Malaria parasites regulate secretion of exosomes carrying distinct cargo

Xavier Sisquella[1,2], Yifat Ofir-Birin[6], Matthew A. Pimentel[1,2], Lesley Cheng[3,4], Natália G. Sampaio[1,2], Thusitha Rupasinghe[4], Jocelyn Sietsma Penington[2,5], Thomas Nebl[1], Jennifer C. Volz[1,2], Benjamin James Scicluna[3,4], Eugene A. Kapp[1], Robyn A. Sharples[3,4], Andreea Waltmann[1,2], Louis Schofield[1,2], Anthony T. Papenfuss[1,2], Malcolm McConville[4], Emily M. Eriksson[1,2], Andrew F. Hill[3,4] Andrew Bowie[5] and Neta Regev-Rudzki[6].

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This work identifies previously unknown molecular players in signalling pathway of malaria parasites, essential for its survival in the host, and provides a new insight into our understanding of how malaria parasites can manipulate their host environment.

Identification of exosomes purified from primary cortical and spinal cord rat microglia

Adriana-Natalia Murgoci[1,2], Luomir Medvecky[3], Michel Salzet[2], and Dasa Cizkova[1,2]

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Exosomes are small extracellular vesicles (EVs) released by CNS cells, which play a key role in pathophysiology of the nervous system. The main goal of present study was to characterize neural primary culture/microglia derived exosomes under normal and stimulated conditions and better understand how neuro-microglia cells communicate. Primary cultures were isolated from neonatal rat (P3) brain cortex, and spinal cord followed by magnetic microglia separation with CD11b + MicroBeads. After reaching confluency both CD11b+ and CD11b- cortical and spinal primary cultures were stimulated with LPS (500ng/mL) for 3 h to activate microglia and enhance release of the exosomes. Based on standard protocols we have used: i) centrifugation and ultracentrifugation steps as well as ii) Total Exosome Isolation Reagent kit to isolate exosomes from each primary cell culture conditioned medium. The exosomes released from different cell cultures were firstly identified by labeling with PKH67-fluorescence dye and observed with Nikon Ti- Fluorescence microscope (100x). To examine morphology of exosomes, we involved field emission scanning electron microscopy analyses (JEOL FE SEM JSM-7000F). As a last step we have performed protein analyses of exosomes by different procedures (SDS- PAGE electrophoresis and proteomics). Our results indicate, that CD11b+ MicroBeads separation yielded a highly purified Iba1 positive microglia population (95-98% of cells). Both, cortical and spinal primary cultures produced similar content of PKH67+ exosomes. While, stimulation of CD11b+ microglia and CD11b- cell populations with LPS resulted in alterations of exosomes revealing differences in terms of protein quantity and content. Furthermore, using SEM we were able to characterize morphology and size of exosomes and make correlations between studied primary cell cultures and LPS stimulation. Present data confirming variations of exosomes released by microglia, are extremely important considering the key roles that microglia play in development and neurological disease. Work was supported by VEGA 2/0125/15, SK-FR-2016-0018/Stefanik.

Characterization and functional prospects of malaria-infected red blood cells-derived exosomes on human endothelial cells

Anna Rivkin[1], Meta Heidenreich[1], Xavier Sisquella[2,3], Matthew A. Pimentel[2,3], Lesley Cheng[4,5], Lisa J. Ioannidis[2], Diana S. Hansen[2], Andrew F. Hill[4,5] and Neta Regev-Rudzki[1]

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We recently discovered that these intracellular parasite transfer information between them via secreted exosomes. Here we demonstrated that parasite-derived vesicles are capable of remodeling the actin cytoskeleton of host endothelial cells and reduce their deformability. Further, we investigate the changes of endothelial adherence-associated proteins after exposure to parasite-derived exosomes. These effects may ultimately be beneficial for the parasite due to alterations of endothelial cell function and behavior.

Exosomes has a role in the ocular drainage system effecting the Wnt signaling - a glaucoma relevant pathway.

Natalie Lerner, Sofia Schraiber Asissar and Elie Beit-Yannai

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Purpose: Canonical Wnt signaling is associated with glaucoma pathogenesis and intraocular pressure (IOP) regulation. Our goal is to provide insights into the influence of Non Pigmented Ciliary Epithelium (NPCE) derived exosomes on trabecular meshwork (TM) cells Wnt signaling. The potential role of exosomes on Wnt signaling in ocular drainage system remains poorly understood.

Methods: We isolated exosomes from media collected from cultured NPCE cell by differential ultracentrifugation. The exosomes were characterized with Dynamic Light Scattering (DLS), Tunable Resistive Pulse Sensing (TRPS), Nanoparticle tracking analysis (NTA), Sucrose sedimentation and Transmission electron microscopy (TEM). The cellular target specificity of the NPCE exosomes was investigated using DiD-labeled exosomes incubated with TM cells as compared to various cell lines and the uptake of exosomes was monitored by confocal microscopy along time. Wnt protein changes in TM cells induced by NPCE exosomes were evaluated using Western Blot technique.

Results: Exosomes derived from NPCE cells were purified and detected as small rounded membrane vesicles within a range of 50-140 nm as defined by DLS, NTA, TRPS and TEM. Western blot analysis indicated that the nanovesicles were positive for classic exosome markers including Tsg101 and Alix. Isolated nanoparticles were found in the sucrose density fractions typical for exosomes (1.118-1.188g/mL). Using confocal microscopy we demonstrated time-dependent specific accumulation of the NPCE labeled exosomes in NTM cells. However other cell lines hardly took up exosomes. We further showed that exosomes induce changes in Wnt signaling proteins expression in the TM cells. Western blot analysis revealed decreased phosphorylation of GKS3β protein and reduced β-catenin protein levels. We found that treatment of the NTM cells with exosomes resulted in a greater than 2-fold decrease in the cytosolic fraction of β-catenin. In contrast, no remarkable difference in the β-catenin nuclear fraction was identified as compared to control.

Conclusions: The data suggest that NPCE cells release exosome like vesicles and that these nanoparticles affect canonical Wnt signaling in TM cells. These findings may have therapeutic relevance since canonical Wnt pathway is involved in IOP regulation. Further understanding of NPCE derived exosomes signaling pathway can provide a new role for pharmacological intervention within the drainage system as a target for glaucoma therapy.

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miRNA sequencing in milk cells and skim milk

CI Schanzenbach[1,2], SE Ulbrich[2], MW Pfaffl[1]

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For veterinary diagnostics milk is an optimal matrix as it is taken routinely and non-invasively. We investigated the somatic milk cell (MC) and skim milk (SM) fraction concerning their miRNA content for early bovine pregnancy detection. While miRNAs in the MC fraction were expected to resemble the physiological state inside blood derived cells, in SM miRNAs were assumed to be present inside extracellular vesicles. Milk samples were taken on three cyclic days and after artificial insemination of the same animals. Whole milk was centrifuged, the fat layer removed and SM samples frozen at -20°C. The remaining MC was washed and stabilized in Trizol at -80°C. Total RNA was extracted and RNA integrity assessed (average RIN=6.1 and 2.3 for MC and SM, respectively). MC and SM samples of n=6 animals were sequenced on a HiSeq 2500 (Illumina). The dataset was processed to align high quality reads to miRBase for mature bovine miRNAs. Resulting read counts were normalized and analyzed with DeSEQ2 and GenEX using principal component analysis (PCA). Selected miRNAs were validated using RT-qPCR. 320`000 and 150`000 reads mapped to miRNA in MC and SM respectively. Although a high number of reads was shorter than 16 nt, in total 132 miRNAs with more than 50 reads were found, of which 87 miRNAs were abundant in both milk fractions. The miRNA profiles were surprisingly similar between MC and SM even though not all miRNAs correlated well. Although the MC sequencing data allowed a separation of cyclic and pregnant cows, we cannot recommend the biomarkers from this study for a diagnostic use as significances were low and the RT-qPCR validation not satisfying. In future studies further interest includes microvesicles present in the milk fat fraction.

Tumor-derived microvesicles facilitate the plasticity of cancer cell invasion.

Crislyn D'Souza-Schorey, Department of Biological Sciences, University of Notre Dame.

The ability of cells to invade into and traverse the extracellular environment is prerequisite for tumor cell dissemination and metastasis. Tumor cell invasion requires the molecular and physical adaptation of both the cell and its microenvironment. Invasive tumor cells release protease-loaded microvesicles into the extracellular environment to facilitate matrix invasion at distal sites. Our studies on the intrinsic and extrinsic factors that regulate microvesicle formation and release will be discussed. We have found that tumor cells assume amoeboid phenotypes on compliant matrices and have a high propensity to shed microvesicles from regions of plasma membrane blebbing. Blocking protease delivery to budding microvesicles at the plasma membrane significantly compromises movement through cross-linked collagen matrices, documenting the importance of vesicle-associated proteases in matrix degradation and cell invasion. These studies underscore the need to better understand the complex motile behavior of tumor cells.

Concentration, sizing and phenotyping EVs using NTA and FFF techniques

Dana Wasserman, Product Manager, Dr. Golik – Scientific Solutions. Tel Aviv.

Within the past decade the vital role of exosomes and microvesicles as mediators of intercellular communication has been increasingly documented. In addition, pathophysiological roles for exosomes and microvesicles are beginning to be recognized in diseases including cancer, infectious diseases and neurodegenerative disorders, highlighting potential novel targets for therapeutic intervention.

Knowing the true size distribution and concentration of EVs in a high resolution technique and in their natural environment is of significant value in elucidating the role these structures play in diseases and the ways in which they may be exploited in diagnostic or therapeutic applications.
Moreover, there is a need for high resolution separation technique, capable of handling with dispersions and that gives the opportunity to collect fractions of these samples for further research and use.

The ability of the NanoSight NTA technique to visualize, size and count exosomes and microvesicles as small as 30nm means it has become an indispensable technology in this field. In addition, the technology has the ability to operate in fluorescence mode allowing visualization, sizing and concentration measurement of fluorescently labeled exosomes and microvesicles.

Field Flow Fractionation brings the ability to separate exosomes and other EVs at a high resolution, fast and gentle technique. Combining FFF to a fraction collector and to multiple detectors [UV, Fluorescence, MALS, DLS etc.] potentially allows a greater understanding of exosome role and function, as well as opening a door to advancements in developing exosome-based diagnostics and therapeutics.

Both FFF and NTA techniques will be presented in this lecture. A focus will be brought on to application examples in which these techniques were used for exosome and extra cellular vesicles research.

Message in a bubble- the role of extracellular vesicles in mediating host-virus co-existence of the bloom-forming *Emiliania huxleyi*

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The coccolithophore *Emiliania huxleyi* forms massive blooms in the oceans and has huge impact on biogeochemical cycles of carbon and sulfur, as well as on global climate regulation. *E. huxleyi*'s intricate calcite exoskeleton accounts for ~1/3 of the total marine $CaCO_3$ production. These *E. huxleyi* blooms are often terminated by infection by a large double-stranded DNA coccolithovirus, the *Emiliania huxleyi* virus (EhV). Although this host-virus interaction has an important ecological role, the cellular mechanisms that govern it are largely unknown. Viral infection led to massive production of extracellular vesicles (ExV's), reflecting their possible involvement in viral replication cycle. The relative concentration of ExV's was higher in uninfected susceptible strains than in viral-resistant ones. We conducted lipidomic analysis and revealed a unique lipidome that greatly differed from that of both host, infected host and purified virion. Transcriptomic analysis of ExV's cargo revealed the presence of mostly host-encoded small-RNAs. Interestingly vesicle production was induced by exposing the *E. huxleyi* cells to the viral free lysate alone, indicating the involvement of small-molecules in cell-cell communication during viral infection. We further investigated the functional role of ExV's on viral infectivity and host defense and susceptibility. We propose that the ExV's may play a novel signaling role that has a major ecological implications to host-virus dynamics within oceanic algal blooms.

Title: **Tumor exosomes determine organotropic metastasis**

Ayuko Hoshino, Bruno Costa-Silva, Irina Matei, Volkmar Muller, Klaus Pantel, Benjamin A. Garcia, Yibin Kang, Cyrus M. Ghajar, Hector Peinado, Jacqueline Bromberg, David Lyden

Metastasis to distant vital organs such as lung, liver, and brain is the most devastating feature of cancer progression, responsible for over 90% of cancer-associated deaths. In 1889, Stephen Paget first proposed that organ distribution of metastases is a non-random event, yet metastatic organotropism remains one of the greatest mysteries in cancer biology. Our recent studies uncovered that tumor-derived microvesicles, specifically exosomes, alter the microenvironment at future sites of metastasis to form pre-metastatic niches, creating a favorable “soil” for incoming metastatic “seeds”. However, by what mechanism this occurs, and the role of exosomes in tumor metastasis, remains unknown. To investigate the role of exosomes in organotropic metastasis, we have used two established organotropic human tumor models: the MDA-231 breast cancer (BC) cell line, and its variants known to metastasize to the lung, brain and bone, respectively, as well as two liver metastatic pancreatic cancer (PC) cell lines, BxPC3 and HPAF-2. We first analyzed the biodistribution of fluorescently-labeled exosomes derived from lung metastatic, brain metastatic or bone metastatic MDA-231 BC variants or PC cell lines, and found that BC exosomes follow the organ-specific distribution of the cells of origin, while PC exosomes home to the liver. In each target organ exosomes are taken up by different cell types: fibroblasts/epithelial cells in the lung, Kupffer cells in the liver, and endothelial cells in the brain. In the organotropic MDA-231 model, prior education with the lung tropic exosomes redirected metastasis of the bone tropic cells to the lung, demonstrating the unique capacity of exosomes to determine the site of metastasis. Unbiased proteomic profiling of exosomes revealed distinctive integrin expression patterns, and analysis of plasma exosomes from BC and PC patients that later developed site-specific metastasis revealed that specific exosomal integrins could predict metastatic spread.

Schistosomiasis is a common parasitic infection caused by blood-flukes helminth of the genus Schistosoma that affects more than 200 million people, mostly in the developing world. Schistosomal infections are also diagnosed in non-endemic areas, in immigrants and travelers coming from endemic countries. The Schistosoma parasites have developed multiple mechanisms for modulating or suppressing host immunity. Despite intensive study over the years, the mechanisms by which adult Schistosoma escape the host immune systems are unknown. Schistosoma was shown to affect Treg cells of the infected patients, suggesting intercommunication with host cells. However, the mechanism by which this communication takes place has not been characterized yet

Using primary schistosomal cultures we were able to isolate a fraction of secreted microvesicles. In preliminary results, we began to characterize these particles applying several tools, including transmission electron microscopy, atomic force microscopy and NanoSight. Further, we performed proteomics analysis of the vesicles contents and altogether the results strongly suggest that these microvesicles, can be classified as exosomes. Importantly, we were also able to isolate exosomes from patients' sera and detected parasite-derived miRNAs in them.

We hypothesize that the adult Schistosoma utilizes secreted exosomes as a mechanism to manipulate and escape the immune system.

'Small talk' in the immune system; heterogeneity of extracellular vesicles and their diverse role in immune regulation

Susanne van der Grein[1], Tom Driedonks[1], Henrike Jekel[1], Marijke Zonneveld[1], Martijn van Herwijnen[1], Ger Arkesteijn[2], Maarten Altelaar[3], Henk Buermans[4], Peter-Bram 't Hoen[4], Johan Garssen[5], Frank Redegeld[6], Marca Wauben[1], Esther Nolte-'t Hoen[1]

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Tight regulation of the magnitude and quality of immune responses is crucial for efficient eradication of pathogens, maintenance of homeostasis, and neonatal development. Hereto, immune cells need to integrate information from the environment and communicate with other cells. Transfer of biologically active molecules by extracellular vesicles (EV) plays a role in this process. We aim to unravel whether and how the immune status affects the molecular contents of EV released by immune cells and how these EV contribute to modulation of immune responses. Strong focus is put on technical aspects of extracellular vesicle purification and flow cytometric analysis for quantitation and multi-parameter characterization of individual EV. Moreover, we focus on small non-coding RNAs as important functional components of EV.
The immune activating or inhibitory activities of dendritic cells (DC) are determined by environmental stimuli and the immune status of an individual. We found that functionally different DC release phenotypically different EV subpopulations. EV from tolerogenic and immunogenic DC not only displayed different miRNA profiles but also differed in the amount and type of other small non-coding RNAs with potential gene regulatory functions. Importantly, our data also indicate that EV contribute to the immune modulatory activity of DC. EV from tolerogenic DC suppressed the release of the pro-inflammatory cytokine IL-17 by T cells, suggesting that these EV can contribute to creating a local immune suppressive milieu to limit potentially harmful Th17 responses. Similarly, we found that human milk contains EV that suppress T cell responses. Proteomic and transcriptomic analysis indicated that milk contains EV subpopulations that are likely released by immune cells and that these milk EV harbor proteins and miRNAs with strong immune modulatory capacities. In summary, diverse immune cell-derived EV transport (antigen-specific) immune information and contribute to regulation of immune responses.

Whispers of the embryo – maternal communication: Monitoring spatio-temporal cell interactions through microRNAs

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The crosstalk between maternal uterine endometrial cells and embryonic trophoblasts is crucial for the establishment of pregnancy. Of the numerous regulators that have been proposed to mediate maternal-embryo interactions, miRNAs standout for their capacity to regulate the cell transcriptome and cellular identity. miRNAs are transported amongst a variety of elements like proteins, nucleic acids and organelles via exosomes. Communication through exosomes has been observed in a wide range of organisms, from Plasmodium and Drosophila to mice, pigs, and human. To gain a better understanding of the maternal-embryo communication, we have devised a method to follow real-time expression of miRNAs potentially transferred through exosomes using an in vitro model of trophoblast – endometrial cells interaction.

We designed a miR-sensor system based on a double reporter plasmid track to visualize the expression of the miRNA of interest. Two endometrial cell lines and HEK293T cells, as a nonspecific control, were transfected with four different miR-sensors and expression of the tagged microRNA was monitored in response to the presence of trophoblast spheroids (TS) which simulated the embryo. The TS affected the expression of four miRNAs examined in the three cell lines. We observed that changes in miRNA expression occurred early and mostly in cells located in close proximity to the TS. Interestingly, we observed a trophoblast specific modulation of the GFP reporter expression, only in the endometrial cell lines, independent of the miRNA expression. Using the miR-sensor system developed, we were able to monitor spatio-temporal intercellular communication mediated by miRNAs using a model of endometrial cell interactions with trophoblast cells. The miR-sensor system could be further applied to monitor microvesicle / exosome-mediated cell communication.

Embryo-maternal interactions: lessons from phylogenetically distant species

Yael Heifetz and Monika M. Kaczmarek, Department of Entomology, The Hebrew University, Israel; Institute of Animal Reproduction and Food Research PAS, Poland

Embryo-maternal communication is crucial to achieve successful pregnancy and for the future health of the progeny. After mating, gamete transport in the female reproductive tract, gamete maturation, fertilization, and early embryonic development are all dependent upon the relay of appropriate molecular signals between the mother, gametes and embryo. Synchronization between developing embryo (egg) and maternal reproductive tract is recognized as a milestone ensuring proper/optimal fertility, and disturbances in maternal-gamete/embryo communication can influence the outcome of pregnancy. While to date studies have identified numerous molecules, including small non-coding RNAs such as microRNAs (miRNAs), that may be important for this process, still there remain many gaps in our understanding of the embryo-maternal communication. In our talk, we will focus on extracellular vesicles (EVs), including exosomes and microvesicles, which may act as essential mediators of embryo-maternal communication, in phylogenetically distant species: the fruit fly *Drosophila melanogaster* and the domestic pig *Sus scrofa domestica*. Analysis of the uterine microenvironment of the pig identified microvesicles, including exosomes, containing miRNAs, which have been suggested as regulators of reproductive tract remodeling and embryo development/implantation. Furthermore, our results indicate possible sources of microvesicles involved in regulating the extracellular environment of the maternal tract and the uterine environment. Our studies also support the concept that gamete/embryo-maternal communication may have not only short (local) but also potential long-range effects on the mother. Finally, we will highlight phylogenetically conserved elements of embryo (egg)-maternal crosstalk.

An insight into the unknown terrain of sperm storage in Drosophila female using correlative microscopy

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Females in taxa ranging from insects to mammals have evolved sperm storage mechanisms. Different organisms store different amounts of sperm for different lengths of time in closed or open reservoirs. Given its wealth of genetic and molecular tools and the high conservation of genes between flies and mammals, Drosophila promises to be an important model system for understanding the molecular basis of sperm-female interactions across animal taxa. Drosophila females have two types of sperm storage organs: paired spherical spermathecae and a single elongate, tubular seminal receptacle. Despite the interest in the evolution and function of the seminal receptacle, the structure of the receptacle epithelium has received little attention in Drosophila. We have used a correlative microscopy approach in which we combined confocal for light, X-ray microtomography for three-dimensional view of the whole organ and *high resolution in vivo* imaging with focus ion beam (FIB). This combination allowed an increased sample throughput gaining insights into the internal structure of the seminal receptacle. Each imaging technique revealed a different level of information about sperm localization within storage, outline of the organ and details about the internal structures. The obtained results open new avenues and allow new questions to be asked about sperm-female interactions post-mating.

Exosome secretion affects social motility in *Trypanosoma brucei*

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The Mina and Everard Goodman Faculty of Life Sciences and Advanced Materials and

Extracellular vesicles secreted by pathogens function in a variety of biological processes. Here, we describe exosomes secretion that is induced by severe stress in *Trypanosoma brucei*. We show that under *trans*-splicing inhibition, the spliced leader RNA (SL RNA), which donates its exon to all mRNAs via *trans*-splicing, is secreted from the cell. Following perturbations in SL RNA biogenesis or under heat-shock, the SL RNA is exported to the cytoplasm and forms a complex with cytoplasmic proteins. The SL RNA is then secreted by exosomes. The exosomes are found on the surface of the parasites and are formed in multivesicular bodies utilizing the endosomal sorting machinery, ESCRT, through a mechanism similar to microRNA secretion in mammalian cells. Silencing of the ESCRT factor, *Vps36*, compromised exosome secretion. Cells secreting exosomes or purified exosomes are sensed by cells engaged in social motility, and repel their migration. This finding has implications to the mechanisms by which parasites control their motility within their hosts and how stress-signals are transmitted from the parasite to the external milieu.

Communication between cells is an essential function in multicellular organisms. The possible transfer of RNA molecules between cells was recently suggested as a form of communication that could have a regulatory role in the acceptor cells. The current model suggests that such transfer occurs by secreting RNA to extracellular fluids, either as a free RNP particle or packed in exosomes. However, most work in the field has been done on whole cell populations, using mostly biochemical methods. Many questions still remain. In particular, can we show a quantitative analysis of RNA transfer and what are the exact mechanism and kinetics of this process.

Here, we used single molecule fluorescent *in situ* hybridization (smFISH) and live imaging of single mRNA molecules to show that mRNAs are transferred in mammalian co-cultures. We found that the transfer of mRNAs occurs in both immortalized and primary cells. Among the mRNAs we followed are those encoding mouse β-actin, human Cyclin D1, human BRCA1, and others. We were unable to detect transfer of HER2 mRNA, suggesting this process has specificity. co-FISH experiments revealed that the transferred β-actin mRNA makes up ~2-5% of the total β-actin mRNA in the acceptor cell. mRNA transfer is quick and is independent of *de-novo* protein synthesis, but modulated by stress conditions.

Contrary to the current model, we found that mRNA transfer requires close proximity between cells and is not mediated by diffusion. Rather, we suggest that mRNA is transferred through membrane nanotubes. These are very long (>100μm) yet thin (50-300nm) cytoplasmic projections that were recently shown to be involved in direct, contact-dependent, intercellular communication. We present both biological and imaging data to support this hypothesis.

The biological significance of intercellular transfer hinges on whether these mRNAs are translated and effect cell physiology. We currently are developing methods to assess this.

A generic approach for isolating and characterizing extracellular vesicles

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Large efforts are currently made to produce reliable samples of extracellular vesicles (EVs) and to develop, improve, and standardize techniques allowing their biophysical characterization.

In a first example, we show how to use ultrafiltration and size-exclusion chromatography for the isolation and a model-free fluorescence fluctuation analysis for the investigation of the physical and biological properties of EVs secreted by mammalian cells. Our purification strategy produces enriched samples of morphologically intact EVs free of extra vesicular proteins and allows labeling of marker molecules on the vesicle surface for single vesicle analysis with single-molecule sensitivity. This novel approach provides information on the distribution profile of both EV size and relative expression level of a specific exosomal marker, deciphering the overall heterogeneity of EV preparations.

To demonstrate a clinically relevant example, we applied our procedures to exosomes derived from human breast cancer cells. The composition of such exosomes depends on the sort and state of the tumor, requiring screening of multiple antigens to reliably characterize the disease. Therefore, we exploited the capacity of surface plasmon resonance biosensing to detect simultaneously multiple exosomal and cancer biomarkers on the exosomes. This method delivered a characteristic molecular signature for each cell type and is ready for implementation in academic research and clinical diagnostics.

We will in addition outline how to monitor GPCR- and channel-mediated trans-membrane signaling reactions in cell-derived vesicles. As the vesicles comprise parts of a cell's plasma membrane and cytosol, they represent the smallest autonomous containers performing cellular signaling reactions. Using fluorescence microscopy, we measured in individual vesicles the different steps of G-protein-coupled receptor mediated signaling like ligand binding to receptors, subsequent G-protein activation and finally arrestin translocation indicating receptor deactivation. Observing cellular signaling reactions in individual vesicles opens new directions for the analysis of EVs.

Characterization of natural and synthetic vesicles

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Extracellular vesicles (EVs) are lipid bilayer enclosed structures released by cells and are classified based on their cellular origin, biogenesis and physicochemical properties, including apoptotic bodies, microvesicles and exosomes. They have emerged as important mediators of intercellular communication and may serve as biomarkers of disease and as potential therapeutic targets. Despite intense investigation, however, many properties and mechanisms remain indefinable due to the lack of standardization of isolation and characterization methods which hinders the translation of EV-based diagnostics into clinical use.

Extracellular vesicles were isolated by centrifugation from human erythrocytes and characterized by freeze-fracture electron microscopy (FF-TEM), dynamic light scattering (DLS), attenuated total reflection Fourier-transform infrared spectroscopy (ATR-FTIR). Total protein and lipid content were determined by Bradford and Stewart bioanalytical assays.

The cell membrane is an ordered environment of lipid molecules, proteins and other signaling molecules. Monitoring membrane orientation by polarized light spectroscopy will receive information on protein conformation and interactions in the lipid bilayer. Small aromatic molecules (pyrene and retinoic acid) were used for determining the lipid bilayer orientation. These data can also be used to identify how the applied shear force can distort the original vesicle morphology - a property that is probably characteristic to the composition of a particular type of vesicle.

miR-4443, a Possible Participant in Mast Cell Activation by T Cell-Derived Microvesicles

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Background: In addition to being pivotal in the elicitation of allergic responses, mast cells were found to be activated in T cell-mediated inflammatory processes. We have recently shown that mast cells can be activated by microvesicles (MVs) derived from activated T cells (mvT*) to degranulate and release several cytokines. These events are associated with internalization of mvT* followed by RAS and sustained ERK activation. The aim of this study was to analyze the possible effect of microRNAs delivered by MVs on mast cell activation.

Methods: The high-throughput microRNA profiling was performed using NanoString technology platform and was validated by real time PCR. The biological role of mvT*-derived microRNA was verified by overexpression of these microRNAs in mast cells using mimic or inhibitor molecules and analyzing their predicted targets.

Results: T cell-derived microvesicles were found to downregulate the tyrosine phosphatase PTPRJ expression, a known ERK inhibitor. Bioinformatics analysis revealed that miR-4443 is predicted to regulate the PTPRJ gene. Indeed, miR-4443 present in mvT*, was also found to be overexpressed in human mast cells stimulated with this MVs. Luciferase reporter assay indicated that 3'UTR of PTPRJ was the target of this miR. Transfection of mast cells with mimic or inhibitor of miR-4443 resulted in decreased or enhanced PTPRJ expression respectively. Furthermore, miR-4443 was found to regulate ERK-phosphorylation and IL-8 release in mast cells activated by mvT*.

Conclusion: Stimulation of mast cells with mvT* leads to overexpression of miR-4443 that serves as PTPRJ negative regulator. This may explain, at least in part, the sustained ERK phosphorylation and mast cell activation in response to stimulation with mvT*. Thus, by carrying a cargo of genetic information from one cell type to another, MVs may play an important role in T cell - mediated inflammatory processes where mast cells were found to be involved.

Regulated delivery of membrane-type proteases to microvesicles in invasive tumor cells

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Cells release multiple, distinct forms of extracellular vesicles including structures known as microvesicles, which are known to alter the extracellular environment. Despite growing understanding of microvesicle biogenesis, function, and contents, mechanisms regulating cargo delivery and enrichment remain largely unknown. Here we demonstrate that in invasive tumor cell lines, the v-SNARE, VAMP3, regulates delivery of microvesicle cargo including the membrane-type 1 matrix metalloprotease (MT1-MMP) to shedding microvesicles. MT1-MMP delivery to nascent microvesicles depends on the association of VAMP3 with the tetraspanin CD9. VAMP3-shRNA expression depletes shed vesicles of MT1-MMP and decreases cell invasiveness. Finally, we describe functionally similar microvesicles isolated from bodily fluids of ovarian cancer patients. Together these studies demonstrate the importance of microvesicle cargo sorting in matrix degradation and disease progression.

microRNA cargo of extracellular vesicles isolated from uterine cavity during early pregnancy

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Efficient embryo implantation into the receptive endometrium requires synergistic endometrial-conceptus interactions within the uterine cavity, crucial for establishing pregnancy. Early pregnancy is characterized by the extensive exchange of a great variety of factors, molecules, particles between the uterus and conceptus. Our studies are focused on the role of microRNAs (miRNA), small non-coding RNAs, able to regulate broad range of biological processes through sequence-specific interactions with mRNAs. Recently, we demonstrated that conceptuses may cross-talk with uterine cells via exosomes containing miR-26a and miR-125b. Here we isolated EVs form uterine cavity of pregnant pigs on day 12, 14 and 16 (n=4/day). Presence of EVs in the uterine luminal fluid (ULF) was confirmed by several visualization methods (electron microscopy, western blot) and nanoparticle tracking analysis (NTA). miRNAs profiling was accomplished with custom made TaqMan Low Density Arrays (TLDA) and quantitative real-time PCR. Out of 84 investigated miRNAs we were able to detect 49, among which 10 miRNAs showed significantly affected profiles between days 12-16 of pregnancy ($p<0.05$). The majority have been already detected in our recent studies performed in pregnant animals. Most importantly, expression profiles of miRNAs detected in EVs resemble those of endometrium or conceptuses (e.g., miR-30a-5p, miR-422b, and miR-193b). Experimentally validated miRNA-mRNA interactions combined with target prediction analysis showed that miRNAs being a cargo of EVs detected in ULFs from pregnant animals are potential regulators of pregnancy related genes, such as inflammatory mediators or growth factors. Our results suggest that miRNA cargo in EVs isolated from uterine cavity during early pregnancy is an important component of maternal-conceptus communication, giving an impetus to further large scale functional analysis investigating the mechanism of action and the impact of their delivery on the processes crucial to successful pregnancy.

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Inter-cellular transport of Ran GTPase through exosomes

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Ran GTPase has a well-established role in nucleo-cytoplasmic transport of macromolecules across the nuclear envelope. Recently, we found that Ran gets transferred between cells in a GTP- and CRM1 dependent fashion. Here we show that the inter-cellular transport of Ran occurs through exosomes, vesicles derived from multivesicular bodies. We find that the recruitment of Ran into exosomes also is GTP- and CRM1-dependent. Moreover, our studies reveal that Ran's ability to get recruited into exosomes is negatively modulated by its SUMO-modification. Furthermore, using enucleated RBCs, our studies uncover a possible role for Ran-CRM1 axis in the recruitment of soluble protein cargos into the exosomes. Together, the findings unravel a unique role for Ran beyond nucleo-cytoplasmic transport and provide a framework for identifying the signals and mechanisms involved in targeting soluble proteins into exosomes.

Bone marrow mesenchymal stem cells microvesicles critically affect multiple myeloma cells in accordance with their normal or pathological source

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Background and Purposes: Myeloma cells' (MM) interaction with the bone marrow (BM) microenvironment critically hinders disease therapy. Previously, we showed that co-culture of MM cells and BM-mesenchymal stem cells (MSCs) caused co-modulation of translation initiation (TI) and cell phenotype and that secreted components are implicated. Here, we studied the role of the MSCs secreted microvesicles (MVs) on the MM cells phenotype, TI, and signaling.

Methods: MVs were extracted from BM-MSCs secretomes (3 days; normal donors (ND-MSCs), MM patients (MM-MSCs)). Basic MVs characterization (electronic microscope), their uptake (fluorescence microscope, FACS) and dose response were verified. MM/ND-MSCs MVs (10-150mg/ml) were added to MM cell lines (U266, ARP1, MM1S and OPM2) for (5min ,1h, 4h, 12h and 3days) assayed for viability (wst1) and live/dead/total cell count (trypan blue); migration (transwell); TI status (factors: eIF4E, eIF4GI; regulators: mTOR, MNK, 4EBP; targets: SMAD5, NFκB, cyclinD1, HIF1, cMyc) (immunoblotting); and MAPKs activation (immunoblotting).

Results: Within 24h BM-MSCs MVs were internalized by MM cells evoking opposite responses according to MVs origin (ND/MM-MSCs). ND-MSCs MVs decreased total and live cell counts (↓15-50%, *p<0.05*), viability (↓22-40%, *p<0.05*), migration (↓>30%, n=1) and TI status (↓>10-80%; *p<0.05*). In contrast, MM-MSCs MVs increased total and live cell counts (↑10-60%, *p<0.05*), PCNA proliferation marker (↑20-120%, *p<0.05*) and TI status (↑20-185%, *p<0.05*). ND-MSCs MVs treated MM cells demonstrated a rapid (5min) activation of MAPKs followed by a persistent decrease (1h, 4h, and 24h). Studies on MAPKs in MM-MSCs MVs treated MM cells are underway.

Discussion: BM-MSCs MVs display a major effect on MM cells TI and phenotype in accordance with source. The rapid induction of MAPKs is consistent with a receptor-ligand interaction between the MVs and the MM cells. The manipulation of TI and significant alteration in phenotype underscore the potential of MVs to MM cells communication with their microenvironment and warrant additional studies.

Microvesicles mediate radioresistance induction in melanoma cells.

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Cells never exposed to radiation can respond to signals from irradiated cells via a process known as bystander effect. In recent years, strengths aimed to identify new players in bystander information exchange have brought to consider exosomes as potential mediators of this crosstalk. Exosomes are microvesicles which diameter ranges between 30 and 130 nm, released by cells in physiological and altered conditions. They have also been found to mediate the radioadaptive response, by increasing radioresistance in recipient cells via paracrin signalling. This resistance induction naturally composes the organism reaction to radiation exposure. It can interfere with the outcome of some important medical tools such as radiotherapy, where cancer cells are target of radiation delivery. Aim of this work is to relate the exosome-mediated communication to the radioresistance occurring in several cases of tumours. To validate this hypothesis we isolated exosomes-enriched microvesicular fractions produced by a neuroblastoma cells before (CTRL cells) and after exposure to 1 Gy of X Rays (IR cells) using filtration and ultracentrifugation techniques. Characterization of vesicular populations by Dynamic Light Scattering, Z potential analysis and Scanning Electron Microscopy revealed physical features similar to the exosome fraction. Data obtained from protein and RNA quantification suggested an increase in microvesicles production by IR cells compared to CTRL cells. After verifying exosome uptake by recipient cells using immunofluorescence, we analyzed radioresistance induced by microvesicles treatment in term of cell viability. We related these results with presence and phosphorilation of apoptotic-related molecules (AKT and ERK) and activation of Nrf2, a transcription factor involved in antioxidant response. All our data suggest an induction of radioresistance in melanoma cells treated with exosomes produced by the same cell type during oxidative stress induced by ionizing radiation.

Angiogenic and anti-inflammatory properties of mesenchymal stem cells from cord blood: soluble factors and extracellular vesicles for cell regeneration

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In a recent work, our group showed the existence of two distinct mesenchymal stem cell (MSC) subsets within human umbilical cord blood (CB): one less proliferative and short-living (SL-CBMSC), the other with a higher growth rate and long-living (LL-CBMSC). Based on these phenotypes, we proposed LL-CBMSC as better CBMSC subset to use in regenerative medicine approches. Therefore, we examined whether LL-CBMSC possessed peculiar paracrine properties able to affect angiogenesis or inflammatory processes. Pro-angiogenic, proliferation-stimulating and tissue repair factors were released at high levels both as soluble cytokines and, shown for the first time, as mRNA precursors embedded in membrane vesicles. The combination of this primary (proteic factors interacting with surface receptors) and delayed (mRNA transferred via vesicle fusion and cargo release) interaction in endothelial target cells resulted in strong blood vessel induction with the development of capillary-like structures. In addition, in an in vitro model of damage, LL-CBMSC could dynamically modulate their release of pro-angiogenic and anti-inflammatory factors, hinting at a regulated cross-talk. In conclusion, LL-CBMSC synthesize and secrete multiple factors that may be attuned in response to the status of the target cell, a crucial requisite when paracrine mechanisms are needed at onset of tissue regeneration.

A novel technique for high throughput plasma proteomics profiling using extracellular vesicles

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The interest in using mass-spectrometry (MS) based proteomics for clinical applications has profoundly increased over the past few years, towards the discovery of potential biomarkers and the development of new diagnostic tests. However, the huge dynamic range of proteins in the plasma, which leads to the masking of potential biomarkers by the highly-abundant proteins, limits the coverage of the plasma proteome. To overcome this challenge we fractionate the plasma by isolating plasma microparticles. Microparticles are extracellular vesicles that are shed by all cell types. They can be released into the bloodstream, while carrying the proteomic signature of the cell of origin. Importantly, both their number and composition change upon disease, indicating their great potential in the biomarkers discovery field. Based on that we have developed PROMIS-Quan (PROteomics of MIcroparticles with Super-SILAC Quantification), which provides a high throughput and unbiased approach for MS-based plasma proteomics, combined with highly accurate relative and absolute quantification of the examined proteins. Using this technique we can reach an unprecedented depth of over 3,000 proteins in single MS runs. We tested PROMIS-Quan on two different datasets. In one we compared samples of healthy donors and prostate cancer patients, and found a predictive signature for prostate cancer diagnosis comprised of 3 proteins with area under the ROC curve (AUC) of 0.84. In a second dataset we examined the response to immunotherapy among stage-IV melanoma patients and got a strong predictive signature with AUC of 0.91. Altogether we propose PROMIS-Quan as an innovative platform for unbiased biomarker discovery based on the isolation of microparticles from the blood.

Circulating EBV-modified exosomes in SLE patients target the renal tubular epithelium delivering inflammatory small RNAs and sensitizing RNA sensors

A possible pathogenic role of Epstein-Barr virus (EBV) in autoimmune diseases such as systemic lupus erythematosus (SLE) is long suspected, but its exact role remains unknown. Recently, we have shown that Epstein–Barr virus-encoded small RNA 1 (EBER1) and some EBV-related microRNAs (miRNA) are selectively released from infected B cells via exosomes and are internalized by human plasmacytoid DCs, expressing TIM1 phosphatidylserine receptor, a known viral and exosomal target molecule.
In this study we focus on SLE patients with renal involvement, known as Lupus Nephritis (LN). We analyzed LN and other kidney disease control biopsies by in-situ hybridization, which revealed a defined EBER1 presence in tubular epithelial cells (TEC) of LN biopsies. Accordingly, high levels of EBER1 and EBV-produced miRNAs were detected in LN biopsies by RT PCRs, consistent with a role of EBV-modified exosomes in SLE and LN pathogenesis. Strikingly, TIM1 is expressed by Tubular Epithelial cells (TEC) and we confirmed in primary TEC cultures that EBV-modified exosomes are internalized in a PtdSer-dependent manner delivering inflammatory RNAs into endosomes. We next analyzed the possible inflammatory effects of EBV-modified exosomes entry into TEC cells by isolating exosomes from EBV-infected B cells. The addition of EBV-modified exosomes, as well as infection of RNA-isolated from the same exosomes into TEC cells, caused an induction of cytokine production such as IL6 and interferon-stimulated genes, as well as RNA sensors such as RIGI and TLR3. The induction of these RNA sensors upon exosome addition was significantly decreased after treating TEC cells with Hydroxychloroquine (known as Plaquenil), an antimalarial drug used for SLE treatment. These data support the hypothesis that EBV-modified exosomes might play a significant role in promoting inflammatory responses in autoimmune diseases including SLE and LN, partially through sensitizing the key RNA sensors in affected tissues and thereby increasing endogenous and exogenous RNA recognition.

Bacterial outer membrane vesicles induce plant immunity and enhance bacterial disease resistance

All Gram-negative bacteria pinch off portions of their outer membrane, releasing outer membrane vesicles (OMVs) to the surroundings. Strangely, the interactions between these highly abundant structures and plants have been mostly overlooked. In this study we sought to examine the interactions of OMVs and the plant immune system. We demonstrate that the cytosolic microbe associated molecular pattern (MAMP) EF-Tu is released to the outer cellular space in OMVs and that OMVs induce typical innate immune responses in *Arabidopsis*. Treating OMVs with proteinase K prior to *Arabidopsis* challenge did not reduce the level of defence gene activation indicating that non-proteinaceous immunogenic factors in OMV are being exposed to plant immune receptors. Nevertheless, *Arabidopsis* knockout lines lacking the immune receptors for peptidoglycan and lipopolysaccharides were sensitive to OMVs as wild type plants. To determine whether the elevated immune response imposed by OMVs would lead to bacterial disease resistance we pretreated *Arabidopsis* plants with OMVs and inoculated it with *Pseudomonas syringe* pv. *tomato* DC3000. A statistically significant reduction in DC3000 cell titer was observed in the OMV-pretreated plants compared with the water-pretreated control. Altogether, our results reveal a new facet in plant-bacteria interactions that may lead to new discoveries of the plant immune system and bacterial pathogenesis.

BAG6 as a novel regulator of exosome release and protein sorting

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Natural killer (NK) cells are a major component of the anti-tumor immune response. We have recently shown that the impaired activity of NK cells in chronic lymphocytic leukaemia (CLL) is dependent on soluble ligands for NK cell receptors (NKRs) that are released by the malignant B-cells. One of these soluble ligands is BAG6, which engages the cytotoxic NK cell-receptor only when expressed on the surface of extracellular vesicles (exosomes). BAG6 is a multifunctional protein also acting as an intracellular chaperone involved in protein targeting and protein degradation. Here, we analyzed the role of BAG6 for the release of immune-activating exosomes upon DNA damage in CLL.

Immunoprecipitation, *in vitro* translation and a yeast-two-hybrid approach revealed that BAG6 binds directly to p53 and forms a ternary complex with the actetyltransferase CBP/p300 in response to doxorubicin-induced DNA damage. Induction of DNA-damage triggered the release of exosomes and the nuclear export of BAG6. The release of exosomes was dependent on BAG6 and p53 under basal and stress-related conditions.

Exosomes from BAG6 wild type cells were characterized by an enriched expression of BAG6-interacting partners and immune regulatory molecules. These molecules were diminished in exosomes collected from BAG6-deficient cells suggesting that BAG6 impacts on exosome cargo. The data presented identify BAG6 as a novel key component in exosome formation and loading.

Oviductosomal Expression of Mouse PMCA1 in Female Fertility of *Pmca4* nulls

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Plasma membrane Ca^{2+} ATPase4 (PMCA4) is the major Ca^{2+} efflux pump in murine sperm, where deletion of its encoding gene (*Pmca4*) leads to male infertility due to loss of hyperactivated motility. PMCA4, which accounts for 90% of PMCAs in sperm, is not only crucial in males but is also expressed in females where extracellular vesicles (EVs) in the oviductal luminal fluid (termed Oviductosomes) are capable of transferring it to sperm, just before fertilization. Acquisition of additional PMCA4 at the fertilization site meets the increased demand for Ca^{2+} efflux in sperm, resulting from the elevated Ca^{2+} levels required for their hyperactivation and acrosome reaction. However deletion of murine *Pmca4* has no effect on female fertility; despite Ca^{2+} efflux requirement which also occurs for oviductal ciliary action, necessary for oocyte transport. Here, we show that the PMCA1 isoform is expressed in reproductive tissues (vagina, uterus, and oviduct) in the epithelial lining, luminal fluids, EVs, with an upregulation in oviductal luminal fluid (OLF) during estrus in *Pmca4*$^{-/-}$, compared to wild-type females. Western analysis showed that PMCA1 levels are significantly higher (P=0.02) in *Pmca4*$^{-/-}$ OLF during estrus, compared to wild-type or null epididymal luminal fluid. In cycling females, PMCA1 expression in OLF of *Pmca4*$^{-/-}$ was significantly (P=0.02) higher than in wild-type during proestrus/estrus; but significantly decreased (P=0.03) during metestrus/diestrus, showing an interaction between genotype and stage. Importantly, during estrus PMCA1 levels were significantly (P<0.05) elevated in *Pmca4*$^{-/-}$ oviductosomes (OVS), compared to wild-type, unlike *Pmca4*$^{-/-}$ uterosomes, or epididymosomes. OVS delivered PMCA1 to sperm following co-incubation of OLF and sperm, and were shown to carry in their cargo nNOS (neuronal nitric oxide synthase) and CASK (Ca^{2+}/CaM- dependent serine kinase), interacting partners of PMCA1 and PMCA4. The data show an important role of oviductosomal PMCA1, which compensates for PMCA4 in *Pmca4*$^{-/-}$ OVS, in female fertility.

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The past, presence and future of extracellular vesicles

Extracellular vesicles such as microparticles and exosomes are common and widely distributed, and are now thought to have a multitude of functions in health and disease. Evidence is accumulating that vesicles in human body fluids are involved in protection, intercellular communication, and disease development and progression. Furthermore, vesicles potentially behold an entirely new level of clinical relevant information for diagnosis and monitoring of therapy.

We have an interest in tissue factor (TF), and have shown that body fluids such as saliva of healthy subjects contain vesicles exposing coagulant TF. Because such vesicles are absent in the blood, we hypothesize that "licking a wound" facilitates blood clotting and wound healing. In patients suffering from meningococcal septic shock or cancer, however, coagulant TF-exposing vesicles can be present within the peripheral blood, and such "blood-borne" vesicles are thought to contribute to development of bleeding and thrombosis.

Furthermore, we have worked on improved isolation and detection of (single) vesicles. With regard to isolation, rediscovery of size exclusion chromatography has increased the general awareness that a decent isolation procedure is essential to reduce the risk of artefacts. Similarly, detection of single vesicles is often more difficult than anticipated. Because detection of both rare (biomarker) and common (reference ranges) vesicles requires comparison of results between instruments and institutes, we have compared, tested and selected reference materials, are developing novel reference materials and software, and have initiated an international standardization study to compare vesicle measurements between instruments and institutes.

Taken together, a whole new research field is now evolving rapidly, and an introduction to and overview of vesicles will be provided, with emphasis on our research.

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Size exclusion chromatography (SEC) is a powerful tool for the separation of macromolecules and biological nanoparticles, and it is useful in the isolation and purification of EVs. Despite the wide variety of SEC macroporous stationary phases only limited number of them was applied in the EV field.

The aim of our study was to compare different stationary phases in SEC regarding their ability of separation and size characterization of EVs.

EVs were isolated from different natural milieus: urine, cell medium, erythrocyte concentrate and plasma, and were compared with biologically relevant reference materials: lipoproteins and liposomes. Separation and size characterization was performed by HPLC-SEC (Jasco HPLC system, PU-2089 pump, UV-2075 UV/Vis detector, supplemented with an on-line coupled W130i DLS from Avid Nano Ltd.), with TSK G6000PW (Tosoh Corp.) pre-packed column, and Tricorn 5/200 glass column filled with Sepharose CL-2B (GE Healthcare) cross-linked agarose gel.

Based on the fractionation ranges of the used columns (TSK G6000PW: up to 8000 kDa for polyethylene glycol, and Sepharose CL-2B: 70-40,000 kDa for dextrans) both of them was found to be suitable for the separation of EVs from soluble proteins and lipoprotein particles. Additionally, slight differences were found in the elution profiles of synthetic liposomes and EVs with different sizes, hence the applicability of SEC for discrimination of different vesicle fraction by size is limited. However, liposomes with different surface characteristics and EVs from different origin resulted slightly different elution times and profiles, revealing, that not only steric interactions governs the retention of these particles in SEC.

SEC using different macroporous stationary phases was found to be suitable for the characterization of the purity of different EV preparations. The use of SEC is limited for the size determination of the vesicles, although, more investigations are needed to reveal the physicochemical background of the observed elution profiles.

Exosomes derived from malaria parasites-infected red blood cells contain distinct human and parasite small RNAs

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Cells use extracellular vesicles to communicate, to coordinate social activities and, in the case of pathogens, to manipulate host target cells. Previously we showed that the lethal malaria parasite, *Plasmodium falciparum* (*Pf*) can transfer episomal plasmids via exosome-like vesicles produced at the ring-stage of the asexual life cycle. Here, we identify *Pf*-derived nanovesicles as exosomes and determine their RNA cargo. Unlike vesicles from uninfected Red Blood Cells (RBCs), parasite-derived exosomes contained high amount of RNA molecules with a length of 4 -150 nt. Small RNA deep sequencing revealed a significant population of host and parasites non-coding RNAs in *Pf*-derived exosomes; The most abundant parasite non-coding region was PF13TR011:ncRNA, found on chromosome 13, with a current unknown function. Surprisingly, this sequence aligns almost perfectly to a portion of the human complement component C2 (C2) mRNA 5'UTR.

Of importance, upon performing enrichment analysis, the functional group involved in regulation of cell adhesion contained the highest number of represented human miRNA targets. These were found to influence various cell adhesion and known human receptors for the major parasite virulence ligand. Although further studies are required, these findings raise the possibility that parasite exosomes act on different host cells to manipulate their gene profile.

THyPRP and KD1 regulate flower abscission by affecting the extracellular vesicle cargo of cell wall degradation enzymes

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Organ abscission is an important cell separation process regulated by endogenous and exogenous signals, and occurring in a layer of functionally specialized cells known as the abscission zone (AZ). Loosening of the primary cell wall and dissolution of the middle lamella are fundamental steps in the abscission process of many plant systems, which involves induction of cell wall associated enzymes and proteins required for their export across the plasma membrane of AZ cells. The differentially expressed genes in the AZs include both cell wall enzymes and secretory pathway proteins such as SNARE-like proteins and syntaxins. To investigate these molecular events occurring during pedicel abscission following flower removal in inflorescences of tomato (*Solanum lycopersicum*), we performed a microarray study and identified two genes, *Tomato Hybrid Proline-Rich Protein (THyPRP*) *and Knotted1-like homeobox protein (KD1*) that are specifically expressed in the AZ. To functionally analyze these two genes, we generated two transgenic tomato lines in which each gene was silenced independently using antisense RNAs under the control of an AZ-specific promoter. We observed a significant inhibition of pedicel abscission in both transgenic lines. A transcriptomic analysis using a tomato AZ-specific microarray revealed that, in addition to other gene families, 28 genes encoding cell wall-modifying enzymes, which were strongly upregulated in the WT, were much repressed in the *THyPRP*- and *KD1*-silenced plants. Of particular interest was a *syntaxin* gene, which encodes a membrane integrated Q-SNARE protein necessary for vesicle trafficking and was induced in the WT. This gene was significantly repressed in both silenced plants. Given that the primary role of SNARE proteins is to mediate vesicle fusion with their target membrane bound compartments participating in exocytosis, our results suggest that THyPRP and KD1 may mediate flower abscission by regulating biosynthesis and exocytosis of cell wall and middle lamella-modifying enzymes of the AZ cells.

Extracellular vesicles (EVs) emerge as important carriers for intercellular communication and they have been implicated in many biological processes. However, due to their small size and the difficulties of manipulating them, the data on EV biology has thus far been based mainly on *in vitro* or indirect *in vivo* evidence, or on experiments involving *ex vivo* manipulations. Thus, the extent and pathogenic role of EV signalling *in vivo*, particularly with regard to the transfer of functional RNA, remains poorly understood. Using the Cre-Lox system we could overcome some of the major shortcomings in this field by establishing a method to trace the functional transfer of RNA by EVs *in vivo*. Expression of Cre recombinase in transgenic mice under the control of a hematopoietic-specific promoter leads to the release of EVs containing Cre mRNA, but no measurable amounts of protein from blood cells. In mice with a Cre reporter background, EV mediated transfer of Cre mRNA induces marker gene expression in target cells. In this way we could identify the direct transfer of functional RNA from blood to neurons in the brain as a novel route of communication between the immune system and the brain. While occurring vey rarely in healthy animals, this process is greatly enhanced by systemic inflammation, affecting multiple structures in the brain and leading to changes in the miRNA profile of targeted neurons. Thus, the brain and the immune system are more intimately interconnected than previously anticipated.

Sterol transport and antifungal drug resistance

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Invasive opportunistic fungal infections of humans are common among those suffering from impaired immunity, and are difficult to treat resulting in high mortality. Amphotericin B (AmB) is one of the few antifungals available to treat such infections. The AmB resistance mechanisms reported so far mainly involve decrease in ergosterol content or alterations in cell wall. In contrast, depletion of sphingolipids sensitizes cells to AmB. Recently, overexpression of PMP3 gene, encoding plasma membrane proteolipid 3 protein, was shown to increase and its deletion to decrease, AmB resistance. Here we have explored the mechanistic basis of PMP3 effect on AmB resistance. It was found that ergosterol content and cell wall integrity are not related to modulation of AmB resistance by *PMP3*. A few prominent phenotypes of PMP3 delete strain, namely, defective actin polarity, impaired salt tolerance, and reduced rate of endocytosis are also not related to its AmB-sensitivity. However, *PMP3* overexpression mediated increase in AmB resistance requires a functional sphingolipid pathway. Moreover, AmB sensitivity of strains deleted in PMP3 can be suppressed by the addition of phytosphingosine, a sphingolipid pathway intermediate, confirming the involvement of this pathway in modulation of AmB resistance by PMP3. We further explored that this PMP3 mediated AmB resistance can be modulated by sterol transport between Plasma membrane (PM) to Endoplasmic Reticulum (ER) which suggest that oxysterol binding protein (OSBP) play importance role in drug resistance.

Background: There is no perfect biomarker in the field of radiation induced biomarkers. Recent results suggest that therapeutic doses of radiation influences exosome abundance, specifically alters their molecular composition (Arscott 2013). To date, no studies have been undertaken on the effect of radiation on exosome production and composition in healthy individuals. As exosomes are easy to enrich and are protected from RNAase and proteinase degradation by their membrane, we believe that they present a possible route to the discovery of radiation-induced biomarker.

Study Aims: 1) To purify exosomes from human or animal plasma. 2) To determine the effect of radiation on protein, mRNA and miRNA profiles of exosomes. 3) To identify possible radiation-induced biomarkers for radiation protection.

Methods and Materials: Both old (26 months old) and young (2 months old) C57bl/6j mice were irradiated with different doses of gamma-radiation. The exosomes in the plasma of mice were purified using an Exoquick Exosomes Purification Kit. The presence of exosomes was confirmed with both dot and western blots of the exosomes markers. The amount of exosomes was quantified with Exocet Quantitation Assays, and the exosomal RNA was purified using SeraMir Exosome Amplification Kit. In addition, the total miRNA from the plasma was purified using miRNeasy Mini Kit. After reverse transcription, miRNA arrays of exosomal and total miRNA were performed using Mouse miScript miRNA PCR Array.

Current Results: We found that there was a slight increase of the amount of exosomes in the plasma of radiation treated mice. The miRNA expression profiles were very different between old and young mice, and with or without irradiation. We identified several candidate miRNAs correlated with radiation exposure, and could be used as radiation induced biomarker.

Current Conclusion: Exosomes could provide a premium platform for biomarker discovery for assessment of internal and external radiation exposures.

Cell-cell communication between *Plasmodium* and host immune via exosomes

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Malaria, kills up to a million people each year, is caused by the protozoa of the genus *Plasmodium falciparum (Pf)*. These vector-born parasites cycle between mosquitoes and humans and, in both contexts, are faced with an unstable and hostile environment. To ensure survival and transmission, the malaria parasite must infect and survive in the human host and differentiate into sexual forms that are competent for transmission to mosquitoes. We found, for the first time that *Pf*-infected red blood cells (iRBCs) directly exchange cargo between them using nanovesicles (exosomes). These tiny vesicles are capable of delivering protected genes to target cells.

Cell-cell communication is a critically important mechanism for information exchange that promotes cell survival. How *Pf* parasites sense their host environment and coordinate their actions, remain one of the greatest mysteries in malaria. Moreover, our understanding in the mechanism regulate human immune response to malaria infection is poor. Here, we found that malaria-derived exosomes carry remarkable cargo providing a secure and efficient mode for signal delivery. We developed an exosome tracking assay and could measure *Pf* exosome uptake by different cell types. Moreover, although early life-stages of *Pf*-iRBC are considered immunologically inert, our initial observations show that ring-stage derived exosomes are immunogenic. We show that exosomes can specifically activate and induce pro-inflammatory responses, resulting in interferon type I response. This is a new area of malaria research which may shed a light on the ability of malaria parasite to manipulate their host response.

“MVBs function to Degrade the Sperm Mitochondria after fertilization in *Drosophila”*

Almost all animals contain mitochondria of maternal origin only, but the exact mechanisms underlying this phenomenon are still unclear. We investigated the fate of the paternal mitochondria after fertilization in *Drosophila*. We demonstrated that the giant sperm mitochondrion is rapidly eliminated in a stereotypical process dubbed paternal mitochondrial destruction (PMD). PMD is initiated by a network of vesicles, resembling multivesicular bodies (MVBs) and displaying common features of the endocytic and autophagic pathways. These dual origin vesicles, which are also known as amphisomes, associate with the sperm tail, secrete their microvesicles inside the sperm flagellum and mediate the disintegration of its plasma membrane. Subsequently, the mitochondrion separates from the axoneme and breaks into smaller fragments, which are then sequestered by autophagosomes for degradation in lysosomes. We further provided evidence for the involvement of the ubiquitin pathway and the autophagy receptor, p62, in this process. Currently we focus on the role of the MVBs through the identification of their contents. We generated transgenic flies expressing the human exosomal marker hCD63 fused with EGFP. We showed that this marker specifically labels the MVBs on the sperm flagellum, allowing the successful isolation of the $hCD63^{+}$ microvesicles, and subsequent proteomic analysis of their contents.

Transmission of miRNA in macrophages-derived exosomes induce drug resistance of pancreatic adenocarcinoma

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Pancreatic ductal adenocarcinoma (PDA) rank fourth among cancer-related deaths. Despite decades of research, cure rate of the disease remains disappointingly low (<5%). This dismal prognosis stems from two sources: late detection, when the disease is already metastatic and resistant to all known systemic therapies. Gemcitabine, the drug of choice for the treatment of PDA, is a cytidine analog that acts to inhibit cell growth by termination of DNA replication. Unfortunately, resistance to the drug develops within weeks from initiation of therapy as a result of intrinsic resistance and extrinsic factors. Despite increasing recognition of this clinical problem, there has been minor progress in overcoming PDA drug resistance. Here we show that tumor associated macrophages (TAMs) in the canecr microenvironment secrete nano-vesicles known as exosomes which are selectively internalize by the cancer cells and not by the tumor stroma. Exosomes secreted by TAMs, deliver a specific miRNA known as miR-365 which upregulates cytidine deaminase (CDA), the rate limiting enzyme that metabolizes gemcitabine. Using in vitro studies and transgenic animal models we show that exosomes-induced drug resistance is reduced in Rab27ab$^{-/-}$ mice which lack exosomal secretion. We suggest a novel mechanism by which the tumor microenvironment regulates drug resistance in PDA cells by transmission of mRNA.

Radiolabeling of extracellular vesicles with ^{99m}Tc-tricarbonyl for quantitative *in vivo* imaging studies

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Introduction

Exosomes, microparticles, and other extracellular vesicles (EVs) have gained particular attention due to their role in biological processes ranging from intercellular communication and angiogenesis to cell survival. The biodistribution of EVs is a fundamental question in the field of circulating biomarkers, which has recently gained attention. Despite the capabilities of nuclear imaging methods such as single photon emission computed tomography (SPECT), radioisotope labeling of EVs and the use of the aforementioned methods for *in vivo* studies cannot be found in the literature.

Methods

We describe a novel method for the radioisotope labeling of erythrocyte-derived EVs using the ^{99m}Tc-tricarbonyl complex. Moreover, the capability of the developed labeling method for *in vivo* biodistribution studies is demonstrated in a mouse model.

Results

Labeling efficiency of 39 ± 6% was obtained for the erythrocyte-derived EVs which is reasonable, especially if the generally low concentration of EVs is considered. After purification of the reaction mixture by size-exclusion chromatography, the radiochemical purity of the labeled EVs was 97 ± 1%. We found that the intravenously administered, ^{99m}Tc-labelled EVs mostly accumulated in the liver, and in the spleen (67% and 5% of the whole-body radioactivity, respectively). The *in vivo* stability of the labeled EVs was assessed by the comparison of the obtained biodistribution of EVs with that of the free ^{99m}Tc-tricarbonyl.

Conclusions

The tricarbonyl method combined with a custom size-exclusion chromatography process enabled us to radiolabel extracellular vesicles with high stability and purity. The radiolabeled EVs are good candidates to elucidate more on EV roles, but they can also be employed for diagnostic purposes.

miRNA sequencing in milk cells and skim milk

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For veterinary diagnostics milk is an optimal matrix as it is taken routinely and non-invasively. We investigated the somatic milk cell (MC) and skim milk (SM) fraction concerning their miRNA content for early bovine pregnancy detection. While miRNAs in the MC fraction were expected to resemble the physiological state inside blood derived cells, in SM miRNAs were assumed to be present inside extracellular vesicles. Milk samples were taken on three cyclic days and after artificial insemination of the same animals. Whole milk was centrifuged, the fat layer removed and SM samples frozen at -20°C. The remaining MC was washed and stabilized in Trizol at -80°C. Total RNA was extracted and RNA integrity assessed (average RIN=6.1 and 2.3 for MC and SM, respectively). MC and SM samples of n=6 animals were sequenced on a HiSeq 2500 (Illumina). The dataset was processed to align high quality reads to miRBase for mature bovine miRNAs. Resulting read counts were normalized and analyzed with DeSEQ2 and GenEX using principal component analysis (PCA). Selected miRNAs were validated using RT-qPCR. 320`000 and 150`000 reads mapped to miRNA in MC and SM respectively. Although a high number of reads was shorter than 16 nt, in total 132 miRNAs with more than 50 reads were found, of which 87 miRNAs were abundant in both milk fractions. The miRNA profiles were surprisingly similar between MC and SM even though not all miRNAs correlated well. Although the MC sequencing data allowed a separation of cyclic and pregnant cows, we cannot recommend the biomarkers from this study for a diagnostic use as significances were low and the RT-qPCR validation not satisfying. In future studies further interest includes microvesicles present in the milk fat fraction.

Tumor-derived microvesicles facilitate the plasticity of cancer cell invasion.

Crislyn D'Souza-Schorey, Department of Biological Sciences, University of Notre Dame.

The ability of cells to invade into and traverse the extracellular environment is prerequisite for tumor cell dissemination and metastasis. Tumor cell invasion requires the molecular and physical adaptation of both the cell and its microenvironment. Invasive tumor cells release protease-loaded microvesicles into the extracellular environment to facilitate matrix invasion at distal sites. Our studies on the intrinsic and extrinsic factors that regulate microvesicle formation and release will be discussed. We have found that tumor cells assume amoeboid phenotypes on compliant matrices and have a high propensity to shed microvesicles from regions of plasma membrane blebbing. Blocking protease delivery to budding microvesicles at the plasma membrane significantly compromises movement through cross-linked collagen matrices, documenting the importance of vesicle-associated proteases in matrix degradation and cell invasion. These studies underscore the need to better understand the complex motile behavior of tumor cells.

Concentration, sizing and phenotyping EVs using NTA and FFF techniques

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Within the past decade the vital role of exosomes and microvesicles as mediators of intercellular communication has been increasingly documented. In addition, pathophysiological roles for exosomes and microvesicles are beginning to be recognized in diseases including cancer, infectious diseases and neurodegenerative disorders, highlighting potential novel targets for therapeutic intervention.

Knowing the true size distribution and concentration of EVs in a high resolution technique and in their natural environment is of significant value in elucidating the role these structures play in diseases and the ways in which they may be exploited in diagnostic or therapeutic applications.
Moreover, there is a need for high resolution separation technique, capable of handling with dispersions and that gives the opportunity to collect fractions of these samples for further research and use.

The ability of the NanoSight NTA technique to visualize, size and count exosomes and microvesicles as small as 30nm means it has become an indispensable technology in this field. In addition, the technology has the ability to operate in fluorescence mode allowing visualization, sizing and concentration measurement of fluorescently labeled exosomes and microvesicles.

Field Flow Fractionation brings the ability to separate exosomes and other EVs at a high resolution, fast and gentle technique. Combining FFF to a fraction collector and to multiple detectors [UV, Fluorescence, MALS, DLS etc.] potentially allows a greater understanding of exosome role and function, as well as opening a door to advancements in developing exosome-based diagnostics and therapeutics.

Both FFF and NTA techniques will be presented in this lecture. A focus will be brought on to application examples in which these techniques were used for exosome and extra cellular vesicles research.

Message in a bubble- the role of extracellular vesicles in mediating host-virus co-existence of the bloom-forming *Emiliania huxleyi*

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The coccolithophore *Emiliania huxleyi* forms massive blooms in the oceans and has huge impact on biogeochemical cycles of carbon and sulfur, as well as on global climate regulation. *E. huxleyi*'s intricate calcite exoskeleton accounts for ~1/3 of the total marine $CaCO_3$ production. These *E. huxleyi* blooms are often terminated by infection by a large double-stranded DNA coccolithovirus, the *Emiliania huxleyi* virus (EhV). Although this host-virus interaction has an important ecological role, the cellular mechanisms that govern it are largely unknown. Viral infection led to massive production of extracellular vesicles (ExV's), reflecting their possible involvement in viral replication cycle. The relative concentration of ExV's was higher in uninfected susceptible strains than in viral-resistant ones. We conducted lipidomic analysis and revealed a unique lipidome that greatly differed from that of both host, infected host and purified virion. Transcriptomic analysis of ExV's cargo revealed the presence of mostly host-encoded small-RNAs. Interestingly vesicle production was induced by exposing the *E. huxleyi* cells to the viral free lysate alone, indicating the involvement of small-molecules in cell-cell communication during viral infection. We further investigated the functional role of ExV's on viral infectivity and host defense and susceptibility. We propose that the ExV's may play a novel signaling role that has a major ecological implications to host-virus dynamics within oceanic algal blooms.

Title: **Tumor exosomes determine organotropic metastasis**

Ayuko Hoshino, Bruno Costa-Silva, Irina Matei, Volkmar Muller, Klaus Pantel, Benjamin A. Garcia, Yibin Kang, Cyrus M. Ghajar, Hector Peinado, Jacqueline Bromberg, David Lyden

Metastasis to distant vital organs such as lung, liver, and brain is the most devastating feature of cancer progression, responsible for over 90% of cancer-associated deaths. In 1889, Stephen Paget first proposed that organ distribution of metastases is a non-random event, yet metastatic organotropism remains one of the greatest mysteries in cancer biology. Our recent studies uncovered that tumor-derived microvesicles, specifically exosomes, alter the microenvironment at future sites of metastasis to form pre-metastatic niches, creating a favorable “soil” for incoming metastatic “seeds”. However, by what mechanism this occurs, and the role of exosomes in tumor metastasis, remains unknown. To investigate the role of exosomes in organotropic metastasis, we have used two established organotropic human tumor models: the MDA-231 breast cancer (BC) cell line, and its variants known to metastasize to the lung, brain and bone, respectively, as well as two liver metastatic pancreatic cancer (PC) cell lines, BxPC3 and HPAF-2. We first analyzed the biodistribution of fluorescently-labeled exosomes derived from lung metastatic, brain metastatic or bone metastatic MDA-231 BC variants or PC cell lines, and found that BC exosomes follow the organ-specific distribution of the cells of origin, while PC exosomes home to the liver. In each target organ exosomes are taken up by different cell types: fibroblasts/epithelial cells in the lung, Kupffer cells in the liver, and endothelial cells in the brain. In the organotropic MDA-231 model, prior education with the lung tropic exosomes redirected metastasis of the bone tropic cells to the lung, demonstrating the unique capacity of exosomes to determine the site of metastasis. Unbiased proteomic profiling of exosomes revealed distinctive integrin expression patterns, and analysis of plasma exosomes from BC and PC patients that later developed site-specific metastasis revealed that specific exosomal integrins could predict metastatic spread.

Schistosomal exosomes: structure, contents and communication with the host immune system

Dr. Dror Avni- Sheba Medial Center

Schistosomiasis is a common parasitic infection caused by blood-flukes helminth of the genus Schistosoma that affects more than 200 million people, mostly in the developing world. Schistosomal infections are also diagnosed in non-endemic areas, in immigrants and travelers coming from endemic countries. The Schistosoma parasites have developed multiple mechanisms for modulating or suppressing host immunity. Despite intensive study over the years, the mechanisms by which adult Schistosoma escape the host immune systems are unknown. Schistosoma was shown to affect Treg cells of the infected patients, suggesting intercommunication with host cells. However, the mechanism by which this communication takes place has not been characterized yet

Using primary schistosomal cultures we were able to isolate a fraction of secreted microvesicles. In preliminary results, we began to characterize these particles applying several tools, including transmission electron microscopy, atomic force microscopy and NanoSight. Further, we performed proteomics analysis of the vesicles contents and altogether the results strongly suggest that these microvesicles, can be classified as exosomes. Importantly, we were also able to isolate exosomes from patients' sera and detected parasite-derived miRNAs in them.

We hypothesize that the adult Schistosoma utilizes secreted exosomes as a mechanism to manipulate and escape the immune system.

'Small talk' in the immune system; heterogeneity of extracellular vesicles and their diverse role in immune regulation

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Tight regulation of the magnitude and quality of immune responses is crucial for efficient eradication of pathogens, maintenance of homeostasis, and neonatal development. Hereto, immune cells need to integrate information from the environment and communicate with other cells. Transfer of biologically active molecules by extracellular vesicles (EV) plays a role in this process. We aim to unravel whether and how the immune status affects the molecular contents of EV released by immune cells and how these EV contribute to modulation of immune responses. Strong focus is put on technical aspects of extracellular vesicle purification and flow cytometric analysis for quantitation and multi-parameter characterization of individual EV. Moreover, we focus on small non-coding RNAs as important functional components of EV.
The immune activating or inhibitory activities of dendritic cells (DC) are determined by environmental stimuli and the immune status of an individual. We found that functionally different DC release phenotypically different EV subpopulations. EV from tolerogenic and immunogenic DC not only displayed different miRNA profiles but also differed in the amount and type of other small non-coding RNAs with potential gene regulatory functions. Importantly, our data also indicate that EV contribute to the immune modulatory activity of DC. EV from tolerogenic DC suppressed the release of the pro-inflammatory cytokine IL-17 by T cells, suggesting that these EV can contribute to creating a local immune suppressive milieu to limit potentially harmful Th17 responses. Similarly, we found that human milk contains EV that suppress T cell responses. Proteomic and transcriptomic analysis indicated that milk contains EV subpopulations that are likely released by immune cells and that these milk EV harbor proteins and miRNAs with strong immune modulatory capacities. In summary, diverse immune cell-derived EV transport (antigen-specific) immune information and contribute to regulation of immune responses.

Whispers of the embryo – maternal communication: Monitoring spatio-temporal cell interactions through microRNAs

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The crosstalk between maternal uterine endometrial cells and embryonic trophoblasts is crucial for the establishment of pregnancy. Of the numerous regulators that have been proposed to mediate maternal-embryo interactions, miRNAs standout for their capacity to regulate the cell transcriptome and cellular identity. miRNAs are transported amongst a variety of elements like proteins, nucleic acids and organelles via exosomes. Communication through exosomes has been observed in a wide range of organisms, from Plasmodium and Drosophila to mice, pigs, and human. To gain a better understanding of the maternal-embryo communication, we have devised a method to follow real-time expression of miRNAs potentially transferred through exosomes using an in vitro model of trophoblast – endometrial cells interaction.

We designed a miR-sensor system based on a double reporter plasmid track to visualize the expression of the miRNA of interest. Two endometrial cell lines and HEK293T cells, as a nonspecific control, were transfected with four different miR-sensors and expression of the tagged microRNA was monitored in response to the presence of trophoblast spheroids (TS) which simulated the embryo. The TS affected the expression of four miRNAs examined in the three cell lines. We observed that changes in miRNA expression occurred early and mostly in cells located in close proximity to the TS. Interestingly, we observed a trophoblast specific modulation of the GFP reporter expression, only in the endometrial cell lines, independent of the miRNA expression. Using the miR-sensor system developed, we were able to monitor spatio-temporal intercellular communication mediated by miRNAs using a model of endometrial cell interactions with trophoblast cells. The miR-sensor system could be further applied to monitor microvesicle / exosome-mediated cell communication.

Embryo-maternal interactions: lessons from phylogenetically distant species

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Embryo-maternal communication is crucial to achieve successful pregnancy and for the future health of the progeny. After mating, gamete transport in the female reproductive tract, gamete maturation, fertilization, and early embryonic development are all dependent upon the relay of appropriate molecular signals between the mother, gametes and embryo. Synchronization between developing embryo (egg) and maternal reproductive tract is recognized as a milestone ensuring proper/optimal fertility, and disturbances in maternal-gamete/embryo communication can influence the outcome of pregnancy. While to date studies have identified numerous molecules, including small non-coding RNAs such as microRNAs (miRNAs), that may be important for this process, still there remain many gaps in our understanding of the embryo-maternal communication. In our talk, we will focus on extracellular vesicles (EVs), including exosomes and microvesicles, which may act as essential mediators of embryo-maternal communication, in phylogenetically distant species: the fruit fly *Drosophila melanogaster* and the domestic pig *Sus scrofa domestica*. Analysis of the uterine microenvironment of the pig identified microvesicles, including exosomes, containing miRNAs, which have been suggested as regulators of reproductive tract remodeling and embryo development/implantation. Furthermore, our results indicate possible sources of microvesicles involved in regulating the extracellular environment of the maternal tract and the uterine environment. Our studies also support the concept that gamete/embryo-maternal communication may have not only short (local) but also potential long-range effects on the mother. Finally, we will highlight phylogenetically conserved elements of embryo (egg)-maternal crosstalk.

An insight into the unknown terrain of sperm storage in Drosophila female using correlative microscopy

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Females in taxa ranging from insects to mammals have evolved sperm storage mechanisms. Different organisms store different amounts of sperm for different lengths of time in closed or open reservoirs. Given its wealth of genetic and molecular tools and the high conservation of genes between flies and mammals, Drosophila promises to be an important model system for understanding the molecular basis of sperm-female interactions across animal taxa. Drosophila females have two types of sperm storage organs: paired spherical spermathecae and a single elongate, tubular seminal receptacle. Despite the interest in the evolution and function of the seminal receptacle, the structure of the receptacle epithelium has received little attention in Drosophila. We have used a correlative microscopy approach in which we combined confocal for light, X-ray microtomography for three-dimensional view of the whole organ and *high resolution in vivo* imaging with focus ion beam (FIB). This combination allowed an increased sample throughput gaining insights into the internal structure of the seminal receptacle. Each imaging technique revealed a different level of information about sperm localization within storage, outline of the organ and details about the internal structures. The obtained results open new avenues and allow new questions to be asked about sperm-female interactions post-mating.

Exosome secretion affects social motility in *Trypanosoma brucei*

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The Mina and Everard Goodman Faculty of Life Sciences and Advanced Materials and

Extracellular vesicles secreted by pathogens function in a variety of biological processes. Here, we describe exosomes secretion that is induced by severe stress in *Trypanosoma brucei*. We show that under *trans*-splicing inhibition, the spliced leader RNA (SL RNA), which donates its exon to all mRNAs via *trans*-splicing, is secreted from the cell. Following perturbations in SL RNA biogenesis or under heat-shock, the SL RNA is exported to the cytoplasm and forms a complex with cytoplasmic proteins. The SL RNA is then secreted by exosomes. The exosomes are found on the surface of the parasites and are formed in multivesicular bodies utilizing the endosomal sorting machinery, ESCRT, through a mechanism similar to microRNA secretion in mammalian cells. Silencing of the ESCRT factor, *Vps36*, compromised exosome secretion. Cells secreting exosomes or purified exosomes are sensed by cells engaged in social motility, and repel their migration. This finding has implications to the mechanisms by which parasites control their motility within their hosts and how stress-signals are transmitted from the parasite to the external milieu.

Cell-to-cell transfer of mRNA via membrane nanotubes

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Communication between cells is an essential function in multicellular organisms. The possible transfer of RNA molecules between cells was recently suggested as a form of communication that could have a regulatory role in the acceptor cells. The current model suggests that such transfer occurs by secreting RNA to extracellular fluids, either as a free RNP particle or packed in exosomes. However, most work in the field has been done on whole cell populations, using mostly biochemical methods. Many questions still remain. In particular, can we show a quantitative analysis of RNA transfer and what are the exact mechanism and kinetics of this process.

Here, we used single molecule fluorescent *in situ* hybridization (smFISH) and live imaging of single mRNA molecules to show that mRNAs are transferred in mammalian co-cultures. We found that the transfer of mRNAs occurs in both immortalized and primary cells. Among the mRNAs we followed are those encoding mouse β-actin, human Cyclin D1, human BRCA1, and others. We were unable to detect transfer of HER2 mRNA, suggesting this process has specificity. co-FISH experiments revealed that the transferred β-actin mRNA makes up ~2-5% of the total β-actin mRNA in the acceptor cell. mRNA transfer is quick and is independent of *de-novo* protein synthesis, but modulated by stress conditions.

Contrary to the current model, we found that mRNA transfer requires close proximity between cells and is not mediated by diffusion. Rather, we suggest that mRNA is transferred through membrane nanotubes. These are very long (>100μm) yet thin (50-300nm) cytoplasmic projections that were recently shown to be involved in direct, contact-dependent, intercellular communication. We present both biological and imaging data to support this hypothesis.

The biological significance of intercellular transfer hinges on whether these mRNAs are translated and effect cell physiology. We currently are developing methods to assess this.

A generic approach for isolating and characterizing extracellular vesicles

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Large efforts are currently made to produce reliable samples of extracellular vesicles (EVs) and to develop, improve, and standardize techniques allowing their biophysical characterization.

In a first example, we show how to use ultrafiltration and size-exclusion chromatography for the isolation and a model-free fluorescence fluctuation analysis for the investigation of the physical and biological properties of EVs secreted by mammalian cells. Our purification strategy produces enriched samples of morphologically intact EVs free of extra vesicular proteins and allows labeling of marker molecules on the vesicle surface for single vesicle analysis with single-molecule sensitivity. This novel approach provides information on the distribution profile of both EV size and relative expression level of a specific exosomal marker, deciphering the overall heterogeneity of EV preparations.

To demonstrate a clinically relevant example, we applied our procedures to exosomes derived from human breast cancer cells. The composition of such exosomes depends on the sort and state of the tumor, requiring screening of multiple antigens to reliably characterize the disease. Therefore, we exploited the capacity of surface plasmon resonance biosensing to detect simultaneously multiple exosomal and cancer biomarkers on the exosomes. This method delivered a characteristic molecular signature for each cell type and is ready for implementation in academic research and clinical diagnostics.

We will in addition outline how to monitor GPCR- and channel-mediated trans-membrane signaling reactions in cell-derived vesicles. As the vesicles comprise parts of a cell's plasma membrane and cytosol, they represent the smallest autonomous containers performing cellular signaling reactions. Using fluorescence microscopy, we measured in individual vesicles the different steps of G-protein-coupled receptor mediated signaling like ligand binding to receptors, subsequent G-protein activation and finally arrestin translocation indicating receptor deactivation. Observing cellular signaling reactions in individual vesicles opens new directions for the analysis of EVs.

Characterization of natural and synthetic vesicles

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Extracellular vesicles (EVs) are lipid bilayer enclosed structures released by cells and are classified based on their cellular origin, biogenesis and physicochemical properties, including apoptotic bodies, microvesicles and exosomes. They have emerged as important mediators of intercellular communication and may serve as biomarkers of disease and as potential therapeutic targets. Despite intense investigation, however, many properties and mechanisms remain indefinable due to the lack of standardization of isolation and characterization methods which hinders the translation of EV-based diagnostics into clinical use.

Extracellular vesicles were isolated by centrifugation from human erythrocytes and characterized by freeze-fracture electron microscopy (FF-TEM), dynamic light scattering (DLS), attenuated total reflection Fourier-transform infrared spectroscopy (ATR-FTIR). Total protein and lipid content were determined by Bradford and Stewart bioanalytical assays.

The cell membrane is an ordered environment of lipid molecules, proteins and other signaling molecules. Monitoring membrane orientation by polarized light spectroscopy will receive information on protein conformation and interactions in the lipid bilayer. Small aromatic molecules (pyrene and retinoic acid) were used for determining the lipid bilayer orientation. These data can also be used to identify how the applied shear force can distort the original vesicle morphology - a property that is probably characteristic to the composition of a particular type of vesicle.

miR-4443, a Possible Participant in Mast Cell Activation by T Cell-Derived Microvesicles

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Background: In addition to being pivotal in the elicitation of allergic responses, mast cells were found to be activated in T cell-mediated inflammatory processes. We have recently shown that mast cells can be activated by microvesicles (MVs) derived from activated T cells (mvT*) to degranulate and release several cytokines. These events are associated with internalization of mvT* followed by RAS and sustained ERK activation. The aim of this study was to analyze the possible effect of microRNAs delivered by MVs on mast cell activation.

Methods: The high-throughput microRNA profiling was performed using NanoString technology platform and was validated by real time PCR. The biological role of mvT*-derived microRNA was verified by overexpression of these microRNAs in mast cells using mimic or inhibitor molecules and analyzing their predicted targets.

Results: T cell-derived microvesicles were found to downregulate the tyrosine phosphatase PTPRJ expression, a known ERK inhibitor. Bioinformatics analysis revealed that miR-4443 is predicted to regulate the PTPRJ gene. Indeed, miR-4443 present in mvT*, was also found to be overexpressed in human mast cells stimulated with this MVs. Luciferase reporter assay indicated that 3'UTR of PTPRJ was the target of this miR. Transfection of mast cells with mimic or inhibitor of miR-4443 resulted in decreased or enhanced PTPRJ expression respectively. Furthermore, miR-4443 was found to regulate ERK-phosphorylation and IL-8 release in mast cells activated by mvT*.

Conclusion: Stimulation of mast cells with mvT* leads to overexpression of miR-4443 that serves as PTPRJ negative regulator. This may explain, at least in part, the sustained ERK phosphorylation and mast cell activation in response to stimulation with mvT*. Thus, by carrying a cargo of genetic information from one cell type to another, MVs may play an important role in T cell - mediated inflammatory processes where mast cells were found to be involved.

Regulated delivery of membrane-type proteases to microvesicles in invasive tumor cells

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Cells release multiple, distinct forms of extracellular vesicles including structures known as microvesicles, which are known to alter the extracellular environment. Despite growing understanding of microvesicle biogenesis, function, and contents, mechanisms regulating cargo delivery and enrichment remain largely unknown. Here we demonstrate that in invasive tumor cell lines, the v-SNARE, VAMP3, regulates delivery of microvesicle cargo including the membrane-type 1 matrix metalloprotease (MT1-MMP) to shedding microvesicles. MT1-MMP delivery to nascent microvesicles depends on the association of VAMP3 with the tetraspanin CD9. VAMP3-shRNA expression depletes shed vesicles of MT1-MMP and decreases cell invasiveness. Finally, we describe functionally similar microvesicles isolated from bodily fluids of ovarian cancer patients. Together these studies demonstrate the importance of microvesicle cargo sorting in matrix degradation and disease progression.

microRNA cargo of extracellular vesicles isolated from uterine cavity during early pregnancy

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Efficient embryo implantation into the receptive endometrium requires synergistic endometrial-conceptus interactions within the uterine cavity, crucial for establishing pregnancy. Early pregnancy is characterized by the extensive exchange of a great variety of factors, molecules, particles between the uterus and conceptus. Our studies are focused on the role of microRNAs (miRNA), small non-coding RNAs, able to regulate broad range of biological processes through sequence-specific interactions with mRNAs. Recently, we demonstrated that conceptuses may cross-talk with uterine cells via exosomes containing miR-26a and miR-125b. Here we isolated EVs form uterine cavity of pregnant pigs on day 12, 14 and 16 (n=4/day). Presence of EVs in the uterine luminal fluid (ULF) was confirmed by several visualization methods (electron microscopy, western blot) and nanoparticle tracking analysis (NTA). miRNAs profiling was accomplished with custom made TaqMan Low Density Arrays (TLDA) and quantitative real-time PCR. Out of 84 investigated miRNAs we were able to detect 49, among which 10 miRNAs showed significantly affected profiles between days 12-16 of pregnancy ($p<0.05$). The majority have been already detected in our recent studies performed in pregnant animals. Most importantly, expression profiles of miRNAs detected in EVs resemble those of endometrium or conceptuses (e.g., miR-30a-5p, miR-422b, and miR-193b). Experimentally validated miRNA-mRNA interactions combined with target prediction analysis showed that miRNAs being a cargo of EVs detected in ULFs from pregnant animals are potential regulators of pregnancy related genes, such as inflammatory mediators or growth factors. Our results suggest that miRNA cargo in EVs isolated from uterine cavity during early pregnancy is an important component of maternal-conceptus communication, giving an impetus to further large scale functional analysis investigating the mechanism of action and the impact of their delivery on the processes crucial to successful pregnancy.

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Inter-cellular transport of Ran GTPase through exosomes

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Ran GTPase has a well-established role in nucleo-cytoplasmic transport of macromolecules across the nuclear envelope. Recently, we found that Ran gets transferred between cells in a GTP- and CRM1 dependent fashion. Here we show that the inter-cellular transport of Ran occurs through exosomes, vesicles derived from multivesicular bodies. We find that the recruitment of Ran into exosomes also is GTP- and CRM1-dependent. Moreover, our studies reveal that Ran's ability to get recruited into exosomes is negatively modulated by its SUMO-modification. Furthermore, using enucleated RBCs, our studies uncover a possible role for Ran-CRM1 axis in the recruitment of soluble protein cargos into the exosomes. Together, the findings unravel a unique role for Ran beyond nucleo-cytoplasmic transport and provide a framework for identifying the signals and mechanisms involved in targeting soluble proteins into exosomes.

Bone marrow mesenchymal stem cells microvesicles critically affect multiple myeloma cells in accordance with their normal or pathological source

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Background and Purposes: Myeloma cells' (MM) interaction with the bone marrow (BM) microenvironment critically hinders disease therapy. Previously, we showed that co-culture of MM cells and BM-mesenchymal stem cells (MSCs) caused co-modulation of translation initiation (TI) and cell phenotype and that secreted components are implicated. Here, we studied the role of the MSCs secreted microvesicles (MVs) on the MM cells phenotype, TI, and signaling.

Methods: MVs were extracted from BM-MSCs secretomes (3 days; normal donors (ND-MSCs), MM patients (MM-MSCs)). Basic MVs characterization (electronic microscope), their uptake (fluorescence microscope, FACS) and dose response were verified. MM/ND-MSCs MVs (10-150mg/ml) were added to MM cell lines (U266, ARP1, MM1S and OPM2) for (5min ,1h, 4h, 12h and 3days) assayed for viability (wst1) and live/dead/total cell count (trypan blue); migration (transwell); TI status (factors: eIF4E, eIF4GI; regulators: mTOR, MNK, 4EBP; targets: SMAD5, NFκB, cyclinD1, HIF1, cMyc) (immunoblotting); and MAPKs activation (immunoblotting).

Results: Within 24h BM-MSCs MVs were internalized by MM cells evoking opposite responses according to MVs origin (ND/MM-MSCs). ND-MSCs MVs decreased total and live cell counts (↓15-50%, *p<0.05*), viability (↓22-40%, *p<0.05*), migration (↓>30%, n=1) and TI status (↓>10-80%; *p<0.05*). In contrast, MM-MSCs MVs increased total and live cell counts (↑10-60%, *p<0.05*), PCNA proliferation marker (↑20-120%, *p<0.05*) and TI status (↑20-185%, *p<0.05*). ND-MSCs MVs treated MM cells demonstrated a rapid (5min) activation of MAPKs followed by a persistent decrease (1h, 4h, and 24h). Studies on MAPKs in MM-MSCs MVs treated MM cells are underway.

Discussion: BM-MSCs MVs display a major effect on MM cells TI and phenotype in accordance with source. The rapid induction of MAPKs is consistent with a receptor-ligand interaction between the MVs and the MM cells. The manipulation of TI and significant alteration in phenotype underscore the potential of MVs to MM cells communication with their microenvironment and warrant additional studies.

Microvesicles mediate radioresistance induction in melanoma cells.

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Cells never exposed to radiation can respond to signals from irradiated cells via a process known as bystander effect. In recent years, strengths aimed to identify new players in bystander information exchange have brought to consider exosomes as potential mediators of this crosstalk. Exosomes are microvesicles which diameter ranges between 30 and 130 nm, released by cells in physiological and altered conditions. They have also been found to mediate the radioadaptive response, by increasing radioresistance in recipient cells via paracrin signalling. This resistance induction naturally composes the organism reaction to radiation exposure. It can interfere with the outcome of some important medical tools such as radiotherapy, where cancer cells are target of radiation delivery. Aim of this work is to relate the exosome-mediated communication to the radioresistance occurring in several cases of tumours. To validate this hypothesis we isolated exosomes-enriched microvesicular fractions produced by a neuroblastoma cells before (CTRL cells) and after exposure to 1 Gy of X Rays (IR cells) using filtration and ultracentrifugation techniques. Characterization of vesicular populations by Dynamic Light Scattering, Z potential analysis and Scanning Electron Microscopy revealed physical features similar to the exosome fraction. Data obtained from protein and RNA quantification suggested an increase in microvesicles production by IR cells compared to CTRL cells. After verifying exosome uptake by recipient cells using immunofluorescence, we analyzed radioresistance induced by microvesicles treatment in term of cell viability. We related these results with presence and phosphorilation of apoptotic-related molecules (AKT and ERK) and activation of Nrf2, a transcription factor involved in antioxidant response. All our data suggest an induction of radioresistance in melanoma cells treated with exosomes produced by the same cell type during oxidative stress induced by ionizing radiation.

Angiogenic and anti-inflammatory properties of mesenchymal stem cells from cord blood: soluble factors and extracellular vesicles for cell regeneration

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In a recent work, our group showed the existence of two distinct mesenchymal stem cell (MSC) subsets within human umbilical cord blood (CB): one less proliferative and short-living (SL-CBMSC), the other with a higher growth rate and long-living (LL-CBMSC). Based on these phenotypes, we proposed LL-CBMSC as better CBMSC subset to use in regenerative medicine approches. Therefore, we examined whether LL-CBMSC possessed peculiar paracrine properties able to affect angiogenesis or inflammatory processes. Pro-angiogenic, proliferation-stimulating and tissue repair factors were released at high levels both as soluble cytokines and, shown for the first time, as mRNA precursors embedded in membrane vesicles. The combination of this primary (proteic factors interacting with surface receptors) and delayed (mRNA transferred via vesicle fusion and cargo release) interaction in endothelial target cells resulted in strong blood vessel induction with the development of capillary-like structures. In addition, in an in vitro model of damage, LL-CBMSC could dynamically modulate their release of pro-angiogenic and anti-inflammatory factors, hinting at a regulated cross-talk. In conclusion, LL-CBMSC synthesize and secrete multiple factors that may be attuned in response to the status of the target cell, a crucial requisite when paracrine mechanisms are needed at onset of tissue regeneration.

A novel technique for high throughput plasma proteomics profiling using extracellular vesicles

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The interest in using mass-spectrometry (MS) based proteomics for clinical applications has profoundly increased over the past few years, towards the discovery of potential biomarkers and the development of new diagnostic tests. However, the huge dynamic range of proteins in the plasma, which leads to the masking of potential biomarkers by the highly-abundant proteins, limits the coverage of the plasma proteome. To overcome this challenge we fractionate the plasma by isolating plasma microparticles. Microparticles are extracellular vesicles that are shed by all cell types. They can be released into the bloodstream, while carrying the proteomic signature of the cell of origin. Importantly, both their number and composition change upon disease, indicating their great potential in the biomarkers discovery field. Based on that we have developed PROMIS-Quan (PROteomics of MIcroparticles with Super-SILAC Quantification), which provides a high throughput and unbiased approach for MS-based plasma proteomics, combined with highly accurate relative and absolute quantification of the examined proteins. Using this technique we can reach an unprecedented depth of over 3,000 proteins in single MS runs. We tested PROMIS-Quan on two different datasets. In one we compared samples of healthy donors and prostate cancer patients, and found a predictive signature for prostate cancer diagnosis comprised of 3 proteins with area under the ROC curve (AUC) of 0.84. In a second dataset we examined the response to immunotherapy among stage-IV melanoma patients and got a strong predictive signature with AUC of 0.91. Altogether we propose PROMIS-Quan as an innovative platform for unbiased biomarker discovery based on the isolation of microparticles from the blood.

Circulating EBV-modified exosomes in SLE patients target the renal tubular epithelium delivering inflammatory small RNAs and sensitizing RNA sensors

A possible pathogenic role of Epstein-Barr virus (EBV) in autoimmune diseases such as systemic lupus erythematosus (SLE) is long suspected, but its exact role remains unknown. Recently, we have shown that Epstein–Barr virus-encoded small RNA 1 (EBER1) and some EBV-related microRNAs (miRNA) are selectively released from infected B cells via exosomes and are internalized by human plasmacytoid DCs, expressing TIM1 phosphatidylserine receptor, a known viral and exosomal target molecule.
In this study we focus on SLE patients with renal involvement, known as Lupus Nephritis (LN). We analyzed LN and other kidney disease control biopsies by in-situ hybridization, which revealed a defined EBER1 presence in tubular epithelial cells (TEC) of LN biopsies. Accordingly, high levels of EBER1 and EBV-produced miRNAs were detected in LN biopsies by RT PCRs, consistent with a role of EBV-modified exosomes in SLE and LN pathogenesis. Strikingly, TIM1 is expressed by Tubular Epithelial cells (TEC) and we confirmed in primary TEC cultures that EBV-modified exosomes are internalized in a PtdSer-dependent manner delivering inflammatory RNAs into endosomes. We next analyzed the possible inflammatory effects of EBV-modified exosomes entry into TEC cells by isolating exosomes from EBV-infected B cells. The addition of EBV-modified exosomes, as well as infection of RNA-isolated from the same exosomes into TEC cells, caused an induction of cytokine production such as IL6 and interferon-stimulated genes, as well as RNA sensors such as RIGI and TLR3. The induction of these RNA sensors upon exosome addition was significantly decreased after treating TEC cells with Hydroxychloroquine (known as Plaquenil), an antimalarial drug used for SLE treatment. These data support the hypothesis that EBV-modified exosomes might play a significant role in promoting inflammatory responses in autoimmune diseases including SLE and LN, partially through sensitizing the key RNA sensors in affected tissues and thereby increasing endogenous and exogenous RNA recognition.

Bacterial outer membrane vesicles induce plant immunity and enhance bacterial disease resistance

All Gram-negative bacteria pinch off portions of their outer membrane, releasing outer membrane vesicles (OMVs) to the surroundings. Strangely, the interactions between these highly abundant structures and plants have been mostly overlooked. In this study we sought to examine the interactions of OMVs and the plant immune system. We demonstrate that the cytosolic microbe associated molecular pattern (MAMP) EF-Tu is released to the outer cellular space in OMVs and that OMVs induce typical innate immune responses in *Arabidopsis*. Treating OMVs with proteinase K prior to *Arabidopsis* challenge did not reduce the level of defence gene activation indicating that non-proteinaceous immunogenic factors in OMV are being exposed to plant immune receptors. Nevertheless, *Arabidopsis* knockout lines lacking the immune receptors for peptidoglycan and lipopolysaccharides were sensitive to OMVs as wild type plants. To determine whether the elevated immune response imposed by OMVs would lead to bacterial disease resistance we pretreated *Arabidopsis* plants with OMVs and inoculated it with *Pseudomonas syringe* pv. *tomato* DC3000. A statistically significant reduction in DC3000 cell titer was observed in the OMV-pretreated plants compared with the water-pretreated control. Altogether, our results reveal a new facet in plant-bacteria interactions that may lead to new discoveries of the plant immune system and bacterial pathogenesis.

BAG6 as a novel regulator of exosome release and protein sorting

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Natural killer (NK) cells are a major component of the anti-tumor immune response. We have recently shown that the impaired activity of NK cells in chronic lymphocytic leukaemia (CLL) is dependent on soluble ligands for NK cell receptors (NKRs) that are released by the malignant B-cells. One of these soluble ligands is BAG6, which engages the cytotoxic NK cell-receptor only when expressed on the surface of extracellular vesicles (exosomes). BAG6 is a multifunctional protein also acting as an intracellular chaperone involved in protein targeting and protein degradation. Here, we analyzed the role of BAG6 for the release of immune-activating exosomes upon DNA damage in CLL.

Immunoprecipitation, *in vitro* translation and a yeast-two-hybrid approach revealed that BAG6 binds directly to p53 and forms a ternary complex with the actetyltransferase CBP/p300 in response to doxorubicin-induced DNA damage. Induction of DNA-damage triggered the release of exosomes and the nuclear export of BAG6. The release of exosomes was dependent on BAG6 and p53 under basal and stress-related conditions.

Exosomes from BAG6 wild type cells were characterized by an enriched expression of BAG6-interacting partners and immune regulatory molecules. These molecules were diminished in exosomes collected from BAG6-deficient cells suggesting that BAG6 impacts on exosome cargo. The data presented identify BAG6 as a novel key component in exosome formation and loading.

Oviductosomal Expression of Mouse PMCA1 in Female Fertility of *Pmca4* nulls

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Plasma membrane Ca^{2+} ATPase4 (PMCA4) is the major Ca^{2+} efflux pump in murine sperm, where deletion of its encoding gene (*Pmca4*) leads to male infertility due to loss of hyperactivated motility. PMCA4, which accounts for 90% of PMCAs in sperm, is not only crucial in males but is also expressed in females where extracellular vesicles (EVs) in the oviductal luminal fluid (termed Oviductosomes) are capable of transferring it to sperm, just before fertilization. Acquisition of additional PMCA4 at the fertilization site meets the increased demand for Ca^{2+} efflux in sperm, resulting from the elevated Ca^{2+} levels required for their hyperactivation and acrosome reaction. However deletion of murine *Pmca4* has no effect on female fertility; despite Ca^{2+} efflux requirement which also occurs for oviductal ciliary action, necessary for oocyte transport. Here, we show that the PMCA1 isoform is expressed in reproductive tissues (vagina, uterus, and oviduct) in the epithelial lining, luminal fluids, EVs, with an upregulation in oviductal luminal fluid (OLF) during estrus in *Pmca4*$^{-/-}$, compared to wild-type females. Western analysis showed that PMCA1 levels are significantly higher (P=0.02) in *Pmca4*$^{-/-}$ OLF during estrus, compared to wild-type or null epididymal luminal fluid. In cycling females, PMCA1 expression in OLF of *Pmca4*$^{-/-}$ was significantly (P=0.02) higher than in wild-type during proestrus/estrus; but significantly decreased (P=0.03) during metestrus/diestrus, showing an interaction between genotype and stage. Importantly, during estrus PMCA1 levels were significantly (P<0.05) elevated in *Pmca4*$^{-/-}$ oviductosomes (OVS), compared to wild-type, unlike *Pmca4*$^{-/-}$ uterosomes, or epididymosomes. OVS delivered PMCA1 to sperm following co-incubation of OLF and sperm, and were shown to carry in their cargo nNOS (neuronal nitric oxide synthase) and CASK (Ca^{2+}/CaM- dependent serine kinase), interacting partners of PMCA1 and PMCA4. The data show an important role of oviductosomal PMCA1, which compensates for PMCA4 in *Pmca4*$^{-/-}$ OVS, in female fertility.

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The past, presence and future of extracellular vesicles

Extracellular vesicles such as microparticles and exosomes are common and widely distributed, and are now thought to have a multitude of functions in health and disease. Evidence is accumulating that vesicles in human body fluids are involved in protection, intercellular communication, and disease development and progression. Furthermore, vesicles potentially behold an entirely new level of clinical relevant information for diagnosis and monitoring of therapy.

We have an interest in tissue factor (TF), and have shown that body fluids such as saliva of healthy subjects contain vesicles exposing coagulant TF. Because such vesicles are absent in the blood, we hypothesize that "licking a wound" facilitates blood clotting and wound healing. In patients suffering from meningococcal septic shock or cancer, however, coagulant TF-exposing vesicles can be present within the peripheral blood, and such "blood-borne" vesicles are thought to contribute to development of bleeding and thrombosis.

Furthermore, we have worked on improved isolation and detection of (single) vesicles. With regard to isolation, rediscovery of size exclusion chromatography has increased the general awareness that a decent isolation procedure is essential to reduce the risk of artefacts. Similarly, detection of single vesicles is often more difficult than anticipated. Because detection of both rare (biomarker) and common (reference ranges) vesicles requires comparison of results between instruments and institutes, we have compared, tested and selected reference materials, are developing novel reference materials and software, and have initiated an international standardization study to compare vesicle measurements between instruments and institutes.

Taken together, a whole new research field is now evolving rapidly, and an introduction to and overview of vesicles will be provided, with emphasis on our research.

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Size exclusion chromatography of extracellular vesicles: comparison of different stationary phases. Robert Deak, Research Centre for Natural Sciences, HAS

Size exclusion chromatography (SEC) is a powerful tool for the separation of macromolecules and biological nanoparticles, and it is useful in the isolation and purification of EVs. Despite the wide variety of SEC macroporous stationary phases only limited number of them was applied in the EV field.

The aim of our study was to compare different stationary phases in SEC regarding their ability of separation and size characterization of EVs.

EVs were isolated from different natural milieus: urine, cell medium, erythrocyte concentrate and plasma, and were compared with biologically relevant reference materials: lipoproteins and liposomes. Separation and size characterization was performed by HPLC-SEC (Jasco HPLC system, PU-2089 pump, UV-2075 UV/Vis detector, supplemented with an on-line coupled W130i DLS from Avid Nano Ltd.), with TSK G6000PW (Tosoh Corp.) pre-packed column, and Tricorn 5/200 glass column filled with Sepharose CL-2B (GE Healthcare) cross-linked agarose gel.

Based on the fractionation ranges of the used columns (TSK G6000PW: up to 8000 kDa for polyethylene glycol, and Sepharose CL-2B: 70-40,000 kDa for dextrans) both of them was found to be suitable for the separation of EVs from soluble proteins and lipoprotein particles. Additionally, slight differences were found in the elution profiles of synthetic liposomes and EVs with different sizes, hence the applicability of SEC for discrimination of different vesicle fraction by size is limited. However, liposomes with different surface characteristics and EVs from different origin resulted slightly different elution times and profiles, revealing, that not only steric interactions governs the retention of these particles in SEC.

SEC using different macroporous stationary phases was found to be suitable for the characterization of the purity of different EV preparations. The use of SEC is limited for the size determination of the vesicles, although, more investigations are needed to reveal the physicochemical background of the observed elution profiles.

Exosomes derived from malaria parasites-infected red blood cells contain distinct human and parasite small RNAs

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Cells use extracellular vesicles to communicate, to coordinate social activities and, in the case of pathogens, to manipulate host target cells. Previously we showed that the lethal malaria parasite, *Plasmodium falciparum* (*Pf*) can transfer episomal plasmids via exosome-like vesicles produced at the ring-stage of the asexual life cycle. Here, we identify *Pf*-derived nanovesicles as exosomes and determine their RNA cargo. Unlike vesicles from uninfected Red Blood Cells (RBCs), parasite-derived exosomes contained high amount of RNA molecules with a length of 4 -150 nt. Small RNA deep sequencing revealed a significant population of host and parasites non-coding RNAs in *Pf*-derived exosomes; The most abundant parasite non-coding region was PF13TR011:ncRNA, found on chromosome 13, with a current unknown function. Surprisingly, this sequence aligns almost perfectly to a portion of the human complement component C2 (C2) mRNA 5'UTR.

Of importance, upon performing enrichment analysis, the functional group involved in regulation of cell adhesion contained the highest number of represented human miRNA targets. These were found to influence various cell adhesion and known human receptors for the major parasite virulence ligand. Although further studies are required, these findings raise the possibility that parasite exosomes act on different host cells to manipulate their gene profile.

THyPRP and KD1 regulate flower abscission by affecting the extracellular vesicle cargo of cell wall degradation enzymes

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Organ abscission is an important cell separation process regulated by endogenous and exogenous signals, and occurring in a layer of functionally specialized cells known as the abscission zone (AZ). Loosening of the primary cell wall and dissolution of the middle lamella are fundamental steps in the abscission process of many plant systems, which involves induction of cell wall associated enzymes and proteins required for their export across the plasma membrane of AZ cells. The differentially expressed genes in the AZs include both cell wall enzymes and secretory pathway proteins such as SNARE-like proteins and syntaxins. To investigate these molecular events occurring during pedicel abscission following flower removal in inflorescences of tomato (*Solanum lycopersicum*), we performed a microarray study and identified two genes, *Tomato Hybrid Proline-Rich Protein (THyPRP*) *and Knotted1-like homeobox protein (KD1*) that are specifically expressed in the AZ. To functionally analyze these two genes, we generated two transgenic tomato lines in which each gene was silenced independently using antisense RNAs under the control of an AZ-specific promoter. We observed a significant inhibition of pedicel abscission in both transgenic lines. A transcriptomic analysis using a tomato AZ-specific microarray revealed that, in addition to other gene families, 28 genes encoding cell wall-modifying enzymes, which were strongly upregulated in the WT, were much repressed in the *THyPRP*- and *KD1*-silenced plants. Of particular interest was a *syntaxin* gene, which encodes a membrane integrated Q-SNARE protein necessary for vesicle trafficking and was induced in the WT. This gene was significantly repressed in both silenced plants. Given that the primary role of SNARE proteins is to mediate vesicle fusion with their target membrane bound compartments participating in exocytosis, our results suggest that THyPRP and KD1 may mediate flower abscission by regulating biosynthesis and exocytosis of cell wall and middle lamella-modifying enzymes of the AZ cells.

Neuro-Immune Interactions by Extracellular Vesicles

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Extracellular vesicles (EVs) emerge as important carriers for intercellular communication and they have been implicated in many biological processes. However, due to their small size and the difficulties of manipulating them, the data on EV biology has thus far been based mainly on *in vitro* or indirect *in vivo* evidence, or on experiments involving *ex vivo* manipulations. Thus, the extent and pathogenic role of EV signalling *in vivo*, particularly with regard to the transfer of functional RNA, remains poorly understood. Using the Cre-Lox system we could overcome some of the major shortcomings in this field by establishing a method to trace the functional transfer of RNA by EVs *in vivo*. Expression of Cre recombinase in transgenic mice under the control of a hematopoietic-specific promoter leads to the release of EVs containing Cre mRNA, but no measurable amounts of protein from blood cells. In mice with a Cre reporter background, EV mediated transfer of Cre mRNA induces marker gene expression in target cells. In this way we could identify the direct transfer of functional RNA from blood to neurons in the brain as a novel route of communication between the immune system and the brain. While occurring vey rarely in healthy animals, this process is greatly enhanced by systemic inflammation, affecting multiple structures in the brain and leading to changes in the miRNA profile of targeted neurons. Thus, the brain and the immune system are more intimately interconnected than previously anticipated.

Sterol transport and antifungal drug resistance

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Invasive opportunistic fungal infections of humans are common among those suffering from impaired immunity, and are difficult to treat resulting in high mortality. Amphotericin B (AmB) is one of the few antifungals available to treat such infections. The AmB resistance mechanisms reported so far mainly involve decrease in ergosterol content or alterations in cell wall. In contrast, depletion of sphingolipids sensitizes cells to AmB. Recently, overexpression of PMP3 gene, encoding plasma membrane proteolipid 3 protein, was shown to increase and its deletion to decrease, AmB resistance. Here we have explored the mechanistic basis of PMP3 effect on AmB resistance. It was found that ergosterol content and cell wall integrity are not related to modulation of AmB resistance by *PMP3*. A few prominent phenotypes of PMP3 delete strain, namely, defective actin polarity, impaired salt tolerance, and reduced rate of endocytosis are also not related to its AmB-sensitivity. However, *PMP3* overexpression mediated increase in AmB resistance requires a functional sphingolipid pathway. Moreover, AmB sensitivity of strains deleted in PMP3 can be suppressed by the addition of phytosphingosine, a sphingolipid pathway intermediate, confirming the involvement of this pathway in modulation of AmB resistance by PMP3. We further explored that this PMP3 mediated AmB resistance can be modulated by sterol transport between Plasma membrane (PM) to Endoplasmic Reticulum (ER) which suggest that oxysterol binding protein (OSBP) play importance role in drug resistance.

. The role of exosomes in the radiation?induced biomarker development

Dr. Yi Wang,Canadian Nuclear Labs

Background: There is no perfect biomarker in the field of radiation induced biomarkers. Recent results suggest that therapeutic doses of radiation influences exosome abundance, specifically alters their molecular composition (Arscott 2013). To date, no studies have been undertaken on the effect of radiation on exosome production and composition in healthy individuals. As exosomes are easy to enrich and are protected from RNAase and proteinase degradation by their membrane, we believe that they present a possible route to the discovery of radiation-induced biomarker.

Study Aims: 1) To purify exosomes from human or animal plasma. 2) To determine the effect of radiation on protein, mRNA and miRNA profiles of exosomes. 3) To identify possible radiation-induced biomarkers for radiation protection.

Methods and Materials: Both old (26 months old) and young (2 months old) C57bl/6j mice were irradiated with different doses of gamma-radiation. The exosomes in the plasma of mice were purified using an Exoquick Exosomes Purification Kit. The presence of exosomes was confirmed with both dot and western blots of the exosomes markers. The amount of exosomes was quantified with Exocet Quantitation Assays, and the exosomal RNA was purified using SeraMir Exosome Amplification Kit. In addition, the total miRNA from the plasma was purified using miRNeasy Mini Kit. After reverse transcription, miRNA arrays of exosomal and total miRNA were performed using Mouse miScript miRNA PCR Array.

Current Results: We found that there was a slight increase of the amount of exosomes in the plasma of radiation treated mice. The miRNA expression profiles were very different between old and young mice, and with or without irradiation. We identified several candidate miRNAs correlated with radiation exposure, and could be used as radiation induced biomarker.

Current Conclusion: Exosomes could provide a premium platform for biomarker discovery for assessment of internal and external radiation exposures.

Cell-cell communication between *Plasmodium* and host immune via exosomes

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Malaria, kills up to a million people each year, is caused by the protozoa of the genus *Plasmodium falciparum (Pf)*. These vector-born parasites cycle between mosquitoes and humans and, in both contexts, are faced with an unstable and hostile environment. To ensure survival and transmission, the malaria parasite must infect and survive in the human host and differentiate into sexual forms that are competent for transmission to mosquitoes. We found, for the first time that *Pf*-infected red blood cells (iRBCs) directly exchange cargo between them using nanovesicles (exosomes). These tiny vesicles are capable of delivering protected genes to target cells.

Cell-cell communication is a critically important mechanism for information exchange that promotes cell survival. How *Pf* parasites sense their host environment and coordinate their actions, remain one of the greatest mysteries in malaria. Moreover, our understanding in the mechanism regulate human immune response to malaria infection is poor. Here, we found that malaria-derived exosomes carry remarkable cargo providing a secure and efficient mode for signal delivery. We developed an exosome tracking assay and could measure *Pf* exosome uptake by different cell types. Moreover, although early life-stages of *Pf*-iRBC are considered immunologically inert, our initial observations show that ring-stage derived exosomes are immunogenic. We show that exosomes can specifically activate and induce pro-inflammatory responses, resulting in interferon type I response. This is a new area of malaria research which may shed a light on the ability of malaria parasite to manipulate their host response.

“MVBs function to Degrade the Sperm Mitochondria after fertilization in *Drosophila”*

Almost all animals contain mitochondria of maternal origin only, but the exact mechanisms underlying this phenomenon are still unclear. We investigated the fate of the paternal mitochondria after fertilization in *Drosophila*. We demonstrated that the giant sperm mitochondrion is rapidly eliminated in a stereotypical process dubbed paternal mitochondrial destruction (PMD). PMD is initiated by a network of vesicles, resembling multivesicular bodies (MVBs) and displaying common features of the endocytic and autophagic pathways. These dual origin vesicles, which are also known as amphisomes, associate with the sperm tail, secrete their microvesicles inside the sperm flagellum and mediate the disintegration of its plasma membrane. Subsequently, the mitochondrion separates from the axoneme and breaks into smaller fragments, which are then sequestered by autophagosomes for degradation in lysosomes. We further provided evidence for the involvement of the ubiquitin pathway and the autophagy receptor, p62, in this process. Currently we focus on the role of the MVBs through the identification of their contents. We generated transgenic flies expressing the human exosomal marker hCD63 fused with EGFP. We showed that this marker specifically labels the MVBs on the sperm flagellum, allowing the successful isolation of the $hCD63^{+}$ microvesicles, and subsequent proteomic analysis of their contents.

Transmission of miRNA in macrophages-derived exosomes induce drug resistance of pancreatic adenocarcinoma

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Pancreatic ductal adenocarcinoma (PDA) rank fourth among cancer-related deaths. Despite decades of research, cure rate of the disease remains disappointingly low (<5%). This dismal prognosis stems from two sources: late detection, when the disease is already metastatic and resistant to all known systemic therapies. Gemcitabine, the drug of choice for the treatment of PDA, is a cytidine analog that acts to inhibit cell growth by termination of DNA replication. Unfortunately, resistance to the drug develops within weeks from initiation of therapy as a result of intrinsic resistance and extrinsic factors. Despite increasing recognition of this clinical problem, there has been minor progress in overcoming PDA drug resistance. Here we show that tumor associated macrophages (TAMs) in the canecr microenvironment secrete nano-vesicles known as exosomes which are selectively internalize by the cancer cells and not by the tumor stroma. Exosomes secreted by TAMs, deliver a specific miRNA known as miR-365 which upregulates cytidine deaminase (CDA), the rate limiting enzyme that metabolizes gemcitabine. Using in vitro studies and transgenic animal models we show that exosomes-induced drug resistance is reduced in Rab27ab$^{-/-}$ mice which lack exosomal secretion. We suggest a novel mechanism by which the tumor microenvironment regulates drug resistance in PDA cells by transmission of mRNA.

Radiolabeling of extracellular vesicles with ^{99m}Tc-tricarbonyl for quantitative *in vivo* imaging studies

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Introduction

Exosomes, microparticles, and other extracellular vesicles (EVs) have gained particular attention due to their role in biological processes ranging from intercellular communication and angiogenesis to cell survival. The biodistribution of EVs is a fundamental question in the field of circulating biomarkers, which has recently gained attention. Despite the capabilities of nuclear imaging methods such as single photon emission computed tomography (SPECT), radioisotope labeling of EVs and the use of the aforementioned methods for *in vivo* studies cannot be found in the literature.

Methods

We describe a novel method for the radioisotope labeling of erythrocyte-derived EVs using the ^{99m}Tc-tricarbonyl complex. Moreover, the capability of the developed labeling method for *in vivo* biodistribution studies is demonstrated in a mouse model.

Results

Labeling efficiency of 39 ± 6% was obtained for the erythrocyte-derived EVs which is reasonable, especially if the generally low concentration of EVs is considered. After purification of the reaction mixture by size-exclusion chromatography, the radiochemical purity of the labeled EVs was 97 ± 1%. We found that the intravenously administered, ^{99m}Tc-labelled EVs mostly accumulated in the liver, and in the spleen (67% and 5% of the whole-body radioactivity, respectively). The *in vivo* stability of the labeled EVs was assessed by the comparison of the obtained biodistribution of EVs with that of the free ^{99m}Tc-tricarbonyl.

Conclusions

The tricarbonyl method combined with a custom size-exclusion chromatography process enabled us to radiolabel extracellular vesicles with high stability and purity. The radiolabeled EVs are good candidates to elucidate more on EV roles, but they can also be employed for diagnostic purposes.