster session*

Targeting the Eph/ephrin system *Chairs: Y. Jones (UK) - M. Mor (Italy)*	
10.20-10.30	Presentation of ULLA network
10.30-11.00	A. Lodola *University of Parma, Italy* **Targeting the Eph-ephrin System with Protein-Protein Interaction (PPI) Inhibitors**.
11.00-11.20	J.P. Himanen *Memorial Sloan-Kettering Cancer Center, New York, USA* **Single-chain anti-Eph antibodies as novel anti-tumor agents**
11.20-11.40	M. Leone *CNR, Napoli, Italy* **Structure-based design of peptides against the Sam domain of EphA2 receptor and its heterotypic interactions**
11.40-12.00	C. Festuccia *University of L'Aquila, Italy* **EphA2 receptor and ephrin-A1 ligand targeting in glioblastoma: function and therapeutic effects.**
12.00-12.30	E. Pasquale *Sanford-Burnham Prebys Medical Discovery Institute, La Jolla, USA* **Targeting Eph receptors with peptides and peptides conjugates**
12.30-12.40	*Farewell*

ORAL COMMUNICATIONS

Eph receptors: a historical overview

Y. Maru
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Southern blotting in a low stringent condition of total human placenta DNAs with a tyrosine kinase domain fragment of viral Fps as a probe revealed additional bands to the cellular Fps gene in 1987. Genomic screening with the same probe in the same condition gave us more than 70 positive clones, among which clone 7 turned out to be an as yet unidentified gene with a putative tyrosine kinase sequence. Overexpression of clone 7 was observed in one nude mouse-transplantable human liver tumor that highly expressed erythropoietin. The full-length cDNAs of clone 7 were isolated from a cDNA library of the tumor. The putative tyrosine kinase, now called EphA1, was of receptor type and named as Eph after Erythropoietin-Producing Hepatoma. An EphA2 ligand, ephrinA1, was originally found as a TNF-induced early response gene in human endothelial cells in 1990. Subsequently, the Eph Nomenclature Committee unified the names of the accumulating Eph-ephrin family members in 1998. Currently there are at least 14 Eph family members in mammals that are sub-grouped into 9 EphAs and 5 EphBs based on sequence divergence. The Eph-ephrin system is involved in development, neuronal transactions, angiogenesis, stem cell affairs and tumor growth. Several features of the Eph-ephrin system expand the scope of traditional understanding of receptor tyrosine kinase, including context-dependent opposite effects on cell proliferation and insulin secretion, functions both dependent on and independent of the tyrosine kinase activity or ligand binding, release of soluble forms after shedding by proteases like ADAMs and proposed coupling with biologically key molecules as exemplified by NMDAR with EphB2 in cognitive functions and ephrinB2 in control of VEGFR2 functions in angiogenesis. Thus elucidation of precise mechanisms is absolutely necessary to target a given Eph in a pathological context by drugs. Although overexpression and mutations have been found in a variety of human tumors, relevance of the Eph-ephrin system in metastasis is largely unknown. We have found that binding between ephrinA1 and EphA1/EphA2 in lung endothelial cells in a tyrosine kinase-independent fashion maintains cell-cell adhesion, which is disrupted by soluble forms of ephrinA1, which is released into the serum by cleavage of the membrane-attached form of ephrinA1 by ADAM12 in the primary tumor, stimulating EphA1/EphA2 in a tyrosine kinase-dependent manner in the pre-metastatic lungs with increased vascular permeability that allows entry of tumor cells.

Role of homotypic and heterotypic cell responses in Eph-ephrin mediated border formation

H. Taylor, A. Khuong, Q. Xu, Z. Wu, W. Taylor and D.G. Wilkinson
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Eph receptor and ephrin signaling has a major role in the formation of sharp borders between adjacent tissues and between subdivisions within tissues during vertebrate development. A key question is the nature of the molecular pathways and cell responses that underlie such border sharpening. We find that border sharpening by Eph-ephrin signaling depends upon N-cadherin, both in the zebrafish hindbrain and in cell culture assays for cell segregation and border formation. We have analysed the role of N-cadherin and cell responses to Eph-ephrin signaling by combining experimental manipulations of signaling, measurements of cell behaviour, and computer simulations of adhesion and repulsion. We find that border sharpening requires regulation of homotypic cell responses of Eph receptor expressing and ephrin expressing cells, as well as heterotypic responses at the Eph-ephrin interface. These findings have general implications for the roles of complementary and overlapping Eph-ephrin expression during tissue morphogenesis.

A structural perspective on ephrin and Eph cell surface signalling assemblies

Y. Jones
University of Oxford, UK

Differential EphA2 signalling induces the segregation of Ras-transformed epithelial cells from normal neighbours

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In epithelial tissues, cells expressing oncogenic Ras (RasV12) are detected by normal neighbours and are subsequently removed from the tissue via an extrusion process. RasV12 cells are extruded apically, suggesting that extrusion may represent a clearing mechanism and may therefore be tumour suppressive. Extrusion requires intact E-cadherin-based cell-cell adhesions with normal neighbours and increased actin-myosin contractility within the extruding cell, however little is known about the initial signals that trigger extrusion. Here, we reveal that differential EphA2 signalling is the mechanism that

triggers detection and extrusion of RasV12 cells from normal epithelial. EphA2, a known transcriptional target of the Ras-Raf-MAPK pathway is overexpressed on RasV12 cells. Cell-cell interactions between RasV12 and normal cells induce an EphA2-ephrin-A signalling cascade that triggers RasV12 cells to tightly cluster, self-aggregate and segregate from normal cells. In the absence of an ephrin-A signal, RasV12 cells remain in epithelial monolayers and adopt a pro-invasive morphology. Our data indicate that expression of RasV12 in single or small clusters of cells creates 'artificial' EphA2 boundaries, which promote the segregation and extrusion of RasV12 cells from normal tissues. This implies that changes in Eph/ephrin expression would allow RasV12 cells to go undetected and expand within an epithelium.

The transforming ability of EphA3 is independent of its tyrosine kinase activity in a Cdkn2a null neural stem cell model of glioma

B. W. Stringer, K. Goasdoué, A.W. Boyd
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Glioblastoma (GBM) is the most common primary malignant brain cancer. Its treatment involves surgery, post-operative radiotherapy and oral temozolomide chemotherapy. Despite this, median survival is <15 months and only ~10% of patients survive two years without disease recurrence. Several factors contribute to the poor prognosis of this disease. Key among them are glioma initiating cells (GICs) a subset of cells within these tumours that exhibit neural stem cell-like features, are resistant to both chemotherapy and radiotherapy, and are responsible for disease progression/recurrence. We have reported previously that EphA3, a receptor tyrosine kinase (RTK) expressed in ~40% of GBM, is highly expressed by GICs and has a functional role in their survival and self-renewal. EphA3 knockdown induced glioma cell differentiation and dramatically reduced tumour formation in vivo. Radio-immunotherapy using an EphA3 monoclonal antibody prevented growth of GBM in pre-clinical animal models. Our findings suggest EphA3 may be a suitable therapeutic target for GICs.
Homozygous deletion of CDKN2A is the most common genetic alteration found in GBM. It is usually detected in combination with amplification or mutational activation of a RTK. We have found that expression of EphA3 in Cdkn2a null murine neural stem cells is sAniliufficient to initiate glioma formation in NOD/SCID mice. As tyrosine kinase inhibitors such as dasatinib are being trialed as a targeted therapy for GBM, we have investigated whether the transforming activity of EphA3 in neural stem cells requires its tyrosine kinase activity. We have found that both kinase dead (K654R) and kinase constitutively active (A972P) mutants of EphA3 initiate glioma formation in Cdkn2a null neural stem cells suggesting the transforming activity of EphA3 in these cells is independent of its kinase

activity. We have followed up these experiments by performing RNA-seq analysis of EphA3 kinase dead (K654R) vs kinase constitutively active (A972P) vs vector control Cdkn2a null murine neural stem cells to investigate EphA3-induced gene expression changes that may contribute to malignant transformation of neural stem cells in GBM.

Day BW, Stringer BW, et al. (2013) EphA3 maintains tumorigenicity and is a therapeutic target in glioblastoma multiforme. Cancer Cell 23(2):238-248.

EphrinB2 drives perivascular invasion and proliferation of glioblastoma stem-like cells

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Glioblastomas (GBM) are aggressive and therapy-resistant brain tumours, which contain a subpopulation of tumour-propagating glioblastoma stem-like cells (GSC) thought to drive progression and recurrence. Diffuse invasion of the brain parenchyma, including along preexisting blood vessels, is a leading cause of therapeutic resistance, but the mechanisms remain unclear. Here, we show that ephrin-B2 mediates GSC perivascular invasion. Intravital imaging, coupled with mechanistic studies in murine GBM models and patient-derived GSC, revealed that endothelial ephrin-B2 compartmentalises non-tumourigenic cells. In contrast, upregulation of the same ephrin-B2 ligand in GSC enabled perivascular migration through homotypic forward signalling. Surprisingly, ephrin-B2 reverse signalling also promoted tumourigenesis cell-autonomously, by mediating anchorage-independent cytokinesis via RhoA. In human GSC-derived orthotopic xenografts, EFNB2 knock-down blocked tumour initiation and treatment of established tumours with ephrin-B2-blocking antibodies suppressed progression. Thus, our results

indicate that targeting ephrin-B2 may be an effective strategy for the simultaneous inhibition of invasion and proliferation in GBM.

The EPHB6 receptor both augments growth and suppresses drug resistance in triple negative breast tumours

B. Toosi, A. Kusalik and A. Freywald
University of Saskatchewan, Canada

Triple negative breast cancer (TNBC) is associated with a high resistance to therapy and early tumour relapse. This is most likely due to the resistance of slowly-proliferating tumour-initiating cells (TICs). Here, we report that a catalytically-inactive member of the Eph group of receptor tyrosine kinases, EPHB6, partially suppresses the epithelial-mesenchymal transition in TNBC cells, while promoting expansion of triple-negative TICs. Its proliferative effect is mediated by the activation of the ERK pathway, predominantly by the ERK2 kinase. In xenograft models, EPHB6 accelerates tumour growth and potentiates tumour initiation. Remarkably, EPHB6 also suppresses tumour drug resistance, most probably by forcing TICs into a more proliferative, drug-sensitive state. In agreement, patients with higher EPHB6 expression in TNBC tumours have a better chance for recurrence-free survival. These observations suggest that it may be beneficial to support EPHB6 action concurrent with applying conventional therapies, as it would decrease TIC-mediated resistance and reduce recurrence of TNBC.

These findings not only reveal a new molecular mechanism that governs the behaviour of tumour-initiating cells (TICs), but unexpectedly, they also imply that the enhanced proliferative activity of TICs is not necessarily a bad factor for cancer patients, since it may make cytotoxic treatments more effective by increasing sensitivity of TICs to DNA-damaging agents. This counterintuitive paradigm cautions against inhibiting molecules that support TIC proliferation, since this treatment is likely to drive TICs into a more dormant, drug-resistant state, especially if it is used in combination with DNA-damaging therapies.

It takes two to tango: both EphA2 receptor and ephrin-A ligands are required for tumor suppression

B. Wang
Case Western Reserve University, *Cleveland, USA*

Eph and Ephrins affect thymus biology by governing thymocyte-thymic epithelial cell interactions

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The thymus is a central, primary lymphoid organ, essential for the functional maturation of T lymphocytes. Interactions between T cells and thymic stromal cells, principally epithelial cells (TECs), are key for organizing a 3D cellular network necessary for T-cell differentiation. We and other authors have demonstrated the relevance of Eph/Ephrins for these interactions. We will review available information on this role of Eph and Ephrins in different processes occurring in the thymus, with special reference to the importance of EphB2/EphB3-Ephrin B interactions for the functional maturation of TECs. Our interest will be then focused to recent results confirming and quantifying a delayed maturation of these cells in EphB-deficient mice, more severe in EphB2 than in EphB3 thymuses. This delay that becomes gradually more important to finally result in adult thymus phenotypes with profound alterations in different TEC subsets and disturbed cortex-medulla organization, seems to be dependent on the low numbers of developing thymocytes, including those that colonize the thymus, and changes in the survival and cell cyclying of the mutant TECs, but also in the lack of adequate thymocyte-TEC interactions that could impede the signaling mediated by other molecules known to govern the epithelial maturation

Ephrin-B1 blocks adult cardiomyocyte's proliferation: impact on cardiac regeneration

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Background: Rebooting the proliferation of adult cardiomyocytes (CM) has recently emerged as a promising avenue in cardiac regenerative medicine. For that purpose, there is an urgent need for identifying molecular mechanisms involved in the natural blockage of adult CM proliferation. Recently, we demonstrated that ephrin-B1 ensures the cardiac tissue cohesion in young mice by stabilizing the adult CM rod-shape (Genet et al, 2008). In this study, we present the role of ephrin-B1 in the control of CM proliferation.
Methods and results: Old (12 months) ephrin-B1 knock-out mice (KO) don't exhibit cardiac functional defects despite huge cardiac tissue disorganization. Interestingly, these mice compensate for cardiac aging stress through a CM hyperplasia, suggesting an atypical proliferation of adult CM. This proliferative ability was confirmed in vitro in isolated 2 months-old CM stimulated with neuregulin-1 growth factor. Remarkably, in KO CM this

treatment resulted in a significant increase in BrdU uptake (9.57% vs 0.17% in WT CM) and in mitotic CM (1.5% vs 0.07% in WT CM). This proliferative ability of 2 month-old KO CM was confirmed in vivo using the apectomy model. Contrary to WT, KO mice almost completely regenerated the cardiac apex as indicated by the significant presence of mitotic CM and reduced fibrosis in the resected area. More importantly, the absence of ephrin-B1 in the CM led to an improvement of cardiac contractile function as assessed by echocardiography. Thus, ephrin-B1 acts as a natural blocker of adult CM proliferation.
Paradoxically, ephrin-B1 is also expressed in proliferative neonatal CM, suggesting distinct role to ephrin-B1 in the CM neonatal/proliferative state versus adult/unproliferative state. Interestingly, in neonatal CM, ephrin-B1 is specifically phosphorylated on its tyrosine 328 whereas in adult CM ephrin-B1 is unphosphorylated. This dephosphorylation occurs more precisely around the 7th day after birth which coincides with the onset of CM proliferation arrest and the downregulation of YAP1, the effector of the Hippo pathway that plays a key role in CM proliferation. Interestingly, compared to WT, KO CM restored cytosolic YAP1 expression, suggesting that dephosphorylated ephrin-B1 acts as a repressor of YAP1 signaling in adult CM.
Conclusion: These results demonstrated for the first time a role for ephrin-B1 in the control of adult CM proliferation though regulation of the Hippo/YAP1 pathway. In the future, the accurate molecular characterization of this pathway will be determinant for cardiac regenerative medicine.

The role of Eph-receptors in viral infections

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Novel mechanisms of Eph/ephrin repulsion via extracellular and intracellular vesicles

R. Klein
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Cells release membranous vesicles known as exosomes that represent a novel mode of intercellular communication. Exosomes are formed by budding into multivesicular bodies (MVBs) and their fusion to the plasma membrane, a process that requires the endosomal sorting complex required for transport (ESCRT). Eph receptor tyrosine kinases and their membrane-tethered ephrin ligands have important functions in neuronal development, plasticity, and pathological processes. Ephrin-Eph signaling requires direct cell contact and is bi-directional: ephrin to Eph signaling is called forward signaling, while Eph to ephrin signaling is called reverse signaling. Biological functions of Eph-ephrin signaling are well understood, whereas our mechanistic understanding is modest. We report the release of

exosomes containing Ephs and ephrins by different cell types including neurons, a process that requires ESCRT activity and is regulated by neuronal activity. Treatment of cells with purified EphB2+ exosomes induces ephrinB1 reverse signaling and causes repulsion of neuronal axons. These results indicate a novel mechanism of Eph/ephrin signaling independent of direct cell contact and proteolytic cleavage, and suggest the participation of EphB2+ exosomes in neural development and synapse physiology.
Cell repulsion induced by ephrin-Eph signaling at cell contact sites is promoted by bi-directional trans-endocytosis of clustered Eph/ephrin complexes at cell interfaces. The underlying intracellular signaling pathways are poorly understood. We identified an actin-regulating signaling pathway which allows ephrinB+ cells to trans-endocytose EphB receptors from opposing cells. Live-imaging revealed Rac-dependent F-actin enrichment at sites of EphB2 internalization, but not during subsequent EphB2 trafficking. Systematic depletion of Rho family GTPases and their regulatory proteins identified the Rac subfamily and the Rac-specific guanine nucleotide exchange factor Tiam2 as key positive regulators of EphB2 trans-endocytosis, a pathway previously implicated in Eph forward signaling. However, unlike in Eph forward signaling, this pathway is not required for uptake of soluble ligands by ephrinB reverse signaling. These results indicate the presence of a conserved signaling pathway for EphB trans-endocytosis that removes the physical tether between cells and thereby likely enables cell repulsion.

The glia-neuron ephrinA/EphA system in major depressive disorder - a novel target for alternative therapeutic approaches?

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Morphometric examinations of post mortem brains of patients with major depressive disorder (MDD) revealed alterations in the density of astrocytes, in addition to neurons. Astrocytes extend processes that wrap around synapses and blood vessels, thereby regulating the formation and functionality of neuronal circuits and of the blood-brain barrier. In a recent work, we showed that long-term potentiation (LTP), a major cellular correlate of memory storage which depends on activation of the ERK/MAPK signalling pathway, was inhibited in the hippocampus by treatment with the antidepressant desipramine (DMI). In addition, we showed that, shortly after LTP induction, an increase in the number of MAPK-positive cells occurred specifically among astrocytes of the stratum radiatum and this astrocytic MAPK activation was selectively hindered by DMI, thereby attenuating synaptic potentiation. The examination of a regulator of LTP located at the astrocyte-neuron interface in the stratum radiatum, namely the ephrinA3/EphA4 signalling pathway, revealed that DMI enhanced EphA4 clustering, which favoured an

increased ephrinA3-mediated EphA4 phosphorylation and elevated EphA4 forward signalling. The co-administration of DMI with the Src inhibitor SU6656, which blocks EphA4 forward signalling, could partially reverse the LTP attenuation, further supporting the targeting of the ephrinA3/EphA4 pathway by DMI. Our findings suggest a putative novel mechanism of action for antidepressants through the regulation of the ephrinA/EphA signalling pathway. Currently, we are exploring the molecular underpinnings of the regulatory effects of antidepressants at the neuron-glia interface and their consequences on adaptive changes in an animal model of depression-like behavior. A further examination of the molecular and behavioral consequences of targeting the ephrinA/EphA system might help to improve the clinical use of antidepressants or develop new drugs with better side-effect profiles, tolerability and efficacy.

Ephrins in the pathogenesis of axonal degeneration

W. Robberecht
University of Leuven, Belgium

Targeting the Eph/ephrin system with Protein-Protein Interaction (PPI) inhibitors

A. Lodola
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The Eph receptor-ephrin system is an emerging target for the development of novel anti-angiogenic, anti-inflammatory and metabolic therapies.[1] Research programs aimed at developing small-molecule antagonists of the Eph receptors are still in their initial stages. Our efforts in the field led to the discovery of lithocholic acid as a “genuine” PPI inhibitor of the EphA2-ephrinA1 interaction.[2] The following hit-to-lead campaign led to identification of more potent antagonists of the Eph-ephrin system.[3] Among the discovered compounds UniPR129 (L-□□homotryptophan conjugate of lithocholic acid) emerged as one the most promising chemical probe being able to block in vitro angiogenesis in HUVE cells thanks to the inhibition of both EphA2 and EphB4 activity at low micromolar concentration.[3] However, the usefulness of this compound as a pharmacological tool has been severely hampered by its low kinetic solubility, high lipophilicity and moderate selectivity.[5]

In the present talk, we report our medicinal chemistry and pharmacological efforts aimed at discovering a new generation of PPI targeting the Eph-ephrin system featured by a good PK profile and devoid of activity on G-protein coupled and nuclear receptors physiologically targeted by lithocholic acid.

Two examples of potential therapeutical activity related to the blockage of the Eph-ephrin system with newly identified small-molecules will be also disclosed for the first time.

References
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Single-chain anti-Eph antibodies as novel anti-tumor agents

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Recombinant antibody phage library technology is a method to generate antibodies without immunization, which provides multiple advantages. For example, human antibodies can be generated against Eph and other receptors that are extremely highly conserved between species. We have used this technology to isolate and characterize an anti-EphA2 single-chain scFv antibody. We show that the antibody binds the antigen with 1:1 stoichiometry and has high specificity for EphA2 over other Eph receptors both in a test tube and on the cell surface. The crystal structure of the complex reveals how the antibody binds EphA2 targeting the same hydrophobic surface cavity as the ephrin ligand. Specifically, a lengthy CDR-H3 loop protrudes deep into the ligand-binding cavity of EphA2, with several hydrophobic residues at its tip forming an anchor-like structure buried within the hydrophobic Eph pocket, in a way similar to the ephrin receptor-binding loop in the Eph/ephrin structures. Consequently, the antibody blocks ephrin binding to EphA2. Furthermore, the antibody induces apoptosis and reduces cell proliferation in lymphoma and leukemia cells lines. Since Eph receptors are important mediators of tumorigenesis, such scFv antibodies could have important applications both in research and therapy.

Structure-based design of peptides against the Sam domain of EphA2 receptor and its heterotypic interactions

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Receptor tyrosine kinase EphA2 is up-regulated in several types of cancers and the processes of ligand-induced endocytosis and subsequent degradation have been explored as potential routes to diminish tumor malignancy. The cytosolic Sam (Sterile alpha motif)

domain of EphA2 (EphA2-Sam) is the site where modulators of these events -such as Ship2(SH2 Domain-containing Inositol Phosphatase 2) and Odin- are engaged through heterotypic Sam-Sam associations1,2. Ship2 works as an inhibitor of endocytosis and its interaction with EphA2 should mainly produce pro-oncogenic outcomes in cancer cells. Odin is a member of the ANKS family of proteins that protects EphA8 and EphA2 from undergoing degradation after ligand stimulation.
Currently, we are working on the design and validation of peptide inhibitors of EphA2-Sam mediated interactions by using several strategies and a multidisciplinary approach3. Our final goal is to explore the potentialities of these molecules to modulate receptor endocytosis and degradation, and eventually work as therapeutic compounds.

References
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EphA2 receptor and ephrin-A1 ligand targeting in glioblastoma: function and therapeutic effects.

C. Festuccia
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The Eph receptor tyrosine kinases and ephrin ligands have been studied extensively for their roles in developmental processes particularly within the central nervous system. In recent years, Eph Receptor and ephrins have been found to be integral players in cancer formation and progression. Some of these proteins, particularly EphA2 and ephrinA1, are of increasing interest in recent years due to their documented or suspected involvement in mediating processes leading to the formation and progression of malignancy. The function of EphA2/ephrinA1 system in tumorigenesis and tumor progression is complex and seems to be dependent on cell type and microenvironment. One of the initial questions surrounding EphA2 as a newly discovered RTK overexpressed in cancer was whether it played a functional role in contributing to the malignant phenotype. This question was directly answered first by Zelinski et al., who showed that expression of EphA2 was sufficient to transform mammary epithelial cells. An intriguing finding in this and subsequent studies is that EphA2, despite its abundant overexpression, is present in some tumor cells in a non–tyrosine-phosphorylated state and is localized to membrane ruffles at the leading edge of invasive cancer cells In nonneoplastic epithelia, however,

EphA2 was expressed at much lower levels, tyrosine phosphorylated, and localized to points of cell-cell contact. It is evident from several studies that the mere presence of EphA2 elicits oncogenic effects. The ligand-independent effects of EphA2 are, thus, important in influencing processes that are critical for malignant progression being, for example, physically associated with FAK and adhesion molecules and participating to movement (endothelial cells) and invasion. EphrinA1 is a tumor necrosis factor-α early-inducible gene product located within chromosomal region 1q21-q22 with a predicted molecular mass of 22 kDa. Interestingly, ephrinA1 was originally identified as a soluble protein but with a sequence of hydrophobic amino acids at the COOH-terminal end reminiscent of those found in proteins that undergo GPI linkage to the plasma membrane. Subsequent studies showed the GPI-anchored, plasma membrane localization of the protein. Therefore, it is possible to modulate the activity of this receptor-ligand system in several manner: (i) small molecules acting to inhibit tyrosine kinase activity (EphA2 target); (ii) soluble agonists (including soluble ligand) or antibodies able to activate tyrosine kinase of EphA2 and induce receptor internalization and degradation and (iii) small molecules or antibodies able to antagonize this receptor system and able to target both EphA2 and ephrinA1 signals. This presentation will specifically focus on the therapeutic effects of different families of compounds targeting EphA2 and ephrinA1 play in preclinical models of glioblastoma..

Targeting Eph receptors with peptides and peptides conjugates

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The 14 members of the Eph receptor tyrosine kinase family and their 8 ephrin ligands have widespread roles in tissue development and homeostasis. Aberrant expression and activity of various Eph receptors and ephrins have been implicated in a variety of disease processes ranging from inhibition of neural repair and neurodegeneration to pathological forms of angiogenesis, cancer malignancy, inflammation and infections. A long-standing interest of our laboratory has been to develop short peptides that bind Eph receptors and inhibit ephrin ligand binding. In phage display screens, we have identified both linear and cyclic dodecameric peptides that bind specifically to individual Eph receptors. This highlights the uniqueness of the ephrin-binding pocket of each Eph receptor, despite the promiscuity of Eph-ephrin interactions. Peptides that can specifically target individual Eph receptors have been used by our group and others as research tools to elucidate receptor function in various biological processes and as potential starting points towards therapeutic leads and for medical imaging applications.

Peptides targeting EphA4 can counteract neurodegenerative processes and promote neural repair in cell culture and in vivo preclinical models of ALS, Alzheimer's disease and nerve injury. Our recent efforts have focused on the structure-guided improvement of an

EphA4 peptide antagonist identified by phage display, APY, which is cyclized through a disulfide bond and is thus particularly suitable for pharmacological development. We have obtained APY derivatives with nanomolar EphA4 binding affinity and dramatically increased resistance to plasma proteases, while ongoing work focuses on improving peptide lifetime in the blood circulation and on further increasing EphA4 inhibitory potency. The linear YSA peptide, which was identified by phage display and targets the EphA2 receptor, functions as an agonists that causes EphA2 activation and internalization through macropinocytosis. YSA and several of its derivatives can be used for delivery of conjugated drugs, siRNAs and nanoparticles to tumors and other diseased tissues with high EphA2 expression. However, YSA derivatives have not yet achieved nanomolar receptor binding affinity. We have also developed TNYL-RAW, a 15-mer derivative of the TNYL peptide identified by phage display, as a nanomolar EphB4 receptor antagonist. TNYL-RAW can inhibit EphB4-dependent angiogenic responses and differentiation of bone forming cells. Furthermore, TNYL-RAW has been used to enhance in vivo delivery of chemo-therapeutics, imaging agents and theranostic nanoparticles to tumors expressing EphB4.

POSTER SESSION

Understanding the Tumour Suppressive Function of Ephrin A5 Signalling in Adult Brain Cancer

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Glioblastoma (GBM) is the most common and aggressive malignant primary brain cancer and is associated with very poor patient outcomes. Standard treatment involves surgical resection, post-operative radiation and temozolomide (TMZ) chemotherapy. This necessitates further research into new therapeutic approaches and, in particular, therapies which target chemo-resistant tumour propagating cells, often referred to as cancer stem cells.

It was recently reported that the EphA3 receptor is frequently elevated in GBM, particularly in the mesenchymal subtype and is most highly expressed in tumour-initiating cells. Our data in GBM tissue shows that tumour cells expressing the high-affinity EphA3 ligand, ephrin A5; are distinct from EphA3 expressing cells. The ephrin A5 positive cells express the known glial differentiation marker GFAP, are less proliferative and less stem cell-like. EphA3 is expressed in the vimentin positive, highly proliferative, mesenchymal cells in GBM. Though we detected both EphA3 and ephrin A5 at elevated levels in GBM tissue; ephrin A5 and GFAP expression was lost when primary GBM tissue were cultured under conditions known to support mesenchymal cells and enrich for the more de-differentiated stem cell-like cells. Conversely, EphA3 expression was lost when GBM cells were forced to differentiate in-vitro.

A significant reduction in tumoursphere proliferation three days post EphA3 activation with either ephrin A5-Fc or an EphA3 activating mAb (IIIA4) was observed. Activation was accompanied by rapid internalisation of receptor complexes and a loss of sphere formation and increased cell attachment indicative of cell differentiation. Our data thus indicates that ephrin A5 could be employed as a soluble differentiation agent in GBM. In order to understand the mechanism of action of ephrin A5 in GBM we are employing a SILAC based quantitative phosphoproteomic approach. This will provide a catalogue of phosphoproteins regulated by ephrin A5, and could result in the identification of novel druggable targets.

Thus, this work explores the potential of soluble ephrin A5-Fc protein to activate EphA3; induce cell differentiation and reduce GBM aggressiveness, and identify novel targets leading to an extension GBM patient survival.

EphrinB trans-endocytosis during contact repulsion is mediated by Tiam/Rac1 signalling

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In the developing nervous system, Eph-ephrin interactions commonly act as cell-contact mediated negative guidance cues, regulating axon guidance and boundary formation. The signalling cascade that induces this response is tightly linked to trans-endocytosis of Eph-ephrin complexes, where the receptor-ligand clusters and part of the opposing cell's membrane are internalised into the activated cell. This process has two proposed functions: Firstly, for efficient cell detachment to occur, the bond between the two cells formed by the Eph-ephrin cluster must be relieved. Secondly, it is possible that the internalised vesicles remain active, enhancing the signal within the cell.

To date, our understanding of Eph-ephrin endocytosis has mostly come from studies looking at forward signalling, and moreover, utilised a soluble ligand fused to Fc to activate the Eph. Here, we focus on EphB2-ephrinB reverse signalling and compare soluble activation to that induced by co-culturing cells expressing EphB2 with those expressing ephrinBs, a system that better reflects the physiological in vivo interaction. By systematically knocking down members of the Rho family GTPases (RhoA-like, Rac1-like and Cdc42-like) in neuroblastoma cells, we show that all members of the endogenously expressed Rac family (Rac1, Rac3, RhoG) are required for reverse trans-endocytosis of membrane-tethered , but not soluble, ephrinB. The requirement of Rac in EphB2 reverse trans-endocytosis was further confirmed in a cell-cell assay using cultured primary neurons endogenously expressing ephrinB ligands. Live-imaging in cells expressing LifeAct showed a Rac-dependent requirement for actin at the site of internalisation, which was lost as the vesicle trafficked internally. We then performed a functional siRNA screen targeting Rho family activators (Guanine Exchange Factors, GEFs) and deactivators (GTPase Activating Proteins, GAPs) utilising our cell-cell assay. We found the Rac-specific GEF Tiam2 was required for EphB2 trans-endocytosis. In confirming this result, we saw that Tiam2 was also required for forward direction ephrinB trans-endocytosis, but not for uptake of soluble EphB2-Fc. We therefore conclude that the mechanism of uptake of EphB2 clusters by ephrinB+ cells differs for cell-cell internalisation as compared to soluble fusion protein uptake, and this is a distinct feature of ephrinB reverse signalling. Moreover, this process requires a spatio-temporal specific Tiam2-activation of Rac family members that subsequently regulate actin activity at the site of contact.

The brain penetrating EphA2 antagonist UniPR1331 shows potent antiangiogenic and anti-invasive effects in glioblastoma preclinical models

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Background. Patients diagnosed with glioblastoma (GBM) relapse after standard treatments with radiotherapy, temozolomide or their combined use. The limited clinical efficacy observed with this approach, combined with the highly vascularized nature of GBM, prompted us to evaluate the antitumoral activity of agents targeting neoangiogenesis in preclinical murine models of GBM. We focused our attention on antiangiogenetic compounds targeting the EphA2/Ephrin-A1 system which has been found upregulated both in GBM cells and in the microvascular endothelial cells recruited by GBM cell to promote vasculogenis.

Materials and Methods: The effect of UNIPR1331, a novel and orally bio-available small molecule targeting the EphA2-ephrinA1 interface and thus able to abolish tyrosyl phosphorylation of EphA2, was investigated in glioblastoma cell lines(U87, U251 and T98G), endothelial cells (HUVEC or brain derived) as well as in chick embryo chorioallantoic membranes. The expression of EphA2 (total and phosphorylated forms), ERK, Akt/pAkt, and VEGF was measured by Western blotting and ELISA assay. The effect of UNIPR1331 on migration, invasion, and vasculogenic mimicry formation (VMF) of U87MG cells was also evaluated. UNIPR1331 was also tested in subcutaneous xenografts of U87, U251 and T98G and in an orthotopical brain-tumor model with luciferase tagged U87MG cells. UNIPR1331 was administered by oral gavage at 30 mg/Kg/3 days/week). Bioluminescence images were taken to visualize in vivo tumors and immunohistochemical staining of VEGF, CD31, EphA2, and HIF1a was performed. The activity of UNIPR1331 was compared to that of antiangiogenetic agents selectively targeting VEGFR (bevacizumab) or blocking both VEFGR and PDGFR (sunitinb).

Results. In vitro UNIPR1331 showed low antiproliferative effects in GBM cells whereas their migratory and invasive capacities were significantly reduced at 5.0 µM. UNIPR1331 dose-dependently reduces the VMF ability of U87MG cells and the secretion of MM2 in the extracellular environment. In addition, reduction of the tube formation of HUVEC and

brain-derived endothelial cells was observed. Chick Embryo Chorioallantoic Membrane Models confirmed the marked anti-angiogentic activity of UNIPR1331.
In vivo UNIPR1331 resulted slightly more effective than bevacizumab (4mg/Kg/1 day/week iv) or sunitinib (40 mg/Kg/5 days/week os) in inhibiting the growth of U87MG xenograft and in luciferase-tagged U87MG intra-brain orthotopic models. We observed that the administration of UNIPR1331 led to significant reduction of tumor growth increasing the time to progression (TTP), disease-free survival (DSF) and overall survival of treated nude mice bearing intra-brain tumors.
Conclusions: Taken together our data suggest that blockage of the EphA2-ephrinA1 interaction by UNIPR1331 may represent a novel therapeutic strategy to tackle GBM tumors.

Identification of a new, functional cross-talk between EphB1 receptor and mu opioid receptor

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EphB1 receptors, and their membrane-bound ligands, ephrinBs, are expressed in the nervous system, where they modulate different processes such as neurogenesis, neuronal migration, axon guidance, synaptogenesis; furthermore, EphB1 receptors and ephrinBs significantly contributes to contact-dependent neuron-glia communication.
Mu-opioid receptors (MOR) are activated by exogenous and endogenous opioids; morphine and related MOR agonists are the most effective and widely used pain killers, but their clinical use is limited by severe side effects, including tolerance and addiction.
Interestingly, EphB/ephrinB system has been recently implicated in the onset and maintenance of different types of pain, (neuropathic, inflammatory, cancer), and in the physical dependence on opiates: activation of EphB1 receptor in nociceptive neurons induces hyperalgesia and allodynia (classic hallmarks of neuropathic pain), whereas its blockade ameliorates hyperalgesic/allodynic responses in animal models of neuropathic pain, and rescues the opioid-mediated analgesia that is lost in a mouse model of cancer-related chronic pain. However, any cross-talk between EphB1 receptor and MOR, and its potential influence on reduced opioid analgesia or tolerance to opiates, has been so far poorly investigated. Therefore, the aim of this research has been to investigate any functional cross-talk between intracellular signaling pathways triggered by MOR and EphB1 receptors in different cell models co-expressing the two receptors.
We found that EphB1 receptor agonist (ephrinB1-Fc) or morphine determined a time-dependent increase in ERK1/2 phosphorylation only when the ligands were administered as single agents, whereas their co-administration occluded ERK1/2 activation. Such cross-

talk, as well as EphB1 and MOR expression, was modified in neuronal cells subjected to in vitro differentiation or to exposure to the pro-inflammatory agent TNFα; thus, suggesting a differential role played by the functional interaction between EphB1 and MOR depending on the physiological state of neuronal cells.
Experiments aiming to unravel the precise signaling pathways responsible for the functional cross-talk between EphB1 receptor and MOR, and to ascertain the ability of novel EphB1 antagonists to counteract such an occlusive interaction, are ongoing and will be presented at the conference. Data obtained within this research shows, for the first time, a functional, occlusive cross-talk between EphB1 receptors and MOR; this interaction is likely to play an important role in the onset and maintenance of different chronic pain states, as well as in the reduction of opioid mediated analgesia or in the development of dependence to opioids.

Role of the Eph/ephrin system in lung metastasis

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Overexpression of ephrin-A1 has been shown the correlation with malignancy and poor prognosis in various tumors such as hepatocellular carcinoma. However, the detailed molecular functions of ephrin-A1 in malignancy and poor prognosis remain unclear. To solve the molecular functions in poor prognosis, we investigated the roles of ephrin-A1 in metastasis that is the most potent factor in worse prognosis. We herein present dual roles of ephrin-A1 in lung metastasis. In HUVEC, membrane anchored-ephrin-A1 maintains cell integrity with cell adhesion molecules such as Integrin and VE-cadherin in an EphA2 tyrosine kinase-independent manner. Moreover, we found that up-regulation of ephrin-A1 expression induced by inflammation was mediated by the S100A8-TLR4 (toll-like receptor 4) axis in primary tumors, and soluble form of ephrin-A1 produced by ADAM12 (A Disintegrin and Metalloproteinase 12) in primary tumors destabilized cell integrity in vascular endothelial cells in the lungs of tumor-bearing mice in an EphA2 tyrosine kinase-dependent manner. In turn, soluble form of ephrin-A1 causes lung hyper-permeability, and it renders circulating tumor cells easily invade metastatic organs and forms metastatic foci. A treatment of a neutralizing antibody against ephrin-A1 successfully showed a significant decrease of lung metastasis.
Taken together, ADAM12 and ephrin-A1 are potential therapeutic targets for lung metastasis. We are currently investigating the recognition fashion of ephrin-A1 by ADAM12 and the effect of an anti-ADAM12 reagent on lung metastasis.

Drosophila epidermal development to investigate Eph/ephrin signaling in epithelia

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Drosophila development has proven an invaluable model to identify the molecular components of intercellular signaling pathways and to study their logic and function. In Drosophila there is one Ephrin homolog (D-Ephrin) and one Eph Receptor (D-Eph or DEK). Their functions have been studied mostly in the context of the development of the nervous system, and probably not with the depth that the importance of this signaling module warrants. This is partly due to the localization of both loci to the fourth chromosome, which is more challenging to work with. Here we present expression data for Dephrin and DEK in imaginal discs (the larval precursors of the adult epidermis and appendages), as well as RNAi-based functional analysis in these tissues. We also present data placing DEK downstream of oncogenic Ras (RasV12) during cell extrusion in early epithelial transformation. Our results indicate that Drosophila larval development will be a good system where to study the complexitites of Eph/ephrin signaling in epithelia.

Eph/Ephrin- family and their role in myocardial infarction

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The Eph-receptor tyrosine kinase receptors and their ephrin ligands are known to be involved in a variety of processes like tumor biology and inflammation. Up to now, little is known about the impact of Eph/ephrin-interaction in the pathophysiology of myocardial infarction. Therefore, the aim of the present study was to investigate the regulation of the Eph/ephrin system in a mouse model of chronic myocardial infarction.
Mice underwent a coronary artery ligation to initiate a myocardial infarction. The occlusion of the ramus interventricularis anterior (RIVA) was done using standard surgical procedures. Echocardiography of the mice revealed a significant decrease of ejection fraction as a sign of lowered heart function due to the infarction. Samples of infarcted myocardium and non-infarcted tissue of the posterior wall were acquired 1, 4, 7 and 21 days after RIVA-ligation. Afterwards screening of mRNA-expression of several Eph-receptors and ephrin-ligands within the myocardial infarction zone in comparison to non-infarcted tissue of the myocardium was done. The most impressive effect could be detected in case of ephrinA1 and ephrinB3. Myocardial infarction leads to decreased expressions within the myocardial zone of ephrinA1 after 7 days and ephrinB3 over the entire observation period. Also, the mRNA-expression of EphA4 and EphB1 was significantly reduced after myocardial infarction (4, 7 and 21 days).
In summary, the present data suppose an important influence of Eph/ephrin interaction during myocardial infarction and might open new perspectives in therapeutic strategies.

EphrinB2 mediated EphA4 forward signaling mediates monocyte adhesion to endothelial cells

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In the last years diverse functions of the Eph/ephrin-system in inflammatory processes were described, such as the receptor EphA4, which plays a crucial role in the process of monocyte adhesion. Since ephrinB2 is known as an interaction partner of EphA4, the aim of the present study was to investigate a possible interplay of EphA4-receptor with ephrinB2 during monocyte adhesion to the endothelium.

As verified by bulk adhesion assays under static and flow conditions and atomic-force microscopy based single-cell force spectroscopy, temporary stimulation of endothelial cells from different sources with the soluble ligand ephrinB2 increased monocyte adhesion to endothelial cells. The receptor EphA4 was identified as the responsible interacting partner of ephrinB2 on endothelial cells as shown by siRNA-mediated silencing. Interestingly, the proadhesive effect of EphA4 forward signaling was independent of an active transcription. But, the monocyte adhesion was mediated via the Rho signaling pathway with subsequent modulation of the actin cytoskeleton. Furthermore, ephrinB2 was induced by TNF-α treatment and silencing of ephrinB2 led to a lowering of the TNF-α mediated effect on monocyte adhesion to endothelial cells. Immunohistochemical staining of human atherosclerotic plaque revealed expression of ephrinB2 in macrophages/foam cells and oxLDL stimulated macrophages show increased ephrinB2 expression.

The results of the present study demonstrate a crucial role of ephrinB2 induced EphA4 forward signaling in the context of monocyte adhesion to endothelial cells, which might be important in inflammatory processes like atherosclerotic plaque development. This transcription-independent effect is mediated by Rho signaling induced changes in actin-filament polymerization.

Regulation of the Eph/ephrin-system during macrophage polarization

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Beside classical macrophages (M1) also alternative activated subsets (M2) were identified representing a more anti-inflammatory phenotype. Both phenotypes were identified

within atherosclerotic plaques. However, until now, little is known about the regulation and function of the Eph/ephrin-system in the process of macrophage polarization.
Human monocyte-derived macrophages (M0) were polarized into M1 and M2a phenotype using IFNɣ/LPS or IL-4 (IL 13) and screened for the expression of all Eph-receptors and ephrin-ligands. This analysis showed that the different macrophage subtypes express a defined pattern of receptors and ligands. The most pronounced effect was seen in the case of ephrinA1. Its mRNA expression was 15fold higher in M2a macrophages compared to M1 or M0. This could be verified on protein level and was also associated with an increased expression at the cell surface. Further analysis revealed that the induction is mediated by STAT6 as proven by ChIP and siRNA-silencing. Furthermore, using siRNA-mediated silencing of ephrinA1 showed that expression of typical M2 marker genes, like MRC1,was reduced in ephrinA1-silenced M2a macrophages. This suggests that ephrinA1 itself can modulate macrophage phenotype, which is further corroborate by reduced expression of STAT6. To prove the hypothesis that ephrinA1 is expressed within atherosclerotic plaques, aortae of 1yr old ApoE-/- mice were explanted and divided in parts with or without macroscopic plaques. Analysis of mRNA expression revealed a higher expression of ephrinA1 in regions with plaque, which correlates with the CD68 expression. These results confirm our previous data showing high expression of ephrinA1 in human plaque macrophages.
In conclusion, the present study showed that different subtypes of macrophages express a defined pattern of Eph-receptor and ephrin-ligands. Furthermore, M2a macrophages express high amounts of ephrinA1, which is regulated via STAT6 and influences the phenotype of M2a macrophages. Analysis of murine and human plaques showed that ephrinA1 might be a potential target to analyze for its atherogenic potential.

Targeting the Eph-ephrin system as anti-inflammatory strategy in a murine model of Crohn's disease

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Background: Inflammatory Bowel Disease (IBD), including Crohn's disease (CD) and Ulcerative Colitis, is a chronic intestinal disorder resulting from an inappropriate inflammatory response to gut luminal antigens: its incidence is increasing worldwide and, up to now, remains incurable. Besides its critical role in embryogenesis and cancer development and progression, the Eph-ephrin system appears increasingly involved in the regulation of inflammatory and immune responses. Interestingly, EphBs-ephrinBs mRNA are overexpressed in the gut epithelium in CD and stimulation of ephrinB reverse

signalling up-regulates epithelial cells wound healing genes in vitro. Our study aims at investigating the role of EphBs-ephrinBs in the local and systemic inflammatory responses induced in a murine model of CD by administering chimeric proteins ephrinB1-Fc (unidirectional activation of forward signalling), EphB1-Fc (activation of reverse signalling), or monomeric EphB4 (interfering with both signals). Methods: Colitis was induced in C57BL/6 mice by enema administration of 5mg/mouse 2,4,6-Trinitrobenzene sulfonic acid (TNBS) in 50% ethanol. Subcutaneous pharmacological treatments started 8 hours after induction of colitis and were applied daily till the sacrifice, 3 days later; control mice (CTR) received only vehicle. Disease Activity Index (DAI), colonic macroscopic score (MS), colon length and thickness and colon and lung myeloperoxidase (MPO) activity, index of leukocyte recruitment, were determined. Splenic CD3+CD4+ and CD3+CD8+ T cells were counted by flow cytometry. All experiments were performed according to the guidelines for the Care and Use of Animals (DL26/2014). Results: Compared to CTR, treatment with ephrinB1-Fc 17µg/kg lowered DAI and lung MPO levels ($P<0.05$), while EphB1-Fc 30µg/kg strongly reduced DAI ($P<0.001$) and MS ($P<0.05$), minimized colon shortening ($P<0.001$) and thickening and curtailed local ($P<0.05$) and systemic ($P<0.01$) neutrophil infiltration by about 80%. EphB4 dose-dependently improved colitis parameters, showing at the highest tested dose of 20µg/kg the same efficacy as equimolar EphB1-Fc. No treatment significantly modified T cells subpopulations compared with CTR. Conclusions: These preliminary findings are the first evidence that endogenous EphBs-ephrinBs forward signalling promotes the local inflammatory response in TNBS-induced colitis, seemingly in a T cell-independent way: pharmacological agents selectively disrupting this pathway might represent a novel strategy for the treatment of intestinal inflammatory conditions like CD.

Structural Studies of EphA2-EphrinA5

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Erythropoietin-producing hepatoma (Eph) receptors constitute the largest subfamily of receptor tyrosine kinases and along with their ephrin ligands play a crucial role in mediating cell-cell communication regulating cell attachment, shape and mobility. EphA2 receptor regulates cell adhesion during embryonic development and also acts as an oncogene by promoting tumour vascularization and metastasis.

Cell-cell signalling from the interaction between EphA2 and EphrinA5 results in the formation of array-like clusters of the receptor thereby initiating downstream signalling cascade. The X-ray crystal structure of the EphA2 ectodomain in complex with ephrinA5 receptor binding domain from our lab highlighted the interfaces of these clusters. Now, we are aiming to further investigate the molecular mechanisms underlying these

structural changes upon ligand binding specially in the intracellular domains as this could provide some useful insights for targeting these receptors for drug discovery.
We plan to determine the structure of full length EphA2 receptor in its unbound form and in complex with its ligand EphrinA5 by using Single particle Cryo-EM and Cryo electron tomography.

Small molecules inhibiting Eph-ephrin interaction improve glucose tolerance in insulin-resistant mice

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Eph receptors and ephrins are expressed in human pancreas, where ephrin-As reverse signaling increases insulin secretion, whilst EphA receptors forward signaling inhibits insulin secretion. EphA5 and its cognate ligand ephrin-A5 seem to be particularly important for this process and indeed the pharmacological inhibition of EphA5 receptor by receptor tyrosine kinase (RTK) inhibitors showed to enhance glucose stimulated insulin secretion (GSIS) from mouse and human pancreatic islets as well as to increase glucose tolerance in mice, providing the proof-of-concept in which Eph inhibitor could represent a new class of drugs for the treatment of Type 2 Diabetes. Unfortunately, the inhibition of Eph forward signal by RTK inhibitors is based on the use of small molecules targeting the ATP binding site in the intracellular kinase domain, with the result to suffer from poor selectivity due to the inhibition of several kinases, with heavy consequences in terms of side effects. An alternative way to inhibit Eph receptor forward signalling and overcome the risk associated to the use of RTK inhibitors is through the inhibition of the ephrin binding site in the extracellular domain of the Eph receptors. In this view, our laboratory has been working since 2009 to develop small molecules able to interfere with Eph-ephrin binding. In particular starting from lithocholic acid (LCA), a weak binder of Eph receptors, we obtained different LCA coniugates active in the low micromolar range when tested in ELISA binding assay. Here we disclosured UniPR500, a new reversible and competitive Eph antagonist able to inhibit EphA5-ephrinA5 binding with an IC50 value of 3.7µM and a Ki value of 1.4µM. Notably UniPR500 binding results were confirmed by SPR technique. Moreover according to the binding study UniPR500 was able to inhibit Eph phosphorylation until to 3µM without affecting cell viability. Finally UniPR500 showed to be bioavailable after oral administration in mice opening the road to the evaluation of this compound as glucose tolerance enhancer. Indeed UniPR500 (30mg/kg x os) was able to improve glucose tolerance after i.p glucose injection (2g/kg) in both wild type mice and in

a non genetic mouse model of insulin resistance. Conversely, as expected by Eph antagonist working on GSIS, UniPR500 wasn't effective to control glycaemia in a non genetic mouse model of type I diabetes. Finally UniPR500 was resulted inactive when tested on other known hypoglycemic targets such as GLP1, PPAR-γ, PTP1B, DPPIV and KATP channel, confirming its specific action on Eph receptors. In conclusion this work gives another proof of the feasibility of Eph antagonism as a new target of Type II diabetes.

Neuron-glia communication in depression- implication of the ephrinA/EphA system

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Proper neuron-glia communication is one of the key factors to determine whether a person may develop major depressive disorder (MDD). Post-mortem studies in MDD patients revealed a reduced number of neurons and glia cells, accompanied by a reduced volume in the prefrontal cortex (PFC) and an impairment of long-term potentiation (LTP), most likely caused by a deregulated glutamate occurrence in the synaptic cleft. Moreover, animal models of depression show neuronal atrophy, a reduced synaptic density and a reduced dendritic spine complexity, as well as decreased levels of synaptic signaling proteins (e.g., synaptophysin, synapsin, GLAST, GLT-1), which are all strong indicators of a miscommunication at the level of synapses in MDD. Interactions between Eph-receptors (on pre- and post-synapses) and ephrins (on astrocytes, surrounding synapses) have been shown to be involved in synapse morphogenesis and dendritic spine stabilization. Therefore, we might hypothesize that any deregulation in one of these factors may result in the development of depressive symptoms.

We are using rats bred for high-anxiety related behavior (HAB), an animal model well characterized for its depressive-like phenotype. Using immunohistochemistry, we proved, for the first time, that an increased H3K4me3 (Histone 3-lysine 4- trimethylation) occurs in astrocytes of the PFC of this model which correlated with a misregulation of ephrinA1. In primary cortical astrocytes, with chromatin-immunoprecipitation, we further showed that differences in ephrinA1 between normal (NAB) and HAB astrocytes depend on H3K4me3. This posttranslational histone modifications (PTM) is known to be increased in the PFC of MDD patients, further validating our animal model at the molecular level. In this study we showed that, under basal conditions, ephrinA1 is upregulated at both the transcriptional and translational levels in HAB rats compared to normal controls. Administration of fluoxetine (FLX, 10 µM for 120h) to primary astrocytes decreases

ephrinA1, suggesting its reversal after a short-term drug treatment. Currently, by using a combination of pharmacological treatments with RNAi, we are examining the effects of blocking ephrinA1 in co-cultures of HAB astrocytes/NAB neurons on the formation of synaptic spines. Our hypothesis is that the endogenous overexpression of ephrinA1 in HAB astrocytes might cause the retraction of spines by interacting with one of the EphA receptors (most likely EphA4), thereby reducing LTP and ultimately resulting in depressive behavior.

Tyrosine kinase Inhibitor GLPG1790, blocks EphA2/ephrin-A1 signaling and is synergistic with radiotherapy and temozolomide i*n vivo* in preclinical models of glioblastoma.

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Background. Ephrin receptors, including EphA2, have been reported to be overexpressed in glioblastoma (GBM), the most malignant and aggressive primary brain tumor, and to be associated with poor survival and cancer stemness. In addition, it has been demonstrated that aggressive standard therapy (radical surgery plus concurrent chemo-radiation treatment) provides palliative treatment only. The infiltrative expansion and aggressiveness of GBM is highly correlated with the level of EphA2 expression, which also promotes resistance to chemotherapy and/or radiotherapy in several tumors. GLPG1790 is a small molecule, nanomolar inhibitor of various EPH receptor kinases and able to inhibit phosphorylation of both Tyr 594 and Ser 897. It has demonstrated preclinical efficacy in several cancer models.

Methods. The aim of the present study was to explore the effects of GLPG1790 p.o. on the regrowth of subcutaneous and intra-brain xenograft tumors in Cd1 nu/nu mice after treatment with radiation (RT), temozolomide (TMZ) or a combination of both. Three different human GBM derived cell lines were used for subcutaneous injection (U87MG, U251 and T98G), whereas luciferase transfected U87MG and patient derived GBM stem cells were injected orthotopically in the brain of mice.

Results. We observed that blockade of EphA2 with GLPG1790 *in vitro* reduced cell proliferation with IC50's ranging between 110 and 400 nM. These concentrations reduced the expression of stem cell associated markers and in combination with TMZ reduced neurosphere forming ability in GBM cancer stem cells. In subcutaneous xenografts, we observed that GLPG1790 increased TTP with hazard ratios of 14.8 and 9.0 in U87 and U251, respectively. GLPG1790 was more effective than RT with HR= 4.8 (U87) and 1.7

(U251). Moreover, increased efficacy was observed in combined treatments with RT (HR=22.4 and 21.6 in U87 and U251 xenografts, respectively). In orthotopic models, treatment with GLPG1790 increased disease free survival and overall survival in both U87MG and PCT8 models when combined to RT, TMZ or RT plus TMZ (standard of cure) of treated mice.

Conclusions. Targeting the EphA2/EphrinA1 signaling pathway with GLPG1790, the stem cell expression signature *in vitro* was reduced, and abolished neurosphere forming ability when dosed with TMZ. Moreover GLPG1790 delayed significantly the regrowth of GBM cell-line derived tumors- including cancer stem cells after irradiation or temozolomide treatment, which resulted in increased mouse survival.

Exploiting surface plasmon resonance in drug discovery for the Eph/ephrin system

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Background: The interactions between Ephrin receptors (Ephs) and their ephrin ligands are implicated in important human diseases including diabetes, neurological disorders, atherosclerosis and cancer, thus representing promising therapeutical targets. The search for new drugs is often pursued by means of "random" screening of large library of compounds. In this context, the choice of the screening assays is critical. Indeed, the systematic evaluation of a huge number of small molecules by means of classical cell culture-based or *in vivo* assays is hardly feasible, calling for simple biochemical assays predictive of the *in vivo* behaviour of the prodrugs screened [1,2].

Materials and Methods: Surface plasmon resonance (SPR) is a handy-user and high-throughput optical technique to evaluate biomolecular interactions largely exploited in biomedical research [1-3]. It typically consists of a laser beam that passes through a prism fitted with a glass slide coated with gold, from which the beam is reflected and detected at the specular angle. When the light hits the glass, an electric field intensity is generated and absorbed by the free electron cloud in the gold layer, causing a reduction of the intensity of the reflected light. The angle corresponding to the sharp intensity minimum (called resonance angle) depends on the refractive index of the material close to the gold surface. In SPR, a molecule (ligand) is chemically immobilized to the gold film and exposed to a putative binder (analyte). The ligand/analyte interaction causes a change of the refractive index at the gold surface resulting in the shift of the resonance angle monitored as a real-time graph of the response units against time (sensorgram). At MIAU, it is operative a BIACORE X-100 SPR apparatus with which we have analyzed libraries of

cholenic acid- or lithocholic acid-conjugates in search of binders of Ephs endowed with antagonist activity [4,5].
Results: We have immobilized EphA2 by means of amine coupling to a CM4 sensorchip composed of a gold surface coated with carboxymethylated dextran. Immobilized-EphA2 retains its capacity to bind to ephrin A1, indicating that it has maintained a correct tridimensional conformation and binding availability after the immobilization procedure. This "biosensor" has been then used to screen the above mentioned libraries of compounds for their capacity to bind EphA2. For each compound has been calculate the association and dissociation rates (that give a measure of how fast the compound attach and detach spontaneously from EphA2) and the dissociation constant (Kd, that is inversely proportional to the affinity of interaction). Relevantly, the Kd values of the various compounds turned out to be proportional to their capacity to displace ephrin-A1 from EphA2.
Conclusions: In our hand, SPR turned out to be a fast, reliable and predictive "first-line" screening assay for the search of inhibitors of the Eph/ephrin system.

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Exploiting binding minima identified by free-energy simulations to design novel EphA2-ephrinA1 inhibitors

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Erythropoietin-producing hepatocellular carcinoma (Eph) receptors are tyrosine kinase receptors activated by membrane-bound peptides called ephrins.1 Dysregulation of the Eph-ephrin system, and in particular of the EphA2-ephrinA1 activity, has been found involved in tumor insurgence and progression. Moreover, inhibition of EphA2 signaling has been shown to disrupt cancer growth and angiogenesis in vivo.2 The increasing role of the EphA2-ephrinA1 system in tumorigenesis and angiogenesis makes it a promising target for new anticancer treatments.3 In this context, recent medicinal chemistry efforts focused at identifying small molecules able to disrupt EphA2-ephrinA1 interaction, identified lithocholic acid (LCA) as physiological inhibitor of EphA2,4 which was successfully employed as a scaffold to discover more potent antagonists such as UniPR129.5 So far, little is still known about the molecular recognition between EphA2 and LCA-based inhibitors, as no X-ray structures of the complexes are currently available.

That said, molecular simulations can contribute to characterize their binding process by allowing the reconstruction of the free-energy surface (FES) of binding.6 The FES provides useful information for drug design, such as position and energetics of free-energy minima and transition states, including minima corresponding to binding geometries distinct from those observed in X-ray structures,7 that could be exploited to chemically modify an active compound to improve its potency.
In this work, we exploited a free-energy minimum of EphA2-LCA complex identified by metadynamics simulations to design a new EphA2 antagonist with improved inhibitory potency.

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Structure-Activity Relationships of Lithocholic Acid Derivatives as EphA2 Receptor Antagonists

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The Eph receptor–ephrin system is an emerging target for the development of novel antiangiogenetic agents. We recently identified lithocholic acid (LCA) as a small molecule able to block EphA2-dependent signals in cancer cells,[1] suggesting that its 5β-cholan-24-oic acid scaffold can be used as a template to design a new generation of improved EphA2 antagonists. We next design and synthesized an extended set of LCA derivatives, which were examined for their ability to disrupt EphA2-ephrinA1 binding.[2] Here, we explore the structure activity relationships of LCA analogues, obtained in the first instance by conjugation of the carboxyl group with a properly selected series of L-β-homo-amino acids and, in the second instance, by introducing a small set of substituents featured by different chemical and hydrophilic properties at position 3. The structure-activity relationships analysis revealed that the presence of an aromatic side chain on the amino acid moiety of the conjugated derivatives is fundamental to achieve good potencies. Indeed, the L-β-homo-tryptophan conjugate of LCA (UniPR129) resulted the most potent antagonist of the series disrupting EphA2-ephrinA1 interaction at low μM concentrations

(IC_{50} = 0.9 μM),[3] thus being significantly more potent than LCA (IC_{50} = 57 μM). As a next step of our investigation, the most promising substituents introduced at position 3 of the 5β-cholan-24-oic-based scaffold of LCA were selected and inserted in the structure of the most potent L-β-homo-amino acid conjugate UniPR129, to give a short series of final compounds. By virtue of this last SAR exploration, we identified two further highly potent EphA2 antagonists, represented by the 3-hydroxyimino and 3α-carbamoyloxy derivatives of UniPR129. The 3-hydroxyimino derivative UniPR500 and the 3α-carbamoyloxy derivative UniPR502 displayed an IC_{50} value of 1.12 μM and 1.07 μM, respectively. UniPR500 and UniPR502 not only demonstrate a potency comparable to that of the parent compound UniPR129, but they are also endowed with improved physical-chemical properties, that made them bioavailable in vivo.

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