Mechanisms of spontaneous mutations in *Mycobacterium marinum*

Carlos T Alvarado¹, Emily A. Williams², and Patricia A. Champion²

We have observed spontaneous point mutations in genomic DNA during routine laboratory growth of the pathogenic mycobacterial species, *M. marinum*. Specifically we identified a premature stop codon, which led to the truncation of protein required for virulence. Surprisingly, we detected robust read-through of this stop codon resulting in a restoration of virulence. How often spontaneous mutations occur in the genome and how the translational machinery can bypass such mutations are unknown. We think that spontaneous mutation frequency in pathogenic mycobacteria grown under defined laboratory settings is underappreciated, giving rise to the necessity of developing studies to better understand the effects that appear *in vitro*. We hypothesize that using rich growth media promotes the loss of genes not required when grown *in vitro*. We also hypothesize that *M. marinum* has a system in place which allows readthrough of premature stop codons. To test our hypotheses we are taking two approaches. First, we are defining the rate of spontaneous mutation in *M. marinum* under laboratory conditions by passaging in growth media with differing sugar sources. Second, we are developing tools to monitor the rates of stop-codon readthrough in *M. marinum* via translational fusion constructs. By characterizing the frequency of spontaneous mutations under laboratory conditions, we hope to develop a clearer understanding of mutations that occur in the genome that can lead to a premature stop codon and the translational machinery that allows for the successful readthrough of a premature stop codon.

Comparison growth rates in cultured malaria parasites

Authors: Khai Q. Pham, Lisa Checkley, Michael T. Ferdig

The emergence of drug-resistant Malaria parasites significantly affects our ability to control human malaria. Artemisinin Combination Therapies are one of the few remaining compound classes that are currently used to cure multidrug-resistant Malaria infections. Artemisinin-based treatments are beginning to lose their effectiveness due to the emergence and spread of artemisinin drug resistant parasites in many parts of Southeast Asia. Even though mutations associated with antimalarial resistance and beneficial for parasites under drug pressure, but it can cause a fitness cost. This experiment presents an *in vitro* model showing how antimalarial resistance mutation effect on parasite growth. We specifically investigate and compare the growth rates between Artemisinin resistant Malaria parasites (ART 1-SBM), drug sensitive ones (NF54 GFP–luciferase) and their progenies of their crosses (1B6 and 1D12). The result shows that there is a significance different in growth rate between the parental lines and their cross progenies. The collected data suggest that growth rate phenotype may be a fitness cost caused by drug resistant mutation. Understanding the development and growth events within the erythrocytic cycle widens our knowledge of killing mechanisms and cellular targets of antimalarial drugs.
Ion channel inhibitors reduce Streptolysin S-Dependent Cytotoxicity in Group A Streptococcal infections of human keratinocytes

Rebecca A. Reis, Rebecca A. Flaherty, Dustin Higashi, Shaun W. Lee

*Streptococcus pyogenes*, or Group A *Streptococcus* (GAS), causes a variety of diseases in humans ranging from mild cases of pharyngitis to invasive forms of necrotizing fasciitis. One of the major virulence factors that contributes to the invasiveness of GAS is Streptolysin S (SLS). SLS is responsible for the characteristic beta hemolysis observed when GAS is grown on blood agar. Recent studies have indicated that the lysis of blood cells occurs when SLS binds to the Band 3 chloride bicarbonate exchange proteins on the surface of red blood cells, causing an influx of chloride ions, which results in osmotic lysis. These results suggest that the cytotoxicity of the SLS toxin may not be attributed to pore formation alone. This evidence is corroborated by studies in human keratinocytes, which have shown an SLS-dependent modulation of pathways leading to programmed necrosis. We propose that SLS initiates a signal transduction event by interacting with an outer membrane transporter present on the surface of keratinocytes during infection, which results in the downstream activation of pathways leading to cell death. Using ethidium homodimer assays, we demonstrate that niflumic acid (NFA), the cyclooxygenase-2 (COX-2) and calcium gated ion channel inhibitor, is capable of reducing SLS-dependent cytotoxicity in HaCaT keratinocytes. The Band 3 specific inhibitors 4,4'-diisothiocyanatostilbene-2,2'-disulfonic acid disodium salt hydrate (DIDS) and 4-acetamido-4'-isothiocyanato-2,2'-stilbenedisulfonic acid disodium salt hydrate (SITS), which dramatically reduced SLS-mediated lysis in red blood cells, provided only minimal protection from SLS-dependent cytotoxicity in keratinocytes. These findings suggest that SLS binds to an outer membrane protein distinct from the Band 3 exchanger in keratinocytes to induce cell death. Because calcium gated channels are known to be targeted by niflumic acid, we hypothesize that in keratinocytes, SLS may interact with a calcium-dependent channel on the surface of the cell to produce cytotoxicity.

---

HPV detection by in situ hybridization

Author: Ashley N. Olguin, Jeffrey Johnson and Dr. Sharon Stack

Human papillomaviruses (HPV) are a family of viruses that cause benign skin lesions and can be oncogenic. Recent data support a link between oral HPV and oropharyngeal squamous cell carcinoma (OPSCC). HPV-associated oral cancer progression proceeds differently from other oral cancer types and patients respond differently to therapy. Therefore, it is of the utmost importance to determine HPV status prior to clinical treatment. Both high risk subtypes HPV 16 and HPV 18 have been detected amongst HPV + oropharyngeal cancers. HPV-associated tumors express the E6 and E7 oncoproteins, leading to loss of various tumor suppressor genes and the upregulation of p16. P16 is a cyclin-dependent kinase-4 inhibitor that is expressed in a
limited range of normal tissues and tumors. The E7 oncoprotein inactivates the RB gene product resulting in the upregulation of p16. Clinically, overexpression of p16 is used as an immunohistochemical (IHC) marker of HPV-associated OPSCC. We have previously reported differential expression of microRNAs observed in HPV+ tumors, including miR-9. The current study compares a newly described in situ hybridization (ISH) methodology to detect E6/E7 mRNA to IHC to p16 in a panel of OPSCC cell lines and human tumors. Results show positive detection of for 5 of 5 HPV+ tissue samples by E6/E7 probe. 4 out of 5 HPV+ tissues produced E6/E7 staining patterns similar or identical to p16. Interestingly, one HPV+ tissue stained in multiple areas where p16 was not detected by IHC. This finding suggests that RNA ISH is more sensitive in determining HPV status then P16 IHC, since a smaller tissue sample may not have continued the p16 + tumor portion. An additional goal of the study was to evaluate mRNA levels of a known target of miR-9, Matrix metalloproteinases family collagenase-3 (MMP-13). MMP-13 is responsible for the breakdown of extracellular matrices (ECM), and contributes to metastasis. With the significant upregulation of miR-9 we hypothesize that MMP-13 will be significantly down-regulated. To test this hypothesis, we will use the novel RNA Scope ISH to visualize the various MMP-13 levels within HPV + and - cells and tumor tissues. We predict that the down regulation of MMP-13 maybe one of the reasons HPV+ or -OPSCC cancers are less aggressive then HPV- cancers.

---

**Provisioning pollution in the population genetics of Blastocystis in Bali’s Long Tailed Macaques**

Erik Rodriguez, Justin Wilcox

*Blastocystis* is the most common intestinal parasite in humans, infecting approximately one third of the world’s population. Although its pathogenicity is unresolved, it has recently been implicated in causing, gastroenteritis, urticaria (hives), irritable bowel syndrome, and opportunistic infections of the immunocompromised. Zoonotic transmission is believed to be an important source of *Blastocystis* infections, but little is known about *Blastocystis*’ life cycle in sylvatic hosts. Here, we assessed landscape and demographic factors that influenced the prevalence and population genetics of *Blastocystis* in a potential reservoir host, the long-tailed macaque (Macaca fascicularis), across 13 sites in Bali, Indonesia. We constructed a model of *Blastocystis* prevalence using 9 landscape and demographic variables to assess how the external environment contributes to *Blastocystis* persistence. We anticipated that water availability would be the primary predictor of *Blastocystis* prevalence, as contaminated water is a common source of protozoan infections. We also assessed how landscape and demographic factors affect the allele richness of *Blastocystis* at each site, with the prediction that this would be primarily predicted by prevalence. To assess the role of host movements in dispersing *Blastocystis*, we compared the population genetic structure of *Blastocystis* and macaques. We found that *Blastocystis* prevalence was associated with decreased water availability, suggesting that water sharing increases *Blastocystis* transmission. Urbanization, macaque population size, and elevation were also found to be major contributing factors towards Blastocystis prevalence, which together suggest that the anthropogenic environment may contribute to increased *Blastocystis* transmission as well. Macaque population size, which is primarily driven by human provisioning, was also found to be the primary contributing factor to the allele richness of
Blastocystis; surprisingly, prevalence was not correlated to the genetic diversity of Blastocystis across sites. Likewise, no relationship was found between the population genetic structure of Blastocystis and that of the macaques. Ultimately our findings highlight the potential for humans to inadvertently increase the transmission of wildlife diseases and supports management strategies that seek to prevent the provisioning of macaques.

Molecular Identification of malaria vectors and novel species- Western Province Solomon Islands and Sulawesi, Indonesia

Emma Troth, Diego Echeverri-Garcia, Honglin Xiao, Lily Yu, Julie Niedbalski, Neil Lobo

Malaria is a parasitic disease caused by species of Plasmodium genus, which are transmitted to humans through the bite of female Anopheles mosquitos. There are five different species of Plasmodium parasite: P. falciparum, P. vivax, P. ovale (subspecies wallikeri and curtisi), P. malariae, and P. knowlesi.

Controlling the malaria vectors can be helpful to decrease malaria burden. The goal is to prevent human-mosquito contact, and thus inhibit malaria transmission. By knowing with confidence the malaria vectors that are present in an area, malaria control interventions can be specific to the area and thus be a more effective intervention.

Multiple species are important malaria vectors. Species often differ in their biologies in ways that make them differentially susceptible to different control interventions. Patterns of behavioral biology tend to be uniform across a species’ range. The most important malaria vectors are members of cryptic species complexes. It is largely recognized that molecular criteria is essential for the identification of most complex members.

The Western Province Solomon Islands and Sulawesi, Indonesia, have been affected by malaria for many years. The goal of this study is to identify all species present in these locations that are malaria vectors. Specific interventions can then be implemented to decrease human-mosquito contact, and thus decrease malaria prevalence overall.

Methods:

Mosquitos are caught using Human Landing Catchings (HLCs) in the Solomon Islands In Indonesia, mosquitos are caught using HLCs, fences, and kelambo traps. All mosquitos are sent to the University of Notre Dame for dissection, DNA extraction, COX-I PCR for sporozoite detection, ITS2 PCR for mosquito species identification, and CO-I PCR for mosquito species identification. All samples are then sequenced at Notre Dame’s Genomic Core Facility.

Results:
Discrepancies were identified between morphological identification and molecular identification. Only 31% of the Indonesia samples were correctly morphologically identified compared to their molecular identification. Mosquitos whose DNA did not match any of the sequences in the
Discussion:
At most sites there was an unexpected degree of misidentification of species. Misidentified specimens result in incorrect species compositions, wrong attributions of disease vectors with potentially negative consequences on intervention selection and efficiency. Misidentifications are due to incorrect/old keys, uncommon/novel mosquitoes, and human error. An identification system that identifies local species compositions using both morphology and molecular biology can be used to make a taxonomic based step-by-step locally adapted methods to reduce error rates.

---

Wnts and tumor cell invasion

Diana Esparza, Alanna Sedgwick, Crislyn D’Souza-Schorey

Tumor cells may develop the ability to leave their site of origin by gaining invasive abilities, a required step in metastasis. Proteolysis of the extracellular matrix is important for tumor invasion and two important cellular structures utilized for this process are invadopodia, formed at the adherent cell surface, and extracellular invasive tumor cell-derived microvesicles. The Wnt family of secreted proteins regulate important events during tissue morphogenesis and development, however, it has been shown that Wnts can also promote invasion and metastasis of cancer cells. Using LOX melanoma cells as a model system, in preliminary investigations, we evaluated the ability of secreted Wnts to affect invasive activity using a fluorescent matrix degradation assay. Future work will include further characterization of how Wnts may alter the invasive behavior of tumor cells.

---

Landscape variability and climate change implications for wild blue lupine emergence across the Indiana Dunes National Lakeshore

Katia J. Cruz1, Lainey Pasternak2, Tamatha Patterson2, Jessica Hellmann2

Climate change poses a threat to species extinction through changes in phenology (timing of an organism life cycle). Specifically, insect host-plant interactions can shift their phenological timing as a response to temperature changes and landscape variability. Toward understanding changes in plant phenology of an important insect-plant relationship, we examine the influence of temperature and other environmental variables on the timing of Wild Blue Lupine (Lupinus perennis). Shifts in lupine phenology will affect larval food availability for the endangered Karner
Blue Butterfly (KBB, *Lycaeides melissa samuelis*). We address the following questions: 1) Have increased temperatures shifted lupine phenology, resulting in earlier emergence and potential mismatch with KBB? 2) Is landscape variability (i.e. canopy cover and distance from Lake Michigan) across the Indiana Dunes National Lakeshore contributing to earlier emergence?

Using growing degree day calculations and multiple regression analysis, we estimated an average lupine emergence degree day of 351.8. We used this estimate to recreate past and project future lupine emergence. We did not find a trend in emergence across the past 63 years. However, climate change projections indicate that lupine will emerge at least one month earlier if abnormal increases in temperature are experienced. We also found that lupine emergence distributions in southern and northern slopes are relatively similar. Therefore, emergence does not appear to be strongly influenced by aspect. Further, we did not find any correlation between canopy cover ($p = 0.3018$) or distance from Lake Michigan ($p = 0.8098$) with lupine emergence. Future research studies should determine the KBB average GDD for egg-hatching stage, as this will help examine the possible asynchrony between the timing of lupine emergence and KBB larvae emergence, potentially explaining KBB’s regional decline.

---

**Using insect odorant receptors to identify potential attractants for malaria mosquito**

Savannah Kounelis and Zain Syed

Sugar feeding (from plants) is critical in all mosquitoes’ lives and olfaction plays critical role in mediating this behavior. A large and divergent family of proteins, Odorant Receptors (ORs), is expressed on the dendrites of Olfactory Receptor Neurons (ORNs) that are housed in sensilla on the antennae and maxillary palps that detect and discriminate the odorants from potential hosts, mates and oviposition sites. Among a handful of ORs that are conserved across various mosquito species, one of highly conserved OR is Or2 and has been implicated in playing a key role in attraction to plant among the disease transmitting mosquitos *Culex quinquefasciatus*, *Anopheles gambiae*, and *Aedes aegypti*. We used bioassay guided methods to isolate and identify active constituents from the two plants, *Lantana camara* and *Parthenium hysterophorus* that are used by *An. gambiae* as a sugar source. These compounds have been identified as benzaldehyde, salicylaldehyde, naphthalene and indole. My ongoing work involves localization of the AgamOR2 using whole mount fluorescent in situ hybridization (WM-FISH) that will further assist in charactering the receptor in its natural milieu.

---

**Developing tools for homology-independent CRISPR/Cas9 mediated integration of a reporter cassette in zebrafish**

Kevin Wilkins, Ryne Gorsuch, David R. Hyde

The development of designer nucleases has greatly improved the ability to edit the genome in a precise and targeted fashion. The latest technology in the field of genome editing is the clustered regularly interspaced short palindromic repeats (CRISPR) system, that consists
CRISPR-associated protein 9 (Cas9) and a chimeric small guide RNA (sgRNA). The sgRNA is composed of a Cas9-interacting scaffold and a nucleotide protospacer complementary to a target DNA sequence to which the sgRNA is directed. The sgRNA interacts with the targeted genomic locus via Watson-Crick base pairing; after annealing to its target, the sgRNA recruits Cas9, which, in turn, induces a double strand break (DSB) in the DNA. The cell can repair this break via non-homologous end joining (NHEJ), an error prone mechanism that creates insertions and deletions at the cleavage site and can be used to create null alleles and to integrate foreign DNA into the genome. This study describes the preliminary work to create knock-in reporters using NHEJ. We generated a donor plasmid containing the fluorescent reporter gene tdTomato fused to an N-terminal self-cleaving E2A peptide. Any endogenous sgRNA target site can be introduced upstream of this reporter cassette via PCR. In this system, both the endogenous locus and donor plasmid can be cleaved by the same sgRNA, resulting in E2A-tdTomato expression from the endogenous locus. In order to design a proof-of-principal experiment, we needed to identify a functional sgRNA for an endogenous gene. We designed a sgRNA targeting the sox2 open reading frame. When compared to uninjected control fish, the majority of the sox2 sgRNA injected fish exhibited morphology ranging from wild type to mild and severe developmental defects, suggesting the disruption of sox2. T7E1 assays showed DSBs induced at the targets site, which was confirmed via sequencing. Future work will involve injecting this sox2 sgRNA with our donor plasmid harboring the sox2 sgRNA target site in order to generate a Tg(sox2:tdTomato) transgenic reporter line.

Validation and characterization of Tg[pax6b:EGFP] zebrafish line

Darby Heard, Kristin Ackerman, David Hyde

In response to damage, zebrafish (Danio rerio) produce hundreds of progenitor cells that can regenerate all retinal cell types. In contrast, mammals generate a limited regeneration response where many exogenous factors are required to generate low numbers of progenitors that differentiate into a few neuronal cell types (Ooto et al 2004, Osakada et al 2007). Upon light induced damage to the zebrafish retina, Müller glia reenter the cell cycle, producing progenitors that differentiate and migrate to the site of damage to replenish the lost retinal cells, restoring vision (Vihtelic et al 2006). Our laboratory has determined that the transcription factor Pax6 is important during the regenerative process. Specifically, quantitative real-time PCR and morpholino mediated gene knockdown of pax6a and pax6b suggested that these homologs play a role in progenitor cell formation and amplification. Recently using BAC transgenesis, our laboratory generated a transgenic zebrafish line, in which GFP is under the control of the pax6b promoter [Tg(pax6b:GFP)]. The overall goal of my project is to characterize the expression of pax6b during neuronal regeneration. Taking an immunohistochemical approach and using cell specific markers, I determined that the undamaged adult retina contains pax6b-positive Muller glia, amacrine cells and ganglion cells. Further more, approximately 44% of Müller glia and 50% of amacrine cells express pax6b. For the first time, we show that throughout regeneration there is a significant increase in total number of amacrine cells corresponding with increased numbers of pax6b-positive amacrine cells and a significant increase in pax6a-positive amacrine cells. The
number of pax6b-positive Muller glia remains unchanged. Finally, both proliferating Müller glia and neuronal progenitors express pax6b with a significant increase in the GFP/PCNA-positive cell populations.

Disruption of the visual system of Aedes aegypti with CRISPR/Cas9

Morgan O. Petersen1, Michelle A. Whaley2, Joseph E. O'Tousa2

The mosquito vector Aedes aegypti integrates information from multiple sensory cues in order to perform routine behaviors that contribute to vector competence. Due to its high level of complexity and ability to adapt to environmental light stimuli, we believe the visual system of the mosquito is an especially promising area for vector control. In order to characterize the role of vision in vector competence, we employ CRISPR/Cas9 gene editing technology to irreversibly mutate Aaop1 Aaop3, Arr1, and Arr2 genes. We constructed two guide RNA plasmids for each gene by cloning guide RNA encoding DNA into pU6-BbsI-chiRNA plasmid, which can be transcribed into RNA upon injection into the animal by use of the U6 promoter. These plasmids were confirmed by DNA sequencing. Next, we obtained homology arms by PCR amplifying regions directly upstream and downstream of the CRISPR cut site for each gene and cloned them into PCR2.1 TOPO vector. These homology arms were then cloned into pH-DsRed-attP vector. The pH-DsRed-attP vector provides the opportunity for homology directed repair to occur, introducing the DsRed and attP sequences, and simultaneously creates a frameshift mutation to make the gene nonfunctional. We have begun a first round of embryo injections to compare the efficiency of guide RNAs, which will be analyzed using the T7 Endonuclease Assay. Finally, our wildtype animals show constant results in visual behavior studies. The Locomotor Activity Monitor (LAM), used to measure the response of mosquitoes to a visual light stimulus, shows a 84% response rate for wildtype WH strain mosquitoes with a 13.5 lux 30 minute light pulse, linking visual cues to activity. Our landing assay studies display an 82% for wildtype WH strain mosquitoes to land on black over white (n=40 p=0.0001). These patterns will be compared to visually impaired animals to characterize the role of vision in behavior.

Ras-mediated evasion of ECM-detachment induced cell death

Jordan Cockfield, Raju Rayavarapu, Zachary Schafer

Breast cancer is the most diagnosed cancer and the second highest cause of cancer-related deaths among women. The primary cause of cancer related death is metastasis. A major hurdle that needs to be overcome by metastatic disease is extracellular matrix (ECM) detachment
induced apoptosis know as anoikis. Found in approximately 30% of all tumors, Ras is the most common oncogene in human cancer. Ras drives a variety of processes including the ability to suppress cell death and promote invasion and metastasis. It has been previously observed that Ras can activate multiple signaling pathways downstream of its activation in order to suppress apoptosis through the regulation of the Bcl-2 family of proteins. These proteins regulate the release of cytochrome c from the mitochondria. Cytochrome c binds to Apaf-1 (apoptotic protease activating factor) and, along with procaspase-9, forms a complex known as the apoptosome. The apoptosome activates caspase-9 which is able to cleave and activate effector (or executioner) caspases which are responsible for carrying out the apoptotic program. The purpose of this study was to investigate the molecular mechanisms utilized by HRas to affect apoptosomal formation and activity in ECM-detached breast epithelial cells. MCF-10A breast cancer cells were engineered to overexpress Bcl-2 and HRas respectively. We used coprecipitation experiments coupled with western blots in order to assay the protein-protein interactions involved in apoptosome formation. It was found that the activation of HRas results in a loss of interaction between Apaf-1 and Procaspase-9 in ECM-detachment. Our data suggest that HRas is suppressing the formation of the apoptosome by inhibiting the interaction between Apaf-1 CARD and the pro-domain of Procaspase-9. Deciphering the molecular mechanisms that enable cell death evasion in ECM-detachment is critical to the development of therapeutics and the inhibition of metastatic disease.