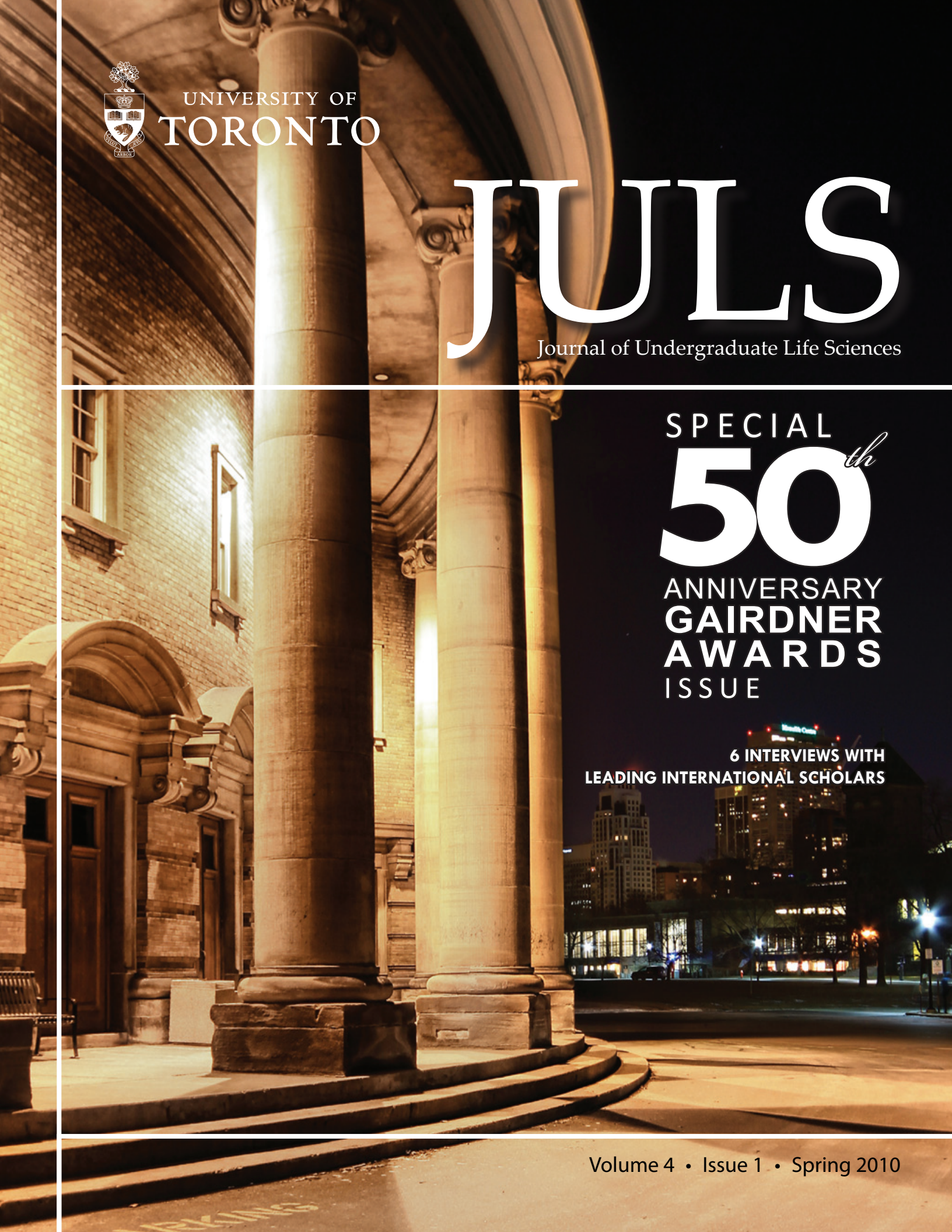

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Call for Submissions

The *University of Toronto Journal of Undergraduate Life Sciences (JULS)* is always looking for submissions that showcase the research achievements of undergraduate life science students. We welcome manuscripts in the form of Research Articles or Reviews. Submissions must come from University of Toronto undergraduate students or undergraduate students outside of U of T who have conducted research for at least three months under the supervision of a faculty member at U of T.

Research articles should present original research and address an area of the life sciences. Mini-reviews should focus on a specific scientific topic of interest or related to the research work of the author. Research articles should be between 2,000-3,000 words and mini-reviews between 1,500-2,000 words. All works must not have been previously submitted or published in another undergraduate journal. The deadline for submissions for each issue will appear on the JULS website at http://juls.library.utoronto.ca.

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Acknowledgements

We thank Dr. George Fantus, and the Principal of University College for their support in the creation of this year's issue of JULS.

Staff List

Letter From the Editor

About the Cover
Convocation Hall - the timeless building that holds the memories of our first science classes and the moment we can look at the last four years of our lives with pride.

Cover photograph by
Dariusz Adamski

Dear readers,

I would like to cordially welcome you to the fourth annual issue of the University of Toronto *Journal of Undergraduate Life Sciences (JULS)*. It is amazing how far *JULS* has come – holding the first ever issue in my hands as a freshman four years ago, I could not have imagined that *JULS* would be where it is right now. Unlike many similar student clubs, it has not waned, but has grown and improved with every year. *JULS* continues to provide a unique avenue for undergraduate students to showcase their accomplishments in research. As in previous years, we have maintained our commitment to publishing the highest quality work by conducting peer student and faculty review. Most importantly, we hope that the *JULS* experience, for authors and for those working on the editorial side, has been integral to their development as science writers.

This year, *JULS* editors have prepared a unique feature on the 50th Anniversary of the Gairdner Awards, held at the University of Toronto in October 2009. We sat down with six leading international scholars to get an insider's perspective on the world of research. Dr. Bruce Alberts, the editor-in-chief of *Science*, talked with us about the practicality of measuring the impact of scientists. Dr. Samuel Weiss from the University of Alberta took us on a tour of adult neurogenesis and offered salient advice for those on the road to becoming scientists. Dr. Sackett from McMaster University, this year's Gairdner Wightman Award winner, introduced us to the world of clinical epidemiology. In addition, we spoke with Dr John Sulston, Dr. Nubia Munoz and Dr. John Dirks about a number of exciting topics conerning science, education and medicine.

In addition to our main feature, over 30 student authors have contributed to the publication of 17 *primary research*, *review* and *news* articles, representing many life science disciplines, such as psychology, neuroscience, bioinformatics and cancer biology. Like last year, *JULS* will recognize the best articles in each category, as selected by our senior editors and faculty reviewers, with awards to be presented at the year-end *JULS* reception.

Lastly, I would like to express my utmost gratitude to all members of the *JULS* team for their part in making the publication of this issue a reality. Their dedication and hard work throughout the year was invaluable. It is only with a foundation of a supportive team, full of ideas, creativity and passion that *JULS* can continue to grow stronger every year.

Sincerely,

Kirill Zaslavsky

Editor-in-Chief, 2009-2010

NOTE: All articles in this issue as well as supplementary information are freely available online at http://juls.library.utoronto.ca/. If you would like to join the JULS team, submit an article or have any comments or suggestions, please feel free to contact us at juls@utoronto.ca.

Lp-PLA2: An Emerging Risk Factor for Cardiovascular Heart Disease

— Tina Binesh Marvasti —

Until recently, traditional cardiovascular heart disease risk factors, such as high cholesterol and blood pressure levels, were considered to be the potential and predictor risk factors of heart disease. However, recent studies show that not all patients suffering from myocardial infarction have had such predicting signs. Studies on blood samples of patients with a cardiac event show that high levels of an enzyme called lipoprotein associated phospholipase A2 (Lp-PLA2) in blood plasma can be associated with future cardiac events. Hence researchers have hypothesized that among patients with no traditional risk factors, abnormal activities of Lp-PLA2, an emerging coronary disease risk factor, can increase the risk of future coronary complications independent of other factors.

This hypothesis was tested in an epidemiological study conducted by the West of Scotland Coronary Prevention Study Group [1]. In this study, patients with hypercholesterolemia were monitored for the levels of Lp-PLA2 activity. The result of the research, which was published in The New England Journal of Medicine, showed that in a sample of 580 patients, high levels of Lp-PLA2 activity have a positive association with the development of arterial plaque and can independently increase the risk of myocardial infarction.

Furthermore, an article published in Nature Medicine in 2008 reported a study done on hypercholesterolemic pig models, which demonstrated that abnormal activities of Lp-PLA2 produce deleterious effects by oxidizing cholesterol and producing coronary atherosclerotic lesions on the arterial walls [2]. This was shown using darapladib, a drug that inhibits the activity of Lp-PLA2 in blood plasma, which lowered the rate of plaque formation by preventing the oxidation process.

As a newly emerging risk factor, Lp-PLA2 must be investigated further for better understanding of its activity in cardiovascular diseases. Hopefully, genetic and environmental factors that play a role in the over expression of this enzyme can be identified, studied, and controlled. By better understanding the factors causing Lp-PLA2's abnormal expression in the blood plasma, more can be learned about the potential risk factors associated with coronary disease - a leading cause of death and disability in Canada and throughout the world.

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Mechanism of swearing as a response to pain

— Nancy Dong —

Given how common swearing is as a response to pain, Richard Stephens and colleagues at Keele University were surprised to find that no neurological mechanism has been established to explain exactly why that is. To investigate, they recruited a group of undergraduate students who were subjected to the cold pressor test while repeating a swear word of their choice, and later as a control underwent the test again repeating a neutral word. It was found that under the swearing condition there was significantly lower pain perception than in the non-swearing condition [1].

In recent years, there has been a growing body of research on the role that emotions play in pain regulation, to allow a better understanding of the brain and its application in a clinical setting. Is it the sensation of pain that is unpleasant or the emotions that accompany it? It was found in a 2006 study by INSERM that under the same conditions, individuals who were shown scenes of human pain had higher pain perception than individuals who were shown equally unpleasant pictures that do not contain references to pain [2]. More recently, a Chinese study done this year showed that contrary to common view, both happy and sad music that are equally emotionally arousing can significantly reduce pain ratings to the same painful stimuli [3].

The regulating effect that emotions have on pain can be explained by the fact that the body's own mechanism for pain modulation is closely tied to the parts of the brain that process emotions [4], such as the hypothalamus and the amygdala (which have been shown to activate when one swears) [5]. Activation of these brain parts in turn activate the periaqueductal gray area in the brain stem, resulting in the release of the neurotransmitter enkephalin that inhibit the release of substance P by binding to the pain transmitting neurons in the spinal cord [6]. Previous research has shown that rats lacking in substance P cannot detect increasing intensities of pain, thus possibly explaining why an individual has a higher pain tolerance when emotionally aroused [7].

The findings made by Stephens' team add to the body of evidence that can be used to answer the mind-brain problem by explaining how the physical actions of the brain can produce our mental experiences. The next step is to gain further understanding of exactly which emotions reduce pain and how it might be applied to emotion-provoking psychotherapy for patients suffering from chronic pain.

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New Research on NK Cells blurs the line between the innate and adaptive immune systems

— Ana Komparic —

Prevention remains one of the sustaining pillars of modern medicine, with vaccination lying at its foundation. Understanding the intricacies of the immune system is essential for the continued development and amelioration of preventive medicine. Traditionally, a clear distinction has been drawn between the innate and adaptive immune systems. The innate immune system is an organism's first line of defense against non-self antigens, requiring no prior exposure to initiate an immunological response. The adaptive immune system, which is believed to have developed after the innate immune system, requires exposure to a particular antigen prior to initiating an effective response and developing 'memory' cells which maintain long-term immunity to the antigen in question [1].

The adaptive immune response has been described as a set of four phases: expansion, contraction, memory maintenance and a recall or secondary response phase. The last two phases were believed to be unique to the adaptive immunes system and to be responsible for the principle of long-term immunity, which vaccination relies on. In 2009, Sun et al documented the first memory maintenance and secondary response phases ever to be observed in natural killer (NK) cells [2]. This suggests that NK cells, which are the effector cells of the innate immune system, may in fact share some key characteristics with cytotoxic T lymphocytes and other cells of the adap¬tive immune system.

Sun et al exposed infected mice with mouse cytomegalovirus (MCMV) and monitored NK cells which display the MCMV-specific Ly49H receptor (Ly49H+). They noticed that the Ly49H+ NK cells proliferated vigorously, and produced 'memory' cells, which were detected in both lymphoid and non-lymphoid organs several months after infection. These 'memory' cells produced IFN-γ ex vivo upon reactivation. Furthermore, following adoptive transfer of MCMV-primed Ly49H+ NK cells into naïve immunodeficient mice, the experienced NK cells were found to be ten times more protective against MCMV then NK cells transferred from naïve mice [2].

These findings add to the growing body of research which proposes that innate and adaptive immune systems share more similarities than previously believed, leading to new understanding of NK cell redundancy and the evolution of the immune system [1,3]. Further research is required to investigate whether a similar NK response persists *in vivo* or to other pathogens and models. If further studies confirm immunological memory in NK cells, novel approaches in preventive medicine, particularly vaccination, will surely be investigated.

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Neuroscience: the key to effective education?

— Sarah Sidky —

Recent technological advances in neuroscience have allowed scientists to gain a stronger understanding of how the human brain actually learns. But why, despite this, do neuroscience and education continue to be kept separate? At Harvard University, Dr. Kurt Fischer and his team strive to bridge the gap between neuroscience and education. He explains that "the relationship between neuroscience and education should be a reciprocal one in which scientific research and educational practice inform and learn from one another, as medicine and biology act symbiotically [1]."

Previous research suggests that an increased performance level in memory-involving activities is associated with physical activity. Usha Goswami, director of Cambridge University's Center for Neuroscience, believes that we can use scientific research such as this to modify our teaching methods. She explains that "learning comprises changes in neural activity, either via changes in potentiation at the synapse or the strengthening of connections [2]." Since successful teaching directly affects brain function by changing connectivity, it is up to us to discover and employ the most effective teaching methods – teaching methods that will allow students to reach their full academic potential.

Sarah Shaw, an advanced skills teacher at Lakeside Primary School of Cheltenham, England, leads one of the few classes in the world taught according to the principles of neuroeducation. She believes that incorporating movement and dance into her curriculum has the ability to intentionally trigger the brain's biological ability to take in and store information, thus strengthening connections in the brain. Shaw's work brings up a principal question in our mission to bridge the gap between neuroscience and education: to what extent does physical movement in the classroom induce or enhance long-term potentiation in the hippocampus? We can now at least attempt to answer this question thanks to recent technological advances such as functional magnetic resonance imaging (fMRI), which allows us to study the brain *in vivo*.

Results obtained from these experiments can offer quite valuable possibilities to education, including early diagnosis of special educational needs, comparison of the effects of different kinds of educational input, and an increased understanding of individual differences in learning. As Trisha Gura explains in her paper titled Big Plans for Little Brains, "we are going to see an explosion of interdisciplinary findings that we had not had before in learning science [3]."

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Accurate Interpretation of Electrocardiograms by Nonexperts: Validation and Identification of Challenges

Celia Lai[1] **and Douglas Lee**[2]

[1]Leslie Dan Faculty of Pharmacy, University of Toronto, Toronto, Canada.
[2]Institute for Clinical Evaluative Sciences, Toronto, Canada.

Abstract

Objective: This study was primarily designed to validate electrocardiogram (ECG) interpretation via nurse abstractors and computational methodology against the interpretations of an expert electrocardiographer.

Method: The ECGs (n=429) were evaluated independently by 3 different subject groups: a cardiologist, nurse abstractors, and computational software. Required interpretative fields included rhythm, rate, PR interval, QRS duration, type of bundle branch block and type of ischemic changes. The ECG interpretations of the cardiologist served as the comparison standard. A library of synonyms equated computer output with corresponding ECG features of interest to facilitate analysis. Kappa analysis and crude agreement were computed for each interpretative field, comparing cardiologist's responses against those of nurse abstractors and computational software. Separate sub-group analyses for selective ECG characteristics (paced rhythm, presence of bundle branch block, and number of lead groups with significant Q waves) were conducted.

Results: The validity criteria of this study was determined to be $\kappa > 0.7$ or crude agreement >70%. It was found that all nurse abstractors' interpretations met the validity criteria. The crude agreement ranged from 84.85% up to 100%, while the kappa values spanned over a wider range (0.00 to 0.97). However, nurse abstractor's ability to detect atrial fibrillation/ flutter was significantly lower when the rhythm was paced –24% lower in the crude agreement between cardiologist's and nurse abstractors' interpretations. Except for PR interval (crude agreement = 69.16%, kappa = 0.67), all other computer interpretations met our validation criteria.

Conclusions: Aside from PR interval, the relative validity of all other computer software's interpretations was established. The relative validity of nurse abstractors' interpretations was also established. This suggests ECG training courses for nurse abstractors can be adapted to focus on their apparent weak areas to further improve their ECG interpretations.

Introduction

Electrocardiography (ECGs) is one of the most useful tools in cardiovascular clinical studies. Kurisu S *et al.* suggested that electrocardiograms may be useful in predicting short-term prognosis in patients with AMI associated with left main coronary artery [1]. On the other hand, incorrect interpretations of ECGs can carry lethal consequences as Bogun et al suggested that incorrect computerized interpretation of atrial fibrillation that is not corrected by the ordering physicians may result in potentially harmful medical treatments as well as inappropriate use of medical resources [2].

The importance of correct interpretations of ECGs is acknowledged in general, and there have been numerous studies conducted to evaluate competencies of nurses and medical doctors as well as computer software in their ability to correctly interpret ECGs. Salerno *et al.* conducted a literary review of articles between 1996 to 2002 and concluded that computer software can identify 58% to 94% of various nonrhythmic abnormalities that had been previously identified by an expert electrocardiographers [3]. Salerno *et al.* also found that noncardiologists identified 87% to 100% of ECGs showing acute myocardial ischemia, correctly classified 72% to 94% of ECGs as meeting criteria for thrombolytic therapy, and diagnosed 95% of ST-segment abnormalities [3].

Although the results obtained by Salerno *et al.* are useful in preliminary evaluations of the accuracies of ECG interpretations by nonexperts, several subjective aspects and possible contributing factors were not fully considered in many of the consulted studies, and may render inaccuracies to the data. Firstly, the experiences and educational backgrounds of the non-experts enrolled in the studies aforementioned varied. Furthermore, although these studies focused on particular aspects of ECG interpretation, no study has yet investigated whether the presence of paced rhythm and bundle branch block would influence ECG interpretation. In addition, no study has yet looked at whether the presence of significant Q waves in multiple lead groups is associated with the ability by noncardiologists to detect significant Q waves in another lead group (anterior, lateral, anterolateral, or inferior).

With the above considerations in mind, this study aims to further evaluate accuracies of ECG interpretations of nonexperts by: 1) validating electrocardiogram (ECG) interpretations conducted by nurse abstractors and computational analysis against the interpretations of an expert human electrocardiographer, and 2) determining whether certain ECG characteristics (paced rhythm, bundle branch block, and significant Q waves) are subjective to interpretation errors (Table 1).

Methods

Population and Sampling

ECGs collected from the Emergent Heart Failure Study arriving at the Institute for Clinical Evaluative Sciences (ICES) no later than June 12, 2009 (n=429) were evaluated independently by 3 different subject

Table 1: Interpretative fields.

Section of Survey	Field Values
Section 1	Rhythm Rate PR Interval QRS duration
Section 2	Bundle Branch Block (BBB)
Section 3	Ischemic changes in lateral, inferior, anterior & anterolateral lead group ST elevation ST depression Significant Q wave T inversion

There were 3 sections that were required to be filled out according to the responses received from each subject group (cardiologist, nurse abstractors, and computational software).The interpretative fields in section 1 included: type of rhythm (atrial fibrillation/ flutter or sinus), rate, PR interval, QRS duration. Section 2 looked at the presence of bundle branch block (BBB). If a bundle branch block was present, it is further classified into right bundle branch block, left bundle branch block and bifasciular block. Section 3 looked at the presence of ischemic changes (ST elevation, ST depression, significant Q wave, T inversion) in the lateral, inferior, anterior, and anterolateral lead groups. A lead group is indicative of the position of the ischemic changes, and consists of multiple leads. Lateral lead groups consists of lead 1 and avL; inferior lead groups consists of lead II, III, avF; anterior lead group consists of lead V1, V2, V3, V4; and anterolateral lead group consists of lead V5 and V6. There were specific criteria that must be met to satisfy the definition of "significant Q waves", "ST elevation", "ST depression" and "T inversion" in a lead group. The predetermined definition required the corresponding ischemic feature to occur more than once: in both leads (lead 1 and aVL) for lateral lead group; in a minimum of 2 out of 3 possible leads (lead II, III, avF) for inferior lead group; in a minimum of 2 out of 4 consecutive leads (lead V1, V2, V3, V4) for anterior lead group ; and in both leads (lead V5 & V6) for anterolateral lead group.

groups: 1) a cardiologist, 2) nurse abstractors, and 2) computational software. One cardiologist was recruited from ICES, and his ECG interpretations served as the comparison standard. Three nurse abstractors were recruited from ICES. The nurse abstractors have all completed an ECG training session and have passed an ECG competency test prior to this study. Computer software used for ECG interpretation varied with hospital sites and the specific software has not been investigated. However, the differences of software used were assumed to be negligible because of the preliminary nature of the study. To facilitate interpretation, a library of synonyms equating computer output with corresponding ECG features of interest was created.

Measurement of Outcomes

Three sections of interpretative fields were entered into a database according to the responses collected from each subject group (Table1). Interpretative fields included rhythm, rate, PR interval, QRS duration, type of bundle branch block and type of ischemic changes. Description and definition for each interpretative field were provided to each nurse abstractor in a reference binder during a training session. It should be noted that the definition of ischemic changes (ST elevation, ST depression, significant Q wave, T inversion) in a given lead group implies that the ECG feature of interest appears in at least 2 consecutive leads within the corresponding lead group.

Statistical Procedures

Kappa analysis and crude agreement were computed for each interpretative field comparing cardiologist's responses against those of nurse abstractors and computer software. The validity criteria of this study was arbitrarily determined to be $\kappa > 0.7$ or crude agreement >70%. These values were assumed to be the minimal degree of validity required for chart abstraction in the Emergent Heart Failure Study. Separate sub-group analyses for nurse abstractors' versus cardiologist's interpretations were conducted for the following: 1) paced versus non-paced rhythm; 2) presence versus absence of bundle branch block, and 3) presence of significant Q waves in 0, 1, 2, 2 or 3 lead groups. Statistical significance was set at $P < 0.05$.

Results

All computational interpretations, with the exception of PR interval interpretative, met the validity criteria (Figure 1). The crude agreement and kappa value comparing computer interpretation versus cardiologist's interpretation for PR interval were found to be 69.16% and 0.67 respectively. On the other hand, all the interpretive fields from the nurse abstractors' interpretations met the validity criteria (Figure 1). Although the kappa values varied a wide range (0.00 to 0.97), the crude agreement consistently passed the 70% threshold (84.85% to 100.00%) for all interpretation fields. However, nurse abstractor's ability to detect atrial fibrillation/ flutter was lower when the rhythm was paced. In fact, there was a difference of 24% in crude agreement between the responses of the cardiologist and nurse abstractors. Additionally, presence of bundle branch block was associated with a lowered ability for nurse abstractors to detect lateral ST depression and anterior ST depression; with difference in crude agreement of 11.35% and 12.25% respectively. In addition, there appeared to be a trend of lowered crude agreement between the nurse abstractors and the cardiologist group in the detection of significant Q waves in a given lead group when significant Q waves were also present in other lead groups (Figure 2). However, this trend is not statistically significant except for the detection of significant Q waves in the inferior lead group (Figure 2). Specifically, the following observations were made for the crude agreement for inferior significant Q waves: 98.06%

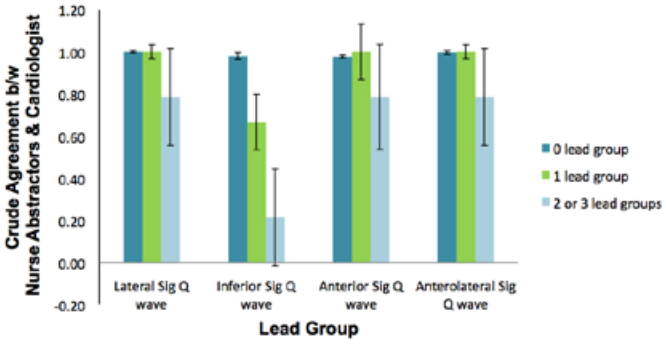

Figure 1: Comparison nurse of abstractors' and the cardioglogist's ability to detect significant Q waves when multiple significant Q waves were present. This sub study compared the crude agreement between nurse abstractors and the cardiologist's interpretations of significant Q waves in the 4 different lead groups (lateral, inferior, anterior, anterolateral) under the following conditions: no actual significant Q waves were present in any lead group; significant Q wave was present in 1 lead group; significant Q waves were present in 2 or 3 lead groups. The results from this study has shown that significant Q waves present in multiple lead groups appeared to show a trend of lowered crude agreement between nurse abstractors and cardiologist in their detection of significant Q wave in another lead group. This trend, however, was not statistical significant except for the inferior lead group.

(96.05-99.22) when no significant Q waves were present; 66.67% (52.53-78.91%) when significant Q wave was present in 1 lead group; 25% (5.49-57.19) when significant Q wave was present in 2 lead groups, and 21.43% (4.66-50.80) when significant Q wave was present in 2 or 3 lead groups.

Conclusion & Discussion

Computerized interpretation, except for PR interval, was found to be valid for the purpose of this study. All the nurse abstractors' interpretations were found to have met the experimental validity criteria. However, there were potential areas that could be improved to increase the accuracy of nurse abstractor's interpretations. Specifically, nurse abstractors' ability to accurately interpret ECG decreased under the following situations: 1) detection of atrial fibrillation/ flutter with paced rhythm, 2) detection of lateral and anterior ST depression with bundle branch block, and 3) detection of significant Q waves in one lead group when significant Q waves were also present in other lead groups.

This study provided preliminary results to study questions that were not being addressed in current literature. However, there are limitations in the study of ECG interpretations. First of all, the validity criterion was arbitrarily set to meet the minimum demands assumed for most abstraction studies. Nevertheless, these criteria may have to be set higher when ECG readings are used for clinical purposes. Secondly, there were ambiguous terms from computer output that could not clearly be correlated with the ECG interpretation fields used in the study. In addition, low numbers of experimental interpreters for certain sub-group analyses did not provide the power to conclusively shown significant differences.

ECG validation is important because many abstraction studies conducted rely on nurse abstractors for ECG interpretations. In addition, ECG interpretation cannot always be cross-referenced with other medical records in a clinical setting; hence valid interpretation is crucial in maintaining high quality data.

There are several implications that can be drawn from this study. First of all, the shown experimental validity of nurse abstractors' interpretations suggests that that the nurse abstractors are able to independently review ECGs without a cardiologist's review. This has economical implications as using nurse abstractors to abstract ECG data would help to save financial resources and cardiologists' time. Secondly, findings from this study can be applied to improve current ECG training courses. It is advised that the content of ECG training courses for nurse abstractors be adapted to focus on their weak areas as discussed to improve the accuracy of ECG interpretations.

Acknowledgement

This project was funded by the Canadian Cardiovascular Outcome Research Team (CCORT) through their Canadian Institutes of Health Research (CIHR) grant program. This study was conducted with study data from the Emergent Heart Failure Study, led by Dr. Douglas Lee at the Institute of Clinical Evaluative Sciences (ICES). Special thanks go to Ms. Alice Chong for assisting with the data analysis, as well as Dr. Douglas Lee and Ms. Audra Stitt for providing invaluable advice.

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Table 2: ECG interpretations.

		Computer Interpretation			Nurse interpretation		
	ECG Interpretation Fields	**% Agreement**	**Kappa**	**Valid?**	**% Agreement**	**Kappa**	**Valid?**
Section 1	Sinus Rhythm	91.82	0.8310	Yes	94.87	0.8940	Yes
	Atrial Fibrillation/Flutter	91.36	0.7887	Yes	94.41	0.8708	Yes
	Paced rhythm	94.86	0.7783	Yes	99.30	0.9669	Yes
	ECG rate	94.39	0.9430	Yes	97.44	0.9739	Yes
	PR interval	69.16	0.6698	No	90.44	0.8874	Yes
	QRS interval	96.50	0.9645	Yes	96.04	0.9598	Yes
Section 2	Type of bundle branch block	91.36	0.7384	Yes	92.07	0.7384	Yes
Section 3	Ischemic changes in lateral leads	78.27 to 99.07	-0.0318 to 0.0611	Yes	84.85 to 100.00	0.0000 to 1.0000	Yes
	Ischemic changes in inferior leads	96.26 to 96.96	-0.0187 to 0.7082	Yes	91.61 to 99.07	0.0000 to 0.2409	Yes
	Ischemic changes in anterior leads	92.52 to 96.96	0.0505 to 0.5863	Yes	91.61 to 97.90	0.0000 to 0.4709	Yes
	Ischemic changes in anterolateral leads	92.52 to 96.96	0.1425 to 0.4537	Yes	89.98 to 98.37	0.0000 to 0.5017	Yes

It can be seen that all of nurse abstractors' interpretations met the validation criteria. Although the kappa values for all the interpretative fields in section 3 were below the cutoff of 0.7, their crude agreement consistently scored above 70%. The low kappa values could be explained by the low study numbers with the specific ischemic changes in the specified lead groups at any one time. Except for PR interval (crude agreement = 69.16%, kappa = 0.67), all other interpretative fields met the validation criteria. Like the nurse abstractors' interpretations, the kappa values for all the interpretative fields in section 3 for computational software were below the cutoff of 0.7, yet their crude agreement consistently scored above 70%.

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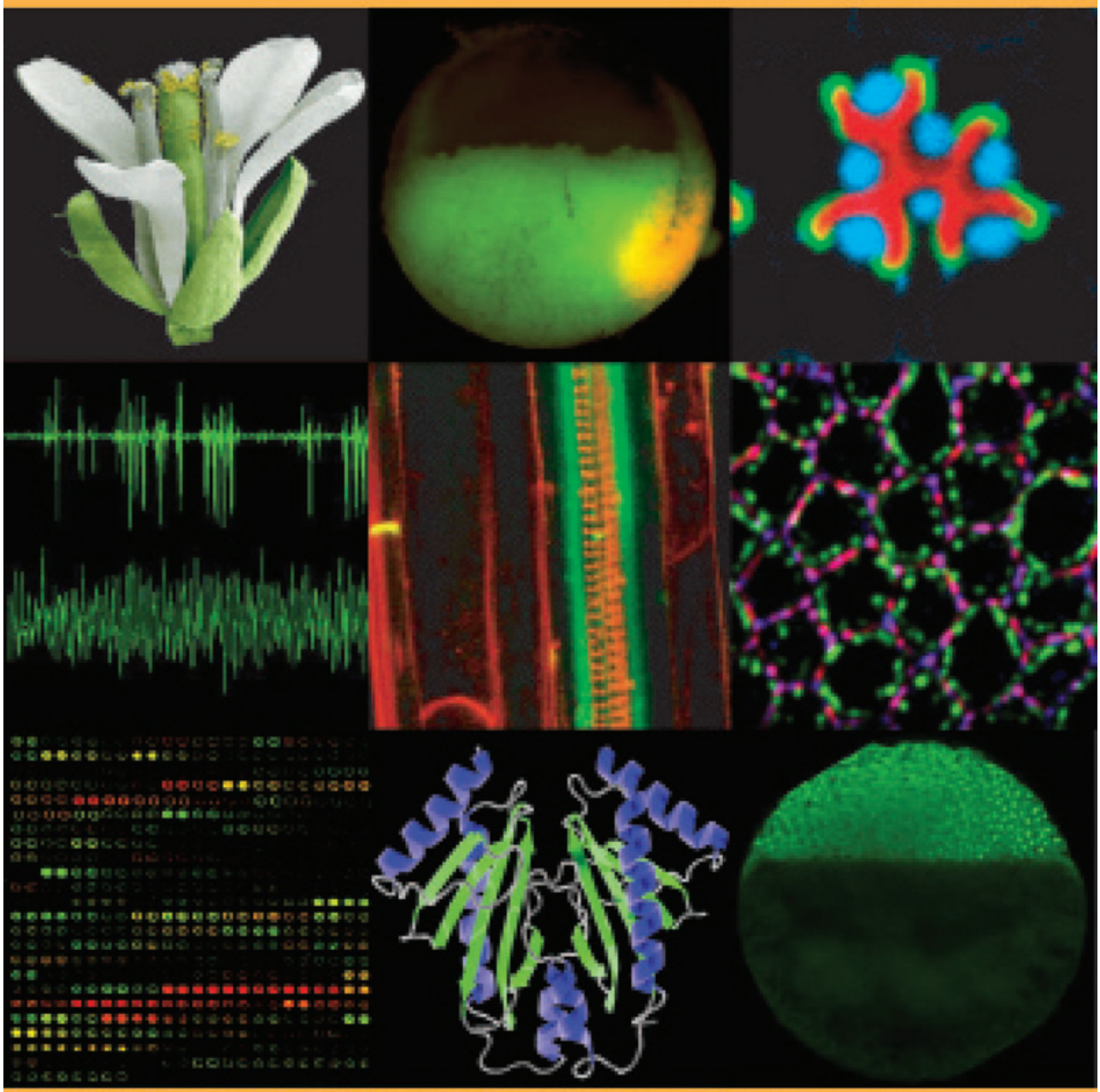

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Research Article

Top-down trophic shifts in the subtidal habitats of Cuba and the role of herbivorous fish and urchins

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Abstract

A healthy coral reef is governed by top-down population control. This type of regulation suggests that algal abundance is suppressed by the presence of herbivores. Low algal cover would thus be expected to accompany the observation of a high abundance of herbivorous fish and urchins. This study aims to detect top-down control in the subtidal zone of a Cuban coral reef system and also aims to reveal any differences when comparing the grazing effects of herbivorous fish to those of sea urchins. Population data on grazers were collected for three habitats – beach, cliffs, and rocks – and then correlated with palatable algal cover for the corresponding quadrat. The comparison of regression lines for each habitat suggested that there was no difference between the grazing effects of herbivorous fish and sea urchins. Also, a significant positive correlation was found in the rocky habitat between urchins and algal cover ($P < 0.05$, $R^2 = 0.177$). This indicates a trophic shift and lack of top-down regulation that can be attributed to recent hurricane damage and mechanisms outside of the study's original design, such as the strong positive dependence of crustose coralline algae on urchins ($P < 0.01$, $R^2 = 0.264$). This study demonstrates that species-level interactions are so diverse in such a highly complex ecosystem that they may not be detectable at a community level, which obscures the overall strength of the studied response.

Introduction

There are many reports of coral reef decline and coral reef health is being affected by a variety of factors. Caribbean coral reefs have lost 80% of their hard coral cover over the past 30 years and have been replaced by turf algae [1]. One of the main stressors in this fragile ecosystem is the decline in herbivorous organisms that are capable of controlling the growth of turf algae [1]. This study will investigate grazers like sea urchins and herbivorous fish and their relationship to coral reef health. Grazers characteristically eat weeds and algae, which tend to inhibit coral growth [2]. As a result, the presence of grazers is often an indicator of good reef health [2]. Reef herbivores help corals survive by reducing competitively superior algae, leading to corals with better overall health that are less inflicted by disease [3]. Such a system is maintained by top-down population control where the herbivores control the populations of the lower trophic levels.

There are two types of density-dependent controls found in coral reef fish populations: top-down trophic interactions (predation and herbivory) and bottom-up trophic interactions (food availability and competition for resources) [4]. Top-down regulation occurs in interactions where the top trophic levels control the balance of the ecosystem – predators indirectly increase plant abundance by suppressing the herbivores [5]. An ecosystem governed by bottom-up regulation would collapse if the bottom trophic level was removed [6]. In this study, top-down regulation would be evident if increased herbivorous fish and urchins accompanied low algal cover, since this means that higher trophic levels are controlling lower ones. Conversely, bottom-up regulation would predict that increased algal cover leads to increased herbivorous fish and urchins, since there are more resources. It is often debated whether top-down or bottom-up control has a greater impact upon coral reef ecosystems. This is an important question for researchers and conservationists, because coral reefs are especially vulnerable to anthropogenic stressors, such as overfishing, which affect top-down interactions and nutrient loading, which affect bottom-up interactions. Understanding which type of population control has a greater impact upon the system can help define the priorities of conservation methods [4]. Many researchers [7,8,9] agree that a healthy marine ecosystem is governed by top-down control and that the removal of keystone predators will result in dramatic shifts in prey and resource abundance. Even though top-down dynamics are the dominant natural state, these processes are still variable within the same ecosystem [8]. For example, according to a study by Kramer and Heck, a survey of patch reefs in the Florida Keys revealed that phase shifts could be attributed in part to natural stressors like frequent hurricanes [10]. It is logical to assume that this may be the case for Cuban coral reefs as well because of their proximity to the Florida Keys and high hurricane frequency.

As for the individual roles of sea urchins versus those of herbivorous fish, there is some debate over which animal is the dominant grazer of coral reefs. Competition for resources has been documented between sea urchins and herbivorous fish [11]. Some studies show that sea urchins are the most important grazing animals [12] and caging experiments where herbivorous fish were excluded showed that they played only a minor role in grazing as compared to the urchins [2]. However,

in spite of strong support from current literature that the urchins play the strongest role, it is important to note that sea urchins are typically studied more often because they are slow-moving and easy to examine, thus there may be discrepancies in the current available data [3].

The purpose of this study is to investigate the relationship between grazer abundance and algal cover on coral reefs in the subtidal habitats of Punta Frances, Cuba. The specific goals are to determine (1) if the three subtidal habitats studied exhibit trends indicative of top-down control, and (2) if there is a significant difference between the grazing effects of sea urchins and herbivorous fish. Ultimately, the goal of this study is to answer the question: Is the observed system representative of a healthy coral reef?

Materials and Methods

Study Site

Herbivorous fish and sea urchin populations were studied in three subtidal zone habitats (beach, cliffs, rocks) on Punta Frances, Isla de la Juventud, Cuba. All quadrats were placed in shallow water levels (<2.2m). The habitats are named after the closest land characteristics but are all located underwater. Thus, "beach habitat" does not refer to the actual beach, but to the subtidal zone of the beach. The beach habitat was characterized by abundant sand and sediment coverage. The cliffs were composed of fossilized coral, and due to their abrupt steepness, quadrats in this habitat were relatively deep. The rocks habitat was characterized by reef cement coverage (*Porolithon pachydermum*), fewer sediments, and greater water movement and turbulence. The areas varied in algae coverage between filamentous or fleshy algae, and calcareous or coralline algae. I surveyed the study site for twelve days in May of 2009. Control of consistency was maintained by performing two trips per day at approximately the same time during the morning and afternoon hours. Weather conditions were excluded from measurement since a comparison of calm and rough weather by Foster (1987) did not result in significant differences. However, it was noted that Punta Frances and the surrounding area recently incurred damage from several hurricanes.

Study Organisms

The study included species of herbivorous fish and sea urchins that graze the algae growing in coral patches. Herbivorous fish forage during the day with a variety of foraging strategies including territorial defense (common in Pomacentrids) and foraging groups (*Acanthuridae, Labridae, Scaridae*) [3]. Herbivorous fish are selective in feeding and prefer algae that can be most readily utilized – the filamentous or fleshy algae [3]. In contrast, sea urchins have adapted the ability to consume calcified algae – the type that is avoided by fish since they cannot ingest it [13]. Sea urchins cannot efficiently digest cellulose and therefore prefer to consume the calcareous and crustose algae [14].

Survey Approach

Twelve line transects were set up in each subtidal habitat at a distance of 25m apart from each other. From each transect, three quadrats were chosen ranging from the most logical shallow point to a depth of up to approximately 2m. In total, there were 36 transects and 108 quadrats. The quadrats were all chosen by selecting the nearest patch of coral from the transect line, thus eliminating researcher's bias. Each quadrat was a semi-circle marked with flags and a radius of approximately 2m. In each study site, the species and abundance of herbivorous fish were recorded during a ten-minute observation. For the same quadrat, data was then recorded for the number of urchins by doing a close survey of the habitat floor and by flipping over movable rocks. This study was conducted under water using snorkeling equipment.

Statistical Analyses

The data collected for fish and urchin abundance was analyzed to identify characteristics of top-down control using algal cover as a dependent variable since the main hypothesis is that algal cover is dependent on grazer abundance. The data for algal cover was collected independently of this study, but correspond to the same time period and quadrats as those in this study.

Correlations across habitats – The algae data was categorized into palatable and unpalatable for either fish or sea urchins using existing literature on the preferred diets of each [13,15,16]. For each habitat, the fish and sea urchin data was standardized by dividing each individual with the highest number of individuals in that species column. For each habitat, the total number of herbivorous fish in each quadrat was correlated against palatable algal cover for that quadrat using the spreadsheet program Excel, and regressions were performed. The resulting R^2 value and Pearson R was tested for statistical significance in SPSS, a statistical analyses program. The regression correlations were repeated for sea urchins using the palatable algae as the dependent variable with the independent variable being sea urchin abundance. In order to reflect a top-down controlled system, the trend between variables is expected to be negative – the more herbivores there are in a quadrat, the less palatable algal cover. The trends obtained regressions were further analyzed for associations between habitats and on a species-specific level (see high-resolution analysis under "Associations between habitats and species-specific correlations" section).

Herbivorous fish versus sea urchins –Next, the regression lines for fish and sea urchins were compared using a two-tailed t-test. The t-statistic was computed as follows:

$$t = \frac{Slope_1 - Slope_2}{\sqrt{SE_{slope_1^2} + SE_{slope_2^2}}} \rightarrow d.f. = (n_1 - 2) + (n_2 - 2)$$

where the summary statistics for slope and standard error (SE) for each of the regression equations were obtained using SPSS. The values n_1 and n_2 are the sample sizes for fish and sea urchins respectively, with both equaling 36 for each habitat.

Associations between habitats and species-specific correlations – A high-resolution multi-dimensional analysis was run relating the three habitats to each other to test for associations between algae, fish, and sea urchins. A Spearman correlation matrix and multi-dimensional scaling (MDS) graph was produced. Also, an MDS graph illustrating the relationships between species of fish and algae and another MDS graph showing the relationships between sea urchin species and algae was produced and correlation tests (Pearson) were run in SPSS to assess likely relationships.

Results

Correlations across Habitats

No significant correlations indicative of top-down control were found in the three habitats (Fig. 1). Only the rocks habitat produced significant correlations, but this trend is not evident of top-down interactions (Fig. 1C). There were no significant correlations for the beach quadrats between sea urchins and palatable algae (Fig. 1A; P=0.298, R^2=0.008) and between fish and palatable algae (Fig. 1A; P=0.430, R^2=0.0009). Likewise, the cliffs habitat showed no significant correlations between sea urchins and palatable algae (Fig. 1B; P=0.446, R^2=0.0005) and between fish and palatable algae (Fig. 1B; P=0.215, R^2=0.018). A significant positive correlation was found between sea urchins and palatable algae (Fig. 1C; P<0.05, R^2=0.177) and a marginally significant positive correlation was found between fish and palatable algae (Fig. 1C; P=0.09, R^2=0.052) for the rocks habitat.

Herbivorous Fish versus Sea Urchins

There were no significant differences between the regression lines of fish and urchins for all three habitats (Table 1 Below). Therefore, the null hypothesis cannot be rejected.

Table 1: Summary statistics for two-tailed t-test comparing regression lines between fish and urchins.

	Habitat		
	Beach	**Cliffs**	**Rocks**
Urchins	Slope = -10.223	Slope = -0.825	Slope = 26.806
	SE = 19.143	SE = 6.000	SE = 9.919
Fish	Slope = -2.117	Slope = -9.803	Slope = 18.049
	SE = 11.953	SE = 12.262	SE = 13.190
t-statistic	-0.359	0.658	0.531
Significance	ns	ns	ns

For n = 36, df = 68, and P = 0.05,critical value is 1.669. ns = not significant.

Associations between Habitats and Species-specific Correlations

The MDS graph (Fig. 2), and the resulting Spearman correlation matrix, revealed biologically relevant and significant results for the rocks habitat: urchins and palatable algae show a strong positive correlation (ρ=0.400). There was a positive correlation between urchins and unpalatable algae for fish (ρ = 0.564) and fish palatable algae and sea urchins unpalatable algae (ρ=0.750). For all three habitats, the amount of palatable algae for both fish and sea urchins is positively correlated with ρ=0.735 for beach, ρ=0.557 for cliffs, and ρ=0.495 for rocks.

Based on the visual assessment of the fish and algae MDS graph for associations on the species level, there was one point of interest that showed many species very closely grouped. This grouping of species included many damselfish (*Abudefduf saxatilis, Stegastus partitus, Stegastus adustus, Stegastus diencaeus, Stegastus planifrons*) and two wrasses (*Halichoeres bivittatus, Thalassoma bifasciatum*). The algae in this grouping were four palatable algae (*Lyngbia lyngbia, Cladophoropsis sp., Dictyota sp., Sargassum natans*) and two unpalatable algae (*Stypopodium*

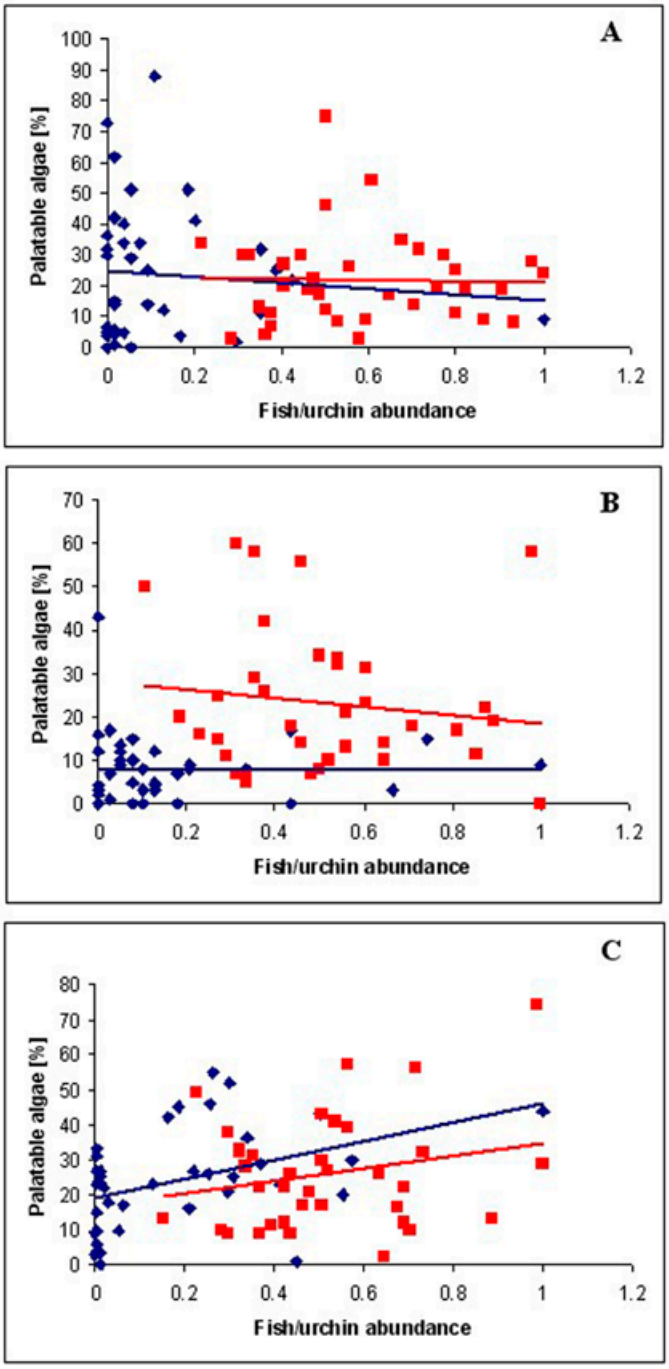

Figure 1: Standardized fish and urchin abundance correlated with percent palatable algae cover. The red line represents the regression line for fish and the blue line represents the regression line for urchins. **(A)** There were no significant correlations for the beach quadrats for urchins (P = 0.298, R2 = 0.008; n = 36) and fish (P = 0.430, R2 = 0.0009; n = 36). **(B)** There were no significant correlations for the cliffs quadrats for urchins (P = 0.446, R2 = 0.0005; n = 36) and fish (P = 0.215, R2 = 0.018; n = 36). **(C)** There was a significant positive correlation for the rocks quadrats for urchins (P < 0.05, R2 = 0.177; n = 36) and a marginally significant positive correlation for fish (P = 0.09, R2 = 0.052; n = 36).

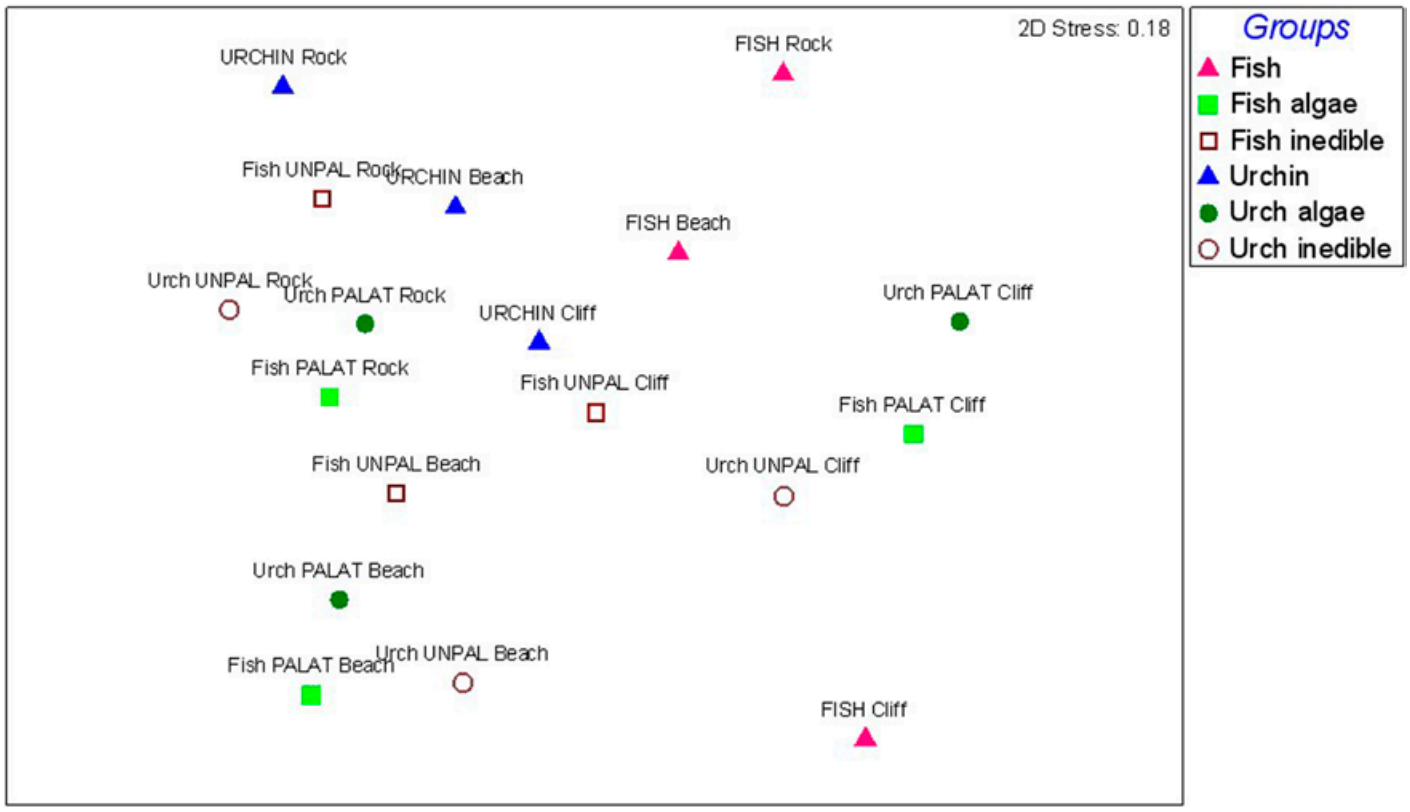

Figure 2: MDS graph illustrating associations between habitats. A high resolution multi-dimensional analysis was run relating the three habitats against each other to test for associations between algae, fish, and sea urchins. The closer two points are on the graph, the more closely correlated they are.

zonale, Tubinaria sp.). See correlation matrix using Pearson's R (Table 2A) for the relationships between these species.

Discussion

The results did not support the occurrence of top-down controlled systems in the subtidal zone habitats of Punta Frances. The main findings were that the beach and cliffs habitat showed an ambiguous trend between grazers and algal cover while the rocks habitat showed a significant positive correlation between grazers and algal cover, particularly with sea urchins (Fig. 1). Previous studies have shown that grazers control algal cover (representing a negative correlation), and this top-down population control is indicative of a healthy reef [2,3,11]. The lack of negative correlations between grazer abundance and algal cover in the study sites indicate a trophic shift from a top-down controlled system. A large-scale disturbance would disrupt the healthy system and cause these trophic shifts to occur, and the recent hurricane damage was most likely the source. As argued by various researchers [10,17,18], phase shifts associated with storm damage and hurricanes could result from fish and urchins death, sediments that smother coral, and mechanical clearing of coral substrate allowing for macroalgae growth. These factors would supersede top-down control by triggering a growth in macroalgal cover, dominating the area after the disturbance.

On Cuban coral reefs and the Caribbean in general, hurricanes are a natural part of the system and do not usually pose long-term risks. Intermediate levels of natural disturbance maintain high biodiversity, and reef ecosystems are both accustomed to these disturbances and capable of recovering pre-disturbance compositions [17]. Algae usually grow over dead coral and exposed patches of calcareous substrate after hurricane damage, and these perturbations can occur several times in a decade [18] so a lack of top-down control is not necessarily a sign of poor coral reef health – the reef may just be in a transitional period. However, the algae growth can sometimes overwhelm the coral after a disturbance and the system is unable to recover to its original coral-dominated state. The reef is particularly susceptible when there are an insufficient number of grazers. This collapse in health has been seen in the Caribbean during a disease outbreak and mass mortality of *Diadema antillarum* [18] and in Jamaican coral reefs where a hurricane combined with mass mortality of *D. antillarum* [17] caused a phase shift towards an algae-dominated reef. In this study, the hurricanes alone may not have triggered a trophic shift since the resulting mortality of herbivores, particularly *D. antillarum*, would have compounded the effects.

Looking specifically at the rocks habitat, the regression line between urchins and palatable algae is significantly positively correlated (Fig. 1C). This is confirmed by the results of the Spearman correlation matrix testing for associations between habitats and illustrated by the MDS graph (Fig. 2).

Table 2: Based on visual assessment of MDS graph of species-specific correlations. Pearson R highlighted in red represents significance at 0.01 level (2-tailed) and Pearson R highlighted in blue represents significance at 0.05 level (2-tailed). **(A)** Pearson correlation matrix for fish and algae species of interest. **(B)** Pearson correlation matrix for urchin and algae species of interest.

A		lyngbia	Cladophoropsis	dictyota	sargassum_natans	stypopodium_zonale	tubinaria
Abudefduf_saxatilis	Pearson *R*	.153	.070	-.005	.051	.140	-.038
	Sig. (2-tailed)	.113	.471	.960	.598	.149	.698
	N	108	108	108	108	108	108
Stegastus_partitus	Pearson *R*	-.001	-.172	.051	-.055	.260**	-.041
	Sig. (2-tailed)	.988	.075	.602	.571	.007	.676
	N	108	108	108	108	108	108
Stegastus_adustus	Pearson *R*	-.080	.002	.051	.085	.020	.062
	Sig. (2-tailed)	.408	.987	.599	.381	.836	.523
	N	108	108	108	108	108	108
Stegastus_diencaeus	Pearson *R*	-.066	-.219*	.126	-.154	-.031	.064
	Sig. (2-tailed)	.497	.023	.192	.111	.754	.511
	N	108	108	108	108	108	108
Stegastus_planifrons	Pearson *R*	.088	-.098	.019	.018	.021	-.049
	Sig. (2-tailed)	.367	.311	.848	.852	.827	.612
	N	108	108	108	108	108	108
Halichoeres_bivattatus	Pearson *R*	-.256**	-.192*	.123	.053	-.365**	-.094
	Sig. (2-tailed)	.007	.046	.206	.584	.000	.331
	N	108	108	108	108	108	108
Thalassoma_bifasciatum	Pearson *R*	-.084	.055	.162	.130	.050	-.046
	Sig. (2-tailed)	.386	.573	.094	.181	.611	.638
	N	108	108	108	108	108	108

B		porolithon	lyngbia	dictyota
Echinometra_lucunter	Pearson *R*	.513**	.235*	-.148
	Sig. (2-tailed)	.000	.014	.126
	N	108	108	108

For sea urchins in the rocks habitat, there is a strong positive correlation showing: as urchins increase, palatable algae increase as well. The correlation matrix also shows a positive relationship between unpalatable algae for fish and palatable algae for urchins, suggesting that there is a prevalence of algae that fish find unpalatable but urchins find palatable. The data suggests that this algae species is *Porolithon pachydermum* (reef cement, a type of crustose coralline algae) and the species-specific correlations show that the sea urchin *Echinometra lucunter* is strongly positively correlated to *P. pachydermum* (Table 2B). Several studies [7,19,20] have shown that sea urproductivity through compensatory productivity (nutrient recycling and removal of senescent cells). Crustose coralline algae are not necessarily an indication of bad coral health. They can contribute to coral health by promoting settlement of coral recruits since it provides a solid substrate to attach to [21,22] and helps to build reef structure by contributing calcium carbonate [7]. The large effect of crustose coralline algae on the rocks habitat may be attributed to its ability to survive in areas of high water movement. This is because sea urchin grazing produces sediments that accumulate in poor water circulation, creating anoxic environments that inhibit algae growth [14]. These results show that it is very difficult to isolate the specific mechanisms associated with herbivory that control algal communities since many factors influence algal growth and not simply the rate of removal. This creates a nonlinear relationship between grazing and algal growth and complicates the basic model for top-down control.

Thus far, this study has emphasized the importance of as-

sessing species-specific interactions. Instead of the most readily apparent observation that the positive correlation between urchins and algae is an indicator of poor reef health and lack of top-down control, the strong dependency of *P. pachydermum* on urchin abundance demonstrated that there was indeed control by higher trophic levels. The activity of the urchins (higher trophic level) stimulated the growth of *P. pachydermum* (lower trophic level). Species-level interactions are very diverse and may not be detectable at a community level, which may mask or decrease the overall strength of a response. Other mechanisms exist in the species-habitat interactions that complicate the top-down control model. For example, damselfish limit grazing by defending their territories and algal abundance is generally higher in damselfish territories [23,24,25]. It was found in this study that the damselfish *Stegastus partitus* was positively correlated with unpalatable algae *Stypopodium zonale* (Table 2A), which may be correlated not as a food source (since it is unpalatable) but for habitat use. Thus, the results did not fit basic models because there is high complexity in such a diverse ecosystem, but could potentially fit more complex models that take the many interactions into account.

It is inconclusive whether sea urchins or herbivorous fish are the superior competitor based on the data obtained. The comparison between the urchin and fish regression lines for all three habitats generated t-statistics that were below the critical value (Table 1) and the null hypothesis could not be rejected. This suggests that there is no difference between the effects of sea urchins and herbivorous fish on algal cover and is further supported by the high-resolution analysis testing for associations between habitats. For all three habitats, the amount of palatable algae for fish is positively correlated with the amount of palatable algae for urchins, indicating no need for interspecific competition, because the prevalence of one organism's food source does not seem to affect the other organism. Numerous studies [2,12,11,14,26] identify sea urchins as the primary grazer, however this was not observed in this study, possibly because of the recent hurricane disturbance in the area. Sea urchin herbivory is shown to be superior where there are more than 20 urchins/m^2 [26]. The hurricanes most likely caused a decline in urchin numbers, and with the exception of the rocks habitat, there were no quadrats with more than 20 urchins/m^2.

Conclusion

The inability to detect a trend towards top-down control does not necessarily indicate an unhealthy reef system and can be attributed to alternative mechanisms outside of the original design. There are many differences in grazing intensity and algal cover at the species level. Other mechanisms could occur between the consumer and the resource or between nonadjacent trophic levels [6]. These nonlinear relationships between grazing and algae abundance can appear to destabilize top-down trophic control. The shallow study site was the source of confounding factors since algal cover is generally high in lower intertidal and shallow subtidal zones because turbulence limits herbivore access [24]. Also, herbivorous fish are more capable of suppressing algae >20 m deep because of a greater abundance and size [24]. Therefore, these unresolved limitations suggest the need for future studies in shallow subtidal zones. The outcome of this research can help us better understand the vulnerabilities of coral reefs to disturbances and its effects on grazers and algal cover.

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Research Article

Development of STAT3 as an accessible target for fluorescence-based inhibition assays

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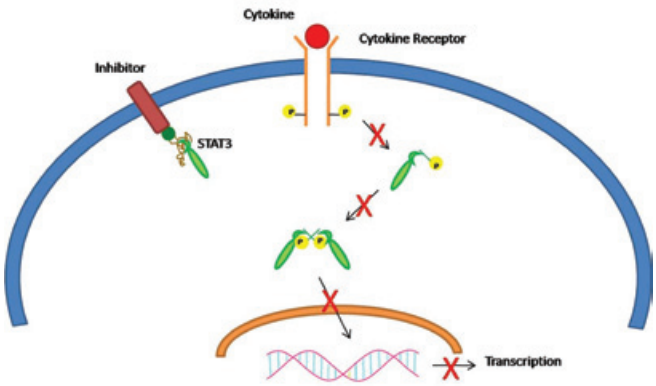
Abstract

Signal-Transducer-and-Activator-of-Transcription 3 (STAT3) is a protein which plays an important role in relay of cytokine signaling pathways. However, hyperactive STAT3 also contributes significantly to human cancers, such as leukemia and lymphoma. We are currently developing a novel therapeutic modality that inhibits STAT3 protein mobility within the cell based on protein anchorage. In order to assess STAT3's localization within the cell we have developed a STAT3 labelling protocol with a tetramethylrhodamine (TMR) fluorescent label. The majority of STAT3 inhibitors target the SH2 domain binding module. Thus, we selected a known peptidic STAT3 SH2 domain binder with a dissociation constant in the range of 100 nM (as estimated via two independent fluorescent techniques, Fluorescence Correlation Spectroscopy (FCS) and Fluorescence Anisotropy (FA)) to determine whether the SH2 domain is structurally compromised by TMR labelling. We herein report successful TMR labelling of STAT3 protein (~2-3 molecules of dyes per protein molecule). Most encouragingly, TMR labelling was shown to confer negligible loss of STAT3 functional activity, as indicated by FCS measurements of the binding of STAT3-TMR and the peptide.

Introduction

STAT3 has been a topic of recent anti-cancer research [1-2] due to its significant role in tumorigenesis [3-5]. Extracellular signal binding by a wide range of receptors such as cytokines, growth factors, or hormones, leads to the activation of cytoplasmic receptor bound proteins such as Janus kinases (JAKs). JAK mediated phosphorylation of a tyrosine residue in STAT3 allows the formation of transcriptionally active STAT3:STAT3 homo-dimers that are mediated by reciprocal phosphotyrosine-SH2 domain interactions. Activated STAT3 dimers translocate to the nucleus where they bind to specific DNA-response elements in the promoters of target genes and regulate gene expression (Figure 1). Although active STAT3 is not commonly harmful to cells, overactive STAT3 activity causes unwanted transcription of anti-apoptotic genes, leading to tumorigenesis [3-5].



Many methods of inhibiting the oncogenic functionality of STAT3 have been proposed which target various steps of the signaling pathway [6]; most commonly the goal is the disruption of STAT3 protein complexes with SH2 domain binders [2, 7]. However, due to the complexity of the activation of STAT3 [6], any approach which only targets one pathway has the disadvantage that activation could be initiated by an alternative pathway. For example, there is evidence [6] that phosphorylation of a serine residue located in the SH2 domain enhances transcriptional activity. An approach which targets this pathway would thus reduce activity, but not prevent it. To address this concern, we have proposed to develop inhibitors of STAT3 mobility that target the SH2 domain and subsequently suppress STAT3 transcriptional activity. The ultimate goal of this approach is to prevent STAT3 nuclear translocation by diverting it to other regions of the cell. This approach is beneficial in that it provides inhibition regardless of the method of activation.

Fluorescence spectroscopy is a powerful technique that can be used to probe dynamics and interactions at a single-molecule level. However, STAT3 is not a natural fluorophore, and thus for suitable candidacy for fluorescent inhibition studies, it must be first labelled with a dye. Fluorescent inhibition

Figure 1: Activation and inhibition pathways of STAT3 in the cell. Competitive binding of p-STAT3 to an anchored ligand disrupts the STAT3 dimerization and translocation into the nucleus. Blue: plasma membrane, yellow: phosphate group (PO4-), green: STAT3, burgundy: lipopeptide ligand/inhibitor, orange: nuclear membrane, arrows: normal activation pathways, X's: inhibited pathways.

assays studying the interaction between STAT3 monomers or STAT3 and drug molecules rely on the proximity of the fluorophores being used. For example, Förster resonance energy transfer (FRET) is effective on distances in the range of ~1-10 nm [8]. More accurate measurements occur in the lower end of this range. Because dimerization occurs at the SH2 domain, and this domain is the most common target for inhibiting drugs, the SH2 domain is the ideal location for fluorescent labelling. Although labelling of this domain allows for more effective fluorescence-based inhibition assays, it is important to avoid compromise of function due to the SH2-dependance of proper STAT3 functionality. Successfully, a functional, TMR-labelled STAT3 protein was prepared which is suitable for measuring SH2 domain inhibition by fluorescent microscopy. Furthermore, we have tested a STAT3-binding peptide sequence and explored its inhibitory interaction with TMR-labelled STAT3 protein.

Materials and Methods

Multiparameter Single-molecule Microscopy

Spectroscopic measurements were carried out with a custom-built confocal microscope capable of multiparameter, fluorescent detection at a single-molecule level [9-10] (Figure 2). Excitation light in the range of 700-1100 nm is produced by a tunable, femtosecond-pulsed laser (Tsunami HP, Spectra Physics, USA), which is frequency-doubled by a nonlinear β-BBO crystal to produce light in the visible spectrum. A portion of the beam is diverted to a sync which allows for time-resolved fluorescence trajectories to be constructed. The light is filtered through a pinhole and then passes through a 1.4 NA/100X plan-apochromat objective (Carl Zeiss, Canada) which excites the coverglass-mounted sample at intensities in the range of 100 W/cm2. The sample is raster scanned in the xy-plane by a three-dimensional translational piezo scanner (T225, MadCity Labs, USA). The emitted light is collected by the same objective and is spatially and spectrally filtered using a 50 μm pinhole and long-pass and interference filters (Semrock, USA) to remove out-of-focus fluorescence and scattering contributions. The fluorescence is then split into two separate pathways by polarization and focused onto avalanche photodiodes (PD5CTC, Optoelectronic Components, Canada).

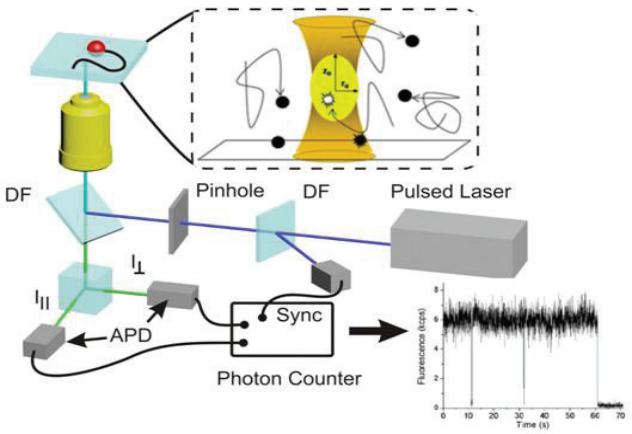


Figure 2: Simplified schematic of the multiparameter confocal microscope used in this study. DF: dichroic filter/mirror, at s = 5.77 and r0 = 231.07 nm.

Fluorescence Correlation Spectroscopy (FCS)

Fluorescence Correlation Spectroscopy is a method of temporally autocorrelating the fluctuations in fluorescence signals emitted by a diffusing molecule [11]. The intensity-normalized correlation at time t can be expressed as [11]:

$$G(t) = \frac{\langle \delta I(t+t_0)\delta I(t_0)\rangle}{\langle I(t_0)\rangle^2} \quad (1)$$

where $I(t)$ is the detected fluorescence intensity as a function of time, $\delta I(t) = I(t) - \langle I(t)\rangle$ is the deviation from the mean intensity, and the brackets denote time averaging. In this paper we make use of two models to describe the physical systems we are studying. For a fluorescent molecule freely diffusing in three dimensions the function describing the temporal autocorrelation of fluorescent signal is expressed as [11]:

$$G_1(t) = \frac{1}{N_{eff}}\left(1+\frac{t}{\tau_{diff}}\right)^{-1}\left(1+\frac{t}{s^2\tau_{diff}}\right)^{-1/2} \quad (2)$$

where τ_{diff} is the characteristic diffusion time of the molecule, $s = \frac{z_o}{r_0}$ is the ratio between the axial (z_o) and lateral (r_o) radius of the ellipsoid detection volume $V_{eff} = \pi^{3/2} \times s \times r_0^2$ and N_{eff} is the average number of fluorescent molecules in the detection volume. From the parameters determined by fitting FCS curves, the translational diffusion coefficient can be determined according to the formula:

$$D = \frac{r_0^2}{4\tau_{diff}} \quad (3)$$

The geometric parameters of the fit, s and r_o, were determined using Rhodamine 110 (Rh110) as a standard sample with a known diffusion constant ($D = 2.7\times10^{-10}$ m^2/s [12]). It was found that $s = 5.77$ and $r_o = 231.07$ nm.

It is often important to study the interaction between two fluorescent species with different sizes, for example, the binding of a small fluorescent peptide to a protein. To this end, measured FCS were fitted to the following model [11]:

$$G_{II}(t) = \frac{1}{\left(\sum_i N_i\right)^2}\sum_i N_i^2 G_i(t) \quad (4)$$

where the index i runs over 1 and 2, representing the two species with respective autocorrelation functions $G_i(t)$ given by eq.(2), and N_i is the number of molecules of each species.

Titration measurements of unlabelled STAT3 and the fluorescently labeled inhibitor allowed for the peptide-protein interaction to be studied. All experiments were carried out after 30 minutes of incubating varying concentrations of STAT3 with 12 nM of F*-peptide in Tris buffer. The sample was excited at 480 nm with pulsed, linearly polarized light. The emitted fluorescence was separated into components with polarization parallel and perpendicular to the excitation light, and detected in two channels.

Fluorescence Anisotropy (FA)

As molecules diffuse in three dimensions, they also undergo rotational movements. If such molecules are fluorophores excited with linearly polarized light, their emission becomes depolarized on a time scale determined by their rotational correlation time [13]. A measure of this depolarization is the fluorescence anisotropy, r, which

is defined as [13]:

$$r = \frac{I_{\parallel} - g \times I_{\perp}}{I_{\parallel} + 2g \times I_{\perp}} \quad (5)$$

Where $I_{\parallel}$ and $I_{\perp}$ are the detected fluorescent intensities with polarizations parallel and perpendicular to the polarization of the excitation light, respectively, and g is a factor correcting for the difference in sensitivities between the two channels, and is a function of the set-up [14]. The *g* factor was determined prior to each measurement by measuring the anisotropy of 10 nM Rh110 in water and assuming $r = 0$ since the dye is small and rotates very quickly.

Epi-fluorescence Imaging

Fluorescence imaging was carried out in a custom built, inverted wide-field microscope [15]. A 473 nm diode-pumped, solid-state laser (Cobolt Blues 25mW, MarketTech, USA) excited the sample at ~250 W/cm^2 by passing through a 1.45 NA/60x oil objective (Olympus, USA). Emitted fluorescence was captured using the same objective and laser scattering was eliminated with a dichroic mirror (FF495-Di02, Semrock, USA), followed by extensive filtering using a combination of long-pass (LP-488-RS, Semrock, USA) and band-pass (HQ520/66, Chroma, USA) filters. Images were captured with an electron-multiplied, charge-coupled device (EMCCD) camera (DU-897BV, Andor, USA) with typical exposure times on the order of 100 ms. To avoid excessive photobleaching of the sample, the camera was synchronized with a shutter in the excitation path (LS6, Uniblitz, USA).

Sample Preparation

Microscope coverglass was cleaned by a plasma cleaner (PDC-32G, Harrick Plasma, USA) for 30 minutes. The surfaces were then coated with a mixture of polyethylene glycol (PEG) and PEG-biotin as described in a protocol published previously [8]. Such coating serves two purposes: first, PEG-biotin provides a stable anchor for the immobilization of large phospholipid vesicles. Second, the hydrophilic PEG surface prevents nonspecific binding of diffusing molecules in solution.

STAT3 (S54-54G, SignalChem, Canada) was labelled with a thiol-reactive maleimide form of tetramethylrhodamide (TMR-maleimide). STAT3 was dissolved to 2 µM in a buffer of 50 mM Tris-HCl, 150 mM NaCl at pH7.5. To reduce disulfide bonds, *tris*(2-carboxyethyl)phosphine (TCEP) was added at 20x molar excess. The solution was then desiccated and fluxed with argon for 30 minutes to remove oxygen. TMR-maleimide was added at 20x molar excess, preceding gentle stirring by a vortex for two hours and kept at 4oC overnight. The excess dye was removed by centrifugation at 1300g for 4-6 minutes using 40,000 kDa cutoff columns (732-6223, Bio-Rad Laboratories, USA). To assess the distribution of the number of dyes attached to each STAT3, 20 µL of 1 nM solution of STAT3-TMR in 2% w/v poly-methyl methacrylate (PMMA) in toluene was spin-coated on the surface of a plasma cleaned microscope coverslip. This allowed for immobilized molecules to be illuminated individually.

Large unilaminar vesicles (LUVs) were prepared using an extrusion method modified from a previously published protocol [16]. As per the protocol, egg phosphatidylcholine (PC) was dissolved in chloroform along with 1% biotinylated 1,2-Bis(dimethylphosphino) ethane (DMPE). Subsequently, the solvent was evaporated under nitrogen gas, and the resulting lipid film hydrated with a buffer of 100 mM Tris, 150 mM NaCl at a pH of 7.4 to create multilamellar vesicles (MLVs). The freeze-thaw cycles using liquid nitrogen of the protocol were avoided as they were found to denature STAT3 and other proteins (unpublished data). Molecules to be encapsulated (STAT3 or free dye) were added at a concentration of 3 µM during hydration. MLVs were extruded 11 times through a 1 µm pore-size polycarbonate membrane filter using a Mini-Extruder apparatus (610000, Avanti Polar Lipids, USA) to produce LUVs.

Results

Protein Labelling

For accurate fluorescent measurements, a dye must be considerably photostable, as well as functional at physiological pH levels. To address these requirements, TMR was selected as the STAT3-label. To couple the dye to STAT3, a maleimide form was chosen because STAT3 contains instrisic thiol groups in its cysteines. TMR labelling efficiency was assessed both qualitatively and quantitatively through FCS experiments (Figure 3). By fitting the data to eq.(2), freely diffusing TMR-maleimide was found to have a characteristic diffusion time (τdiff) of 58 µs, corresponding to a diffusion coefficient (D) of 2.31×10-10 m^2/s, close to the reference value for Rh110 (see above). The dye bound to STAT3 is part of a much larger complex, and so it diffuses much more slowly. After the labelling protocol, the fluorescent complex diffused with a τdiff of 0.61 ms and a resulting D of 2.19×10-11 m^2/s. This data was fitted assuming only two species (TMR and STAT3-TMR) and using eq.(4). Only a minor fraction of free dye was found (15-20%), thus indicating that STAT3 was successfully labelled and purified.

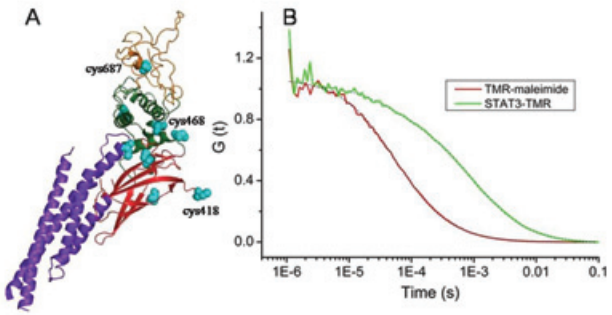


Figure 3: A) Dye-labelling of the STAT3 protein at Cys residues (cyan). Image generated in Pymol **B)** Normalized FCS curves of the free dye and of the dye-labelled STAT3. In both cases, the sample concentrations were 400 pM and pulsed 527 nm laser light was used for excitation. Curves were fit with eq.(2)

Structural analysis of STAT3 has shown that the protein has a total of nine cysteine residues [17], constituting nine possible binding locations for the dye. Individual proteins were immobilized on microscope coverslips via spin-coating and sequentially illuminated until all dyes were irreversibly photobleached (Figure 4A). The photostability of each molecule of TMR varies, and so the steps shown in Figure 4A correspond

to the photobleaching of each individual dye molecule. Thus the number of steps corresponds to the number of dyes bound to that particular protein.

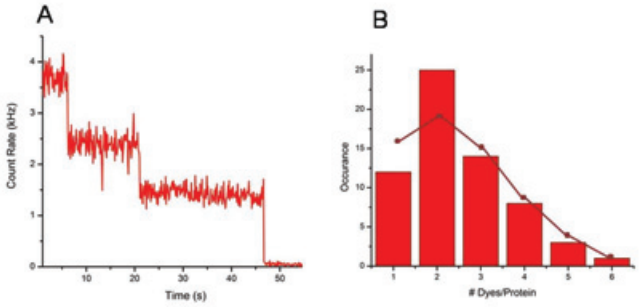


Figure 4: A) Intensity-time trajectory of a single dye-labelled STAT3 protein spin-coated in a thin PMMA film. **B)** Histogram of the number of dye labels per protein recorded for 63 proteins (bars) and fitted with a Poisson distribution (line; λ = 2.38).

The statistics performed on the single-molecule data (Figure 4B) revealed that on average, each STAT3 molecule had 2.4 fluorophores covalently attached, up to a maximum of six. This agrees with the expectations based on the crystallographic structure, indicating that three of the cysteines are buried inside the STAT3 structure and are therefore inaccessible to TMR-maleimide labelling.

Peptide-Protein Interaction

In order to assess the functional integrity of STAT3's key phospho-tyrosine binding SH2 domain, we conducted a series of binding experiments with known STAT3 phosphopeptide binding sequences. We reasoned that successful binding of the phosphorylated ligands would confirm that TMR labelling had not affected SH2 domain function. We selected the truncated gp130 [2, 18] peptide sequence, pYLPQTV, due to its potent binding affinity for the SH2 domain. To assess the binding potency, two independent fluorescent techniques, FCS and FA, were employed.

FCS titration can be a powerful tool for studying ligand-protein interaction dynamics provided the ligand is fluorescently labelled and has a much smaller size than the protein [10]. To this end, the peptide sequence was coupled with 5-carboxyfluorescein (F*) and unlabelled STAT3 was used. The concentration of peptide bound to protein increases with STAT3 concentration, shifting the FCS curve to longer diffusion times. The normalized FCS curves are shown in Figure 5.

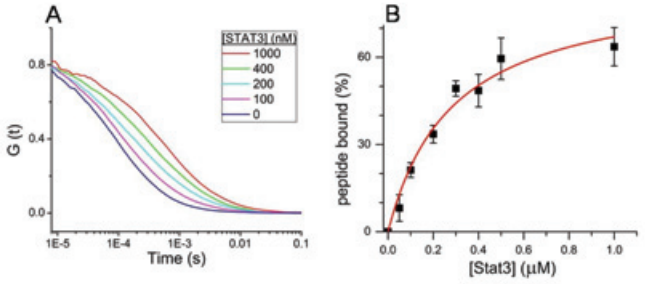


Figure 5: A) Normalized FCS curves measured for the titration of the binding reaction between F*-pYLPQTV and STAT3. The concentration of the peptide was 12 nM and the concentration of STAT3 is given in the legend. Data was fit with eq. (4) **B)** The fraction of peptide bound to STAT3 as a function of STAT3 concentration, fitted using eq.(6) with R^2=0.986

By modelling the FCS with a two-state system (free and bound peptide), the characteristic parameters of the peptide-STAT3 binding interaction can be found via the following equation:

$$\frac{[peptide-STAT3]}{[peptide]} = \frac{f[STAT3]}{k_d + [STAT3]} \quad (6)$$

Where [peptide] and [STAT3] are the total peptide and STAT3 concentrations, respectively, [peptide-STAT3] is the concentration of bound peptide, and k_d is the dissociation constant. Since hydrolysis of the phosphate group of the peptide can inactivate its binding capabilities, a fraction of active peptide (f) is assumed. From the fitting of the FCS curves with this equation, it was determined that k_d = 285±84 nM and f = 0.86±0.07.

A similar titration of binding can be made employing fluorescence anisotropy (FA), as bound peptide would exhibit higher anisotropy values due to slower rotation of the much larger host protein. Due to the multiparameter functionality of the set-up, the FA at each data point in Figure 5 was measured simultaneously as per eq.(5) (Figure 6). Making use of the additive property of anisotropy [12], the following equation can be employed to determine k_d:

$$r = f_f r_{min} + f_b r_{max} = \frac{k_d}{k_d + [Stat3]} r_{min} + \frac{[Stat3]}{k_d + [Stat3]} r_{max} \quad (7)$$

Where f_f and f_b are the fraction of bound and unbound peptide, respectively, rmin is the minimum measured anisotropy (from free peptide), and r_{max} is the maximum measured anisotropy (from bound peptide). Note that r_{min} and r_{max} were assumed to be constant. Curve fitting of anisotropy data with this model resulted in $k_{d\ fit}$ = 209±36 nM. Correcting for the fraction of active peptide measured by FCS (k_d=$k_{d\ fit}$ /f), this value becomes k_d = 243±46 nM.

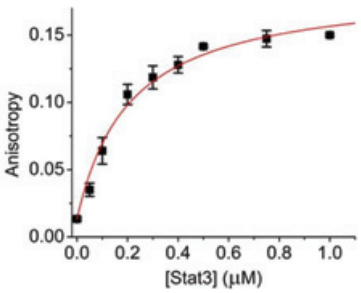


Figure 6: Fluorescence anisotropy (FA) of the titration of F*-peptide-STAT3 binding as a function of protein concentration. Data was fit with eq.(7) with R^2=0.978

Labelled STAT3 was found to exhibit similar binding affinity to gp130, as seen by the FCS analysis shown in Figure 7. Samples of 6 nM F*-peptide with 250 nM STAT3-TMR were incubated for 30 minutes prior to measurements.

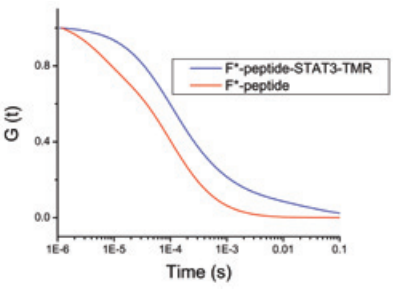


Figure 7: Comparison of the normalized FCS curves of 12 nM F*-peptide and 6 nM F*-peptide mixed with 250nM STAT3-TMR. Data was fit using eqs.(2) and (4), with R^2=0.999 And R^2=0.977 respectively. Only fits are shown.

The shift of the FCS curve to a longer diffusion time implies the binding of the peptide to STAT3-TMR. In addition, aggregation of protein-peptide complexes was apparent, and so although the binding is evident qualitatively, further experiments are currently being conducted in our lab to evaluate the dissociation constant of the reaction between F*-peptide and STAT3-TMR.

Liposome Model System

Having labelled STAT3 protein, we subsequently conducted both fluorescent imaging and spectroscopy measurements in a liposome model system. To simulate cells, large unilamellar vesicles (LUVs) were created as per the protocol mentioned in the Materials and Methods section (Figure 8A). To characterize the LUVs, free molecules of F-NAc [9], a dye with suitable photostability and pH invariance, were captured in vesicles. The size of the fluorophore filled vesicles could be determined by their diffusion time from the fluorescence autocorrelation decay. Using a 1 µm-pore size polycarbonate membranes for extrusion, the vesicles were found to have a diffusion time of 59.6 ms. Relative to a standard fluorescent polystyrene bead (F8784, Invitrogen, USA) having a diameter of 24 nm and an FCS measured diffusion time of 1.23 ms, the hydrodynamic diameter of the prepared liposomes was determined to be ~1.16 µm, in agreement with the expected size of 1 µm (due to the pore size). Labelled STAT3 was also encapsulated to show the application of this model to our assay. Figure 8 shows a wide-field fluorescence image of encapsulated STAT3-TMR in 1 µm LUVs.

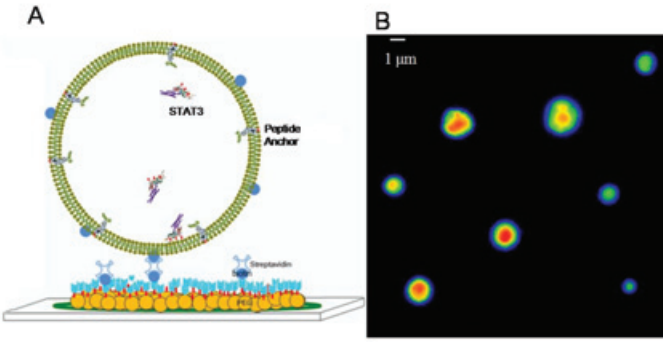


Figure 8. A. Liposome model system showing the proposed inhibition of STAT3 by peptide anchors B. Wide-field fluorescence image of STAT3-TMR encapsulated in 1 µm phospholipid unilamellar vesicles. Sample was prepared following the protocol described above and immobilized on a PEG-biotin surface mediated by streptavadin molecules.

As seen in Figure 8B, the distribution of protein within the vesicle is relatively uniform, as the fluorescence intensity (indicated by a colour scale) is homogeneous in the center of the liposomes. It is also important to note that there does not appear to be considerable amount of signal coming from the liposome wall, indicating a lack of significant interaction between STAT3 and the lipid bilayer.

Discussion

Structural observation of STAT3, as shown in Figure 3A, indicates the most likely candidates for label binding sites. Due to steric effects, it can be hypothesized that certain residues such as cys251, cys328, and cys550 are inaccessible to TMR-maleimide. This would account for the apparent maximum of six labels per protein as seen in Figure 4B. By this logic, the most likely cysteine residues that interacted with the maleimide are cys418, cys468 and cys687, since these are the most exposed to the solvent. It is important to note that the location of cys687 in the SH2 domain may potentially affect STAT3 functionality if the maleimide binds there. The FCS data in Figure 7 reveals that F*-peptide binds to STAT3-TMR at comparable strength to STAT3 with no dye. Since the truncated gp130 ligand is known to bind to STAT3's SH2 domain, the FCS data strongly suggests that the TMR-labelled STAT3 retains its SH2 domain binding functionality. This binding also implies that cys687 is an unlikely location for high efficiency dye-labelling due to steric effects as TMR has a similar size to the labelled peptide. Therefore, fluorescently labelled STAT3 can be used in a number of molecular inhibition strategies including the development of STAT3-SH2 domain binders.

The purity of the sample is dependent on the number of iterations of centrifugation in the mass chromatography columns. However, protein is lost in each cycle, and so a balance must be found between the purity of the solution and the protein content. With this in mind, the TMR content within the solution (15-20%) was deemed acceptable for the current study, although further improvements of the purification protocol will be done to ensure minimal loss of protein. This TMR content was considered acceptable due to its insignificant contribution to the FCS curves and FA as revealed by fitting.

Our approach to STAT3 inhibition focuses on inhibiting the cellular mobility of this molecule. The STAT3-TMR can be used to identify the precise location of STAT3 protein within liposome models. As part of our investigation we successfully encapsulated TMR labelled STAT3 without denaturing the protein (by avoiding freeze-thaw cycles). This modified protocol allows for protein-ligand interactions to be studied *in vitro* in both ensemble and single-molecule experiments. The liposome system, along with the development of a protocol for labelling STAT3, contributes to powerful fluorescence technique employment for studying the inhibition of STAT3 oncogenic activity.

Conclusion

Due to its significant contribution to the onset of cancers such as leukemia and lymphoma, the STAT3 signaling pathway is an ideal target for molecular therapeutics. The results reported here show that STAT3 protein can be readily labelled with a TMR flourophore and still retain its SH2 domain binding functionality. Moreover, TMR-labelled STAT3 can contribute to the design of new *in vitro* fluorescence-based inhibition assays to study the spacial distribution and the binding partners of this oncogenic protein.

Acknowledgements

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Research Article

The Effect of Tranilast Treatment on Breast Cancer Cell Growth, Migration and Invasion

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Abstract

Breast cancer, and particularly the resulting metastases, continues to pose a major health risk for women. Studies in both in vitro and in vivo models have shown that tranilast has the potential to be used in the treatment of breast cancer as an anti-metastatic agent. This study looks at tranilast treatment response in relation to the particular receptor (ER, PR and HER2) statuses of breast cancer. The objective of the study is to determine the effect of tranilast on the growth and migration of two human breast cancer cell lines BT 474 (receptor positive) and MDA MB 231 (receptor negative). Both cells lines showed a negative correlation between tranilast concentration and quantified growth inhibition at concentrations ranging from 50 µmol/L to 200 µmol/L. In the examination of migration, BT 474 and MDA MB 231 cells also exhibited slower migration rates after tranilast treatment (200 µmol/L). Additionally, the extent of colony growth in a soft agar assay was reduced after tranilast treatment in both cell lines. Immunocytochemistry of tranilast and vehicle treated cells appeared to show downregulation of two metastatic markers, endoglin and MMP-9, in BT 474 cells; however, this was not observed with MDA MB 231 cells. While tranilast treatment does exert inhibitory effects on both cell lines tested, BT 474 cells showed greater sensitivity with a larger reduction of growth and slower migration, as compared with MDA MB 231 cells. Tranilast may therefore be a potential therapeutic agent for breast cancer, and possibly functions through a receptor pathway.

Introduction

With the current rates of breast cancer occurrence and mortality, there is an urgent need for the development of novel drugs and therapies. Canadian cancer statistics show that 1 in 9 women are expected to develop breast cancer and 1 in 28 women are expected to die from the disease [1]. In particular, metastases to the bones, lungs and liver continue to pose major obstacles in breast cancer treatment [2, 3].

The small, orally administered compound tranilast (N-[3,4-dimethoxycinnamonyl]-anthranilic acid) has recently been investigated and shown to be an anti-tumour and anti-metastatic agent in various models of cancer, including breast cancer [3, 4]. This drug has the advantage of having a clinical history in Japan for the treatment of allergy and fibrotic disorders, and has few side effects [5]. Previous in vivo studies have shown that tranilast is effective in reducing the growth of the primary tumour, as well as significantly reducing metastases to the lungs and liver in a mouse model [3]. In vitro, a number of mechanisms have been elucidated regarding tranilast's function, including its role in inhibiting the TGF-β (transforming growth factor beta) pathway [6]. TGF-β is a cytokine that suppresses cancer in its early stages, but later promotes tumor and metastasis formation [6]. Additionally, tranilast has been shown to block cell cycle progression, as well as increase apoptosis of tumour cells [3].

The current study aims to investigate the effect of tranilast treatment with respect to the different subtypes of breast cancer, as characterized by estrogen receptor (ER), progesterone receptor (PR), and human epidermal factor receptor 2 (HER2) statuses. The overexpression of ER and PR are established prognostic markers, as well as indicators of response to hormonal therapy [7]. Breast cancers cells that are positive for these receptors are stimulated to divide and proliferate when the particular hormone binds, and are sensitive to drugs targeting these specific receptors. Amplification of the HER2 proto-oncogene also plays an important role in breast cancer development and can be targeted for therapy [7]. The presence or absence of these receptors categorizes cancers into various subtypes, such as basal (triple negative), luminal (ER+), and HER2+ [7]. Since tranilast functions through a number of different pathways, it was hypothesized that it would be effective against different subtypes of breast cancer. In this study, the subtypes were represented by two human breast cancer cell lines, BT 474 (ER, PR, and HER2 positive) and MDA MB 231 (ER, PR, and HER2 negative).

Methods and Materials

Cell Lines

BT 474: Human breast cancer cell line isolated from a solid invasive ductal breast carcinoma. ***MDA-MB-231***: Human breast cancer cell line from an adenocarcinoma. Both cell lines obtained from the ATCC.

MTT Assay

Cells were plated at a density of 2000 cells per well on a 96 well plate and treated with 50 µmol/L, 100 µmol/L or 200 µmol/L tranilast or vehicle (0.8% DMSO) respectively. 72 hours after treatment, MTT dye (from Promega) was added and incubated for two hours. The

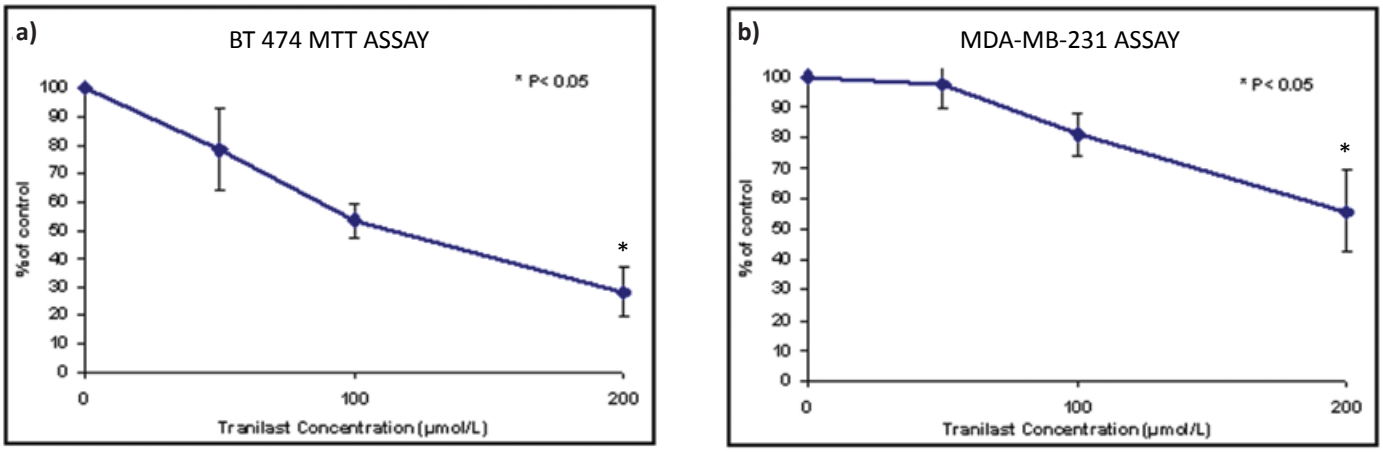


Figure 1: Tranilast treatment reduces cell growth in both BT 474 and MDA MB 231 breast cancer cell lines. By MTT assay as detailed in Materials and Methods. Tranilast treatment resulted in 70% growth inhibition in BT 474 **(a)** and 45% inhibition in MDA MB 231 **(b)**. The results of 5 independent experiments in BT 474 and 4 independent experiments in MDA MB 231 cells.

reaction was arrested using a stop solution and the color developed was read using a spectrophotometer at 540 nm. Experiment was conducted in nine replicates for each group.

Soft Agar Assay

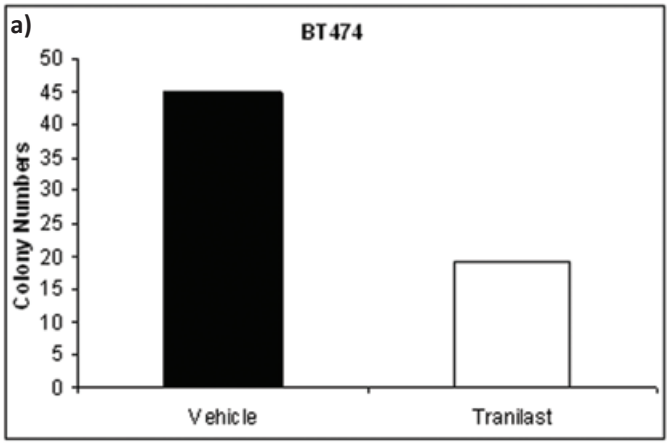
20 000 cells in 0.5% agar was layered on 0.8% agarose in a 35 mm Petri dish either with tranilast (200 µmol/L) or vehicle (0.8% DMSO) respectively. After plating, DMEM containing 10% FBS was added to each plate. Experiment was conducted in triplicates for each group.

Wound Assay

Cells were plated at 150 000 cells per well in a 6 well plate and allowed to grow to confluency. A wound was created with a pipette tip. Floaters were washed away with PBS before the addition of 200 µmol/L tranilast or 0.8% DMSO to the cell lines respectively. Experiment was conducted in triplicates for each group. Photographs were taken at 10x between days zero and day three and wound closure was quantified by Image J Software from NIH.

Immunocytochemistry

5000 cells per chamber were plated using an 8 chamber slide. The cells were treated with 200 µmol/L tranilast or 0.8% DMSO 24 hours after plating. The cells were fixed with -20° C methanol at the end of 24 and 48 hours respectively. Before immunocytochemistry, the cells were hydrated with PBS, permeabilized with 0.1% Triton X-100 and incubated with 5% goat serum for 1 hour. The cells were then



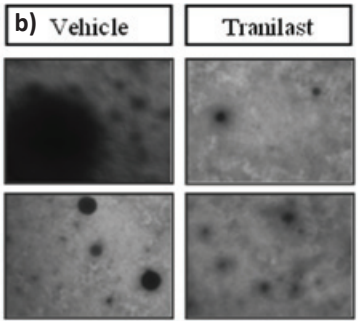
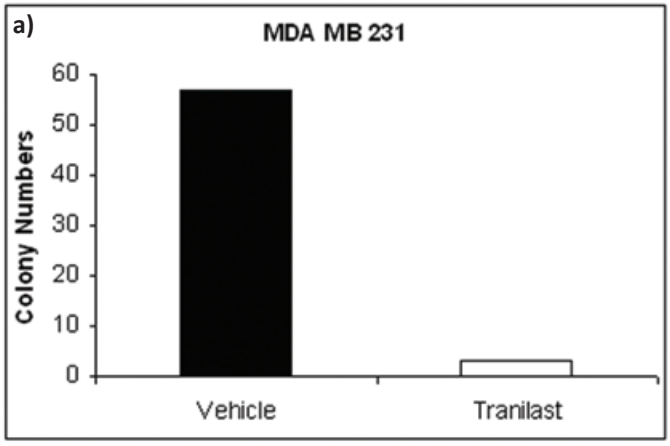


Figure 2: Tranilast treatment reduces colony numbers in BT 474 cells. In BT 474 cells (day 12), colony numbers **(a)**, as well as colony size **(b)**, were reduced in tranilast treated cells as compared to the vehicle control.



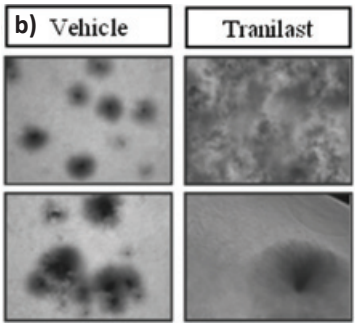


Figure 3: Tranilast treatment reduces colony in MDA MB 231 cells. In MDA MB 231 cells (day 10), tranilast treatment reduced colony numbers as compared to vehicle control **(a)**. Also, the tranilast treated colonies were smaller and not as well defined as the vehicle control colonies **(b)**.

incubated with the primary antibody overnight (rabbit α Endoglin or rabbit α MMP-9) or rabbit IgG as control. Goat α Rabbit FITC conjugated secondary antibody was added and the cells were incubated for 45 min. After washing, the cells were coverslipped using permafluor mounting medium.

Statistical Analysis

Treated and control groups were analyzed using two tailed t-tests with a $p < 0.05$ being considered significant.

Results

Tranilast Inhibits the Growth of Breast Cancer Cells

At concentration ranging from 50 µmol/L to 200 µmol/L, cell growth was inhibited in both cells lines when compared to control, as shown in Fig. 1A for BT 474 and Fig. 1B for MDA MB 231. At 200 µmol/L, significant decreases in cell growth (70% for BT 474 and 45% for MDA MB 231) were observed as compared to the 0.8% DMSO control.

In assessing cell growth in soft colony agar assays, both

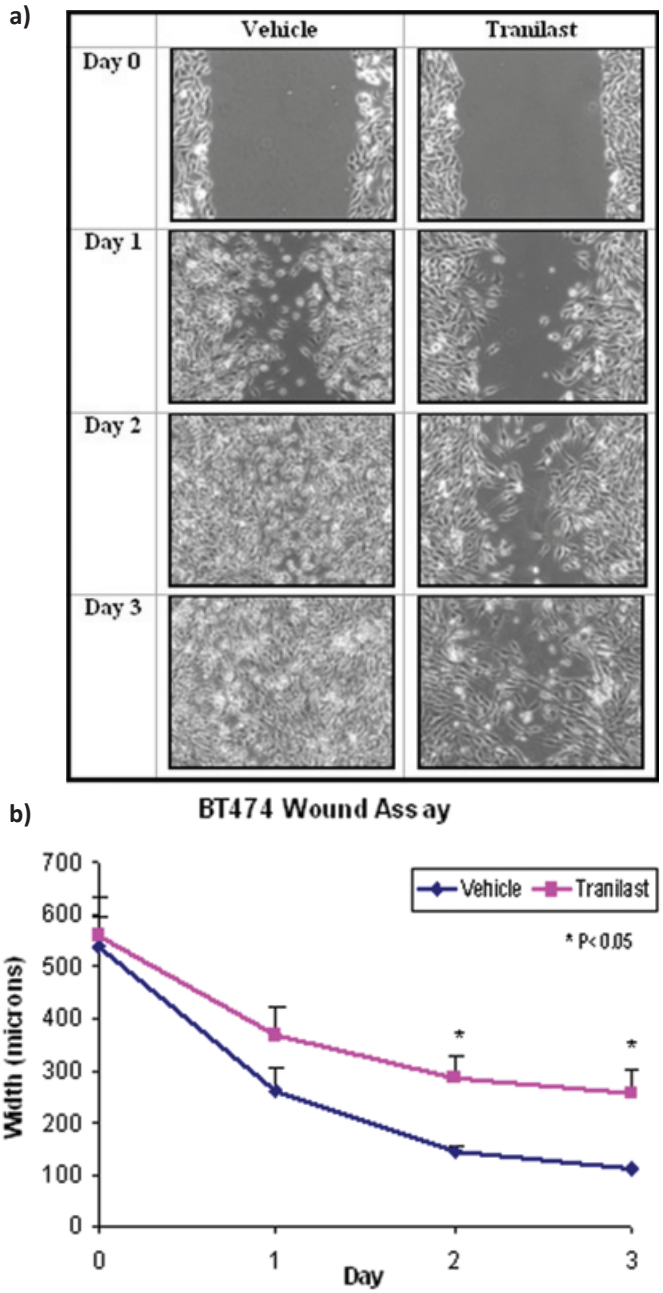


Figure 4: Tranilast treatment reduces cell migration in BT 474 breast cancer cells. a) Results of BT 474 cell migration in vehicle treated group (left panels) and tranilast treated group (right panels). **b)** The results from panel A were quantified by Image J Software. Tranilast treatment showed a significant decrease in cell migration between day 2 and 3 when compared to the vehicle control. Results obtained from three independent experiments in triplicates.

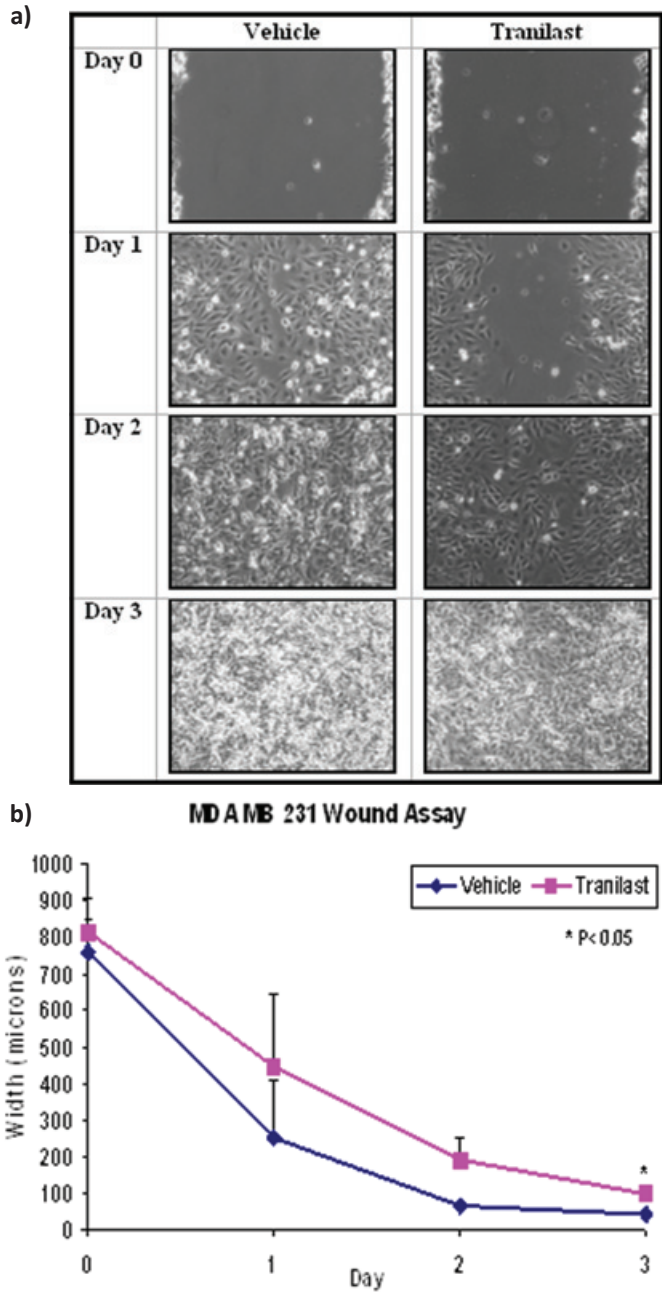


Figure 5: Tranilast treatment reduces cell migration in MDA MB 231 breast cancer cells. a) Results of MDA MB 231 cell migration in vehicle treated group (left panels) and tranilast treated group (right panels). **b)** The results from panel A were quantified by Image J Software. Tranilast treatment showed a significant decrease in cell migration when compared to the vehicle control on day 3. Results obtained from two independent experiments done in triplicate.

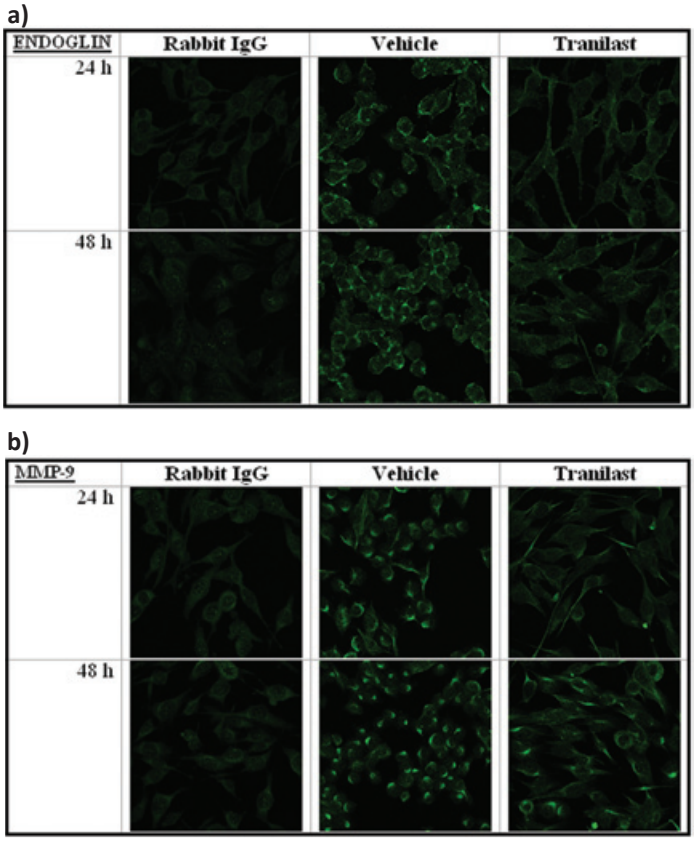


Figure 6: Tranilast treatment reduces the levels of endoglin and MMP-9 in BT 474 cells. Immunocytochemistry was performed as detailed in Materials and Methods. **A)** Tranilast treatment appeared to reduce the expression of endoglin at 24 and 48 hours (top right panel) **B)** MMP-9 levels also appeared reduced by tranilast treatment at 24 h and less at 48 h (bottom right panels). Pictures taken at 80x.

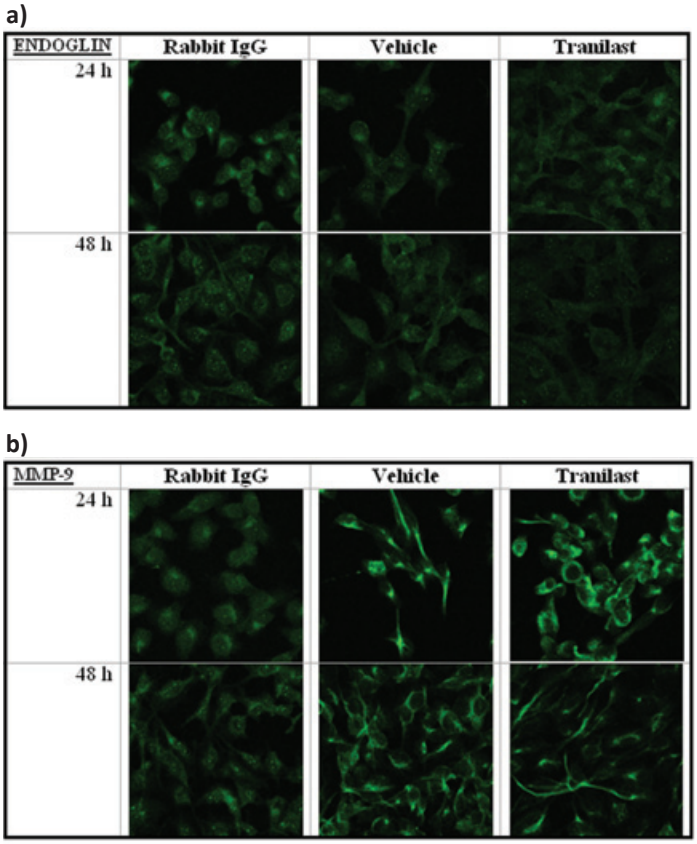


Figure 7: The effect of tranilast treatment on the levels of endoglin and MMP-9 in MDA MB 231 cells. A) Endoglin appeared little expressed in MDA MB 231 cells (same as control). **B)** MMP-9 levels appeared slightly increased at 24 hours with tranilast treatment and slightly reduced at 48 h. Pictures taken at 80x.

cell lines showed a decrease in colony numbers with tranilast treatment, Fig. 2A for BT 474 and Fig. 3A for MDA MB 231. Additionally, tranilast treatment reduced the size of the colonies, as well as the definition of the boundaries in BT 474 (Fig. 2B) and MDA MB 231 (Fig. 3B).

Tranilast Inhibits Breast Cancer Cell Migration

In scratch wound assays, BT 474 showed significantly slowed migration at day 2 and 3 with 200 µmol/L tranilast treatment as compared to vehicle control (Fig. 4A,B). Although MDA MB 231 showed significantly slowed wound closure between the tranilast treated and vehicle treated groups by day 3, migration was more aggressive than in BT 474 cells (Fig. 5A,B).

Tranilast Downregulates Endoglin and MMP-9

Immunocytochemistry of BT 474 breast cancer cells appeared to show downregulation of endoglin at 24 and 48 hours; MMP-9 also appeared reduced at 24 hours and to a lesser extent at 48 hours (Fig. 6A,B). In MDA MB 231 cells, endoglin expression appeared limited and MMP-9 levels seemed to show a slight increase at 24 hours and a minor reduction in expression at 48 hours with tranilast treatment (Fig 7A,B).

Discussion

It was found that tranilast inhibits cell growth of both BT 474 and MDA MB 231 human breast cancer cells at concentrations as low as 50 µmol/L and significantly at 200 µmol/L when compared to vehicle control. This is consistent with previous results that showed the inhibition of growth with tranilast treatment in a number of different breast cancer cell lines [3]. Additionally, the effect of tranilast treatment on BT 474 cells was greater than on MDA MB 231 cells, with a larger inhibitory effect. This difference was most notable at a tranilast concentration of 200 µmol/L, where inhibition was 70% in the BT 474 cell line compared to the 45% inhibition in the MDA MB 231 cell line.

The effect of tranilast treatment in reducing breast cancer cell growth is supported by the data from the soft agar assay, showing a reduction in colony formation for both cell lines. The tranilast treated cells in both cell lines also showed dispersed colonies indicating cell death, as opposed to the defined boundaries and larger size of vehicle treated colonies.

Similar to data pertinent to other breast cancer cell lines, tranilast treatment slowed wound closure and hence, migration rates, in both BT 474 and MDA MB 231 cells extending to three days after treatment. MDA MB 231 cells generally had

a faster rate of migration, and the effect of tranilast treatment was less pronounced compared to BT 474 cells. It is notable; however, that a significant reduction in migration was still observed in MDA MB 231 cells by day 3, indicating tranilast treatment does inhibit the migration of triple negative cells. This same trend was observed with the MTT assay, and is also a reflection of the more aggressive nature of triple negative breast cancer cell growth.

Immunocytochemistry was performed to look for the expression of two cancer markers, endoglin and MMP-9. Endoglin is a glycoprotein that is a co-receptor for TGF-β and a potential metastatic marker, while MMP-9 is an invasion marker due to its role in the degradation of the extracellular matrix [8, 9]. Immunocytochemistry confirmed the effect of tranilast treatment on BT 474, with an apparent reduction in the expression of endoglin and MMP-9 at both 24 and 48 hours after treatment. Previous Western blot data (with the mouse breast cancer line 4T1) supports the down regulation of these markers as part of tranilast's mechanism of function [Unpublished data]. In MDA MB 231 cells, there appeared to be little expression of endoglin with and without tranilast treatment. Additionally, the levels of MMP-9 appeared to increase slightly with tranilast treatment at 24 hours, yet decrease a small amount by 48 hours, which can be explained by uneven cell numbers in the field of focus or potential temporal expression. This suggests again that this cell line is less affected by tranilast treatment as compared to BT 474 cells, but also highlights the need for more quantitative experiments to further investigate this result.

Conclusion

Taken together, these assays show the anti-cancer effect of tranilast treatment in the observed reduction of cell growth and migration in both BT 474 and MDA MB 231 cells. The inhibition of both cell lines suggests that tranilast is a multi-action drug, and functions through pathways affecting different subtypes of breast cancer.

The receptor status in these two cells lines is important in determining the response to tranilast treatment. BT 474 cells, expressing ER, PR and HER2, showed a greater reduction in cell growth, migration and expression of endoglin and MMP-9, as compared to MDA MB 231 cells, which do not express ER, PR, or HER2. The involvement of receptor status is expected given that tranilast is known to function through the inhibition of the TGF-β pathway, which also involves these same receptors. However, this pathway may not be the only one involved, and some of tranilast's modes of action remain unclear. Overall, tranilast continues to show promise as a novel breast cancer therapy for different subtypes of breast cancer, although further elucidation of receptor involvement is necessary to decide its potential role.

Acknowledgments

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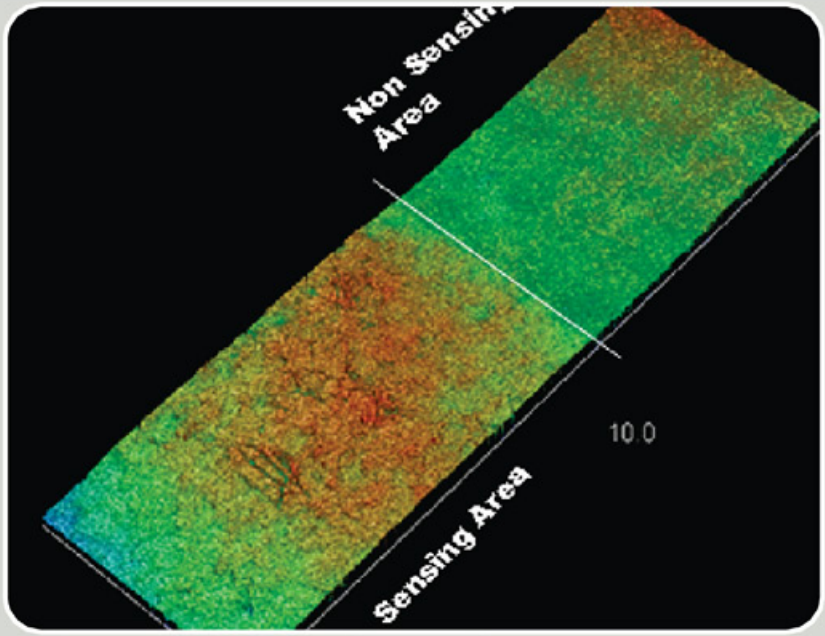
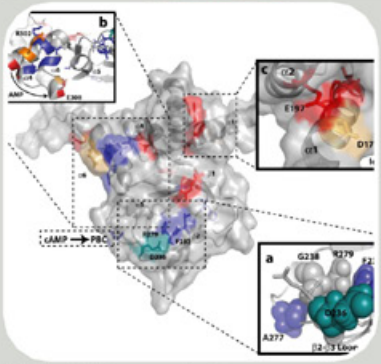
BIOMOLECULES



Bio-Active Paper for Cheap, Colorimetric Detection of Pathogens

The group of John Brennan, in the Department of Chemistry and Chemical Biology, recently invented a process to produce novel paper-based solid-phase biosensors that utilize piezoelectric inkjet printing of biocompatible, enzyme-doped, sol-gel-based inks to create colorimetric sensor strips. Acetylcholinesterase inhibitors, including paraoxon and aflatoxin B1, were detected successfully using this sensor, with toxin concentrations determinable by eye, without any expensive equipment.



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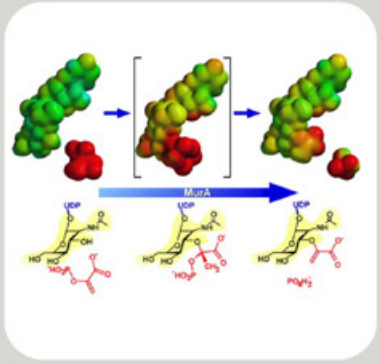

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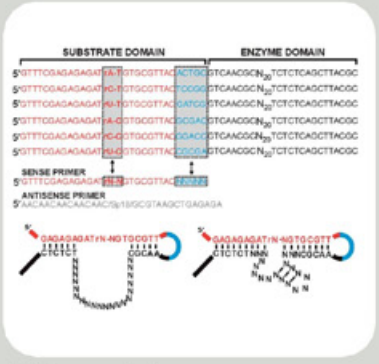

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Research Article

The Detection of an Ovarian Cancer Protein Using Thickness Shear Mode (TSM) Acoustic Wave Sensor

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Abstract

Heat-shock protein 10 (HSP-10) is a protein found in ovarian cancer patients that functions as an immunosuppressant. Currently, there is a need for a cost-effective and highly sensitive technique for the detection of HSP-10. The thickness shear mode (TSM) acoustic wave biosensor may present a suitable method. The TSM is widely used for the detection of protein-surface interactions on a piezoelectric gold coated quartz crystal. A change to the surface of the crystal is primarily detected through changes in the resonant frequency "fs" of the generated shear wave measured in Hz. To form a viable ovarian cancer detection system, the biosensing surface must be tailored to specifically bind the HSP-10 with a high degree of sensitivity and selectively. This specificity is influenced by the type of bioreceptor and bifunctional organic linkers used to tether the bioreceptor to the sensor surface. This paper compares and presents suitable HSP-10 biosensing surfaces through the construction of mixed self-assembled monolayers (SAMs) – consisting of 100, 75, 50, and 25% linker surface coverage, alongside two carboxyl activation schemes (EDC/NHS and sole EDC) – onto which a single stranded DNA aptamer is immobilized. The aptamer employed specifically binds to HSP-10 and reduces non-specific adsorption. From an avidin-biotin model system, an understanding of the TSM response was characterized and the 75% EDC/NHS and all EDC surfaces were proven most viable for protein detection. The 75% EDC/NHS surface proved optimal for HSP-10 protein detection when solvated in water rather than in buffer. This paper introduces the TSM as a valuable screening method for ovarian cancer

Introduction

Ovarian cancer is the leading cause of death from all gynaecologic cancers, largely due to a lack of early and reliable screening tools. Approximately 1700 Canadian women die of the disease each year [1]. Current screening methods employed are only capable, and that too inaccurately, of detecting ovarian cancer in late stages when the tumour is fairly advanced and the five year survival rate is as low as 20 % [1]. Conversely, if ovarian cancer is found early and treated the survival rate climbs to a substantial 90% [1]. With this in mind, we aim to introduce acoustic wave biosensor technology as a new tool for ovarian cancer detection that could overcome the many drawbacks of current screening techniques.

Current screening methods utilized for ovarian cancer detection are fairly time consuming, expensive, inadequate for early diagnostics, and yield significant amount of false positives and negatives. The method most frequently used is the detection of the müllerian-derived glycoprotein antigen designated CA-125, which has failed to reflect the size of the tumour and has low predictive values, especially in early screening [2]. Furthermore, in addition to ovarian cancer, CA-125 appears at elevated levels in a variety of physiological states, benign disorders, and other cancers [3]. In turn, diagnosis based on CA-125 levels is generally cautioned due to the nonspecific and inaccurate behaviour of the antigen.

Recent ovarian cancer proteome studies have led to the discovery of elevated levels of Heat shock protein 10 (HSP-10) in the circulatory system of ovarian cancer patients. Aside from primarily functioning as a co-chaperone in protein folding, it is believed that HSP-10 functions as an immunosuppressant in ovarian cancer patients and thus allows the progression of the cancer [4-5]. Unlike CA-125, HSP-10 concentration levels are more specific to ovarian cancer and thus ensure an accurate screening. Therefore, establishing a detection method for the new promising tumour maker HSP-10 is more likely to ensure success in diagnostics of ovarian cancer.

Biosensors are capable of detecting and measuring the levels of a specific protein, such as HSP-10, in a biological or environmental sample [6]. The basic components of a biosensor are a sampling area, a transducer, and a signal processing unit. When a sample is bound onto a surface changes in the properties of the sensing device are used to detect or quantify an analyte, such as an oligonucleotide or protein, within the sample. Biosensors are well sought for the purposes of protein detection and measurements since they offer many operational advantages: rapidity, ease-of-use, low cost, simplicity, portability, and ease of manufacture. Herein we aim to introduce a specific biosensor, the thickness shear mode (TSM) acoustic wave biosensor, as a means of detecting HSP-10 in ovarian cancer patients.

The TSM device offers a sensitive, label-free, and real-time detection system of biochemical interactions at the sensor surface. The TSM functions through the propagation of bulk acoustic waves through a piezoelectric material such as quartz. The quartz is mechanically strained via voltage application and thereafter oscillates at a fundamental frequency. Biochemical

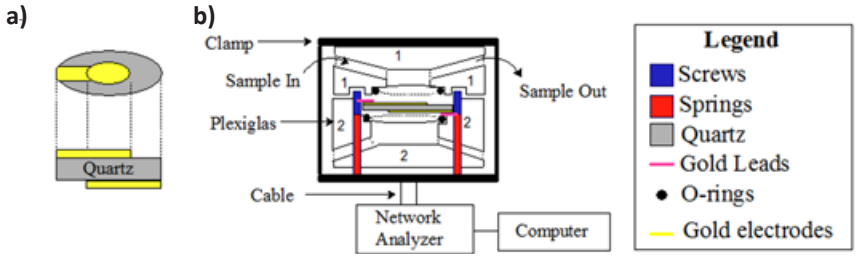


Figure 1: Schematic of crystal and cell holder. a) Piezoelectric quartz crystal with gold electrodes. Only a single side of the crystal is shown. **b)** Cross-section of a flow through cell holder, consisting of two Plexiglas blocks (1 and 2), clamped together.

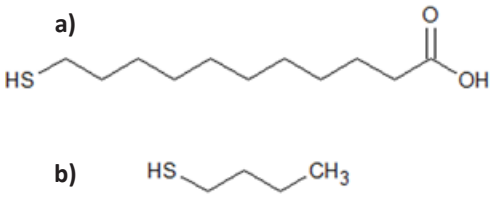


Figure 2: Linker and Spacer Molecules. Structures of the a) linker 11-MUA and **b)** spacer 1-butanethiol.

interactions at the sensor surface such as the binding of the analyte, any non-specific adsorption, changes in viscosity and density of the liquid, and a multitude of other phenomena are reflected through the changes in the resonant frequency "f_s" of the generated shear wave measured in Hz, and through changes in the motional resistance "R_m" measured in Ohms of the quartz crystal [7-10].

Biosensors need to avoid non-specific adsorption (adsorption of interfering species) onto the sensor since this would distort the results and inaccurately depict the levels of analyte present. This is accomplished through the use of biological recognition elements (or bioreceptors) that are placed onto the biosensor surface such that the analyte of interest, in this case HSP-10, will specifically bind within a matrix and the adsorption of interfering species is ideally minimized. Some examples of these elements are enzymes, antibodies, and the new promising group aptamers. Unlike antibodies, aptamers are oligonucleotides synthesized by chemical means and can bind with high affinity to their respective target molecules [6, 11].

Bioreceptors are covalently bound to the transducer surface through the aid of self assembled monolayers (SAMs). These monolayers are highly ordered molecular assemblies of bifunctional organic linkers. The head group functionality has specific affinity for the sensor surface (such as a thiol group chemisorbs onto a gold substrate), and the terminal functional group provides a means by which the bioreceptor can be bound. These SAMs can be varied in terms of linker density (through the incorporation of a spacer), and through variations in the activation schemes of the terminal functional groups [12]. Through experimentation, the SAMs most suitable for the detection of HSP-10 in terms of sensitivity and selectivity can be found.

The goals of this paper are to present and compare a variety of SAMs which could be applied to the TSM biosensor in order to monitor the interactions between HSP-10 and its aptamer. This is accomplished through (1) use of four ratios of linker to spacer alongside two terminal group activation schemes to form a total of eight SAMs, (2) using an avidin-biotin model system to verify the transferability of the SAMs to the HSP-10 to aptamer system, and to begin optimizing the sensitivity, selectivity, and reproducibility of the SAMs, and through (3) monitoring the TSM response as HSP-10 interacts in real-time with its aptamer using the SAMs, while comparing the effects of two HSP-10 solvents: water and buffer.

Materials and Methods

Reagents and Materials

The quartz crystals with gold electrodes were purchased form Lap-Tech incorporation® (model #: XT2905). Dulbecco's phosphate buffer saline (PBS), 1-ethyl-3-(3 dimethylaminopropyl)carbodiimide (EDC), N-hydroxysuccinimide (NHS), 11-mercaptoundecanoic acid (11-MUA), 1-butanethiol, biotin, avidin, and the various solvents used were purchased from Sigma-Aldrich®. Biotinthiol was synthesized from biotin in five steps with an overall yield of 34% [13-15]. The 2-(N-morpholino)ethanesulfonic acid (MES) buffer utilized was 10 mM in deionized water and brought to a pH of 5.98 using NaOH.

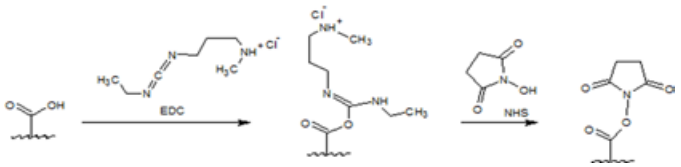
The 10 kDa, 102 amino acid, Heat shock protein 10 (HSP-10) was purchased from Abnova Antibody Innovation® [16]. The SELEX synthesised HSP-10 aptamer was purchased from Integrated DNA Technologies® of sequence: 5'-5ThioMC6-D/TTT TTG TCT TGA CTA GTT ACG CCA ACT TGT GCG GGG TGG TGG GGA TGG ATG TTG CTT GAG GGG TCT CAT TCA GTT GGC GCC CTC-3'

Equipment - TSM Biosensor

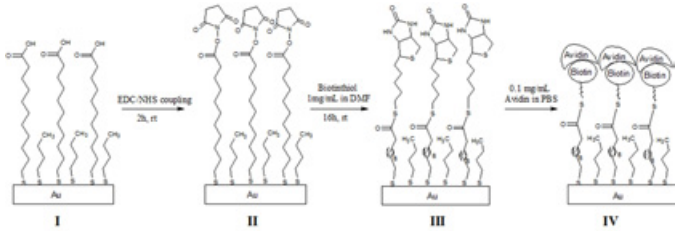
AT-cut 9.0 MHz piezoelectric quartz crystals with gold electrodes, referred to as slides and shown in Figure 1(A), were incorporated into a flow through system. One side of the slide was exposed to liquid, while the other to air. The liquid was introduced using a syringe pump. Experiments were performed with the slide in the horizontal position and at ambient temperature. The slide was secured in the holder using two O-rings. The gold electrodes were kept in contact with the gold leads in the holder as shown in Figure 1 (B). Continuous monitoring of surface interactions was achieved by a network analyzer (HP4195A Network/Spectrum Analyzer, Hewlett Packard, Colorado Springs, USA). The network analyzer measures the impedance changes on the gold electrodes in real-time. Prior to each run, the analyzer was internally calibrated using the circuit elements: open, short, and load. Changes in resonant frequency (f_s) and motional resistance (R_m) were documented during the course of protein and PBS/H_2O introductions.

Procedure

Preparation of SAMs. Sets of nine gold coated quartz slides were cleaned with sodium dodecyl sulphate (SDS), acetone, ethanol, methanol, deionized water, and nitrogen gas respectively. One slide



Scheme 1: Carboxyl activation scheme. Using 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC)/N-hydroxysuccinimide (NHS) coupling.



Scheme 2: Monolayer with 50% 11-MUA coverage. Formation of the mixed SAMs onto the gold electrodes of the TSM slides proceeds through the chemical adsorption of the linker, 11-MUA, and spacer, 1-butanethiol ontto the cleans slides to form **I**, this is followed by an esterification reaction to form **II** via EDC/NHS chemistry, next the slides are biotinylated to form **III**. The on-line introduction of avidin onto the SAMs with the biotin bioreceptors ultimately form **IV**, representing one molecule of avidin per biotin molecule.

within each set was set aside as a control (bare gold).

A 10 mM solution of 11-mercaptoundecanoic acid (11-MUA) in ethanol (to be used as the linker) and a 10 mM solution of 1-butanethiol in ethanol (to be used as the spacer) were prepared. The linker and spacer molecules are shown in Figure 2. Four samples of 25%, 50%, 75%, and 100% 11-MUA were prepared using the 1-butanethiol solution as the diluent. The eight clean slides were divided into two subsets of four; each slide within a set was immersed in approximately 1.5 mL of one of the four different 11-MUA samples in separate test tubes, capped, and left in solution for 24 hours to form the different SAMs. The slides were then rinsed three times with methanol, dried with nitrogen gas, and stored in vials for subsequent carboxyl activation.

The acquired two subsets were then each exposed to a different esterification scheme to activate the carboxyl terminal group on the linker molecules. The first scheme employed is the well known 1-ethyl-3-(3 dimethylaminopropyl)carbodiimide (EDC)/N-hydroxysuccinimide (NHS) coupling reaction (Scheme 1) [17]. The second scheme employed was the sole use of EDC. The activating EDC (60mM) and NHS (50nM) solutions were prepared in MES buffer. The slides were immersed in approximately 1.5 mL of their respective solution in separate test tubes, capped, and left in solution for two hours. The slides were then rinsed three times with deionized water, dried with nitrogen gas, and stored in vials for either subsequent biotinylation or HSP-10 aptamer attachment.

With biotinylation, the slides were then immersed in approximately 1mL of biotinthiol (1 mg/mL in dimethylformamide) in separate test tubes, capped inside a glove box, and left in solution for 16 hours; after which they were rinsed three times with methanol, dried with nitrogen gas, and stored in vials for TSM measurements.

A representation of the complete binding pattern for avidin-biotin is shown in Scheme 2. For HSP-10 aptamer attachment, the slides were immersed in 1mL of a 309 nM solution of the HSP-10 aptamer in separate test tubes, capped, and left in solution for 16 hours; after which they were rinsed three times with deionised water, dried with nitrogen gas, and stored in vials for TSM measurements.

TSM measurement procedure. Prior to the introduction of an analyte, the instrument is left to establish a steady baseline through a pre-wash. Subsequent to protein binding, a post-wash step is used to remove any proteins that are non-specifically or loosely bound to the bioreceptor. For avidin-biotin experiments, all biotinylated slides plus the control, were flushed through with PBS at a rate of 60 µL/min until a stable baseline was achieved (20-30 minutes), avidin (0.1 mg/mL in PBS) at a rate of 30 µL/min for 15-20 minutes, and finally PBS once again at a rate of 60 µL/min to examine the reversibility of protein binding.

Similarly, for HSP-10 to aptamer experiments, all aptamer attached slides plus two types of controls (bare slide and sole aptamer) were each flushed through with PBS, followed by HSP-10 (92 nM in PBS), and finally PBS once again, all at a rate of 30µL/min. The sole aptamer control slide was prepared by immersing a clean slide directly into the aptamer solution. In addition to this, a second series of measurements were performed in water rather than PBS. This was done to determine the solvent that best facilitates protein binding and minimizes interference from multilayer formation which would complicate the analysis in such preliminary studies. Changes to the resonant frequency and motional resistance were noted for all cases

Statistical Analysis

Outliers were rejected using the Q-test at the 95% confidence limit. Microsoft excel was used to calculate the mean and standard deviation. The F-test was employed to determine any statistical difference in variance. The independent two sample t-test was performed to determine any statistical significance between the eight different activated SAMs. The slopes were noted from the least-squares plots.

Results

Response of the TSM Acoustic Device to Avidin

The avidin-bitoin model system was used to begin assessing the selectivity, sensitivity, and reproducibility of the TSM and surface chemistries employed. This model system was chosen since avidin is widely known to form a very strong bond with biotin and both reagents are readily available. The changes in the resonant frequency (f_s) and motional resistance (R_m) upon avidin introduction and PBS re-introduction generally followed the same trends across all SAMs and controls, i.e., decrease in f_s with a minimal increase in R_m upon avidin introduction and an increase in f_s with a minimal decrease in R_m upon PBS re-introduction. The ideal representative TSM response is shown in Figure 3. However, some of experiments showed periods of drift or a decrease in frequency after PBS re-introduction (see sample graphs in supplementary material for control, 100% and 25% NHS/EDC, and 100% and 25% EDC).

Minimal changes in motional resistance are observed for all instances (0-3Ω). The change in frequency upon avidin introduction, Δf_s, is shown comparatively in Figure 4 for all

possible SAMs and the control. The amount of wash-off is subtracted from the Δf_s in order to achieve the corrected frequency shift $\Delta f'_s$, shown comparatively in Figure 5 for all SAMs plus the control. The error bars are the calculated standard deviations. From a series of F-tests, the 75% 11-MUA EDC/NHS activated surface's variance is statistically significant from all other surfaces with the exception of the 100% and 25% EDC surfaces. No other surfaces show statistically significant difference in their variances. From the series of independent two sample t-tests, only the 100% and 25% EDC surfaces are statistically significant from one another. However, the t-test only accounts for the mean and variance of the shifts, whereas for our purposes the pattern of the TSM response curve is a better test for significance as will be explained in the discussion.

TSM Response to HSP-10

Once an understanding of the TSM response was gathered from the avidin-biotin model system, the response from HSP-10 binding was examined. The TSM response for the majority of measurements performed in constant buffer media is shown in Figure 6 where the frequency continuously drifts downwards upon HSP-10 introduction (with the exception of SAMs NHS/EDC 100% and 50% 11-MUA which reached a stable baseline upon HSP-10 introduction). The measurements in constant water media yielded the pattern of Figure 6 for surfaces NHS/EDC 100%, 50% 11-MUA, and the control (bare gold).

The preliminary washes with buffer or water result in the acquisition of stable frequency and resistance baselines; however the buffer has shown a higher tendency to drift. The subsequent introduction of HSP-10 results in a linear decline in the resonance frequency Δf_s, with the exception of the aforementioned surfaces. Subsequent introduction of water or buffer yielded a wide array of responses: instantaneous stabilization of the drift pattern observed from HSP-10, a sudden increase/decrease followed by stabilization, gradual increase/decrease followed by stabilization, or no change in the f_s and/or R_m. The various changes observed in the resonant frequency and motional resistance upon HSP-10 introduction for the buffer and water media are shown in Tables 1 and 2, respectively.

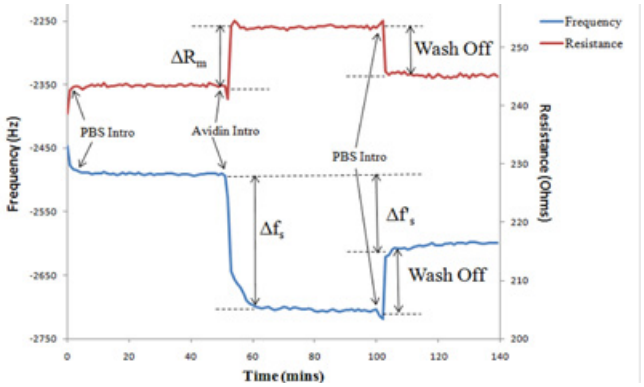


Figure 3: Representative TSM response for avidin-biotin. Δf_s represents the frequency shift upon avidin introduction and $\Delta f'_s$ represents the corrected frequency shift. The red and blue curves represent the changes in motional resistance and resonant frequency over time, respectively. Points of PBS and avidin introductions are shown.

Discussion

A new and reliable ovarian cancer screening method using TSM technology to detect the HSP-10 tumour marker is approached once an understanding of the TSM response is gained through the widely known avidin-biotin model system. The avidin-biotin model system was primarily studied in order to begin optimizing the selectivity, sensitivity, and reproducibility of the TSM and surface chemistries employed; especially since the avidin-biotin biochemistry has been largely studied and the reagents are readily available.

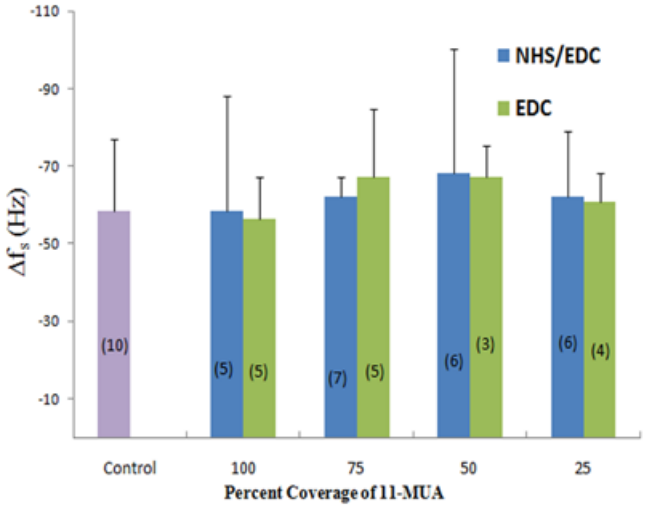


Figure 4: Frequency shifts upon avidin introduction (Δf_s). Numbers in parenthesis indicate the number of replicates. The error bars represent the calculated standard deviations.

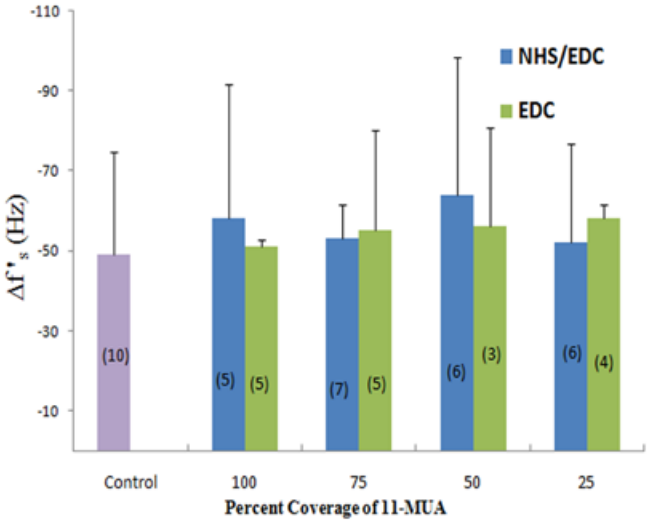


Figure 5: Frequency shifts corrected for buffer wash-off ($\Delta f'_s$) for avidin-biotin experiments. Numbers in parenthesis indicate the number of replicates. The error bars represent the calculated standard deviations.

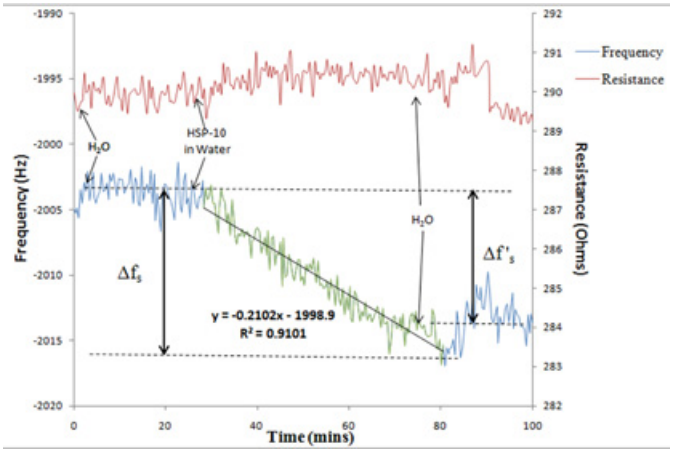


Figure 6: TSM response curve for HSP-10 in water for the control (bare gold surface). This response for HSP-10 to aptamer experiments was also seen for the majority of PBS experiments and some surfaces in the water experiments. The red and blue curves represent the changes in motional resistance and resonant frequency over time, respectively. The green curve outlines the region of drift observed upon HSP-10 introduction; its corresponding equation for the line of best fit is shown.

Magnitude of Avidin Protein Adsorption

Avidin appears to absorb rapidly onto all the possible SAMs, as a sudden decrease in frequency is observed upon avidin introduction and saturation is achieved almost instantly. The controls (bare gold slides) also exhibit a sudden decline in f_s, but are followed by prolonged patterns of declining drift (see supplementary figure). This behaviour suggests that the binding of avidin molecules onto the bare gold surface is non-specific, and that this freedom permits them to constantly undergo rearrangements from a native to a denatured state. These properties are not as influential in the SAMs since specificity is provided by the biotin molecule and since avidin forms a very strong non-covalent bond with biotin (dissociation constant of 10^{-15} M) [18]. The SAMs effectively reduce the number of possible random orientations of the protein onto the surface [19].

The change in frequency upon avidin introduction, Δf_s, for all possible SAMs and the control, as outlined in Figure 4, shows somewhat greater shifts for the NHS/EDC activated slides than for the EDC activated slides, with the exception of the 75% sample. This suggests that the NHS/EDC synergy supplies avidin with more available binding sites (i.e., biotin molecules) by providing more activated 11-MUA functionalities. Previous studies have shown that the addition of NHS results in the formation of a more hydrolysis resistant intermediate, and thus improves the overall yield of the esterification reactions [20-21].

According to the TSM frequency shifts, Figure 4, samples of 75% NHS/EDC and 50% EDC exhibit the largest, most precise, Δf_s values prior to wash-off. However in accordance with this, Figure 5, samples 50% NHS/EDC and 25% EDC exhibit the largest $\Delta f'_s$. Although the Δf_s represents an ease in detection (i.e., sensitivity to analyte binding), the $\Delta f'_s$ represents the selectivity of the sensor. Hence, both were equally considered when choosing optimal surfaces. The differences in wash-off observed amongst the different SAMs, though minimal, is likely due to the removal of weakly adhered avidin proteins and can be a direct consequence form the differences in packing efficiency. Evidently, an intermediate percent coverage of the linker onto the gold (50% or 75%) provides the highest packing efficiency and is the most reproducible surface. Full coverage of the gold surface with 11-MUA (100%) is quite irreproducible since the biotin molecules are too closely packed and likely held askew. The 25% 11-MUA coverage yields reasonable Δf_s values, but is also fairly irreproducible since the biotin molecules are unable to attain optimal surface density and possibly bend due to the substantial amount of free space available.

Aside from choosing the optimal surfaces based on reproducibility, those samples with TSM response curves with drifting patterns or a decrease in frequency upon post-washing (100% and 25% EDC/NHS, 100% and 25% EDC) were cautioned against for protein detection purposes (see supplementary figures). It is important to note that the TSM is a real-time detection system and is responsive to a multitude of changes happening at the sensor surface, such as protein adsorption, conformational changes, multilayer formation, or changes in solution viscosity. We are primarily interested in specific protein adsorption as this represents the concentration of analyte in solution. TSM response curves that show drift, a decrease in frequency upon post-washing and generally deviate from the ideal scenario shown in Figure 3 are deemed unworthy towards protein detection since the response from analyte adsorption is buried within the response generated from the interference effects. Therefore, even though the t-tests show no statistical significance among majority of the surfaces, it is irrespective to the pattern of the TSM response curves. This must be taken in account to determine which surface is most optimal towards protein detection with minimal interference effects.

After careful consideration of the Δf_s and $\Delta f'_s$ values, reproducibility, and TSM response pattern for all surfaces, it is deemed fit to choose five out of the eight possible SAMs as the most viable surfaces for protein detection: 75% NHS/EDC and all EDC possibilities.

Unlike the resonant frequency, the motional resistance is generally known to be unaffected by rigid mass loading, and is rather sensitive to solution viscosity and density changes [7]. Therefore, the minimal changes in R_m observed upon avidin introduction or PBS re-introduction can be attributed to changes in the solution viscosity.

HSP-10 Instigated TSM Responses

The TSM is responsive to HSP-10 introduction for all surfaces, i.e., SAMs, control, and sole aptamer, but to varying degrees. The measurements performed in buffer media yielded incomplete stabilization (slight drift) during the pre-buffer wash; whereas the measurements performed in water media yielded almost instantaneous stabilization during the pre-

Table 1: Changes in frequency observed for HSP-10 (in buffer) and PBS re-introductions. Slopes are shown for those frequency curves that had a linear decline upon HSP-10 introduction. Notes on superscripts: (a) Although a drift pattern is observed upon HSP-10 introduction, the Δf_s could not be computed since stabilization was not achieved upon post-washing. (b) The total time of drift was 20 minutes for the control.

Activation Scheme	Percent 11-MUA	HSP-10 (IN PBS) INTRODUCTION		ΔFrequency upon PBS re-introduction ± 1 (Hz)
		$\Delta f_s \pm 1$ after 50 mins (Hz)	Slope of drift observed (Hz/min)	
NHS/EDC	25	-19	-0.32	25
	50	0	-	-2
	75	-[a]	-0.87	0
	100	0	-	-11
EDC	25	-21	-0.40	0
	50	-29	-0.25	0
	75	-	-0.22	0
	100	-13	-0.31	5
CONTROL		-17[b]	-0.63	0
SOLE APTAMER		-37	-0.64	-

Table 2: Changes in frequency observed for HSP-10 (in water) and water re-introductions. Slopes are shown for those frequency curves that had a linear decline upon HSP-10 introduction.

Activation Scheme	Percent 11-MUA	HSP-10 (IN WATER) INTRODUCTION		ΔFrequency upon water re-introduction ± 1 (Hz)
		$\Delta f_s \pm 1$ after 50 mins (Hz)	Slope of drift observed (Hz/min)	
NHS/EDC	25	-5	-	-2
	50	-17	-0.33	0
	75	-14	-	-12
	100	-20	-0.50	9
EDC	25	-2	-	0
	75	-10	-	1
	100	0	-	-8
CONTROL		-10	-0.23	3
SOLE APTAMER		-6	-	3

water wash. The aptamer experiences larger resistance to flow in the PBS medium than in water due to the differing solution viscosities (PBS is more viscous than water). In order for the aptamer to alleviate the stress caused by the onset of the viscous PBS medium, it is suggested that the aptamer undergoes structural changes from a native extended conformation to a clenched one so that the amount of surface area exposed to the viscous medium is reduced; resulting in the observed drift.

The introduction of HSP-10 generated a linear decline in the resonance frequency Δf_s, for the 100% and 50% NHS/EDC and control surfaces within the water media, and for the majority of surfaces within the buffer media, as shown in Figure 6. This linear decline is indicative of protein multilayer formation, i.e., successive HSP-10 adsorption onto the preceding monolayer. The slopes are representative of the extent to which multilayer formation occurs and are summarized in Tables 1 and 2 for the buffer and water media, respectively. Generally, a steeper slope indicates rapid multilayer formation. Multilayer formation is greatest for the sole aptamer surface, followed by the control, EDC activated SAMs, and finally the NHS/EDC activated SAMs. This trend illustrates that the surfaces with randomly distributed aptamers are more prone to multilayer formation, where NHS/EDC provides higher uniformity than the EDC activated slides. As with the biotin-avidin model, the adsorption of HSP-10 onto the control is influenced primarily due to the hydrophobicity and electrostatics of the protein. The extent of multilayer formation is less for the runs conducted in water media, and is only observed for the 100% and 50% NHS/EDC and control surfaces. The surfaces with 100% 11-MUA are congested and do not provide an energetically favourable state for the bioreceptors to bind. Thus with few aptamers on the surface, the HSP-10 negative surface charges interact with the head functionalities of the 11-MUA molecules in a non-specific manner. It is suggested that the HSP-10 then undergoes structural changes to spread itself across the linker surface, exposing positive surface charges to which subsequent HSP-10 molecules can adsorb on top of; thus resulting in multilayer formation. Alternatively, surfaces 100% NHS/EDC in buffer and 100% EDC in water surfaces also have few aptamers on the surface, but do not show a response to HSP-10 introduction. Here it is hypothesized that since few aptamers are on the surface no binding sites are available for the HSP-10, and thus no response is observed. Irrespective of the mechanism, the 100% 11-MUA surfaces are inadequate for HSP-10 protein detection.

The majority of the measurements run in water media were characteristic of a sudden decrease in frequency upon HSP-10 introduction to stabilization, much like Figure 3. The initial sudden drop in frequency is indicative of aptamer-HSP-10 binding. Although a rapid adsorption event is apparent for the 75% NHS/EDC surface, the sole aptamer and 75% EDC surfaces gradually approached stabilization upon HSP-10 introduction. This gradual decline is due to possible rearrangements of the HSP-10 onto the surface in order to maximize surface binding with the aptamer.

It is concluded that binding of HSP-10 to its aptamer is facilitated in the water media rather than in buffer. HSP-10 is known to solvate better in water than in buffer, explaining the decrease in signal fluctuation observed. A large degree of non-specific adsorption leading to multilayer formation is observed for the buffer experiments. It is suggested that the adsorption of the HSP-10 onto the surface is more energetically favourable than remaining in the buffer solution irrespective of the aptamer to HSP-10 binding energy considerations, and thus the HSP-10 molecules adsorb onto the sensor surface somewhat biased. On the other hand, when HSP-10 is in water, it will selectively bind to the immobilized aptamers if the surface conditions are more energetically favourable. In turn, the experiments performed in water media are less complicated and provide better accuracy in deducing the concentration of HSP-10 with minimal interference effects. These results highly suggest that preliminary HSP-10 detection optimization ex-

periments should be performed in water media rather than in buffer.

The optimal surface for HSP-10 detection was chosen based on three requirements: rapid protein adsorption with a quantifiable change in the resonant frequency, minimum wash-off resulting in a substantial $\Delta f'_s$, and minimal interference patterns associated with HSP-10 rearrangement and multilayer formation. The surface 75% NHS/EDC, when run in water, best fulfills these requirements. This surface is most sensitive and selective towards HSP-10 binding. Second to this would be the EDC surfaces in water. The information presented is useful in the detection of HSP-10 and in determining the binding chemistry between the aptamer and HSP-10 in terms of conformational and/or interfacial changes.

We are currently investigating the use of a similar instrument, the ElectroMagnetic Piezoelectric Acoustic Sensor, towards the detection of HSP-10. Since this instrument can operate at ultra-high frequencies, it provides greater sensitivity in detecting proteins at minimal concentrations with reduced interference from matrices and solvent viscosity and density effects as was a limitation with the TSM technology. Furthermore, this study could have benefitted from a set of linker and spacer molecules which further prevent any non-specific electrostatic binding. Consequently, we intend to employ novel linkers and spacers with oligoethylene glycol backbones, which are known to highly resist non-specific adsorption [22]. Finally, we hope to utilize this biosensor in real-world samples such as serum, urine, and blood.

Conclusion

The thickness shear mode (TSM) acoustic sensor is a highly sensitive and selective device for the detection and measurement of protein-bioreceptor interactions. The use of self-assembled monolayers (SAMs) allows the specificity of this interaction to be greatly enhanced. Variations of the monolayer, such as the employment of a spacer and development of a highly activated terminal group, permit optimal protein immobilization. From the avidin-biotin interaction it was determined that the SAMs that provide the greatest selectivity and sensitivity are all the EDC possibilities and NHS/EDC 75% 11-MUA. Changes in frequency were primarily used to interpret the extent of avidin adsorption. The observed changes in motional resistance were attributed to solution viscosity and density changes. The interaction between HSP-10 and the aptamer showed definite regulation of the HSP-10 binding properties for the SAMs versus the control or sole aptamer surfaces. Aside from HSP-10 adsorption, a significant portion of the response is attributed to conformational changes in the protein or in the SAM with various interfacial phenomena. The increased solvation of HSP-10 in water media yielded the NHS/EDC 75% 11-MUA surface as optimal for HSP-10 detection. This study has shown positive responses to HSP-10 binding using the TSM. Further characterization of the TSM responses to aptamer-HSP-10 interactions would allow the development of an inexpensive, relatively simple, and reliable screening method for ovarian cancer detection. In addition, conformational studies regarding the stability of the aptamer-HSP-10 bond is approachable

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The influence of wind and light exposure on the extent of lichen coverage in an alpine environment

Daniel Anstett

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Abstract

Lichens are important components of arctic and alpine ecosystems. Their abundance and diversity are high despite the harsh conditions, yet little is known about the microclimates that favour lichen growth. Using a 2 x 2 factorial design, we classified rock faces at the high alpine Barcroft Field Station in the White Mountains of California into high and low wind exposure and high and low light exposure categories. We then measured the extent of coverage of two alpine crustose lichen species, *Caloplaca ignea* and *Pleosidium flavum*, on the rock faces. The data show that lichen coverage is greatest on rock faces that are protected from wind but exposed to light (median = 60 m2, range = 1.8 - 647.1 m2), and lowest on faces that are exposed to wind but protected from light (median = 7.4 m2, range = 0.15 - 142.5 m2). The lack of success in high wind conditions may be due to desiccation-induced inhibition of photosynthesis, leading to reduced carbon balance in wind-exposed lichens. By characterizing favourable microclimates, this study provides observational data that help to establish the environmental dependence of photosynthesis and respiration in cold climate lichens. This is particularly important for informing estimates of ecosystem-level carbon balance in regions like the boreal, where lichens represent 50-90% of vegetation cover.

Introduction

Alpine regions are characterized by a number of environmental stressors, including temperature extremes, high irradiance, and strong winds. Some of the most successful organisms in this environment are lichens, which are a symbiotic union of a fungus and a photosynthetic photobiont that is usually either a green algae or cynobacteria [1]. In most lichen systems the fungi forms a complex tissue structure with algal cells carrying out photosynthesis just above the cortex. In exchange for nutrients, the fungi provide shelter and UV protection; however, the fungi only form a thallus structure when joined with a photobiont [2]. Lichens are usually classified by their fungal component, but the organismal ambiguity has left them understudied both by mycologists and botanists [3].

These peculiar organisms play a unique and important role in alpine as well as in arctic ecosystems, providing 90% of the winter food source for arctic caribou and reindeer, and the majority of the winter food source for many other organisms [4]. These "nature's pioneers" are proficient at colonizing the barren substrates that are common in high latitude and altitude landscapes [4]. In so doing they help create soil by weathering rock, trapping dust and debris, and by contributing their own organic matter when they die, which is an important first step in succession [4]. Cyanobacterial lichens can also fix nitrogen, which favours plant growth [4].

What makes lichens so successful in harsh cold environments is their ability to tolerate desiccation, extreme temperatures and high light stress. Even so, little is known about the microclimatic conditions most suitable for lichen growth and photosynthesis. Lichens are poikilohydric organisms, which means that their water content depends on environmental conditions [4, 5]. Lichens that have green algae as the photobiont are unique in that they can absorb water vapour directly from the air [4], which means that they can thrive in areas with little or no precipitation [5]. Still, photosynthesis in lichens requires hydration, so lichens should grow where desiccation stresses are not too extreme.

Temperature is not as likely to influence habitat selection, as lichens can tolerate temperature extremes as cold as -196°C and as hot as 60°C as long as they are desiccated [1]. They are more vulnerable when hydrated, but still seem to be able to operate at a wide range of temperatures [1]. High light intensities are also unlikely to limit growth because alpine lichens generally possess photoprotective mechanisms that allow them to tolerate high light intensities and UV exposure typical of alpine areas [6]. Thus, we hypothesize that alpine lichens will favour areas that are sheltered from the wind to minimize desiccation, but exposed to light to maximize photosynthesis. We test this hypothesis by measuring the extent of lichen coverage on rock faces exposed to high and low levels of wind and light in the White Mountains of California.

Methods

Study Site

The study was conducted from August 25th to 28th 2009 several hundred meters northwest of Barcroft Station, (37° 35' 17" N latitude, 18° 14' 26" W longitude, 3800m a.s.l.,White Mountains Research

Figure 1: Study site. Rock mounds just northwest of Barcroft Station in the White Mountains in California.

Station, Bishop, California). The study area was a dry alpine tundra plateau with scattered rock mounds (Fig. 1). Wind records for the period 2003-2009 obtained from the White Mountain Research Station (website) indicate that the prevailing wind direction is westerly (Fig 4), with mean (± s.d.) bulk wind speeds of 5.0 ± 0.2 m/s in the winter (DJF) and 3.0 ± 0.4 m/s in the summer (JJA) (data not shown) [16].

Sampling Design

Rock faces were classified into four categories according to high and low wind exposure and high and low light exposure, resulting in a 2 x 2 factorial design. Historical records of wind direction obtained from the White Mountain Research Station indicated that the prevailing wind direction was westerly [7] (Fig 2). Thus, exposed faces were defined as sites that faced westward and were not sheltered by another rock formation. Sheltered sites were defined as sites that face away from the west or were sheltered from west-blowing prevailing winds by another rock formation, or by the particular angle of the face. Similarly, high light sites were defined as rock faces exposed to sunlight for at least a few hours a day, while low light sites were exposed to little or no direct light. Faces where the light and/or wind condition was unclear were excluded. Two transects were laid across four rock piles in an approximately straight east-west line. Sampling was conducted approximately 0.5 m on either side of the transect. Each category of rock face was sampled 130 times for a total of 520 samples, whereby a rock face was considered the unit of replication.

Environmental Measurements

To characterize microclimate characteristics of the four categories of rock faces, wind speed and temperature were measured at the rock faces. Wind speed was measured using a thermoanemometer (Sper Scientific, model #840002, Scottsdale, AZ). The tip of the device was held at arm's length a few centimeters away from the rock face. Thirty typical exposed and sheltered rock faces were sampled at 3 PM, when the wind speed was greatest. Rock face temperature, a proxy for sun exposure, was measured using an infrared thermometer (Omega Engineering, Inc., Model # OS685, Stamford, CT). Thirty typical exposed and sheltered rock faces were sampled approximately four hours after sunrise.

Extent of Lichen Coverage

Two crustose lichen species, *Caloplaca ignea* (Fig 2a) and *Pleosidium flavum* (Fig 2b) were selected for coverage measurements [4]. These two species were the most abundant and easy to recognize on the rock mounds in the study area. The extent of lichen coverage on each rock face was estimated by applying an appropriate geometric shape to approximate the shape of the talus. Lengths, widths and radii measurements were taken with a ruler.

Statistical Analysis

To verify microsite differences between exposed and unexposed rock faces, wind speed and temperature measurements were tested using two-tailed Student's t-tests in Microsoft Excel 2008 (v. 12.1.0). The extent of lichen coverage was log-transformed and modeled using a linear model with wind speed, temperature, species, and their interactions as predictors in R [8].

Results

Microsite Characteristics

Exposed and unexposed rock faces differed in both wind speed and temperature. At 3 P.M., exposed rock faces experienced a mean wind speed (± s.e.) of 0.88 ± 0.6 m/s, while unexposed rock faces experienced 0.2 ± 0.04 m/s (Fig 3, $t_{\upsilon=59,\alpha/2=0.025}$=9.97, p<0.0001). Exposed rock face temperature was 16.9 ± 0.7 °C while unexposed rock faces were 3.8 ± 0.5 °C (Fig 5, $t_{\upsilon=59,\ \alpha/2=0.025}$=15.93, p<0.0001).

Lichen Coverage

The extent of lichen coverage varied widely (overall range = 0.15-791.8 m2), with no significant difference between species. Microsite characteristics explained 17.8% of the total variance in the extent of lichen coverage (Table 1). Coverage was greatest on rock faces that were protected from the wind but exposed to the sun (Fig 6, median = 60 m2, range = 1.8 - 647.1 m2), and lowest on rock faces that were exposed to the wind but sheltered from the sun (Fig 6, median = 7.4 m2, range = 0.15 - 142.5 m2). Wind speed alone explained 11.2% of the total variance (Table 1, F(1,515)=70.2, p<0.0001), while temperature, a proxy for light exposure, explained 3.1% of the total variance (Table 1, F(1,515)=19.1, p<0.0001). The interaction between wind speed and temperature explained an additional 3% of the variance (Table 1, F(1,515)=18.8, p<0.0001).

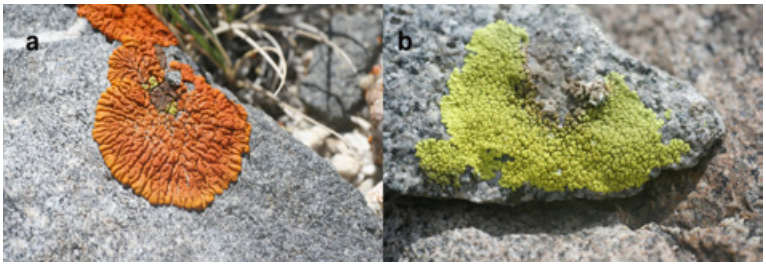

Figure 2: Study species. a) *Caloplaca ignea* is recognizable by its dark orange/red colour and radial growth with many ridges and bumps on the talus. **b)** *Pleosidium flavum* is bright green/yellow. Its bumps are less pronounced and of a more circular shape.

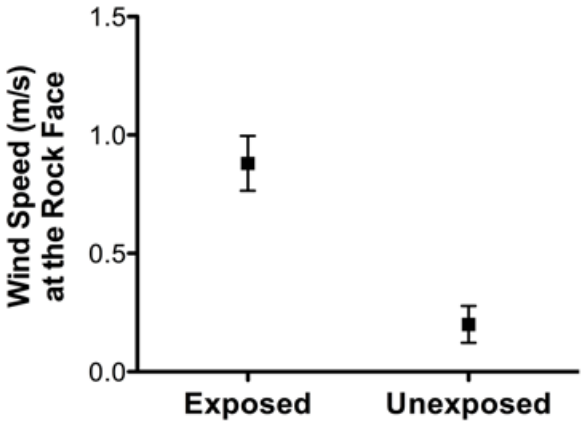

Figure 3: Mean wind speed at exposed and unexposed rock faces. The wind on exposed rock faces is significantly higher (tv=59,a/2=0.025=9.97, p<0.0001). Error bars represent standard errors.

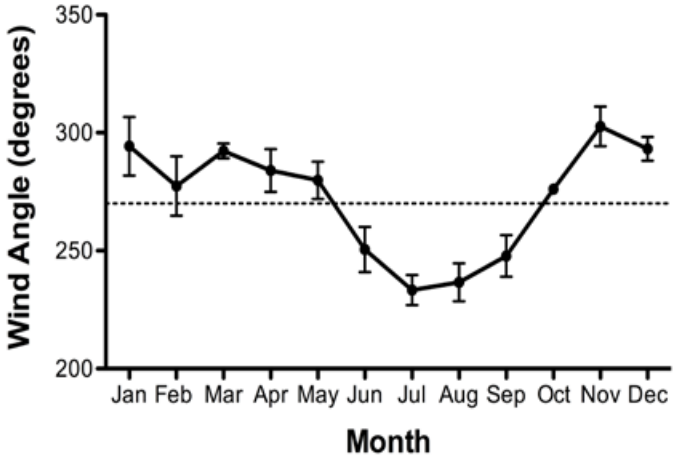

Figure 4: Mean monthly wind direction from 2003-2009. The direction of the wind (in degrees) is given on the y-axis. The values are always close to 270 (horizontal dotted line), or due west. Error bars represent standard errors.

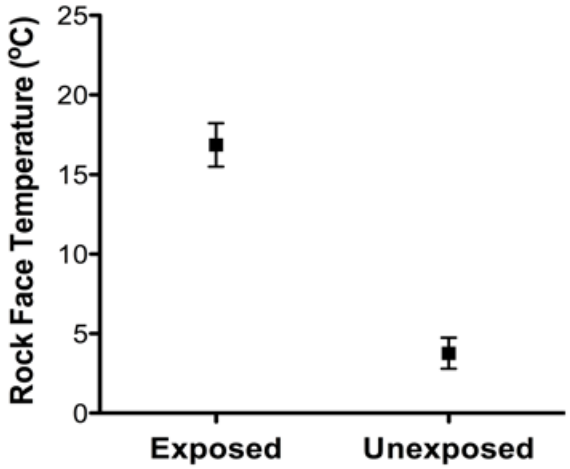

Figure 5: Mean temperature on exposed and unexposed rock faces. Temperature, a proxy for light exposure, was significantly higher on sun exposed (high light) faces (tv=59,a/2=0.025=15.93, p<0.0001). Error bars represent standard errors.

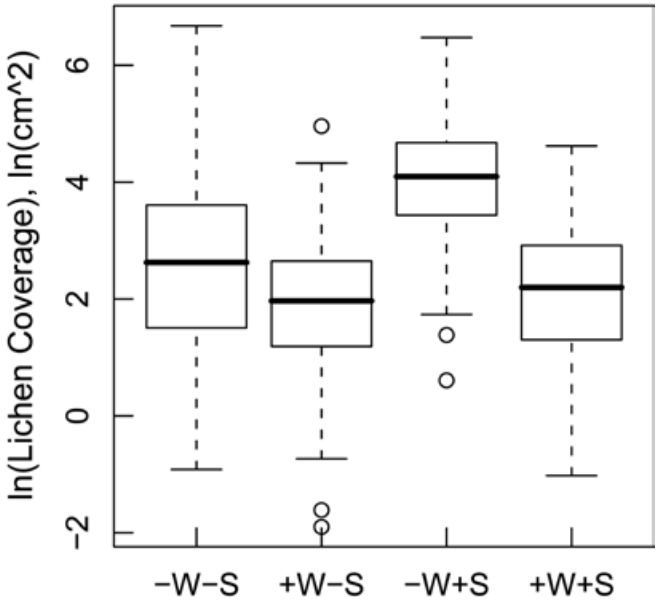

Figure 6: Log-transformed extent of lichen coverage on exposed and unexposed rock faces. W represents wind and S represents sun (light). The signs stand for presence (+) or absence (-). Black lines are medians, while the boxes show the interquartile range.

Discussion

The extent of lichen coverage on alpine rock outcrops is clearly influenced by microsite characteristics, whereby lichens of both species were more successful in microsites that are protected from the wind, but exposed to the sun. One possible explanation is wind abrasion, but lichens are known to tolerate abrasion [1]. Wind would also remove snow on windward surfaces, exposing the lichens to intensely cold temperatures. However, the exposure would also result in the loss of water from the vapour layer of the snow [1], leading to a desiccated state. Since lichens are known to survive temperatures as low as -196°C when dehydrated [4], exposure due to snow removal does not appear to explain the effect of wind in reducing lichen area on windward rock surfaces.

A third way wind could influence lichen area is by changing the water status of the lichens. Lichens are extremely desiccation tolerant, with some surviving under laboratory conditions down to 5% humidity [1], but in order to initiate photosynthesis, lichens need to rehydrate. The extreme fluctuations in water status that are typically experienced by lichens would exert a strong influence on photosynthesis, and thus their ability to grow. Curiously, lichen photosynthesis is maximal not when saturated with water, but rather over a narrow range of intermediate water contents [9, 10, 11]. This is because saturation prevents carbon dioxide from reaching the photobiont [12]. In the absence of strong desiccating winds, water loss would be slow, extending the period of intermediate water content where photosynthesis is high. On an exposed surface, the moisture would be lost much more quickly, leading to reduced photosynthesis and less carbon available for growth. As well, alpine lichen growth is likely highest not on hot midsummer days, but in the period during and just fol-

lowing snowmelt [4] or on cool mornings before temperatures on the dark rock faces climb too high [13].

It seems likely that the main effect of wind is influencing some aspect of lichen water balance. To test whether desiccation rate or simply low water status is limiting, various wind speeds and percent humidities could be applied to alpine lichens in the lab. If the rate of water loss is what inhibits lichen photosynthesis, then well-hydrated lichens regularly exposed to high wind speeds should reduce photosynthesis. If the problem was due to a general lack of water, then an increase in humidity at certain times would ameliorate the condition regardless of whether or not the lichens were later exposed to high wind speeds.

Understanding the temperature and water dependence of photosynthesis is particularly important for lichens because lichens have a larger respiration load than other photosynthetic organisms. This is because only the photobiont can fix carbon, but both the photobiont and the fungus respire carbon, which limits their maximum carbon assimilation rate [14]. In addition, in most lichens, the temperature optimum for carbon assimilation is often lower than the optimum for respiration [4]. There is some evidence that lichens acclimate respiration in response to seasonal changes, which would reduce the carbon lost at high temperatures in the absence of acclimation; however, net carbon assimilation is still likely highest when temperatures are cool [14]. In summary, lichens growing in microsites exposed to wind would have very limited opportunities for photosynthesis due to hydration state, and even fewer opportunities in which photosynthesis will more than compensate for carbon lost to respiration. This poor carbon balance would lead to reduce growth on windward surfaces, which is consistent with the findings of this study.

This study provides observational data that help to establish the dependence of lichen photosynthesis and respiration on the interacting factors of water status and temperature in cold climate lichens. This is particularly important for informing estimates of ecosystem-level carbon balance in regions like the boreal, where lichens represent 50-90% of vegetation cover [4]. A better understanding of lichen respiration may also help improve estimates of soil fungal respiration, which is notoriously difficult to measure but is nevertheless a substantial source of carbon respired to the atmosphere [14].

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JULS

Phylogenic and Evolutionary Analysis of Glutamate Receptors Based on Extant Invertebrate Genes

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Research Article

Abstract

The ionotropic glutamate receptors, iGluRs, play diverse but important roles in the central nervous system. Specific iGluRs were found to be implicated in neurological pathways that modulate memory and behaviours in lower species including *C. elegans*. In this project, a bioinformatics approach involving protein BLAST, multiple sequence alignment and phylogenetic analyses was used to examine the evolutionary relationship between *C. elegans* and other invertebrate species. While most *C. elegans* iGluRs shared close lineages with their human counterparts, some, including iGluR subtype 5-8 did not share significant lineages with specific human orthologs. Interestingly, phylogenetic trees constructed using full-length and partial sequences specifically showed that *C. elegans* iGluR-8 remained on a lineage that is noticeably different than that of NMDA and non-NMDA families. Such evolutionary lineages may elucidate important information regarding neurological development in different species and may facilitate the development of therapeutic agents for the treatment of neurological disorders.

Introduction

The ionotropic glutamate receptors (iGluRs) form a major class of ligand-gated ion channels that participate in excitatory neurotransmission in the central nervous system [1]. Based on pharmacological specificity, members of the iGluR family can be classified as either belonging to the N-methyl-D-aspartate (NMDA) class or the non-NMDA class. A total of 18 iGluR subtypes have been identified in *Homo sapiens* [2] and 10 have been identified in the worm, *Caenorhabditis elegans* [3]. In humans, seven of the iGluR subtypes belong to the NMDA class (NR1, NR2A - NR2D, NR3A, NR3B) while the remaining belongs to the non-NMDA class. The non-NMDA class could be further divided into the α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA; GluR1 - GluR4), kainate (GluR5 – GluR7), and delta subtypes (δ1 and δ2), which are sometimes referred to as orphan receptors as it is unclear which ligands they bind and whether they could form functional ion-gated receptors [4, 5]. The NMDA receptors require both glutamate and glycine (and/or D-serine) for activation, whereas non-NMDA receptors are activated by glutamate alone [6].

Unlike the metabotropic glutamate receptors (mGluRs) which are G-protein coupled receptors that are thought to form functional units as homodimers [7], iGluRs form functional units in tetramers[8]. In the central nervous system, glutamate acts on postsynaptic iGluRs to mediate fast excitatory synaptic transmission and acts on mGluRs to modulate neuronal excitability via G-protein coupled receptor signaling [9]. A typical structure of mGluRs consists of an extracellular amino-terminal domain also known as the Venus flytrap domain, a seven-transmembrane hydrophobic domain, and a cytoplasmic carboxy-terminal tail [10]. A typical structure of iGluRs consist of an amino-terminal domain (NTD), an S1-S2 ligand-binding domain, three transmembrane domains, a re-entrant pore loop and a short carboxy-terminal cytoplasmic tail (Fig. 1).

Crystal structures of the S1-S2 iGluR domains are available for GluR2 [11], GluR0 [12] and NMDA NR1 [13] receptors in different agonist and antagonist bound forms. It was reported that closure of the S1-S2 domains directly correlate with the extent of receptor activation and desensitization [14]. The currently accepted mechanism of iGluR channel dynamics is that ligand-induced closure of the S1-S2 domains led to conformational changes that trigger iGluR activation [15-18]. In addition, it was found that a conserved SYTANLAAF sequence exists in the second transmembrane loop in iGluRs [19, 20]. Amino acid substitutions in the SYTANLAAF motif strongly altered channel function [21, 22].

Interestingly, it was reported that the mGluR Venus flytrap domain as well as the iGluR NTD domain shared sequence homology to the leucine/isoleucine/valine-binding protein (LIVBP) of the bacterial periplasmic binding proteins (PBPs) [23-27]. it has been reported that the S1-S2 domain in iGluRs forms a pocket that shares mechanistic similarity with the PBPs in ligand recognition [28, 29]. Therefore, a reasonable question to ask is whether a common primordial receptor existed and if so, was it more similar to the iGluRs or the mGluRs, especially within the smaller Venus flytrap domain in the extracellular region common to both receptors. Ancestral reconstruction of the ligand-binding pocket of Family C GPCRs involving the mGluRs had previously been performed [30].

Phylogenetic studies in invertebrate species such as *C. elegans* may have important biological implications for functional conservation and variation in protein families. Studies have shown that information from phylogenetic analyses could be used to produce diagnostic assays specific for disease

conditions and vaccine production[31]. Phylogenetic information may also be useful for analyzing comparative experimental data involving structural, functional and behavioural relationships in a given species[32]. For example, phylogenetic analyses on the AMPA and kainate subtypes provided insight on crucial developmental pathways in invertebrate species including fish[33] and insects[34].

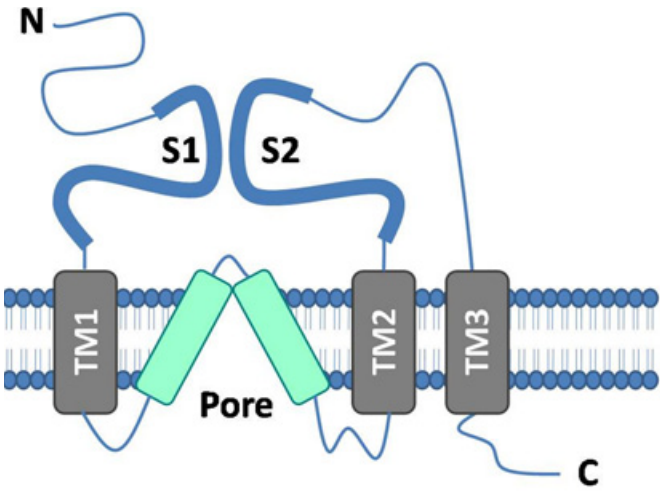

Figure 1: Schematic diagram of a typical iGluR. The ligand-binding region in the iGluR is formed by two separate extracellular loops containing the S1 and S2 domains. There are three hydrophobic transmembrane domains, TM1, TM2 and TM3, which fully span the membrane. A re-entrant membrane loop forms the pore that lines an ion channel in iGluRs. The amino (N) and carboxy (C) termini situate extracellularly and intracellularly, respectively.

Phylogenetic analysis on *C. elegans* iGluRs may have essential pharmaceutical implications. It has been reported that analysis of receptor diversity may provide important information regarding stereochemical specificity in ligand-binding to members of the iGluR family[35]. Moreover, genome informatics of iGluRs have reported polymorphisms among orthologous members that have important neurobehavourial consequences[36]. Results from a study examining the S1-S2 binding pockets in NMDA receptors via an evolutionary analysis showed that disruption of domain closure could affect channel activation and may affect normal behaviour in animals[37]. The non-NMDA AMPA and kainate as well as NMDA receptors may be therapeutic targets for the treatment of neurological diseases such as neuronal death schizophrenia[38], mental retardation[39], depression[40], epilepsy[41], Alzheimer's Disease[42] and Huntington disease[43] as they mediate fast synaptic transmission in the CNS and have been implicated in physiological processes involving learning and memory in early development[44-47]. A phylogenetic analysis on *C. elegans* iGluRs may therefore provide important information for directing de novo drug discovery processes with implications for the ligand-binding regions in iGluRs in humans.

Hypothesis

Since members in the *C. elegans* iGluR protein family contain amino acid regions that share similarity with the bacterial periplasmic binding protein, PBP, it could be hypothesized that proteins from invertebrates, including those without an intact nervous system such as plant species, would contain domains that also share similarity with the PBP. In addition, it could be hypothesized that these proteins may not necessarily contain the hydrophobic regions commonly shared among iGluR members.

Rationale

This project represents the first phase of a long-term, collaborative study between Drs. Chang and Hampson that aims to examine the evolution of iGluRs and mGluRs among vertebrates and invertebrates. This project aims to examine the evolutionary relationship between C.elegans iGluRs and similar proteins from other species. Later phases of the study would involve analysis of results from both the iGluR and mGluR groups to investigate common evolutionary lineages that may be shared between the iGluR and mGluR protein families. The goal of this project is to perform a phylogenetic analysis of the aligned glutamate receptor sequences to identify a common ancestral lineage among iGluRs among lower species in the evolutionary process. Ultimately, ancestral reconstruction may be performed to analyze structural and functional characteristics of an ancestral primordial glutamate receptor.

Objectives

The objectives of this project are: 1) to perform BLAST searches using the protein sequences of each iGluR subtype from *H. sapiens* and *C. elegans*, 2) to compare the pairwise sequence homologies between query iGluR proteins and each returned BLAST result, 3) to perform sequence alignments on interest protein targets against all iGluR subtypes from *H. sapiens* and *C. elegans*. This project is performed in collaboration with Prof. Belinda Chang of the Department of Zoology whose efforts will be focused BLAST searches and protein sequence alignments on mGluRs.

Materials and Methods

Protein BLAST

The C.elegans iGluR genes used in this study were obtained from GenBank. Table 1 lists all of these *C. elegans* genes and their respective GenBank accession numbers. Protein BLAST searches were performed using each C.elegans iGluR receptor subtype as amino acid sequence probes via the National Center for Biotechnology Information online database. Each query sequence was specified by entering the respective GenBank accession number for individual *C. elegans* iGluR receptor. Parameters for all protein BLAST searches were as follows: the number maximum target sequences for algorithm parameters was set to 250, expected threshold was set to 10, word size was set to 3 and automatic adjustment of parameters for short input sequences was selected under short queries. As a strategy to maximize search results from invertebrate species, the following command was entered under the entrez query option: "all[filter] NOT mammalia [orgn] NOT drosophila". This command eliminates all sequence results originating from mammalian and Drosophila species. It was found that results from Drosophila species crowded protein BLAST results and minimized the probability of

retrieving orthologous proteins from other species (results not shown). Using this strategy, a total of 10 protein BLAST data sets were produced. Protein results with a pairwise homology greater than 80% or less than 20% than the probe protein sequence were all eliminated from each data set.

Data Selection, Sequence Alignment and Phylogenetic Tree Construction

All protein sequences in this study were aligned using the Vector NTI® (Invitrogen Corporation) program. Phylogenetic trees were generated using the Molecular Evolutionary Genetic Analysis (MEGA), which contains a Clustal alignment core, was previously shown to be a feasible program for analyzing alignment data for the generation of phylogenetic trees [48, 49]. Only full length protein sequences from *C. elegans* were used in this study as protein BLAST query items.

Protein sequences from all iGluR subtypes in *C. elegans* and *H. sapiens* (Table 2) were included in the construction of the alignment and phylogenetic tree.

The top 20 protein hits for each data set were collected and pooled into a separate module with a total of 200 protein sequences. Identical protein sequences in this module were subsequently

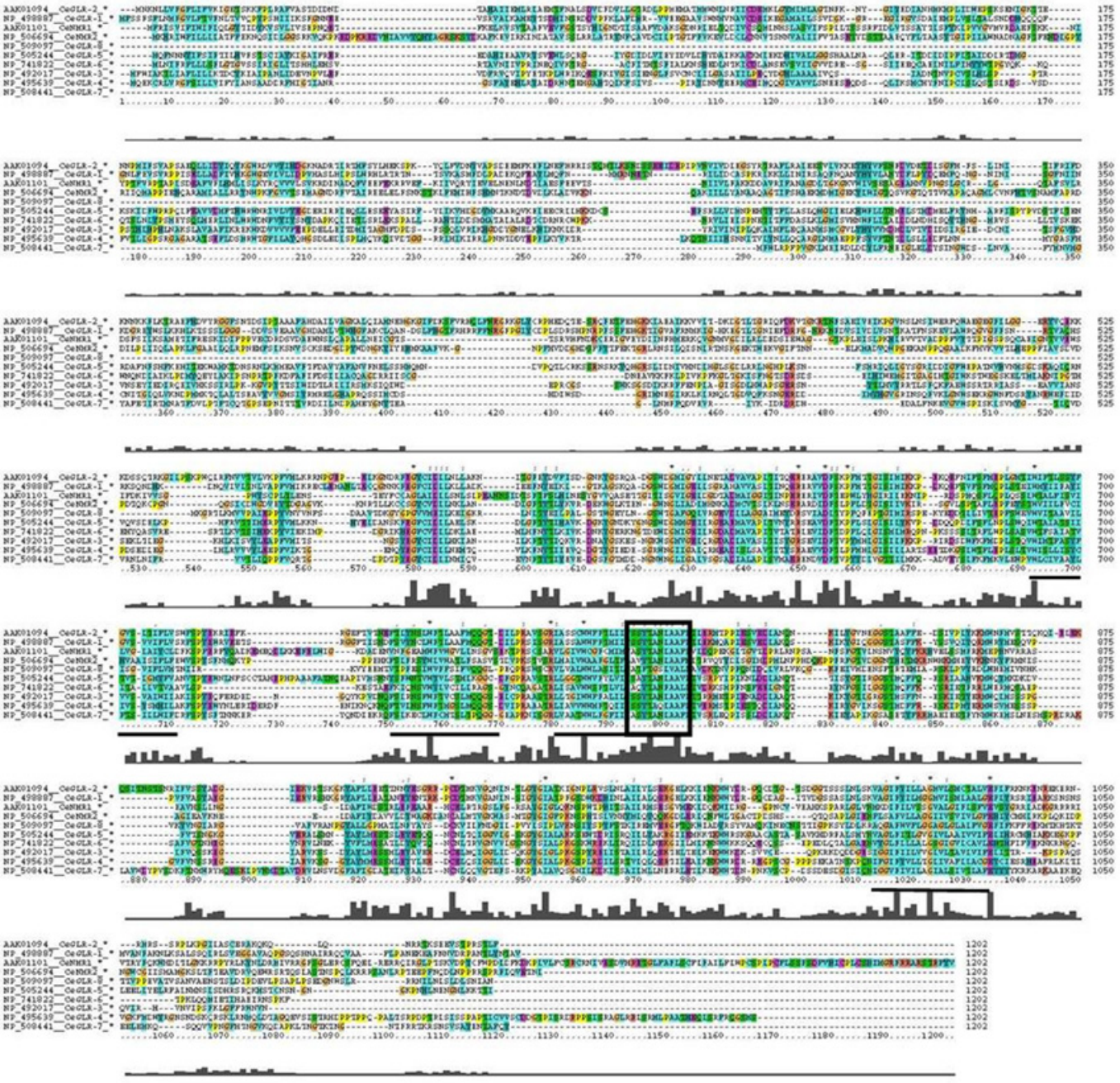

Figure 2: Sequence alignment of all iGluR protein members in *C. elegans*. The SYTANLAAF motif is boxed and the hydrophobic regions are underlined.

eliminated and the remaining full length sequences were used for generating an alignment and a phylogenetic tree. Full length protein sequences from all iGluR subtypes in *C. elegans* and *H. sapiens* were included in the construction of both the alignment and phylogenetic tree.

Subsequently, a separate phylogenetic tree was constructed using amino acid sequences from the protein BLAST results that aligned with the bacterial periplasmic LAOBP protein region in C.elegans CeNMR1 (vvtvadppfvyttpigspsqcaelgntvvewsifdkivvsgpwyscpltlensteyfccaglaidllsnlslpeannsidtsftfslhlnesygvvqasettgitisgvigeldgdtadmaiggitinpererivdftepwlyhgirilekniprdspmqsflqplqsslwtalfisvilvglaiycldfkspferfyqadkemeqdlkkefelwigkdadenvnfgeamwfvwgvllnsgvsektprscsarvlgivwcgfcmimvasytanlaaflvldqpekgltgvtdprlrnpsanfsfgtvlnsnvyqyfkrhvelssmfrkmephnvrraseavhsllngsldafiwdstrlefeaarhcelrtrgslfgrsaygiglqknspwtphitsailrmsesgvmekldqkwidrggpncvveahksparlglvnmkdifilvssgvalgiflsfv, amino acids 399-978).

Results

Following the elimination of identical proteins from the pooled sequence data, a total of 128 unique protein sequences were collected (results not shown).

As a strategy to ensure validity and accuracy of the methods used in this study, construction of sequence alignments and phylogenetic trees were performed, using all 10 *C. elegans* and 18 *H. sapiens* iGluR protein sequences, individually and in combination between the two species (Fig. 2-3, sequence alignments; Figure 4 phylogenetic tree using both human and *C. elegans* sequences). Both alignment and phylogenetic analyses among *C. elegans* iGluRs showed results similar to those reported in the literature. Sequence alignment showed a consistent pattern that aligned the hydrophobic regions as well as the relatively conserved SYTANLAAF motif among *C. elegans* iGluR members. Based on the results, it could be observed that CeGLR-5 shares a close lineage with CeGLR-6, CeGLR-3 with CeGLR-4 and CeGLR-1 with CeGLR-2. These proteins all belong to the non-NMDA group of iGluR and collectively represented the AMPA, kainate and delta subclasses. The exact identity of iGluR proteins representing individual non-NMDA iGluR subclass could not be conclusively deduced based on these results. This finding is consistent to reports in the literature indicating that CeGLR-1 to CeGLR-7 proteins

Table 1: Summary of *C. elegans* iGluR proteins used in this study.

Accession Number	Protein Description	Abbreviation
AAK01101	NMDA	CeNMR1
NP_506694	NMDA	CeNMR2
NP_498887	non-NMDA	CeGLR-1
AAK01094	non-NMDA	CeGLR-2
NP_492017	non-NMDA	CeGLR-3
NP_495639	non-NMDA	CeGLR-4
NP_505244	non-NMDA	CeGLR-5
NP_741822	non-NMDA	CeGLR-6
NP_508441	non-NMDA	CeGLR-7
NP_509097	non-NMDA	CeGLR-8

Table 2: Summary of *H. sapiens* iGluR proteins used in this study.

Accession Number	Protein Description	Abbreviation
EAW88359	NMDA1	hNMDA-1
EAW85187	NMDA2A	hNMDA-2A
EAW96311	NMDA2B	hNMDA-2B
EAW89202	NMDA2C	hNMDA-2C
EAW52346	NMDA2D	hNMDA-2D
NP_597702	NMDA3A	hNMDA-3A
NP_619635	NMDA3B	hNMDA-3B
NP_000818	AMPA1	hAMPA1
NP_001077089	AMPA2	hAMPA2
CAI95683	AMPA3	hAMPA3
EAW67066	AMPA4	hAMPA4
EAW67511	Kainate KA1	hKainate-KA1
EAW57090	Kainate KA2	hKainate-KA2
CAC80546	Kainate GluR5	hKainate-GluR5
CAC67487	Kainate GluR6	hKainate-GluR6
CAC80548	Kainate GluR7	hKainate-GluR7
NP_060021	Delta 1	hDelta1
NP_001501	Delta 2	hDelta2

exhibited ligand-binding and pharmacological profiles that encompass mixed AMPA, kainate and delta receptor characteristics. Interestingly, CeGLR-8, which represented a member of the C.elegans iGluR family with unknown functions, was found to belong in a different lineage among other iGluR members, a result that is consistent with the literature.

Alignment and phylogenetic results on iGluR members in H.sapiens are also consistent with those in the literature, indicating that the methodologies used in this study produced valid results. It was found that among the NMDA receptors, hNMDA-2A associates most closely with hNMDA-2B, hNMDA-2C with hNMDA-2D, and hNMDA-3A with hNMDA-

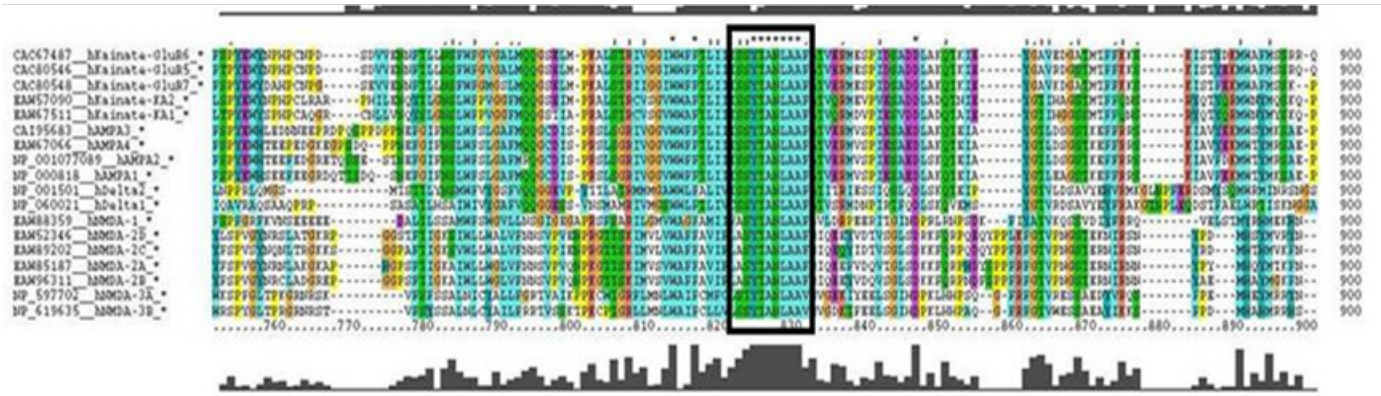

Fig. 3: Sequence alignment of all iGluR protein members in *H. sapiens*. Shown here is the partial sequence alignment illustrating the SYTANLAAF in human iGluR receptors (boxed). This motif is conserved for all iGluR members in *C. elegans* and human.

3B. The hNMDA-2 and hNMDA-3 receptors appeared to share a common lineage while hNMDA-1 diverged. Among the non-NMDA receptors, hAMPA2 and hAMPA4 shares a common lineage while both hAMPA3 and hAMPA1 diverged from the former two. The human kainate receptors have two major lineages, one consisting of hKainate-KA1 and hKainate-KA2 and the other hKainate-GluR5, hKainate-GluR6 and hKainate-GluR7, the latter two were more closely associated with one another than the former. Consistent to reports in the literature, the delta receptors, hDelta1 and hDelta2, diverged from the kainate and AMPA receptor subclasses but are more closely associated with non-NMDA receptors than with NMDA receptors.

Based on phylogenetic analyses (data not shown), it was found that CeGLR receptors share a common lineage with human AMPA receptors, CeNMR2 with human NMDA2 receptors and CeNMR1 with human NMDA receptors. Interestingly, based on this phylogenetic analysis, some C.elegans iGluRs including CeGLR-5, CeGLR-6, CeGLR-7, CeGLR-8 did not share significant lineages with specific human orthologs. CeGLR-8 was relatively distal in the phylogeny with other iGluR members, either possessing characteristics of both NMDA and non-NMDA receptor subclasses or could belong to a completely different class on its own. Based on the sequence alignment, it appears that the SYTANLAAF motif is also conserved among human iGluR receptors.

Phylogenetic analysis of the protein BLAST results using full-length protein sequences showed that more proteins belong to the non-NMDA than to the NMDA lineage (Figure 5). It should be noted that remarkably few sequences shared lineages with CeGLR-5, CeGLR-6, CeGLR-7 and CeGLR-8 proteins. Phylogenetic analysis of the protein BLAST results using only those amino acid sequences that aligned to and spanned the conserved LAOBP region in CeGLR-1 was constructed (data not shown). Most proteins remained on similar lineages as those constructed using the full length sequences. Interestingly, some proteins previously belonged to the non-NMDA lineage were now listed under the NMDA lineage. Specifically, the CeGLR-7 receptor is now listed under the NMDA lineage. In both phylogenetic trees, CeGLR-8 remained on a lineage that is noticeably different than that of NMDA and non-NMDA.

Discussion

The construction of protein sequence alignment and phylogenetic using Vector NTI® and Mega® appears to be valid methods of analyzing evolutionary relationships between C.elegans and other invertebrate species. Amino acid sequences from *H. sapiens* iGluRs were included in this study as comparative markers that facilitate the interpretation of phylogenetic analyses. Some protein BLAST sequences were clustered at phylogenetic lineages that are clearly distal to both C.elegans and H.sapiens iGluRs. This finding may depict the intermediate steps in iGluR evolution among lower invertebrate species. More stringent statistical tests that employ phylogenetic analysis using parsimony or bootstrapping are needed to examine these clustered lineages.

The phylogenetic and sequence alignment data presented in this study showed that CeGLR-5, CeGLR-6, CeGLR-7 and CeGLR-8 were more distally related to not only other iGluR members from C.elegans and H.sapiens but also to other invertebrate species. Interestingly, protein BLAST searches were able to identify iGluRs in lower invertebrate species with relatively simple nervous systems including Ciona intestinalis, Strongylocentrotus purpuratus[50, 51].

It is interesting that no protein sequences included in the alignment and phylogenetic analyses in this study were found to share close lineages with the delta iGluR subtypes. The delta receptors have unknown functions and ligand-binding characteristics. Recently, it was suggested that the delta receptors might have lost channel properties during evolution but acquired adhesion molecule-like functions to modulate depolarization in Purkinje cells[52].

Although phylogenetic analyses may provide important information such as ligand-binding, strategies to deduce evolutionary relationships among homologs and orthologs might not be concluded based on phylogeny alone[53]. For example, NMDA receptors and non-NMDA AMPA receptors, which belong to different lineages on phylogenetic trees have both been found to be implicated in the transmission of fast currents at excitatory synapses in the CNS[45].

Phylogenetic studies in iGluRs may provide insights in the emergence of diversified species in the evolutionary process. For example, lineage information deduced from iGluR phylogeny aided to design mutagenesis studies that elucidated the evolution of RNA editing in the pore-lining region of AMPA2 receptors among two species of cartilaginous fishes[54]. Gene duplication was found to be a potential mechanism for generating alternatively spliced AMPA receptor subunits in vertebrate, Drosophila and C.elegans species via phylogenetic analysis[55]. Indeed, it was shown that a strategy that includes multiple sequence alignment techniques such as BLAST and phylogenetic tree programs with calculation packages such as Clustal may be useful in protein analysis[32, 56].

A shortcoming for this study is that the inclusion method for collecting data from individual protein BLAST searches resulted in the inclusion of protein sequences that, although unique in amino acid sequences, repeatedly originated from a limited number of species. This outcome led to the exclusion of potentially interesting protein hits from lower species from sequence alignment and phylogenetic analysis. For example, protein sequences that originated from Trichoplax adhaerens, a primitive organism belonging to the Placozoa phylum that lacks organs and a nervous system, were excluded from alignment and phylogenetic analyses because they share low sequence identity with C.elegans probe sequences and appeared low in the protein BLAST list. Exclusion of proteins from lower species such as T.adhaerens may affect phylogenetic analysis and interpretation of the results for this study, especially when it was recently reported that glutamate receptors exist in T.adhaerens. In addition, protein sequences of plant iGluR species including those from Arabidopsis were not identified, perhaps to the study method. Proteins with iGluR-like properties including GLR proteins in Arabidopsis

have previously been found in plant species that lack nervous systems with unknown functions[57, 58].

There seems to be a disparity of absolute, standardized methods of conducting phylogenetic analyses in elucidating evolutionary lineage relationships among proteins in different species. A future study could use a propensity score system that accounts for 1) sequence identities of the hit finds to the probe C.elegans queries and 2) uniqueness of species to re-examine the protein sequences generated in this study. This way, proteins from species that are commonly found in protein BLAST queries may not crowd the data while proteins from unique or lower species may be more likely to be included in a list that will be used to perform multi-sequence alignment and phylogenetic analyses.

It will be interesting to examine whether inclusion of plant iGluRs will yield protein sequences that enrich the phylogenetic trees presented in this study. In turn, more proteins from species unidentified in this study may be found that share lineage similarity with CeGluR5, CeGluR6, CeGluR7 and CeGluR8. In addition, alignment analyses could be performed and combined with those with mGluR BLAST searches. Both iGluRs and mGluRs share sequence homology with the bacterial PBPs. A phylogenetic analysis combining iGluR and mGluR protein BLAST data may lead to the discovery of evolutionary lineages novel to glutamate receptor research. Lastly, mutagenesis experiments could be used to test potential ligand-binding targets in a primordial glutmate

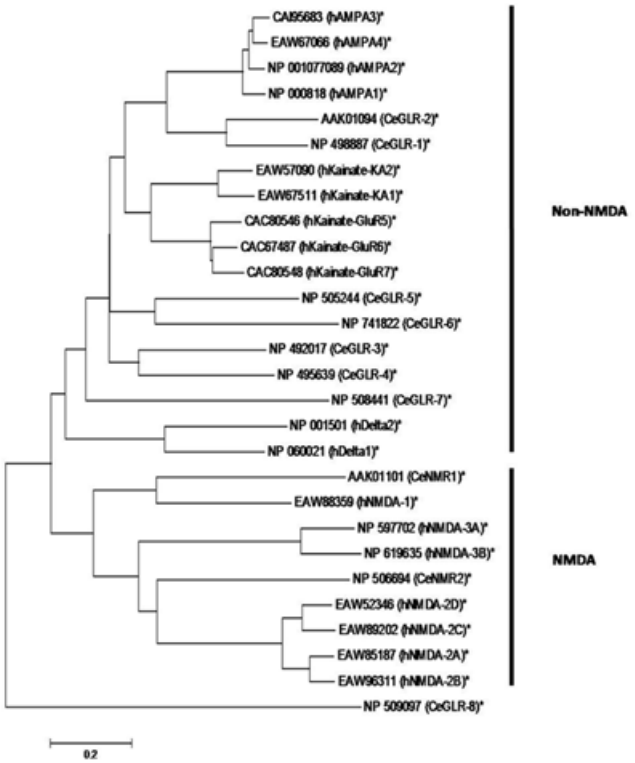

Figure 4: Phylogenetic analysis of all iGluR protein members in *C. elegans* and *H. sapiens*. Receptors could be divided into NMDA and non-NMDA groups. This result shows that the construction of a phylogenetic tree using Mega® produces results similar to that in the literature. Abbreviations of individual receptor subtype were shown as previously indicated.

Figure 5: Phylogenetic analysis of full length protein BLAST sequences. This tree was constructed based on those amino acids that aligned the conserved LAOBP region in CeNMR1. Sequences from *C. elegans*, human and other invertebrate species were used in the construction of this phylogenetic tree.

receptor cloned using data generated from iGluR and mGluR phylogenetic analyses.

Conclusion

Based on results from this study, it can be concluded that CeGluR-8 could either share both non-NMDA and NMDA receptor characteristics or belong to a completely different protein lineage. In addition, it can be concluded that, consistent to the current understanding of C.elegans iGluRs, CeGluR5, CeGluR6 and CeGluR7 share evolutionary lineages with both AMPA and kainate receptors and are more distally related to the delta subclass. Furthermore, phylogenetic analyses of iGluRs using amino acid sequences that contain the conserved LIVBP bacterial periplasmic binding protein categorized CeGLR-7 into the NMDA lineage rather than in the non-NMDA lineage. Lastly, an evolutionary study using protein BLAST searches together with multiple sequence alignment and tree analysis consisting of Clustal calculation packages could be valid and accurate methods for deducing phylogeny among different species.

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JULS

First Principle Molecular Computational Study of Peptide Models and Point Mutations

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Research Article

Abstract

The fundamentals constituents of peptide structure, such as hydrogen bonding and disulfide bridge formation, as well as the affects of point mutations in structure, were explored in the present first principles computational investigation. In this study, the consequences of changing elements of molecular axial chirality in peptide dimerization have been analyzed by observing the the carboxyl-carboxyl hydrogen bonding interactions between two free glycines. To further explore dimerizations, a model was built to study cystine, a dimer of two cysteines linked by a disulfide bond. These dimerizations are known to help stabilize the secondary, tertiary and sometimes the quaternary structure of the protein. Taking such observations into consideration, in order to understand biological phenomena from a quantum mechanical perspective, two peptide models were built for point mutations. A substitution study of a penta-alanine peptide model with subsequent replacement of the central alanine residue by each of the other 19 standard amino acid residues, as well as the mutation of a model of the tri-peptide, Pro-Glu-Glu to Pro-Val-Glu were analyzed. It should be noted that the latter point mutation is present in Sickle Cell β-globin at the sixth position from the N-terminus end. All calculations were performed by first principles computational methods using Hartree-Fock and Density Function Theory (DFT) at the 3-21G level of theory. The underlying objective of the present computational study is the geometry optimization of the peptide models for future studies involving reaction mechanisms.

Introduction

The Importance of Cystine Structure in Relation to Cancer Therapy

Cysteine is an essential amino acid for certain cells under disease and its transporter-mediated uptake of cystine from the micro-environment is vital for their growth and viability [3]. The x-c cystine/glutamate antiporter is a major plasma membrane transporter for the cellular uptake of cystine in exchange for intracellular glutamate. This is a potential target for treatment of cancer and other diseases. By specifically inhibiting the x-c transporter, monosodium glutamate can drastically reduce or completely arrest the proliferation of malignant cells, for which growth is dependent on x-c –mediated uptake of cystine *in vitro* [5]. However, glutamate cannot be used as a therapeutic to inhibit cellular uptake of cystine *in vivo* since it is neurotoxic [7]. It is thus important to understand the structure-function relationship of cystine. Once fully explored, it would then be feasible to find or design an inhibitor for the x-c transporter.

Hydrogen-Bonding between two COOH Groups

In lipid bilayer of plasma membrane of a living cell, helices are commonly associated with one another through hydrogen bonds that form between two protonated carboxyl groups (C=O---H-O)[8]. Such polar interaction is unusually strong and capable of driving transmembrane-transmembrane association, resulting in the formation of quaternary protein structure of very stable dimers or trimers [9]. It has already been suggested that the resonance-assisted hydrogen bonds (RAHB) is a major contributor to dimerization of acetic acid, formic acid and formamide [10]. We revisit this explanation with rigorous theoretical analysis to obtain a full understanding of the phenomena involved in the process of dimerization of two COOH groups.

In particular biological context, an example of dimerization via COOH tandem hydrogen bonding is observed in the trans-membrane helix of Glycophorin A. Having a hydrophobic seven-residue LIxxGVxxGVxxT motif in its transmembrane domain, close packing of Gly-79 – Gly-79 and Gly-83-Gly-83 occurs across the dimer interface [11]. This close packing creates a short interhelical distance, placing the β-hydroxyl of the only polar amino acid in the motif, Thr-87, within hydrogen-bonding range of the backbone carbonyl of Val-84 on the opposing helix [12]. Such interactions contribute to the structure of the glycophorin A homodimer in membrane bilayers [13]. For us, ab initio modeling of biological systems is an ultimate goal but since it is computationally prohibitive, we seek to have an adequate model in order to accurately investigate the polar interaction in membranes. Therefore as a major aspect of the paper, we present and assess a variety of appropriate models for the process of COOH dimerization.

The Effects of Point Mutations on Protein Structure through a Pentapeptide Model

In addition to intermolecular hydrogen bonds, point mutations also cause a significant change in the primary structure of the peptide, influencing its secondary, tertiary and quaternary structure and ultimately protein folding, including over-packing, alteration of structural strains on backbone, and consequently, a loss of protein stability [14]. Although it is important to investigate specific point mutations, it would be beneficial to develop a more basic and fundamental model for the effect of point mutations on oligopeptides using first principles computational studies. By substituting the third residue of a pentaalanine model with each of the 19 natural amino acids, and tabulating the resulting optimized dihedral angles, the impact of amino acid substitution can be studied.

While significant empirical evidence has shown the significance of point mutations on peptide tertiary structure, the greater level of detail and analysis offered by a computational approach cannot be underestimated and this approach has been little pursued.

Two key studies have contributed useful data on backbone dihedral angles of amino acids. One investigation presented the optimal bond lengths, bond angles and dihedrals for all standard amino acids and some non-standard variants, such as selenocysteine, at various levels of theory [15]. A subsequent study proposed a standardized numbering system to characterize the most energetically stable conformer in mono, di-, tri-, and tetrapeptide models [16]. The present study, which applies a similar modeling system to a pentapeptide, can thus help to further comprehend mutational effects on protein structure and their involvement in diseases like sickle cell anemia.

Inquiry of a Specific Point Mutation – Sickle Cell Anemia

Moving from a modelling to a particular case-study perspective, this portion of the study focuses on the point mutation causing sickle cell anemia, in which a glutamic acid residue in the sixth position of both β-globins is replaced by valine [17]. Consequently, cells form abnormal, crescent-shaped erythrocytes, which resemble "sickles". These cells induce intermittent paroxysms of pain (a symptom known as acute painful crisis) as they circulate throughout the body [18].

"Sickling" is a product of hemaglobin tetramer-tetramer interactions resulting in the polymerization of numerous sickle hemaglobin (HbS) into lengthy chains. Normal adult hemaglobin (HbA) is comprised of two α-globin and two β-globin subunits. In an HbS polymer, the tetramers are bound to each other in a 'lock-and-key' mechanism via hydrophobic interactions of the side chain of valine in β-globin, and the complementary hydrophobic patches in α-globin [19]. Atypical of hydrophobic molecules, the valine residue does not aggregate with other non-polar molecules but instead protrudes into the hydrophilic, aqueous environment acting as the "key" [20]. The sickling phenomenon occurs exclusively

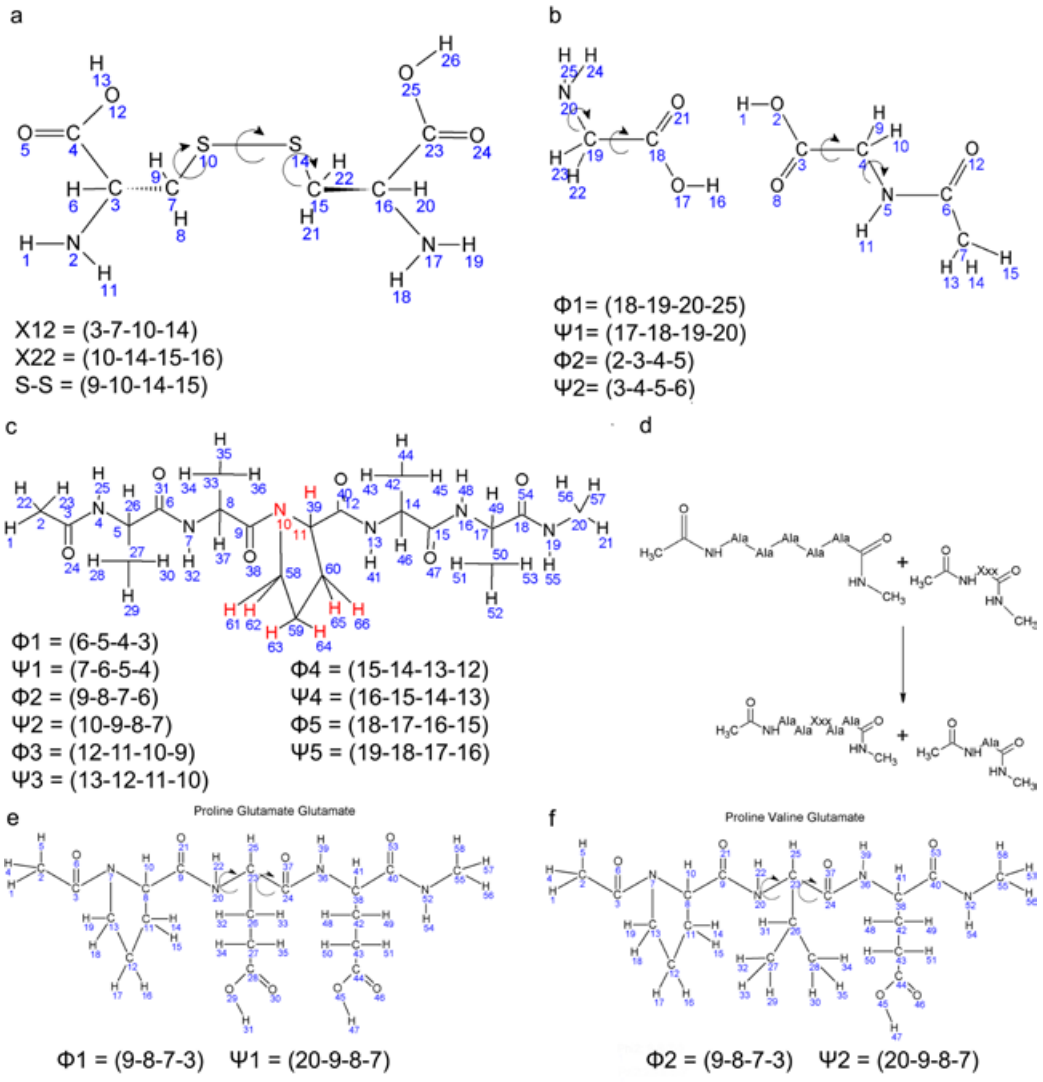

Figure 1: Illustrations of molecules in study with numbered atoms and labeled dihedrals. a) cystine; **b)** acetyl-glycine and free glycine; **c)** pentaalanine with alanine → proline substitution; **d)** point mutation isodesmic reaction; **e)** proline-glutamine-glutamine; **f)** proline-valine-glutamine.

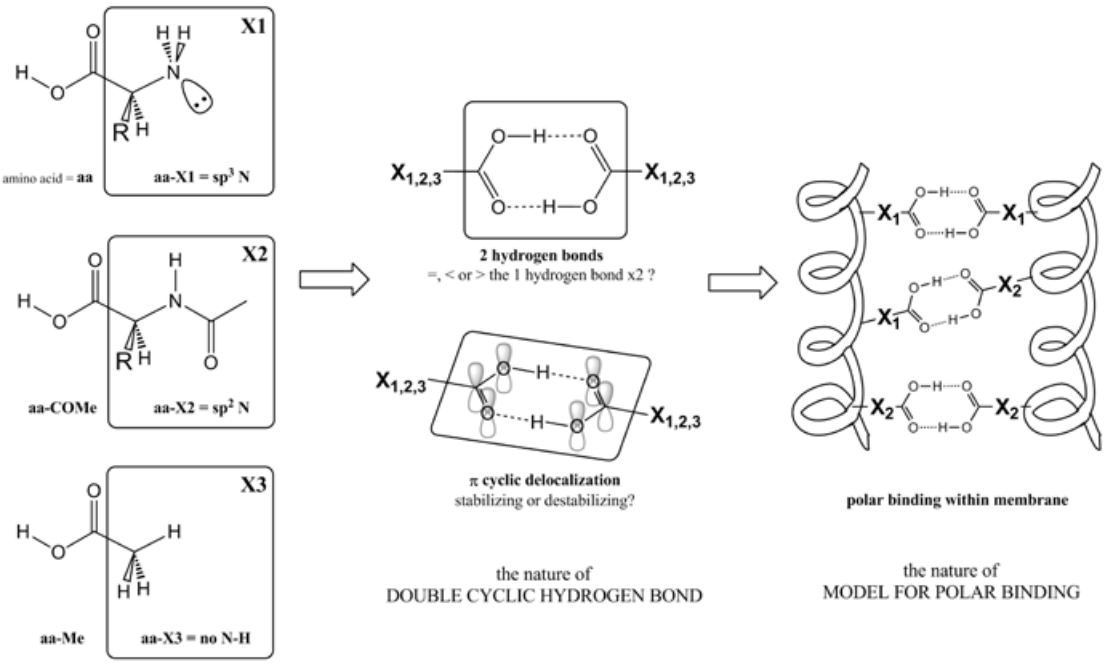

Figure 2: Three types of structures used (left) to model COOH dimer (middle) between two amino acids within cell membranes (right).

in deoxygenated HbS, and in the presence of oxygen, erythrocytes resume their normal, disc-like shapes, suggesting that these conjoining hydrophobic patches become unavailable in the presence of oxygen. Moreover, the original glutamic acid residue has the highest propensity to form α-helices, whereas the mutated valine residue has the highest propensity to form β-pleated sheets, perhaps suggesting that the energy minima for the Pro-Val-Glu and Pro-Glu-Glu tripeptides will differ [20].

The point mutation causing sickle cell anemia is an exemplary model of the cascading nature of protein structure – a single amino acid change in the primary structure induces changes in the upper hierarchies of protein structure, which further alter intermolecular interactions. Acute painful crisis, the hallmark symptom of sickle cell anemia, is a result of this miniscule yet profound change. Adopting a reductionist approach, conformations of the Pro-Val-Glu (HbS) tripeptide are compared with the Pro-Glu-Glu (HbA) tripeptide, which represents the fifth, sixth, and seventh residues in HbS and HbA respectively. This investigation was conducted in belief that knowledge of the primary structure would allow insight into the tetramer-tetramer interactions of the sickling phenomenon.

Scope

Investigating the Disulfide Bridge Conformation of Cystine

The lowest energy conformation of a cystine molecule was determined. To effectively investigate the energy levels, the cystine model (Figure 3a) was constructed only after determining the lowest energy conformation of L-cysteine by investigating its χ11, where the g+ conformer was the global minimum. Note that the cysteine was studied in L-configuration since this is the enantiomer that exists in nature. In this study, one dihedral of the disulfide bond (C7-S10-S14-C15) and two chi dihedrals on the side chain χ12(C3-C7-S10-S14) and χ22(S10-S14-C15-C16) were arranged into the three potential energy minima, a, g-, g+ configurations, producing 27 different expected optimizations to be calculated.

Carboxyl-carboxyl Dimer between Amino Acid Residues

The nature and the process of formation of the double hydrogen bond in a cyclic dimer of two COOH groups are investigated. Having COOH in focus, we use glycine as a model amino acid to avoid COOH-residue interactions. It would be a straight forward process if a COOH-dimer of two amino acids existed with only a single hydrogen bond between two COOH. In that case, the stabilization energy of a double hydrogen bond of a dimer can be compared to the single hydrogen bond multiplied by two. This would be clear measurement how much stabilizing is tandem COOH hydrogen bonding. Since we cannot have a dimer with only one bond between two amino acids we investigate the reaction coordinate of the process of forming COOH dimer. The models with both sp2 and sp3 nitrogen as well as without nitrogen and –CH3 instead we considered as ligands to COOH. Once the dimer is formed, we analysed its electronic properties of the cyclic COOH-HOOC structure (Figure 2).

Point Mutations of Pentapeptides in an Alanine Environment

The present study aimed to create a generalized model of point mutations using first principles computational chemistry. The focus was on the effect of an amino acid substitution in a pentaalanine environment (Figure 1c), in which the central alanine is replaced by another amino acid through a point mutation isodesmic reaction (Figure 1d). The study was then extended to the conformational analysis of the alanine substitution with the other 19 standard amino acids. The aim was to determine optimized values for the defined dihedrals of interest (φ, ψ) in the pentapeptide environment (Figure 1c). For the purpose of the following study, the original and mutant pentapeptides were initially set with all backbone dihedrals in the anti (a) conformation. This peptide will be referred to as the "anti-input conformation" hereafter. To ensure correct identification of the optimized conformer, the

optimized φ and ψ dihedrals surrounding each amino acid from a previous investigation [15] were applied to the dihedrals of interest in this study. This will be referred to as the "monopeptide-optimized-input conformation". The following ranges were used to classify the resulting dihedral angles: anti (a) (120˚-240˚), gauche plus (g+) (0˚-120˚) and gauche minus (g-) (240˚-360˚).

Proline-Glutamate-Glutamate and Proline-Valine-Glutamate Tripeptides

This portion of the study includes the computation of energies for φ1, ψ1, φ2, and ψ2 dihedrals surrounding the central residues for the Pro-Val-Glu (Val) and Pro-Glu-Glu (Glu) tripeptides. The isodesmic reaction from Pro-Glu-Glu to Pro-Val-Glu can be visualized in figures 1e and f respectively. Arranging the desired dihedrals in a, g+ or g- configurations and setting the backbone at the anti-anti conformation, (with the exception of the proline residue which must be set at phi = g+), 18 potential conformations result after geometry optimization. It should be noted that because calculations were performed in the gas-phase in vacuo, glutamate was used in place of glutamic acid.

Materials and Methods

All computations were carried out using the Gaussian 03 program package (G03) [21]. Each structure was initially optimized using the ab initio restricted Hartree-Fock (RHF) method [22] using the standard split-valence 3-21G basis set that is incorporated in G03 [21], except for the COOH interaction analysis which was done in optimized at the B3LYP/6-31+G(d) level of theory. Total energies were given in Hartrees, and relative energies were given in kilocalories per mole (with the conversion factor: 1 Hartree = 627.5095 kcal.mol-1. Dihedral angles studied were sampled at the suggested minima of anti (a), positive gauche (g+), and negative gauche (g-) configuration, to find out all probable conformers in the gas phase in vacuo- at zero Kelvin. The optimized geometries and energies were computed from first principles using G03. The relative energies of the different stable conformations were then calculated. The Z-matrices associated with the local minima are described in the supplementary material published online.

Results and Discussions

Investigating the Disulfide Bridge Conformation of Cystine

After completing optimization for the predicted stable conformers, it was found that only 18 of 27 expected conformers exist (Table SS 1). Computations of cystine model by varying dihedrals χ12, the S-S bond and χ12, yielded the (a, g+, g-) conformer and the (g+,g-,g+) conformer as global minima and the (g+,a,a) conformer and (a,g-,a) conformer as global maxima. Approximately 14 kcal/mol of energy difference between the lowest energy conformers and the highest was observed. It is possible that intramolecular hydrogen bonding between amino group and carboxyl group of cystine leads to a preference for certain conformers over others. As can be observed in Figure 3, the lowest energy conformers (Figures 3a and 3b) exhibit possible hydrogen bonding, whereas the highest energy conformer (Figures 3c) does not.

Carboxyl-carboxyl Interactions between Amino Acid Residues

The reaction pathway of two COOH monomers associating into a dimer is presented in Figure 4. The energy values are presented in Supplementary Materials (Table SS 2). Since the reactions are in the gas phase and only bond formation is involved, it is assumed that there are no energy barriers. It is interesting to note that the dimer with only one hydrogen bond, II is a transition state and is thus most likely not included in the mechanism of dimerization leading to the final product with two hydrogen bonds. The final product V is most likely formed through the intermediate dimmers III and IV. From the values of stabilization energies of V and intermediates, one can see that V is approximately twice as stable as II or IV. Since each of the intermediates has at least two hydrogen bonds, we can conclude that two tandem COOH hydrogen bonds in V are more stabilizing than two separate hydrogen bonds. In other words, because the stabilization energy of intermediates is the energy of one H-bond between COOH and the other H-bond not between two COOH groups, the H-bond between COOH groups is less than half the energy of the two H-bonds between two COOH groups.

The second conclusion comes from Natural Bond Orbital Analysis and Nuclear Independent Chemical Shift of the COOH dimer V presented in Figure 5. [23] RAHB indicates that π electrons are involved in the stabilization. However we calculated the occupancy of all p Atomic Orbitals which could be involved in formation of π Molecular orbitals [24], [25]. The occupancy numbers are similar for the COOH, isolated as a monomer and a dimer. Since tandem H-bond is also cyclical, there is a chance of de/stabilization by anti/aromaticity [26], [27], [28]. NICS value indicates that the cycle is non-aromatic. Additionally, we measured the charge on each atom and bond lengths in COOH. The results suggest slight bond length equalization when COOH of a monomer is compared to COOH of a dimer. However NBO and NICS indicate that this is not due to dramatic electron redistribution of π electrons. Consequently, the

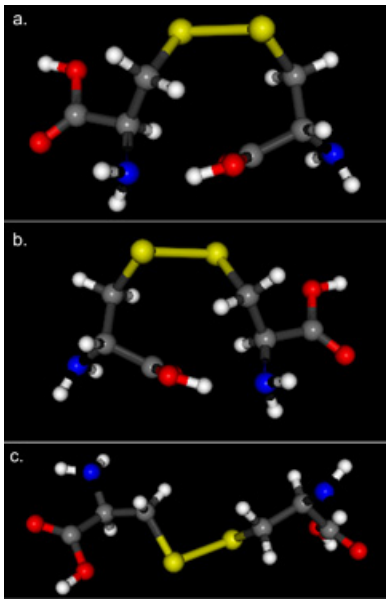

Figure 3: 3D visualization of important optimized conformers of cystine. a) a,g+,g- conformer, one of the two global energy minima; **b)** g+,g-,g+ conformer, the other global energy minimum; **c)** g+,a,a conformer, one of the two global energy maxima.

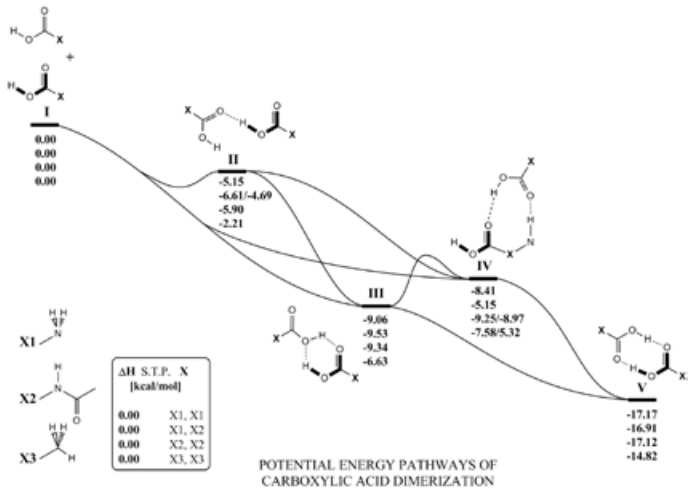

Figure 4: Energy reaction pathway of dimerization process for model amino acids (HOOC-X, X=X1, X2, X3) at B3LYP/6-31+G(d) level of theory.

amplification of hydrogen bond is due to, equally important, simultaneous and synchronous charge transfer along σ bonds between a pair of carboxyl C=O and hydroxyl O-H groups. Finally, we add that, RAHB needs to be considered as a consequence of sink-and-source type inductive effect rather than a cause for unusually strong stabilization of tandem COOH dimmers [29], [30].

The Effects of Point Mutations on Protein Structure through a Pentapeptide Model

All optimized backbone dihedrals for each of the central amino acids were determined and tabulated (Table SS3). For all amino acids other than proline, arginine, glutamine and methionine, the a conformer was achieved, following the criterion indicated previously.

The proline pentapeptide experienced the greatest difference in ψ3 and φ3 between the two conformational models. In addition, there wass a significant deviation in φ3 from the a conformation in both the anti-input and monopeptide-optimized-input conformations. However, while the ψ3 result was a in the anti-input conformation, it was g+ in the monopeptide-optimized-input conformation. Structurally, the deviation from the anti conformation allows proline to take on a conformation that causes the entire pentapeptide to turn at this residue (Figure 6). This solidifies the idea that proline is prone to form β-turns [23], which allows peptides to turn and form anti-parallel and parallel β-sheets. The φ3 value remains relatively consistent between the two studied conformations because the peptide bond is locked into a ring structure, reducing its flexibility; however, ψ3 is unrestricted, allowing it to take on other angles. This variation in ψ3 between the two studied conformations suggests that two different conformers of the mutant peptide were identified. The difference in energy of the two optimized conformers, that is 2.561 kcal/mol, can be explained by observing the bond distance between Oxygen-38 and Hydrogen- 41 (Figure 1c); in the monopeptide-optimized-input conformation, the bond length is approximately half of that of the anti -input conformation (2.097 Å versus 4.152 Å, respectively). This closer bond distance, possibly indicating the presence of a stabilizing hydrogen-bond, and lower energy associated with the monopeptide-optimized-input conformation suggests that this is the preferred conformation of proline in a pentapeptide model.

While arginine, glutamine and methionine do not experience significant deviations from the a conformation for the ψ3 and φ3 dihedrals, they consistently show a change in the optimized φ4 dihedral to the g- conformation for both anti¬-input and monopeptide-optimized-input conformations. These residues are highly polar and have significantly longer side chains extending away from the backbone compared with other amino acids, enhancing steric interactions between the side chain and the peptide backbone. Furthermore, in all three peptides, the side chains were oriented towards the φ4 dihedral, allowing for asymmetry in the overall peptide and causing this φ4 deviation. This seems to suggest that side chain conformation rather than the presence of the side chain itself plays a bigger role in determining the backbone angles in mutant peptides. Furthermore, specific properties pertaining to the central residue, such as hydrophobicity or acidity, may also play a role in determining the backbone and side-chain conformation and will be explored in future investigations.

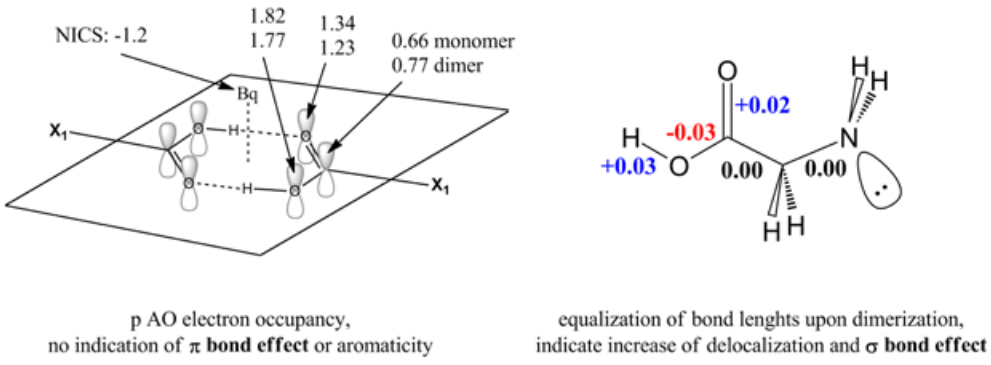

Figure 5: Electron occupancy of COOH cyclic dimer (left) and change of bond lengths in COOH group between a monomer and a dimer of glycene (right).

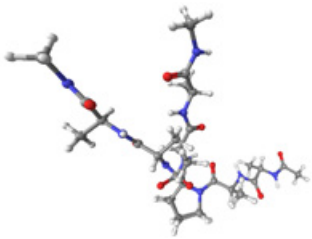

Figure 6: Monopeptide input conformation and the anti-input conformation overlayed over one another. The proline pentapeptide experienced the greatest difference in ψ3 and φ3 between the two conformational models.

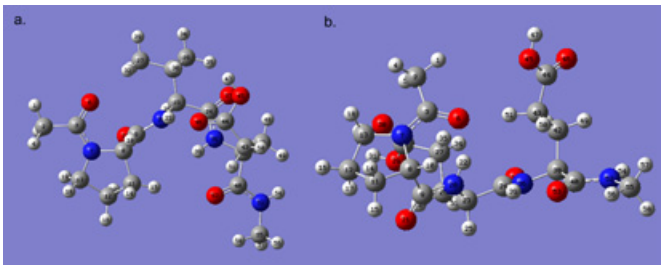

Figure 7: 3D visualizations of a) Most energetically favourable Pro-Val-Glu conformer at g-+g+ configuration. **b)** Most energetically favourable Pro-Glu-Glu conformer at ag+ configuration.

Further analysis will be also done to investigate all possible combinations of a, g+ and g – for dihedrals φ3 and ψ3 of the pentaalanine model to further understand the effect of specific changes in the backbone dihedral of a peptide on peptide structure and folding.

Inquiry of a Specific Point Mutation – Sickle Cell Anemia

Among the 9 expected conformations for each tripeptide, 9 energy minima were discovered for Pro-Val-Glu and 8 energy minima were discovered for Pro-Glu-Glu, but most optimized dihedral angles did not converge to values within the idealized range (Table SS4). Preset g-g-, g-g+, and ag+ conformations for the Pro-Val-Glu tripeptide converged to φ2 = g-+ (bordering -120) and ψ2 = g+. This energy minima annihilation is most likely explained by the fact that this conformation permitted hydrogen bonding between side chains and the backbone. For example, it appears as if the valine twisted to create a potential for hydrogen bonding at 37-31 (O--H) and 37-35 (O--H) for the preset g-g- conformation and 37-30 (O--H) for the ag+ conformation.

The most energetically favourable Pro-Val-Glu tripeptide conformation was the preset g-g+ conformation, which converged to g-+ and g+ dihedral angles (Figure 7a). In this conformation, the glutamate side chain bent inwards, towards the valine side chain, and they seemed to be juxtaposed in such a way to permit hydrogen bonding between atoms 46-22 (O--H). Furthermore, hydrogen bonding appeared to be a large source of stabilization within the most energetically favourable conformation of the normal Pro-Glu-Glu tripeptide; ag+. As seen in Figure 7b, it is speculated that potential for hydrogen bonds existed both within the backbone, and between glutamate side chains and the backbone at atoms 6-22 (O--H), 6-51 (O--H), 30-19 (O--H), 30-5 (O--H).

Conclusion

The results obtained from the present conformational analysis of cystine models would be of value in future molecular docking studies with the x-c antiporter. They could also serve as the basis for future studies investigating the role of cystine disulfide bridge conformation in determining the secondary, tertiary, and quaternary structure of a protein. Conversely, the analysis of the COOH interactions via hydrogen bonds will contribute to the future study of a model of glutathione which is believed to form similar COOH interactions between glutamate and glycine residues, leading to a "basket" conformation. Finally, point mutation anaylysis of pentapeptides and tripeptide of Pro-Glu-Glureveals that hydrogen bonding is also a significant source of stabilization for the ultimate energy minima of both normal (Pro-Glu-Glu) and mutant (Pro-Val-Glu) tripeptides though further investigation is required to confirm this hypothesis. Computational analysis at a higher basis set would most likely preclude energy minima annihilation and provide a more thorough investigation of the conforms with the assumption being that more energy minima would be discovered within preset, idealized dihedral angles. Furthermore, an extension of the current study could be to investigate not only anti backbone conformations but also gauche - and gauche + backbones as well as side chain conformations.

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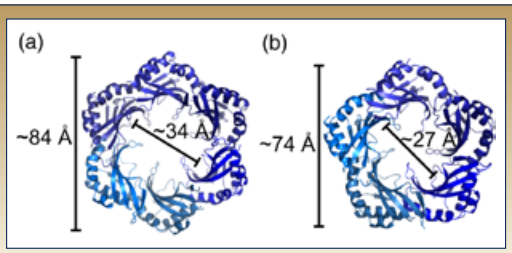

Crystal structure of the mutant gpU D75A tail terminator protein hexamer from bacteriophage λ (a) and wild-type gpU pentamer (b). Pell LG, Liu A, Edmonds L, Donaldson LW, Howell PL, Davidson AR. (2009) *J Mol Biol*. **389**(5):938-51.

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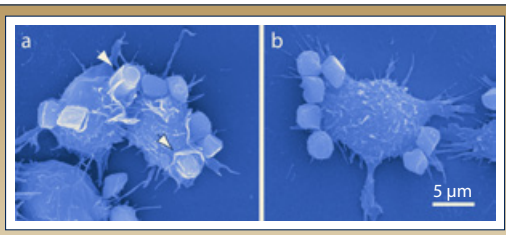

Macrophage cells extend lamellapodia (arrows) around opsonized red blood cells during phagocytosis (a). Inhibition of the coronin1 protein blocks lamellapodia formation but not binding (b). Yan M, Collins RF, Grinstein S, Trimble WS. (2005). *Mol Biol Cell*. **16**(7):3077-87.

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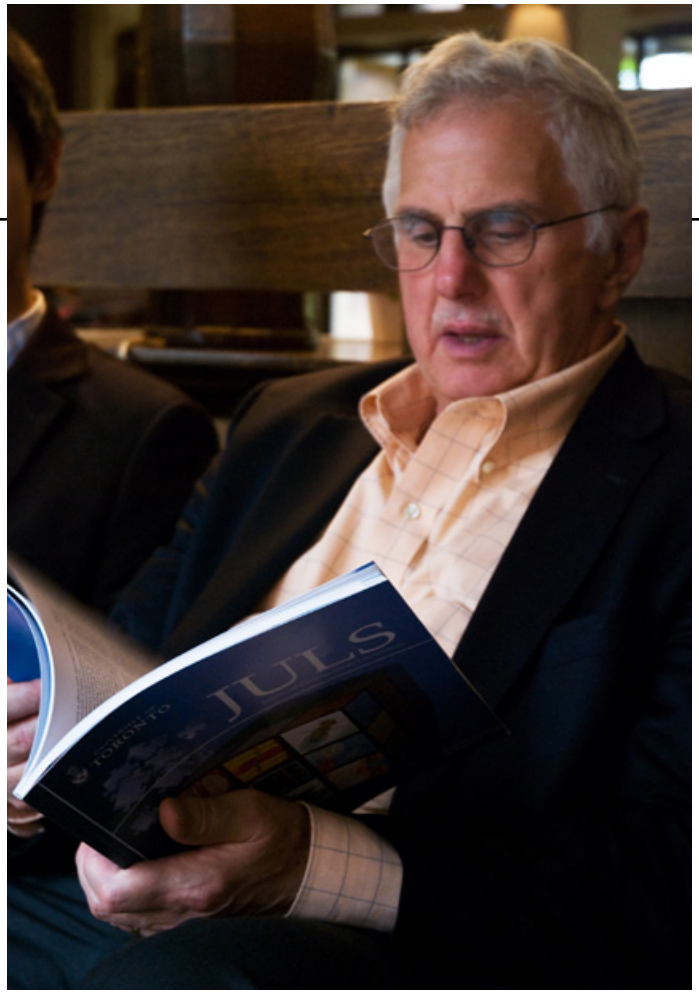
Interviews at the 50th ANNIVERSARY GAIRDNER AWARDS

In 1957, Canadian philanthropist James Arthur Gairdner created the Gairdner Foundation to recognize investigators whose discoveries in the biomedical sciences have played a significant role in improving human life. Since the first Gairdner Awards were given out in 1959, they have become recognized as one of Canada's most important international awards. Reception of an award is traditionally considered an overture to receiving a Nobel Prize in Chemistry or a Nobel Prize in Physiology or Medicine. As of 2009, there were 79 Nobel Prize winners among prior Gairdner awardees.

2009 marks the 50th anniversary of the Gairdner Awards. This year, the Foundation has expanded to include the Canada Gairdner Wightman Award, which recognizes Canadian innovators whose work has made significant contributions to medical science, as well as the Canada Gairdner Global Health Award, which recognizes those who have made significant advances for improving health in the developing world. The seven Gairdner Award Recipients of 2009 were:

- **Dr. Richard Losick, Ph.D.** (Harvard University, USA) and **Dr. Lucy Shapiro, Ph.D.** (Stanford University, USA) – "mechanisms that define cell polarity and asymmetric cell division"
- **Dr. Kazutoshi Mori, Ph.D.** (Kyoto University, Japan) and **Dr. Peter Walter, Ph.D.** (University of California, San Francisco, USA) – "key pathway in the unfolded protein response which regulates protein folding in the cell"
- **Dr. Nubia Munoz, M.D.** (National Cancer Institute, Colombia), awarded the Canada Gairder Global Health Award – "essential role of the human papilloma virus in the etiology of cervical cancer on a global level which led to the development of successful prophylactic vaccines"
- **Dr. David Sackett, M.D. OC, FRSC, FRCP** (McMaster University, Canada), awarded the Canada Gairdner Wightman Award – "leadership in the fields of clinical epidemiology and evidence-based medicine"
- **Dr. Shinya Yamanka, M.D., Ph.D.** (Kyoto University, Japan) – "demonstration that the key transcription factors which specify pluripotency may reprogram somatic cells to pluripotent stem cells"

On the following pages, we sat down with six leading international scholars to reflect on their careers in research and discuss what the future of science holds.



Dr. Bruce Alberts

Dr. Bruce Alberts earned his Bachelor and Doctorate degrees from Harvard University. He was a professor at Princeton University until he joined the faculty at the University of California, San Francisco, where he became the Chair of the Department of Biochemistry and Biophysics. He served as the President of the National Academy of Sciences for 12 years. He is one of the authors of Molecular Biology of the Cell, and he is currently the editor-in-chief of the journal Science. He is a past recipient of the Canada Gairdner International Award.

Exposure as a young person to science makes a huge difference – just one positive experience can shape your entire career.

JP: You are one of the authors of Molecular Biology of the Cell, a textbook that is widely used in introductory biology courses in universities across North America, and many of our readers are familiar with the book. How did your undergraduate education influence your career?

BA: Everybody's undergraduate education has a profound effect on their career. I had taken high school chemistry, which I very much liked, and I wanted to see what I could do with chemistry as a career. There were two speakers who came to my high school who addressed my question. One of them was a chemical engineer who talked about processing huge volumes of materials and showed these big factories of pipes and I thought that was very boring; the other one was a very articulate doctor who talked about medicine from the point of the view of science, and how science contributed to medicine. At that point, I decided I'd be a premedical student. A lot of people do this, at least in the United States, because there's no real appreciation for a career in science. I never knew a scientist, and you read about Einstein having to work in a patent office because he couldn't support himself. That may have changed now but this was the 1950's.

I went to Harvard as a premedical student, and as a premedical student you have to take a lot of science courses. That involved a lot of afternoons in the laboratory, three days a week I'd be in the laboratory. I found these laboratories very tedious actually, in retrospect, it was nothing like science. It was: follow directions, take some measurements, weigh out some stuff, write up in your notebook, and turn it in to see if you've got the right answer. But of course we would all compare notebooks with everybody else so we were learning how to cheat, among other things! It was more like cooking than science. After two years of this, I got into a physical chemistry course, which I really liked, but the laboratory for that course was really bad. It was the worst. So after one semester I wondered if I could continue to take the course but drop the laboratory, because the laboratory had nothing to do with the lecture part of it. It was only then that I learned that I could do this, but only if I went into a research laboratory.

And so I did go into a research laboratory, the laboratory of Paul Doty, a physical chemist. I discovered that science was nothing like those laboratory courses I took! I was actually quite lucky: I stayed over the summer of my junior year to work in the laboratory. After that I decided I wasn't going to apply to medical school, I would go to graduate school instead. By being in a laboratory I realized that there was such a thing as getting a PhD and then getting a job afterwards.

So my undergraduate studies and especially my exposure to a research lab made a huge difference. Ever since then, I've been a great advocate for getting students into research labs and out of those cooking labs very early on.

JP: As President of the National Academy of Sciences, you became very active in promoting science education, not only in universities but all the way down to elementary schools. And this year's inaugural issue of *Science* had a special focus on science education. What led you to get started in that field and why do you think it's so important?

BA: In part, it was the experience I just told you about, which emphasizes that exposure as a young person to science makes a huge difference - just one positive experience can shape your entire career. I've seen that repeatedly in young students who come, for example, to UCSF where my laboratory was for many years. They come from high school and they spend two months in the laboratory in the summer and it really doesn't make a difference what they accomplish, but they see that it's a different life and a different culture They see that there is a new possibility for this kind of life if you study and are successful in college.

We've done this over and over with students from backgrounds that normally have low college enrolment rates, and they are dramatically affected. Then you ask yourself, well why can't you do this as part of learning in school? Why do you have to make school so boring? I guess my first exposure to the wider opportunities for science education came from when I was in San Francisco and going to a workshop for teachers given by the Lawrence Hall of Science. It was on 4th grade science kits that teach something on pendulums, and on the idea that you can investigate the world successfully if you keep everything else constant and change one variable at a time. Seeing that piece of curriculum made me recognize that there is great opportunity to teach science differently not only in elementary school, middle school and high school but also in college. It fit perfectly with my own experience that studying science as a bunch of facts you learn and then give back on an examination is quite different from doing science. If you want a generation of young people to be excited about learning broadly, but also about science, and excited about school, this kind of science education is a great way to change their attitude about education.

Any system that measures the total number of papers published is going to distort science in a very bad way.

I see this as a very powerful opportunity that's still being largely missed in most places in the United States. So when I went to the National Academy of Sciences, they convinced me that I should take this job - which I didn't really want initially - because I can do something in education with it, and I did try to do that. We had a manual of science education standards, we published over a hundred reports on how people learn that tried to make the point that education is really important, that it's worthy of the greatest scholars' attention as the National Academy of Sciences, and that there are real opportunities to do things differently. Then I finished at the Academy after 12 years, and I got a call from some recruiting company asking me whether I would be interested in being considered as the editor-in-chief of *Science*, and I said "Of course not, I'm too old!" Then maybe six months later I got a call saying, "Well look, we'd like it for you to be the editor-in-chief of *Science*", and they knew how to get me to do it, after all this is a place where you could promote the education agenda that you failed on, because we didn't really finish at the Academy. It's a hard problem and we didn't really succeed to revolutionize the nation. So after giving it about a weekend of thought, I said "Well, I might as well keep trying!"

At *Science* I had the promise that I could use it for the purpose of science education. Of course we had to keep it going as a great magazine for science research, news, and commentary, but one of the simple things we've been doing is to have a major special issue every year on education. Last year it was about education and computer technology, and this year it's going to be about science education as an avenue to powerfully teach language and communication skills, such as reading, writing, and reasoning skills. The kind of writing that's important for adults is not the kind of writing that I learned in school, which was writing for assignments by making up some kind of story, a fairy tale, and I was terrible at this. Most literacy is reading those kinds of stories as young children and writing stories that are imaginary narrative texts. But there's another kind of text called informational text, which is what you need to read when you're trying to do a science experiment, even as a young child in school, or if you're trying to write up your experiments or you're trying to communicate with other people to try and enable them to do what you did. So part of this issue is going to be talking about these two different kinds of texts, and the opportunities to use science to motivate young people to learn how to communicate more effectively.

The other thing that we're excited to do is to publish really outstanding education research along with the research in *Science*. Then there are of course all the editorials that I and other people write. I've had maybe 10 people in the last year write about education, and maybe something like 20% of the editorials have something to do with education. I also think one of the major avenues for change in the United States for science education comes from the fact that the only national curriculum is the Advanced Placement (AP) exam. The Academy did a study in 2002, and they concluded that the three science AP exams needed major changes. Those changes are now starting to happen: the new standards for the AP exams that are still not out yet but they're planned. I included

an article by a senior Academy member, Bill Wood, about a month ago about the new AP exam in Biology and why he thought that it was a good change. So in those ways and others, that I probably don't have the time to talk about, I'd like to see if *Science* can help steer science education in a new direction.

JP: As the editor-in-chief of *Science*, you have enormous influence over what research has an impact on society and the scientific community. What are some of the responsibilities and challenges that you face and how do you meet them?

BA: We now have something like 14,000 submissions a year, so how do you choose which 850 papers to publish? This is a high stakes, difficult task. Obviously there is no way that I could personally do this. We have over 20 highly skilled and highly trained scientist editors and specialists in all kinds of different fields who've all had post-doctoral experience. When we hire new editors I always talk to their research advisors, since we're looking for people who would have been great scientists but who want to go into scientific editing. The reason is that it takes tremendous talent to make a good choice for papers, and of course you're always going to make mistakes, potentially terrible mistakes! Publishing things you shouldn't or not publishing things you should have published.

We have tried to develop processes at *Science* that don't involve me personally because there's no way that I could look at 14,000 papers a year, even looking at their abstracts. So one of the things we're doing – we're all scientists, we're trying an experiment – is we're organizing the editors into teams. Currently there are five teams of four editors, three teams in biology and two in the other sciences that jointly make the decision. Some papers are so obviously bad that I don't take them and others are so obviously good that everybody wants them, but most papers are more difficult choices, so now it's a team decision.

It really is a big responsibility once you realize that papers that get published in *Science* very often get published in newspapers all around the world, so in a way the things that you select and highlight are a way of communicating (small "s") science to the world. It's not only us of course, there are others that do this, but a few journals seem to have a lot of the attention of the news media. It's also an opportunity if you think about it: why should you have a journal like Science? Why not just have everyone put everything out on the web, or have journals like *PLoS One*, which I think is a great idea, where it is very easy to publish as long as it makes sense, it doesn't really have to be important? Well, one of the arguments for having a journal like *Science* is to encourage the right kind of science to be called to the attention of the public.

The *New York Times*, *Newsweek*, around the world, they're very effective in getting science correspondents to think of writing about different kinds of science, but they're overwhelmed by press releases from universities and research hospitals, so they need some help in trying to pick out what's worth reporting on. This is another important function that *Science* has, because otherwise you might have the press just full of press releases from biotech companies hyping something that they're trying to sell. In fact, we have an annual meeting now with major editors, that we initiated – it first started with Phil Campbell, the editor-in-chief of *Nature* (we're all friends!), myself and Randy Schekman, the editor-in-chief of *Proceedings of the National Academy of Sciences*. We have a great responsibility to try to make sure that science is functioning as well as it should be.

What kind of journal policies, or what kinds of things can journal editors do, that will help to make science work well? One of the things we came up with at our first meeting, which is about to be experimentally initiated, is the idea of having an author ID for every scientist who is publishing. With the increased research activity in China and many individuals having the same name, you can't really figure out what this person has published. This of course has also been a problem with some English names.

We have also been very disturbed about the occasional times when data is manipulated by somebody in the laboratory under some kind of pressure. Now with digital imaging, it's very tempting for somebody under pressure to make it look better than it is. The traditional thing is to have one author take responsibility for everything, but I don't think that works anymore for many papers because they have a large number of authors in different groups, some doing mass spectrometry, others doing electron microscopy, other doing biochemistry. No one person has the expertise to do everything, and secondly, it's unreasonable if you have a group in San Francisco and another group in Manitoba to expect one person to control everything. So we are going to initiate a policy at our three journals where there has to be one senior author for each set or type of result, and that person has to sign off that they received the original data. If there's a figure with the photograph of a gel in it, they are obligated to go ask the student who prepared that figure to show how they actually prepared it and where the original gel is that they took this from.

I think this will help everybody, because if I were to see myself as a lab supervisor, and a student gave me a figure that they made, and I challenged them myself to see the original

One of the really eye-opening modern changes in the world that all national leaders need to look at is what China is doing and how they're talking about science.

data, it's almost as if I don't trust that student. But under the new operating system I can say that I have to sign this paper saying that I've seen the original data, and obviously I can't do that without actually seeing it, so although I trust you, please let me see the original data. These small adjustments to our system are the kind of things that I think the major scientific journals could do that would be helpful to the scientific enterprise as a whole.

KZ: Do you think the introduction of metrics, such as the impact factor or the h-index, has played a role in increasing this pressure to publish?

BA: Well certainly, especially in some countries, where they have this highly productive system of giving you points according to the impact factor of the journal that you publish your paper in. I've actually written an editorial about this. I think that it's very important how you evaluate scientists; otherwise you have perverse incentives on publishing. I said in the editorial that I'm very much in favour of a kind of system that the Howard Hughes Medical Institute and many other institutions in the United States use, where if somebody's being evaluated you don't ask them just to provide their list of 30 publications and sort of guess how good that is by the names of the journals that they were published in, because you can't possibly read those 30 papers.

Instead you ask them to just give five papers that they've published, write a paragraph about why each one of them is important and send the papers. This way the reviewer has an obligation to actually read those papers. I think that system has a very different way of affecting the values of young people and scientists in general. If I have to do five good papers, it doesn't make any difference if I do a hundred bad ones, I just have to do five good things in five years. Sydney Brenner, who's here and who's very imaginative, actually once wrote on this subject, saying that everybody should be given a license to publish 50 papers in their life, so every time you publish one you're really careful about what you put in it. Any system that measures the total number of papers published is going to distort science in a very bad way.

JP: You won your Gairdner Award in 1995. Reflecting on your experience then, and your experience here at the 50th Anniversary, what makes this year's event special?

BA: This year is obviously special because they've invited so many past winners of the Gairdner Awards here, and they've had this huge series of talks in Toronto, plus all kinds of things around Canada. I very much enjoyed the scientific talks because I don't get that many chances to hear my colleagues talk about really interesting things. I also enjoyed some of the people, Oliver Smithies for example, talking about his life, and that's fantastic for young people because we never have the chance to do this. Yamanaka talking about setting up his first lab, and having to think of something imaginative to do because he didn't have any resources, he wasn't in a first-rate university, and the only resource he had was getting graduate students, so he had to think big. What if he had gone to a first-rate university and didn't have to think big? He'd probably still be doing boring things! All of those are really important lessons.

I've really enjoyed the three days of talks. I think the Gairdner Awards have been very successful, by and large-excluding myself, they've chosen well, and it helps give Canada a focus on science. I think Canada could benefit from more focus on science at the highest level. One of the really eye-opening modern changes in the world that all national leaders need to look at is what China is doing and how they're talking about science. The leaders in China are mostly engineers and scientists, and their top priority for the future, thinking long-term, is the development of science and technology. They're talking about this in national and international forums with Presidents of nations and leaders in industry, in ways that haven't been talked about much at those levels. I think China, in its example and its passion for science and technology as the source of its future success, can help drive other national governments to rethink their own policies.

Dr. John Sulston

Dr. John Sulston graduated with a B.A. and Ph.D. from the University of Cambridge, did his postdoctoral training at the Salk Institute for Biological Studies, and later joined the MRC Laboratory of Molecular Biology. He served as the Director of the Sanger Centre for 8 years, overseeing a major component of the Human Genome Project and championing the public effort. He is the Chair of the Institute for Science, Ethics, and Innovation at the University of Manchester. He is a recipient of the Canada Gairdner International Award and the Nobel Prize in Physiology or Medicine.

It was dedicated work, doing one thing everyday, the same thing, looking down the microscope. It was great!

JP: How did you first get involved in working with *C. elegans*?

JS: Oh! Well that's way back. You've just been listening to my boss, Sydney Brenner, and it all started in 1969 when I joined him. It was my second post-doc; I had been in California and then came back to the UK. I had no particular idea what I was getting into or how long I would stay, but somehow after the first year I renewed, and eventually I became a staff member in his group. I just kept going, because it was a tremendous group of people. Bob Horvitz was there after a very short time, and there were a number of other people who were really eager. We were working on something which was almost untouched. People had worked on the nematode before, but Sydney had some ideas about this very small worm with its more or less fixed numbers of cells. *C.elegans* has a fixed number of cells, it is clear, has a rapid development, and the ability to slice it up and put it under a microscope (because it is so small), meant that we could really find out an awful lot of stuff at the cellular level. So we all started working and finding things out about its basic processes. The important thing, the key thing, was the genetics. The whole point was to find out how genes control the development of an animal, which, of course, we haven't discovered but we took a lot of steps towards it, which is why it was a good project.

JP: How did you decide to pursue a career as a scientist?

JS: Ah well the thing is, you must realize, I've never had a career. I've never taken it seriously. I always find it strange when people say "what's the right way, what choices should I make to have a career in science and to be successful?" I've never done any of that. I was always very doubtful. The only *constant* thing is that I always was interested in science and that goes way back to childhood, probably to babyhood, that I was a manipulator. I was somebody who loved to mess around with all kinds of stuff, go all the way from physics to biology. Messing around with all sorts of things I could find at home, taking them apart into pieces, finding out how they work, eventually putting them back together again. Then at school, taking science and then naturally continuing with science at university.

So in that sense, I was never in any doubt that I was a scientist. I think the approach of understanding ourselves and everything about us, which is the scientific method as far as I'm concerned, is just the way we go. We don't, for example, pay a lot of attention to what people have said in the past. Doesn't matter what people have said. The point is: what can you prove

with the evidence of your own eyes and the experiments you can do with your own hands? That's the scientific method and it appealed to me, I suppose, because I'm a sceptic of people's views. That is probably characteristic of all scientists. The motto the Royal Society in the UK, the academic society, is *Nullius Inverba*, which is Latin for "Don't take anybody's word for it."

TJ: Do you think that the way you progressed with your career, just accidentally, or not in any way planned, is an anomaly? How important do you think is planning out your career, or working for those grades or that particular internship, in getting you somewhere?

JS: I don't think it matters. And I think it still doesn't matter. I think people pay far too much attention to that kind of thing. Now, it depends obviously on what you want to achieve. I'm sure there are some jobs where you do have to plan quite far ahead, you say I'm going to do this, you're absolutely determined, you go and get all the qualifications. But you see, I was never like that. I didn't care where I ended up. I didn't care if I got paid; I obviously would have found a way to avoid starvation somehow or other.

Of course growing up in a developed society, that's not hard. If I'd been born in a poor family, in a very low income country, then I don't know, obviously it would have been different. But actually, I would probably have taken a random walk in just the same way. I don't think I would have put myself in a box and tried to emigrate illegally. I suspect I'm too cautious to have done that. But I might have thought and experimented and climbed my way up to some position where I could have some security. But, anyway, growing up as I did in a developed society, you know you sort of say, well I can just try it out, I can do what I like, which actually is very powerful when you think about it. However, having said all that, I think I do come with a certain measure of natural competitiveness, so I actually like succeeding and I like doing better than somebody I'm competing with. So when it comes I will run the course. But in my own way.

JP: Do you feel that the explosion of information in biology over the last couple of decades has affected undergraduates at all in their outlook on science, about what to pursue, how to go about pursuing it?

JS: I don't know. I would hope they're not particularly influenced by it. I mean what happens is that, as Sydney Brenner was indicating, one handles the explosion of information through computers. He joked about it and said he was reading the whole thing. Obviously he's not. What he means is he's going through it very carefully, using the computer tools no doubt. I think that one shouldn't be intimidated by the information. After all, the internet is full of information. People deal with their lives, with their Facebooks, with everything else. I'm just astonished at the amount of information that kids actually know and tell all the time. I think what you would do, surely, is to select in your own way, what you really want out of that, what you think is important. You can use other people to help you with that, maybe, just like you'd use Google search to find things, and then you still make your choices.

I suppose now, growing up as a young scientist, you tend to be in a larger field because the number of working scientists in the world today is much, much larger than when I was growing up as a young scientist. But I suspect the solution is the same now as it was then, which is to find your way into some sort of niche where you have a relatively small group and you're doing something new. Going back, for example, to Yamanaka's talk about iPS cells, he had an idea, pursued it with a really small, dedicated group. And that was good science! He wasn't doing something big and planned from the beginning. Once he succeeded, of course, then the thing spreads out. Then he got his own institute and they start doing all sorts of things in parallel.

TJ: Do you think that because of this surge in information, possibly making science more commercialized, it negatively impacts the dedication that scientists have for their work? If something is not giving them an immediate payback, they might not be as dedicated?

JS: If you're dealing with something that has a commercial goal, then of course the rules are quite different. Obviously, when you just doing science out of curiosity, you still have a goal, which is to find something which is interesting, otherwise you don't get funded. I was talking to my very old colleague who was working, as I mentioned earlier, when I was first with Sydney Brenner and Bob Horvitz. He was saying how much he admired the chemists in the companies. He started one company and now he's involved with a couple more, and they are producing drugs involved in curing degenerative diseases. He said it's just extraordinary to see these scientists, how effective and efficient they are, far more efficient than anybody in university. But they have a clear goal: they've got to modify this drug. They're producing all the possible modifications of a molecule with a goal, and that in itself is fascinating.

I was not a biologist in the beginning; I was an organic chemist by training which I did as an undergraduate and as a research student. I did exactly what the scientists are doing in these companies, I made molecules. It's lovely! It's a real engineering job, putting atoms and devising ways of getting atoms into position. I think that in itself is a hugely satisfying thing to do. But obviously quite different from wandering around, picking the flowers as somebody said in one analogy for doing more exploratory research. But there's no reason why you have to decide which of these you're going to do. At different times I channelled myself differently... the thing that I eventually got specifically awarded for, I suppose, in so far as it was anything specific, was getting the cell lineage of the nematode and that was two years on my own part as well as some colleagues. It

was dedicated work, doing one thing everyday, the same thing, looking down the microscope. It was great! That was complete channelling at that point. At other times I was trying to find ways of doing different things. It's great if you can get yourself into a position where you have some choices. I've always been very fearful of selling myself too hard and of being too concerned about a particular career. I want the space, I do not want to be committed to a line of action for the rest of my life and it seems to have worked out quite well for me.

JP: You talked about channelling in your talk yesterday at the Global Health Symposium in the context of channelling the innovation chain to the right place to do good. I was wondering what led you to go from basic science in the nematode worm to global health.

JS: It was through the genome project. It started out with joining Sydney Brenner's group, having the whole 1 mm long worm to explore, which is a whole universe of life. And then through various accidents, getting the cell lineage job, and then working with Bob Horvitz and others, and beginning to get mutant worms which had interesting effects on the cellular development. At that point it was not only us, but every nematode lab in the world, because by now there were at least a hundred of them scattered around the world, they all were faced with the same problem, which was isolating their genes (the genes causing the mutations and interesting phenotypes). And so for me, that's the thing I'm most happy about, I'd hesitate to say proud, but you know I'm kind of a little bit proud of having decided that the right thing to do was to do something about the genome, at that point, to map the genome. This provided the tools for people to isolate their genes of interest fast.

When I was working with *C. elegans*, I was working with cell lineages, seeing cells divide, getting the mutants that affected that and then being frustrated at not being able to get a hold of the molecules fast enough! Armies of research students were really just wasting their time. So I said "We've got to find a mechanism with which they can do better." And the answer was a map of the genome. This way you're not looking through the whole genome for your gene, you're looking at just the right bit, because my colleagues and I have already mapped it out, in little bite size chunks, which enables you to find the location of your gene much quicker. Then you are able to go on and do interesting things and study the gene product go on, instead of doing heavy-duty molecular biology to isolate your gene.

There was a direct link, I got into genomics, having got the genome mapped, then as sequencing technology advanced, definitely getting into sequencing it, because that was an even more powerful method of searching. Once you have the sequence, you can search with computers for genes that look like one another. Now I can find a whole family of genes that are similar. Then getting involved in the human genome project, into all that business, finding out that some people wanted to keep data private, and then getting really concerned about what's happening with the commercial control of this information we've got and how it's being used. So you see what I mean? It's completely logical progression!

TJ: This is relating to the controversy where certain companies wanted to commercialize the human genome. How big a danger does commercialization pose to science and access to information?

JS: Well, I think we need a balance. If things become too commercialized, then we can only do things that have immediate market potential and we exclude important areas of science. In global health, we exclude working on sleeping sickness, for example, by that mechanism. The only way you can work on sleeping sickness, in terms of putting serious funding so that you have a chance of developing a drug all the way to something that works, is to use charitable money or uncommitted government money. But uncommitted government money is getting scarcer and scarcer because government money has now drifted more and more to putting pressure on scientists to do what they call high impact research, which means that it is pre-commercial. I think it is fine to do it to a point, there's no harm at all in satisfying the market, but you should not put all of science into that channel.

JP: You won your Gairdner Award in the early nineties. Coming back this year for the 50th Anniversary, what has been special about this event?

JS: In fact, I have two! The second one was a little different, it wasn't an individual one, but a whole bunch of us, about a dozen, got a collective prize for the human genome, in 2002. My earlier one was really nice. Sydney Brenner was here as well, we got that for the early nematode worm research. Coming back to the 50th Anniversary, it's terrific! Walking in here, I've seen a number of people that I haven't seen in quite a while. It's a great celebration. And as you know the Gairdner is an extraordinary foundation and getting awards from them is something to be very, very proud of.

I always find it strange when people say "what's the right way, what choices should I make to have a career in science and to be successful?" I've never done any of that.

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Dr. David Sackett

Dr. David Sackett is a medical doctor with degrees from the University of Illinois and Harvard University. He was the founding Chair of Clinical Epidemiology & Biostatistics at the McMaster University School of Medicine, and he served as the Director of the Oxford Centre for Evidence-Based Medicine. He is an Officer of the Order of Canada. He pioneered the disciplines of clinical epidemiology and evidence-based medicine, establishing a firm foundation for many aspects of applied clinical research and medical practice. He was awarded the 2009 Gairdner Wightman Award.

"Do I want to be in the field zoology... or would it be medicine? And I decided on medicine."

JP: What is clinical epidemiology and how did you first get involved with it?

DS: Clinical epidemiology is taking some public health sciences, like the study of epidemics and biostatistics, and taking them out of the public health realm and into the clinical realm of individual patients. And the two key questions are: how accurate is a diagnostic test and how effective is a treatment?

It came about when I was drafted. I was a bench scientist as part of my postgraduate training in internal medicine, Khrushchev put the missiles in Cuba, and we all got drafted. I was put in an epidemiology field station where I learned immunology and biostatistics from a public health perspective, but then got the idea that you might translate that over into patient care and I decided to call it clinical epidemiology.

JP: You are a medical doctor by training. Many of our readers are interested in pursuing degrees in medicine, science or both. How did your medical background influence your career and your work?

DS: I suppose it's a combination of always being curious and being interested in science, but even more so, trying to have a prepared mind. There are going to be a series of events, people you run into, over which you have no control at all, and the exciting careers that come out when that lightning strikes, might open up an area that you may want to pursue. In my case, the Cuban missile crises opening up this area, a few years after that, the fact that there was a new medical school starting up at McMaster, and the Dean there had heard about me and called me up. This series of unanticipated events, over which you have no control, they offer opportunities to work in particular labs, to meet particular people, to find someone who's interested in you and your career development.

JP: How did your undergraduate education set the stage for your career?

DS: I grew up in the United States and I went to a tiny college. After my second year I was never in a class bigger than 9 people. In that kind of setting, the interaction between students and faculty became very blurred because we were carrying out mostly discussions and seminars. At that point, I was interested in biology in general, but my main interest, up until my third year of university, was in field zoology and ecology. I was terribly interested in insects. And being in Wisconsin, with all of the aquatic insects, it was a particularly interesting area. But then I took a course in physiology, and because very fascinated with physiology and human physiology. Then at that point there was a battle: do I want to be in the field zoology at a university post, or go into medicine? And I decided on medicine.

JP: You served as the Director of the Oxford Centre for Evidence-Based Medicine. What is evidence-based medicine and what led you to get involved with it?

DS: In clinical epidemiology, we began generating good evidence about what are really good diagnostic tests and what are really good treatments. But that wasn't enough, and what we had to do was work harder and harder, taking the results of that research, combining it with the best clinical skills we could have (you really needed to be a good diagnostician) and then incorporating what patients felt, expected and deserved.

There are three arms: very good evidence, seen by a very good clinician and integrated with patients' expectations and wants. For example, we had seen someone who was at risk of having a stroke, because there had been an irregular heart rate that might send a blood clot up to their brain. We could treat them with a blood-thinner like warfarin, which would reduce their risk of stroke, but if we treated them with warfarin we would increase their risk of bleeding, usually from their GI tract. And the question is: how do we then incorporate the patient in that decision making? We had the evidence, we could sort out what the problem was, but then we wanted to integrate that and present it to the patient so that they could have their input into the decision. And in this case, it comes down to saying, from the patient's point of view, how bad is a stroke and how bad is one of these bleeds? And how do they relate to each other? f you have a stroke from this condition it's usually awful and you wind up with a permanent disability. You might not be able to walk again, have trouble reading or speaking, in other words, it would be very devastating. If you have the bleed, there's a small chance you might die, but in general you're going to be scared to death, sick as hell for about 2 weeks, and then slowly recover and be back to normal in about 3 months.

I hate being singled out for what is obviously a group activity. So my standard approach has been, frankly, to turn down awards and to turn down honorary degrees and that sort of stuff.

Against that background, we describe the stroke and the bleed to our patients, and then ask "how do you weight these things against each other?" In general, a patient would say, "well, as you describe it, the stroke is about 5 times as bad as the bleed." So this then gives us this 5 to 1 business. We can then sort out their individual risk of having the stroke or the bleed, and we could put that together in a very simple formula, and then be able to tell the patient, here are the likelihoods we're going to help versus harm you if we use this treatment on your own terms, in terms of what you think is important. We decided that we went far beyond just clinical epidemiology into front-line patient care, and we needed a new name for it. In 1992, a chap named Gordon Guyatt in our unit said "let's call it evidence-based medicine." It took off from there and other fields then began to adapt it into evidence-based nursing, physio, decision making and so on.

JP: Do you see a crucial role for evidence-based medicine in the health care reform going on in the United States, and how would you like to see it applied?

DS: I gave up on the United States in 1967 when we moved to Canada. It seems to me that in the United States, health care is about money, not about health care. Here, health care certainly has huge financial implications, but the basic view in Canada is people deserve health care, and that the rest of us shouldn't be selfish about it. That's not so in the States. When I was practicing in the States, before I moved here, I practiced in the poor parts of town, and I could never send a bill! I just never felt it was right to ask people for money when their situation was already so awful. Whereas up here, with universal health care, I could give so much better care to people, that I see no comparison. And as long as the States' health care is going to be driven by money, rather than by the health needs of the people, it's not going to be solved. Rich folks will get better and better health care, poor people will get worse and worse.

JP: You've been awarded the Order of Canada and numerous other awards. What does the Gairdner Wightman Award mean to you?

DS: Someone like me always has trouble with awards. I'm here because of all the simply brilliant medical students, house staff, colleagues, graduate students, the people who have come to work with me, and we've done so many things together. I hate being singled out for what is obviously a group activity. So my standard approach has been, frankly, to turn down awards and to turn down honorary degrees and that sort of stuff. This one was special in that when my colleagues and students heard about it, they said, "Well wait a minute, in a way you're being kind of selfish. This is an award for us, not just for you." This award recognizes that what we, my colleagues and I, have been doing collectively is important. In addition, this particular award has a physician's name associated with it. Kager Wightman, who was this outstanding clinician teacher here in Toronto, and so revered by everybody who knew him, and to have an award in his name is an additional honour.

Dr. John Dirks

Dr. John Dirks received his medical degree from the University of Manitoba. He has held Professorships at McGill University, the University of British Columbia, and the University of Toronto, and he has served as the Dean of Medicine at the University of Toronto. He is a Senior Fellow at Massey College, a Member of the Order of Canada, and the President and Scientific Director of The Gairdner Foundation.

"Gairdner is Canada's only international science award, in fact, I think it's Canada's only international award of standing of any kind."

JP: What first led you to get involved with the Gairdner Foundation?

JD: In 1982 I was at UBC as Chairman of the Department of Medicine. I was invited to be a member of the Gairdner Foundation Medical Advisory Board (the group that makes the final selection every year for the Gairdner International Awards) by the then president Charles Hollenberg. So I started coming here (Toronto) every year thereafter, so when I came in 1987 as Dean of the Medical School, I continued my membership and in 1993, I took on the Presidency of the Gairdner Foundation. So I have a certain longevity with the Gairdner Foundation and I've enjoyed it a lot.

They would send the Gairdner winners of that year to Vancouver before they came to Toronto to receive their awards and actually the first person I hosted in Vancouver was Harold Varmus, who won a Nobel Prize a few years later. Back in 1993, when I took on the Presidency, the Gairdner Awards were a quieter affair. We always had the awardees going to Canadian cities first, they would have an awards day like we have now, present the reasons for giving the awards, and visit some of the hospitals.

The Gairdner Foundation already had a very good record, but when I took it on, we decided to expand and broaden several components. First, we gradually expanded the medical advisory board from exclusively Canadian to international. Now the group is half Canadian and we have members from the United States, Great Britain, Germany, Hong Kong, Australia, and soon Japan. There are also 5 Nobel Laureates on our committee who also have Gairdner Awards and an additional couple of Gairdner awardees.

Now it is a very active and internationally represented community that meets in mid-January and looks at the long-list and short-list prepared by our medical review panel. The committee has quite a few people from Toronto but it is also national, with members from Halifax to Vancouver. We've tried to make Gairdner national and international, and I think we've succeeded. Industry Canada said that Gairdner is Canada's only international science award, in fact, I think it is Canada's only international award of standing of any kind.

JP: As President and Scientific Director for the last 16 years, you expanded the advisory board and I know that you also launched a series of high school lectures and expanded the national program.

JD: Exactly. The national program has been growing every year, and every year we bring back, in addition to the current year's awardees, former awardees. This year, which is proudly our most extensive year, we were at 22 academic centres in Canada. And because this is our 50th Anniversary, we had 7 major symposia starting in March in Vancouver, then Edmonton on cancer, York on entrepreneurship in health research, a symposium dealing with the new RNA explosion in Sherbrooke and in Montreal on neuroscience.In June we were in Ottawa, dealing with genetics of heart disease and a session called the brain and the mind, and a genetics symposium in Halifax.

On the high school front, we started this in Toronto, in 2000 and it has become a traditional event. Our model is quite simple: what we take is usually 2 awardees to speak for about 15-20 minutes each on how they became a scientist. Was this something that was spurred on at home, or was there a great mentor in their early years, or an illness in the family that focused the person on wanting to do something? Then they talk about the scientific question they have worked on and made discoveries in.

I describe it as a single encounter inspirational moment, which often can turn students around. Most of us, if we look at our own lives, will find that there are certain key moments when we got inspired and those became turning points. It is based on a very simple formula: how I became a scientist and what have I been doing, and that inspirational moment. It is in the hope that some of the students will say, "you know I heard Phil Sharp and Sydney Brenner speak, and maybe I'd like to try this," or maybe "I want to go into health sciences to become a specialist or a general physician because I'll also get the spin-offs from what's happening in modern medicine". This year we had the high school program in 15 academic centres, and it likely helps recruit students to biology programs and health professional schools.

JP: This year there was a new Gairdner Award, the Canada Gairdner Global Health Award, which was given to Dr. Nubia Munoz. Why did the Gairdner Foundation create this award?

JD: Part of it comes from my own personal experience, from 1994 to 2006, I co-chaired and then chaired a commission for the International Society of Nephrology. This was directed at presenting education programs, making fellowships available, having collaborative research studies, and developing relationships between institutions from the developed to the developing world among over 70 countries. As a result of that, I was often invited to more general and larger meetings from institutions such as Institute of Medicine, where science that was to be applied in developing countries with major health problems, was the primary agenda. And I became very committed to that idea.

We also had a number of people suggest that we should have a global health award, which I was naturally interested in, and so we went about doing this. When we made our proposal to the federal government, we put in this part of the proposal (to establish the first Canada Global Health Award) and the government actually liked it a lot. It's an opportunity to honour individuals for whom there are almost no awards in the world, and none on an international scale that honour people who do the kind of science like Nubia Munoz, our winner, does. Nubia Munoz has taken on a problem which may have been initially started in the West, but has conducted her epidemiological studies in many poor African, Asian and South American countries, creating a tidal wave to stimulate immunologists and vaccinologists to develop the HPV vaccine. And as she says, if this is well applied in the world in the next decade, it could save a quarter of a million lives every year, which is a substantial contribution.

So the award has been very well received; in fact, this very morning, I got a letter from John Sulston from Cambridge, and let's not forget that he was one of the key players in elucidating the human genome, and now has become a great exponent for global health. So here's a basic scientist, Sulston, and he views this as a milestone moment. I feel confident that the Global Health Award is going to be widely recognized. Our nominations doubled from last year already. We hope to build on this and we've asked the federal government to give us resources for a second award. One award is for science relating to the developing world and another which deals with the advancement, innovation or policy configuration. It will be aimed at someone who has developed a strategy for the application of something like the HPV vaccine, for example. It is under consideration in Ottawa, and hopefully being well received.

I visualize that this will become an important part of the Gairdner Awards. Our symposium this year on global health

Most of us, if we look at our own lives, will find that there are certain key moments when we got inspired and those became turning points. It is based on a very simple formula: how I became a scientist and what have I been doing, and that inspirational moment.

was extremely well received and as long as we keep the global health award with the others, we'll probably have another symposium next year on a global health topic, depending on the nature of the winners. I was amazed, that room where we held the global health symposium was overflowing.

Now we're trying another experiment on the global health front. Professor Munoz is from Colombia, so Sheila Robinson, my colleague, and I, are going to Bogota a week from now. We will talk at El Rosario University about the Gairdner Foundation and then Dr. Munoz will give her perspective. Also coming with us is a McGill professor, Eduardo Franco, who's also in the HPV field and is of Spanish speaking origin. It is good for Canada to show that it's also active in the (HPV) field and to present ourselves in developing countries.

JP: This year was especially big for the Gairdner Awards as it was the 50th Anniversary. There was a series of very high profile events across Canada and especially in Toronto. What was your personal vision for the anniversary this year and how was it realized?

But just think of Dr. Smithies, he's 85 and he hates to travel because then he's not in his lab doing things. Let's all hope that when you and I are 85 that our minds are as good as that! It's a joy to behold, really!

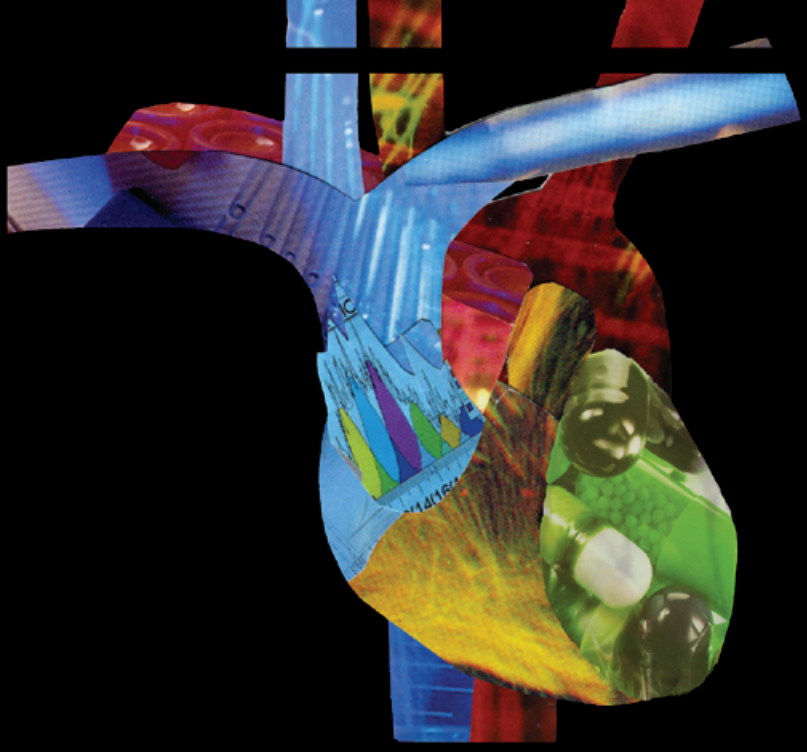
JD: The vision was to make this a very high profile event of international science in Canada. And I think that it goes without saying that last week in Toronto was the largest gathering of widely known, high profile biomedical scientists that's ever occurred in our country. And we really appreciate the good partnership with the University of Toronto, President Naylor, and Vice-Dean Lewis, who has been particularly helpful. Our vision was to have these scientists together and to talk to each other, because almost all of them are still pretty active researchers. Sometimes when people win major awards, like the Gairdner or the Nobel Prize, it's late in life and they're not doing science anymore. But just think of Dr. Smithies, he's 85 and he hates to travel because then he's not in his lab doing things. Let's all hope that when you and I are 85 that our minds are as good as that! It's a joy to behold, really!

We also wanted to address various communities. We have the academic, scientific research community, which started off with a bang with Yamanaka's talk because he is at the cutting edge as much as anybody right now. Then we addressed the private sector in-part during our terrific industry breakfast, where we had a highly intellectual presentation and practical discussion by three American leaders: from Boston in the case of Phil Sharp, David Baltimore from the LA-San Diego area, and Corey Goodman from the Bay Area in San Francisco. These are the hotspots in commercial development of medical discoveries, and Toronto is quickly catching up. When they gave their insights, you could hear a pin drop in that room. We had to turn people away. And then the high school students: 500 high school students at the University of Toronto, 300 students at York University, in Winnipeg, 800 high school students, to hear David Sackett.

This is a contribution to Canadian science culture. If we don't get young people interested in science, how are we going to have the talent that's available in the long run to bring new knowledge economy, or good health care? And of course we wanted the awards dinner celebration to be something truly memorable. And I think we made an impact across the country and we got much more media coverage than we ever did.

JP: What was your favourite moment of the Gairdner Awards in Toronto?

JD: Well, there were many, including listening to Shinya Yamanaka and Sydney Brenner at dinner. What was particularly touching was when the Minister of Health Canada, Leona Aglukkaq, had made a few introductory remarks before Nubia Munoz came up, and they embraced each other - it was terrific. Also when Elizabeth Blackburn spoke to that dinner audience; she's a very modest person, and everybody realized that here is a person who has achieved so much, while being a woman scientist is something that is very special, and she has done it all! And yet she wasn't talking in any exultant way about herself; she was very modest about it. And I think the video from the Prime Minister, who couldn't come (because of the Olympic flame which was coming from Greece), was very well done.



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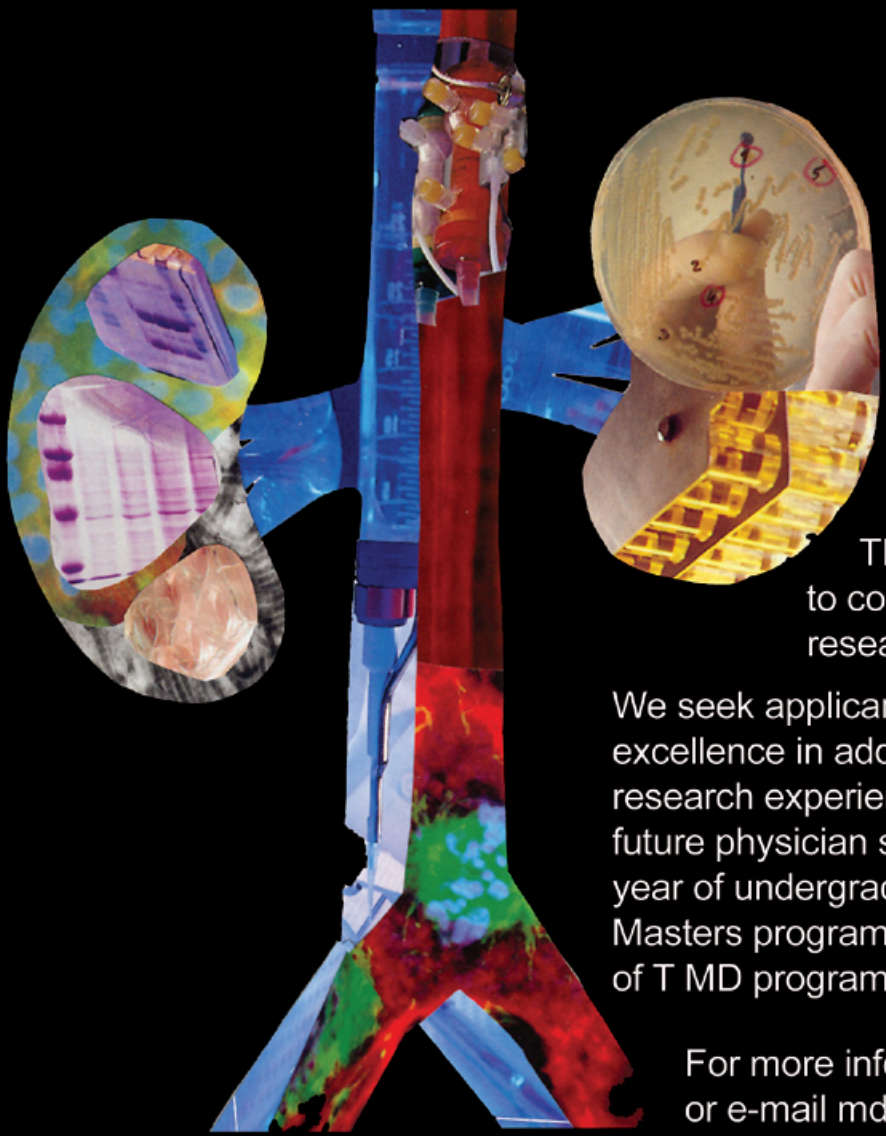

We live during a time of remarkable advances in the sciences that span the expanse of biomedical to health services research. Indeed, there has never been a time when it is so exciting to do science; never has there been such potential for discovery and application of these discoveries for the benefit of human health.

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Dr. Nubia Munoz

Dr. Nubia Munoz is a graduate of the School of Medicine at Universidad del Valle and the Johns Hopkins School of Public Health. She spent most of her career at the International Agency for Research on Cancer in Lyon, France. Her work helped establish the link between human papillomavirus and cervical cancer. She is the inaugural recipient of the Canada Gairdner Global Health Award.

JP: How did you first get involved with your research in cancer epidemiology?

NM: Well I did medicine in my home country of Colombia. I liked clinical medicine very much but I had a problem: when my patients didn't do well, when my patients died, I was very depressed, because I identified very much with my patients. So I decided that despite liking clinical medicine, I better go into research, which I really liked. I started doing research as a medical student with a professor of pathology who was the most active professor in research at my university at that time. I got involved in several studies even before graduating from medicine, but since he was a pathologist, I continued training in pathology for 3 years. I didn't like pathology as a discipline to work in for my whole life because I found that part of it was too routine and I decided that I should use this basis to do something that will benefit more people than one given patient.

I got a fellowship from the International Agency for Research on Cancer to go to John Hopkins University and do epidemiology. I did my Master's in Public Health, and I was supposed to go back to Colombia, but I met the director of the agency and I said, "I need another year to finish my training." He said that fellowships are only two years but if you wish to come to Lyon to complete your training then you are welcome. And I said "Yes, of course!" So I went to Lyon for one year in the epidemiology unit and after one year they said, "You are performing well, if you wish to stay here we can give you a contract for two years, and two years, and two years" … until I ended up staying there for 31 years!

Since I was practically the only senior staff from Latin America, my main interests were always cancers that were important health problems for developing countries, like gastric cancer, cervical cancer, and liver cancer. I did research projects on these different cancers in about 30 countries around the world. Research on cervical cancer I started over 30 years ago with the idea that it might be caused by Herpes Simplex Type 2. But after more research we knew that this was probably not the case. I became interested in the human papillomavirus (HPV) in 1974, but at that time the technology was not available to measure papillomavirus. So later on, once the technology was available, I started conducting epidemiological studies using assays allowing for the detection of HPV DNA. The studies that I did in around 30 countries around the world are considered today as the most solid epidemiological evidence showing that certain types of HPV are not only the main cause but also a necessary cause for cervical cancer. In addition, I am now helping former colleagues of mine to unveil the role of this virus in other tumours, like in cancer of the otopharynx.

JP: What do you see as the greatest challenge that is ahead for you?

NM: I think the greatest challenge before was to allow these discoveries to be translated into preventative tools. But the problem now is to bring these tools, such as vaccines and new HPV assays, to the countries that need it most, in Latin America, Africa, and Asia. These are the poorest countries and these tools are very expensive. So there has to be a lot of effort to facilitate the introduction of vaccines and HPV assays into the countries that need it most.

JP: You've traveled extensively for your research. If you had a favourite country, which one would it be?

NM: Well, I am from Latin America, but I've lived for more years in France than in Colombia. My husband is French and I like life in France very much. I still like my country, and I am discovering more and more about Canada. I found it very interesting touring Quebec and Montreal and different universities, it has been a wonderful experience.

Dr. Samuel Weiss

Dr. Samuel Weiss is a neurobiologist in the Department of Cell Biology & Anatomy/Pharmacology & Therapeutics at the University of Calgary. He is the director of the Hotchkiss Brain Institute and a member of the Genes and Development Research Group in the Faculty of Medicine. In 1985, Dr. Weiss, along with Dr. Sladeczek, discovered the metabotropic glutamate receptor (mGluR), a breakthrough that birthed a major research area and provided clues about the etiology of neurological disorders such as schizophrenia, Creutzfeldt-Jakob disease and Fragile X syndrome. In 1992, Dr. Weiss and graduate student Brent Reynolds isolated stem cells from the adult mouse brain, suggesting that certain regions of the brain generate can generate new neurons throughout adulthood. This discovery has provided another possible mechanism for brain plasticity and a possibility of developing regenerative therapies for the central nervous system.

KZ: How do you feel your undergraduate education prepared you for a career in science?

SW: I was an undergrad at McGill doing biochemistry. Between 2nd and 3rd year, I almost quit because I felt pure biochemistry was too dry – it became tiring tracing all those cyclocarbons. When I returned to school from summer holidays, I took a course in neurochemistry of the brain and everything changed. It became clear that there was a good rationale for understanding biochemistry and applying it to understanding of the brain, its normal biology and pathophysiology. I was lectured by eminent neurochemists - among them Leonard Wolfe - and it was immediately made it clear why biochemistry was important inunderstanding the CNS. This motivated me to understand the chemistry of the CNS and ultimately the signaling systems in the brain.

KZ: And that's what put you on the path to the discovery of the metabotropic glutamate receptor.

SW: Yeah, absolutely. When I was doing my PhD, I was interested in 2nd messengers in invertebrate muscle and nerve-muscle interaction. As a post-doc, I studied signaling in mammalian neurons and the discovery was serendipitous. The metabotropic glutamate receptor was not something we were looking for – it was an accident.

KZ: A lot of discoveries mentioned here at the talks have been ascribed to accidents.

SW: It depends. Take what people recognize from development of new technologies – at least 50% of the time, they stumble upon them. The most important discoveries are not hypothesis-driven, dare I say.

KZ: It is interesting also that you entered the field of neurogenesis in 1992, but the first reports of neurogenesis were in 1965 by Altman and Das…

SW: Oh, even earlier! Leblond and Smart at the Montreal Neurological Institute identified the phenomenon, but it was thought to be a rodent-specific phenomenon (as the rodent brain keeps growing throughout adulthood) so it wasn't seen as important. It was ignored for 30 years, but interest was renewed again when we began to identify the phenomenon in the human brain. And now the whole world was looking at it! It has tremendous untapped potential – we still have a long way to go to understand how it influences behaviour or how it can be harnessed for repair.

KZ: One argument that can be levied against the importance of neurogenesis is that it's a very small population of cells that is adding to the hippocampal circuitry and several studies which have looked at neurogenesis have not shown deficits in behavioural performance.

SW: Certainly, some behaviours are unaffected. However, some adaptive behaviours are. Is it critical in a human? In a rodent? If it were removed from the rodent, the species would cease to exist.

KZ: Your research shows that there is a concurrent increase in neurogenesis in the olfactory bulb and in the hippocampus which appears critical for establishing social memo-

ries. However, there is a residual population of cells that is still proliferating. What do you think is the function of these cells?

SW: That's a great question. Why is there this baseline neurogenesis? What's the difference between neurons born in response to sensory stimulation and those born as part of the ongoing process? We don't know the answer to that. I think that the ongoing process is there to allow for quick responses to adaptive circumstances that would allow for the formation of certain types of memories. But what role does it play intrinsically? It is hard to say. If you knock it out or reduce it, at least in the olfactory bulb, then the ability to form memories is significantly reduced - we have done those experiments, as well. If you have a steady-state reduction in olfactory bulb neurogenesis, your ability to effectively retain and act on noxious vs. non-noxious odours is blunted.

Presumably, the ongoing process is there for everyday activity. Enhanced neurogenesis may be there for specialized seasonal or other types of behaviours that are only transient. But we still don't know – the focus has not been on behaviour, it has been on repair and pathology (and I'm guilty of that myself). However, the really interesting stuff is behaviour. As we learn more about what regulates neurogenesis - what turns it on and what turns it off – we can begin to do up and down regulation experiments and look at behavioural outcomes.

Some behaviours appear to be particularly well-regulated by hippocampal neurogenesis, like fear conditioning, for example. If you think about it, this can confer an adaptive advantage. We've managed to modify – permanently – hippocampal neurogenesis, by doubling its baseline rate. The animals with increased neurogenesis perform much better on fear conditioning trials are able to more readily adapt to fearful stimuli, which means they have a greater likelihood of surviving. Just by regulating baseline neurogenesis, we're also making the individual more likely to survive. So increases in neurogenesis are interaction-dependent and are probably more important for reproduction; baseline is for individual survival.

KZ: That is a very interesting view.

SW: Well, it's why we have PhDs! It's the doctor of philosophy – so we can profess and wonder about phenomena – how much of it is true depends on the results of the experiment you do!

KZ: You also mentioned in your talk the possibility of harnessing the process of neurogenesis in regenerative therapy and restorative therapy. It's also known that, for example, in aging, neurogenesis decreases profoundly. Does this decrease correlate with a decline in behavioural performance?

SW: Certainly. Olfactory discrimination is blunted significantly, which is also known to happen in humans. Is that a consequence of something physiological, hormonal? We don't know. We can mimic it in a number of ways by reducing endogenous signaling systems – but we don't know what is the chicken or the egg in this case. My reckoning is that decline in neurogenesis is important in aging. If there is a way to ensure that these processes are not diminishing with age, it may allow for more active social activities and as well as the ability to function effectively in a number of environments. Hippocampal neurogenesis really provides a sense of self in a changing environment. In aging, people can get very nervous about change, about being in a foreign environment and it's possible that adaptive behaviours in part are diminished because of the reduction in neurogenesis.

You got to love what you do. It can't be a job. Challenge the dogma. Don't listen to your supervisor! These are critical ingredients of success in science.

Of course, this is not the only cause, but I feel that neurogenesis can have very important roles that we haven't been able to appreciate yet. It may be discrete, the numbers may be small, but they are there and the phenomenon is evolutionarily conserved over many species. However, it's hard to study in humans. You can't just ablate neurogenesis in humans!

KZ: One final question. What advice would you have for undergraduates willing to undertake a career in science? What personal qualities should they develop?

SW: You got to love what you do. It can't be a job. Challenge the dogma. Don't listen to your supervisor! These are critical ingredients of success in science. I did that throughout my graduate career. Obviously, it all changes. When you go from being a kid to becoming a teenager, you suddenly get a sense of "Wow, I'm intelligent enough to make decisions and choices". Same thing when you go from being an undergraduate student to being a graduate student – you go to being able to intelligent to make decisions and not having to abide by the principles of others. You learn from them by example: they'll show you their passion, their innovation, but you need to find a way to develop that on your own, independently. So early on, you have got to challenge them, challenge what's in the literature and it's got to be - as Oliver Smithies said – no problem coming on Sunday, New Year's Eve. If it ever becomes a job, don't do it. Science is a huge amount of work and the rewards are not so often there. Yes, there are long-term rewards, but the short-term gratification? It's a culture of rejection, right? It's all too often that papers, grants are rejected. But when they're accepted, and when they have an impact, it is as good as it gets.

KZ: Thank you very much!

JULS

Ageing and Toxicology Research: Current Issues and Applications

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Review Article

Abstract

Two fields of modern research are discussed in this review article: ageing and toxicology. Ageing has been the subject of many studies, including those of in the field of neurodegenerative diseases. More often than not, it is a homeostatic alteration in the body's normal metabolic function that underlies the process of ageing. Comparatively, the study of toxicology explores the detrimental effects of chemical agents on the body. However, the research areas of ageing and toxicology are not completely independent of each other. This review article examines the connections between the two fields, and the current issues in the subject areas. Additionally, an overview of ageing theories is provided, with the focus on ROS-derived effects in ageing. Also, the use of animal models and relevant techniques are discussed as means of future treatment and prevention methods.

Introduction

The study of toxicology encompasses both the mechanisms and the effects of exogenous and endogenous agents as they pertain to cell, organ, and organism. Ageing may be defined as a homeostatic alteration in body's normal metabolic processes [1], resulting usually in an increased risk of death with time [2]. Therefore, it is important to know the mechanisms inflicted by age-related toxicology in order to prevent it.

Many theories have been proposed to explain the nature and biology of ageing [3]. However, many of them are intertwined in a complex mesh of molecular pathways. The section below summarizes key ageing theories as they relate to toxicology.

Theories of Ageing

Within the cell, free radicals that contain the oxygen atom are often called reactive oxygen species (ROS). It was not until the 1950's, when Denham Harman developed the free radical theory of ageing [4], that the toxic nature of ROS was understood. Advocates of the free radical theory argue that the accumulation of oxidative damage within the cells drives the process of cellular senescence.

Having an unpaired electron makes ROS an excellent electrophile [5]. Hence ROS attack cellular components, such as membrane lipids, proteins, DNA and RNA [6]. In turn, damage of these macromolecules disturbs the balance in cell communication, protein synthesis, DNA replication and transcription and other chemical pathways [7]. For example, proponents of membrane theory of ageing argue that membrane lipid damage increases with age, leading to impaired sodium-potassium transfer [3], and thus, minimal cell-environment communication. On the other hand, various forms of DNA mutations lead DNA damage theorists [8] to argue this macromolecule's involvement in ageing.

This review is mainly focused on ROS-derived effects in toxicology and ageing, since ROS is an important factor in many ageing theories.

Toxicology Causes Ageing

Generation of ROS within the cell may be attributed to both endogenous and exogenous sources [9]. One source of the latter is the exposure to singlet oxygen. Being less stable than the ground state triplet oxygen [10], it causes oxidation of membrane lipids and enzymes, including aconitase and glutamate decarboxylase [11]. Such chronic oxidative stress may underline the development of dementia associated with ageing [12, 13]. Other exogenous ROS sources include: UV and gamma radiation [14], car exhausts and smoking [15], drugs and xenobiotics. The latter category is especially important since many xenobiotics produce ROS as by-products of their metabolism [16, 17, 18]. For example, MPP+, an MPTP metabolite that generates hydroxyl radicals in the striatum [19], may serve a model for Parkinson's disease [20]. Moreover, glutathione (GSH) depletion [17] caused by many drugs [21], including acetaminophen [18], decreases cytoprotective pathways, resulting in further development of age-related neurodegenerative diseases [22], such as Alzheimer's disease [23]. Interestingly, nutrition may also contribute to ROS production [14]: foods packed with oxidized fatty acids and aldehydes may contribute to carcinogenesis and ageing [24, 25].

Endogenous ROS production is no less important for ageing processes. ROS are continuously generated within the body, having both essential functions in enzyme regulation [26] and detrimental characteristics. Mitochondrial electron transport chain (ETC) is one of the sources of ROS generation [3]. Sometimes, ROS penetrates inside the mitochondrial environment [27]. Mitochondrial DNA has no histone protection

like nuclear DNA, and thus is more susceptible to free radical damage [28] and 8-OHdG lesions. The mitochondrial theory of ageing [3] employs these observations to understand the underlying causes of degenerative diseases, including those of heart and CNS [29]. Furthermore, endogenous cells, including neutrophils and macrophages produce ROS [30] to destroy invading micro-organisms. Interestingly, chronic inflammation is now being associated with age-related pathology and oxidative imbalance [7].

Overall, oxidative stress and ROS toxicity predispose individuals to higher risks of age-related degenerative diseases and decline in physiological functions over time.

Ageing Causes Toxicology

Age-associated changes in cellular processes affect pharmacokinetic and pharmacodynamic profiles of many drugs [1]. In addition, ageing processes change physiological functions in the elderly, resulting in increased susceptibility to drug-related toxicities [31].

Oral drug absorption is guided by several factors, but mainly the gastrointestinal pH [31]. A close association between achlorhydria [32], lack of hydrochloric acid in the stomach, and age has been shown to exist [33]. Therefore, the absorption of weakly basic drugs increases with age. In addition, a decrease in total body water [34] and skeletal muscle mass [35] with age results in a reduced volume of drug distribution for water-soluble drugs, such as digoxin [36]. Moreover, drugs requiring biotransformation to be detoxified and excreted are affected by the decline in P450 levels and hepatic blood flow [1], as well as progressive decrease in renal perfusion and glomerular filtration with increasing age [37]. In general, drug absorption, distribution, metabolism and elimination change in the elderly population, thereby augmenting the probability of drug toxicity and interactions.

Changes in pharmacodynamic effects are also involved in age-related drug sensitivity. For example, anti-coagulant effects of warfarin, and anti-hypertensive effects of both diltiazem and verapamil are increased with age [36], while cognitive function due to scopolamine is decreased [36]. Furthermore, decline in beta-adrenergic activity with age [36, 37, 41] is observed. This minimizes the sensitivity to several respiratory and cardiovascular drugs [36, 1], including propranolol. Similarly, the risk of adverse effects due to psychotropic drugs is elevated in elderly [36, 38]. For example, ageing is correlated with increased sensitivity to benzodiazepines [40].

Overall, advancing age inflicts physiological changes in both pharmacokinetic and pharmacodynamic properties of the drugs. Therefore, the effect and toxicity of a given drug may not be easily predicted.

Research Techniques and Models

The risk of developing common neurodegenerative disorders, like Parkinson's (PD) and Alzheimer's (AD) diseases, increases with age. However, the etiology of these diseases is not fully understood [42], and requires novel research techniques for both drug-development and molecular mechanism studies [42, 43]. Use of animal models, such as P301L tau mice [44] for AD and parkin KO mice [45], allowed researchers to identify changes in proteins associated with the diseases. In addition, predictive and sensitive techniques are required for drug-development to identify mechanisms of toxicity. For example, advancements in microarray analysis allow for more comprehensive gene expression studies and toxicological evaluations [46, 47]. Also, *in vitro* systems using human cells provide an advantage over using those from animals [43]. The field of tissue engineering shows the most promise. In this field, cells are promoted to grow and differentiate in well-organized three-dimensional structures with defined tissue characteristics [48, 49]. Assessing drug toxicities in these tissues may serve as an alternative for animal models [50].

Like any biological research, ageing and toxicology research relies on novel computerized technologies to characterize and study age-related changes and pathologies.

Conclusion

The above discussion focused on the endogenous and exogenous toxicities resulting in ageing, as well as ageing itself producing toxic effects. Often, it is hard to differentiate between the two. Nevertheless, ageing and toxicology research moves ever onward to reveal new and exciting discoveries.

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JULS

Review Article

The Portion Size Effect: A Review

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Abstract

Obesity rates have been growing exponentially over the past two decades in North America warranting a label of epidemic, and many fingers have been pointed at the food industry for its alarming increases in portion sizes. Generally speaking, the portion-size effect describes the principle that participants ingest more calories when a larger portion size is served to them. This paper summarizes current knowledge in the field of eating psychology regarding the portion-size effect. The portion-size effect has been empirically demonstrated in a diversity of participants and situations. These demonstrations have led to the development of theories regarding the mechanisms by which portion sizes affect energy intake. An important controversy in the literature concerns the empirical evidence (or lack thereof) for weight differences in terms of response to portion-size manipulations. This controversy has direct effects on the credibility of the hypothesis that the portion-size effect contributes to the obesity epidemic. Finally, the portion size literature discusses possible applications of the portion-size effect in the real world. These potential applications are thought to have links to obesity control.

What is the Importance of the Portion-Size Effect?: A Review

The Portion Size Effect is Robust

In a typical portion size experiment, the independent variable is portion size and the main dependent variable of interest is energy intake. The effect is observed when increasing the portion size served causes an increase in energy intake. Is this effect an epiphenomenon of an evolutionary trait that helped ensure survival in an environment where food was scare, a result of learning of societal norms, or perhaps both? This effect has been demonstrated in a variety of contexts, with a variety of foods and among a diverse sampling of participants. It has been observed in adult women and men [1] and in children as young as five years old [2]. Although most portion size experiments occur in a laboratory, there is plenty of evidence that this effect occurs in the real world [3]. The foods used in portion size experiments have varied from having an amorphous shape such as macaroni and cheese [1], to those with a well-defined shape such as sandwiches [4]. The portion size effect has been observed with foods low in energy density (calories per gram) such as soup [5] as well as foods high in energy density such as a heavy pasta bake [3]. It has been observed with packaged snacks [4], unpackaged snacks [6], and full meals [7]. Although the perceived palatability of the food can interact with the portion size effect, it cannot eliminate it. For example, Wansink *et al.* (2005) manipulated the freshness of popcorn served to participants as well as the portion size. Although the stale popcorn was rated as unfavourable by participants, a portion size effect was observed in both the fresh and stale conditions, however the effect was smaller in the stale condition [8]. In addition, socioeconomic status (SES) and education variables do not eliminate the portion size effect as it has been observed in both high SES and low SES individuals [9], and university educated individuals [3]. The literature also shows that the effect is not time limited. Rolls *et al.* (2007) showed that the portion size effect lasts up to 11 days without any compensation on the part of the participant [10]. The portion size effect has been observed when individuals eat on their own [4] as well as when individuals eat in a group [5]. The implications of such a robust effect are twofold. First, the many different contexts and types of foods used for these experiments have helped to generalize the effect and rule out the possibility that this effect is limited to specific environments and foods. It appears that this there is a very real tendency for people to increase their energy intake when served more food, regardless of context and type of food. Second, the diversity of participants within which the effect has been observed has suggested possible mechanisms that mediate the portion size effect. These mechanisms and the empirical evidence supporting them are the focus of the next section.

Possible Mechanisms

The Role of Learning

A study by Rolls *et al.* (2000) found that children who are about three years old do not show a portion size effect while children around the age of five do show this effect [2]. The authors conclude that susceptibility to the portion size effect develops at some point between these two ages. The authors propose that children are taught to focus on external eating cues by being told to "clean their plate" and they learn to disregard internal cues of hunger and satiety. This proposed mechanism for the development of the portion size effect implies that it occurs because participants are trying to "clean their plate", yet this seems highly improbable. In fact, Rolls

et al. (2004) demonstrated a portion size effect in those who reported frequently being told to clean their plates and those who reported rarely being told to clean their plates [4]. Indeed, if participants were simply trying to eat as much as they could, one would expect an equal amount to be eaten in both the standard and large portion size conditions. This is not the case in a typical portion size experiment, where participants eat more when they are served more food. It may be that parents unintentionally teach children to ignore internal hunger cues, but it is unlikely that children learn to eat more when a larger portion size is served to them. Furthermore, there is correlational evidence suggesting that children as young as two do not regulate their own energy intake. Mrdjenovic *et al.* (2005) had parents record what they served to their children and how much of it their children ate over a two week period. The authors found that the best predictor of how much the children ate was dependant on how much they were served [11].

Perceptual Shortcuts

Wansink *et al.* (2007) have proposed that the portion size effect occurs because participants rely on consumption norms (which are influenced by external factors such as portion size) to decide how much to eat [12]. Reliance on consumption norms occurs because people are not able to estimate with accuracy the size of large portions [13], and because it would consume time and cognitive resources to pay attention to how much one eats, or to estimate how much one should eat. Instead, people rely on perceptual indicators of portion size (among other things). There is certainly evidence that people do use perceptual heuristics when eating. Wansink *et al.* (2006) found that participants eat more when the same portion size is placed in a larger bowl relative to a smaller bowl [14]. Wansink *et al.* (2006) also found that participants eat more when they scoop ice-cream with a larger spoon relative to a smaller spoon [14]. In summary, it appears that participants do use visual shortcuts when eating and these shortcuts may cause them to over eat. These visual shortcuts make one susceptible to the portion size effect because participants tend to underestimate large portion sizes and do not take the time and energy to determine how much energy is appropriate for intake. Instead, they rely on consumption norms, which are influenced by these shortcuts.

Is Susceptibility to the Portion Size Effect Adaptive?

Perceptual shortcuts and reliance on consumption norms may be mediators of the portion size effect, but these perceptual shortcuts are unlikely to be learned. There is some evidence that susceptibility to the portion size effect starts early. The biggest predictor of how much children as young as two years old eat is how much they are served [11]. We propose that susceptibility to the portion size effect is genetic in origin and is a trait that has been naturally selected for because it was adaptive at some point during man's existence. This implies that at some point in man's existence, food was scarce. When individuals did encounter food, those who ignored their internal cues of satiety and kept eating and storing energy for less prosperous times were more likely to survive, reproduce, and pass on their genetic information. In this manner, the population of human beings became susceptible to the portion size effect and this trait was transmitted from generation to generation. This would explain the robustness of the effect and the wide diversity of participants in whom a portion size effect has been observed. Portion size studies on cultures with different values, experiences, and beliefs might provide evidence that there is no one childhood experience that causes participants to disregard their internal hunger and satiety cues and rely on perceptual heuristics, because it may be in their genes.

Obese and Normal-Weight Differences in Susceptibility to the Portion Size Effect

Nisbett *et al.* (1968) manipulated the number of sandwiches served to overweight and normal-weight participants [15]. The authors found that overweight participants ate more when served 3 sandwiches than when served 1 sandwich, whereas normal-weight participants did not show this pattern. Studies following this finding have not found any differences between obese and normal-weight individuals in susceptibility to the portion size effect. For example, Rolls *et al.* (2002) found a portion size effect in both obese and normal weight individuals when the energy from a serving of macaroni and cheese serving was manipulated [1]. Furthermore, the underlying mechanism that mediates the portion size effect does not differ between obese and normal-weight individuals. Both obese and normal-weight individuals are unable to estimate large portion sizes [13], and probably rely on consumption norms, making them equally susceptible to the portion size effect. There is, however, evidence from food diaries that women who are obese take larger portions of foods with a high energy density than do women who are not obese. Furthermore, women who are obese take smaller portions of foods low in energy density than do women who are not obese [16]. This is an interesting correlation because it provides an avenue for the portion size effect to contribute to the obesity epidemic. It may be the case that although both obese and normal-weight individuals are susceptible to ignoring their internal satiety cues and relying on external cues such as portion size, obese individuals are more likely to seek out large portions in the first place. It may be that although obese and normal-weight individuals do not differ in their susceptibility to the portion size effect, they differ in their exposure to large portion sizes.

Applications of the Portion Size Effect

It appears there may be ways for people to take advantage of the portion size effect. If participants eat more when served larger portion sizes, than participants also eat less when served smaller portion sizes. Indeed, limiting the size of portions served has been shown to be an effective way to treat obesity [17]. In another example of an application of the portion size effect, Wansink *et al.* (2005) proposed that since a portion size effect has been observed in foods low in palatability, serving children large portions of vegetables may be a way to increase their intake of these undesirable food items [5]. It has also

been proposed that that the existence of a portion size effect suggests that individuals are more influenced by the volume of the serving and less influenced by the actual energy of the serving. Therefore, eating foods low in energy density results in lower energy intake relative to eating foods higher in energy density and this effect occurs regardless of portion size [7]. The authors therefore suggest that individuals fill up on foods low in energy density (such as salad, soup, and water) before eating the main course in order to limit their energy intake.

Directions for Future Research

The portion size effect is indeed very robust. It has been observed with a diverse range of participants, foods and contexts, however it has not been demonstrated cross-culturally. A demonstration of this effect (or a lack thereof) in a variety of different cultures may help in understanding the origins of susceptibility to the portion size effect and the mechanism by which the portion size effect is mediated. The differences between obese and normal-weight individuals' response to changing portion sizes seems minimal. More work should look to explore what real effect, if any, the portion size effect has on the obesity epidemic. Finally, the portion size effect operates in so many people and in so many contexts that there must be multiple ways to use it to our dietary advantages. This review has touched on only a few of them, but more work should focus on the real-world applications of the portion size effect.

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Gender Differences Associated with Social Phobia: A Developmental Perspective

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Review Article

Abstract

Social phobia, a psychological disorder marked by intense fears in social situations, affects 6.7% of Canadians. Recent literature has found a strong and unique association between social phobia and behavioural inhibition (BI) (a temperamental factor). BI is believed to be a necessary precursor for the development of social phobia. Interestingly, BI appears to be more prevalent in females, which is also the case for the sex ratios in social phobia. Men and women also have different risk factors and outcomes with BI and social phobia. While men with BI are more likely to have a psychological and social burden than women, women tend to have more environmental risk factors for social phobia than men. Researchers have hypothesized that gender differences are likely due gender stereotype scripts, which affects caregivers' and clinicians' judgments of providing help in boys and girls who are vulnerable to social phobia. Beyond identifying behavioural characteristics, environmental risk factors and outcomes for boys and girls who have BI and social phobia, this review takes a developmental perspective to examine the developmental trajectory from BI to social phobia. As such, a study which measures BI, personality factors, and social phobia will be explored to understand how BI directly impacts personality, and thus social phobia. This developmental perspective suggests that BI and social phobia are connected at a dimensional level, with socio-environmental factors mediating boys' and girls' outcomes.

Introduction

As stated in the fourth revised edition of the Diagnostic and Statistical Manual of Mental Disorders [1], social phobia is a psychological disorder marked by evident and constant fear and anxiety in situations involving social evaluation or unfamiliar people. This anxiety must affect the person's normal routine. Individuals with social phobia undergo great distress in their daily functioning, because meeting new people is nearly inevitable in normal social interactions. The onset of social phobia is estimated to occur between the ages of 12 to 15 [2], and usually affects 3 to 5% of youths [3]. When individuals with social phobia go untreated, it impacts their interpersonal, academic, and work development. Having social phobia also increases an individual's chance of comorbidity with one or more other psychological disorders including depression, substance abuse or dependence, and other chronic conditions [2, 4]. Unfortunately, even though it is the third most common psychiatric disorder [5], social phobia often goes undetected by health care professionals [6]. Furthermore, it is the least studied psychological disorder in the literature, despite its debilitating impact on the lives of individuals with social phobia [7].

Although there are many potential factors involved in the development of social phobia (e.g., temperamental factors, abnormal linguo-physiological factors, child-parent interaction factors, and genetic factors) [8], temperamental factors in particular are noteworthy to discuss. Temperament is the set of individual characteristics anteceding personality development and remains fairly stable throughout lifetime [9]. One temperamental factor in particular, behavioural inhibition (BI) has a specific and strong relationship with social phobia compared to other anxiety disorders. BI is the persistent tendency for fear and withdrawal in unfamiliar social situations, but in a less severe degree of distress than social phobia [10]. Individuals with BI respond to unfamiliar or novel social situations with restraint, caution, low rates of approach, withdrawal, shyness, and timidity [10]. BI affects 10 to 15% of children [11]. Even though the gender ratio for BI has not been confirmed in any single study, the prevalence of both social phobia and BI appears to be higher in women than men [2], and those with social phobia reported that they had BI when they were younger [6]. Men and women are also usually treated differently due to gender roles, which facilitate the stability in individual differences. Therefore, gender differences may affect the developmental trajectory of individuals with BI, and thus social phobia.

Looking at specific gender factors involved in BI may assist in prevention of social phobia by allowing more health care professionals and parents to be educated about the different socio-environmental risk factors associated with this disorder in boys and girls. Hence, this review will reveal the striking relationships between gender, BI, and social phobia since these relationships point to the role of gender stereotypes in the development and detection of social phobia. First, the relationship between BI and social phobia, along with demonstrating the need for exploring this relationship with a gender perspective will be revealed. After, research that investigated BI and social phobia separately with gender will be presented. Lastly, this review will link all these variables with the results of a cross-sectional study that analyzed BI, personality factors, social phobia, and gender to show a developmental transition from temperament, personality and psychopathology.

The Relationship between Behavioural Inhibition and Social Phobia

BI and social phobia differ in severity of distress but are similar according to their behavioral definitions. Both conditions involve avoidance strategies, passive withdrawal, inhibition when approaching strangers, and delayed development in expressing self with words [12]. Apart from their theoretical similarities, experimental interviews and observations provide evidence to support the strong positive association between BI and social phobia. Specifically, researchers have found that children with an inhibited temperament are more likely to develop traits of social phobia [13-15]. Adolescents who demonstrated inhibited behaviours as children were four to five times more at risk for adolescent social phobia than those who were not identified with having BI [8, 16]. With research, the literature has supported that BI is a strong precursor in developing social phobia.

Even though BI is a factor in predicting social phobia, it has been found that a significant proportion of children characterized as behaviourally inhibited do not develop social phobia. For instance, one study showed that 61% (n = 26) of adolescents who demonstrated BI as toddlers had social phobic characteristics (i.e., sub-threshold diagnostic social phobia), whereas 27% (n = 7) of adolescents who did not demonstrate BI as toddlers had social phobia [18]. The significant percentage of adolescents who do not show a continuous development from BI to social phobia appears to contradict the role of BI as a necessary precursor to social phobia. Some researchers feel that this inconsistent finding may be due to the use of an older paradigm for evaluating symptoms of BI, therefore masking the effect of BI on social phobia [12]. However, further review on this study revealed that when the threshold was raised for the diagnosis of social phobia to require definite impairment in people's daily routine, 44% (n = 10) of female adolescents and 22% (n = 4) of male adolescents who were inhibited toddlers were affected by social phobia, as opposed to 6% (n = 1) female adolescents and 13% (n = 2) male adolescents who were not inhibited toddlers yet were affected by social phobia [18]. In summary, females who were inhibited toddlers were nearly seven times more likely to have social phobia compared to females who were uninhibited toddlers. This seven-fold difference suggests that females appear to be especially vulnerable to social phobia if they were behaviourally inhibited when they were young. Despite a caveat of this study's low sample size and retrospective design, it is noteworthy to understand that perhaps the masked effect may have been due to the study's lack of attention on gender differences.

Gender Differences in Behavioural Inhibition and Social Phobia

Gender Differences in Behavioural Inhibition

While it has been found that more girls than boys have an inhibited temperament [18-19], there is a scarcity of research on gender differences in BI. Schwartz *et al.* explored temperament or personality characteristics in different gender [18]. This study found that inhibited girls made fewer spontaneous comments (i.e., comments that were unrelated to the interviewer's questions or explanations) than inhibited boys and uninhibited individuals during the interviews. The authors suggested that boys who are inhibited are more noticeable to health care professionals and parents because stereotypical gender scripts delineate boys to be active and out-spoken instead. As a result, inhibited boys are less likely to develop social phobia because of direct or subliminal help from their environment.

Furthermore, BI tended to be manifested differently in males and females. Shy boys were more likely to delay romantic development (i.e., marriage and parenthood) and occupational development (i.e., obtaining achievement in work and stable careers), whereas shy girls were more likely to follow stereotypical gender roles (i.e., homemaking and endorsing motherhood) [20]. Shy men were less likely to move out of their home and independently expand their social networks. They also felt more emotional distress and unpleasant moods [21]. Thus, although girls were more likely to have an inhibited temperament, boys demonstrating an inhibited temperament tended to be more affected in their daily functioning than girls.

Gender Differences in Social Phobia

Much like the sex ratios seen in BI, more girls are diagnosed with social phobia than boys [2]. Various studies have shown the differences in behaviour and environmental stimuli for girls and boys with social phobia. Girls with social phobia or generalized anxiety disorder have different behavioural characteristics than boys. Warren *et al.* investigated 72 children to look at the characteristics of toddlers with certain anxiety disorders [15]. The study found that girls displayed significantly more general anxiety, imaginary play, and fine motor activity compared to boys.

Gender is also associated with different environmental factors in individuals with social phobia. Specifically, social phobia in girls is associated with parental conflicts, childhood physical abuse, maternal mania [6] and failure to complete high school [22]. In contrast, social phobia in boys is linked with the absence of a parent or adult confidant [6]. It appears that girls are more likely to develop social phobia than boys because there are many more risk factors for girls, which increase their vulnerability to the disorder.

The rationale that was provided to account for gender differences in BI is also used to explain gender differences in social phobia. Specifically, gender differences in middle childhood are likely due to cultural expectations, such that the inhibited behaviours that characterize social phobia are more tolerated in girls than boys. As a result, these cultural expectations increase the risk of developing social phobia for girls [23]. The interplay between the gender socialization and the shaping of a child's reaction to stressors appears to impact the development of both BI and social phobia. However, despite the clear results showing on gender differences link with BI and social phobia, a major limitation in concluding how these gender differences in BI directly link to gender differences in social phobia because of the disregard of measuring transitions throughout development. A cross-sectional study

resolves this issue by measuring the relationship between temperamental factors, personality factors, and social phobia in middle childhood [19].

Temperament, Personality, and Gender Differences in Symptoms of Social Phobia

One study that linked temperament, BI, and social phobia investigated personality factors in middle childhood. Personality factors are considered the mature and sophisticated form of temperament, and unlike temperament that emerges a few months after birth, personality emerges a few years after birth [9]. Generally, individuals can be classified under a constellation of levels with five personality traits, as known as the Big Five Personality factors. These five personality factors are openness to experience, conscientiousness, extraversion, agreeableness, and neuroticism. In fact, three temperamental dimensions can be mapped onto three of the Big Five Personality factors: the extraversion/surgency dimension onto extraversion, the negative affectivity dimension onto neuroticism, and the effortful control dimension onto conscientiousness [9].

BI was predicted to be linked to two personality factors: neuroticism and extraversion [19]. High neuroticism was predicted to be associated with inhibition, because individuals who are inhibited are fearful and anxious in novel environments and respond more intensely to environmental stressors [24-25]. Low extraversion was predicted to be linked with BI because of traits such as shyness and low engagement in social activities [25].

To test their hypothesis, the authors conducted a cross-sectional study, using questionnaires to collect data on 226 children aged 9 to 12 years [19]. As hypothesized, it was found that BI is best represented by high neuroticism and low extraversion, and not by the other personality factors. More girls than boys self-reported to be more behaviourally inhibited and anxious, and these girls with higher levels of neuroticism and lower levels of extraversion were associated with more symptoms of anxiety. Through statistical analyses, BI remains a stronger factor than personality factors in predicting anxiety symptoms. This result confirms another study's findings that adult self-reports of BI in childhood, along with adult self-reports of current traits of BI strongly predict the incidence of social phobia [26]. Thus, the results from this cross-sectional study support that BI transitions into personality through childhood development and increases the vulnerability of developing of social phobia [19]. Furthermore, these results provide the evidence that female toddlers, children, and adolescents are more vulnerable to social phobia than the male population, which shows that gender differences affect the prevalence of social phobia across childhood development.

Discussion and Conclusion

In summary, literature has shown that a temperamental factor, BI, can be used to predict social phobia in individuals. The similar pattern of prevalence of BI and social phobia across gender suggest gender socialization generated a diversion in the way boys and girls are affected developmentally by BI, and increasing girls' risk in developing social phobia. These gender differences probably include varying environmental and behavioural factors. Moreover, detrimental effects are likely to occur for men and women with BI in different ways. Although males have a lower prevalence rate than females in BI and social phobia, they tend to have greater burden than females with BI in social development. Females with BI appear to be more susceptible in developing social phobia than males with BI because they have more risk factors and do not stand out from female norms. Therefore, health care providers and parents should be more conscious of the social roles and environmental risk factors associated with each gender, which can influence the development of social phobia. Being conscious of these identified risk factors and social roles are especially crucial because they could instead provide the solution for developing feasible protective factors (e.g., promoting girls to complete high school, setting up peer support programs for boys to find adult confidents, educating children about social roles).

Unfortunately, the lack of longitudinal studies makes it difficult to capture the entire picture of the effects of BI on social phobia [8]. Many studies use a retrospective design, a research design in which the results depends on memory recall [6, 15]. The two studies that explored the variables of temperament, personality, and anxiety symptoms used a cross-sectional method, a research design that takes participants from different age strata [19, 26]. Unless more rigorous longitudinal studies are implemented, the conclusion that BI is a necessary precursor to the development of social phobia cannot be strengthened.

Future research might also consider investigating how gender roles affect the development of social phobia. The effect of gender difference has not been proven in this review because no studies have explicitly explored this factor. Secondly, as mentioned in this review, researchers only postulated instead of finding that gender stereotype socialization is the reason for the sex differences in the prevalence BI and social phobia. Given that there are limited studies which suggests the relationship between gender, BI, and social phobia, a mixed-method approach in a longitudinal study is needed. A mixed-method approach is critical in understanding the research question because the qualitative aspect captures the information in this unknown area of research, and the quantitative aspect can be used to determine the relationship's validity and reliability in a robust analysis.

Although social phobia is one of the least studied psychopathological disorders [7], finding a consistent gender difference in the small number of available studies suggests that the effect of gender is not a coincidence, but rather a remarkable factor in the developmental trajectories and outcomes of individuals with social phobia. Therefore, researchers, health care professionals, and parents alike should not devalue this factor, but instead, embrace it to better understand the mental well-being of individuals.

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Review Article

The Evolution of Immunotherapy; Looking at Anti-CD20 Monoclonal Antibodies

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Abstract

Over the past 10 years, immunotherapy has emerged as one of the most promising fields in neoplastic research. Immunotherapy involves introducing an antibody into the blood that recognizes and flags a particular cell marker expressed by malignant cells, allowing the immune system to destroy them. In 1997 the first immunotherapeutic drug, rituximab, a CD20 monoclonal antibody, was approved by the FDA for treating Non-Hodgkin's Lymphoma. Since then, more CD20 therapeutics are appearing on the market along with many immunotherapies targeting other cancer and autoimmune cell markers. Their popularity and potential for future use comes from their specificity when binding to target cells, leaving most healthy cells untouched. Immunotherapies can also have significantly reduced side effects compared to traditional chemotherapies. Rituximab in particular has enjoyed great success in treating B cell lymphomas, since CD20 is only expressed on early B cells and more differentiated B cells, but not on healthy precursor B cells. More recent CD20 therapies are designed to improve the efficiency of targeted cell death and further decrease side effects. Such an approach is used by the drugs ibritumomab tiuxetan and tositumomab, which target CD20 cells with conjugated radioactive isotopes, delivering radiation to specific cells. Another potentially effective strategy is conjugating chemotherapeutic agents to CD20 antibodies to deliver chemotherapy only to target cells. This has already been accomplished for a CD33 antibody, Mylotarg®, in treating acute myeloid leukemia. The future prospects for antibody oncology are bright.

Introduction

The recent surge in cancer research has resulted in many novel therapies unlike traditional chemotherapy, including a very promising new option called immunotherapy. Traditional cancer therapeutics kill rapidly dividing cells by damaging the DNA or other vital cell components during cell replication, resulting in non-specific targeting of the rapidly dividing cancer cells [1]. Unfortunately this approach also targets the body's healthy rapidly dividing cells, including cells of the immune system, hair cells, and several other cell types [2]. This produces many side effects including a compromised immune system and hair loss [2]. Traditional chemotherapies also have a significant risk of causing secondary cancers due to their mutational effects on DNA [3]. In contrast, immunotherapeutic strategies target a specific group of cell surface markers, called a cluster of differentiation (CD). Most cell types will express unique combinations of CDs depending on their function and developmental status. This allows immunotherapies to target a very specific group of cells. Tumor cells often evade the immune system by altering the expression of surface markers, thereby preventing the immune system from recognizing them since the immune system isn't designed to recognize the particular markers in the context of the tumor cell. By binding a specific CD that is exclusively expressed by the tumor cell with a monoclonal antibody (MAb), the cell is then flagged and can be recognized and killed by the immune system [4]. Furthermore, the MAb binding can directly kill the cell by creating an internal signaling cascade [4, 5]. This results in very specific cell killing, with reduced side-effects and a decreased risk of developing secondary cancers [6].

For these reasons immunotherapy has become a fundamental treatment for B-cell lymphomas and leukemias as well as autoimmune conditions. B cell cancers lend themselves particularly well to immunotherapy due to the existence of very specific CD markers at different stages in B cell development. This allows a particular stage in lymphocyte development to be targeted, depending on which stage is affected. This target-specific method leaves the healthy stem cells and early precursor cells in place, allowing them to replace tumor cells with new healthy cells. Since only a specific set of cells are removed during most MAb therapies, the individual's immune system often remains much more robust compared to treatment with traditional chemotherapy. Similarly, in autoimmune diseases, which are the result of an over-reactive immune system, MAbs help to reduce the number of auto-reactive cells [7]. This results in fewer autoimmune reactions and, subsequently, reduced symptoms.

MAb Targets: CD20

One CD of particular interest is CD20 due to its distinct expression from pre-B cell to the mature B lymphocyte cell stage. CD20 is not expressed on stem cells, pro-B cells (pre-B cell precursors), immunoglobulin(Ig) secreting plasma cells, or other normal tissues. Also, CD20, a component of the B cell receptor, is exclusively membrane bound which helps maximize efficiency of the MAb binding to a cell [8, 9]. This

allows drugs like rituximab (Rituxan®) to specifically target tumor cells in cancers which have altered CD20 expressing B cells, such as in chronic lymphocytic leukemia (CLL) and various B cell lymphomas. Rituximab has also been found useful in various non-neoplastic immune-mediated diseases, such as rheumatoid arthritis [10] and Lupus erythematosus [11]. Rituximab is a CD20 chimeric murine/human MAb, meaning the MAb is a fusion of mouse and human protein.

The molecular mechanism through which rituximab mediates cell death has yet to be determined, but has been narrowed down to three potential pathways [12]. The first, and most likely, mechanism of action is antibody-dependent cell-mediated cytotoxicity (ADCC) in which the two Fab segments of the rituximab Ab bind CD20 and the Fc segment binds to an immune effector cell, which then lyses the B cell. The second proposed mechanism is complement-dependent cytotoxicity (CDC) where the complement cascade is activated, leading to holes being made in the cell membrane and cell lysis. The last mechanism is apoptosis, which is directly induced by rituximab binding CD20, resulting in increased calcium levels and the activation of the caspase cascade [12]. It is also known that the rituximab-CD20 interaction results in the inhibition of the NF-kappaB pathway, which is involved in cell survival [13]. This increases the cell's sensitivity to chemotherapy resulting in synergistic effects [13]. For this reason immunotherapies are often used in conjunction with traditional chemotherapies.

Although CD20 MAbs have improved treatment of many diseases, there are still many areas to be improved on. The main side effects of rituximab have primarily been acute allergic reactions during infusion which could be a result of the mouse portion of the MAb. If an individual has human-anti-mouse-antibodies (HAMA), the rapid delivery of rituximab via infusion directly into the blood can overwhelm the immune system causing a massive allergic reaction. In severe cases this can lead to anaphylactic shock or rituximab intolerance [14]. For this reason researchers looked to fully humanize the rituximab MAb, hypothesizing that the immune system should react less to a foreign human protein than a foreign murine protein. As a consequence of reducing the allergic reaction, the drug could potentially be administered in one quick dose as opposed to rituximab which must be infused over a period of a few hours to reduce the chances of a severe reaction. One such potential candidate is a newly emerging drug called ocrelizumab. In addition to improved drug delivery methods, ocrelizumab, a fully humanized version of rituximab, could decrease the amount of MAb neutralized by the immune system before binding to CD20 due to immunogenicity, thus potentially increasing the efficacy of MAb binding [15]. Ocrelizumab has been proven effective in rheumatoid arthritis and is currently being examined for potential therapeutic use in Non-Hodgkin's Lymphoma [16].

Another way of improving the rate of rituximab-CD20 binding is to modify the actual binding portion of the MAb. The exact fit of the MAb to the Fc receptor expressed by immune effector cells in ADCC has been shown to significantly alter clinical response [17]. For example, in Waldenström's macroglobulinemia, a form of Non-Hodgkin's Lymphoma, response rates to rituximab treatment can vary based on a single nucleotide polymorphism (SNP) at amino acid 158 on the FCγRIIIa gene, which codes for the Immunoglobulin G Fc receptor (CD16a). Response rates vary from a 40% response to treatment for individuals who are homozygous for valine (V) versus 9% of individuals who are homozygous for phenylalanine (F) [17]. In essence this boils down to basic biochemistry; V is a small, branched amino acid whereas F is a bulky, aromatic amino acid. The size of F hinders the ability of rituximab to bind immune effector cells, which results in reduced ADCC and poorer clinical results for F/F individuals [17]. Subsequently, it is possible to do a simple test to determine if a specific treatment will likely work based on the specific amino acid sequence of the individual's CD16a molecules. In addition this finding has pushed the field to search for a similar MAb to rituximab which is better suited for the F polymorphism [17].

One way to help get around the problem of the V/F SNP is to use a MAb with low fucose content. MAbs with a lower fucose content are proposed to increase the amount of ADCC by out-competing IgG in binding the Fc receptor, due to a higher binding affinity [18]. This has resulted in the development of afutuzumab (GA101) which is currently in trials. It should be noted, however, that even with an afucosylated MAb V individuals still achieve slightly higher response rates [19].

Another method to try and improve binding responses is to create a MAb which binds an entirely different portion of the CD20 molecule. Such an approach was taken in developing ofatumumab (HuMax-CD20, Arzerra®), a CD20 human MAb. Ofatumumab differs from rituximab in that it binds a small and large loop on the CD20 molecule which is closer in proximity to the cell membrane than the rituximab binding site [20]. This increases the efficacy of signal transduction into the cell when the MAb binds. By binding CD20, which is postulated to be a calcium ion channel, ofatumumab has also been shown to block B-lymphocyte activation [21]. This past October ofatumumab was approved by the Food and Drug Administration(FDA) for use in treating chronic lymphocytic leukemia [22]. It is currently being extensively studied for use in treating Non-Hodgkin's Lymphoma, rheumatoid arthritis, and several other diseases, as well as being under consideration for use in combination with chemotherapy.

Even though rituximab is often very efficient in conjunction with chemotherapy and radiation, it would theoretically be much better if chemotherapy or radiation could be attached to the rituximab MAb, giving the chemotherapy and radiation the same specificity as rituximab. Although radiation increases the chances of serious long term complications, this method of delivery could help reduce the risk of secondary cancers by not unnecessarily exposing healthy cells to carcinogens or other mutagens. One such family of drugs includes ibritumomab tiuxetan (Zevalin®) and tositumomab (Bexxar®), which are CD20 murine MAbs with attached radioactive isotopes, yttrium-90 and radionuclide iodine-131, respectively. When the MAb binds the CD20 cell receptor, the cell is exposed over

an extended period of time to a lethal dose of radiation from the radioactive isotope [23]. Due to the way the lethal radiation is released it also kills the other cells in close proximity, which can be beneficial in eliminating solid tumors. Solid tumors can form clusters, preventing MAbs like rituximab from binding and killing cancerous cells in the middle of the tumor. The downside of this effect is that ibritumomab tiuxetan shouldn't be given to individuals with 25% or more bone marrow involvement since it could totally eliminate the remaining healthy bone marrow [24]. The attached radiation also eliminates the need for the immune system to respond by killing the cell and reduces exposure of healthy cells to radiation [23]. Ibritumomab tiuxetan has preliminarily been found to be more efficient than rituximab in various types of B-cell lymphoma while also showing promise in individuals who no longer respond to rituximab [25].

The Way of the Future?

Although a CD20 MAb with an attached chemotherapeutic agent has yet to be brought on the market for treating B-cell lymphomas and leukemias, a CD33 MAb, Mylotarg®, has been developed for therapeutic use in treating acute myeloid leukemia. Mylotarg® has been developed by attaching a CD33 MAb with calicheamicin, a chemotherapeutic agent isolated from bacteria which binds and cleaves DNA [26, 27]. This has not yet been accomplished for CD20 MAbs but may be coming in the future, perhaps leading a new generation of rituximab style drugs.

Rituximab is also advantageous in that it can often work again after relapse to the initial rituximab treatments whereas most traditional chemotherapies don't work nearly as well the second time due to built up resistance. However, in some instances individuals do develop resistance to a specific MAb which creates a future need for multiple MAbs which can be used in succession. This is also a good reason for combining rituximab with traditional chemotherapies, since the chance of a cancer simultaneously acquiring mutations which protect it from both rituximab and the chemotherapy are much slimmer than acquiring a mutation against rituximab or chemotherapy alone.

In addition to forming new MAbs, the acquisition of specific MAb resistances raises the idea of trying to keep only the functional part of the MAb and eliminating the non-binding part. The non-binding excess part of the MAb could potentially be responsible for the initial immune reaction, which can neutralize rituximab before it actually binds its target cells. A MAb is composed of four peptide chains, but only part of each peptide chain actually binds. This fueled the creation of a single chain polypeptide, called TRU-015, in which the entire MAb binding site is condensed into one polypeptide capable of binding CD20 and inducing cell death [28]. So far TRU-015 has been shown effective in rheumatoid arthritis and shows promise in rituximab refractory lymphoma but is still undergoing extensive study [28].

Another way that may merit further exploration is looking at polymerized MAbs which could recognize a cell expressing two or more different markers. This could greatly enhance the specificity of immunotherapy by introducing targeting of even more specific cell development stages, although there are no reports that this has been attempted.

It should also be noted that immunotherapy is beginning to be used in many diseases other than just B-cell neoplasias and autoimmune disorders. Important strides have been made in breast cancer therapy with the development of trastuzumab (Herceptin®) and bevacizumab (Avastin®) [29, 30]. Immunotherapy is also making inroads in treatment of melanoma, kidney cancer, and many other solid tumors.

Largely due to the huge success of rituximab, many pharmaceutical companies are now developing MAbs in the hope of more commercial successes. As a result, there are many new MAbs finding their way into clinical trials and many may soon become everyday treatments. With the development of numerous functional MAbs being used in immunotherapy, we may soon arrive at personalized treatment. In many ways immunotherapy treatment is already heading that way with the ability to test for SNPs, as in FcγRIIIa-158, and therefore determine which MAb would work best. With time we will be able to better understand which cell surface markers are expressed where and with what genetic variation, enabling us to make even more selectively targeted MAbs. We may even get to the point where specific, personalized MAbs can be made to exactly complement an individual's cell markers, thereby accounting for any mutations or polymorphic variations.

Although rituximab has drastically changed most B cell cancer treatment regimens, there is still a long way to go with potential for much more efficient, safer, and economical treatments.

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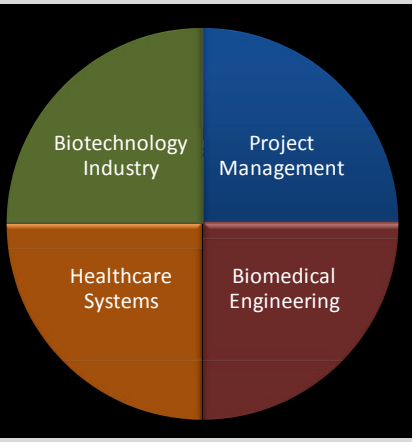

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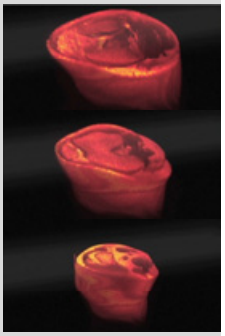

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Applications of Epigenetic Technologies in the Understanding of Complex Diseases

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Review Article

Abstract

Epigenetics is a relatively new field of research that focuses on the regulation of gene expression without changes in the DNA sequence. DNA methylation and histone modification play a major role in epigenetic mechanisms occurring in mammalian cells. Since there has been much evidence linking epigenetic abnormalities to a wide array of human diseases, including cancer and other complex disorders, the study of epigenetic processes can lead to a better understanding of disease etiologies. Furthermore, the examination of epigenetic events may also shed light on the future diagnosis and treatment of related diseases. In this paper, emphasis is placed on DNA methylation, and two complementary technologies used to interrogate methylation profiles, microarray and pyrosequencing, are described.

Introduction

In the early 19th century, the French biologist Jean-Baptiste Lamarck proposed an insightful idea that organisms have the capability to alter their characteristics in response to environmental changes and subsequently pass them on to future progeny. This suggestion of a potential ability of self-adaptation, which leads to direct evolution, is known as the Lamarckian inheritance of acquired traits [1]. In contrast, Darwin spoke of an indirect evolutionary process that involves stochastic mutations giving rise to advantageous phenotypes to account for viability and survival of the individual. Through the remainder of the 19th century, as Darwin's idea on natural selection received approval and recognition in the academic world, Lamarck's theory was largely disregarded by the scientific community. However, in the past 10 years, Lamarck's theory has been attracting more interest, as there is new evidence that phenotypes can be modified by environmental influences [2]. Currently, studies performed in the field of epigenetics may provide insight into the integrative effects of genetics and environment, offering a new perspective for understanding the etiologies of human disorders.

The term "epigenetics" literally means "above the genetic sequence," and it refers to the regulation of genes through processes that do not involve alteration to the DNA sequence [3]. Epigenetic changes are thought to be reversible and heritable through mitosis and meiosis, and they play a crucial role in cellular differentiation and development [4]. In other words, this non-sequence based information, which is essential for normal cellular function, is preserved during DNA replication and cell division and can even be passed on from one generation to the next.

Epigenetic Mechanisms – DNA methylation

Epigenetic regulation of gene expression is generally thought to occur in two ways: DNA methylation and histone modifications (e.g. post-translational transformations that include acetylation, methylation, phosphorylation and ubiquitylation) [5], both of which act together to influence the architecture of chromatin, and ultimately the expression and function of genes [4]. Another process that is also being considered as a form of epigenetic mechanism is RNA interference (RNAi). Widely occurring in eukaryotes, RNAi is a method of gene silencing, involving the creation of double-stranded RNA, which eventually can lead to translational repression or even mRNA degradation [6]. However, whether RNAi is a part of the epigenetic machinery is still up for debate; thus, the main focus of this review will be on DNA methylation.

DNA methylation refers to the addition of a methyl group (CH3) onto the C5 position of a cytosine ring, usually in CpG dinucleotides, which are regions in a linear sequence of DNA where a cytosine nucleotide is adjacent to a guanine. This covalent transformation is performed and maintained by a family of enzymes named DNA methyltransferases (DNMTs) [5]. It has been found that, in humans, around 80% of all CpG dinucleotides are methylated [7]. The remaining unmethylated sites are mostly located in CpG islands, which are long stretches of DNA (of at least 500 base pairs) that contain clusters enriched in CpG sites [4]. In humans, CpG islands are normally located on the promoter region of genes [8]. DNA methylation in the CpG islands triggers a complex series of events downstream (Fig. 1), recruiting various chromatin modifying complexes (such as the methyl-CpG-binding proteins and histone deacetylases), which interact to remodel chromatin conformation [9]. The structure of chromatin becomes more condensed with the formation of heterochromatin, which might eventually diminish the amount of transcription in that region. At the same time, the methyl groups on CpG sites also interfere with the recruitment of transcription factors, which may also

reduce transcription to a large extent and consequently result in downregulation of genes [10].

Effects of Environment on Epigenetic Modifications

There is compelling evidence that the epigenetic processes can be strongly influenced by a number of environmental factors, including diet and stress amongst many others, which could result in phenotypic and behavioral changes [11]. For example, the regulation of Agouti gene, which causes changes in fur color from a spectrum of dark-brown to yellow in mouse, is determined by the methylation status of the upstream region of the transcription start site [12] [13]. The expression of Agouti is inversely correlated with CpG dinucleotide methylation. Therefore, if the gene is heavily methylated, the expression of the Agouti gene is turned off, leading to a dark fur color [14]. What is interesting about Agouti is that the methylation status of the gene (and hence the coat color of the mouse), can be artificially altered by either supplementing or subtracting certain vitamins that act as methyl donors, such as folic acid and vitamin B12, from their diet. Increasing folic acid in the animal's diet leads to increased methylation and consequently darker fur color. The opposite is true if methyl donor is subtracted from their diet [13] [15]. Although there are numerous other examples of environmental influences on epigenetic modifications, there are already many review papers on this topic, and therefore, it will not be discussed in further detail in this paper.

Epigenetics and Human Diseases

Studies have shown strong evidence that suggests epigenetic mechanisms are closely associated with a variety of human diseases, especially complex diseases with non-Mendelian patterns of inheritance, where the proportions of various observed phenotypes do not match the expected values predicted using Mendel's Laws [16]. For instance, there has been compelling evidence that suggests several types of cancer, such as breast cancer and prostate cancer, are associated with epigenetic alteration [17] [18] .

Other human diseases, including Prader-Willi and Angelman syndrome, are also shown to have causes related to epigenetics [16]. These disorders are thought to be imprinting disorders caused by the misregulation of imprinted genes. Imprinting refers to the regulation of genes by epigenetically silencing one copy of either the paternal or the maternal gene by DNA methylation, which results in mono-allelic expression of that particular gene. Around 90 imprinted genes have been identified so far, and they play an important role in helping us understand relevant human pathologies due to their susceptibility to epigenetic changes [19].

It has also been suggested that epigenetic factors may be involved in a number of psychiatric disorders, including schizophrenia, bipolar disorder and major depression [11] [20] [21] . Several key genes that are thought to be related to the etiology of psychiatric disorders have been found to be differentially methylated between affected and control subjects [22]. Variations in methylation patterns have been identified in loci associated with glutamatergic and GABAergic neurotransmission pathways, which is consistent with previous findings on the pathogenesis of such psychotic diseases [23].

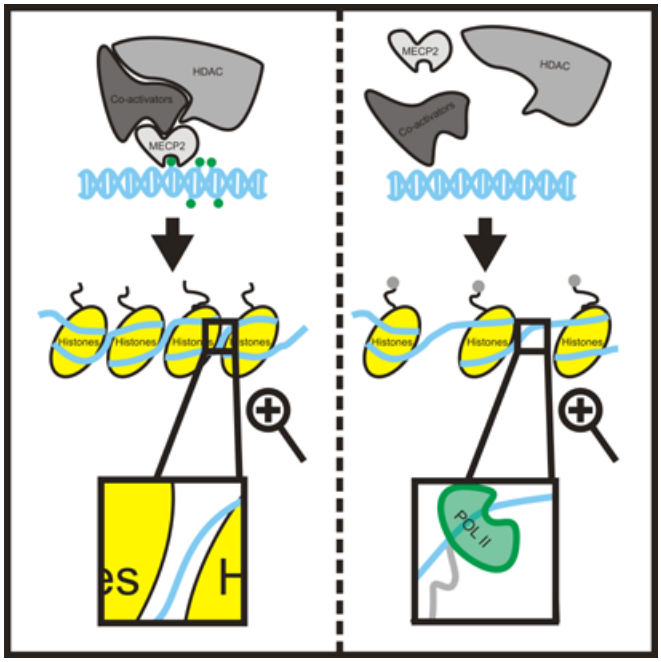

Figure 1: DNA methylation and its effect on chromatin structure. Methylated DNA results in the recruitment of methyl-CpG binding protein 2 (MECP2) and histone deacetylase (HDAC), as shown on the left. This leads to the formation of a compacted chromatin structure, or heterochromatin, which presents little accessibility for RNA polymerase (POL II), thus resulting in gene silencing. POL II activity is not hindered when the DNA is unmethylated, as shown on the right.

Epigenetic changes are pertinent to human health and disease. Thus, alongside the study of 'traditional' DNA sequence variations and environmental factors, epigenetic mechanisms add a new perspective in the investigation of disease etiology.

Epigenetic Technologies

DNA methylation is an important player in the epigenetic machinery, and it is generally thought to be a stable and long lasting modification [24]. Here, two commonly used high-throughput techniques for examining DNA methylation will be discussed.

Microarray Studies

The use of a microarray study allows for a large-scale investigation of a methylation profile. To obtain constructive epigenetic information and understand the methylation status, the DNA samples must be prepared in a specific way prior to being hybridized onto array chips. There are a number of approaches that can be taken to prepare the samples, depending on the objectives of the particular study. One such method involves the enrichment of either the hypomethylated or hypermethylated regions of genomic DNA. For example, enzyme-based enrichment of hypomethylated genomic fractions is achieved through a series of steps [7] (Fig. 2). First, the

genomic DNA is cleaved by methylation-sensitive restriction enzymes (e.g. HpaII), which can only cut at unmethylated CpG sites. Double-stranded adaptors are then ligated to both ends of the digested DNA fragments, and are later used in an adaptor-specific PCR reaction, during which the attached adaptors serve as primers to initiate the amplification process. However, before the PCR amplification, the samples are treated with a restriction enzyme (e.g. McrBC), which selectively cuts at the methylated CpG sites; this results in the disruption and cleavage of adaptor-ligated fragments that contain CpG methylation to ensure that only unmethylated fragments, which have adaptors attached on both ends, are enriched in the amplification step. Following the preparation of samples, the enriched products are labeled with fluorescent dyes. Different dyes (e.g. Cy3 and Cy5) are added to the control and experimental samples to differentiate between the two methylation profiles. The enriched samples can be hybridized onto the microarray chip, which contains thousands of spots of DNA oligonucleotides known as "probes" [25]. After hybridization, the arrays are scanned and statistical analysis identifies the regions of differential methylation between experimental and control samples.

Pyrosequencing

Pyrosequencing is another high-throughput technology that is often used in epigenetics research. Unlike microarray experiments, which aim to analyze a genome-wide methylation profile, pyrosequencing is capable of detecting the precise methylation status of CpG dinucleotides within relatively short sequences of around 100 base pairs. Pyrosequencing is thus a fine-mapping tool that provides quantitative information about CpG methylation that is useful in studying epigenetics-related disease.

When using pyrosequencing to analyze a methylation profile, the DNA samples go through treatment with sodium bisulfite, which converts all unmethylated cytosines into uracils, while methylated cytosines stay unaffected. The efficiency of this step is critical, since theoretically all remaining cytosines after treatment should be methylated, and through the detection of the sequence the status of methylation at each CpG site can be identified. Following treatment with sodium bisulfite, the converted DNA is amplified by PCR, during which all uracils are replaced by thymines. Lastly, PCR amplified samples are placed in the pyrosequencer, and by determining the percentage of cytosine integration instead of thymine at each CpG site, the precise methylation level can be quantified [26].

Pyrosequencing, a luminometric assay that uses a sequencing-by-synthesis principle (Fig. 3), is based on the detection of pyrophosphate molecules, which are released from the incorporated deoxynucleotides (dNTPs) by DNA polymerase during elongation of the strand [27]. First of all, a single-stranded bisulfite-treated template is hybridized with a sequencing primer and incubated in a reaction mixture, which contains four enzymes (DNA polymerase, ATP sulfurylase, luciferase, and apyrase) as well as two substrates (adenosine 5-phosphosulfate (APS), and luciferin). The nucleotides are

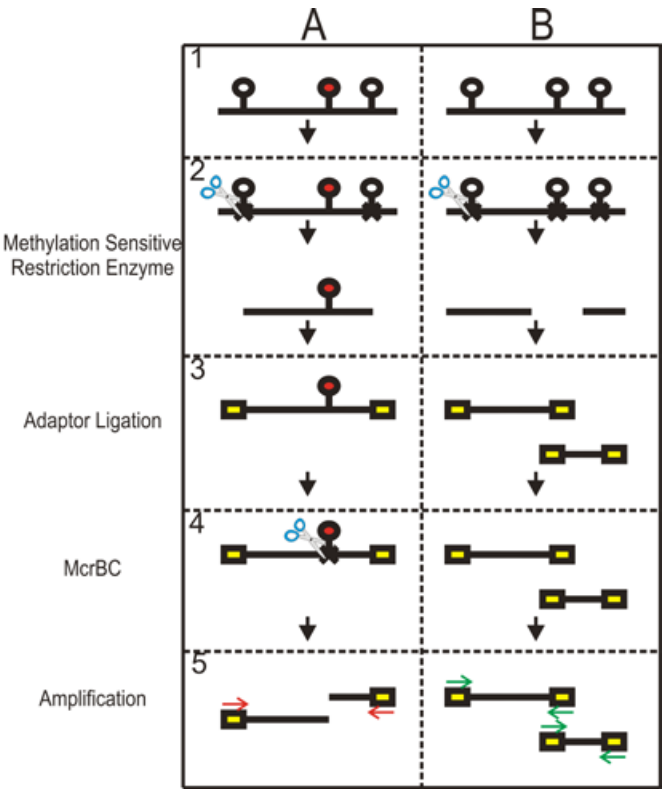

Figure 2: Procedures in the enrichment of hypomethylated regions for CpG microarray hybridization. 1) A methylation-sensitive restriction enzyme is used to cut at unmethylated CpG sites (indicated by white circles). **2)** Double-stranded adaptors are ligated onto both ends of each of the resulting fragments. **3)** McrBC, a methylation-specific restriction enzyme, cuts at methylated CpG sites (represented by red circles). 4) Adaptor-specific PCR amplifies only the fragments containing both adaptors. Therefore, the hypomethylated regions can be enriched.

added into the reaction mixture in a specific dispensation order complementary to the template sequence. One at a time, the four nucleotides are dispensed, and after a nucleotide has been incorporated into the growing strand by the activity of DNA polymerase, a molecule of pyrophosphate is cleaved. This free pyrophosphate becomes the substrate of ATP sulfurylase, which converts a molecule of APS into adenosine triphosphate (ATP). The product ATP from the previous enzymatic reaction then provides energy to luciferase to oxidize luciferin, generating light in the process. A third enzyme, apyrase, functions by continually degrading unincorporated dNTPs as well as the ATP produced by sulfurylase. Subsequent integration of nucleotides will occur only when the degradation process is complete. The emitted light, proportional to the amount of ATP generated, is detected by a charge-coupled device (CCD), which produces a pyrogram that specifies the type and number of nucleotide integration [26, 28]. Therefore, pyrosequencing provides accurate data concerning the methylation level at each CpG site, and can be utilized as both a fine-mapping and validation technology.

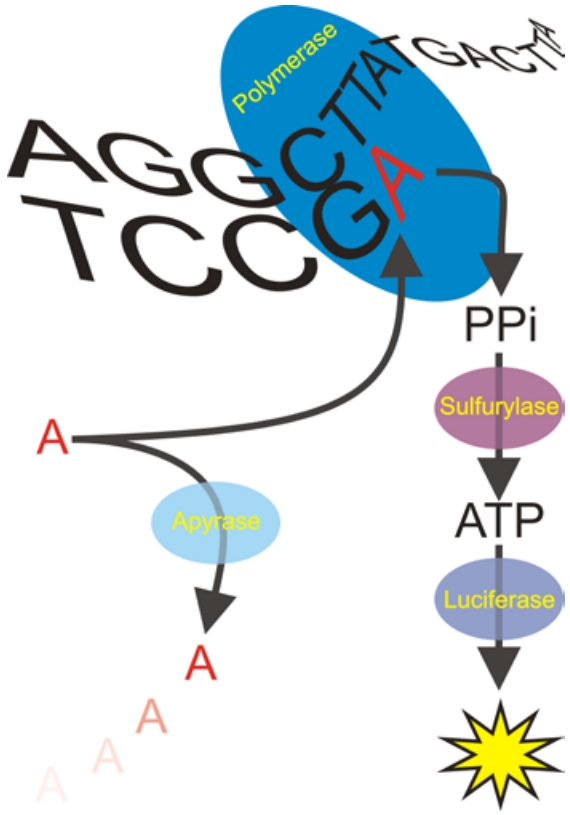

Figure 3: The principles behind pyrosequencing. During the incorporation of a nucleotide into the nascent DNA strand, a pyrophosphate (PPi) molecule is cleaved and released. This free PPi is used as a substrate for sulfurylase to generate energy in the form of ATP. ATP is then utilized by luciferase to oxidize luciferin, emitting light as an outcome of the chemical process. The amount light produced is proportional to the number of nucleotides integrated during the amplification. Apyrase continually degrades unincorporated nucleotides and ATP in preparation for the dispensation of the next nucleotide sequence.

Technological Limitations

Although microarray undeniably can provide us with much useful information concerning large-scale methylation profiling, it also has its limitations. Since each cloned sequence on the microarray chip usually contains multiple CpG sites, it is often impossible to distinguish exactly which of these sites is hypomethylated versus hypermethylated compared to a normal sample [29] [30] . Therefore, microarrays do not specify the methylation status at each individual CpG site, as they only provide a broad overview of the amount of methylation that exists in the entire sequence being assessed. To overcome this problem, a fine-mapping technology such as pyrosequencing is used in conjunction, as a way to zoom in onto a particularly interesting region that has been identified by the microarray. Since methylation of even one single CpG site could potentially have a large impact on the resulting phenotype, by determining the methylation status at specific sites we will be able to acquire a better understanding of the disease etiology and its underlying mechanisms [24]. This knowledge can facilitate the identification of markers for a particular type of disease. Pyrosequencing is also utilized to determine the exact percentage of methylation at each CpG site in order to provide quantitative data, as well as to validate the outcome obtained from the microarrays.

However, there are also drawbacks associated with pyrosequencing. One major limitation is that, when used for methylation detection, only short sequences of 100 base pairs can be examined at a time, which can make large-scale detection quite costly and labour intensive. Typically in experimental studies, several technologies have to be applied in concert to ensure accurate results and a more thorough understanding of the data.

Applications of Epigenetics

Through the study of epigeneticss of human disorders we are able to gather large amounts of information, which can be highly relevant in many applications including the determination of diagnostic markers as well as the identification and development of novel therapeutic targets and agents. For example, hypermethylation of tumor suppressor genes, resulting in their silencing, is found to occur in many types of cancers, some of which include cancer of the prostate, bladder, ovary etc. Thus, these genes that are silenced through epigenetic modifications can be used as diagnostic markers, enabling both the screening and prognosis of cancer patients [31]. One class of drugs called DNA methylation inhibitors has been developed to reverse the irregular methylation of various genes. One such example is azacytidine, a nucleoside analogue that can be incorporated into replicating DNA to interfere with the methylation process, shown effective in treating myelodysplastic syndrome and leukemias [32]. These agents can potentially be targeted to tumor suppressor genes, reversing their methylation to reactivate their function in pathological situations [5]. Additionally, another class of therapeutics termed histone deacetylase (HDAC) inhibitors can be used in combination with DNA methylation inhibitors to kill cancer cells in a synergistic manner [32]. Epigenetic therapy may also shed new light on the treatment of neuropsychiatric disorders. For example, a known HDAC inhibitor, valproic acid, is a drug that is developed to treat schizophrenia [33].

Currently, the therapeutic effects of epigenetic drugs are short-lasting and relapse occurs in some cases [5]. In addition, most epigenetic drugs tend to be non-specific regarding their targets. Thus, advancements should be made to increase the effect duration and improve the specificity of these novel therapeutics [5].

In conclusion, the study of epigenetics has cultivated new approaches to examine the etiological factors contributing to disease phenotypes, especially those that exhibit a non-Mendelian inheritance pattern. However, a great deal of complexities regarding the exact mechanisms has yet to be elucidated. Therefore, future expansion of the research in this field will allow us to fully map the epigenome and acquire a more complete picture of the underlying epigenetic events that contribute to disease development. It is expected that with this kind of etiological knowledge in the future, we will be able to screen patients for epigenetic disorders with a higher degree of

sensitivity and specificity. Furthermore, careful investigations at the molecular level of the integrative effects of epigenetic processes, DNA sequence variation, and environmental factors are likely to offer useful tools for generating prevention and intervention strategies.

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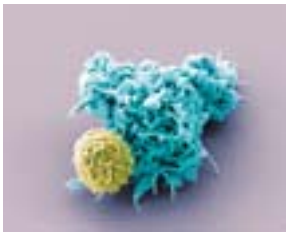
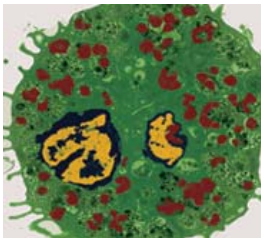
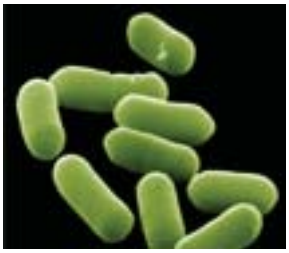
Immunology
UNIVERSITY OF TORONTO

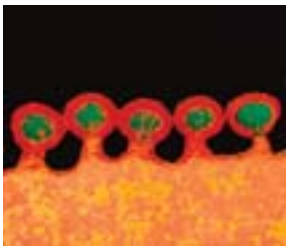
The Department of Immunology offers study programs in a wide range of immunological disciplines. These disciplines include molecular mechanisms of lymphocyte development and selection, T-cell and B-cell receptors, cell interactions, growth factor receptors, cytokine networks, antigen processing and presentation, signal transduction in lymphocytes, V(D)J recombination, anergy, apoptosis, transgenic and knock-out models, immuno-targeting and vaccine design, autoimmunity, AIDS, diabetes, and transplantation.


Adaptive Immunity

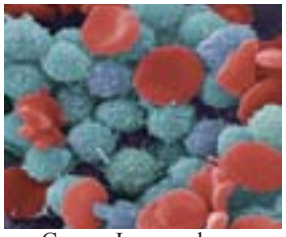

Innate Immunity

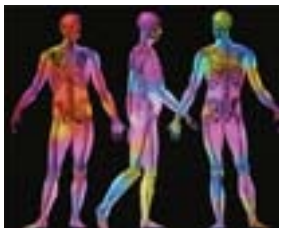

Infectious Disease


Developmental Immunology


Immunodeficiency


Autoimmunity


Cancer Immunology


Transplantation Immunology

UNDERGRADUATE PROGRAM

The Department of Immunology in collaboration with Trinity College co-ordinates a specialist and a major program in Immunology.
Apply via the Faculty's Subject POST website.
http://www.artsci.utoronto.ca/current/undergraduate/subject-post-enrolment

Immunology Specialist Program

The emphasis of the specialist program is to provide students with a sound theoretical understanding of the cellular and molecular basis of non-self recognition, together with sufficient laboratory experience to enable the students to consider embarking on a career in the discipline.

Immunology Major Program

The major program offers students fundamental training in immunology and gives the student the opportunity to combine immunology with another program in Life Sciences, Basic Sciences, or within the Arts.

GRADUATE PROGRAM

The department offers study programs towards the Masters of Science and Doctor of Philosophy degrees in a wide range of immunological disciplines.

M.Sc.

The purpose of the M.Sc. degree is to provide advanced training in an area of specialization, with a particular emphasis on the acquisition of experience in the strategies and experimental methods of modern, original, scientific research.

Ph.D.

The Ph.D. degree is an advanced research degree intended to reflect a level of training consistent with the ability of the candidate to function as an independent research scientist. This involves successful completion of course work reflecting a knowledge of modern Immunology, as well as a demonstrated ability to carry out research of publishable quality.

For more detailed description of our programmes offered, please visit our website:
www.immunology.utoronto.ca

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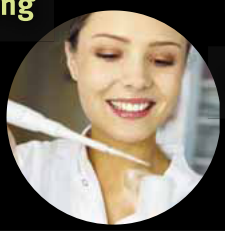
cancer

endocrinology

biochemistry

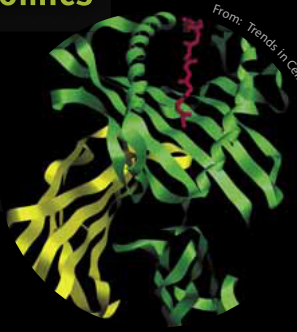
medical imaging

stem cells



proteomics

structural, molecular & cell biology



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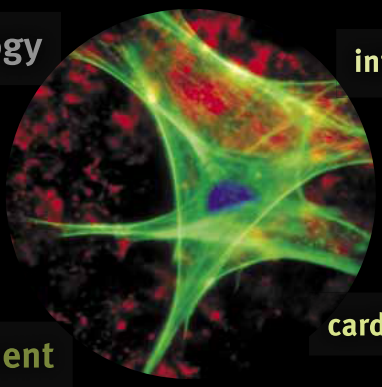
neuroscience

reproduction

immunology



infection & immunity

genetics

nutrition

pharmacology & toxicology

cardiovascular sciences

development

- Eight basic science departments in the Faculty of Medicine
- A community of internationally renowned researchers
- State-of-the-art facilities on campus and at affiliated hospitals and institutes
- Guaranteed financial support

UNIVERSITY OF TORONTO
FACULTY OF MEDICINE