
The 1st International Conference
Notch Targeting in Cancer
22- 24 June 2011, Mykonos, Greece

Organisers:

Agamemnon Epenetos (London UK)
Charles Coombes (London, UK)

Scientific Organising Committee:

Aleksandra Filipovic (London, UK)
Bin-Bing.Zhou (Pearl River, USA)
Lucio Miele (Jackson, USA)
Marc Vooijs(Maastricht, Netherlands)
Raymond Moellering (San Diego, USA)
Robert Clarke (Manchester, UK)
Ronan O'Hagan (Boston, USA)
Spyros Stylianou (Nicosia, Cyprus)

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4.00-4.25 p.m. Registration

4.25-4.30 p.m. Welcome: Agamemnon Epenetos

SESSION 1
Chairman: Charles Coombes

4.30-5.00 p.m.

Cancer stem cell regulation-something out of Notching

Robert Clarke, University of Manchester, UK

We and others have established that the developmental Notch receptor signalling pathway is active in breast cancer cell lines as well as in pre-invasive and invasive primary samples. Recently, a role for Notch in regulating the hierarchy of stem and progenitor cells in both normal and cancer epithelium has been elucidated. Since inhibiting the Notch receptor signalling pathway is a possible future breast cancer therapy, the expression and activity of the different ligands and receptors will be reviewed. How the pathway's activity can be inhibited and the effects of inhibition will be summarised.

5.00-5.30 p.m.

Early clinical trials with gamma-secretase inhibitors

Lucio Miele, Division of Cancer Therapeutics Development, National Cancer Institute, National Institutes of Health, Bethesda, USA and University of Mississippi Cancer Institute, Jackson, MS, USA

The development of embryonic pathway inhibitors, such as the γ-secretase inhibitors (GSIs) or γ-secretase modulators (GSMs), is challenging. Selection of diseases to study and appropriate biomarkers to demonstrate molecular targeted effects, proof of concept and demonstration of cancer stem cell modulation are compromised by an expansive but clinically non-validated set of biomarkers and an even more limited ability to identify and quantify disease-specific-CSC populations in tumor explants. NCI/DCTD approached the development of the GSIs by trying to validate pathway-specific bioassays and generate an extensive human data set that will over time be used to better plan and implement clinical trials. We approached this task by developing improved assays to show changes in cancer

stem cell populations and establish reliable predictive and prognostic markers for disease response when Notch signaling is inhibited. The rationale for disease selection has been largely based on limited and unpublished pre-clinical data. NCI recommended that investigators provide pre-clinical data either from published references, unpublished observations, or both. These include: i) rationale based on clinical biopsy specimen: *e.g.* the tumor contains an increased expression of ICN for one or more Notch receptors in clinical tumor samples and /or increased expression of Notch pathway effectors such as *Hes-1* and/or *Hey-1, Hes-5, Deltex1,* and Jagged-1, -2 in the tumor stroma or tumor vascular endothelial cells. These effectors can be measured by whole-genome arrays, quantitative RT-PCR, Western blotting, or other compelling methods (e.g. quantitative immuno-histochemistry or quantitative in-situ hybridization). Ideally, the expression components of the pathway should include increased expression of ICN and selected Notch target genes such as *Hes-1*, or *Hey* in a sufficient number of tumor samples from a specific disease type (*e.g.* 30 clinical biopsy samples with high ICN expression would be preferred over only 1-2 clinical samples, or cell line studies as evidence for tumors over-expressing a Notch pathway component; ii) i*n vivo* animal model based rationale: *In vivo* pre-clinical models that demonstrate ligand-driven and/or paracrine/autocrine-driven or putative cancer stem cell pathway activation, showing the effect of Notch signaling inhibition by GSI agents against the tumor cells (human xenograft or transgenic models). The disease/tumor selection criteria, which depend significantly on data from patient- derived tumors and /or *in vivo* pre-clinical models, rather than the traditional *in vitro* cell line approach, represent a departure from traditional drug development strategies used for cytotoxic or cytostatic chemotherapy. The true *in vivo* effect of GSI/GSM inhibition or modulation of this pathway in cancer cells cannot be gauged using traditional *in vitro* assay systems.

An additional complication is that pathways downstream of developmental signals such as Notch are highly context-dependent and integrated with other therapeutically relevant pathways. Hence, the biological consequences of Notch inhibition in bulk tumor cells versus cancer stem cells may differ. This has 3 important implications: 1) One cannot always assume that the canonical effectors identified in one disease (e.g. Hes1 for T-ALL) are mechanistically relevant and/or clinically informative in other diseases. Indeed, there is significant evidence that non-canonical effectors play pathogenetic roles in solid tumors; 2) The true usefulness of Notch inhibitors can best be evaluated in mechanism-based combination treatments that target pathway cross-talk specific to a particular disease, compared to traditional single-agent approaches and 3) biological effects on tumors may not translate into radiologically detectable tumor shrinkage (e.g., by RECIST criteria), but may include inhibition of tumor recurrence and/or metastastasis. These effects are potentially more clinically important than short-term tumor volume reductions, but they are harder to track without innovative study designs and/or appropriate surrogate endpoints. NCI/DCTD asked for preclinical data or appropriate references if the proposed rationale for a clinical trial includes a "cancer stem cell" hypothesis: i) the cancer stem cells of the tumor type that will be proposed express high levels of ICN and/or exhibit paracrine/autocrine activation of the Notch pathway; ii) the method used to identify/isolate putative cancer stem cells and the method used to quantify cancer stem cell activity, e.g. cancer stem cell quantitative functional assays etc. Tumor types of interest

were selected to provide more definitive human data addressing one or more of the hypotheses outlined above:

- Breast cancer:Neoadjuvant; Residual risk post chemotherapy, stage 3; Inflammatory breast cancer; Triple negative breast cancer; Numb-negative breast cancer
- Melanoma resected but node + by biopsy, where MRD is of concern (if high ligand expression, pre-clinical data should be provided); Multiple Myeloma, post auto-transplant to eradicate residual disease
- Pancreatic carcinoma, adjuvant setting (Phase 1b/2 combination with gemcitabine, see below)
- Metastatic prostate cancer (Phase 1b/2 combination with hormonal therapy)
- Colorectal cancer or APC
- High grade gliomas (GBM)
- Non-small cell lung cancer, particularly if adenocarcinoma with documented expression of
- Notch-1 or squamous carcinoma with documented expression of Notch-3.
- Renal cell carcinoma
- Choroid plexus carcinoma
- Kaposi's sarcoma

This presentation will provide an overview, preliminary data and emerging results from these studies.

5.30-6.00 p.m.

Rational targeting of Notch in cancer: Combination of γ-secretase Inhibitor MK-0752 and Endocrine Therapy for Early Stage ERα positive Breast Cancer in a Pre-surgical Window Pilot Study

Albain KS, Czerlanis C, Rajan P, Zlobin A, Godellas C, Bova D, Lo SS, Robinson P, Sarker S, Gaynor ER, Cooper R, Aranha G, Czaplicki K, Busby B, Rizzo P, Chisamore M, Demuth T, Blackman S, Watters J, Stiff P, Fuqua SAW, **Miele L.** Loyola University Chicago Cardinal Bernardin Cancer Center, Maywood, IL; Merck Oncology, North Wales, PA; Baylor Breast Center, Houston, TX; University of Mississippi Cancer Institute, Jackson, MS

Breast tumor initiating cells (TIC) use Notch receptors/ligands with other pathways for self renewal, resulting in tumor proliferation and progression. However, successful development of Notch inhibitors in oncology will require a mechanistic understanding of how Notch cross-talks with other therapeutically relevant pathways. In the course of our efforts to dissect the Notch signaling network in breast cancer, we showed that estrogen decreases Notch activation in ERα-positive breast cancer cells. Cells treated with estrogen deprivation or tamoxifen (tam) re-activate Notch signaling and are highly dependent on it

for survival. This makes them more sensitive to pharmacological Notch inhibitors. Treatments with γ-secretase inhibitors (GSIs) potentiate the effects of tam in T47D xenografts (Rizzo et al. Cancer Res 2008). It is unknown whether GSIs plus endocrine therapy result in modulation of Notch and other proliferation markers in human breast cancer. Our objective was to add short exposure of the GSI MK-0752 to ongoing tam or letrozole (letr) during the presurgical window to determine 1) feasibility, 2) safety/tolerance, and 3) impact on biomarkers. We report the initial cohort of this pilot study (ClinTrials.gov NCT00756717). Patients (pts) with early stage ERα+ breast cancer were treated with 25 days of tam or letr. On day 15 MK-0752 was added to endocrine therapy (350 mg orally 3 days on, 4 days off, 3 days on), with definitive surgery day 25.
Formalin fixed, paraffin embedded biopsies were obtained at baseline, day 14 and final surgery, with histologic confirmation of tumor content >50% and RNA extraction by standard methods.
Q-PCR was done for Notch1, Notch3, Notch4, Deltex, Jagged1, c-myc, HEY1, HEY2, HES1, PS2, C-Myc, Cyclin A2, NOXA (pro-apoptotic protein), Ki67, Dicer-1, RPL13 (internal control). Ct averages for 3 replicates were used and mRNA levels were calculated by the 2ΔΔCt method. Baseline gene expression levels were used as comparators for days 14 and 25 levels in each pt. The first cohort of 10 pts was analyzed to determine if enough signals were present to justify expanding the cohort at this dose to 20 pts and possibly test a second cohort an alternate MK-0752 dose/schedule.

The initial cohort of 10 pts completed all therapy (4 tam, 6 letr), all biopsies and definitive surgery on schedule. One other pt withdrew prior to starting MK-0752 due to hypertension. Toxicity was minimal: grade 1 periorbital edema/cough, nausea, and axillary paresthesias in 1 pt each; grade 1 facial rash, 2 pts; and grade 2 fatigue, 1 pt. There was no diarrhea or surgical complications. Significant changes occurred in molecular marker levels after MK-0752 plus tam/letr (day 25) vs. end of tam/letr alone (day 14) as follows: Ki67 mRNA decreased in 9/10 pts; Notch4 decreased, 10/10; NOXA increased, 6/10; and Notch1 decreased, 6/10. Other markers showed inter-individual variations.

The preliminary results from the second cohort are consistent with these findings. The addition of a short exposure of the GSI MK-0752 to ongoing endocrine therapy was feasible, safe, and well tolerated in pts with ERα+ early breast cancer prior to definitive surgery. It results in anti-proliferative and pro-apoptotic effects at the molecular level. Notch4, which plays a key role in breast TIC, was the most consistent molecular marker of response in this setting. This suggests a potential anti-TIC effect of this combination and a role in overcoming endocrine resistance. “Canonical” Notch target genes such as HES1 or HEY1 are not likely to be pharmacodynamically informative when measured in ERα-positive tumors treated with endocrine therapy. Notch targets specifically identified in breast cancer cells, such as Notch-4 and NOXA, and additional candidates emerging from gene expression profiling, are more likely to be pharmacodynamically informative and mechanistically relevant in clinical studies studies with GSIs in this clinical setting. Accrual to the expanded cohort is now complete. If findings are confirmed, the second study with alternate MK-0752 dose/schedule may commence.
Funding: Swim Across America, Inc. (clinical trial costs); Merck (drug supply, profiling)

8.00-10.00 p.m. Welcome Reception Cocktail

SESSION 2
Chairman: Ronan O'Hagan

9.00-9.30 a.m.

Resistance to anti-angiogenic therapy induced by Notch

Adrian Harris, Department of Medical Oncology, Churchill Hospital, UK

Hypoxia is a major driver to tumour angiogenesis, inducing vascular endothelial growth factor, VEGF, and many other growth factors. Bevacizumab has shown activity in early and late recurrent breast cancer, enhancing the effectiveness of chemotherapy in delaying disease progression but resistance is common. This may be either *de novo* with failure to respond at all to initial therapy or may be induced during treatment, and they are likely to have different mechanisms.

We have completed a clinical trial of neoadjuvant Bevacizumab in a window study before neoadjuvant chemotherapy and this shows three patterns of response to Bevacizumab; a clear reduction in tumour vascularity, permeability and perfusion evenly across the tumour, a pattern of reduction of perfusion and permeability but increase in central necrosis and thirdly no response at all. We think these may reflect different types of resistance. We have developed in vivo models for each type and show that upregulation of notch ligands, such as delta-like 4 [Dll4] in the tumour, can change the biology of the endothelial cells making them resistant to anti-VEGF therapy. This can be reversed with notch inhibitors and recently we have shown that this is true with Ephrin B2 blockade. Our analysis of a randomised trial of bevacizumab in breast cancer showed Dll4 expression was associated with resistance clinically. However, little is still known about the mechanisms/genes involved in the downstream responses following Dll4/Notch signalling, their subsequent role in tumour vascular biology and how they are coupled to other angiogenic signalling pathways. Our aim is to investigate the roles of Jag1 versus Dll4, to identify and analyse these downstream genes and mechanisms, to understand their clinical relevance and potential as therapeutic targets. Our data show both stimulte tumour growth and angiogenesis, but different types of vasculature.

Deep sequence analysis (CAGE array) of Human Umbilical Vein Endothelial Cells (HUVEC) stimulated with human recombinant tethered Dll4 (rhDll4) for 16h was validated by real time QPCR in HUVEC and a glioblastoma cell line U87. RHOQ was observed to be up-regulated in HUVEC cells only. Loss of expression of RHOQ in HUVEC reduced sprouting in the hanging drop assay. *In situ* expression profiles show these genes are expressed in blood vessels in zebrafish. Thus this is a new downstream pathway mediating notch signalling.

Thursday, June 23rd, 2011

9.30-10.00 a.m.

The role of the DllA-Notch pathway in tumour angiogenesis

Frank Kuhnert, Regeneron Pharmaceuticals, USA

Delta-like ligand 4 (Dll4) is an emerging anticancer target given its vasculature expression and its role in regulating tumor angiogenesis. We have previously demonstrated that pharmacological blockade of Dll4-Notch signaling results in an excessive production of aberrant non-functional tumor vessels, and these changes were associated with reduced tumor growth. Using VelocImmune® mice, we identified a fully human IgG1 monoclonal antibody, termed REGN421/ SAR153192, which binds human Dll4 and potently neutralizes Dll4-Notch signaling. In mice bearing established human tumor xenografts, administration of Dll4 antibody caused potent dose-dependent inhibition of tumor growth, which was associated with a marked decrease in tumor perfusion as early as 24 hours post treatment. Combined treatment with Dll4 antibody plus various standard chemotherapeutic agents significantly enhanced anti-tumor effects in several xenograft tumor models compared to single agents. Further, co-administration of Dll4 antibody with an inhibitor of VEGF (aflibercept, VEGF Trap) significantly inhibited the growth of tumor xenografts and also induced a greater decrease in tumor perfusion compared to single agent administration. Histologically, the combined blockade of Dll4 and VEGF resulted in a hybrid tumor vascular phenotype characterized by areas of vessel pruning and aberrant vessel branching, respectively. These studies lend further support for the therapeutic targeting of Dll4 as a promising new angiogenesis-based anticancer strategy, particularly in combination with chemotherapy or anti-VEGF agents. REGN421/ SAR153192 is currently under investigation in a Phase 1 study in patients with advanced solid tumor malignancies.

10.00-10.30 a.m.

Targeting tumor angiogenesis with Notch ligand inhibitors

Jan Kitajewski, Columbia University, USA

The concept that blood vessel recruitment is essential for tumor growth helped initiate rationale therapeutic targeting of cancer with anti-angiogenic agents. From that initial concept to the present day, an appreciation of the diverse and complex mechanisms of tumor angiogenesis has evolved. This complexity is evident from studies of the Notch signaling pathway and recent findings show that Notch regulates tumor angiogenesis by diverse mechanisms. Notch is fundamental to proper cardiovascular development, playing prominent roles in arterial-venous specification and endothelial tip cell selection during sprouting angiogenesis. Two key Notch ligands, Delta-like 4 and Jagged1, have been implicated in both developmental and tumor angiogenesis. Inhibition of Dll4-mediated Notch signaling in tumors results in hyper-sprouting of non-functional vasculature. This Dll4 inhibition may paradoxically lead to increased angiogenesis but poor tumor growth because the newly growing vessels are not functional. In contrast, Jagged1 has been described as influencing angiogenesis in a variety of ways.
Endothelial Jagged1 may act as a non-functional Notch ligand, interfering with Dll4/Notch signaling, and thus opposing the sprout restricting activity associated with Notch. Jagged1 can also be expressed on tumor cells, where it can have a positive influence on tumor angiogenesis. To explore the potential for targeting Notch in tumors we discuss a Notch inhibitor, the Notch1 decoy, which blocks both Dll4 and Jagged1. The Notch1 decoy is composed of the signal peptide and EGF-like repeats of Notch1 fused in frame with Fc fragment of human IgG and has been shown to block Notch1 signaling mediated by either Dll1, Dll4, or Jagged1. This pan-Notch ligand inhibitor can disrupt tumor angiogenesis and growth in several murine tumor models and the activity of this inhibitor illustrates the utility of inhibiting several Notch ligands in the tumor microenvironment. Notch1 decoy use results in reduced vascular growth without eliciting the hypersprouting phenotype typical of Dll4 blockade. Variants of the original Notch1 decoy design have been developed that allow for selective inhibition of Notch ligands and there utility will be described.

10.30-11.00 a.m. Coffee break and Poster review

SESSION 3
Chairman: Robert Clarke, University of Manchester, UK

11.00-11.30 a.m.

Modulation of Notch receptor signalling by extracellular proteolysis

Marc Vooijs, Maastricht University, the Netherlands

Notch receptors are type-I transmembrane receptors that interact with membrane bound ligands on adjacent cells. Notch signaling is triggered by unfolding of the extracellular

domain which triggers proteolytic cleavage leading to receptor activation. The extracellular S2 cleavage, which removes the entire extracellular domain, is one of the rate-limiting steps and is executed by ADAM metalloproteases. Others and we previously identified ADAM10 as one of the proteases implicated in ligand dependent Notch1 signaling. In ligand independent Notch signaling other proteases are also implicated. We have developed several in vitro (peptide based) and in vivo approaches to monitor and inhibit cleavage of Notch1. We have extended this analysis to S2 regulation of other Notch receptors of which virtually nothing is known. Here we present new mechanistic insights into the regulation of Notch S2 cleavage and its importance for Notch activation in normal and cancer cells.

Thursday, June 23rd, 2011

11.30-12.00 a.m.

Notch signalling and goblet cell differentiation in cancer stem cells from colorectal cancer derived cell lines

Walter Bodmer, Oxford University, UK

We have shown that single cells derived from certain colorectal cancer cell lines can initiate tumors *in vivo* and differentiate into all of the lineages found in the original cancer, and thus have all the features expected for Cancer Stem Cells(CSCs). Our results further suggest that the homeobox gene CDX1 and Notch ligands, respectively and separately, control the differentiation of CSCs into columnar and goblet cells. Thus, notch pathway inhibition stimulates the development of goblet cells from CSCs. Hypoxia blocks CSC differentiation, but does not prevent notch inhibition from stimulating the differentiation of CSCs into goblet cells.This indicates that the hypoxic block acts up stream of the effects of Notch inhibition. Similar results for the action of the CDX1 gene indicate that this also acts upstream of the hypoxic effect and that the two pathways of differentiation from CSCs diverge at some time after the initial division of the CSCs.

12.00-12.30 p.m.

Gamma-secretase inhibitors and gemcitabine target tumour vasculature in pancreatic cancer

Natalie Cook, CRUK and Merck USA

Pancreatic ductal adenocarcinoma (PDA) is a highly lethal disease that is refractory to medical intervention. Notch pathway antagonism prevents pancreatic tumorigenesis in

mouse models, but potential benefits in the setting of an established tumor are unknown. We demonstrate that the gamma secretase inhibitor MRK003 effectively inhibits intra-tumoral Notch signalling in the KPC mouse model of advanced PDA. While MRK003 monotherapy fails to extend the lifespan of KPC mice, the combination of MRK003 with the chemotherapeutic gemcitabine prolongs survival. Combination treatment synergistically kills tumor endothelial cells to decrease the vascular patency and density and cause widespread hypoxic necrosis. These results indicate that the pauci-vascular nature of PDA can be exploited as a therapeutic vulnerability, and the dual targeting of the tumor endothelium and neoplastic cells by gamma secretase inhibition constitutes a rationale for clinical translation.

1.00-2.00 p.m. Lunch

2.00-3.00 p.m. Open Air workshops:

1.Notch Targeting. Good, bad or ugly? Walter Bodmer & Lucio Miele
2. Which Clinical Targets? Charles Coombes & Adrian Harris

SESSION 4
Chairman: Spyros Stylianou

4.30-4.50 p.m.

Notch activation in mammary tissue and breast cancer

Sean Egan, The Hospital for Sick Children, Canada

Basal breast cancer has been associated with mutations in a number of specific tumor suppressor genes, however, the mechanism by which these tumors express a basal lineage remains unknown. Notch signalling suppresses mammary stem cell (MaSC) self-renewal, while promoting luminal cell fate specification. We report that Lfng, a sugar transferase that facilitates Notch activation, suppresses mammary stem/bipotent progenitor cell proliferation. Targeted deletion of Lfng in mammary epithelium induces basal tumors with reduced expression of Notch targets, amplification of the Met/Caveolin gene locus, and elevated Met and Igf-1R signaling. Human basal breast cancer, a disease associated with elevated MET receptor signaling and Caveolin protein, express low levels of LFNG. Thus, reduced LFNG expression cooperates with a Met/ Caveolin amplicon to promote basal breast disease.

4.50- 5.10 p.m.

Role of the PI3K/Akt signaling in T-ALL: Activation by Notch via IGF1R

Andrew P. Weng, BC Cancer Research Centre, Vancouver, Canada

T-cell acute lymphoblastic leukemia (T-ALL) is an aggressive cancer of immature T cells that often shows aberrant activation of Notch1 and PI3K/Akt pathways. PTEN mutations that activate PI3K/Akt signaling have previously been reported to relieve T-ALL cells of their dependence on Notch signaling, thus raising doubts as to the potential efficacy of Notch inhibitor therapy. Using a genetically defined mouse model, we demonstrate that primary mouse T-cell leukemias remain dependent on Notch signaling despite Pten loss, with or without additional deletion of Ink4a/Arf. We also observe no correlation between Notch-independence and PTEN loss in a panel of primary human T-ALL samples. These data suggest genetic alterations other than PI3K/Akt activation are required to confer resistance to Notch inhibition.

Despite the prevalence of mutations in PTEN, PI3K, and Akt which constitutively activate signaling, the relative contribution of growth factor-dependent pathway activation is unclear. We report that pharmacologic inhibition or genetic deletion of IGF1R blocks the growth and viability of T-ALL cells, but that moderate diminution of IGF1R signaling compromises leukemia-initiating cell (LIC) activity as defined by transplantability in syngeneic/congenic secondary recipients. Furthermore, IGF1R is a Notch1 target and Notch1 signaling is required to maintain IGF1R expression at high levels in T-ALL cells. These findings suggest Notch effects on LIC activity may be mediated in part by enhancing the responsiveness of T-ALL cells to ambient growth factors, and provide strong rationale for use of IGF1R inhibitors to improve initial response and to achieve long-term cure of patients with T-ALL.

5.10-5.30 p.m.

Notch signalling and angiogenesis in primary breast cancer

Panagiota Touplikioti, Theagenion Cancer Hospital and Aristotle University of Thessaloniki, Greece

Notch signaling is an evolutionary conserved pathway that plays an important role in stem cell biology, tumor formation, angiogenesis and cell fate decisions. VEGF is a key mediator-regulator of breast tumor angiogenesis. Preclinical studies have indicated that Notch signaling plays a pivotal role in the control of vascular morphogenesis during development and in tumor angiogenesis. In the present study we assessed the expression of Notch ligands and receptors and VEGF in primary breast cancer specimens and evaluated their correlation to pathologic features. We analyzed the mRNA expression of Notch1-4 DLL1, 3, 4, Jagged1, 2 and VEGF in 200 fresh-frozen breast tissues. Gene expression was evaluated with qReal-time PCR assays with beta-actin as reference gene. A pool of five normal breast specimens from reduction mammoplasty was used for evaluation of controls mRNA

expression. Data analysis was done using the $\mathbf{2}^{-\Delta\Delta Ct}$ method. Statistically significant differences were established in Notch1 ($p=0.005$) and Notch3 ($p=0.023$) expressions only in triple negative (TN) tumors. Notch4 expression was higher in hormone receptor (HR) positive tumors ($p=0.055$). HR+ve tumors was also significantly associated with Jagged1 ($p<0.001$) and DLL4 ($p<0.01$). VEGF expression was higher in TN tumors ($p=0.08$). We found a strong positive correlation between the variables Notch1 and Notch4 in all subgroups except for TN tumors.
Only in HR+ve tumors there was a strong positive correlation between the variables VEGF and DLL4. Notch receptors and ligands are differentially expressed in breast cancer subgroups. Notch1 and Notch3 seem to play an important role in TN oncogenesis. Notch4, DLL4 and Jagged1 seem to be implicated in HR+ve oncogenesis. Although TN tumors have enhanced angiogenesis and high levels of VEGF this does not seem to be regulated by Notch signaling.

5.30-5.50 p.m.

Kinome screening for regulators of the estrogen receptor identifies LMTK3 as a new therapeutic target in breast cancer

Giorgios Giamas, Imperial College London , UK

Therapies targeting estrogen receptor (ER, encoded by *ESR1*) have transformed the treatment of breast cancer. However, large numbers of women relapse, highlighting the need for the discovery of new regulatory targets modulating ER pathways. An siRNA screen identified kinases whose silencing alters the estrogen response including those previously implicated in regulating ER activity (such as mitogen-activated protein kinase and AKT). Among the most potent regulators was lemur tyrosine kinase-3 (LMTK3), for which a role has not previously been assigned. In contrast to other modulators of ER activity, LMTK3 seems to have been subject to Darwinian positive selection, a noteworthy result given the unique susceptibility of humans to ER+ breast cancer. LMTK3 acts by decreasing the activity of protein kinase C (PKC) and the phosphorylation of AKT (Ser473), thereby increasing binding of forkhead box O3 (FOXO3) to the *ESR1* promoter. LMTK3 phosphorylated ER, protecting it from proteasomal degradation *in vitro*. Silencing of LMTK3 reduced tumor volume in an orthotopic mouse model and abrogated proliferation of ER+ but not ER− cells, indicative of its role in ER activity. In human cancers, LMTK3 abundance and intronic polymorphisms were significantly associated with disease-free and overall survival and predicted response to endocrine therapies. These findings yield insights into the natural history of breast cancer in humans and reveal LMTK3 as a new therapeutic target.

8.00 p.m. Gala Dinner

SESSION 5
Chairman: Aleksandra Filipović

9.00-9.30 a.m.

Top-Notch Targets: Progress and Challenges in Developing Notch Antibody Therapeutics

Robert Hollingsworth, MedImmune, USA

The four human Notch receptors play important but distinct roles in normal physiology and pathogenesis. The efforts of multiple laboratories, including our own, have begun to elucidate various different functions of the Notch receptors. The expression of the genes for these receptors and pathway perturbation experiments suggest non-overlapping activities in the differentiation and development of various embryonic and adult tissues. Among other diseases, aberrant Notch function contributes to multiple cancers, and so the receptors and their ligands are attractive therapeutic targets. The four Notch receptors also play different roles in stem cell proliferation and function, and Notch hyperactivation has been implicated as a driver in some cancer stem cells. MedImmune is studying the Notch receptors and their ligands to better understand their different contributions to cancer, and is applying expertise in antibody generation to develop research tools and candidate drugs. My talk will describe our progress and some of the challenges we've faced in dissecting mechanisms, validating targets, and developing therapeutic antibodies for the various Notch pathways.

9.30-10.00 a.m.

Development of therapeutic antibodies targeting Notch 3 for the treatment of lung cancer

Stacey Huppert, Vanderbilt University Medical Center, USA

Substantial evidence from cell culture, preclinical and clinical studies support an oncogenic role for Notch signaling in multiple human cancers. Notch signaling is evolutionarily

conserved as a mechanism for regulating cell lineage diversification and stem cell maintenance. Therefore, activation of Notch signaling can disrupt the normal balance of a cell's maturation leading to the initiation and progression of cancer. Notch3 is overexpressed in about 40% of non-small cell lung cancer (NSCLC) and reduced Notch3 activity results in the loss of malignant phenotype both *in vitro* and *in vivo*. Therefore, we hypothesize that development and optimization of therapeutic strategies that specifically target the individual Notch3 receptor will be a beneficial addition to the treatment of NSCLC.

The pharmacological gamma-secretase inhibitors (GSIs) that have progressed for use in the clinic to inhibit Notch activity are not optimal as they unselectively block proteolysis of many substrates and cause significant Notch1- and Notch2-dependent gastrointestinal toxicity. Here we report the early results of a strategy to inhibit Notch3 signaling through the development of Notch3 monoclonal antibodies. Using a peptide library, we discovered two ligand-binding regions in the Notch3 extracellular domain. Recombinant proteins representing these two regions, respectively, were used as antigens to immunize mice. We have demonstrated that antisera from these mice immunized with portions of the receptor extracellular domain inhibit Notch3 activation. Hybridoma clones were screened with ELISA, immunoprecipitation and their ability to inhibit Notch3 GSI-dependent cleavage. Six hybridoma clones were found to inhibit Notch3 activation, 5 of which are IgG isotype and one is IgM isotype. These antibodies can also reduce Notch downstream targets, *Hes1* and *Hey1* mRNA expression. Further testing is ongoing to validate these findings as well as to determine affinity and anti-tumor activity of these antibodies.

10.00-10.30 a.m.

NOTCH3 Signaling is Essential for Neuroblastoma Cell Survival

Tim van Groningen, Alvin Chan, Jan Koster, Peter van Sluis, Rogier Versteeg and **Johan van Nes**
Department of Oncogenomics, Academic Medical Center, Meibergdreef 9,
PO box 1105 AZ Amsterdam, The Netherlands

Neuroblastoma is a paediatric tumour of the peripheral sympathetic nervous system with a variable prognosis ranging from spontaneous regression to aggressive metastatic disease. Despite intensive treatment regimens, tumours often relapse and become refractory to therapy, urging the identification of molecular pathways essential for cell survival and suitable for therapeutic intervention. The NOTCH pathway plays a key role in proliferation, differentiation and apoptosis in normal development and cancer. After ligand binding, NOTCH receptors are proteolytically cleaved by the γ-secretase complex.
This allows translocation of the NOTCH intracellular (IC) fragment to the nucleus, where it acts as a transcription factor. We performed mRNA expression profiling of a series of 88 human neuroblastoma tumours. NOTCH receptors, -ligands, -co-activators and –modifiers

are expressed in primary neuroblastomas. High expression of the NOTCH3 receptor was associated with poor outcome. Silencing of NOTCH3 by shRNA resulted in apoptosis in all neuroblastoma cell lines tested. This phenotype could be rescued by inducible expression of a NOTCH3-IC transgene. In addition, silencing of the γ-secretase complex member nicastrin triggered apoptosis in all tested neuroblastoma cell lines, which could be partly rescued by NOTCH3-IC. Targeting of the NOTCH pathway and the γ-secretase complex by treatment of neuroblastoma cells with γ-secretase inhibitors reduced NOTCH3-IC signalling. These findings prioritize the γ-secretase complex and the NOTCH3 pathway as targets for pharmacological inhibition in neuroblastoma.

10.30-11.00 a.m.

Antibodies against Notch receptors

Ronan O'Hagan, AVEO Pharmaceuticals, USA

AVEO has developed a series of inducible mouse models of cancer which, through the preservation of critical tumor/stromal interactions, facilitate identification of cell-surface and secreted proteins that represent viable targets for therapeutic antibodies and other biologics. Functional genetic screens performed *in vivo* in these models identified the Notch pathway as a critical regulator of tumor maintenance. This finding is consistent with emerging evidence that activation of Notch signaling via receptor point mutation, receptor amplification, and elevated receptor and ligand expression, plays a key role in various human cancers. Moreover, the Notch pathway controls diverse aspects of tumorigenesis and tumor maintenance, regulating tumor autonomous processes and interactions with the microenvironment, including angiogenesis. To further understand the role of the Notch pathway in tumor maintenance, and to assess the therapeutic potential of targeting the Notch pathway in cancer, we have generated monoclonal antibodies that inhibit various Notch receptors. Characterization of monoclonal antibodies targeting Notch1 or Notch3 through cell-based and biochemical studies demonstrated that these antibodies bound with high affinity and high specificity to the ligand binding domains of the Notch receptors, prevented ligand mediated activation of the target receptor, and specifically repressed Notch-dependent signaling with high potency. Effective inhibition of functional angiogenesis was observed upon anti-Notch1 antibody treatment in both *in vitro* and *in vivo* models. Significantly, specific inhibition of Notch1 by this antibody did not result in the dose-limiting gut toxicity observed with pan-Notch inhibitors such as gamma-secretase inhibitors. Humanized versions of the Notch1 monoclonal antibodies have been generated and characterized. Inhibition of tumor growth by the Notch3 monoclonal antibody was effected through tumor cell autonomous mechanisms. To identify tumors that are dependent upon tumor autonomous Notch signaling, gene expression profiles were correlated with Notch pathway dependence in human cancer cell lines. Expression of specific downstream targets was highly correlated with sensitivity of human cancer cell lines to inhibition of ligand-dependent Notch signal. This biomarker of Notch pathway

dependence successfully predicted that a subset of Kras mutant pancreatic and colon cancer cell lines would be highly sensitive to Notch pathway inhibition. Moreover, identification of Notch1 and Notch3-specific target genes further enables selection of tumors that will respond to monoclonal antibodies specifically targeting one or other, or both of these receptors.

11.00- 11.30 a.m. Coffee break and Poster review

SESSION 6
Chairman: Marc Vooijs

11.30-12.00 a.m.

Regulation of apoptosis in breast cancer by Notch signalling

Keith Brennan, University of Manchester, UK

We have shown that sustained Notch signalling is required to maintain the transformed phenotype in breast cancer cells. It does so, in part, by blocking apoptosis in breast epithelial cells in response to a wide range of stimuli, including DNA damage following treatment with a chemotherapeutic agent, growth factor withdrawal, and detachment from the extracellular matrix. Mechanistically, this appears to be through the activation of Akt by a Notch-induced secreted intermediate. The activation of Akt subsequently prevents apoptosis. For example, in response to DNA damaging chemotherapeutic agents, Akt prevents apoptosis in breast epithelial cells by phosphorylating and inhibiting ASK1. This prevents the subsequent activation of JNK and p53, and thus the accumulation of the pro-apoptotic proteins PUMA and Noxa. Recently, we have been able to show that this also occurs in vivo using a transgenic mouse strain that expresses an activated form of RBP-Jκ/CBF1 specifically in the mammary gland. These mice display phenotypes that suggest apoptosis is disrupted and develop high grade adenocarcinoma. Furthermore, cells isolated from these mice have a marked resistance to apoptosis.

12.00-12.30 p.m.

Computational modelling and molecular optimization of stabilised a-helical peptides targeting NOTCH-CSL transcriptional complexes

Raymond Moellering, Scripps, USA

Direct targeting of transcription factor complexes represents an attractive yet challenging opportunity for therapeutic intervention in many diseases. We recently demonstrated that hydrocarbon stapled α-helical peptides derived from the MAML1 co-activator are capable of interfering with NOTCH transcription factor complex (NTC) formation, leading to Notch pathway suppression *in vitro* and *in vivo*. However, many details surrounding the biophysics of NTC formation and stapled peptide structure activity relationships remain poorly understood. Furthermore, the utility of traditional medicinal chemistry approaches to improve stapled peptide activity is presently unclear. Here we employed all-atom molecular dynamics (MD) simulations to quantitatively describe the critical contacts mediating NTC formation and stability. These simulations identified the residues in MAML1, NOTCH1 and CSL that contribute strongly to complex formation as well as underutilized contacts that might be optimized by introduction of alternative amino acids. Combining these insights with the development of a stapled peptide allatom MD model, we computationally identified several promising residues for optimization in existing and novel stapled peptide scaffolds. Characterization of resulting natural and non-natural amino acid- containing stapled peptide analogs with a novel NOTCH complex formation competition assay identified several classes of analog peptides with improved biochemical potency. Subsequent testing in reporter assays and NOTCH1-driven human acute lymphoblastic leukemia cell lines revealed that several optimized stapled peptides mimicking the E21-T36 and E21-Y41 MAML1 contact surfaces are improved inhibitors of NOTCH-driven transcription and cellular proliferation. These results provide insights into the biophysics of NTC formation, stapled peptide structure activity relationships and demonstrate the application of MD simulations for the design of stapled peptides.

12.30-1.00 p.m.

Targeting of Mastermind

Agamemnon Epenetos & Spyros Stylianou, Imperial College London, UK, and Trojantec, Cyprus

It has been widely demonstrated that the Notch receptor signaling pathway is required for regulation and differentiation of stem cells in many cell types. It is also well established that aberrant Notch signaling can lead to several human forms of cancer, including leukaemia, breast, colon, skin, cervical, and lung cancer, making Notch an attractive target for new cancer therapies. One of the major therapeutic targets in the Notch pathway is the cleavage of four Notch receptors, in which gamma-secretase inhibitors play a role. Unfortunately inhibition of γ-secretase complex can be accompanied by significant side effects, indicating that a more selective approach to Notch inhibition is desired. We report on the development of a new class of therapeutic proteins that interfere with intracellular targets in both cancer cells and cancer stem cells ("CSC"). We used a delivery platform based on Drosophila transcription factor Antennapedia (ANTP) which allows for intracellular and intranuclear delivery as well as enabling the transport of agents across

the blood-brain barrier. We have genetically engineered a fusion protein, consisting of the *Drosophila* transcription factor Antennapedia (ANTP) and the truncated version of Mastermind-like (MAML) that behaves in a dominant negative (DN) fashion and inhibits the Notch transactivation complex. This fusion protein, (ANTP/DN-MAML) or TR4, has been tested for its ability to target tumor cells in vitro and *in vivo.* TR4 has been shown to: i) have a cytotoxic effect at clinically meaningful IC50; ii) act and inhibit specifically the Notch pathway at the nuclear level; iii) be stable when stored & freeze/thawed. In summary, TR4 translocates into the nucleus, targets Notch signaling and suppresses Notch activation, leading to inhibition of tumor growth in mice, without significant organ or systemic toxicity.

1.00-2.00 p.m. Lunch

2.00-3.00 p.m.
Open Air workshop:

1.Notch Targeting. Good, bad or ugly? Robert Clarke & Lucio Miele
2. Which Clinical Targets? Charles Coombes & Adrian Harris

SESSION 7
Chairman: Agamemnon Epenetos

4.30-4.50 p.m.

Notch signalling mediates myofibroblast differentiation of carcinoma-associated fibroblasts

Ahmet Acar, University of Manchester, UK

Carcinoma-associated fibroblasts (CAFs) consist of large proportions of myofibroblasts, a hallmark of activated fibroblasts often found in wound healing and fibrosis. We recently developed human immortalised breast CAFs, designated experimentally generated CAFs (exp-CAFs), by extracting them from human mammary tumour xenografts. These cells stably maintain their tumour-promoting myofibroblastic phenotypes during the propagation in vitro. Such phenotypes are mediated by the establishment of TGF-β and SDF-1 autocrine signalling in a self-sustaining fashion acquired during tumour progression. The tissue remodelling processes involved in wound healing and fibrosis physiologically resemble those occurring within the tumour-associated stroma. Given crucial roles of Notch signalling for promoting myofibroblast differentiation during the tissue remodelling, we hypothesized that activation of this signalling pathway may contribute to give rise to CAFs in the tumour-associated stroma.
To assess this possibility, we have checked expression levels of various Notch ligands and their receptors in exp-CAFs. Here we show that these cells express increased levels of Notch ligands

Jagged1 (Jag1) and Delta-like 4 (Dll4), and their receptors (Notch 1-3). These Notch ligands present on the signal-sending CAFs are also required for transactivation of Notch3 expressed on the signal-receiving CAFs. Importantly, this ligand-activated Notch3 stimulates the canonical Notch signalling, as exemplified by increased expression of Hes-1 and Notch intracellular domain 3 (NICD3). Activation of Notch signalling also collaborates with TGF-β signalling to further boost myofibroblast differentiation in CAFs. Moreover, immunohistochemistry reveals that stromal myofibroblasts within human invasive breast carcinomas are positively stained for Jag1, Dll4, and NICD3. Collectively, these findings suggest that both Notch and TGF-β signalling are crucial for generation and maintenance of myofibroblasts within the tumour-associated stroma.

4.50-5.10 p.m.

Intranuclear targeting of Notch with Monoclonal Antibodies

Silvia Colucci, Imperial College London ,UK

Using the cell-penetrating shuttle protein antennapedia (Antp), a dominant-negative version of Mastermind-like 1 (MAML-1) can be transported intracellularly, leading to inhibition of Notch signalling both *in vitro* and *in vivo*. Notch expression is deregulated in several cancers and has implications in cancer stem cell maintenance.
Based on this innovative approach, we aim to show that intracellular signalling can be interfered using a universal approach of delivering membrane-translocating antibodies and/or ScFv as exemplified in this study of an antennapedia-ScFv fusion directed at the Notch signalling pathway. The strategy for signalling inhibition consists of blocking tripartite complex formation by targeting MAML-1 interactions with NIC and CSL. So far, research was focused on generating the essential tools including production of Antp and selection of a specific Notch pathway inhibitory scFvs through phage display. This novel approach of signalling inhibition, consisting of targeting intracellular/intranuclear pathways with antennapedia-transported antibodies (fragments), may provide effective tissue and cellular penetration leading to improved efficacy of existing antibody therapies as well as opening up new therapeutic avenues of using antibodies directed against intracellular and intranuclear targets.

5.10-5.30 p.m.

Biological and clinical implications of nicastrin expression and function in breast cancer

Aleksandra Filipović, Imperial College London, UK

Nicastrin is an essential component of the gamma secretase (GS) enzyme complex, required for its synthesis and recognition of substrates for proteolytic cleavage. The purpose of this

study was to investigate whether nicastrin has prognostic value or potential as a therapeutic target in breast cancer (BC). Tissue microarrays (TMAs) (n = 1050), and BC cell line analysis confirmed that nicastrin expression was up-regulated in BC compared to normal breast cells. In TMA patient samples, high nicastrin expression was observed in 47.5% of cases and correlated with worse BC specific survival in the ERα negative cohort. *In vitro* transient and stable gene silencing of nicastrin resulted in disruption of the GS complex activity and a decrease in Notch1 cleavage and Akt pathway activation. Nicastrin silencing in invasive MDA-MB-231 and HCC1806 cells resulted in loss of vimentin expression and a marked reduction in cell invasion; which was concomitant with the de novo formation of cell-cell junctions, as well as cellular repolarisation. In a population of breast cancer cells harboring the cancer stem cell $CD44^{+}/CD24^{-}$ phenotype, nicastrin depletion abrogated expression of EMT-markers vimentin, SIP1 and Snail. These data indicate that nicastrin can function to maintain epithelial to mesenchymal transition during breast cancer progression. In order to dissect the role of nicastrin within the GS complex from its presumed independent signaling role in breast cancer cells, and thereby answer the question of why we think that inhibiting nicastrin using a monoclonal antibody will be beneficial even though GS inhibitors (GSI) already exist, we have performed a full genome array and identified a subset of genes that are nicastrin-dependent and are not affected by silencing the Notch receptors. We have developed anti-nicastrin polyclonal and monoclonal antibodies and have shown that they are able to decrease the proliferative and invasive capacity of breast cancer cells *in vitro*. This supports our hypothesis that a nicastrin blocking antibody could be used to limit metastatic dissemination in invasive breast cancer.

5.30 p.m. Farewell

Agamemnon Epenetos

POSTER PRESENTATIONS

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

Incorporation of Dll4 into exosomes and their Effects on Notch Signalling and Angiogenesis.

Helen Sheldon, Emily Heikamp, Helen Turley, Rebecca Dragovic, Russell Leek, Benedikt Kessler, Ian Sargent, Ji-Liang Li, and Adrian L. Harris
Cancer Research UK, Department of Molecular Oncology, University of Oxford, Oxford, UK, OX3 9DS

The Notch ligand Dll4, has an important role in angiogenesis. It is up-regulated in tumour vasculature and is therefore an attractive therapeutic target. Dll4-Notch signalling is thought to require cell-cell contact, however we have recently described a mechanism by which Dll4 can inhibit Notch signalling via its incorporation into exosomes. Consistent with this inhibitory function, Dll4-exosomes can increase vessel branching and vessel density *in vitro* and *in vivo*. Interestingly these exosomes can transfer Dll4 to other cells incorporating it into their cell membrane. They also decrease the level of Notch receptor in endothelial cells resulting in a tip cell phenotype with a high Dll4/Notch ratio and increased filopodia. This transfer of Dll4 also occurs *in vivo* from tumour cells to host endothelium. Dll4 was detected in red blood cells in the xenograft model, which suggests that the Dll4-exosomes are entering the circulation. Dll4 has been detected in the lung and liver of the xenograft models supporting this theory. This is under further investigation as it may have important

implications in metastasis by enhancing the angiogenic potential of other tissues. Dll4-exosomes are detectable in the plasma of breast cancer patients. Dll4 is an endothelial specific protein and therefore could be used to specifically capture endothelial exosomes. We have initiated collaboration with Caris Life Sciences to develop a diagnostic assay for breast cancer. This utilises their Carisome Platform ™, which detects circulating microparticles in plasma and determines the level of exosome components to link them with disease. To optimise the capture and detection of endothelial exosomes we have exposed endothelial cells to angiogenic treatments (hypoxia, FGF, VEGF and Notch signalling) and analysed their protein content using quantitative/comparative mass spectroscopy. Hypoxic exosomes contain a large number of enzymes/proteins involved in energy metabolism (including PFK1, PKLR, AK1, ALDOA, ATP5A1 and SLC25A6), FGF treatment incorporates ephrin receptors (EPHA2, B2, B3 and B4) and Notch signalling via JAG1 increases the number of G-protein signalling molecules (including gustducin and transducin). Some proteins are present under all angiogenic treatments and may be a useful marker of angiogenesis (e.g POTEF). Changes in endothelial exosomes may report on the endothelial cells environment (e.g. the presence of EPHB suggests FGF stimulation) and help with the choice of anti-angiogenic therapy. They may also be an important tool to monitor active angiogenesis and the effectiveness of anti-angiogenic therapies.

NOTCH signaling induces Delta Like-4 expression in endothelial cells through a positive autoregulatory loop.

Vincenza Caolo[1], Nynke M.S. van den Akker [2], Sanne Verbruggen[1], Geertje Swennen[1] , Mark J. Post[1], Daniel G.M. Molin[1]
[1]Dept. of Physiology, [2]Dept. of Cardiology, CARIM, Maastricht University, the Netherlands

Delta like-4 (DLL4) belongs to the conserved NOTCH-family and is specifically expressed in endothelial cells (EC). DLL4 expression is increased by VEGF-A and regulates crucial processes in vascular growth. In the present study we show that VEGF-A induced DLL4 expression is NOTCH-dependent. NOTCH-signaling blockage with γ-secretase and ADAMs inhibitors abolished the positive effect of VEGF-A on DLL4. Similar to VEGF-A but independent of it, recombinant DLL4 itself stimulated NOTCH-signaling, resulting in up-regulation of DLL4. Stimulating NOTCH-signaling in human cardiac microvascular ECs (HCMvECs) by NOTCH Intracellular Domain (NICD)-1 or -4 underlined this positive effect. To discriminate between NICD/RBP-Jk and FOXC2 regulated DLL4 expression DLL4 promoter-activity was assessed in PAECs. DLL4 promoter analysis confirmed involvement of NOTCH/RBP-Jk signaling, whereas FOXC2 had no effect. To study NOTCH induced intercellular communication and propagation of DLL4 expression between cells, HCMvECs were transfected with DLL4-eGFP or eGFP and analyzed for endogenous DLL4 expression

by immunofluorescence. In contrast to eGFP-transfected cells, DLL4-eGFP ECs were able to increase DLL4 expression of non-transfected ECs. Our data provide evidence for a mechanism by which NOTCH-signaling up-regulates DLL4 independently of VEGF-A, and which, through an auto-regulatory loop, propagates its own expression and enables synchronization of NOTCH expression and signaling between ECs.

The role of Notch in non small cell lung cancer (NSCLC) and its implication in epidermal growth factor receptor (EGFR)

D. Kotsirilou[1,2], **E. Giannopoulou[1]**, E. Papadimitriou[2], T. Makatsoris[1] and H.P. Kalofonos[1]

[1]Clinical Oncology Laboratory, Division of Oncology, University Hospital of Patras, Patras Medical School, Rio 26504, Greece; [2]Molecular Pharmacology, Department of Pharmacy, School of Health Sciences, University of Patras, Rio, 26504, Greece

The Notch signaling pathway is evolutionary conserved and has crucial roles in the development and maintenance of embryonic and adult tissues. Recently, Notch has attracted the interest of many researchers regarding its role in cancer. Indeed, there are data for the role of Notch signaling in leukemia, breast, lung and cervical cancers. It has been shown that the functions of Notch pathway are highly cell-type dependent in different embryonic and adult tissues, as well as in cancers. Depending on the cell type, Notch may behave as an oncogene or a tumor suppressor gene.

Both behaviors for Notch have been described in lung cancer. It is suspected that Notch has a growth promoting function in NSCLC, whereas in SCLC exerts an inhibitory effect. Although, the importance of Notch pathway in lung cancer is under investigation, the contribution of EGFR signaling in lung cancer development is well established. So far, it has been found that the cross-talk between the Notch and EGFR signaling can function in either an antagonistic or synergistic fashion, depending on tissue and developmental context. The aim of this study is to investigate the interaction between the pathways of Notch and EGFR in human NSCLC cell lines. **Material and Methods**: *In vitro* experiments were performed on H23, H661, HCC827 and A549 human NSCLC cell lines. HCC827 cells express mutated EGFR and all the other cell lines express wild type EGFR. Notch pathway was blocked using DAPT, an inhibitor of gamma-secretase enzyme which participates in enzymatic cleavage/activation of Notch. Cell proliferation was determined using the MTT (methyl tetrazolium) assay. The Notch protein levels were determined using western blot analysis. **Results:** Notch was differentially expressed in human NSCLC cell lines: H23 and A549 cells express the maximum and the minimum levels of Notch protein, respectively, with H661 and HCC827 cells expressing median, equal amounts of Notch protein. DAPT decreased H23, H661 and A549 cell number in a concentration-dependent manner, 48 hours after its addition in cells but had no effect on the number of HCC827 cells. Furthermore, DAPT reversed the stimulatory effect of EGF in H661 proliferation in a concentration-dependent manner, an effect that was not observed in H23 and A549 cells. In H661 cells, DAPT decreased Notch protein levels 24 h after treatment. In the same cells, EGF activated Notch receptor 60 min after its addition in cells and this effect was reversed at basal levels 90 min

after EGF treatment. **Conclusion:** Previous data have shown that Notch interacts with molecules like ERK, mTOR and Wnt; however, this study is the first that presents indications of a possible direct interaction between EGFR and Notch signalling, at least in some types of NSCLC cells. Further research is required for the evaluation of Notch as a therapeutic target for the NSCLC.

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