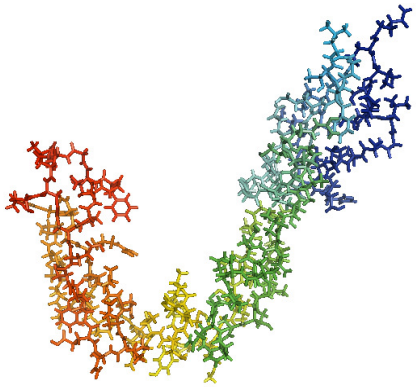
PROGRAM



The 3rd International Conference
'Notch Targeting in Cancer'
Santa Marina Hotel, Mykonos, Greece
26 - 28 June 2013

WE ARE GRATEFUL TO OUR SPONSORS FOR THEIR SUPPORT

The Company of Biologists - *Development*, *Journal of Cell Science*, *The Journal of Experimental Biology*, *Disease Models & Mechanisms* and *Biology Open*

This event has been granted European endorsement by the UEMS and awarded 13 European Continuing Medical Education Credits (ECMEC)

Roche

Trojantec

Development | DMM Disease Models & Mechanisms | Journal of Cell Science | The Journal of Experimental Biology | Bio Biology Open | The Company of Biologists

Wednesday 26th June 2013

4.00-4.30pm **Registration**

4.30-4.35pm **Welcome: Agamemnon Epenetos**

SESSION 1 Chairman: **Lucio Miele**

4.30-4.50 pm **Aleksandra Filipovic, Imperial College London, London UK**

Highlights of the 2nd Meeting, Notch Targeting in Cancer, June, 2012

4.50-5.10 pm **G. Paolo Dotto, Department of Biochemistry, Lausanne University, Lausanne, Switzerland**

Field cancerization and dormant epithelial cancer : Notch/CSL stromal signaling takes the stage.

The vast majority of epithelial cancers is limited to *in situ* lesions that, for internal organs like breast, prostate or lung, can remain undetected for the whole life of an individual. The reason(s) why only a minor fraction of these lesions progresses into malignancy is not understood. In fact, many if not most of genetic changes found in invasive and metastatic tumors can be already present in pre-malignant lesions, raising the question of whether such changes are of primary causative significance or merely permissive for later cancer-spreading events. A related key issue raised by deep sequencing analysis of tumors is which of the many identified mutations has a driver initiating function in the carcinogenic process. An extreme view is that none of these mutations is by itself a driver of cancer development and that it is the "ecological cellular environment" that restrains or unleashes tumor growth. Changes in tumor stroma are most frequently viewed as secondary to changes in the epithelium. However, recent evidence indicates that they may play a primary role. Such a possibility would help explain not only dormancy of most epithelial cancers, but also *field cancerization*, a condition of major clinical significance linked with multifocal and recurrent tumors and broader tissue changes beyond areas of tumor development [5]. In this presentation, I will overview our work on the role of Notch/CSL signaling in this context.

5.10-5.30 pm **Benedetta Ubezio[1], Raquel Blanco[1], Ilse Geudens[2], Martin Jones[1], Thomas Mathivet[2], Fabio Stanchi[2], Katie Bentley[1,3], Holger Gerhardt[1,2]**

[1] Vascular Biology Laboratory, London Research Institute – Cancer Research UK, Lincoln's Inn Fields Laboratories, 44 Lincoln's Inn Fields, London WC2A 3LY, UK

[2] Vascular Patterning Laboratory, Vesalius Research Center, VIB3, Department of Oncology, KU Leuven, Belgium

[3] current address: Beth Israel Deaconess Medical Center, Department of Pathology, Harvard Medical School, Boston, MA

Branch or Expand? New insights into Dll4/Notch dynamics driving vascular patterning

The formation of a hierarchically branched network of blood vessels is critical tissue supply during development and in physiology. What determines whether activated endothelial cells form a new vessel branch, or rather expand the existing vessel has remained unclear. Here we show that the expression of the Notch ligand Dll4 fluctuates in individual endothelial cells within a sprouting vessel in correlation with dynamic cell movement. Studying a novel genetic Dll4 reporter in mouse ES cells, together with the polarity and speed of cell migration, as well as the coordination of individual cell movement and expression levels within the cell population, we find that sprout elongation and branching associates invariably with a highly differential phase pattern of Dll4 between the endothelial cells. Stimulation with pathologically high levels of VEGF, or overexpression of Dll4, leads to Notch dependent synchronization of Dll4 fluctuations within clusters of endothelial cells both in vitro and in vivo, and to corresponding synchronization in collective cell migration. In oxygen-induced retinopathy and in glioblastoma vessels, the loss of vessel diameter control associates with local synchronization of Dll4/Notch signalling. Dynamic imaging in zebrafish tumour vessels identifies cycles of synchronous sprouting and retraction from neighbouring endothelial cells, which leads to vessel expansion instead of branching. Our results demonstrate that the VEGF-Dll4/Notch feedback system normally operates to generate heterogeneity between endothelial cells driving branching, whilst synchronization drives vessel expansion. Based on insights derived from computational simulation, we propose that the sensitivity of this phase transition between differential and synchronized Dll4 dynamics could be a key modulator that is influenced by a variety of environmental factors and signalling pathways, and that therapeutic modulation could be exploited to regulate vessel patterning and therefore vessel network functionality.

5.30-5.50pm **R Charles Coombes, Monica Faronato, Ylenia Lombardo, Aleksandra Filipovic and Simak Ali, Imperial College London, London , UK**

Targeting Notch to Subvert Endocrine Resistance in Breast Cancer

Background: Tamoxifen resistance affects at least 30% of patients receiving this treatment and remains a major hurdle in the clinical management of breast cancer (BC) patients. Tamoxifen resistant BC cells are characterised by constitutive activation of pathways: EGFR, Src, FAK, CD44 etc., providing cells with a mechanism to bypass the requirement for estrogens. These features also confer increased cellular invasiveness. Contribution of Notch signalling has also been demonstrated in developing tamoxifen resistance. Notch receptors are cleaved by the gamma-secretase enzyme composed of nicastrin, presenilin, PEN2 and Aph-1. Gamma secretase inhibitors are being tested as novel therapeutics for cancer treatment, predominantly in combination with anti-hormonal agents and chemotherapy. Here, we show that nicastrin may be a relevant therapeutic target in tamoxifen resistant breast cancer cells and demonstrate the effects of monoclonal anti-nicastrin antibodies.**Materials and Methods:** We used TAMR cells derived by long term culture of MCF7 cells in Tamoxifen that have retained ERα expression. Anti-nicastrin monoclonal antibodies (MAbs), named MAb1 and MAb2, have been raised against Nicastrin extracellular domain. Western blotting, RT-qPCR, transwell invasion, mammosphere formation and 3D matrigel growth assays were used to demonstrate their molecular and functional effects. Gamma secreatse inhibitor RO4929097 and PF-03084014 were used for comparison.**Results:** We demonstrate a novel finding that Nicastrin is overexpressed at the gene and protein levels in TAMR cells (> 4-fold). These cells concomitantly exhibit elevated CD44 (> 2-fold), EGFR (> 4-fold), Vimentin (3-fold), IQGAP1 (2-fold), IQGAP2 (>100-fold), Hey1 (4-fold) and Notch4 (>2-fold), while Notch1 expression is suppressed (> 2-fold). Anti-nicastrin MAbs and RO4929097 were equally potent in inhibiting the Notch target genes Hey1 and Hes1. Inhibitory effect on CD44, IQGAPs and Notch4 was only observed using anti-nicastrin MAbs and not RO4929097, which even further increased Notch4 mRNA and protein. Anti-nicastrin MAbs and PF-03084014 were potent inhibitors of TAMR cell invasion, whereas RO4929097 had no effect. Moreover, using the 3D Matrigel growth conditions, we show that anti-Nicastrin MAb2 is particularly potent in inhibiting outgrowth of acini like structures of tamoxifen resistant cells.**Conclusion:** Nicastrin and Notch4 are over-expressed in endocrine resistant breast cancer cells (TAMr) Anti-Nicastrin monoclonal antibodies inhibit invasion but not proliferation of TAMr cells. Anti-Nicastrin MAbs attenuate Notch4 expression, as well as expression of pro-invasive molecules such as Vimentin, IQGAP1, and CD44. Conversely, RO4929097, which has no effect on Nocth4, also fails to affect the invasive potential of TAMr cells. PF03084014, and its combination with the anti-Nicastrin MAbs, reduces both invasion and mammosphere formation efficacy. Interestingly, over-expressing Notch1 down-regulated Notch4 and Nicastrin expression. This indicates a delicate balance of Nicastrin and Notch1/4 in endocrine resistance. Furthermore, our findings confirm the importance of choosing the appropriate modality of GS/Notch inhibition in BC patients. Finally, we propose that anti-Nicastrin MAbs should be further evaluated as single agents and in combination with GSIs in the setting of endocrine resistance.

8.00 - 10.00 pm

Welcome Reception Cocktail

Thursday 27th June 2013

SESSION 2 Chairman: Aleksandra Filipovic

9.00-9.20am **Mirko HH Schmidt, PhD, MD Professor of Anatomy & Biochemistry, Molecular Signal Transduction Laboratories ,Institute for Microanatomy & Neurobiology,Focus Program Translational Neuroscience, Johannes Gutenberg University, School of Medicine,Bldg. 708, Suite 2.006 Langenbeckstr. 1,D-55131 Mainz, GERMANY**

The novel notch ligand EGFL7 in health and disease

EGFL7 drives the formation of neurons from neural stem cells. In the embryonic and adult brain this process is essential for neurogenesis and homeostasis of the nervous system. The function of adult neurogenesis is not fully understood but potentially it supports life-long learning and brain repair after injuries such as stroke. One of the essential signaling pathways governing this process is the Notch pathway, which controls metazoan development. We identified the novel non-canonical Notch ligand, EGFL7, and described its impact on neural stem cells and explored the molecular mechanisms, which this molecule affects to regulate the self-renewal capacity of neural stem cells and to promote their differentiation into neurons (Schmidt et al., NCB 2009).
Further, we found that EGFL7 stimulates integrin □v□3 to trigger angioegenesis thus providing a mechanistic insight into EGFL7s proangiogenic actions *in vitro* and *in vivo* (Nikolic et al., Blood 2013). This led us to the analysis of the role of EGFL7 in human neural diseases and we found that the expression levels of EGFL7 in human specimens relied largely on the remodeling state of the existing vasculature but not on the origin of the disease, as EGFL7 was upregulated in blood vessels of human brain pathologies and of the penumbra of stroke upon reversible mouse middle cerebral artery occlusion (MCAO). Our work sheds a novel light on the molecular mechanism EGFL7 engages to govern physiological and pathological angiogenesis and the role the novel Notch-ligand EGFL7 plays in health and disease.

9.20-9.40 am **Caolo V[1], Swennen G[1], Chalaris A[4],Wagenaar A[1], Verbruggen S[1], Donners MMPC [2], Rose-John S[4],Molin DGM[1], Vooijs M[3], Post MJ[1]**

[1]Dept. of Physiology, CARIM, [2]Dept. of Molecular Genetics, CARIM, [3]Department of Radiotherapy MAASTRO/GROW, Maastricht University, the Netherlands [4]Institute of Biochemistry, Christian Albrechts University, Kiel, Germany

ADAM10 and ADAM17 regulate Notch activation in ECs, but affect differently angiogenesis

During angiogenesis endothelial tip cells (ECs) start sprouting and express DLL4 downstream of VEGF. However, we have previously shown that VEGF requires activated-Notch to induce DLL4-expression. VEGF also activates ADAMs, which mediate ligand-dependent Notch-activation but

potentially also cleave Notch in absence of ligand. We hypothesize that in tip cells, VEGF, through ADAMs, triggers Notch-activation in a ligand-independent fashion. Soluble-Notch1 (sNOTCH1) was used to block ligand-dependent Notch-signaling in ECs. sNotch1 addition reduced basal and ligand-induced HES1 and DLL4 expression. However, VEGF-stimulation resulted in HES1 and DLL4 upregulation despite sNotch1 presence by activating ADAM10 and ADAM17. DLL4-expression was downregulated following Notch-inhibition in tip cells, where a more pronounced ADAM-activity was detected compared to stalk cells. Intriguingly, ADAM10 or γ-secretase inhibition induced vascular sprouting and density, whereas ADAM17-inhibition produced the opposite phenotype. These observations describe a new mode of ligand-independent and ADAM10/ADAM17-mediated Notch-activation, which might be essential in tip cell selection. Moreover ADAM17-inhibition rescued the hypersprouting phenotype observed following ADAM10/Notch Signaling blockage in stalk cells.

9.40-10.00 am **Rui Benedito, Centro Nacional de Investigaciones, Cardiovasculares,Madrid,Spain**

Regulation of angiogenesis by Notch through VEGF-independent mechanisms

During angiogenesis endothelial cells sense different extracellular molecular cues that change their behaviour. The vascular endothelial growth factor (VEGF) family of secreted ligands and receptors are known to influence endothelial cell sprouting, proliferation and survival in a variety of organs and pathological processes. Besides being influenced by external factors, endothelial cells have also endogenous signalling mechanisms that modulate their response to the surrounding environment. One such mechanism is the Notch signalling pathway that upon cell-to-cell activation leads to a reduction in angiogenesis. Mechanistically it was thought that this decrease in angiogenesis is mainly dependent on the negative regulation of VEGFR2 transcription. By using a combination of different genetic and pharmacological approaches we found that Notch inhibition *in vivo* has no significant impact on VEGFR2 expression and induces deregulated endothelial sprouting and proliferation even in the absence of VEGFR2, which is the most important VEGF-A receptor and normally indispensable for these processes. In contrast, VEGFR3, the main receptor for VEGF-C, was strongly modulated by Notch. VEGFR3 kinase inhibition but not ligand–blocking antibodies suppressed the sprouting of endothelial cells with low Notch signalling. Our results suggest that Notch changes the relative importance of VEGFR2 and VEGFR3 for endothelial sprouting. We propose that successful anti–angiogenic targeting of these receptors and their ligands will strongly depend on the status of endothelial Notch signalling.

10.00-11.00 am ***Coffee break and poster review***

SESSION 3 Chairman: Robert Clarke

11.00-11.20 am **Aleksandra Filipovic, Mahendra Deonarain, Ylenia Lombardo, Monica Faronato and RC Coombes. Imperial College London, UK**

Antinicastrin antibody for the treatment of breast cancer

Nicastrin is a member of the gamma secretase enzyme complex and a therapeutic target on its own merit in breast cancer patients. We have previously shown that Nicastrin is overexpressed in breast cancer tissue where it confers worse overall survival in estrogen receptor negative the breast cancer cohort. Furthermore, nicastrin gene is amplified in a large proportion of basal like breast cancers. We have now developed, purified and characterised anti-nicastrin monoclonal antibodies and implemented them for treatment of invasive breast cancer *in vitro* and *in vivo*. Our study demonstrates that anti-nicastrin monoclonal antibodies inhibit proliferation, alter the morphology of 3D acini of invasive breast cancer cells to compact and small structures, inhibit invasion (> 60%) in transwell assays, inhibit mammosphere forming efficacy of breast cancer stem cells and impinge on Notch, as well as RhoGTPases and Akt pathways. BIACore and peptide based epitope mapping were used to elucidate binding epitopes of our lead antibody candidates. Importantly, using MDA-MB-231 Luciferase labeled cells we conducted an orthotopic breast cancer model, as well as a metastatic model by tail vein injection of cancer cells in BalbC nude mice. The 2H6 antibody candidate was potent to induce a 50% tumour growth reduction in the orthotopic model, while it conferred > 80% inhibition of secondary deposits in the metastatic model. In the same model, the gamma secretase inhibitor RO4929097 had negligible effects. We did not observe any treatment related toxicities, while we reproduced the *in vitro* inhibitory effects of the anti-icastrin McAbs on pro-invasive molecules in tumours. We conclude that anti-nicastrin antibodies are valid novel therapeutics for the treatment of invasive breast cancer.

11.20-11.40 am **Adrian L Harris, Helen Sheldon, Esther Bridges, Russell Leek, Ji-Liang Li, Alison Banham, Massimo Masiero, Len Seymour, Michael Koukourakis.**
Departments of Oncology and Pathology, Oxford University, UK and Democritus University, Alexandropolis, Greece.

Targeting Jagged 1 and Delta-like 4 for anti-tumour therapy

We have investigated the effects of baseline expression of Delta-like 4 [Dll4] and induction of Dll4 after radiotherapy in head and neck cancer to elucidate whether our preclinical observations of Dll4 potentiation of radiotherapy would be applicable in clinical trials. These results showed that high Dll4 expression in tumour associated vessels predicted for favourable radiotherapy outcome in locally advanced squamous head and neck cancer. We hypothesised this was due to good perfusion through well-formed vessels. Vessel positivity ranged from 17-100% with a mean of 71%. We suggest the classification using combined CD31 and Dll4 staining

to classify patients in to different groups relating to angiogenesis and hypoxia for targeting agents. To develop suitable agents we have developed a monoclonal antibody against Jagged 1 and created one to a novel epitope which is particularly effective in blocking Jagged 1 signalling in human tumour cells and endothelial cells to a level comparable to direct blockade of gamma secretase. These results show blockade of growth of MDA231 as both xenograft sand in vitro in three-dimensional growth. For MDA231 cells the effects in vitro are equivalent on spheroids to DBZ and imply that Jagged 1 is the major Notch ligand in the cell line. Future challenges will be to try and develop the biomarkers for patient selection for clinical studies and may involve mutations and amplifications in signalling pathways that directly impact Notch signalling. It will also be necessary to compare Jagged 1, Jagged 2 and Dl4 expression, and these studies are on going. Window of opportunity studies are likely to be critical for development of these reagents. However, they may have significant benefits over global Notch blockade in terms of toxicity. Other approaches we have taken also involve using adenoviral gene therapy to deliver soluble Flit 1 as a VEGF trapand dominant-negative soluble Dll4. These showed significant inhibition of tumour growth in vivo of MDA231 and also ZR751 breast cancer cell lines, particularly with promoters regulated by oestrogen receptor and hypoxia. There are synergistic effects with adenoviruses that are replication defective but cytotoxic. Overall, therefore, Dll4 expression seems to be a critical aspect of human tumour angiogenesis relevant to therapy and new agents, particularly antibodies, which show promise in preclinical studies. The challenge now is patient selection for phase I and phase II studies and biomarkers. Vascular imaging and repeat biopsies are likely to yield the most information.

11.40-12.00 **Keith Brennan, Stephanie Jobling, Lorna Wilkinson, Michael Leverentz, Sally Wood, Olivier Meurette, Giovanna Collu,**
Wellcome Trust Centre for Cell Matrix Research,Faculty of Life Sciences,University of Manchester,Michael Smith Building,Oxford Road,Manchester,M13 9PT

In vivo model for transformation of breast epithelial cells

Aberrant Notch signalling has been shown to play a causal role in human breast cancer. Increased signalling is seen in a wide variety of breast cancers, including ductal carcinoma in situ where it is indicative of lesions that are likely to recur. Also sustained Notch signalling has been shown to increase proliferation, prevent apoptosis, promote stem cell self-renewal and drive the adoption of a mesenchymal morphology in breast cancer cells. However it is not clear how increased activity of the Notch receptors is linked to changes in these cellular properties. Typically the Notch receptors are thought to signal via the nuclear protein RBPj, but there is clear evidence that an active form of Notch4 can transform mammary epithelial cells in the absence of RBPj in the mouse. To address the possible role of RBPj-dependent Notch signalling in breast cancer, we have generated a transgenic mouse strain expressing an activated form of RBPj under the control of the mouse mammary tumour virus promoter. These mice display a failure in ductal outgrowth during puberty with increased ductal branching and enlarged terminal end buds, as the ductal lumen is slow to clear. They progress normally through lactation but display a delayed involution. They also develop high grade papillary adenocarcinoma with a latency of 10-12 months that are associated with ductal carcinoma in

situ lesions. By isolating primary mammary epithelial cells and tumour cells from these mice, we have been able to demonstrate a significant resistance to a range of apoptotic stimuli when RBPj-dependent Notch signalling is activated. There is also an increase in Akt signalling that can explain the acquired apoptosis resistance. Together these data indicate that RBPj-dependent Notch signalling can cause tumour formation in the mammary gland and that this in part driven by an acquired apoptosis resistance.

12.00-12.20 pm

Marina Badenes[1,2], Alexandre Trindade[1,2], Ren Liu[4], Valery Krasperonov[3], Parkash S. Gill[4,5] and Antonio Duarte[1,2]

1 Centro Interdisciplinar de Investigação em Sanidade Animal (CIISA), Lisbon Technical University, Lisbon, Portugal. 2 Instituto Gulbenkian de Ciência, Oeiras, Portugal. 3 Vasgene Therapeutics, Los Angeles, CA, USA 4 Department of Pathology, University of Southern California, Los Angeles, USA. 5 Department of Medicine, University of Southern California, Los Angeles, USA.

Dll4/Notch signaling blockade inhibits the development of chronic colitis-associated colorectal cancer in mouse model

Colorectal cancer (CRC), one of the most frequent cancers worldwide, can develop as a complication of inflammatory bowel diseases. The Notch pathway plays a central role in intestinal homeostasis and its deregulation contributes to intestinal neoplasia. Our objective was to analyze *Dll4* expression in normal and tumoral intestine and characterize the effect of Dll4 genetical (*Dll4*$^{+/-}$) and pharmacological (Dll4-Fc) blockade using a chronic colitis-associated CRC mouse model. Dll4 expression was detected in the vascular endothelium and in the both the absorptive and secretive epithelium of the normal intestine and CR polyps. Both genetical and pharmacological inhibition of Dll4 led to a significant reduction in the average number, volume and burden of CR polyps, promoting immature and dysfunctional tumoral angiogenesis, inducing apoptosis and reducing proliferation and inflammation. Additionally, it appears to inhibit the macrophage switch from M1 to M2 that occurs during tumor progression. Moreover the blockade of Dll4 signalling led to a reduction of the Lgr5 positive stem cells associated with an increase of secretory cell differentiation. In conclusion, Dll4-Fc is likely to be beneficial in the treatment of chronic colitis-related CRC.

Key words: chronic colitis-associated CRC, AOM, DSS, Dll4 expression, *Dll4*$^{+/-}$, Dll4Fc.

1.00-2.00 p.m. ***Lunch***

2.00-3.00 p.m. **Open Air workshops:**

i) Complexities of a simple pathway Gerhardt Holger & Marc Vooijs
ii) Which clinical target? Dennis Hughes & Lucio Miele

SESSION 4 Chairman: Antonio Duarte

4.30–4.50 pm

Jan Theys[1], Ph.D, Sanaz Yahyanejad[1], M.Sc., Arjan Groot[1] Ph.D., Ludwig Dubois[1] Ph.D., Roger Habets[1], M.Sc, Kim Paesmans[1], Ph.D, Kasper Rouschop, Ph.D,, Philippe Lambin[1] Ph.D, Paul Span[2] Ph.D, Jan Bussink[2] , Ph.D, and Marc Vooijs[1], Ph.D [1]Dept. of Radiation Oncology (MAASTRO lab), GROW - School for Oncology and Developmental Biology, Maastricht University Medical Centre, Maastricht, The Netherlands. [2]Dept of Radiation Oncology, Radboud University Nijmegen Medical Centre, Nijmegen, The Netherlands.

NOTCH ACTIVITY INDUCES RADIATION RESISTANCE IN NON-SMALL CELL LUNG CANCER (NSCLC).

Lung cancer is the leading cause of cancer mortality and 5-year survival rates in advanced disease are less than 5%. The NOTCH pathway plays important roles during lung development and physiology and is often deregulated in lung cancer. Notch signaling is also crucial for tumor angiogenesis making it a potentially attractive therapeutic target. We investigated NOTCH signaling in NSCLC and determined its role in resistance to ionizing radiation (IR). We hypothesized that high NOTCH activity in NSCLC promotes resistance to radiotherapy.
First we determined the expression patterns of NOTCH receptors, ligands and target genes in 89 NSCLC patient samples by qPCR. These data show a heterogeneous expression pattern of NOTCH pathway components among patients. Particularly, patients with high tumor NOTCH activity showed worse disease-free survival ($p<0.01$). We detected a comparable heterogeneous NOTCH activity profile in NSCLC cell lines, as demonstrated by transcriptional reporter assays and target gene expression analysis. Next we created NSCLC cells expressing ligand independent constitutive active NOTCH1 (N1ΔE) or a dominant-negative mastermind (dnMAML) to block endogenous NOTCH signaling. Intriguingly, expression of neither N1ΔE nor dnMAML affected the in vitro proliferation or the intrinsic radiosensitivity as measured by clonogenic survival. Next we addressed the role for Notch signaling in the growth and response to radiotherapy of NSCLC in vivo. NSCLC-N1ΔE tumors grew significantly ($p<0.05$) faster than the wild type (wt) tumors whereas the doubling time of dnMAML-expressing tumors was significantly lower ($p<0.05$). Next we addressed the influence of constitutive NOTCH signaling on IR response. Upon a single dose IR (10Gy), N1ΔE tumors showed a significantly ($p<0.01$) reduced response ((specific) growth delay) compared to wt. The cause of this radiation resistance is currently being investigated and point to a potential role for Notch signaling in hypoxia response. Data on the hypoxia tolerance and the role of the tumor microenvironment of wt versus NOTCH-overexpressing tumors on RT resistance will be discussed.
We demonstrate an important role for NOTCH in tumor growth and correlate high NOTCH activity with a radioresistant phenotype in NSCLC in vivo. These data suggest that blocking NOTCH signaling in tumors is potentially a promising way to improve NSCLC outcome after radiotherapy.

4.50-5.10 pm **Tian-Li Wang, Johns Hopkins Medical Institutions, Baltimore, USA**

NOTCH3 target genes in ovarian cancer

NOTCH3 signaling amplification appears to be involved in malignant behaviors of many ovarian, lung and breast cancers, but the targets of NOTCH3 signaling are unclear. We report the use of an integrated systems biology approach to identify direct target genes for NOTCH3. Transcriptome analysis showed that suppression of NOTCH signaling in ovarian and breast cancer cells led to downregulation of genes in pathways involved in cell cycle regulation and nucleotide metabolism. ChIP-seq analysis defined promoter target sequences, including a new potential CSL binding motif in addition to the canonical CSL binding motif, that were occupied by the NOTCH3/CSL transcription complex. Integration of transcriptome and ChIP-seq data demonstrated that the ChIP target genes overlapped significantly with the NOTCH-regulated transcriptome in ovarian cancer cells. From the set of genes identified we determined that the mitotic apparatus organizing protein DLGAP5 (HURP/DLG7) was a critical target. Both the new motif and the canonical CSL binding motif were essential to activate DLGAP5 transcription. DLGAP5 silencing in cancer cells suppressed tumorigenicity and inhibited cellular proliferation by arresting the cell cycle at the G2/M phase. In contrast, enforced expression of DLGAP5 partially counteracted the growth inhibitory effects of a pharmacological or RNAi-mediated inhibition in cancer cells. Our findings define direct target genes of NOTCH3 and highlight DLGAP5 in the tumor-promoting function of NOTCH3**.**

5.10-5.30 pm **Dennis P. M. Hughes, MD, PhD, Associate Professor of Pediatrics Department of Pediatrics – Research,UT MD Anderson Cancer Center,Houston, Texas, USA**

The vital role of Notch signaling in sarcoma metastasis

Signaling pathways vital to early development and organogenesis are frequently usurped by cancers to promote the malignant phenotype and impede maturation and development. The Notch pathway is one of the most frequently hijacked pathways, and is particularly important in sarcoma biology. Several groups, including ours, have shown that Notch signaling promotes osteosarcoma tumor growth and metastasis, while inhibition of Notch or Hes reduced proliferation in some systems and impeded invasion in others. Similar results have been obtained with rhabdomyosarcoma tumor models. Since Hes1 and DXT1 have reciprocal regulatory effects on Notch pathway activity, the impact of Notch pathway manipulation can be complex. In Ewing sarcoma, Notch pathway signaling, mediated by DLL4, is essential for recruitment of pericytes to the developing vasculature of developing tumors, and blockade of DLL4 with antibodies prevented the development of a mature, organized vasculature in Ewing sarcoma tumors and reduced tumor growth. We now show that, for osteosarcoma, the tumor cells themselves express relatively little in the way of Notch ligands, but that exposure to Jag1 or DLL4 can induce as much as a 50-fold increase in Notch pathway activity. Interestingly, a major impact of this upregulation of Notch pathway signaling is transient growth arrest. In

other systems, cell cycle arrest has been shown to be essential for tumor cell invasiveness. Since both pericytes and endothelial cells have higher expression of Notch ligands than osteosarcoma cells do, the expectation would be that Notch pathway activity is not uniform across a tumor, varied geographically, with the highest levels near vasculature. In these locations, high levels of Notch signaling would mediate transient cell cycle arrest, increased invasiveness and migration of tumor cells into the endovascular space, providing a cellular mechanism for the association of Notch pathway signaling with increased metastasis.

5.30-5.50 pm **Stefano Indraccolo Istituto Oncologico Veneto - IRCCS, Padova, Italy**

Notch1 targeted therapy for T acute lymphoblastic leukemia

T acute lymphoblastic leukemia (T-ALL) is characterized by several genetic alterations and poor prognosis in about 20-25% of patients. Notably, about 60% of T-ALL shows increased Notch1 activity, due to activating *NOTCH1* mutations, or alterations in the *FBW7* gene, which confer to the cell a strong growth advantage. Therapeutic targeting of Notch signaling could be clinically relevant, especially for chemotherapy refractory patients. We investigated the therapeutic efficacy of a novel anti-Notch1 monoclonal antibody by taking advantage of a collection of pediatric T-ALL engrafted systemically in NOD/SCID mice and genetically characterized with respect to *NOTCH1/FBW7* mutations. Anti-Notch1 treatment greatly delayed engraftment of T-ALL cells bearing Notch1 mutations, including samples derived from poor responders or relapsed patients. Notably, the therapeutic efficacy of anti-Notch1 therapy was significantly enhanced in combination with dexamethasone. Anti-Notch1 treatment increased T-ALL cell apoptosis, decreased proliferation and caused strong inhibitory effects on Notch target genes expression along with complex modulations of gene expression profiles involving cell metabolism. Serial transplantation experiments suggested that anti-Notch1 therapy could compromise leukemia initiating cell functions. Our results show therapeutic efficacy of Notch1 blockade for T-ALL, highlight the potential of combination with dexamethasone and identify surrogate biomarkers of the therapeutic response.

8.00 pm - until late
Conference Dinner

Friday 28th June 2013

SESSION 5 Chairman: Freddy Radtke

9.00-9.20 am **Dimitris Skokos, Regeneron Pharmaceuticals, Inc. 777 Old Saw Mill River Road, Tarrytown, 10591, NY, USA**

Notch Signaling at the Crossroad of Immuno-metabolism

Delta-like ligand 4 (Dll4)-Notch signaling is essential for T cell development and alternative thymic lineage decisions. Further, ongoing Dll4-Notch signaling is required for T-cell development in the adult thymus. In the current study, using both genetic inactivation models and anti-Dll4 antibody treatment we show that Dll4-Notch signaling blockade promotes *de novo* natural regulatory T cells (Treg) expansion by a dendritic cell (DC)-dependent mechanism that requires major histocompatibility complex II expression on DCs. We further demonstrate that anti-Dll4 treatment fully prevents type 1 diabetes (T1D) and importantly it reverses established hyperglycemia not only via a Treg-mediated mechanism that inhibits $CD8^+$ T cell pancreatic islet infiltration but also by a robust metabolic effect. The therapeutic implications of Notch signaling blockade in diabetes open new horizons in the treatment of autoimmunity.

9.20-9.40 am **Cathy Zhang, Principle Scientist, Oncology Research Unit, Pfizer Global Research and Development, San Diego, California, USA**

The biomarker and combination assessment of γ-secretase inhibitor PF-03084014 in breast cancer models

Aberrant Notch signaling pathway is highly implicated in breast cancer disease progression. Upon Notch pathway activation, γ-secretase catalyzes the cleavage of Notch receptors and results in the release of Notch intracellular domain (NICD). PF-03084014 is a small molecule γ-secretase inhibitor (GSI) that is currently under clinical investigation. We investigated the biomarker and combination strategies in breast cancer xenograft models to aid the clinical development of PF-03084014. By impairing Notch signaling, PF-03084014 exhibited pleiotropic biological properties including anti-proliferation, apoptosis and anti-angiogenesis. Significant antitumor efficacies were observed in a subset of breast cancer xenograft models. The Notch pathway target gene expressions correlated with the antitumor efficacy of PF-03084014 and can potentially serve as biomarkers for patient enrichment. In the combination assessment, PF-03084014 enhanced the antitumor efficacies of standard of care agents and target agents. In particular, PF-03084014 and docetaxel were highly synergetic. Mechanistic insights revealed the abilities of PF-03084014 to suppress cancer stem cell self-renewal; reverse endothelial mesenchymal transition and reduced drug resistance. This work provides guidance for the clinical investigation of PF-03084014 in breast cancer patients.

9.40-10.00 am **Pannuti A[1*], Yun J[2*], Espinoza I[1], Zhu H[1], Hicks C [1], Tonetti DA[3], Means-Powell[4], Miele L[1] [1]*University of Mississippi Cancer Institute, Jackson, MS;* [2]**Korea Research Institute of Bioscience and Biotechnology (KRIBB), Ochang, Republic of Korea;** [3]*University of Illinois at Chicago Department of Biopharmaceutical Sciences, Houston, TX;* [4] *Vanderbilt-Ingram Cancer Center, Nashville, TN*
***Contributed equally**

Role of PKCα-Notch crosstalk in endocrine-resistant, ER+ breast cancer: preclinical and early clinical studies

BACKGROUND: Notch-1 has been reported to maintain an estrogen-independent phenotype in ERα+ breast cancer cells. Notch-4 expression correlates with Ki67. Notch-4 also plays a key role in breast cancer stem-like cells. Estrogen-independent breast cancer cell lines have higher Notch activity than estrogen-dependent lines. Protein kinase Cα (PKCα) overexpression is common in endocrine-resistant breast cancers and promotes tamoxifen-resistant growth in breast cancer cell lines. We tested whether and how PKCα overexpression affects Notch activity, and whether Notch signaling contributes to endocrine resistance in PKCα overexpressing breast cancer cells. We also tested PKCα expression in tissue specimens from patients enrolled in a phase 1b study of GSI R04929097 plus exemestane in the metastatic setting, which showed safety, tolerability and clinical response in 7/14 evaluable patients.
RESULTS: Analysis of published gene expression data from ERα+ breast carcinomas shows that PKCα expression correlates strongly with Notch-4. Real-time RT-PCR and immunohistochemistry on archival specimens confirmed this finding. In a PKCα-overexpressing, tamoxifen-resistant T47D model, PKCα selectively increases Notch-4, but not Notch-1 expression *in vitro* and *in vivo*. This effect is mediated by AP-1 occupancy of the Notch-4 promoter. Notch-4 knockdown inhibits estrogen-independent growth of PKCα-overexpressing T47D cells, whilst Notch-4IC expression stimulates it. Gene expression profiling shows that multiple genes and pathways associated with endocrine resistance are induced in Notch-4IC and PKCα-expressing T47D cells. In PKCα-overexpressing T47D xenografts, an orally active γ-secretase inhibitor (GSI) at clinically relevant doses significantly decreased estrogen-independent tumor growth, alone and in combination with tamoxifen. In metastatic, endocrine-resistant ER+ breast cancers, the combination of GSI R04929097 and exemestane was well tolerated overall, but MTD was not reached due to auto-induction of drug metabolism. There was a strong correlation (r=.857) between quantitative RT PCR expression of PKCα and Notch-4 expression in FFPE tumor specimens from enrolled patients. PKCα overexpression (>2+) was detected by immunohistochemistry (IHC) in 100% (10/10) evaluable primary tumors. Notch4 overexpression was detected by IHC in 70% (7/10) of specimens in the nucleus and 30% (3/10) in the cytoplasm.
CONCLUSIONS: 1) PKCα and Notch4 expression may identify a subset of endocrine-resistant patients who could benefits from treatment with Notch inhibitors; 2) Pathways activated by Notch4 and PKCα offer additional therapeutic opportunities for this group of tumors.
Funding: NCI (D.T., L.M., J. M).

10.20-10.40 am Tim van Groningen, Nurdan Akogul, Marloes Broekmans, Jan Molenaar, Bart Westerman, Jan Koster, Rogier Versteeg and Johan van Nes
Dept. of Oncogenomics, Academic Medical Center, University of Amsterdam, The Netherlands

Neuroblastoma contains a dedifferentiated subpopulation with mesenchymal identity that can be induced by Notch signaling and is inherently resistant to therapy

Cancer is a heterogeneous disease with cellular subpopulations that are different in many cancer-relevant traits like e.g. drug-resistance. Neuroblastoma is an embryonic tumour from the peripheral sympathetic nervous system and is responsible for ~15% of pediatric cancer deaths. We study intra-tumoral heterogeneity of neuroblastoma and identified two major cell types. One type is mesenchymal and shows a high motility, while the other type characterizes as neuro-epithelial (NE) and expresses more mature lineage differentiation markers. The two cell types differ in 1) activation of many molecular pathways including Notch and 2) sensitivity to therapeutic drugs. Immunohistochemical analysis shows that all neuroblastoma tumours include both cell types *in vivo*. *In vitro*, these cell types can spontaneously interconvert. We identified Notch signaling as a major switch of this transition. In addition, we have sequenced the full genomes of >100 neuroblastoma tumours and thus identified the full mutation spectrum of genes in this tumor. Remarkably, some recurrently mutated genes relate to pathways and functions associated with mesenchymal and neuro-epithelial cell types. As tumor heterogeneity, epithelial-mesenchymal transition and stemness are highly relevant for metastasis and drug-sensitivity, our data provide a rationale for pharmacological Notch inhibition to overcome therapy-resistance.

10.20-11.00 am ***Coffee Break and poster Review***

SESSION 6 Chairman: Charles Coombes

11.00-11.20 am Ozden Yalcin, Duje Buric, Cathrin Brisken
ISREC, School of Life Sciences, Ecole Polytechnique Fédérale (EPFL), Switzerland

Is it safe to inhibit Notch in breast cancer patients?

Notch4 has long been identified as an oncogene in the mouse mammary gland. In recent years evidence has accumulated that deregulated Notch signaling is associated with poor prognosis in human breast cancer. During normal mammary gland development Notch signaling appears to have a dual role. On the one hand it is important in mammary stem cells, a function mediated by Notch4. On the other hand Notch signaling has emerged as a control factor of differentiation of progenitor cells into luminal cells. We show that in primary human breast epithelial cells, maintenance of basal/progenitor cell characteristics depends on continued expression of the p63 isoform, □□p63, which is expressed in the basal compartment. Notch signaling down modulates □□p63expression and mimics □□p63 depletion resulting also in growth arrest, whereas forced expression of □□p63partially counteracts the effects of Notch. Consistent with Notch activation specifying luminal cell fate in the mammary gland, Notch signaling activity is specifically detected in mice at sites of pubertal ductal morphogenesis where luminal cell fate is determined. Basal cells in which Notch signaling is active show decreased p63 expression. Both constitutive expression of □□p63 and ablation of Notch signaling are incompatible with luminal cell fate. Thus, the balance between basal and luminal cell compartments of the breast is regulated by antagonistic functions of □□p63 and Notch. These pro-differentiation effects of Notch need to be considered as Notch signaling is targeted in breast cancer patients.

11.20-11.40 am Rajwinder Lehal[1], Viktoria Reinmüller[1], Viktoras Frismantas[3], Vincent Zoete[3], Olivier Michielin[3], Gerardo Turcatti[1], Jieping Zhu[1,] Jean-Pierre Bourquin[2] and Freddy Radtke[1]
[1]Ecole Polytechnique Fédérale de Lausanne (EPFL), School of Life Sciences, ISREC, Station 19, 1015 Lausanne, Switzerland
[2] University Children's Hospital Zurich, Division of Pediatric Oncology, August-Forelstrasse 1, 8008 Zürich, Switzerland
[3]Swiss Institute of Bioinformatics, Quartier Sorge - Batiment Genopode, 1015 Lausanne, Switzerland

Identification and pre-clinical validation of a novel Notch inhibitor

Cancer can be seen as disease of perturbed self-renewal. In the last decades it became clear that many of the signaling pathways known to be important during embryonic development also play important roles in regulating self-renewing tissues. Deregulation of the self-renewal process results in sustained proliferation, evasion of cell death, loss of differentiation capacity, invasion and metastasis all of which are hallmarks of cancer. The major question is how do

these deregulated signaling cascades mechanistically contribute to cancer and are they suitable for targeting therapy? The Notch pathway is one such cascade required for normal stem cell maintenance and development of different organs. Over activation of this pathway due to mutations in the Notch receptor are found in more than 50% of human T-cell leukemia and deregulated Notch signalling has been shown to promote tumour progression of various organs. In addition aberrant activation of this pathway also correlates with poor survival due to chemoresistance in human cancers such as chronic lymphoid leukemia (CLL). Studies in mice and humans over the last two decades highlighted the critical need of targeting the Notch pathway in order to treat human malignancies. Thus, the importance of Notch signaling in human cancers and the lack of effective therapeutic intervention justify the development of novel inhibitors. In order to identify Notch inhibitors, we have developed a coculture assay to screen small molecule as well as peptide libraries. This exercise has led to the identification of several Notch signaling inhibitors. Here we will present a novel small molecule inhibitor of the Notch pathway (referred to as I3) that blocks Notch signaling by interfering with the Notch transcriptional activation complex. The compound I3 has shown remarkable ability to block Notch signaling in human cancer cell lines as well as primary human T-ALL cells, thus abrogating their proliferative properties. In addition, compound I3 exhibit *in vivo* activity as demonstrated by its ability to impinge upon Notch dependent developmental processes and by impeding tumour growth in xenograft models of human leukemia.

11.40-12.00 **Hannah Harrison, Bruno Simões, Lynsey Rogerson, Sacha Howell, Göran Landberg and Robert Clarke, Breast Biology Group, Institute of Cancer Sciences, University of Manchester Paterson Institute for Cancer Research**

Estrogen Increases the Activity of Estrogen Receptor-Negative Breast Cancer Stem Cells Through Paracrine Notch Signalling

Estrogen is essential for the development of the normal breast but mammary stem cells are estrogen receptor alpha (ER) negative and rely on paracrine signals for their activity. However, little is known about how systemic oestrogen regulates breast cancer stem cell (CSC) activity. We tested the effects of oestrogen on CSC activity in vitro and in vivo and investigated the Notch signalling pathways as a paracrine mediator of oestrogen effects.

CSC-enriched populations from ER-positive patient-derived and established cell lines have low or absent ER expression. However, estrogen stimulated CSC activity demonstrated by increased mammosphere formation in vitro and tumour formation in vivo. This effect was abrogated by the anti-oestrogen tamoxifen or ER siRNA. These data suggest that the oestrogen response is mediated through paracrine signalling from non-CSCs to CSCs. We demonstrate that Notch ligands are highly expressed in non-CSC compared to CSC populations and that gamma secretase inhibitors block oestrogen-induced CSC activity in vitro and in vivo and reduce CSC frequency.

These data establish that Notch receptor signalling pathways operate downstream of oestrogen in the regulation of ER negative CSCs. However, our results suggest that anti-estrogens will not directly target ER-negative cancer stem cells in ER+ breast tumours and that they may enrich for breast cancer stem cells by selective depletion of ER+ cells.

12.00-12.20 pm James West, Ph.D., CytomX Therapeutics, Inc., USA

Probodies: protease activated antibodies for disease-specific inhibition of Notch signaling

The expression of Notch receptors and their ligands has been shown to be dysregulated in solid tumors, T-cell leukemia and multiple myeloma. In addition, inhibition of Notch signaling by GSIs or neutralizing antibodies has demonstrated a central role for Notch signaling in the initiation, progression and metastasis of cancer. Furthermore, inhibition of notch signaling in the pre-clinical and clinical setting, has revealed the therapeutic potential of targeting this pathway. However, although inhibition shows therapeutic benefit, systemic inhibition of Notch signaling leads to toxicities that limit therapeutic utility. We have developed a human/mouse cross-reactive, neutralizing Jagged1/2 antibody which shows potent anti-neoplastic activity in xenograft and mouse models of cancer. However, treatment with this antibody results in toxicities associated with anti-jagged activity in the skin, gut and immune system. In order to retain potent anti-neoplastic activity and spare healthy tissues, we have engineered a novel proteolytically activated antibody scaffold, the Probody™ platform. Probodies are comprised of a masking peptide that inhibits the activity of the antibody in healthy tissues, which is linked to the antibody through a protease sensitive linker engineered for activation by specific proteases that are up-regulated in the tumor microenvironment. When the Probody enters the tumor the linker is cleaved, resulting in dissociation of the masking peptide, thereby releasing an active form of the antibody. The biological activity and therapeutic potential of an anti-Jagged antibody will be discussed as will how application of Probody™ technology will open its therapeutic window. Probodies represent a new class of antibody-based therapeutics that specifically target activity to cancer thereby expanding the universe of drugable targets.

1.00-2.00 pm ***Lunch***

2.00-3.00 pm Open Air workshops:

i) Complexities of a simple pathway Gerhardt Holger & Marc Vooijs
ii) Which clinical target? Dennis Hughes & Lucio Miele

SESSION 7 Chairman: Agamemnon Epenetos

4.30-4.50 pm Jason Sagert, CytomX Therapeutics, South San Francisco, USA

An Anti-Jagged Probody Enables Tumor-specific Inhibition of Jagged-Dependent Notch Signaling

The Notch pathway has been an attractive target for the treatment of cancer using both small-molecule and biological drugs. However, toxicities associated with the systemic inhibition of the pathway have limited the clinical translation of these findings. Here we demonstrate the efficacy of a human/mouse cross-reactive Jagged 1/2 blocking antibody. Such a Jagged 1/2 blockade was efficacious in xenograft models, but systemic inhibition of Jagged-dependent Notch signaling resulted in toxicities that preclude therapeutic development. To effect tumor-specific Jagged inhibition, while sparing signaling in healthy tissues, we developed a unique approach to alleviating systemic toxicities: a protease-activatable anti-Jagged1/2 Probody™ therapeutic. The anti-Jagged Probody is engineered to be inactive in circulation and is activated by the dysregulated protease activity in the tumor microenvironment. Here we show that an anti-Jagged 1/2 Probody can maintain parental antibody efficacy while reducing systemic toxicities. Further, we show that human tumor samples are capable of activating anti-Jagged Probodies. These data demonstrate the potential of the Probody platform to enable the drugging of a broad range of targets that have been avoided due to systemic toxicities.

4.50-5.10 pm Mahendra Deonarain Honorary Reader in Antibody Technology, Imperial College London, UK, Silvia Colucci, Imperial College London, Aleksandra Filipovic, Imperial College London, Spyros Sylianou, Trojantec Ltd, Christina Kousparou, Trojantec Ltd & Agamemnon Epenetos, Imperial College London and Trojantec Ltd.

Novel Strategies for Inhibiting Notch Signaling

Deregulation of Notch signaling is implicated in many diseases, particularly cancer. Our strategy to inhibit oncogenic Notch signaling involves blocking the activation of the Notch/Mastermind-Like(MAML)/CSL transcription complex using a dominant-negative (DN) peptide derived from the MAML protein. Using Trojantec's Antennapedia (Antp) cell-penetrating peptide delivery system, we show that a Antp-DN-MAML fusion peptide, known as TR4 is able to passively enter cells and inhibit Notch signaling in vitro, in breast cancer MDA-Mb231 cells. Furthermore, pharmacodynamic studies in vitro and in human tumour xenografts in vivo show that TR4 inhibits tumour cell growth with biomarker readouts indicative of reduced cell proliferation (Ki-67) and Notch target genes (Hes-5 and Hey-2) downregulation, supporting the proposed mechanism of action. TR4 is easily produced as a synthetic peptide that acquires a pharmacologically-active form easily and rapidly greatly facilitating its development as a therapeutic drug. Next-generation Notch inhibitors, analogous to TR4 could consist of more

specific and higher affinity antibody fragments, delivered using the Antp-system. Some progress towards this goal will also be described.

5.10-5.15 pm **Farewell: Agamemnon Epenetos**

POSTER PRESENTATIONS

These will be displayed in the Hall area during the whole period of the conference

- **Horimoto Y[1], Igari F[1], Kadowaki N[2], Arakawa A[3], Saito M[1]**

Departments of Breast Oncology[1], Pathology and Oncology[2] and Human Pathology[3], Juntendo University School of Medicine, Tokyo, JAPAN

Notch signalling in ductal carcinoma *in situ* of human breast

Background and purpose

Several studies have shown notch signalling to promote tumour progression, while others have obtained evidence of suppressed tumour growth. However, roles of this pathway in ductal carcinoma *in situ* (DCIS) have not been adequately investigated. We previously showed Jagged1-dependent Notch3 receptor activation to be responsible for the development of invasive breast cancer and that this is involved carcinoma-associated fibroblasts. In this study, we examined the expressions of Jagged1 and Notch3 in DCIS by immunohistochemistry (IHC).

Objectives

Twenty DCIS operative specimens, including 13 with microinvasion, were examined by IHC. Correlations between IHC and clinicopathological features were determined.

Results

All tumours were successfully removed surgically and none showed lymph node involvement. Sixteen (80%) were estrogen receptor positive, while HER2 protein was overexpressed in seven (35%). None of the routine biomarkers correlated with notch signalling. In all but one case, Jagged1 was strongly expressed in DCIS. Notch3 was positive in 7 tumours, all of which were comedo type with microinvasion. In the stroma, Notch3 expression was often detected in fibroblasts around ductal lesions. However, the possibility of Notch3 reflecting only a stromal reaction, often seen in DCIS, could not be ruled out. There was no difference in notch signalling expression between *in situ* and invasive nests in the same lesion. Though further studies (e.g. of other notch ligands and receptors) are needed, our results raise the possibility of notch pathway involvement in the progression of *in situ* disease. Furthermore, different roles of notch ligands in DCIS, such as maintenance of cancer cells in ducts under conditions of hypoemia and hypoxia, were suggested.

- **Helen Sheldon[1], Esther Bridges[1], Evelyn Ramberger[2], Esther Kleibuker[3], Massimo Maseiro[4] and Adrian L Harris[1]**

[1]Department of Medical Oncology, Weatherall Institute of Molecular Medicine, University of Oxford, Oxford, United Kingdom, , [2]The University of Applied Sciences, FH Campus Wein, Vienna, Austria, [3]VU University Medical Centre, Amsterdam, The Netherlands, [4]Nuffield Department of Clinical Laboratory Sciences, John Radcliffe Hospital, Oxford, United Kingdom.

The Role of RHOQ in Notch Signalling and Angiogenesis.

Delta-like 4 (Dll4)–mediated Notch signalling is a key pathway essential to angiogenesis. We are interested in identifying novel transcription targets of Dll4 stimulated Notch signalling to establish their role in vascular biology and their interactions other angiogenic signalling pathways. Deep sequence analysis (CAGE array) of Human Umbilical Vein Endothelial Cells (HUVEC) stimulated with human recombinant tethered Dll4 (rhDll4) for 16h identified a number of genes that were up-regulated. RHOQ was identified in this array and this was confirmed in HUVEC. RHOQ is a member of the Rho family of small GTPases, which switch between inactive GDP-bound and active GTP-bound states and function as molecular switches in a number of signal transduction pathways. Rho proteins promote reorganization of the actin cytoskeleton and regulate cell shape, attachment, and motility. RHOQ has been shown to play a significant role in the exocytosis of glucose transporter member 4 (GLUT4) and other proteins, possibly acting as the signal that turns on the membrane fusion machinery. It has also been described in neurite outgrowth and membrane expansion in developing neurons. The role of RHOQ is currently unknown in endothelial cells, so to assess its function we have transiently knocked out RHOQ using siRNA and over-expressed it using lentivirus. It appears that RHOQ levels need to be tightly regulated within the cells as too much or too little leads to a marked reduction in HUVEC growth, migration and network formation in the Matrigel^{BD} assay. Although RHOQ is a Notch target gene it is also appears to be involved in the signalling process. Transient loss of RHOQ prevented the induction of Hey1 and Dll4 when HUVEC were stimulated with Dll4. Over-expression of RHOQ enabled the cells to react quicker to Dll4 stimulation with Notch target genes increasing as early as 2 hours after plating the cells onto the ligand. Western blotting revealed that the Notch receptor is still cleaved to produce the Notch intracellular domain (NICD) in siRHOQ treated cells but the NICD is not degraded as rapidly as the siControl cells. This suggests that RHOQ is involved in ensuring proper NICD function and experiments are still on-going to elucidate the mechanism. Consistent with a role in Notch signalling, loss of RHOQ leads to hyper-sprouting in a hanging drop assay *in vitro* but over-expression results in the formation of longer sprouts. *In vivo* matrigel plugs impregnated with siRNA targeting RHOQ had a reduced vessel density compared to siControl plugs. These results suggest that RHO proteins may have a central role to play in Notch mediated angiogenesis and as such be a potential target in Notch inhibition strategies.