PP protein found in skeletal muscle of several specimens: A graduate school story.

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Abstract

As a graduate student, many things can happen to your life. As many have already experienced, the PhD path can be pleasant and satisfactory, and sometimes painful and stressful. According to a large number of former students, achieving the success in the PhD path is all about the topic of research one is asked to conduct as a 1st year graduate school fellow. For others, the main factor is luck, as most of your accomplishments depend upon your adviser's capacity and will to submit your papers. Supplementary opinions attribute the graduate student's success to the environment in the laboratory, based on everyday life. No matter how and in which order we consider these options, the formula for a brilliant outcome as a graduate student is probably a combination of determination, love for the field we work in, a good portion of luck, and healthy mentorship and guidance.

This work will briefly report some of the most crucial aspects in my PhD experience as a graduate student, and how, partially by chance, I discovered my dissertation track.

Please, note that, for privacy purposes, only names of institutions are reported in this paper, not those of professors, instructors, lab technicians, postdoctoral fellows or PhD students.

Introduction

Since I was very young, I was very curious and prone to experimentation. I have always believed that even beyond the most stunning and unconfutable evidence there is still something to be revealed. Getting older, I realized that when we find something new, no matter how sure we are of what we have discovered, it takes a whole lot to be "approved" and definitively accepted by the audience or most commonly, by the people surrounding us. One of the first scientific examples history teaches us is Galileo Galilei and his heliocentric theory (National_Academy_of_Sciences, 1998, O'Leary, 2006). At nighttime, on January, 7th 1610, Galileo Galilei started to record his observations on stars and planets, inaugurating modern astronomy, and setting a path for reaching scientific conclusions based upon the use of scientific examination tools (Neubarth, 2010).

Nowadays, we can find many other examples of disproved truths. For instance, since the end of World War II, high fructose corn syrup has been widely employed in the United States. High fructose corn syrup was highly available because of US subsidies and surplus stocks of maize (Wallinga et al., 2009). After decades of intense consumption, high fructose corn syrup has been found to be more harmful to humans than regular sugar, and one of the main causes of body weight increase, causing inhibition of satiety (Moran, 2009). In addition, it has been shown to be a source of mercury in certain foods, and thus highly toxic (Dufault et al., 2009, Wallinga et al., 2009). Another crucial case is the possibility of following a ketogenic diet (low or absent component of carbohydrates, high percentage of proteins and fats) (Signore, 1973) as a treatment. A ketogenic diet could potentially cure cancer via tumor starvation and mitochondrial function improvement (Seyfried et al., 2012), epilepsy via reactive oxygen species reduction (Lambrechts et al., 2012) , or Alzheimer's disease through reduction of A-β amyloid plaques (Stafstrom and Rho, 2012),, although the prevailing attitude in society encourages us to be properly fed during periods of sickness.

As a matter of fact, humanity changes, and so do habits, beliefs and hopes. What I personally experienced in my graduate school was the process of "changing", of bringing a new concept to my dissertation committee and to the scientific community I was working with.

My experience

I started my graduate school in 2005, in a physiology lab in central Italy, after successfully achieving my Master degree in Pharmacy. This choice was driven by my increasing interest in cellular biology, proteomics and fluorescence microscopy following the completion of my experimental thesis in physiology, a delightful experience that allowed me to spend 12 months as a cellular physiology intern.

My initial project was to study the *in vitro* effects of guanine-based purines (mainly guanosine and guanosine tri-phosphate) on neuro-muscular growth and development, by using immortalized human neuroblastoma, rat pheocromocytoma and mouse skeletal muscle cells. During my second year in graduate school, we decided to test the effect in parallel on neuro-muscular primary cultures derived from *Xenopus Laevis* embryos, a south American amphibian specimen. Embryos from this frog are known to be among the best candidates to test neuro-muscular development and primary cultures are easy to obtain. Hence, in order to acquire proper training and be able to plate neuro-muscular primary cultures on my own, my supervisor allowed me to spend about one year in Paris. That decision made me particularly happy, since I was familiar with France and the French language, having spent 14 months in another French city during my Masters degree in Pharmacy in 2003-2004 for the Erasmus project (Bennhold, 2005).

My first couple of months in Paris were intensely dedicated to the primary culture training, with a learning curve particularly steep after the first month. After the first proper *Xenopus Laevis* neuro-muscular primary cultures were obtained, we started labeling them with primary and secondary antibodies, in order to distinguish neurons from myoblasts (undifferentiated skeletal muscle cells) and myotubes (mature muscle cells). Surprisingly, we found that the antibody we were using, which ostensibly bound a specific neuronal protein (I will call this protein "PP", for privacy reasons), was also recognizing PP in the soma of the primary muscle cells.

Thus, we repeated the immuno staining on more aged and differentiated neuro-muscular cultures and observed that the antibody anti-PP stained both neurons and myotubes, but this time in the muscle striations, structures typically present during and after the differentiation of a muscle cell.

Based upon this first main finding, we started an extensive literature search, and were not able to find much evidence of PP in the muscle. For over 50 years, PP had been considered exclusively a neuronal protein, necessary for a number of functions such as neurite outgrowth, cell development and regeneration, synaptic plasticity, growth cone guidance and neurite branching, actin polymerization, nervous structure formation and even regulation of apoptosis. In addition, a few groups generated knock-out mice for the genes coding for PP and found that in the absence of this protein, pups could not survive for longer than few days.

Only 3 relatively recent papers (1992, 1995 and 1998) described PP to be temporarily expressed in embryonic chick limb and in regenerating human muscle, identified via electron microscopy. However none of these studies described its distribution in the muscle fiber, nor its function. It looked like the groups had no further reason to continue investigating in that direction. For these reasons, I started believing that such a dissertation topic could have been perfect for me. Day by day, with my supervisor and tutor's approval, I dedicated my efforts to the identification of PP at several stages of development. After a number of immunofluorescence stainings, we confirmed that PP was present in the nuclei of over 90% of myoblasts (undifferentiated muscle cells) and in almost 100% of striations in myotubes (differentiated muscle cells).

When I came back from France, we decided to continue this interesting investigation in other species, so I was instructed to dissect skeletal muscle fibers from mice and rats. At the same time we received some skeletal muscle human quadriceps biopsies from the hospital associated with our department of physiology and two skeletal muscle primary cell lines (from mouse and rat)

Immunofluorescence assays, using four different isoforms of anti-PP antibodies, on both mouse and rat undifferentiated muscle cells revealed that the PP protein was localized in the nucleus, similarly to the undifferentiated *Xenopus Laevis* muscular primary cultures. In addition, as in *Xenopus Laevis*, murine and rat differentiated muscle cells expressed the PP protein among the muscle striations, underlining an evolutionary conservation between species. Moreover, fluorescent immunostaining assays on mature mouse, rat and human skeletal muscle fibers revealed a very specific localization of PP protein in the mitochondria, thus suggesting one or more roles in mature muscle's excitation-contraction machinery and a novel functional role of this protein in skeletal muscle activity.

In addition, we performed a series of western blot assays and revealed the presence of four different PP isoforms in a variety of skeletal muscle fibers in mice and amphibians. Notably, we collected and homogenized samples of quadriceps, tibial muscle, *extensor digitorum longus* and *flexor digitorum brevis*. Despite the fact that the PP protein had different molecular weights according to the analyzed isoform, we were able to confirm its presence in all skeletal muscle samples.

Moreover, in order to confirm what we observed in western blot, we performed a number of bi-dimensional western blot (2-DIGE), thus detecting the PP protein in the muscle samples described above according to pH and isoelectrical point. As previously observed, the 2-DIGE provided us with further evidence of PP protein in murine and amphibian skeletal muscles.

When the time came, three months prior to the final discussion date, I started working on my dissertation. During the last two months, I went back to my hometown to work as a pharmacist (I had obtained a short-term contract), and at the same time completed the dissertation. I have to admit that working while writing my dissertation was quite challenging, but at the same time very enriching. In fact, during that time, I was able to collect great feedback from my colleagues, who were not researchers, and thus reanalyzed the whole topic of my dissertation from a non-academic perspective. This provided the initial design of my dissertation with significant improvement.

While everything seemed to go in the right direction, from a research perspective, the idea of publishing this astonishing evidence still appeared to be impossible, according to multiple discussions we had within the department. Some of our collaborators and colleagues, as always happens during scientific round tables, kept suggesting the addition of more evidence before submitting the final manuscript. Most of them, however, suggested publishing the evidence of PP protein in the skeletal muscle as soon as possible. However, because we were only able to define the PP protein expression in the skeletal muscle, but not to identify its functions, no paper was submitted before the discussion of my dissertation. Nevertheless, I was assured many times that a manuscript would have been submitted within a few months.

About six months earlier, I had received an interesting postdoc offer in hyperbaric physiology from my current P.I., at University of South Florida, Tampa, Florida. After giving it some thought, I accepted with enthusiasm. However, because I did not have any spare time between the discussion of my dissertation and the beginning of my postdoctoral fellowship, I left the United States only two days after my graduation.

Upon my arrival, all members of the lab warmly welcomed me, and after a short but intense period of settling in, we started running experiments and got straight to the core of our hyperbaric physiology research. Despite being very busy even after the first month of my postdoctoral experience, I kept in touch for over two years with my former advisers and mentors, asking for monthly updates about our PP protein manuscript, but unfortunately, responses did not always satisfy

my curiosity. Sometimes there was no news, because nobody from the lab had been able to work on the last set of experiments. Other times, it seemed that the project was stuck somewhere between teaching activity and lack of resources. At some point, something seemed to move towards the right direction because my department decided to present the data from my dissertation at the annual Italian Physiology Society conference.

In addition, few months later, the expression of PP protein in the skeletal muscle was selected as topic for one of the undergraduate students in the lab.

However, by that time, I had still not been informed of any development for the final version of the manuscript. As now, about two and half years have passed by since my departure for the United States, and I am still patiently waiting for some development.

I am confident that some good news will soon come up, but I can say that what happened taught me a good lesson: whenever you leave your laboratory for a new position without completing your work, even if your advisors/tutors promise it will be done soon, and that you will stay in touch, do not take for granted that the project will be finished and the results published exactly the way you have discussed with them. Unfortunately, this is one of the most common reasons why students are sometimes willing to spend some extra time in the laboratories they have been formed in, rather than leaving towards a new step in their career.

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