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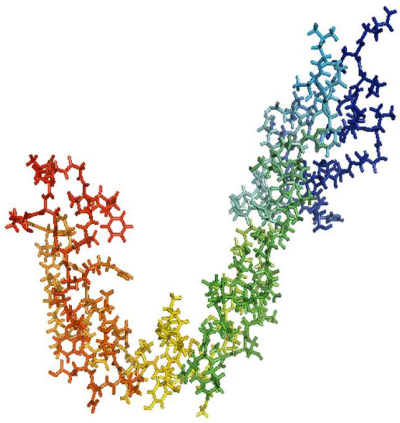

The 7th International Conference

Notch Targeting in Cancer

Grecian Park Hotel, Konnos Bay, Cyprus

14th- 16thJune 2017

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Wednesday 14th June 2017

4.00 - 4.30pm **Registration**

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

SESSION 1- Chairperson: Kim Dale

4.30 - 5.00pm Keith Brennan, University of Manchester, UK.

Highlights of the 6th Meeting, Notch Targeting in Cancer, Mykonos, June, 2016.

5.00 - 5.30pm Lluís Espinosa, Cancer Research Program, IMIM-Hospital del Mar PRBB Dr. Aiguader, 88, 08003, Barcelona. Spain
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Jagged1 addiction in intestinal cancer cells. Why is that important?

Delta ligands are required to induce Notch signalling in the intestinal stem cell (ISC) niche, whereas the Jagged1 ligand specifically activates Notch in intestinal adenomas carrying active β-catenin.
We will now show that intestinal-specific Jag1 deletion or antibody targeting of Jag1 prevented tumour initiation in the ApcMin/+ mouse model, tumour spheroid formation and tumour growth associated with reduced Notch and stem cell-related gene expression. Addiction of adenoma cells to Jag1 is likely associated with low levels of the glycosyltransferase Manic Fringe.

5.30 - 6.00pm Mateusz Antonszweski[1], Ute Koch[1], Tara Sugrue[1], Nadine Zangger[1], Christelle Dubey[1] & Freddy Radtke[1]
[1] Ecole Polytechnique Fédérale de Lausanne, Swiss Institute for Experimental Cancer Research, Lausanne, Switzerland
Tcf7 – A crucial cell lineage regulator and decisive mediator in Notch driven T-ALL

T-cell acute lymphoblastic leukemia (T-ALL) is an aggressive hematological cancer caused by the malignant transformation of immature thymocytes. Notch1 signalling is essential for normal thymopoiesis. *Notch1* gain-of-function mutations are present in >50% T-ALL cases resulting in the activation of Notch1 intracellular domain (N1ICD), leading to aberrant Notch1 signalling in thymocytes. However, the mechanisms by which deregulated Notch1 signalling leads to T-ALL development are only incompletely understood. One hallmark of Notch-induced T-ALL is the repression of non-T hematopoietic lineages. How Notch mediates lineage repression on the molecular level and whether this is important for T-ALL development is currently unknown.
T cell factor-1 (TCF-1) is a transcription factor encoded by *Tcf7*. As well as playing important roles in Wnt signalling, *Tcf7* (i) is a direct Notch1 target gene and (ii) is required for driving early T lineage commitment of hematopoietic progenitors. How TCF-1 implements T cell lineage commitment and whether it also plays

additional roles in non-T lineage repression remains elusive. Herein, we investigated whether *Tcf7* may function as a mediator of Notch1-driven T-ALL. We show that *Mx1*Cre-mediated conditional loss of *Tcf7* in hematopoietic progenitors prevents T-ALL development in both genetic- and retroviral-based mouse models of N1ICD over-expression. Interestingly, conditional *Tcf7* deficiency also partially restored the B lineage potential of N1ICD over-expressing bone marrow progenitors. Transcriptomic analysis of LSKs has revealed that NICD-expressing bone marrow progenitors fail to engage the T cell lineage program in the absence of *Tcf7*. In addition, ATAQ-seq. analysis indicates that TCF-1 is functioning together with Notch1 to influence the accessibility of regulatory regions within lineage specific genes.
Similar to *Tcf7*$^{-/-}$ mice, conditional *Tcf7* deficiency in bone marrow progenitors abrogated T cell development in adult thymi. However, unexpectedly, *Tcf7 deficient* hematopoietic progenitors adopted a B cell fate in adult thymi, a phenotype found to be comparable to conditional loss of *Notch1*. Overall, our results show that *Tcf7* is an important mediator of Notch1-driven T-ALL. Furthermore, our data suggest that, in addition to promoting T lineage commitment, *Tcf7* is also likely to function as a Notch1-dependent transcriptional repressor of B cell development in adult hematopoiesis. Thus, TCF-1 mediated lineage repression/specification seems to be part of and necessary for Notch mediated induction of T-ALL through regulating chromatin accessibility.

6.00- 6.30pm Jon Aster, Brigham and Women's Hospital, Boston, MA, USA.

Biomarkers of Notch Activation in Cancer

Alterations in Notch signalling have been implicated in many cancers, suggesting that Notch is an attractive therapeutic target; however, clinical trials of Notch inhibitors to date have been largely disappointing, initially because of toxicity but ultimately because of lack of response of most cancers. Emerging data from laboratories across the world indicate that Notch has widely varied roles in cancer, including oncogenic and tumour suppressive cell autonomous functions as well as non-cell autonomous effects on the tumour microenvironment, suggesting that use of biomarkers to identify Notch 'addicted" tumours is critical to enrich for cancers that are likely to respond to Notch inhibitors. As of this writing, trials of Notch inhibitors using biomarkers to select patients for trial entry have yet to

be performed. This talk will focus on clinically applicable biomarker tests that can be used to select patients for clinical trials, and also will touch on new data pertaining to mechanisms of resistance to Notch pathway inhibitors.

8.00 - 10.00pm *Welcome Reception Cocktail and Canapes*

Thursday 15th June 2017

SESSION 2- Chairperson: Gian-Paolo Dotto

9.00-9.20 Boguslawa Korona[2], Richard Suckling[1], Pat Whiteman[2], Thomas Rowntree[2], Christina Redfield[2], Susan Lea[1] and Penny Handford, UK.

New insights into receptor/ligand architecture and interplay with lipid
Recent data have increased our knowledge of the molecular basis of the Notch receptor/ligand interaction. It is now known that Notch EGF 11-12 interact with the DSL and C2 domains of ligand to form a complex stabilized by protein-protein, as well as protein-O-glycan interactions, and additional ligand-specific interactions occur along the longitudinal axis of the complex. We report new structures of the N-terminal region of hJagged-2, and hDll-4 which, together with previously

published structures of hJagged-1, rat Dll-4 and hDll-1, suggest that membrane interactions with the C2 domain are likely to be important in optimizing the Notch signal. We demonstrate that the Delta and Jagged ligand families show different preferences for ganglioside- or sphingomyelin-rich liposomes, and disease-causing mutations associated with extra-hepatic biliary atresia (EHBA) affect membrane recognition and reduce Notch activation in cell-based assays. These data suggest that lipid binding is important for fine tuning ligand-dependent Notch signaling, and changes to the membrane environment have the potential to modulate the Notch signal in normal and pathological states.

Email address: penny.handford@bioch.ox.ac.uk; **Keywords:** EGF domain, C2 domain, Lipid, O-glycans, EHBA

9.20 - 9.40 Marc Vooijs, Department of Radiation Oncology, University of Maastricht Medical Centre, Maastricht , The Netherlands. Email: marc.vooijs@maastrichtuniversity.nl

Normal lung toxicity to radiation; a role for NOTCH signaling?

Lung cancer is the leading cause of cancer death. New treatments that complement standard chemoradiation and surgery are urgently needed. Deregulation of the NOTCH signalling pathway is associated with poor outcome and treatment resistance in patients and in preclinical models (Theys et al., 2013) suggesting NOTCH signalling as a novel therapeutic target.

Normal tissue effects to treatment are dose-limiting and negatively affect tumour control and quality of life. Reducing side-effects may improve tumour control by dose-escalation and treatment-time. What is currently lacking are primary human lung tissue models that enable robust evaluation of normal lung tissue effects of combination treatments prior to clinical studies.

Here I will discuss the application of primary human lung tissue models from patients to study the consequences of RT and NOTCH/g-secretase inhibition on normal lung epithelial cell renewal and differentiation.

9.40 - 10.10am Jennifer Yu, Cleveland Clinic, USA.

Hypoxic Regulation of Notch1 Turnover in Glioma Stem-like Cells

Tumour hypoxia selects for highly malignant cells and is associated with poor patient survival. Glioblastoma is an incurable brain cancer characterized by hypoxia. The Notch pathway is implicated in maintaining glioma stem-like cells (GSCs) in the hypoxic niche, but the underlying mechanisms are unclear. We have identified Vasorin as a critical link between hypoxia and Notch signalling in GSCs. Vasorin is preferentially induced in GSCs by hypoxia through a HIF1a/STAT3 co-activator complex. Vasorin binds to and stabilizes Notch1 at the cell membrane by reducing its lysosomal degradation. Vasorin therefore acts as a switch to augment Notch signalling under hypoxic conditions. In mouse models of glioblastoma, Vasorin promotes tumour growth and reduces animal survival. In human IDH1 wt gliomas, Vasorin expression inversely correlates with patient survival. We provide mechanistic insight into hypoxic regulation of membranous Notch1 turnover in GSCs and identify Vasorin as a prognostic marker and potential therapeutic target.

10.10-11.10 Coffee Break

SESSION 3-Chairperson: Lucio Miele

11.10 – 11.300 Lisa M. Minter, University of Massachusetts, USA.

Notch1-PKCtheta Interactions in T cells: At the Cross-roads of T cell Differentiation

The Notch1 transmembrane protein is a central arbiter of T cell activation and differentiation. We and others have demonstrated the importance of Notch1 signalling during CD4 T cell differentiation into various T helper (Th) subsets, including those with Th1, Th2, Th17 and iTreg phenotypes. The T cell-specific kinase, Protein Kinase C theta (PKC□) has also been shown to be required for full activation of CD4 T cells, acting as a signal-amplifying relay for antigens that provide a weak stimulus through the T cell receptor. We have further demonstrated that Notch1 and PKC□ can physically interact and functionally operate within the same signalling pathway in CD4 T cells. Here we used multiple experimental approaches to further explore the dynamic relationship between Notch1 and PKC□. Our results show that in the absence of fully-functional PKC□ during CD4 T cell activation and differentiation, cellular localization of the signalling competent, Notch1 intracellular domain is altered and diminishes pro-inflammatory cytokine production. We also provide evidence of Notch1-PKC□ interactions in CD8 T cells and demonstrate a requirement for PKC□ in these cells to mediate Notch1-dependent IFN□ production. These findings shed additional light on the mechanisms that regulate CD4 and CD8 T cell activation and differentiation. Further understanding the complex interactions between Notch1 and PKC□ in CD4 and C8 T cells may pave the way for developing novel T cell-based therapies for the treatment of hematologic and solid malignancies.

11.30 - 11.50 Haoyu Liu, Tom Barnes and Walter Bodmer
Weatherall Institute of Molecular Medicine, Department of Oncology, University of Oxford

Control of goblet cell differentiation in colorectal cancer cell lines

Blocking differentiation is a key step in the development of carcinomas. A reflection of this is the very frequent down regulation of goblet cells in colorectal carcinomas(CRC), and indeed often their complete absence. It is well known that Notch signalling plays a key role in the maintenance of stem cell turnover in CRC and that blocking Notch activation induces increased levels of goblet cell differentiation in CRC derived cell lines. I will describe some of our new results on the control of goblet cell differentiation in our CRC lines, the relationship of this to Notch activation, and the recognition that CRCs may often differ with respect to the presence of immature goblet cells.

11.50- 12.10 Sandro Goruppi[1] and G. Paolo Dotto[1,2]
[1]Cutaneous Biology Research Center, Massachusetts General Hospital, MA and [2]Department of Biochemistry, University of Lausanne, CH

Multistep process of Cancer Associated Fibroblast activation :
A Notch/CSL – Gli Regulatory Axis

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 tumours 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.

Changes in tumour 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 tumours and broader tissue changes beyond areas of tumour development that expand over time.

We will present our recent progress in this area, with specific focus on the role of the CSL protein – the key effector of Notch signalling – in a multistep process of cancer associated fibroblast (CAF) activation and cancer/stromal cell expansion.

Surprisingly little is known on how multiple pathways converge in transcriptional control of CAF activation. The transcriptional repressor CSL is key to suppress fibroblast senescence and CAF activation. Shh-Gli signalling also contributes to CAF conversion. Here we report that decreased CSL induces autophagy and concomitantly promotes CAF effector gene expression through a Gli1/2-dependent mechanism separate from induction of cellular senescence. Thus, a CSL-Gli regulatory axis involved in CAF activation represents an attractive target for stroma-focused anti-cancer prevention.

12.10-12.30 Rasmus Niemi, , Malin Berg, , Cecilia Sahlgren,

Åbo Akademi University, Department of Biology, Turku , Finland

Notch in tumor cells – angiogenic regulation from afar

Notch is a cell contact-dependent signaling pathway comprising four receptors (Notch1-4) and five ligands (Jag1-2, Dll1, 3, 4), through which it regulates cell fate decisions both in physiological settings and in cancer. Notch is an important player in stem cell maintenance as well as in cancer stem cell development and self-renewal. In addition, it has been ascribed a role in (tumor) angiogenesis where it regulates sprouting and vessel development by regulating endothelial cell differentiation. We show that cancer cells with overexpressed Notch form more vascularized tumors *in vivo*, suggesting a role for Notch in the tumor angiogenic context other than that on the endothelial-endothelial level. *In vitro* studies of the secreted protein levels of known angiogenic regulators revealed an increased secretion of a number of potent angiogenic growth factors from cancer cells overexpressing Notch, compared to controls and GSI-treated cells. Additionally, since other groups have reported that cancer stem cells (CSCs) may regulate angiogenesis through various mechanisms, we also studied the angiogenic output from CSCs with differential Notch levels. These analyses showed that CSCs secreted higher amounts of a handful of angiocrine proteins, compared to the corresponding cancer cell line controls. Interestingly, when culture medium from cancer cells and CSCs (containing cell-secreted growth factors) was harvested and used to treat

endothelial cells in an *in vitro* sprouting assay, culture medium from CSCs (with Notch overexpression, in particular) induced more endothelial sprouting than medium from cancer cell controls. This increase was diminished in GSI-treated samples.

In sum, we show that Notch overexpression in cancer cells promotes angiogenesis *in vivo*, that it increases the secretion of soluble angiocrine growth factors from cancer cells and CSCs, and that these factors are capable of inducing endothelial sprouting *in vitro*. These findings suggest a mechanism through which Notch in cancer cells may regulate angiogenesis from afar, in a paracrine manner.

12.30 – 2.30 Lunch Break

2.30 – 3.30 pm Open Air workshop: Notch activation in cancer
Jon Aster, Freddy Radtke

SESSION 4- Chairperson: Jon Aster

4.00-4.20 pm Nancy Papalopulu
Division of Developmental Biology and Medicine, Division of Molecular and Clinical Cancer Sciences, School of Medical Sciences , Faculty of Biology, Medicine and Health, The University of Manchester ,
Manchester, UK

A role for transcriptional dynamics in regulating stem cell quiescence and plasticity

Understanding the molecular mechanisms by which cells make transitions between slow dividing stem cells, rapidly amplifying progenitor cells and differentiated cells is a problem of shared interest between basic cell biology and cancer biology. In particular, reactivation from a quiescent cell state is a crucial cell biological process, which underlies the problem of cancer relapse.

Within breast cancer tissue, stem cells exist in distinct states, characterised by different proliferation potential and ability to reinitiate a tumour. These distinct states are also known to be plastic and inter-convertible, a process which is not well understood. A novel hypothesis is that cell state conversions from proliferation to differentiation or to reversible quiescence, are not simply driven by genes being on (or off), but by a change in the *dynamics* of gene expression, for example, from fluctuating or pulsatile expression to a more stable state or to a pulsatile expression with different characteristics (such as frequency or amplitude). We focus on the gene expression dynamics of Hes1, a transcriptional repressor activated by Notch signaling, because it is involved in maintaining stem cells both during development and during breast carcinogenesis. Drawing parallels from our findings in quiescent and proliferating neural stem cells, our central hypothesis is that gene expression pulsing of Hes1 takes place in Breast Cancer Stem cells and that the characteristics (e.g. frequency, amplitude) of pulsing may be causally related to their proliferation and re-activation potential. The ultimate aim is to be able to manipulate the expression dynamics for therapeutic gain.

4.20-4.40 Helen Sheldon and Adrian Harris, CRUK Molecular Oncology Laboratories, Weatherall Institute of Molecular Medicine, John Radcliffe Hospital, University of Oxford, UK

Breast Cancer Exosomes as Potential Biomarkers of □-Secretase Inhibitors

Triple negative breast cancer (TNBC) is aggressive and associated with a poor prognosis. No targeted therapies exist for this tumour type and they are currently treated with chemotherapy. Approximately 13% of these tumours have mutations that lead to up-regulation of the Notch signalling pathway and this has leadto the investigation of □-secretase inhibitors for their therapy. Direct monitoring of the effectiveness of these inhibitors in tumours during trials is difficult, therefore the

development of a minimally invasive method to achieve this goal would be advantageous.
Exosomes are small vesicles derived from endocytosis. They contain protein, RNA and DNA and reflect the composition of their parent cells. They are released from cells into biological fluids and there has been much interest in these vesicles as potential biomarkers. To investigate the changes in breast cancer exosomes after □-secretase treatment, MDA-MB-231 triple negative breast cancer cells were cultured in the presence of DBZ and the exosomes harvested by ultracentrifugation. DBZ treatment inhibited Notch signaling, as assessed by QPCR of Notch target genes, and slightly reduced cell number over the period of the experiment but it did not reduce exosome production or exosome size. Western blot analysis revealed that □-secretase components are found in exosomes. Most remain unaltered but Nicastrin levels are slightly increased after DBZ treatment and only the mature form is present. Interestingly the S2 cleaved NEXT fragment is present in exosomes but it is approximately 50kDa larger that the band detected in whole cell lysates.
Notch- 4 is also present as a larger protein and DBZ treatment leads to the reduction of both of these proteins. The S3 cleaved Notch intracellular domain (N1ICD) fragment is undetectable. Jagged-1 is also present in exosomes and this is reduced after DBZ treatment suggesting some Jagged-1 regulation by □-secretase. These effects are not restricted to the MDA-MB-231 cell line as the same changes occur in another triple negative breast cancer cell line, HCC1806, and with other □-secretase inhibitors. Mass spectroscopy analysis is being carried out to identify the composition of the Notch proteins present in exosomes and further work is in progress to establish if the changes observed could be used as a biomarker.

Funded by Cancer Research UK, Breast Cancer Research Foundation and NHS Biomedical Research Centre

4.40-5.10 Kim Dale ,Reader and Associate Dean International
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J.K.Dale@dundee.ac.uk | +44(0)1382 386290

Investigation into the regulation of NICD stability and its role in determining the periodicity of the vertebrate segmentation clock

Somites are bilaterally paired blocks of paraxial mesoderm that form along the head to tail axis of the developing embryo in segmented animals.
The periodicity of somite formation is regulated by a molecular oscillator called the segmentation clock which drives cyclic gene expression in the unsegmented paraxial mesoderm (PSM) from which somites derive.
Three signalling pathways have been proposed to underlie the molecular mechanism of the oscillator: Wnt, Fgf and Notch. In particular, the Notch signalling pathway has been demonstrated to be an essential piece in the intricate somitogenesis regulation puzzle.
Notch signalling does not use second messengers, but rather the receptor becomes cleaved upon ligand activation and this liberates the Notch intracellular domain (NICD) which translocates to the nucleus and activates target gene expression – which includes a number of so-called clock genes.
The stability and turnover of NICD is inextricably linked to the regulation of the pace of segmentation clock. NICD stability is a key requisite for its activity and to date the regulation of stability has been attributed to phosphorylation of the PEST domain by two kinases namely cyclin-dependent kinase-8 (CDK8) and GSK-3β.
NICD turnover is reported to be regulated by the SCF Sel10/Fbxw7 E3 ubiquitin ligase complex. Exposure of PSM explants to the small molecule inhibitor MLN4924, which inactivates SCF E3 ligase complexes by suppressing neddylation of Cullin-1, leads to elevated NICD levels and a delay in clock oscillations.
It is reported that NICD recognition by SCF ubiquitin ligase complex is phosphorylation-dependent, however there is very little data describing the phosphosites involved. Given how pleiotropic this signalling pathway is both in development and in disease and that the fine regulation of its activity is still poorly understood, we aim to elucidate this regulation.

8.30pm until late Conference Dinner

Friday 16th June 2017

SESSION 5- Chairperson: Cathrin Brisken

9.00-9.20 Hossain, F., Peng, Y., Sorrentino, C., Peng, Y., Bilyeu, A., Crabtree, J., Pannuti, A., Matossian, M., Burow, M., Golde, T.E., Osborne, B. A. and Miele, L.
LSU Health Sciences Center, School of Medicine,
New Orleans, USA.

Notch affects mitochondrial metabolism in triple-negative breast cancer

Triple negative breast cancer (TNBC) is a heterogeneous group of clinically aggressive diseases. TNBC patients have high risk of recurrence and metastasis, and current treatment options remain limited. Cancer stem-like cells (CSCs) have been linked to cancer initiation, progression and chemotherapy resistance. Therefore CSC-targeted therapies are keenly sought. There is strong evidence for the involvement of Notch signaling in TNBC. Notch1 is highly expressed in Basal-like 1 (BL1) and especially Mesenchymal-Stem-Like (MSL) TNBCs. Expression of Notch1 and its ligand Jagged1 correlates with poor prognosis. Moreover, strong evidence supports key roles of different Notch paralogs in breast CSCs. However, the role of non-canonical Notch signaling in TNBC remains poorly understood. Here, we provide evidence for a two-pronged non-canonical axis whereby Notch1 promotes cell survival in MDA-MB-231 cells, representative of MSL TNBC: 1) A nuclear pathway mediated by the activation of NF-κB-dependent transcription and 2) A mitochondrial pathway whereby Notch1 activates glycolysis and respiration. Both pathways require IKKα. Notch activation by Jagged1-expressing stromal cells enhances transcription of the anti-apoptotic gene cIAP-2 (BIRC3), a known NF-κB target. This event is dependent on recruitment of NF-κB subunits, IKKα and Notch1 to the cIAP-2 promoter. Short term exposure of MDA-MB-231 cells (MSL, PTEN wild-type), but not MDA-MB-468 cells (BL1, PTEN-null) to

recombinant Jagged1 leads to AKT phosphorylation. This is suppressed by dual mTORC1/2 inhibitors, AKT inhibitors and IKKα inhibitors but not Everolimus (mTORC1-selective inhibitor). These observations support a model where canonical and non-canonical mechanisms downstream of Notch1 trigger AKT phosphorylation and NF-κB activation in PTEN wild type TNBC cells. This suggests a bidirectional crosstalk between the IKKα and AKT arms of this Jagged1-activated pathway. Recombinant Jagged1 increases the cellular metabolism of TNBC cells and knockdown of Notch1 or IKKα by siRNA decreases mitochondrial respiration and glycolysis. Association of cleaved Notch1 with mitochondria was documented by biochemical and morphological analyses. CSCs derived from MDA-MB-231 cells have increased Notch1, p-AKT, and mitochondrial respiration. AKT inhibition or IKKα inhibition decreases both mitochondrial respiration and glycolysis of TNBC derived CSCs. Pharmacological inhibition of Notch cleavage by gamma secretase inhibitor (PF-03084014) in combination with AKT inhibitor (MK-2206) or IKKα inhibitor (Bay11-7082) blocks $CD90^{hi}$ or $CD44^{+}CD24^{low}$ sorted secondary mammospheres formation. These results were replicated in a patient-derived TNBC PDX model. These data suggest that combination treatments affecting Notch, NF-kB or AKT pathways have potential therapeutic importance in targeting CSCs of TNBC at least in part through interference with mitochondrial metabolism.

9.20 – 9.40am Robert S. Haltiwanger[1,2], Michael Schneider[1], Hideyuki Takeuchi[1,2]

[1]Department of Biochemistry and Cell Biology, Stony Brook University, Stony Brook, NY USA

[2]Complex Carbohydrate Research Center, University of Georgia, Athens, GA USA

Modulation of Notch Glycosylation as a potential cancer therapy

Notch can function as either an oncogene or tumour suppressor, depending on context. Thus, mechanisms to either increase or decrease Notch activity are needed to develop appropriate therapies. Glycosylation of the Notch extracellular domain can activate or inhibit Notch depending on context, so modulation of

Notch glycosylation may provide a novel therapy for Notch-related cancers. Towards this goal, we have recently solved the structures of two enzymes involved in addition of O-glucose glycans to Notch: Rumi/POGLUT1 and XXYLT. Rumi/POGLUT1 adds O-glucose to Notch EGF repeats, which is required for Notch function in most contexts. Inhibitors of Rumi/POGLUT1 should reduce Notch activity. XXYLT1 adds a terminal xylose to O-glucose, and genetic studies suggest this reduces Notch activity. Inhibitors of XXYLT1 should enhance Notch activity. In addition to potential inhibitors of the enzymes that add sugars to Notch, we have examined sugar analogs as potential modulators of Notch. This is especially important for fucose, since recent co-crystal structures reveal that O-fucose residues on both EGF8 and 12 of Notch1 are in physical contact with ligand, and binding studies confirm the importance of these modifications. We have shown that some fucose analogs are utilized by POFUT1 and incorporated into Notch EGF repeats. Interestingly, some of these analogs inhibit binding of Delta-like ligands, but not Jagged ligands, to Notch1, leading to inhibition of Notch1 activation by Delta-like ligands. These results suggest that in addition to development of inhibitors of enzymes responsible for addition of sugars to Notch, fucose analogs may be useful as modulators of Notch activity. Supported by NIH grant GM061126.

9.40 - 10-00 Keith Brennan, Michael Leverentz, Abigail Edwards, Matthew Jones, Rajeharish Rajendran, Olivier Meurette, Spyros Stylianou
Faculty of Biology, Medicine and Health, University of Manchester, Manchester, UK

Notch regulates apoptosis in breast cancer cells through IL1α

We have shown that sustained Notch signalling can transform normal breast epithelial cells and 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. Here, we will describe our current data seeking to identify the Notch-induced secreted intermediate. We have identified a medium fraction produced by breast epithelial cells in response to sustained Notch signalling that can activate Akt. Use mass spectrometry, we have identified a number of secreted proteins within this fraction, including G-CSF, IL1α, IL6 and IL18. We have subsequently gone on to determine which of these secreted proteins is relevant and will provide evidence that IL1α is the Notch-induced secreted intermediate that prevents apoptosis in breast cancer cells.

10.00 -10.20 Fortini F, Vieceli Dalla Sega F, Aquila G, Caliceti C, Miele L, Pannuti A, Ferrari R and Rizzo P . Department of Morphology, Surgery and Experimental Medicine
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Is Notch involved in the association between aromatase inhibitors and coronary artery disease in women with breast cancer?

Estrogens inhibits Notch signalling in breast cancer cell lines, and breast cancer (BC) biopsies following treatment with antiestrogens show Notch activation. Combination treatment with γ-secretase inhibitors (GSIs) and antiestrogens is more effective than either treatment alone in preclinical cancer models. Two phase 1

clinical trials of combinations between GSIs and anti-estrogens in ER+ breast cancer have shown molecular and radiological signals consistent with efficacy. However, Notch and estrogens have significant roles in endothelial cells and angiogenesis, and the effects of these combinations treatments on endothelial biology needs to be explored.

Both estrogens and Notch protect endothelial cell (EC) against apoptosis caused by TNFα, which is elevated in the serum of BC patients. EC apoptosis caused by inflammation is one the first steps toward the onset of atherosclerosis. Aromatase inhibitor (AI) treated- BC patients show increased risk of coronary artery disease (CAD) compared to tamoxifen-treated women. It is not known whether this difference is due to lack of endothelium-protective activity by estrogens, which tamoxifen mimics, or to direct effects of AIs on the endothelium. The identification of molecular mechanisms by which estrogens and Notch, independently or together, protect the endothelium could help explaining the association between AIs and CAD. The goal of our study was to establish whether: 1) estrogens modulate Notch in EC, and 2) Notch is required for the endothelium-protective activity of estrogens in the presence of elevated levels of TNFα.

Human umbilical vein endothelial cells (HUVECs) were treated with 17 β estradiol (E2) and/or TNFα and the effects on apoptosis and on the Notch pathway were investigated. We found that E2 increased the levels of active Notch 1 (N1IC) and partially counteracted the decrease in N1IC caused by TNFα. TNFα induced apoptosis, which was counteracted by E2 only in the presence of Notch1. Moreover, siRNA knockdown of the estrogen receptor β (ERβ), but not ERα, abolished E2 effects on active Notch1 and apoptosis.
In summary, we show that E2, through a mechanism involving ERβ, activates Notch1 in the endothelium, which is required to protect cells against TNFα-induced apoptosis. These findings suggest that the endothelium-protective effects of estrogens are Notch-mediated, and that the increased risk of CAD observed in patients treated with AIs may be related to low levels of Notch1 in the endothelium. Hence, long-term treatment with combinations including antiestrogens and Notch inhibitors may increase of the risk of developing CAD.

10.20 - 11.20am Coffee Break

SESSION 6 Chairperson: Christina Kousparou

11.20 -11.40

Laura Simons[1,2]*, Kuiying Ma[1,2]*, Corinne de Chappedelaine[1,2], Marina Cavazzana[1,2,3], Isabelle André-Schmutz[1,2,3]

1. Human Lymphohaematopoiesis Laboratory, INSERM U1163, Paris, France

2. University of Paris Descartes-Sorbonne Paris Cité, IMAGINE Institute, Paris, France

3. Biotherapy Clinical Investigation Centre, Necker Children's Hospital, Paris, France

***: These authors contributed equally.**

Characterization of human T-lymphoid progenitors generated from adult hematopoietic stem cell precursor cells (HSPCs) in a feeder-cell-free Delta-like-4 culture system

Prolonged T cell deficiency with subsequent high risk of infection and relapses is a major complication of non-HLA identical hematopoietic stem cell transplantation (HSCT). Complete restoration of a polyclonal T cell repertoire takes up to 2 years and may never reach pre-transplant levels. Exploiting the Notch signalling pathway for the *in vitro* generation of T cell precursors provides a promising approach to overcome this hurdle and speed up T cell reconstitution after HSCT.
Considering the known role of Notch signalling in T cell lineage differentiation in mouse and human, we have implemented a feeder cell-free system based on the

immobilized Notch ligand Delta-like-4 (DL-4) in combination with a specific set of cytokines. Compared to previously described feeder-free systems, our DL-4 culture is more efficient and highly reproducible when applied to cord blood (CB) hematopoietic stem and progenitor cells (HSPCs). Moreover, our system is currently in the process of being approved for clinical application.
In contrast to CB derived HSPCs, HSPCs from mobilized peripheral blood (mPB) are widely used in allogenic HSCT as they are available in large quantity and exhibit several advantages in clinical settings. However, their intrinsic properties of differentiation, survival and proliferation have been little investigated.
Here, we tested the T lineage differentiation capacity of adult HSPCs in a feeder cell-free culture based on the use of a modified DL-4 Notch ligand and T cell cytokines. Within 7 days, adult HSPCs were able to produce $CD7^+$ T cell precursors expressing T lineage master genes and with high *in vitro* T cell differentiation potential. Compared to CB, adult HSPC DL-4 cultures were associated with lower rate of proliferation, increased apoptosis and differential expression of NOTCH receptors. Most importantly, DL-4 T cell precursors derived from adult HSPCs expressed chemokine receptors implicated in thymus homing and efficiently produced polyclonal T cells upon transplantation in NOD/SCID/γc−/− (NSG) mice. These results provide insights into pathways of Notch-based T cell differentiation and demonstrate that adult HSPCs provide an effective and available source of *in vitro* cultured T cell precursors in the context of future clinical applications directed to shorten T cell recovery after HSCT.

11.40-12.00 Marika Rossini, Paola Rizzo, Fernanda Martini, Mauro Tognon
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Metformin down-regulates Notch1 and induces apoptosis in malignant pleural mesothelioma cell lines.

Introduction: The human malignant pleural mesothelioma (MPM) is a fatal neoplasm that exhibits a strong correlation with exposure to asbestos fibers. At present, there are no effective therapies for MPM and patients after diagnosis die in approximately 1-2 years. Apoptosis or programmed cell death is part of a natural mechanism that regulates cell populations. Several therapeutic agents capable to modulate the apoptosis process are without effect in MPM. However, no data are available for the Metformin. Dysregulation of Notch signaling was reported in MPM cell lines grown in vitro.

Purpose: The aim of this study was to investigate the effect of Metformin on the apoptosis of MPM cell lines and to verify whether the Notch signalling is involved in the mechanism of action of this drug during the apoptosis.

Methods: MPM cell lines, MMP89 and IST-Mes2, which are sarcomatoid and epithelioid histological subtypes, respectively, were treated with different concentrations of Metformin, at distinct time points. The apoptosis in MPM cells was investigated by propidium iodide (PI) and Annexin V staining, followed by flow cytometry analysis. Controls were represented by untreated MPM and normal mesothelial cells (HMCs.)

Results: The expression of Notch1 protein and its active form, known as Notch intracellular domain 1 (NICD1), were investigated in MPM and HMC cells. Higher level of NICD1 and the transmembrane (TM) Notch 1 proteins were detected in MPM cell lines compared to the control, HMCs. The apoptosis was studied in Metformin treated MPM cells. Data indicated that Metformin down-regulates Notch1 and induces apoptosis in MPM cells, as revealed by propidium iodide and Annexin V staining. MPM cells were treated with Metformin at the concentration of 25-50 mM for 24 hours.

Discussion: MPM is a fatal tumor due to the lack of effective therapies. A better understanding of the molecular mechanisms involved in the MPM onset is needed to design successful therapies that could offer MPM patients a real clinical benefit. In the present work, we confirmed that Notch1 pathway is activated in MPM sarcomatoid and epithelioid cell lines. Herein, we demonstrated that the Metformin treatment induces the apoptosis in MPM cells, while it down-regulates Notch1. These preliminary data provide new insights into the apoptosis effect exerted by the Metformin in MPM cells by modulating the Notch1 signaling.

12.00 - 12.20 Luca Tottone1, Mattia Mori1, Nadezda Zhdanovskaya2, Rocco Palermo1, Isabella Screpanti2.
1Center for Life Nano Science@Sapienza, Istituto Italiano di Tecnologia, Rome, 00161 - Italy;
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Novel approaches to inhibit Notch signalling in T-Cell Acute Lymphoblastic Leukemia .

Notch signaling deregulation is linked to development and progression of a broad range of solid and hematological human malignancies. Thus, targeting Notch signaling in cancers represents a compelling therapeutic strategy to overcome chemo-resistance and to reinforce conventional therapies. In the last decade, several Notch inhibitors have been developed and some of them have been evaluated in clinical trials as single agent or in combination with conventional chemotherapeutics in Notch-driven cancer, including T-Cell Acute Lymphoblastic Leukemia (T-ALL). Actually, most Notch inhibitors under development target the Gamma Secretase complex(GS) and although these molecules exert efficient anti-tumor activity, they are not effective in all the patients and often cause severe side-effects.
In our study, a library of about 1000 natural products has been clustered through a chemical informatics approach. By screening, through biological and biochemical analysis, the representative compounds of the 8 most populated clusters of the library for their strength in inhibiting Notch signaling and cell growth in T-ALL cell lines, we identified a potential novel naturally occurring Notch inhibitor, we called C. Several chemical derivatives of the hit C have been designed and synthesized to afford structure-activity relationships (SAR) and to develop a more potent Notch inhibitor, we named compound 8. Treatments with low micromolar concentration of compound 8 was able to inhibit Notch signaling activity and to abrogate proliferation in several human T-ALL cell lines.
Overall, we identified a novel Notch inhibitor endowed with a naturally occurring scaffold, and provided novel guidelines for future development of bioactive Notch inhibitors, useful for the treatment of T-ALL.

12.20-12.40 Raj Lehal, CSO, Cellestia Biotech AG

Non-clinical pharmacology, pharmacokinetics and safety profiling of CB-103: A novel first-in-class small molecule inhibitor of the NOTCH pathway.

NOTCH signalling is a developmental pathway known to play critical roles during embryonic development as well as for the regulation of self-renewing tissues. Aberrant activation of NOTCH signalling leads to deregulation of the self-renewal process resulting in sustained proliferation, evasion of cell death, loss of differentiation capacity, invasion and metastasis, all of which are hallmarks of cancer. Over activation of NOTCH in human cancers can be a consequence of over expression of NOTCH ligands/receptors, GOF mutations in NOTCH receptors as well as chromosomal translocations leading to constitutive activation of the pathway by producing truncated version of NOTCH1 or NOTCH2. Over activation of NOTCH and its oncogenic role in various cancers is prognostically relevant with shorter survival seen in patients harbouring these genetic alterations. Given the importance of Notch signalling in human cancers, several therapeutic approaches have been utilized to block NOTCH signalling. Two of these strategies are; a) the use of monoclonal blocking antibodies (mAbs) against NOTCH ligands and receptors and b) the use of small molecule □-secretase inhibitors (GSIs). However, these approaches can only be effective if tumour cells express full-length ligand or receptor molecules. On the contrary, in human cancers harbouring NOTCH gene fusion due to chromosomal translocations, the use of mAbs and GSIs will have very limited clinical benefits. A third, yet not fully explored approach would be the blockage of NOTCH signalling by targeting the most downstream complex in the NOTCH signal transduction cascade, the NOTCH transcriptional activation complex, using small molecule inhibitors.
Previously we have reported the discovery and characterization of CB-103, a novel first-in-class orally-active small molecule inhibitor, of the NOTCH pathway. CB-103 inhibits NOTCH signalling by targeting the NOTCH transcriptional activation complex in the nucleus. CB-103 has shown the ability to block NOTCH signalling in various human cancer cell lines with active NOTCH pathway. CB-103 has also been tested in *in vivo* models. In a GSI/mAb resistant model of human triple negative breast cancer, CB-103 has demonstrated excellent anti-tumour efficacy. In this study we present additional pharmacology, pharmacokinetics data and provide an overview of the good safety and

tolerability seen with CB-103 in the non-clinical studies. Based on these findings CB-103 has been selected as clinical candidate to be investigated in a FIM, phase I/IIA clinical study in advanced cancer patients.

12.40-1.00 Raphaela Schwentner[1], Gunhild Jug[1], Max Kauer[1], Ingrid Simonitsch-Klupp$_2$, Wolfgang Holter[1,3], Caroline Hutter[1,3]
[1] Children's Cancer Research Institute, St Anna Kinderkrebsforschung, Vienna, [2] Clinical Institute of Pathology, Medical University of Vienna, Vienna, [3] St Anna Children's Hospital, Department of Pediatrics, Medical University of Vienna, Vienna

THE NOTCH SIGNALLING PATHWAY IN LANGERHANS CELL HISTIOCYTOSIS

Langerhans cell histiocytosis (LCH) is a rare histiocytic disorder that may affect any age group, although its most severe clinical course affects predominantly young children. LCH is characterized by the accumulation of Langerin positive histiocytes of unknown origin that are surrounded by a prominent inflammatory infiltrate. One intriguing feature of LCH is the wide spectrum of clinical manifestations that encompasses both single lesions, which can regress spontaneously, as well as severe multisystem disease that requires intensive chemotherapy.

In our previous work we have demonstrated that LCH cells selectively express the Notch ligand Jagged2 (JAG2) and are the only dendritic cells that express both Notch receptor and ligand. In addition, staining for NICD in biopsies of LCH lesions indicates activation of Notch1 in LCH cells, suggesting that the Notch pathway could contribute to LCH pathogenesis.

In vitro the presence of recombinant JAG2 induces the differentiation of primary human CD14+ monocytes in cells that harbour key features of LCH cells, both phenotypically and on gene expression level. Furthermore inhibition of Notch signalling with the γ-secretase inhibitor RO-4929097 abrogated the capacity of CD14+ monocytes to differentiate into LCH like cells.

We are currently exploiting this in vitro system to further study induced signalling pathways. The interplay between the NOTCH and MAPK pathway is of special interest as the MAPK pathway is constitutive active and thereby a driving force in LCH.

This might also be of clinical relevance, as the beneficial effect of combinatorial administration of GSI and MAPK inhibitor was previously demonstrated, therefore we suggest targeting Notch signalling in LCH as a potential new treatment option.

12.30 – 2.30pm Lunch break

2.30-3.30pm Open Air workshops: *Notch Therapeutics*
Lucio Miele, Aleksandra Filipovic

SESSION 7 Chairperson: Agamemnon Epenetos

4.00-4.20 Martin Baron, University of Manchester, Manchester, UK

Tuning Notch signalling through an endocytic regulatory network.

The Notch gene encodes a fundamentally important, highly conserved cell signalling receptor and there are few biological processes that are not impacted on by its activity, from organogenesis to stem cell regulation, from brain development to memory formation, with widespread implications for human health. Recent work has shown that there is an unexpected diversity of different mechanisms that can lead to Notch activation that involve trafficking of Notch through an endosomal network. The latter tunes signal activity by regulating Notch flux towards inhibitory or signal activation outcomes. We have explored the connections between the diversity of Notch mutant phenotypes and mis - regulation at different endocytic control points, and have examined the mechanistic basis by which Notch trafficking can be directed to different endpoints within the endosomal subdomain architecture. Through computational simulation, cell biology and whole genome RNAi studies, we have shown that the setting of Notch signalling levels is deeply embedded in the physiology of the cell.

4.20 -4.40 Christina Kousparou , Novartis , Nicosia, Cyprus.

Pre-clinical drug development of a ' mastermind' inhibitor, AB4, for the treatment of Notch aberrant human neoplasms

We have designed and generated recombinantly and synthetically a novel compound AB4 that can penetrate all cells and inhibit Notch signaling at the mastermind / DNA level and thereby impacting the activity and survival of cancer cells and possibly CSCs.

Our results indicate that AB4:

- has the ability to penetrate cancer cells in vitro and localize to the nucleus
- can penetrate most normal organs, in a non-toxic manner
- penetrates xenografted and orthotopic human tumours

- can be monitored in real-time using a conjugated technique and state-of-the art imaging method
- blocks Notch activation which is sometimes upregulated during oncogenesis and tumour progression
- reduces tumour size
- can be produced synthetically and recombinantly and identified by a His-tag antibody

4.40-5.00
Aleksandra Filipovic , ICL and Puretech , UK and USA.

Puretech model of bringing life to science: venture creation of breakthrough academic science

Abstract awaited

5.00 Agamemnon Epenetos
Farewell and Adjourn to June 2018

Posters

There will be posters displayed and available to view during the whole conference period. Everybody is most welcomed to contribute a poster in addition to/ or complement their presentation. The following have been accepted as poster presentation:

- **Capodanno Y[1], Buishand FO[2], Pang LY[1], Kirpesteijn J[2], Mol JA[2] , Argyle DJ[1]**

[1] Royal (Dick) School of Veterinary Studies and The Roslin Institute, University of Edinburgh, EH25 9RG, United Kingdom.

[2] Department of Clinical Sciences of Companion Animals, Faculty of Veterinary Medicine, Utrecht University, Utrecht, The Netherlands.

A top NOTCH treatment to target human and canine Pancreatic Neuroendocrine Cancer Stem Cells

Insulinoma (INS) is the most commonly diagnosed pancreatic neuroendocrine tumour in humans and dogs. Despite current treatment modalities, malignant canine and human INS have a poor prognosis as patients most often develop metastases in liver and lymph nodes that do not respond to current therapies. We hyphothesise that the aggressive behaviour of malignant INS is driven by cancer stem cells (CSCs). Our aim was to isolate INS CSCs and to identify targets for novel therapies.

Putative CSCs within human (CM) and canine (canINS) INS cell lines were isolated using spheroid culture and then characterised by qPCR, western blotting, chemosensitivity, colony formation, and chicken embryo chorioallantoic membrane (CAM) assays.

Putative human and canine INS CSCs expressed stem cell markers including *OCT4*, *SOX9, CD133 and CD34*. INS CSC-like cells exhibited greater resistance to

chemotherapeutics and formed more substantial and disseminated tumours in the *in vivo* CAM model compared to non-CSCs. Notch pathway components (*NOTCH2* AND *HES1*) were found to be upregulated in INS CSCs and the Notch pathway is activated in INS cell line. It is well documented that Notch signalling operates a key role in pancreatic embryogenesis, influencing the balances between pancreatic endocrine progenitors and differentiated beta-cells. Using a γ-secretase inhibitors, we demonstrated that inhibition of the Notch pathway not only significantly decreased the viability of the INS CSC population *in vitro* but also reduced INS CSC clonogenicity when used in combination with chemotherapeutics. In summary, these findings suggest that the Notch pathway is a key regulator of INS CSC survival and of their resistance to chemotherapeutics. We conclude that this pathway has high potential for the development of targeted INS therapies, which may improve the prognosis of INS patients.

- **Marika Rossini, Paola Rizzo, Mauro Tognon, Fernanda Martini.**
  Department of Morphology, Surgery and Experimental Medicine, Section of Pathology, Oncology and Experimental Biology, Laboratories of Cell Biology and Molecular Genetics, University of Ferrara, Ferrara 441221, Italy. E-mail: mrf@unife.it

Metformin down-regulates Notch1 and inhibits malignant pleural mesothelioma cell proliferation.

Introduction: Malignant pleural mesothelioma (MPM) is a fatal tumor, which is resistant to radio-and chemo-therapies. MPM is increasing in frequency throughout the world. Notch signaling is attracting attentions, because its pathway is involved in cancer cell proliferation. Notch dysregulation has been reported in primary MPM cells, indicating a role for Notch in

mesothelial cell transformation and/or survival. Among several anti-cancer drugs, the Metformin has been proposed as a compound active in many tumors of different histotypes. At present, several clinical trials are ongoing with Metformin in patients affected by different cancers. Dysregulation of Notch signaling is found to be crucial in a multitude of solid tumors.

Purpose: The aim of this study was to investigate the effect of Metformin on MPM cell proliferation and to verify whether the Notch signaling is involved in the mechanism of action of this drug.

Methods: MPM cell lines, MMP89 and IST-Mes2, which are sarcomatoid and epithelioid histological subtypes, respectively, were treated with different concentration of Metformin, at distinct time points. MPM cell proliferation was investigated by Alamar Blue assay, whereas the expression of Notch1 receptor and its active form was studied by western blotting, after Metformin treatment. Controls were represented by untreated MPM and normal mesothelial cells (HMCs).

Results: The expression of transmembrane (TM) Notch1 proteins, together with its active form (NICD1), was investigated in MPM and HMCs. Higher level of NICD1 and TM, were detected in MPM cell lines compared to control, HMCs. The Metformin treatment on MPM cells showed a proliferation inhibition, in a dose and time dependent manner. HMCs were resistant to the Metformin treatment up to 48 hours, at 25 mM concentration.

Discussion: MPM is an aggressive tumor due to the lack of effective therapies. In the present work, we confirmed that Notch1 pathway is activated in MPM cells. We demonstrated that Metformin exerts a proliferation inhibition, in a dose-dependent manner, in MPM cells. Taken together our data are contributing to elucidate relevant issues of MPM, such as the identification of new therapeutic targets and biomarkers. Our results will be useful to develop novel therapies, transferred to the clinic, to cure MPM.