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ACTIN DYNAMICS REGULATE MYOSIN ASSEMBLY IN MUSCLE CELLS

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Abstract:

During muscle cell (i.e. myocyte) development, organization of actin and myosin into the contractile unit (i.e. sarcomere) is required for proper muscle contraction. By disrupting the processes of muscle cell development, I am able to see which steps play important roles in proper maturation of myocytes. Elucidation of the key pathways in muscle development could lead to a better understanding of human cardiac hypertrophies and muscle myopathies. Since actin filament formation precedes myosin organization, I am using actin assembly inhibitors to determine if actin filaments are a necessary prerequisite for myosin organization. It is hypothesized that disruption of actin will disrupt myosin organization. In these experiments Jasplakinolide (Jasp) is applied to cultured embryonic myocytes during sarcomere assembly. Jasp binds, stabilizes, and induces polymerization of actin filaments (i.e. F-actin) making it a useful compound for determining if actin dynamics or precise thin filament length are necessary for myosin incorporation into the sarcomere. My results indicate that normal actin organization is required for correct sarcomere development. Myosin organization was reduced by more than 90% in all treatment regimes (50, 100, and 500 nanomolar Jasp). Sarcomeres failed to form and myosin appeared diffuse throughout the myocytes. My results indicate that intact, precisely regulated thin filaments are a prerequisite for normal myosin assembly. It remains to be determined if this requirement stems from actin-titin interactions, actin-myosin cross-bridge formation, or some other intermolecular interactions. Hopefully a better understanding of how sarcomeres form will provide insight into diseases involving the improper assembly of muscle such as myopathies or hypertrophies.

Introduction:

Striated muscle is a highly specialized tissue with one major function: contraction. Contraction of cardiac muscle causes the blood to pump throughout the circulatory system; contraction of skeletal muscle allows the body to move. Disruptions in the development or regulation of striated muscle can result is serious conditions such as cardiac hypertrophies or skeletal myopathies. So, a better understanding of how muscle cells develop could lead to treatment or prevention of such conditions. In skeletal muscle actin and myosin are organized into structures called sarcomeres, which contract by sliding of filaments not the compression or stretching of them. Actin filaments extend perpendicularly from a protein region called the Z line, while myosin thick filaments extend from the M line. A sarcomere is defined as the portion of the muscle that runs Z line-M line-Z line. Hundreds of sarcomeres are organized into strands called myofibrils, which run parallel to the long axis of the cell. All the aspects of this extensive organization is not completely understood, but new discoveries are always occurring

The organization of actin and myosin into the contractile unit (i.e. sarcomere) during development is a very complicated process requiring numerous actin and myosin associated proteins along with various kinases and phosphatases. For example, actin must associate with Z line proteins like alpha-actinin and CapZ for proper orientation and polymerization of the actin thin filament. Interestingly both actin and myosin can form filaments independently *in vitro*, but does this hold true *in vivo*? In other words, are interactions between actin and myosin necessary for proper assembly inside the muscle cell (i.e. myocyte)? Interactions between myosin A bands and actin I bands appear unnecessary for the organization of actin thin filaments in both vertebrates (Ferrari in prep) and invertebrates[4]. But, are I bands needed for A band formation?

During the formation of myofibrils, F-actin is organized into I bands prior to the incorporation of myosin thick filaments into A bands. Therefore, it is possible that intact I bands are required for proper A band formation. Mature F-actin may be needed to support actin-myosin cross-bridge interactions, which appear necessary to fine tune sarcomere organization[4]. Titin, a massive elastic filament that extends from Z line to M line whose role in muscle development and contraction is not fully understood, may play a role as well. Thin filaments could be

involved in actin-titin binding and subsequent titin dependent myosin organization. Therefore, to determine if proper actin organization is necessary for myosin A band formation, actin inhibitors are applied to embryonic myocytes during sarcomere formation. In this study, I examined the effects of Jasplakinolide (Jasp), which disrupts normal actin dynamics by stabilizing F-actin and inducing polymerization[3].

Methods:

Cultured myocytes were prepared from stage 15 embryos of Xenopus laevis as previously described[6]. Cells were treated at 6 hr in culture (approximately stage 19) with various concentrations of Jasp (50, 100, and 500 nM). This treatment time was selected because it is before the start of rapid organization of actin and myosin into sarcomeres. Some cultures were treated at 24 hr in culture (approximately stage 32) with 50nM Jasp. By 24 hr, sarcomere assembly is minimal and present only at the end of the cells. At 48 hr cultures containing more than 100 myocytes were fixed with .37% formaldehyde in (mM) 80 NaCl, 10 EGTA, 10 $MgCL_2$, 10 Pipes, pH 6.5 and permeabilzed with .37% formaldehyde/.1% Triton. Tris-buffered Saline (TBS) (in mM 80 Tris HCL, 10$MgCl_2$, 10NaH_3, pH 6.7) was used to rinse the cells before blocking with 1% milk in TBS. After blocking, cells were rinsed and left in TBS until stained for immunocytochemistry.

Myosin was labeled with MF20 supernatant (Developmental Studies Hybridoma Bank, DSHB), which recognizes sarcomeric myosin. Visualization of the antibody was achieved with Alexa-Fluor 488 goat anti-mouse IgG (Molecular Probes, MP). Tropomyosin, an actin-associated protein, was labeled with CH1 (DSHB) and a fluorescent secondary antibody, Alexa-Fluor goat anti-mouse 568 IgG_1(MP), as an indirect assessment of F-actin organization. In some experiments, actin was labeled with JLA20 (DSHB) and Alexa-Fluor 488 goat anti-mouse IgM (MP). All antibodies were left on for at least 2 hr at room temperature or overnight at 4°C. TBS washes were used between each antibody treatment to remove unbound antibodies.

An Olympus Microscope using a xenon light source with appropriate filters was used to visualize cells. Digital images were acquired with a Quantix cooled CCD camera and MetaMorph/MetaFluor imaging software (Universal Imaging Corp.). Image analysis was also done with MetaMorph/MetaFluor.

Data for sarcomere organization were recorded in both a qualitative and quantitative manner. In blind sessions, qualitative analysis was done by rating myocytes on a scale of 0-5: 0 corresponding to complete disorganization and 5 to perfect assembly. For quantitative analysis, first the area (in microns) of the myocyte from just outside the perinuclear region to the cell tip was determined. Then the total number of actin I bands and myosin A bands in this area were recorded to yield a density value. All values of both a qualitative and quantitative manner are presented as a percent of the control value. For example if control cells have an average Qualitative Tropomyosin rating of 5 and treated cells average only 1, then treated cells have an Qualitative rating that is 20% of controls. Statistical significance was determined using students unpaired two-tailed t-tests for values < .05. In figures, * indicates .05 > t-value < .001, and ** indicates t-values < .001.

Calcium imaging was preformed as previously described[6]. Myocyte cultures were treated at 2 hr in culture with 100 nM Jasp for at least 2 hours prior to imaging. After incubation in Jasplakinolide, Fluo-4 calcium indicator (MP) was added for 1 hr. The indicator was rinsed out with standard saline (mM 117 NaCl, .07 KCl, 1.3 $MgCl_2$, 2 $CaCL_2$ 4.6 Tris, pH 7.8) and 100 nM Jasp was reapplied. Myocytes were then imaged for 30 minutes.

Results:

Our results suggest that actin filament dynamics are necessary for proper A band formation. Jasplakinolide disrupts actin dynamics but has no known effect on myosin formation or incorporation into A bands. Yet, 6-hr treated cultures show almost complete disruption of myosin based on both qualitative and quantitative analysis. (All data are presented as a percentage of the control cell's value. See Methods paragraph 4). For cells treated with 50 nM Jasp (n = 31) the Qualitative rating (QR) for tropomyosin (TR) was 8.77% ±2.3 while the QR for myosin (MY) was 7.61% ± 2.2. I band density (IBD) was very low at only 3.35% ±1.7 along with A band density (ABD), which was 2.53% ±1.1. When 500 nM Jasp (n=50) was used, there was even more disruption of both actin and myosin. The QR TR was only 3.86% ±1.6, and the QR MY was 1.24% ±.9. Values for band density showed the same pattern: IBD 2.00% ±1.0 and ABD .23% ±.2. For all concentrations of Jasp used, values of I band and A band densities as well as qualitative ratings for both tropomyosin and myosin were significantly less than controls (Figure 1 & 2). Based on our data, actin organization is needed for proper myosin A band development.

Cultures treated at 24 hr showed some disruption but not near the levels seen in 6-hr cultures. Treated cultures (n = 51) had a QR TR that was 57.28% ± 5.6, QR MY 78.57% ± 5.0, IBD 72.16% ± 10.9, and ABD 79.36% ± 4.7 (Figure 3 & 4). These results are not surprising because mature actin filaments undergo constant remodeling,[9] and new sarcomeres are still being added to the ends of cells. Jasp would certainly disrupt these processes. However, F-actin was less organized than myosin A bands. The Wilcoxon sign-rank test showed that in treated cells the QR TR was significantly lower than QR MY as well as that IBD was significantly less than ABD. Both nonparametric tests had significance levels less than .05. This experiment shows that after the bulk of sarcomere formation has occurred, Jasp has significantly less affect on myocyte organization.

To insure results were not because Jasp prevents basic cellular function, controls were preformed to test for proper cell function. Calcium imaging served to show that myocytes maintain normal calcium dynamics in the presence of Jasplakinolide. This is important for two reasons. First, healthy myocytes will generate spontaneous calcium transients[6]; secondly, if Jasp alters normal calcium transients then myocyte organization could be disrupted because transients are necessary for sarcomere assembly (calcium transients are spontaneous increases in the levels of cytoplasmic calcium, mediated by calcium release from the sarcoplasmic reticulium)[6]. Dishes treated with 100 nM Jasp had an incidence (% cells active/ .5 hr) of 37.4% ± 7 while control cells had an incidence of 34.3% ± 4.9[6]. Also the frequency of calcium transients in myocytes was slightly lower but close to control cells (Control 6 ± .4[6]: Treated 4.2 ± .8). Treated cells were able to maintain normal calcium transients based on data for incidence and frequency in the presence of 100 nM Jasplakinolide (Table 1). Therefore, the disruption of actin and myosin was not because of a loss of cell viability but rather because of the action of Jasp on actin organization.

Discussion:

Direct visualization of actin filaments with Phalloidin, a traditional actin label, was not possible at the concentrations used in this experiment because Jasplakinolide and Phalloidin compete for the same binding site on F-actin[2]. Tropomyosin, which twists around the actin filament, was used as an indirect assessment of actin organization. Tropomyosin antibody staining and Phalloidin labeling was used in the presence of low Jasp concentrations to show colocalization of the two stains. Also some cultures were treated with concentrations of Jasp used in experiments and stained with tropomyosin and actin antibodies to show colocalization of tropomyosin and actin in the presence of high levels of Jasplakinolide.

The data show that when actin is disrupted myosin is disrupted as well, but the exact pathway by which this disruption occurs is unclear. The increased stabilization caused by Jasplakinolide could lock F-actin into an unnatural orientation. In this scenario, normal cross-bridge interactions may not occur, preventing consolidation of myosin thick filaments into A bands. In addition, Jasplakinolide-induced polymerization might generate longer than normal actin filaments. These long filaments may cause steric hindrance of myosin thick filament incorporation into the A-band region. Alternatively thin filaments may be required for titin stability. Experiments have shown that actin does interact with titin[8], and titin is known to have myosin binding domains[1,7]. In addition, some titin binding domains are thought to act as a template for myosin organization. Therefore, disruption of titin via disruption of actin would prevent normal myosin organization.

Experiments with 24 hr treatment show that after the bulk of sarcomere formation has occurred, treatment with 50 nM Jasp does not severely alter myocyte organization. The interesting result is that actin was significantly more disorganized than myosin. This result may suggest that once thick filaments are consolidated (perhaps by titin binding or M-Line binding). A band organization can be maintained in the absence of proper actin band organization. At 24 hr titin is also organized and could support myosin; therefore, this idea supports the argument that actin-titin interactions are important for proper myosin and subsequent sarcomere assembly.

Further experiments must be done to examine through which pathway actin disruption leads to myosin disruption. To determine titin's possible role in this pathway, experiments will be done in which cells are treated with Jasp and stained for tropomyosin, myosin, and titin. Secondly, myocytes will be stained for M-line and Z-line proteins to see where actin and myosin congregate when Jasp is applied. This may help us understand if steric hindrance by actin prevents the proper formation of myosin A bands. Finally, fluorescently labeled actin monomers can be used to track actin dynamics in the presence of Jasp, which may allow us to better understand how the inhibitor affects sarcomeric organization.

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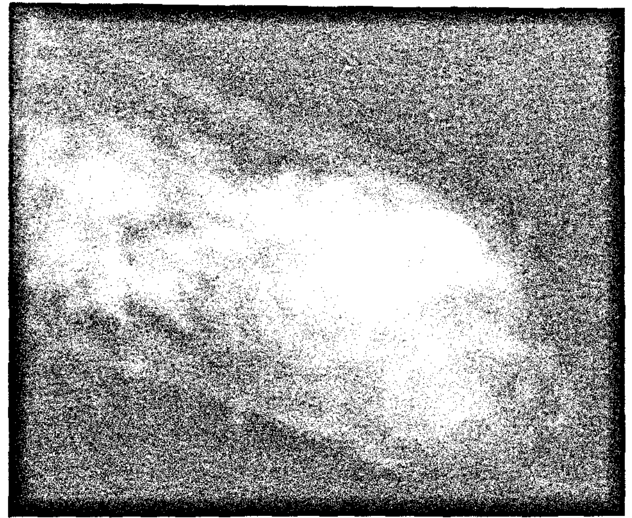
A



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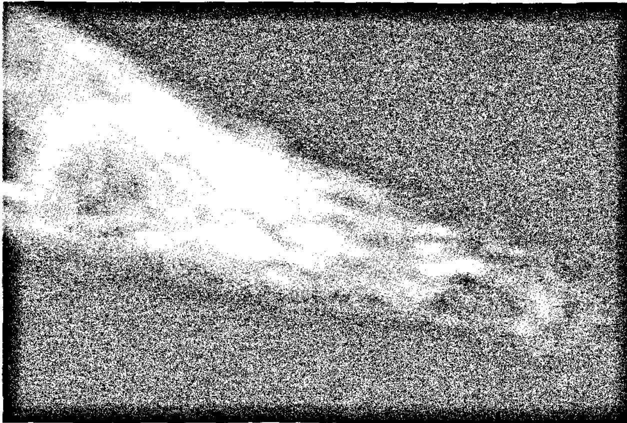

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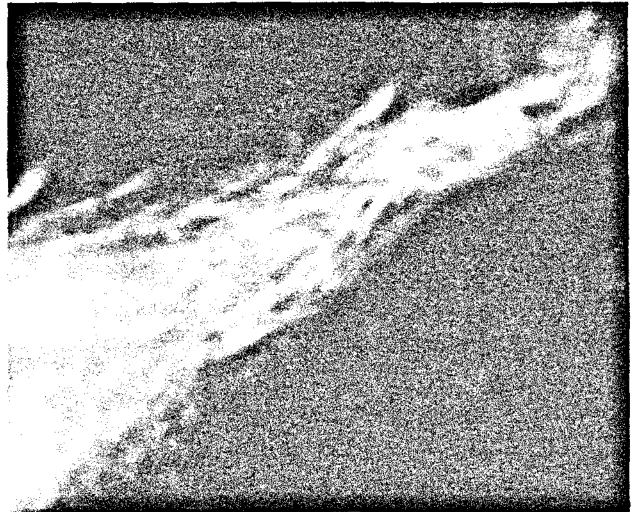

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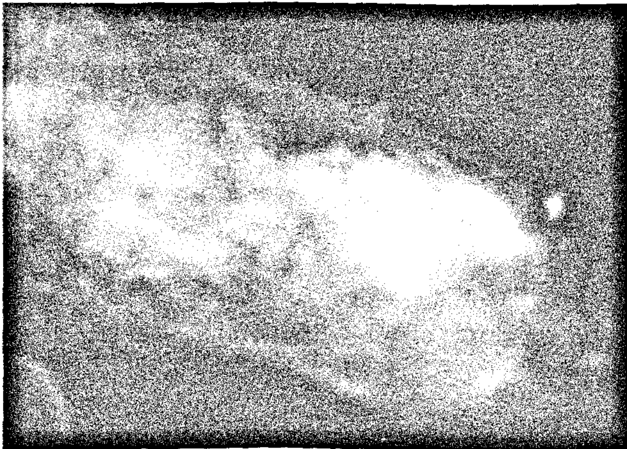

E

F

Figure 1. Jasplakinolde Disrupts Formation of Both A and I Bands. A. Troopomyosin 50nM Jasp. B. Tropomyosin 100nM Jasp. C. Tropomyosin 500nM Jasp. D. Myosin 50nM Jasp. E. Myosin 100nM Jasp. F. Myosin 500nM Jasp.

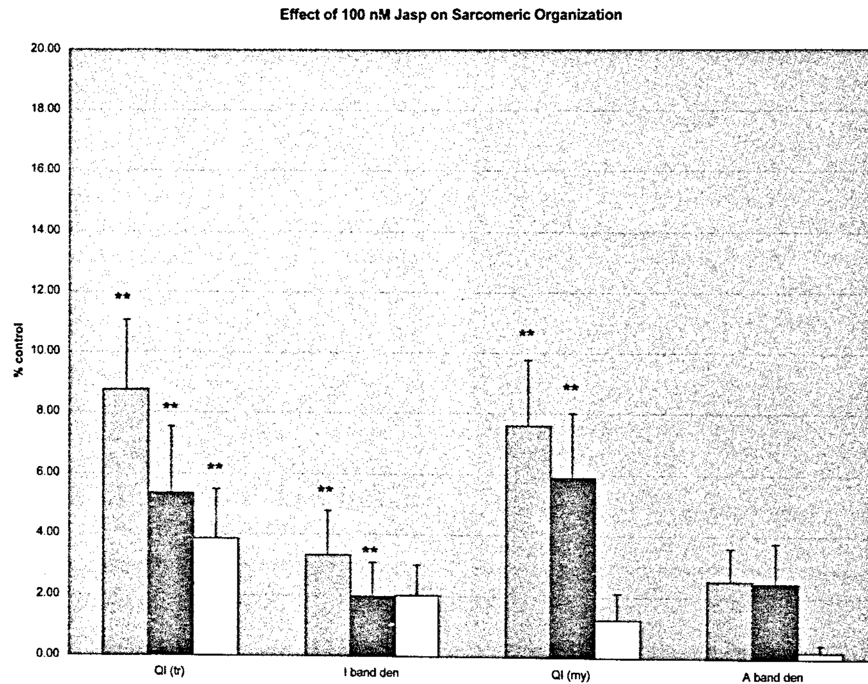

Figure 2. Graph comparing the qualitative and quantitative data from 6hr experiements.

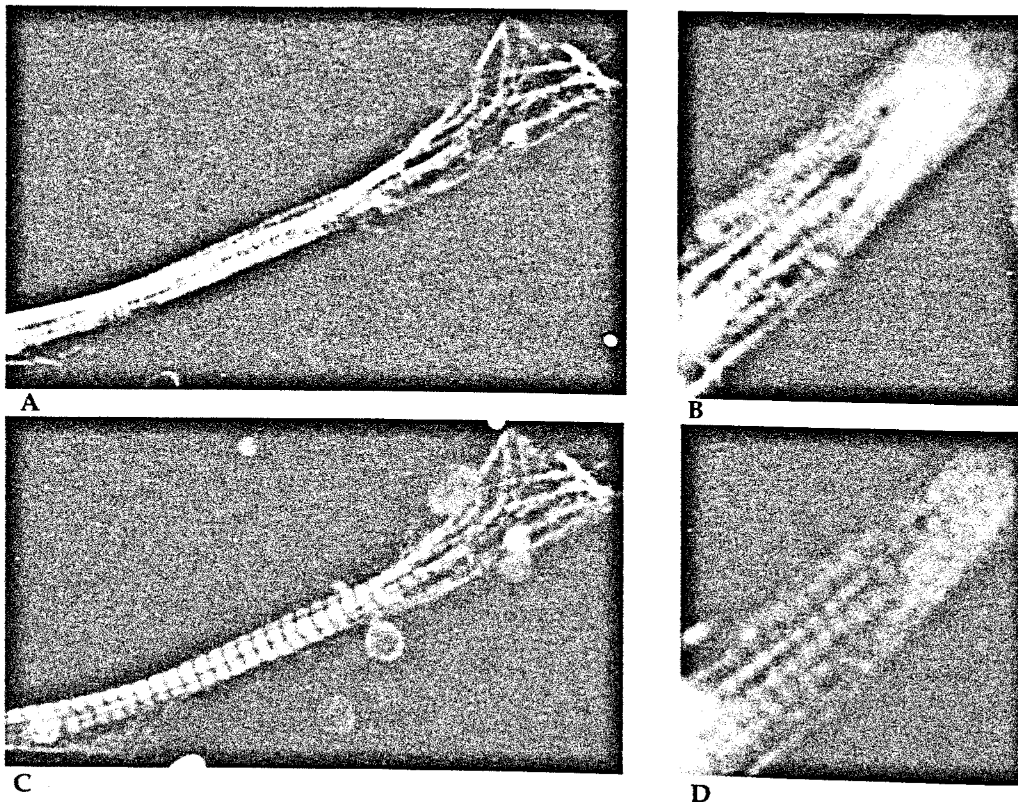

Figure 3. Treatment with Jasplakinolde at 24hr Results in Less Disruption. A. Tropomyosin in control cell. B. Tropomyosin in 24hr treated cell. C. Myosin in control cell. D. Myosin in 24hr treated cells. (For 6hr cells see Figure 2.)

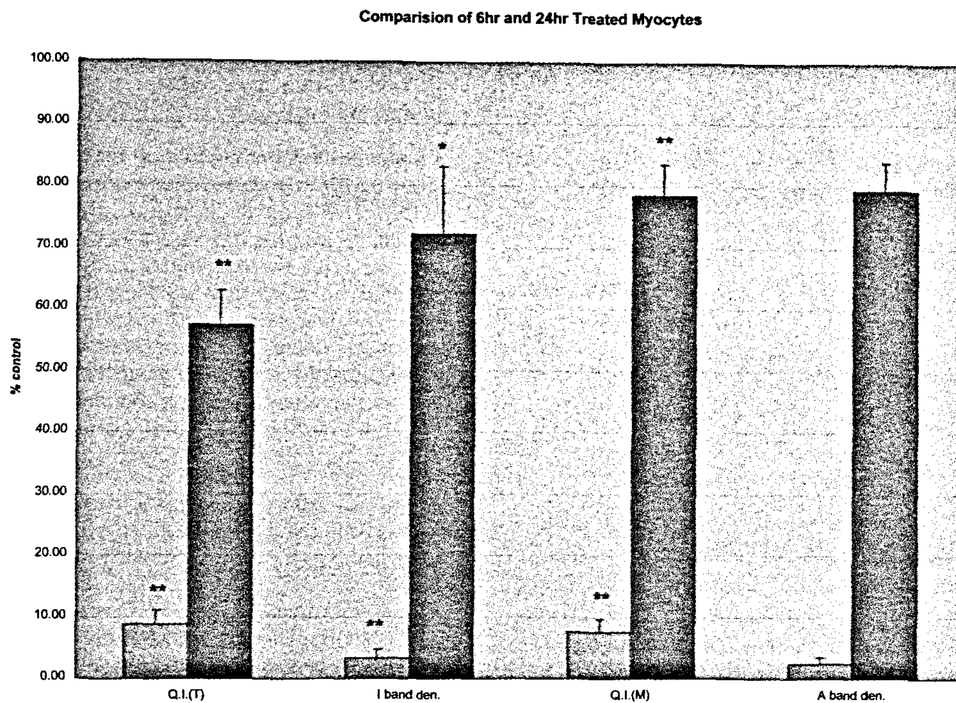

Figure 4. Graph showing the difference om affect of Jasplakinolide when applied at 24 hr as opposed to 6 hr.

Treated Cells have Normal Calcium Dynamics

	Control[6]	Treated (100 nM)
Incidence	34.3 ± 4.9	37.4 ± 7
Frequency	6 ± .4	4.2 ± .8

Table 1. Calcium imaging was done on cells treated with 100 nM Jasp to show that Jasplakinolide treated cells can still maintain normal calcium dynamics. Incidence (% cells active/0.5 hr), Frequency (# transients/hr).

John Dylan Cook

Faculty Comments:

Mr. Cook's faculty mentor, Michael Ferrari, wrote a very enthusiastic letter supporting the publication of Mr. Cook's article. In it he says the following:

> Mr. Cook impressed me tremendously in the year he worked in my laboratory as a research assistant. Although the basic biomedical research in my lab did not relate to his career goal of becoming a practicing veterinarian, he nevertheless undertook his project with intelligence, diligence, and perseverance. Most astonishing, due to his excellent work ethic, he generated enough data to present a poster at both a regional (Midwest Developmental Biology meeting held at UM Columbia) and national (American Society for Cell Biology meeting held in Washington, DC) scientific meeting last year. This is a rare achievement for any undergraduate, especially within a year! Mr. Cook had no previous lab experience, yet in this short time he learned several advanced techniques, generated excellent data, and became familiar enough with the field to present the work with confidence and composure at the aforementioned two meetings.
>
> My lab studies how the internal cables of muscle cells, which generate contractile force, are constructed. John made a major contribution by showing that assembly of one component of these cables, called myosin, is entirely dependent on the normal assembly dynamics of another major component, called actin. He used a specific disruptive compound of actin assembly, called jasplakinolide, to show that myosin cannot assemble properly if actin assembly is compromised. Besides presenting this work at meetings, it is currently being written up for publication in a high profile journal. John was able to achieve so much because of his high intelligence and excellent work ethic. He spent many a late night, and even weekends, in the laboratory working on his experiments. This is a level of dedication rare for graduate students, let alone undergraduates!
>
> I imagine the letter could stop here. However, Mr. Cook's performance was even more noteworthy in an important regard. While I did "hand" the jasplakinolide project to Mr. Cook, he rapidly became familiar with the techniques and literature. This resulted in a rather amazing thing; he came to me not once, but many times with questions about trying new methodologies and suggesting alternative ways to visualize the actin and myosin. In other words, he was very creative in thinking about ways to approach the project, and he made it his own rather than simply performing a given set of directives.
>
> I will not dwell on Mr. Cook's academic record, which is excellent. I will say that, given his performance in the lab, he has the right personality to truly capitalize on his intelligence, self-discipline, and work ethic in his chosen career. From my observations, Mr. Cook has both the intellectual aptitude and emotional attitude necessary for success. Mr. Cook was very well-liked by others in the lab, and was even a bit of a "go-to" person, even though he was just an undergraduate! He has a good sense of humor, is well-balanced, and a pleasure to have around. To be honest, I am disappointed I could not lure him here to Kansas City for graduate school; I would love to have retained him. However, he is committed to his career path. The strongest recommendation I can give Mr. Cook is that, were it not for my wife (who is a veterinarian), I would certainly have had him treat my animals after his graduation (as it stands, I'll have my wife try to hire him as an associate after he graduates!).

Mr. Cook is a well-rounded student, interested in a range of subjects. Classics professor Daniel Levine had this to say about Mr. Cook:

> Mr. Cook was my student in three classes. He is a solid worker who never tries to take the easy way out. He always comes to class prepared and always learns what he is supposed to learn. I have recommended him for several scholarships with great enthusiasm. I believe that this young man is one of the finest students we have seen at the University of Arkansas in years.