University of Puerto Rico

Río Piedras Campus

Department of Biology

Holothurian primary muscle cell culture: Optimization of cell culture techniques and elucidation of molecular pathways involved in muscle cell dedifferentiation

by:

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Abstract

Members of the phylum Echinodermata possess an impressive regenerative potential that could further develop the field of regenerative medicine. Holothurians have one of the most developed musculatures in terms of the myogenesis that leads to regeneration of muscle tissue. We aimed to develop an *in vitro* model for dissociated muscle tissue that could be used for regenerative studies. We focus on molecular candidates that have been previously associated with regeneration, to define their roles in the dedifferentiation of muscle cells. To do this, we optimized protocols for intestinal tissue cultures by changing parameters for ones that best suited our muscle tissue. Then, we applied pharmacological agents and analyzed the presence of SLS's and dedifferentiating fibers, structures that have been associated with the process of dedifferentiation, to elucidate which molecular pathways could induce the dedifferentiation of muscle cells. We found specific parameters such as changes in the enzymatic dissociation media, the adherence protocol and the number of cells cultured, that yielded healthy muscle fibers. Our cultures also showed a cellular component that was added to structures that indicated that dedifferentiation was occurring. For pharmacological assays, we found an increase in dedifferentiating fibers when we treated cultures with LiCl and an increase in SLS's when treated with DPI, EGTA and UK-383,367. Our results indicate that our *in vitro* model serves as an effective way to determine the effects that pharmacological agents have on dedifferentiation. We conclude that pathways involving Wnt/β- catenin and GSK-3, reactive oxygen species, Ca^{2+} and BMP-1 modulate *in vitro* dedifferentiation of muscle fibers.

Keywords: echinoderms, sea cucumber, regeneration, longitudinal muscle dedifferentiation

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My graduate journey was a rocky road, and my feet were mostly tired of all the climbing I had to do just to advance a few steps. Even thought my experience was atypical, I did meet some of the most wonderful people in my life and consider myself lucky if years passed and I still show up in their minds.

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Special shoutout to my family, to Michelle and "Chino" the greatest friends that a person can have, to Natalia who always picked me up when I was down (A lifetime is not enough to thank you) and to Watson "El Sato", a dog that has change my life in ways that I can't put into words. Finally, I would like to thank the most special person that this program provided me, Alejandra, who I love and is now starting her Ph.D., which I know she will exceed at.

A page is not enough to write what you all mean to me.

Introduction

Model system

Echinodermata is a phylum of marine non-chordate deuterostomes consisting of five taxonomic classes: Asteroidea, Echinoidea, Crinoidea, Ophiuroidea and Holothuroidea. The earliest fossil of this phylum dates to 560 million years ago, to the Cambrian period. The phylum's name comes from early observation of the spiny protuberances in their mesoderm-derived biomineralized calcareous endoskeleton that all species in this phylum present, either in the form of plates or represented by scattered tiny ossicles (L. echinatus, prickly, + Gr. derma, skin, + ata, characterized by). They are the second most diverse group within the deuterostomes, with approximately 6,500 extant species and 13,000 fossils (Reich A. *et al.*, 2015; Smith A. *et al.*, 2006). Echinoderms are believed to have descended from a bilateral ancestor due to the bilaterality presented in the larval stage. It is believed that chordates and echinoderms share a common ancestor that gave rise to deuterostomes 540 million years ago. There are five prominent characteristics that make echinoderms the only pentaradial organisms with such complex organ systems: (1) Pentaradial symmetry only found in adults; (2) Water vascular system, a coelomic compartment mainly used for locomotion, feeding, respiration and excretion; (3) Dermal branchiae, for tactile stimulation; (4) Spiny endoskeleton of plates that protects them from predators; (5) Pedicellariae that protects the organisms' surface from debris and aids in food capture (Hickman C., 2020). In addition to the fact that their gametes are abundant and easy to collect and that they present great diversity in developmental mechanisms, echinoderms are widely useful in the laboratory for their phylogenetic closeness to chordates (Satoh N. *et al*., 2014). For this study we focused on the mesmerizing regenerative

potential of echinoderms, specifically those in the class Holothuroidea, known as holothurians or sea cucumbers.

Unlike other echinoderms, the holothurian body presents a second bilaterality. Moreover, they have a soft body that presents a reduction in ossicles. Holothuroidea encompasses about 1,150 living species. They possess both circular and longitudinal muscles along their body length and in their ambulacra. Their coelomic cavity is filled with fluid containing many coelomocytes and serves as a hydrostatic skeleton for locomotion. The digestive system and respiratory tree serve for nutrient uptake and gas exchange and excretion, respectively, and are connected to a muscular cloaca. Upon stress, due to predators or lack of oxygen, sea cucumbers can employ a defense mechanism that consist of the expulsion of their viscera by a strong muscular contraction that may either rupture the body wall of the organism or empty its contents through the anus. Evisceration is soon followed by regeneration of the digestive system, with many of the mechanisms that are involved in said regeneration remaining to be illustrated. During regeneration, the free end of the mesentery, an organ that attaches the digestive system to the body wall, thickens forming a rudiment that eventually expands to form the new intestine (Garcia- Arraras J. *et al*., 2018). Figure 1 shows anatomy of sea cucumbers, using *Sclerodactyla sp*. as the example.

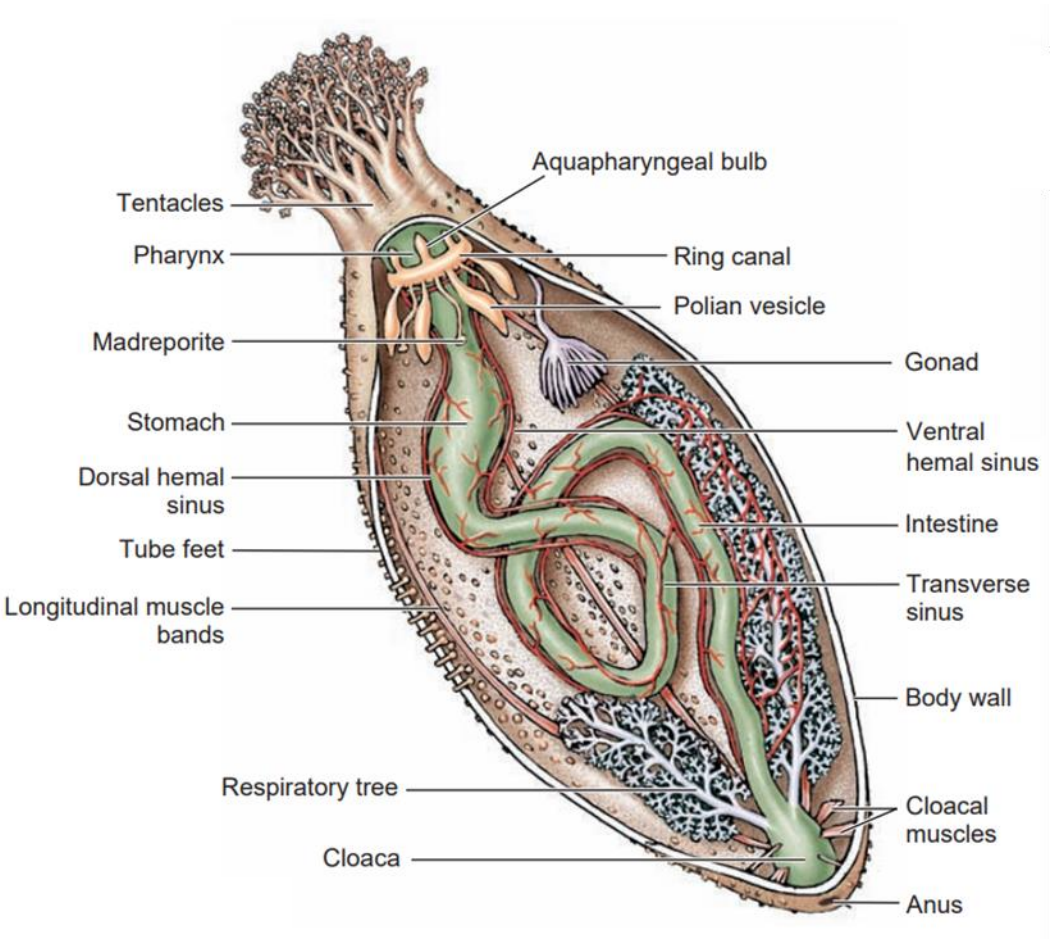

Figure 1 Hickman C. (2020). Anatomy of a sea cucumber *Sclerodactyla*. [Digital image]. Retrieved December 20, 2022. The following image presents the internal anatomy of the sea cucumber *Sclerodactyla sp.*

Our laboratory has been characterizing the process of regeneration, using the sea cucumber *Holothuria glaberrima* as a model system. This species is abundant in coastal areas from Puerto Rico, is easy to maintain in the laboratory, and can form an exact fully functional digestive system following an evisceration event. The regenerative processes have been previously and rigorously documented (Garcia- Arraras J. *et al*., 2018). Thus*, H. glaberrima* provides an ideal model organism for elucidating molecular processes associated with regeneration.

Regeneration

The capacity of an organism to replace various components, from cells to forming a completely new organism is known as its regenerative potential. All organisms in the phylum Echinodermata possess a striking regenerative potential. Crinoids can regenerate internal organs like gonads, digestive system, and external organs like arms, pinnules and cirri (Di Benedetto C. *et al.*, 2014). Members of the classes Asteroidea, Ophiuroidea and Holothuroidea can undergo partial or complete regeneration leading to the regeneration of multiple organs, or to the formation of a new organism by cloning in a process called fission, a way of asexual reproduction (Carnevali C., 2006; Kamenev Y. and Dolmatov I, 2015). Sea urchins (Echinoidea) have been documented to have the least regenerative potential in the echinoderms, with regeneration limited to external appendages like spines and tube feet (Carnevali C., 2006), while in sea cucumbers (Holothuroidea) it is most developed (Garcia-Arraras J. *et al*., 2018). Regeneration capacities of sea cucumbers extend to most of their internal and external organs. Some species contain Cuvierian tubules attached to the posterior end of the respiratory tree, that when expelled become sticky, some of them contain toxins that can distract or deter its predator. These structures are regenerated when expelled. However, it is following the process of evisceration when the sea cucumber regenerative potential can be best appreciated. All the eviscerated organs are eventually regenerated. Digestive tract regeneration is one of the most studied processes.

In organisms with the ability to regenerate tissue, the regenerative mechanism involves either the recruitment of stem-like progenitor cells or the dedifferentiation of existing cells. The former consists of undifferentiated cells, with a respective level of potency, that are recruited

to the injury site where they proliferate and differentiate forming a lump known as a blastema. The latter consist of cells that lose their differentiated state, reprogram, and are recruited to the blastema, where they can proliferate and eventually differentiate (Garcia-Arraras J., 2017). The ability of cells to differentiate into another phenotype varies in spectrum depending on the organism in question. Both amphibians and holothurians rely on transition from epithelial to mesenchymal cells that have multipotency ability of changing phenotype, for limb and intestine regeneration respectively (Anton W. *et al*., 2011; Garcia- Arraras J. *et al*., 2011).

Once an organism's tissue has suffered damage, the healing or regeneration that ensues, depends on the type of tissue, size of the wound, the age of the organism and its regenerative capacities. In animals that do not use stem cells, the process of regeneration consists of time-regulated events of cell dedifferentiation, proliferation, apoptosis, migration, and the remodeling of extracellular matrix (Candelaria A. *et al.*, 2006; Chablais F. *et al.*, 2011; García- Arrarás J. *et al.*, 1998; Junji I. *et al.*, 2012 Quinones J. *et al.*, 2002). Although regeneration seems like a quintessential trait for any organism, it is not fully present in complex organisms, and trying to elucidate treatments for degenerative diseases is one of the more complex challenges in modern regenerative medicine.

The mechanism that an organism employs for regeneration can also depend on its life stage. For example, it has been found that larval newts are able to regenerate amputated limbs using Pax7+ cells, a marker for satellite cells (-muscle stem cells) (Morrison J. *et al.*, 2006). In contrast, limb regeneration in metamorphosed newts takes place by dedifferentiation of skeletal muscle fibers (Tanaka H. *et al.*, 2016). In comparison with the newt's regeneration strategy, planarian's, *Schmidtea mediterranea*, uses neoblasts (dividing adult stem cells)

throughout their life cycle for regenerating damaged tissue. These are the only type of cells that form the blastema, and upon laboratory-induced depletion regeneration is blocked (Reddien P. *et al*., 2005).

Mammalian regeneration studies using mice as a regenerative model have shed information on the limited regeneration found in higher- order animals. Elucidation of molecular pathways involved in the limited regenerative properties of mammals have shown parallels with regeneration pathways found in lower vertebrates. Mammals are not completely lacking in their regenerative abilities, for example, rabbits and mice are able to partially or completely regenerate holes punches inflicted on their ears (Metcalfe *et al.*, 2006); deers regenerate their antlers annually (Kierdorf *et al.*, 2007); rodents can regenerate amputated fingertips (Han *et al.*, 2005); humans can regenerate colon mucosa and hepatic tissue (Melnyk C. *et al.*, 1967; Pu W. & Zhuo B., 2022). Even though there's a handful of regenerative tissues found in higher-order animal, the more common process that follows a laceration is the other aspect of the regenerative process, the formation of scar tissue (Henry L. & Hart M., 2005; Neves J. *et al.*, 2015; Roy S. & Gatien S., 2008). This process consists of the formation of an acellular and avascular area in which fibrocytes and fibroblasts deposit collagenous fiber

bundles. (Verhaegen P. *et al.*, 2009). However, in animals with a large regenerative potential, such as echinoderms, the ECM is remodeled and reformed to limit the deposition of collagenous fiber bundles and, as a consequence, the formation of scar tissues is greatly reduced or non- existent (Grieb G. *et al.*, 2011; Klinge U. *et al.*, 2000).

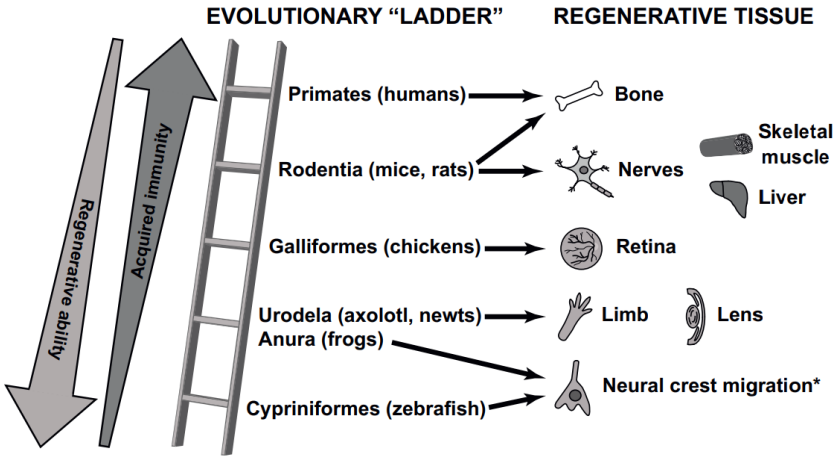

Figure 2 Mastellos DC, et al. (2013). *Complement- associated regenerative processes in evolutionary distinct organisms.* [Digital image]. Retrieved July 6, 2020. The following image presents the inverse correlation between the level of complexity of an animal immune system development and its regenerative potential with examples of the tissues that they're able to regenerate.

As implied in this thesis, regenerative processes are more developed in less complex organisms while they have been replaced in more complex ones with the formation of scar tissue. Since all cellular life descended from a last universal common ancestor, and most of the fundamental properties are maintained during evolution, the study of the properties that one organism exhibits may be fundamental to the understanding of properties that resemble another. As mentioned before, regenerative potential varies depending on the type of wound, organism and its life stage. The ability for cells from certain tissues to reprogram their phenotype and assume a pluripotent progenitor state to regenerate lost tissue is present in most mammals only during fetal state, being lost in most organs during adulthood (Tanaka E. & Ferreti P., 2009).

It has been proposed that the loss of the regenerative potential is due to the development of the immune system. Mastellos and colleagues (2013), present an inverse correlation of the abilities of an organism to regenerate and how well-developed its immune system is (Figure 2). In this work, they compared phenotypic plasticity and tissue remodeling and how tissue damage employs the release of a plethora of chemoattractants that arises from activation of the complement pathways resulting in inflammation. Indeed, inflammatory processes are inhibitory to regeneration processes, as suggested by studies using Xenopus (King M. *et al.*, 2012). Regression of the regenerative potential is seen upon maturation of the immune response from young larvae to adult organism (King M. *et al.*, 2012; Mescher A. *et al.*, 2013). Organisms lacking an adaptive immune system, such as echinoderms, are not restrained by immunosurveillance, lymphocyte activation and histocompatibility restraints that might inhibit the regeneration process (Godwin J. & Brockes J., 2006; Sanchez- Alvarado A., 2000).

Regeneration in *H. glaberrima* has been shown to mainly proceed via the process of dedifferentiation. As in most echinoderms, cell dedifferentiation is more evident in myocytes close to the amputated area and, in other echinoderm classes, in mesodermal cell types such as sclerocytes and fibrocytes (Candia-Carnevali *et al.*, 2009). The underlying molecular pathways by which a regenerative organism can go from receiving a signal of tissue homeostasis disruption and its regenerative potential remains a mesmerizing question in regenerative medicine and is the topic of this thesis.

Muscle cell regeneration

Echinoderm muscle systems are divided into visceral and somatic muscle systems. However, it is not clear if there are extensive molecular differences between both. The visceral muscle system is composed of a mesothelium, that can be further divided into an outer layer of peritoneal cells, and an inner layer of myoepithelial or muscle cells (Garcia-Arraras J. *et al.*, 2018). During regeneration, these cells show a dramatic change in morphology, associated with the process of dedifferentiation (Candelaria A. *et al.*, 2006). Following wounding, the cell's myofibrils become disorganized and are eventually ejected to the extracellular space in membrane bound structures named Spindle- like structures (SLS's) (García-Arrarás J. & Dolmatov I., 2010). Contractile apparatus can be visualized in *H. glaberrima* by fluorescent microscopy using fluorescently-labelled phalloidin, a toxin that stains polymerized actin (García-Arrarás J. & Dolmatov I., 2010; Faulstich H. *et al.*, 1993). The formation of SLS's in dedifferentiating cells, although initially described in sea cucumbers, has also been shown in other echinoderm classes. Research into crinoid arm regeneration showed that a marker in the process of dedifferentiation is also the disorganization, and then expulsion, of the contractile apparatus as a SLS's (Di Benedetto *et al.*, 2014). Another marker for cell dedifferentiation is the activation of the nucleus as seen by a decondensed chromatin (García-Arrarás J. & Dolmatov I., 2010). After dedifferentiation, cells assume a rounded morphology and migrate to the wound site where they proliferate and then re-differentiate, as seen by changes in morphology and appearance of a new contractile apparatus, forming the new mesothelium.

In sea cucumbers, the second division of the muscle system, the somatic musculature, is composed of longitudinal muscle bands (LMB) and retractor muscles of the aquapharyngeal complex (AC). Both components of somatic musculature are myocytes embedded in an

extracellular matrix of connective tissue (Figure 3). In this division, myocytes and myoepithelial cells also contribute to muscle regeneration by dedifferentiation, migration, and immersion into connective tissue (Garcia- Arraras J. & Dolmatov Y., 2010). Upon damage to the LMB, the site where the wound occurs begins to contract until it eventually closes. Coelomocytes are responsible for closing the wound area, where epithelial cells migrate to form a clot (Dolmatov I. & Ginanova T., 2001). Regeneration follows with coelomic epithelial cells, myoepithelial cells and peritoneocytes, that begin to dedifferentiate and migrate into the terminal areas of LMBs where they sink into the connective tissue, and they re-differentiate to form muscle bundles. It has been proposed that the process by which myocytes contribute to form somatic muscle in regeneration depends on the extent of the damage. If wounding is minor, myocyte dedifferentiation is observed by expulsion of SLS's. If wounding is major, then myocytes separate most of their cytoplasm containing contractile apparatus from the nucleus without the formation of a SLS (García-Arrarás J. & Dolmatov I., 2010). Myocytes do not seem to migrate or proliferate on regeneration of LMBs, suggesting that they can differentiate into new myocytes restoring the function of old muscles. Our laboratory has developed dissection protocols of explants and primary muscle tissue from *H. glaberrima* and propose to use this organism for research in LMB's cell dedifferentiation. This thesis will explore the two types of muscle dedifferentiation processes proposed by previous studies.

A

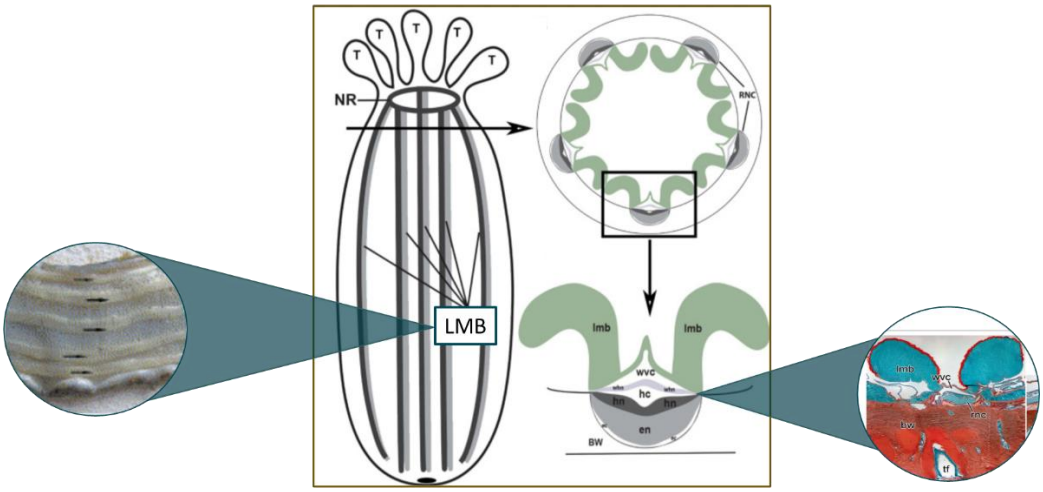
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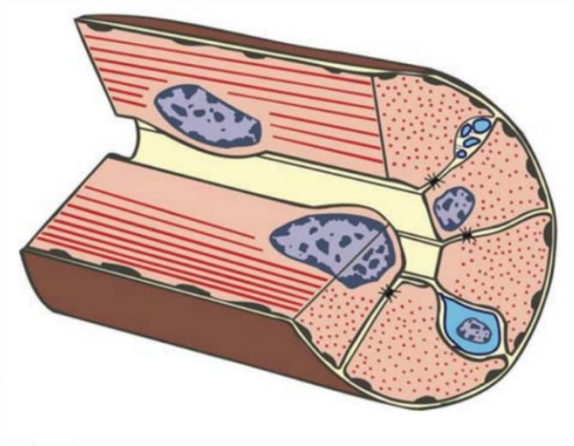
Figure 3 Mashanov V. *et al.* (2009) & Mashanov V. (2015); Garcia-Arraras J. & Dolmatov I. (2010). *Organization of holothurians somatic muscle* [Digital image]. Retrieved December 12, 2022. **A.** Representation and image of longitudinal muscle bands and its components. Left magnification is an image of the interior of a sea cucumber, showing how longitudinal muscle bands run from the anterior part of the organism to the posterior; right image shows staining of a transversal cut. **B.** Schematic representation of a longitudinal muscle bundle composed of myocytes (pink), basal lamina (brown), putative neurons (blue) and myofilaments inside the cells (red spots and lines).

B



Regeneration triggering pathways

While the cellular process of muscle dedifferentiation has been well described, the underlying molecular events remain largely unknown. For this, it is important to focus on the signaling mechanism that might be associated with the process of dedifferentiation. When reviewing the scientific literature, several mechanisms have been proposed for both the dedifferentiation/reprogramming of cells, and for the maintenance of the dedifferentiated states. Here we review several signaling pathways that have been documented, and that could provide important candidates for dedifferentiation signaling in the holothurian muscle. Among these are the following:

Wnt/ β-catenin & GSK-3- Wingless-type family proteins (Wnts) are molecules secreted by several cell types that act as a local mediator or morphogen to control animal cell function, development, and survival (Alberts B. et al., 2002). The Wnt/ β-catenin, also known as canonical Wnt pathway, activates the transcription of Wnt targeted genes by stabilizing cytoplasmic β-catenin, which translocates into the nucleus binding to transcription factors LEF1/TC1 and serving as a transcription coactivator (Figure 4). It has been stated that the echinoderms share about 85% of the molecular components for the Wnt signaling pathways with humans (Croce J. et al., 2006). About 55% of the Wnt signaling proteins found in invertebrates have an ortholog in echinoderms, meaning that data obtained from expression profiles may be useful in comparing functionality of Wnt proteins function between our model organisms and distantly placed taxa, including vertebrates and invertebrates (Khadra B. *et. al.*, 2018). Finally, in the holothurian *Eupentacta fraudatrix*, Wnt homologs and downstream Wnt pathway molecules

are upregulated as early as 3 days post evisceration and can last up to 10 days (Girich A. *et al.*, 2017).

Proteins from the Wnt pathway are found to be up- regulated in mesenchymal precursor cells in adipose tissue from both, mouse cell line 3T3L1 and human tissue. Application of Wnt3a-conditioned medium induced migration, proliferation, and downregulation of adipogenic markers while increasing markers for undifferentiated cells (Gustafson B. & Smith U., 2010). In mesenchymal stem cells that differentiate into osteoblasts, this pathway governs the repression of differentiation pathways and cell death (Krishnan V. *et al.*, 2006). As a more closely related taxa, urodeles have presented expression of most of the proteins that we are interested in working on, and the pathways for these proteins have been found to have a role in the process of dedifferentiation. Limb amputations on both the distal and proximal area in the axolotl *Ambystoma mexicanum* induced upregulation of the protein's variants Wnt5a and Wnt5b in the blastema, specifically in mesenchymal cells, for both amputation sites during the dedifferentiation stage, and remained upregulated for the duration of regeneration, culminating on the stages where re-differentiation of cells is known to happen (Gosh S. *et al.*, 2008). An alternative role for Wnt pathway is observed for zebrafish cardiac tissue regeneration. In the previous examples, Wnt signaling activation suggest to either induce a dedifferentiated state or represses the re-differentiation of cells, while in zebrafish cardiac regeneration, Wnt signaling is inhibited by the upregulation of Wnt inhibition genes and the expression of cardiac embryonic genes such as cardiac transcription factor gata4 and embryonic-specific cardiac myosin heavy chains (emCMHC), which serve a pluripotency marker, are induced (Zhao *et. al.*, 2019; Peng X. *et. al.*, 2020).

It is worth mentioning the findings obtained from our laboratory using our model organism. Bello S., and colleagues 2019 researched the effects of putative disruptors of Wnt signaling and their ability to arrest regeneration of *H. glaberrima's* intestine regeneration. They showed that Wnt pathways are linked to gut rudiment regeneration, and that inhibition of this pathway leads to a reduced size of gut rudiment by almost 50%. This reduction can be attributed to a delay in muscle regeneration from Wnt signaling inhibition, but activation of Wnt signaling due to putative activator LiCl didn't show a change in the dedifferentiation gradient in the mesentery relative to the gut rudiment. The same can be appreciated with proliferation, where inhibition of Wnt signaling led to less proliferation while activation had no significant difference. These results were further corroborated in our lab using a double stranded interference RNA system (dsiRNA) where knockdown of β-catenin did not affect neither dedifferentiation nor apoptosis but did change the rate of muscle cell proliferation, leading to the conclusion that the Wnt pathway regulates regeneration only through proliferation (Alicea-Delgado M. & García-Arrarás, 2021). *In vitro* studies using gut rudiment explants showed that Wnt signaling inhibition using iCRT14 was able to delay dedifferentiation, as measured by ejection of contractile apparatus by muscle cells. Using LiCl led to a significant increase of 169% of SLS's per nucleus found on *in vitro* studies (Bello S. *et al.*, 2019). Finally, it was inferred that the effects of LiCl were through the serine/threonine protein kinase glycogen synthase kinase-3 (GSK-3). LiCl inhibits the function of GSK-3, leading to an increase in cytosolic β-catenin (Kaidanovich-Beilin O. & Woodgett J., 2011). GSK-3 modulates the Wnt pathway by phosphorylating β-catenin, marking it for ubiquitin-mediated proteasomal degradation (Aberle H. *et al.*, 1997). Since GSK-3 can also modulate other pathways and molecules, such as PI3K and

ROS, and data from our laboratory on *H. glaberrima* muscle tissue dedifferentiation suggest that neither Wnt nor β-catenin influence the dedifferentiation of cells from muscle tissue (Frismantiene A. *et al.*, 2016; Guo R. *et al.*, 2017; Bello S. *et al.*, 2019; Alicea-Delgado M. & García-Arrarás, 2021). It can argue that the effects of LiCl in dedifferentiation of muscle tissue are independent on Wnt signaling

It's worth noting the relevance that GSK-3 may be having in our experiments. As mentioned before, GSK-3 can modulate the Wnt pathway by targeting β-catenin for degradation, inhibiting its function on gene expression (Figure 4). In cells, GSK-3 present higher activity during lack of stimulus, and it's inhibited by factors that phosphorylate its serine residue like growth factors, insulin, or serum treatments (Sutherland *et al.*, 1993; Welsh and Proud, 1993; Stambolic and Woodgett, 1994; Kaidanovich-Beilin O. & Woodgett J., 2011). Inhibitory effects of growth factors and other agonists work through phosphorylation of kinases, like PI3K/AKT, that in turn phosphorylate GSK-3 for inactivation (Alessi *et al.*, 1996). Conversely, GSK-3 can phosphorylate, and activate PI3K/AKT pathway, which in turn activates MAPK pathways (Lu S. *et al.*, 2011). The importance of these pathways is reviewed in this work.

The organismal wide range of Wnt signaling in the process of tissue regeneration ranging from mammalian to holothurians, as well as other model organisms, like Xenopus tadpole, hydra and planaria, makes Wnt pathway signaling a possible candidate for dedifferentiation studies (Adelle T. *et al.*, 2009; Lengfel T. *et al.*, 2009; Yokoyama H. *et al.*, 2007). Also, the effects our pharmacological treatment have on Wnt through GSK-3, and the effects of GSK-3 on other signaling pathways that may be affecting the dedifferentiation process can lead us to decipher a signaling cascades involved in this process. Finally, the

established functions of LiCl and iCRT14 in our organism allow us to use these pharmacological treatments as positive control for comparing the effects of other pharmacological treatments in the induction of the dedifferentiation process in our cell cultures.

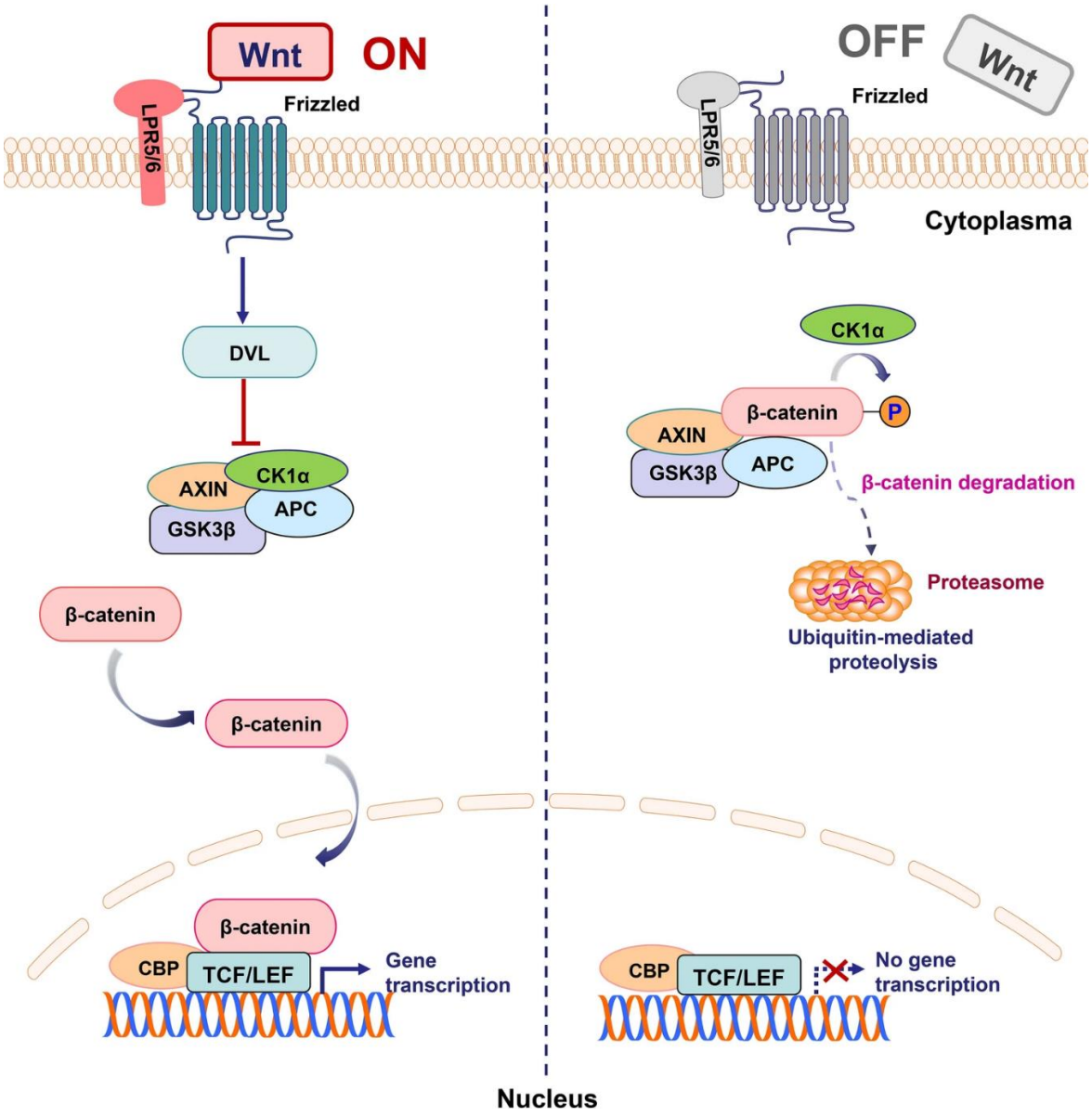


Figure 4 Xang Y. & Wang W (2020). *Schematic representation of activated and inhibited Wnt/β-catenin pathway* [Digital image]. Inhibition of GSK3β by a "WNT ON state" allows β-catenin translocation into the nucleus for gene transcription. "WNT OFF state" leads to GSK3β-mediated β-catenin degradation.

Reactive oxygen species- A second signal that might be involved in triggering dedifferentiation processes is the production of Reactive Oxygen Species (ROS). ROS are widely

known for their intrinsic properties as intra- and intercellular signaling molecules. The majority of endogenous ROS comes as a by-product or as an intermediate of redox reactions. For example, the major source of ROS in animals originates from cellular respiration. The mitochondria produce large quantities of the superoxide ion ($O_2\bullet-$) when it has a high protonmotive force, caused by the membrane gaining charge due to electron transport, and when the NADH/NAD+ ratio is high. Other sources of ROS are attributed to the function of enzymes such as superoxide dismutase (SOD), xanthine oxidoreductase, uncoupled nitric oxide synthase and ROS Generating Nox-Family NAD(P)H Oxidases (Figure 5)(Touyz, R. M., 2012). Some marine invertebrates, respond to environmental stress, such as changes in temperature, by regulating ROS production (Murphy M., 2009; Lockwood, B., *et. al.*, 2010). Depending on the organism, the proteome may change from ROS-generating NADH-producing to ROS-scavenging NADPH-producing pathways Tomanek L. & Zuzow M., 2010). Protein such as superoxide dismutase, glutathione peroxidase and glutathione reductase serve to scavenge ROS, while NADH dehydrogenase, succinate dehydrogenase increases ROS production in response to environmental factors (Dilly G. *et. al.*, 2012; Schafer F. & Buettner G., 2001; Ellis E., 2007). To counteract the increase in ROS production, cells may also reduce NADH-producing pathways, which generates ROS, by decreasing aerobic metabolism (Murphy M., 2009).

A plethora of molecular events are governed by having ROS as an upstream signaling molecule. Examples of these are the activation of kinase/phosphatases, transcriptional factors, ion mobilization transporter and pathogen defense mechanisms, by upregulating host defense genes. Two important cellular processes in regeneration, apoptosis, and proliferation, have been directly linked to having ROS as an upstream signaling molecule (Zhang Q. *et al.*, 2016).

For example, ROS-Generating Nox Family NAD(P)H Oxidases, specifically NADPH homolog NOX-2, are upregulated in geckos 3 days after tail amputation, and pharmacological inhibition of ROS production resulted in significant reduction of regeneration (Zhang Q. *et al.*, 2016). Likewise, Xenopus tadpole tail regeneration reveals that upon inhibition of NADPH homologs, proliferation is decreased following amputation (Love N., 2013). In zebrafish, inhibiting production of ROS on amputated fins disrupted blastema formation in response to a reduced number of apoptotic cells (Gauron C. *et al.*, 2013). In the freshwater planarian, *Schmidtea mediterranea,* ROS production mediates activation of the MAP kinase pathway, which when inhibited results in a dormant blastema, that can be rescued after exogenous introduction of ROS (Jaenen V. *et al.*, 2021). As with planarian, *Drosophila melanogaster* also requires a burst of ROS to induce regeneration. In this case, the homeostatic machinery responds to cell death by releasing a burst on ROS to the extracellular medium, activating JNK/STAT pathway and leading to compensatory proliferation for regeneration of imaginal disc epithelium on *Drosophila sp*. (Santiabarbara-Ruiz P. *et al.*, 2015). In mice cells, such as hepatocytes and mammary epithelial cells, ROS induces the differential expressions of proteins associated with epithelial-to-mesenchymal transition through the induction of matrix metalloproteases (Gou R. *et al.*, 2017; Cichon M. & Radisky D., 2014). The effects of ROS on regeneration, and the widely available sources and inhibitors of ROS make it a candidate for regeneration studies.

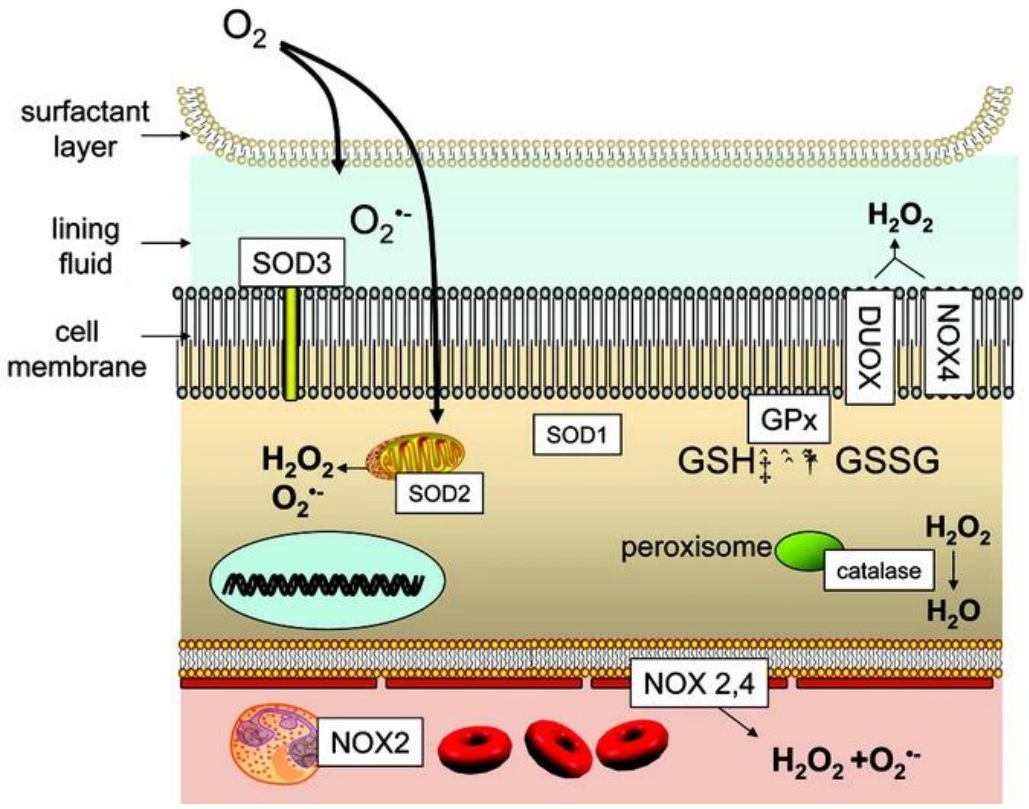


Figure 5 Richard L Auten & Jonathan M. Davis (2009). *ROS generation and detoxification in alveolar epithelium.* [Digital image]. Besides being generated by normal cellular processes, such as cellular respiration, NADPH oxidases (NOXes and DUOXes) and SOD proteins can also serve as an endogenous source of ROS production.

Calcium- Calcium ions function as one the most versatile second messengers for extracellular signals. It is an ubiquitous signal mediator found in low concentration on the cytosol and upon many extracellular signals, a concentration gradient quickly raises the ion's concentration from basal levels (10^{-3}mM) to an increase of 10-20 fold, activating calcium - responsive proteins (Albert B. *et al.*, 2015). An example of calcium responsive proteins is shown in figure 6. Several studies have linked the regenerative process with transient elevation of the ion during tissue damage, even governing the process of dedifferentiation and re-differentiation in cardiac muscle cells from adult mouse cardiomyocytes (AMCMs) and vascular smooth muscle cells (O'Meara C. *et al.*, 2015; Clunn G. *et al.*, 2010). Indeed, mammalian

regeneration is regulated by these calcium increases, and the dedifferentiation process has been assessed by using cardiomyocytes that can dedifferentiate, remodel and re-differentiate (Porrello E. *et al.*, 2011; Szibor M. *et al.*, 2014). Although mammalian hearts cannot regenerate, cardiomyocytes can dedifferentiate and upon dissociation and culture, the cells dedifferentiate into an immature phenotype, with one characteristic being the downregulation of calcium regulatory genes (Szibor M. *et al.*, 2014; O'Meara C. *et al.*, 2015). *In vitro* mammalian cardiomyocytes need calcium to re-differentiate into mature myocytes because calcium is needed to maintain an organized myofibrillar structure. Vascular smooth muscle cells (VSMCs) phenotype from mammalian tissue, which retain some plasticity, is regulated by transient calcium elevations (Halayko A. *et al.*, 1997; Kaimoto T. *et al.*, 2010). Pharmacological ablation of calcium is enough to downregulate markers for cardiomyocyte phenotype and to induce the upregulation of markers for dedifferentiation (Kaimoto T. *et al.*, 2010). In *C. elegans,* transient calcium elevations mediated by epidermal transient receptor potential (TRP) channels have been reported to influence regeneration of tissue. Mutant worms that cannot produce these transient calcium elevations are unable to survive the laboratory produced wounds (Xu and Chisholm, 2011). In zebrafish, these elevations have been observed following amputation of their fin, and upon blockage regeneration was impaired (Yoo S. *et al.*, 2012). Calcium's role in regeneration and dedifferentiation on a wide range of organisms, as well as its ability of being an effective signal mediator makes calcium an interesting target for cellular dedifferentiation in *H. glaberrima*.

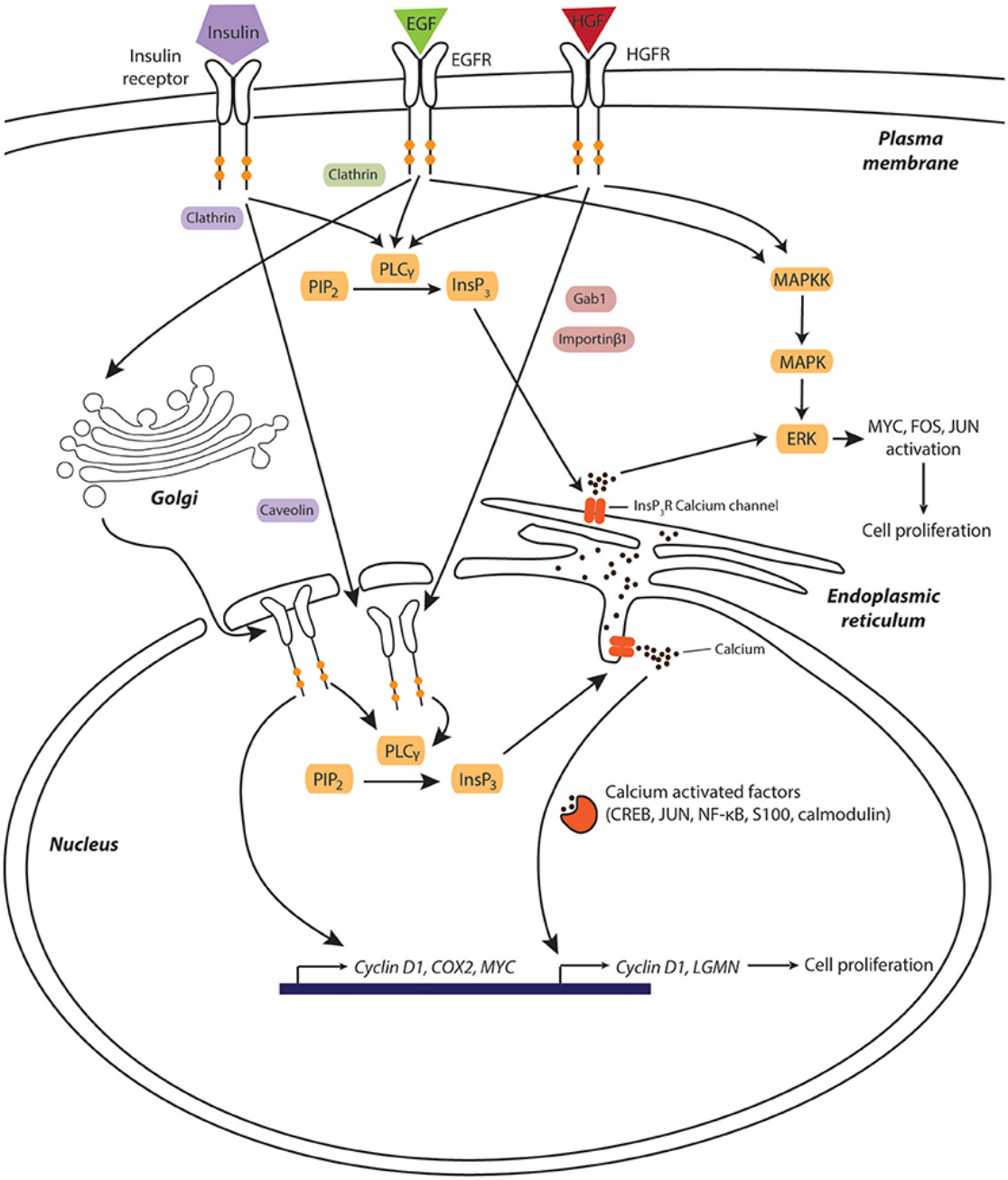


Figure 6 Oliva-Vilarnau N. et al., (2018). *Calcium is a key component of hepatic growth factor signaling during liver regeneration after physical injury* [Digital image]. Liver regeneration results from the mitogenic effects of growth factor through the activation of calcium responsive proteins leadings to an increase in transcription.

PI3K/ AKT pathway & growth factors – AKT/ PKB pathway is a serine/threonine protein kinase that regulates cell survival and proliferation through response of growth factors and other extracellular signals (Song G. *et al.*, 2007). Two regulatory phosphorylation sites, Thr308 and Ser473, are needed for complete activation of this pathway, and activation leads to cell's survival in the presence of apoptotic stimuli (Risso G. *et al.*, 2015; Song G. *et al.*, 2007). The AKT pathway is activated by upstream activator PI3K and has been associated with cancerous properties by its stimulation of proliferation, survival, and cell growth (Brazil D. *et al.*, 2002; Song G. *et al.*, 2007). AKT pathway exerts its effects through phosphorylation of cell machinery components. Among these are, pro- death proteases and members of the forkhead family of transcription factors (FKHR), IκB kinase (IKK) and p53- binding protein MDM2 (survival) (Oren M., 2003; Mayo L. & Donner D., 2001); cyclin D1 kinase glycogen synthase kinase-3β (GSK3β), FKHR leading to repression of CDK inhibitors (CKIs), and modulation of WAF1 affecting its binding to proliferating cell nuclear antigen (PCNA)(proliferation) (Peter M., 1997; Li Y. *et al.*, 2002); mTOR protein (cell growth)(Takuwa N. *et al.*, 1999) (Figure 7). Activation of this pathway by vascular endothelial growth factor (VEGF) has been linked to cell survival, and it has been found to be part of sea urchin development (Tu Q. *et al.*, 2012). Our laboratory has also found differential expression of VEGF during regeneration in *H. glaberrima* (Quispe- Parra D. *et al.*, 2021).

PI3k/ AKT can be activated by fibroblast growth factor 4 (FGF4). Investigation of these molecules has yielded promising results for the treatment of neurodegenerative diseases by inducing astrocyte dedifferentiation into neural stem/progenitor cells (NSPCs) (Feng G. *et al.*, 2014). Inhibition of this pathway led to reduction and, in some cases, complete suppression in

the expression of Sox2, a transcription factor considered the master regulator of astrocyte reprogramming (Yang H. *et al.*, 2019). Conversely, activation of this pathway can lead to redifferentiation of dedifferentiated cells. Inhibiting the phosphorylation of AKT or PI3k has led to an attenuated chondrogenesis from primary chick bud cultures (Park E. *et. al.*, 2008).

Most growth factors work through receptors tyrosine kinase (RTK's) which upon activation by autophosphorylation exerts its effects by activation of downstream effectors like RAS-MAPK, PI3K-AKT, and phospholipase C-Ca^{2+} pathways (Hubbard S. & Till J., 2000; Goetz R. & Mohammed M., 2013; Koch S. and Claesson-Welsh L., 2012). The two growth factors mentioned in this work, VEGF and FGF, have homologs in sea urchin, brittle stars, sea stars and in our model organism, and their functions throughout Eumetazoa has been recently reviewed (Kipryushina Y. *et al.*, 2015). Their expression has been implicated in the development of sea urchin, promoting cell survival, proliferation, and migration through the AKT/PKB, MAPK and p38MAPK or FAK pathways, respectively (Robertson A. *et al.*, 2013; Mulden- Lorillon O. *et al.*, 2017; Röttinger E. *et al.*, 2008). Pharmacological inhibition of VEGF led to malformation of skeletal elements in sea urchin embryos (Adomako-Ankomah A. & Ettensohn C., 2013). In regeneration, it has been inferred that growth factor may have a role in maintaining the pluripotent identity of blastema cells during regeneration and, in later stages, in the differentiation process (Thorndyke M. *et al.*, 2001). The developmental regulations of these pathways, as well as the versatility of the factors that activate them makes them a suitable candidate for dedifferentiation studies.

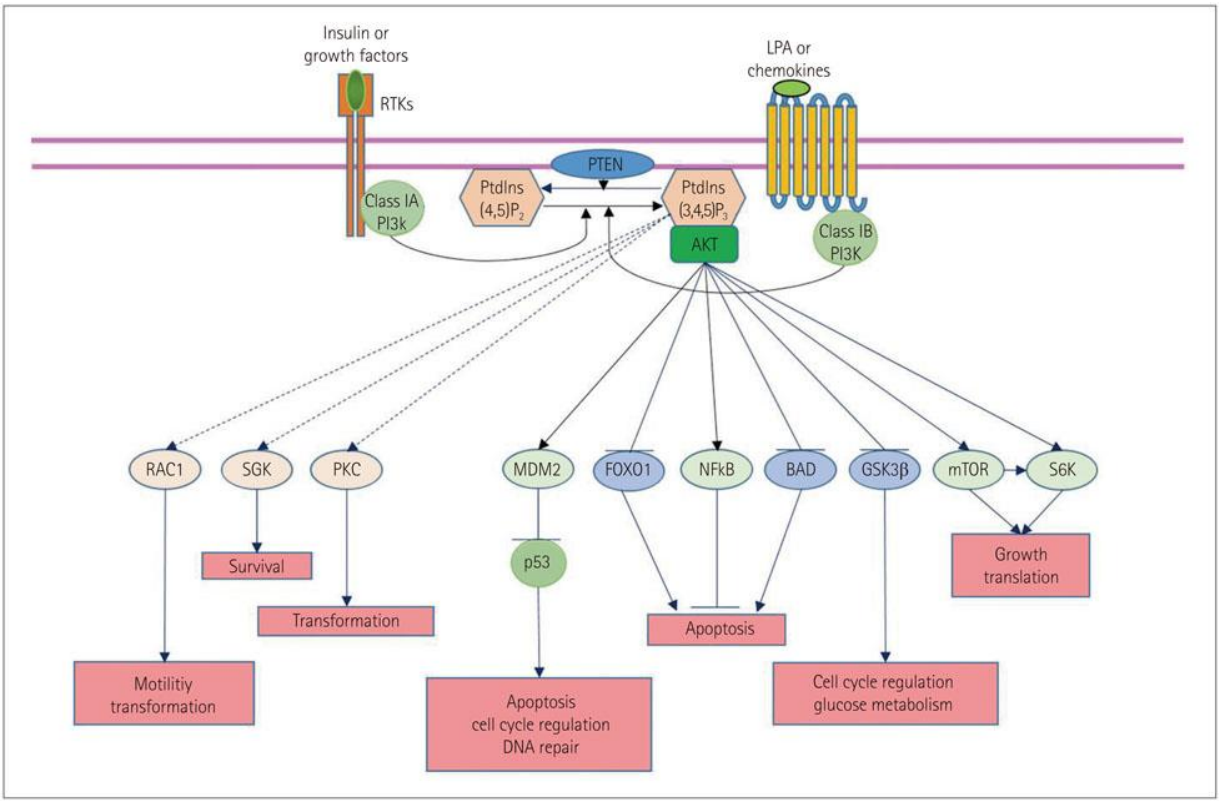


Figure 7 Seong-Ho K. & Eng L. (2015). *Role of the phosphatidylinositol-3-kinase (PI3K) pathway in cells.* [Digital image]. A schematic representation of PI3K signaling pathways including downstream effector molecules and the elicited cellular response.

Autophagy- Cells have developed a process by which they degrade obsolete structures or molecules and recycle the components as "building blocks" for other cellular functions. The process begins with the enclosure of a targeted protein or molecule in a phagophore that matures into an autophagosome, a double membrane structure that fuses with the lysosome for degradation of its contents (Bruce A. *et al.*, 2014). There are several drugs that have been developed to study the effects of autophagy in cellular processes. Of interest, chloroquine diphosphate is a pharmacological agent that works by inhibiting the fusion of autophagosome to the lysosome (Mauthe M. *et al.*, 2018). Inhibition of autophagy during the process of

regeneration using these pharmacological agents have paved the ways for elucidating its function on these processes. For example, this process has been responsible for cytoplasmic remodeling, a process which has been found to dedifferentiate human mesenchymal stromal cells (MSCs) into an early mesoderm-like state and can restore regenerative capacity *in vivo* (Pennock R. *et al.*, 2015). Autophagy also seems to be a crucial process for the formation of induced pluripotent stem cells (IPSC) from mouse embryonic fibroblasts (MEFs) (Wang S. *et al.*, 2013). In this case, for dedifferentiation of MEFs, a series of events must occur with one of them being somatic cell restructuring, in which autophagy seems to play a major role (Folmes C. *et al.*, 2011). As with other molecular pathways, vascular smooth muscle cells dedifferentiation seems to be regulated by the activation of autophagy. Indeed, and keeping with its role as a cytoplasmic restructuring mechanism, autophagy promotes the transition of VSMC into a synthetic phenotype, also known as dedifferentiated phenotype, by removing the contractile apparatus of these muscle cells (Ji Z., Li J. & Wang J., 2021). Mammalian cells are not the only cells that depend on autophagy for dedifferentiation/ regeneration. Pharmacological inhibition of autophagy in zebrafish leads to impairment of caudal fin regeneration by regulating cytoplasmic restructuring (Vargas M. *et al.*, 2013; Saera-Vila A. *et al.*, 2016). Finally, in regeneration of newt lens, damage to the cornea follows the appearance of autophagosomes that sequester cells organelles, degrade them, and induce the dedifferentiation of iris epithelial cells for the regeneration of the tissue (Yamada T. et al, 1978). Therefore, autophagy is an interesting target for the dedifferentiation assays conducted in this work.

In summary, there is a critical need to understand the role of these aforementioned factors and their possible roles in the dedifferentiation pathways (Hempel M. & Terak M. 2017;

Houschyar K. 2019; Morgan M. & Liu Z., 2010). Using primary cell culture from muscle tissue and different pharmacological treatments, we believe that we can provide some insights into the mechanisms involved in muscle regeneration.

Tissue culture

The use of primary cell culture has been widely used for elucidating molecular mechanisms in regeneration processes that may serve as a critical tool for paving the way to new therapeutic approaches in regenerative medicine. To explore molecular mechanisms, it is often important to establish dissociation protocols for cells isolation that can be used for cell suspensions, flow cytometry, or *in vitro* cultures. Our laboratory has designed a protocol to dissociate cells from muscle tissue and culturing them. Thus, we can now have isolated muscle cells adhere to a microscope slide, stained them, and observe them under a microscope. Conversely, we can maintain them in suspension for other studies. We've also developed protocols for immunohistochemistry assays for both muscle tissue explants and from disassociated cells. Upon disassociation with collagenase Type 1A, cells were separated into two homogenous populations by centrifuge gradient (Rodriguez J., 2020). Populations obtained by centrifuge gradients were deemed light and heavy phases. An initial cellular analysis suggested that some of these cells were undergoing dedifferentiation since their contractile apparatus were also found in the centrifugation media and in cultures. The identity of these dedifferentiated cells in both phases has not yet been clearly established. We have the tools, mainly antibody markers, to identify the cellular components of our dissociation experiments, and to determine if these components are changing (i.e., undergoing dedifferentiation) during

the dissociation procedures. Moreover, we propose that we can use these dissociation and culturing processes to determine which factors are involved in muscle dedifferentiation.

Specific Aims

Aim 1. To optimize cell culture techniques for dissociated longitudinal muscle cells of the sea cucumber *Holothuria glaberrima*.

Aim 2. To determine the signaling pathways responsible for holothurian myocyte dedifferentiation in primary cell cultures.

Materials and Methods

Animals- H. glaberrima specimens were collected from coastal areas in the northern part of Puerto Rico. Organisms were placed in oxygenated seawater aquaria in the lab at temperatures of 20 -25 ^{o}C until used.

Tissue dissection- Animals were placed in 500 mL of anesthetic solution (Chlorobutanol 0.2% in sea water; Sigma 112054) for 45-60 minutes prior to dissection. Organisms were then transferred to containers with 10% Clorox solution for 1 minute, 95% ethanol for 5 minutes and finally to distilled water for 1 minute. The anterior part of the animal was removed with a razor blade, cut just below the calcareous ring, and a second cut was made just adjacent to the cloaca (see figure 1 for reference). A third cut was made dorsally, extending from the anterior to the posterior end and, using pin-needles, the body wall was extended on a dissection plate. Internal organs were discarded. Using the scalpel, the mesenteries were gently removed, exposing longitudinal muscle bands. At this point, five pairs of muscle bands were visible. Muscle bands were removed individually using scissors and forceps and, either fragmented into smaller pieces, or placed directly into the dissociation media.

Muscle dissociation- Each muscle band was individually placed in a 15 mL test tube with 10 mL of sea water supplemented with an antibiotic/antifungal solution: 1.5 mL of Penicillin/streptomycin (10,000 U penicillin, 10 mg/mL streptomycin; Sigma P0781), 750 µL of Neomycin (10mg/mL; Sigma N1142), 150µL of Amphotericin B (2mg/mL; Sigma PHR1662) and 10 µL of nystatin (20 µg/mL; Sigma N4014). Test tubes were placed in a platform shaker for 30 minutes at 20-40 rpm. In a sterile culture hood, the contents were then placed into a 15 mL test

tube with 5 mL of 0.15%, 0.20% or 0.30% collagenase type 1A (Sigma C9407) supplemented with 1 mL of Penicillin/streptomycin, 500 µL of Neomycin, 100 µL of Amphotericin B and 10% of FBS (Sigma F2442; optional), and then placed in a platform shaker for 6 or 24 hours. After this, dissociated muscle tissue was centrifuged for 15 minutes in a high speed refrigerated centrifuge (Eppendorf® Centrifuge 5810/5810R) at 1,500 rpm, 25°C, to remove the collagenase solution from the disassociated tissue. Two washes were performed by resuspending the precipitate with 10 mL of SCCM supplemented with 10 µL of nystatin. After the second wash, the precipitate was resuspended in 10mL of SCCM. At this point, the cells were a mixed population of dissociated cells that were cultured.

An alternative separation method was used to separate cells into two different populations (Figure 8). To do this, cells were centrifuged at 400 rpm for 15 minutes to separate the media into a light and a heavy phase. The heavy phase appeared to be denser, resembling a pellet, while the light phase was suspended in the medium. The light phase was then transferred to a new centrifuge tube and the denser heavy phase resuspended with SCCM to the same volume as the light phase.

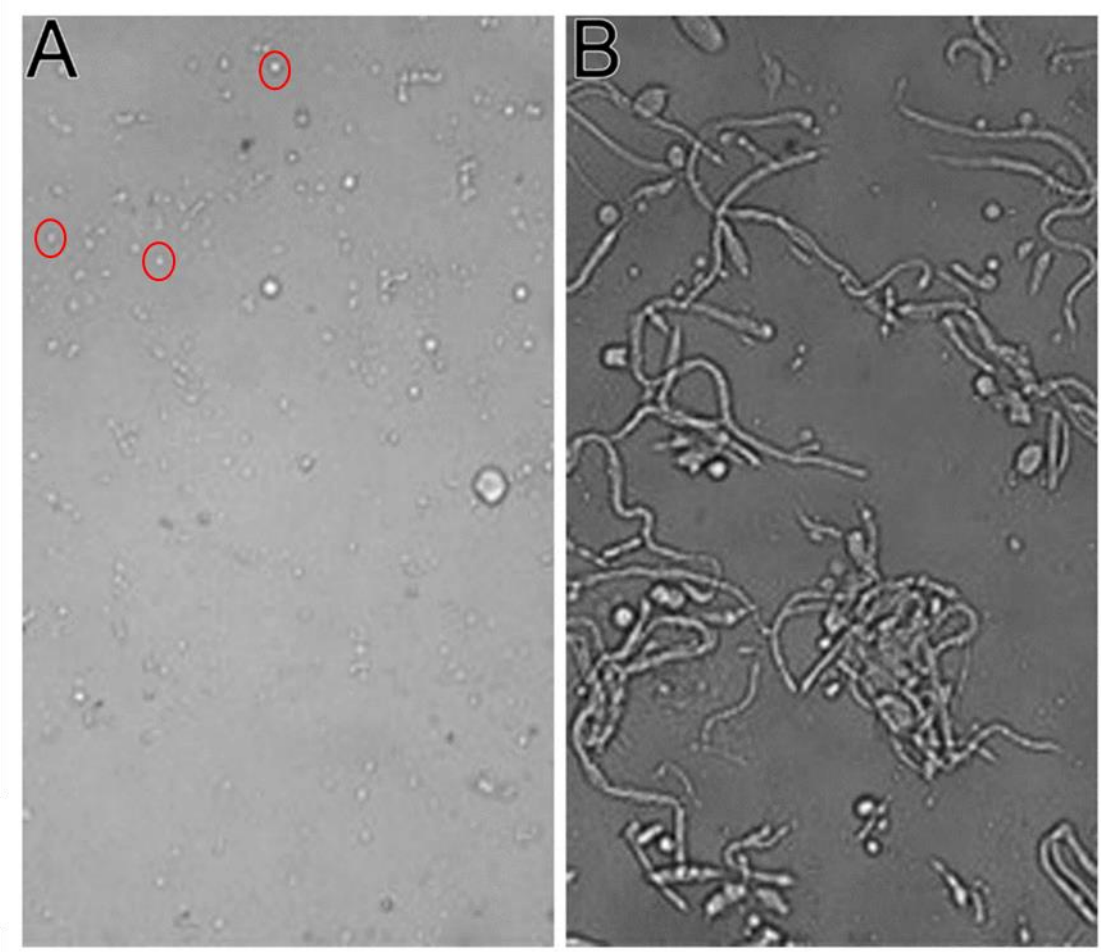


Figure 8 *Wet mount slide containing samples of dissociated muscle tissue prior to culture.* Magnified samples (20X) of dissociated tissue after 6 hours in collagenase type 1A and centrifuged at 400 rpm. Light phases **A.** showing a depiction of morphologically distinctive cells, believed to be dedifferentiated cells (red circle), **B.** while a heavier phase contains long muscle fibers.

Culture preparation- After separating both phases into different test tubes, we proceeded to count cells under a light microscope. For this, a dilution of 1:10 was made using dissociated cells in SCCM and counted using a hemocytometer. Next, we cultured 2.0 X 10^6 cells in Nunc™ Lab-Tek™ Chamber Slide™ wells and completed to a final volume of 300 µL with SCCM. After 2 days, a layer was observed at the bottom of each well.

Culture plate preparation- Recent experiments in our lab have shown that the substrate or treatment of the glass slides, where cells are plated, is a critical step in successful holothurian cell culturing. Therefore, we tested two different substrates to determine the most efficient method for culturing holothurian muscle cells.

Acetic acid treatment protocol- Rodriguez A., 2019 developed a protocol in which undiluted glacial acetic acid was used as a treatment for Nunc™ Lab-Tek™ Chamber Slide™ System to increase adhesion of *H. glaberrima* dissociated muscle tissue. For this, we placed 10 µL of glacial acetic acid to the center of each well where cells were going to be cultured and left to dry in a fume hood overnight. Once the glacial acetic acid evaporated, each well was washed with 600µL of distilled water for 15-20 minutes. Washing of chambers was performed at least 6 times. Following this, SCCM was placed in the wells for 10 minutes to see if there was a change in color of the medium. If it changed color, we applied additional washes with distilled water.

Polydopamine coating protocol- Alternatively, we use polydopamine to adhere cells, a method inspired by a mussel's ability to bind to wet surfaces (Nielsen S. *et al.*, 2013). To polymerize dopamine into polydopamine and to treat culture chamber, Tris- HCl buffer was prepared at a molar concentration of 0.5M and adjusted to a pH of 8.5. We then dissolved 2 mg/mL of dopamine hydrochloride (Sigma H8502) into Tris-HCl buffer and used the solution with the polymerized dopamine to treat the wells. Wells were left with polydopamine overnight. The following day, the wells were washed three times with ultrapure water for 15 minutes each wash.

Assessment of cell identity with cell markers- Cultured cells were fixed in 4% paraformaldehyde. After fixation, the paraformaldehyde was removed, and each chamber was washed 3 times with 0.05M PBS for 15 minutes each wash. Antibodies and other markers to be used (Table 1) were diluted in RIA buffer (40.5 mM K_2HPO_4; 9.5 mM KH_2PO_4; 154 mM NaCl; 75.3 µM BSA; 1.54 mM NaN_3) and added to cover the surface area (100 µL). Cells incubated with primary antibodies were left at RT for 24 hours. The next day, we washed each chamber 3 times for 15 minutes with 0.05 M PBS solution. Secondary antibody was applied along with phalloidin-FITC (Sigma P5282), each at a dilution of 1:000 in RIA buffer. We added 100 µL, or enough to cover the whole area of each chamber and incubated for 1 hour at room temperature. After 1 hour of incubation, 3 washes were done with 0.05 M PBS buffer for 10 minutes each wash. We removed the polystyrene medium chamber using the slide separator and added approximately 12µL of 2.86 mM DAPI solution to each well (Sigma D9542) for nuclei staining. Lastly, we used 24 X 55 mm microscope cover glass to cover each slide with mounting medium (1 mL of 2.86 mM DAPI Sigma D9542; 25 mL glycerol; 25 mL of 0.1 M PBS), sealed the borders with nail polish and analyzed them using a fluorescent microscope (Nikon® ECLIPSE Ni-U upright microscope; DS- Qi2 monochrome microscope camera).

Table.1 Immunohistochemical assessments of cells from disassociated muscle tissue

Stains	*Dilution in RIA buffer*
Phalloidin (Toxin)	1:1000
DAPI (DNA stain)	1:50*
8d10 (Ab)	1:5
5H11 (Ab)	1:1

* Dilution was done in glycerol and PBS

In vitro pharmacological treatments – Cells were cultured with the pharmacological agents listed in Table 2. Concentrations for each drug were selected based on previous articles where they used *in vitro models* for pharmacological assays on organisms phylogenetically closest to echinoderms. The cell cultures for these experiments were done with the adhesion protocol using polydopamine and we cultured 2.0 X 10^6 cells. Two experiments were performed for the pharmacological assays. The first, would have the drug in the dissociation media for 6 hours and would be removed along with the dissociation media by washing it 3 times with culture medium. These cells were then cultured for 3 days in culture media lacking the drug. In the second protocol we did not apply the drugs during the dissociation, but treated cells with drugs at the time of culturing for 3 days. After fixation, culture slides were analyzed for: (1) the number of cells (nuclei), (2) the total number of muscle fibers, (3) the number of fibers presenting a nucleus, (4) the number of fibers in the process of dedifferentiation and (5) the number of SLS's per field of view. The latter two correspond to structures associated with dedifferentiation. Slides were analyzed without knowledge of the pharmacological treatment applied and results were corroborated by a second investigator. Statistical analysis was performed using R studios. Differences among groups were assessed by one-way analysis of variance (ANOVA) followed by Tukey's test.

Table 2 Pharmacological agents and the pathways they modulate

Drugs	*Pathways*	*Mode of action*	*Doses*	*References*
Hydrogen peroxide	ROS	Exogenous source of ROS	2.0µM	Oh S. *et al.*, 2020

DPI	ROS	Inhibition of flavoenzymes; NOXes and DUOxes Reduction of ROS production	25µM	Oliveira J. *et al.*, 2011
Vas2870	ROS	NADPH oxidase inhibitor Reduction of ROS production	25µM	Larroque-Cardoso P. *et al.*, 2013
Lithium Chloride	Wnt signaling	Putative activator	20mM	Bello S. *et al.*, 2019
iCRT14	Wnt/β-catenin	Putative inhibitor	50µM	Bello S. *et al.*, 2019
Calcium Ionophore A23187	Calcium ions	ion-carrier	1µM	Przygodzki T. *et al.*, 2005; Hyatt H. *et al.*, 1984
EGTA	Calcium ions	Chelating agent selective for Ca^{2+}	1mM	Chia, F. & Xing J., 1996
UK-383,367	BMP-1	Inhibitor of BMP-1	5µM, 10µM, 30 µM	Canty E. *et al.*, 2004
LY-294,002 hydrochloride	phosphoinositide 3-kinase (PI3K)	PI3K inhibitor	10µM	Perez J. *et al.*, 2010; Pramanick K. *et al.*, 2014
Chloroquine diphosphate	Increase of intravesicular pH (Endosomes, Golgi vesicles, and lysosomes)	Enzyme inhibitor	1mM	Watchon M. *et al.*, 2017

Results

Optimization of cellular cultures from muscle tissue explants

Our research focuses on the possibility of culturing muscle cells in order to being able to reproduce the process of cellular dedifferentiation *in vitro*. For this, optimization of the cell culture protocol is essential. Our experimental procedure involves dissecting out the longitudinal muscle from the animals, dissociating these muscles enzymatically, placing them in a culture and culturing them for several days. Methodological improvements of any of these steps should yield a higher number of cells and muscle fibers while minimizing the presence of structures associated with the process of dedifferentiation and degradation. Thus, we assessed several steps to improve culture preparation, among these: (1) use of polydopamine to adhere cells to the culture dishes, (2) the time of exposure to collagenase (the dissociating agent), (3) the concentration of collagenase and (4) the addition of fetal bovine serum (FBS) to the dissociation media.

We present the results obtained for each of the modifications for optimizing muscle fiber culture efficiency.

Adhesion of dissociated cells to chamber systems (Hac vs. PDA) - Our laboratory has previously developed a technique for adhering dissociated cells to hydrophilic surfaces, using undiluted acetic acid (Rodriguez A., 2020). The reason for enhanced adherence has yet to be deduced, but viability assays, such as MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide), showed that the seeded cells were metabolically active for up to three days after being

dissociated by collagenase Type IA and plated (Data not shown). Recently, a novel adherence technique, using catechol-based chemistry, has been developed (Figure 9). This technique has been proven to increase adherence and viability in a variety of cells, such as neural stem cells (Nielsen S.R. *et al.*, 2013; Yang K. et al., 2012).

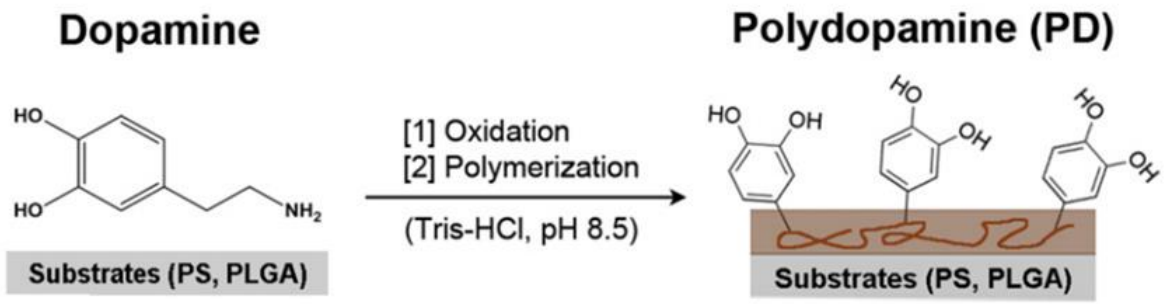


Figure 9 Yang K., *et al.*, 2012. *Chemical reaction and structure of pDA- treated wells [Digital image].* Shows the chemical reaction that leads to the polymerization of dopamine, resulting in polydopamine.

Therefore, we assessed the efficacy of using this method of adherence against acetic acid treatment protocol. Dissociated muscle cells grown in culture wells treated with Hac for 72 hours, showed high concentrations of cellular debris, leading us to believe that acetic acid may be inducing the degradation in muscle fibers *in vitro* (Figure 10A-B). Hypothesizing that acetic acid may be responsible for cellular degradation, we reduced the amount of acetic acid applied to each well from 100µL to 10µL to see if we could increase the number of muscle fibers that were present and reduce the levels of cellular debris believed to appear from the degradation of said fibers. As shown in figure 10C, healthy muscle fibers were obtained.

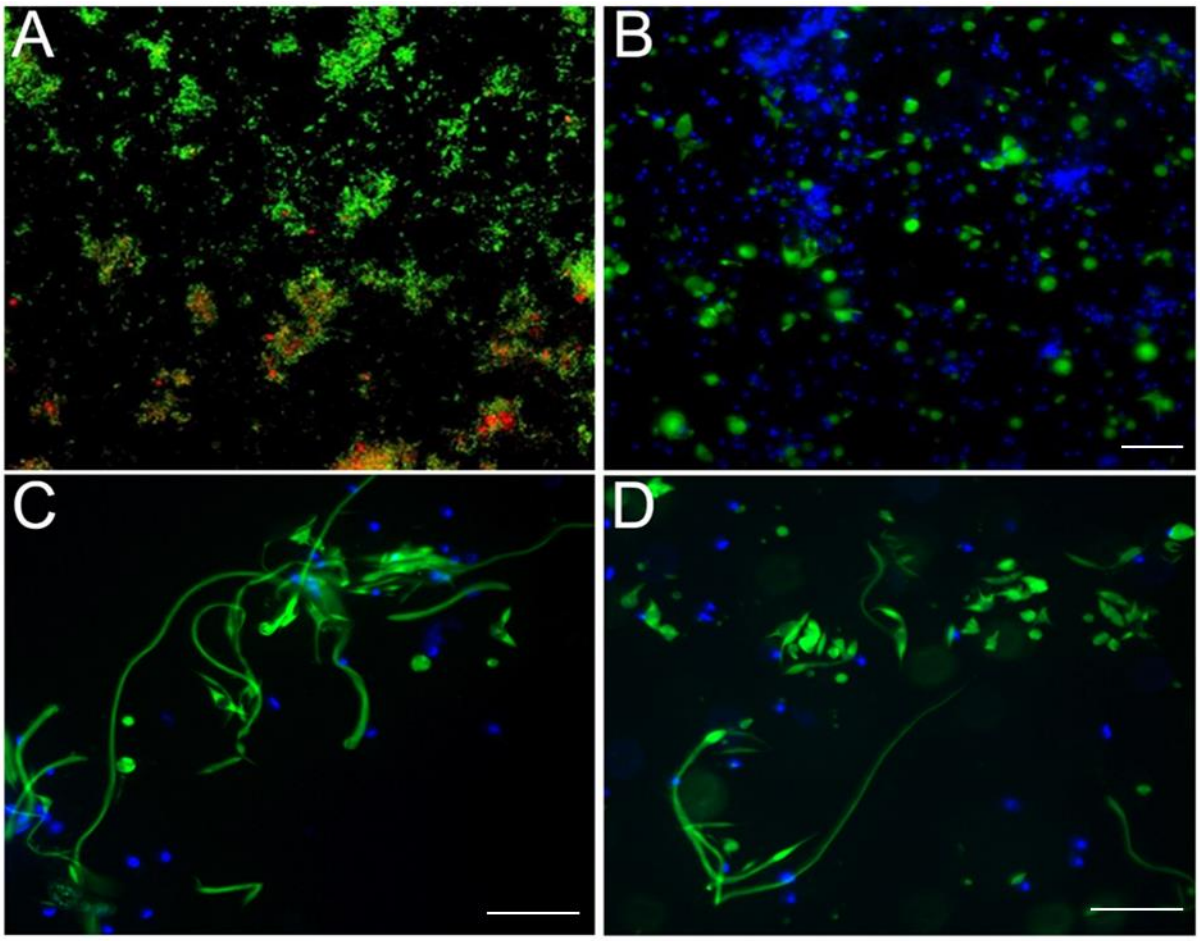

A
B
C
D

Figure 10 *Fluorescent imaging of two adhesion techniques.* Phalloidin staining of cultures after 24hrs. in collagenase treatment from both polydopamine and acetic acid adhesion techniques. **A.** Rodriguez A. (2019) treatment of 100µL of undiluted acetic acid leads to increase degradation of dissociated muscle tissue, **B.** even when 10% FBS was added to dissociation media. **C.** Reducing the volume of the treatment of acetic acid to 10µL yielded healthy looking long muscle fibers. **D.** Adhesion of cells with polydopamine treatment showed the same muscle tissue components as 10µL of acetic acid with the addition of an abundance of nucleated muscle fibers, while still maintaining the presence of SLS's and dedifferentiating fibers, leading to the speculation that exposure to collagenase may be inducing the degradation and/or dedifferentiation of muscle cells. Cultures were labeled with phalloidin-FITC (green for **B, C, D**) (red for **A**) and DAPI (blue for **B, C, D**) (red for **A**). Scale bar = 1µM **(B)** and 10µM **(C, D).**

When comparing acetic acid and polydopamine for adhesion of cells, we found that the number of cells that adhere to wells were not significantly different one with respect of the other. Nuclei staining with DAPI shows no significant difference between the number of stained nuclei between the two adherence treatments as shown in figure 11A, leading us to believe that neither *Hac* nor PDA affect the number of cells that can interact with the treated wells. Since our investigation focuses on the presence of muscle fibers, the appearance of structures associated with the dedifferentiation of those muscle fibers and their response to treatments with different pharmacological agents, we sought to compare the quantity of muscle fibers that we could obtain through our adherence procedures. We found that polydopamine assays significantly increased the presence muscle fibers (Figure 11B). We found an increase in both the total number of muscle fibers and that of nucleated muscle fibers (Figure 11B-C). These observations and quantitative analyses lead us to focus only on using polydopamine.

Having found this, we then asked how much time is needed for cells cultured in polydopamine to firmly adhere to the culture slide. This is particularly important for our subsequent experiments where cultured cells needed to be stained and counted. For this, we

quantified number of cells and muscle fibers in a period of 24 to 72 hours. We found that increasing the time of adherence from 24 hours to 72 hours significantly increased the overall number of cells, the total number of muscle fibers and the number of nucleated muscle fibers that were present on our cultures, as shown in figures 12A-C. Overall, these results suggest that muscle fibers adhere to polydopamine-treated wells in a time dependent manner.

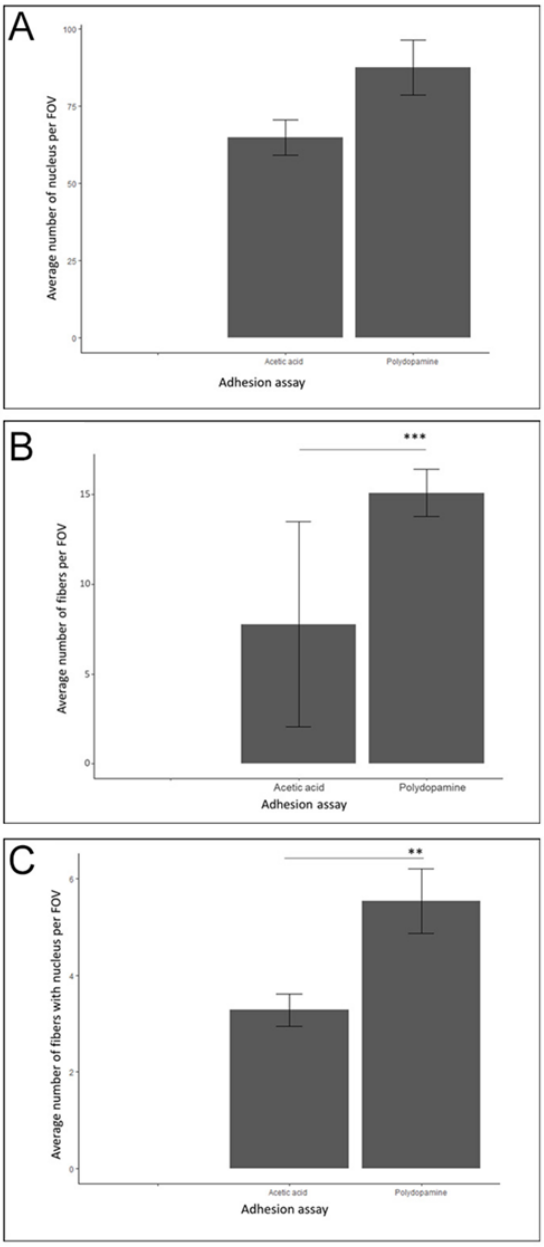


Figure 11 *Polydopamine adhesion assay increases the presence of nucleated muscle fibers that adhere to culture wells when compared to acetic acid, but doesn't change the total number of cells* **A.** No significant difference was observed for the total number of cells that adhere to either acetic acid or polydopamine adherence treatments, although a slight increase can be observe for the latter **B.** Acetic acid, our principal adhesion assay, showed less muscle fibers that can adhere to culture when compared to polydopamine. **C.** There was a significant improvement in the number of nucleated muscle fiber present in our cultures. Reduced number of muscle fibers may be in part from the degradation by acetic acid's acidic pH. Approximately, 10^6 cells were seeded per well. Field of view from 20X magnification. Bars are Mean ± S. E. N= 8. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

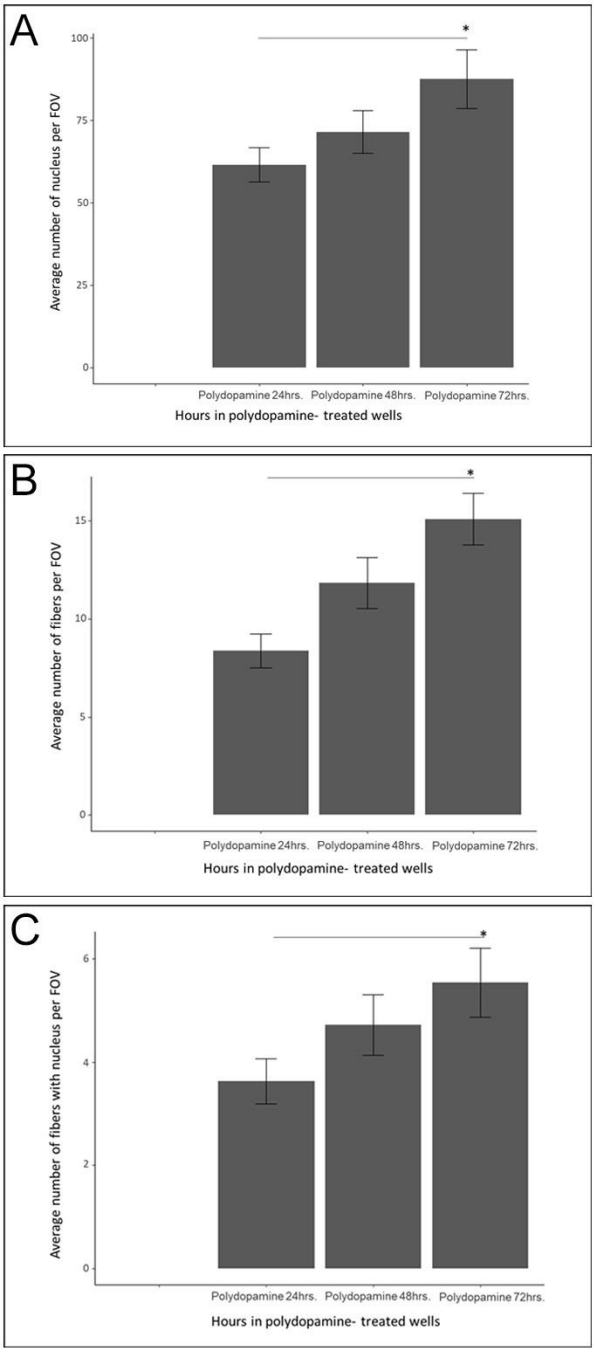


Figure 12 *Number of cells that adhere to polydopamine-treated wells is time-dependent* **A.** We found that the total number of cells gradually increased as cell were left in polydopamine-treated wells for 24-, 48- and 72-hours **B.** This gradual increase was translated in the total number of muscle fibers present in polydopamine-treated wells that remained in culture longer times before paraformaldehyde fixation. **C.** The increasing number of muscle fibers present in our cultures also presented a nucleus. Approximately, 10^6 cells were seeded per well. Field of view from 20X magnification. Bars are Mean ± S. E. N= 8. **P* < 0.05, ***P* < 0.01, ****P* < 0.001.

Collagenase treatment-

1. *Media vs. SCCM*- First, we compared the medium in which collagenase type 1A was diluted, either in sea cucumber culture medium, as it was previously done by Rodriguez A. (2019) for holothurian longitudinal muscle explants, or in calcium and magnesium free sea water, as it was done by Bello S. and colleagues (2019) for the dissociation of holothurian gut rudiment. Qualitative analysis was enough to deduce that CMFSW was a better dilutant for collagenase type 1A, as little to no fibers were observed in the dilution of collagenase in SCCM.
2. *Collagenase concentration*- Continuing the possibility that the dissociation process using collagenase type 1A may be inflicting cellular stress that leads to damage on our muscle fibers, we sought to investigate the effects of different concentrations of collagenase type 1A and the time of exposure of this media in our dissociation protocol. Two concentrations have been previously used in our laboratory for longitudinal muscle tissue and intestine dissociation, 0.06% and 0.12% respectively. We used these concentrations as reference and increased them to 0.15%, 0.20%, 0.30% and 0.70%. Qualitative analysis from the cultures showed that the higher concentration induced the degradation of all fibers in our culture, leading us to exclude it from our experiments. Therefore, our working concentration were 0.15%, 0.20% and 0.30%. Phalloidin staining showed a gradual increase in the presence of dedifferentiating structures as muscle tissue was exposed to higher concentrations of collagenase (Figure 13A-C). Our results showed no difference in the number of nuclei present in our cultures, but the number of nucleated muscle fibers did

increase in 0.20% and 0.30% (Figure 14A). Although we obtained a higher number of nucleated fibers at higher concentrations of collagenase, our results showed a relevant increase in the structures associated with dedifferentiation, SLS's and dedifferentiating fibers (explained below), as collagenase concentration was increased (Figure 14B-C). We found a significant increase in the number of SLS's of 125% and 136% when comparing concentration of 0.15% with 0.20% and 0.30%, respectively (Figure 14D).

3. *Addition of FBS*- Since the start of our investigation, we kept aspiring to improve the state and number of our muscle fibers, and while researching possible ways to do this we came upon a series of experiments where murine muscle was dissociated with collagenase (Selvin D. *et al.*, 2013). In these experiments, the muscle fibers showed sign of degradation and a "super coiled form" that was reversed by the application of FBS to the dissociation media. Scientific literature states that FBS activates serum response factor, a transcription factor that responds to fiber damage and stress activating genes responsible for the maintenance of the sarcoplasmic reticulum as well as the sarcomere (Chai J. & Tarnawski S. 2002; Greenberg M & Ziff E. 1984; Lamon S. *et al.*, 2014). Therefore, we tested whether the presence of 10% FBS in the dissociation media was able to decrease muscle fibers from damage or loss during dissociation. No significant change was observed in the number of muscle fibers present in our cultures (Figure 15).

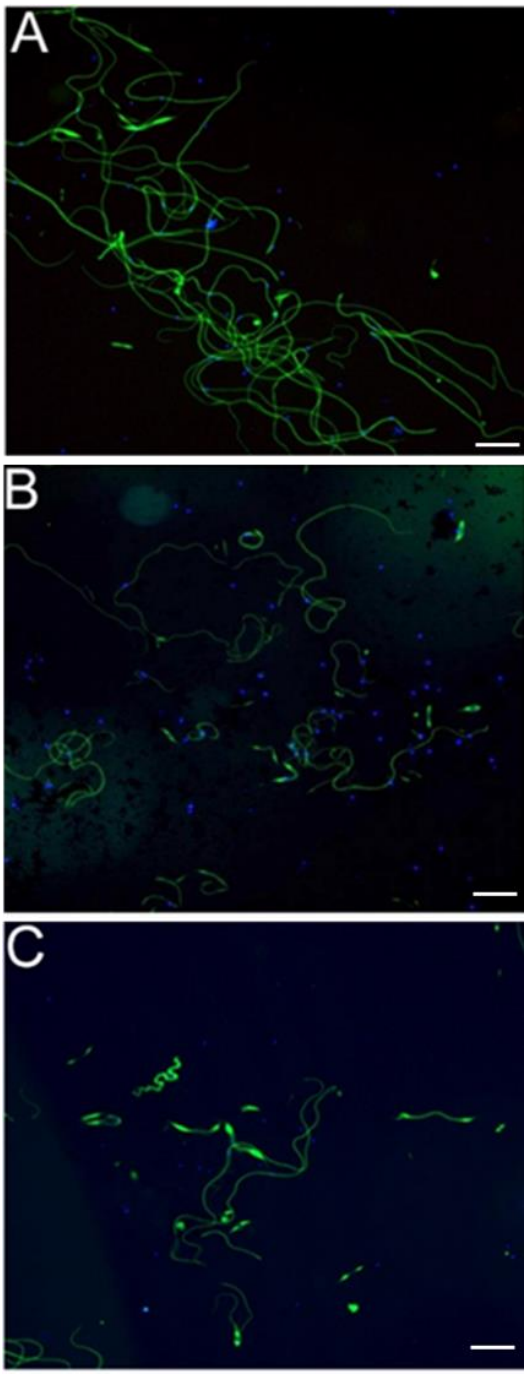


Figure 13 *Phalloidin staining of the cellular components found in different concentration of collagenase type 1A solution* **A.** Lowest experimental concentration of 0.15% was able to produce isolated muscle fibers with nucleus while **B.** signs of dedifferentiation, like SLS's and dedifferentiating fibers, begin to appear in 0.20% of collagenase **C.** These signs were more pronounced at 0.30%, the highest concentration tested in our dissociation protocol. Approximately, 10^6 cells were seeded per well. Cultures were labeled with phalloidin-FITC (green) and DAPI (blue). Scale bar = 10μM.

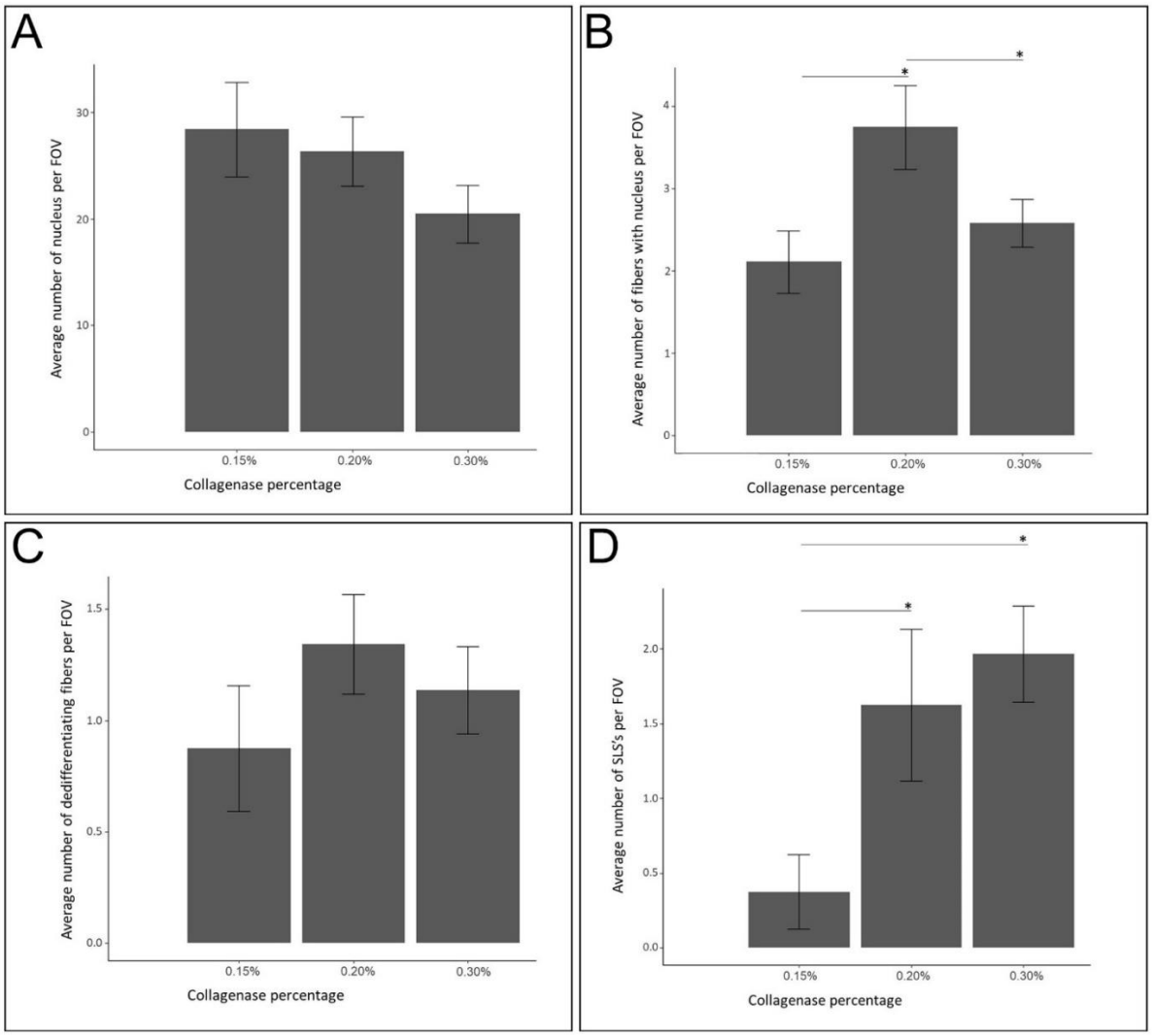


Figure 14 *Statistical analyses of the cells and cellular components found in different concentration of collagenase type 1A solution* **A.** No marked difference was obtained in the number of nuclei presented in our cultures. **B.** There was a significant increased of nucleated muscle fibers at 0.20% of collagenase when compared to 0.15% and 0.30%. **C.** A trend can be seen in the quantity of dedifferentiating fibers without statistical significance. **D.** There was significant concentration-dependent increase in the presence of SLS's, with the highest number of SLS's found at 0.30% of collagenase. Approximately, 10^6 cells were seeded per well. Field of view from 20X magnification. Bars are Mean ± S. E. N= 8. *P < 0.05, **P < 0.01, ***P < 0.001.

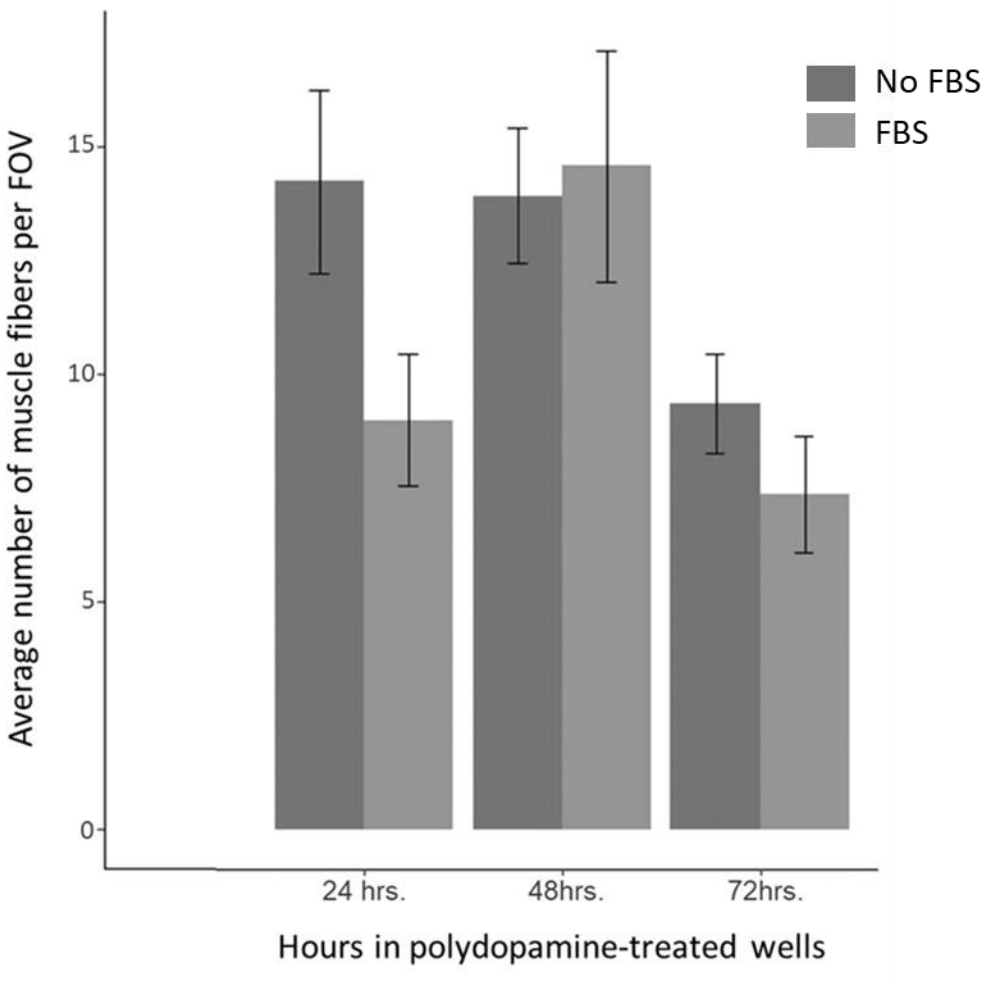


Figure 15 *Fetal bovine serum does not affect muscle fibers in cultures.* Given the time in culture, prior to fixation, we didn't see significant difference between those treated with FBS vs. those that did not have FBS in the dissociation medium. Number of muscle fibers that adhere to polydopamine-treated wells seemed to only be influenced by time. Approximately, 10^6 cells were seeded per well. Field of view from 20X magnification. Bars are Mean ± S. E. N= 8.

Identification of cellular morphologies in dissociated cells from longitudinal muscle tissue

During the course of our experiments, we noticed the presence of various morphologies of cells and cell-derived structures in our wells. To determine if these represented any particular process, we focused on their identification. We identified cell types by their morphologies and their labeling with certain markers. Cells and their components were stained with fluorescently- labeled phalloidin and/or antibodies. We were able to identify

muscle fibers, SLS's and a new structure that was added to our analyses and named "fibers in the process of dedifferentiation" or "dedifferentiating fibers", which were hypothesized to be a stage prior to the formation of SLS's. These results were analyzed, and we propose that these different structures might represent various stages of the process of muscle fibers dedifferentiation. We show here the cells and structures observed and how these were used to create a scheme that might reflect the different phases of the dedifferentiation process. The presence of these structures, along with the use of pharmacological activators and inhibitors, were used to determine which mechanisms are involved in the dedifferentiation of cells. Also, the number of fibers presenting a nucleus compared with those that are a missing one may inform us if the molecular pathway targeted by an activator or inhibitor is involved in either major or minor wounding.

After fixing the cells with paraformaldehyde and staining them with fluorescently-labeled phalloidin, we perceived different structures that were repeated in each slide and arrange them in an order that seemed to best fit the process that starts with a muscle fiber containing a nucleus and ending with the formation of two SLS's and a dedifferentiated cell. The presence of an intermediate morphology was found to correlate with the increase of SLS's. This structure seemed to precede the formation of SLS's and was named a "dedifferentiating fiber". This structure is characterized by the presence of rhomboid shape at each extremity of the dedifferentiating fiber and the loss of polymerized actin starting from the middle of the muscle fibers and extending to each extremity (Figure 16A-B). This led us to hypothesized that for each muscle fibers, two spindle- like structures are formed and released into the culture medium (Figure 16C). Finally, we manage to create both, illustrated and photographic schematics of the

process of dedifferentiation (Figure 17). The process proceeds as follows for a single muscle fiber: 1) A long muscle fiber presents an elongated single nucleus, 2) the fiber eventually starts to condense its contractile apparatus to each extremity of the fiber and eventually expels its nucleus as a dedifferentiated cell and 3) two SLS's are released. The product of the differentiation process is observed as two SLS's and one dedifferentiated cell. It is important to mention that other morphologies were considered for the formulation of the schematics presented here but were discarded due to considerations that these morphologies may correspond to malformation of cells or errors in the dedifferentiation process. Such morphologies are presented in figure 18.

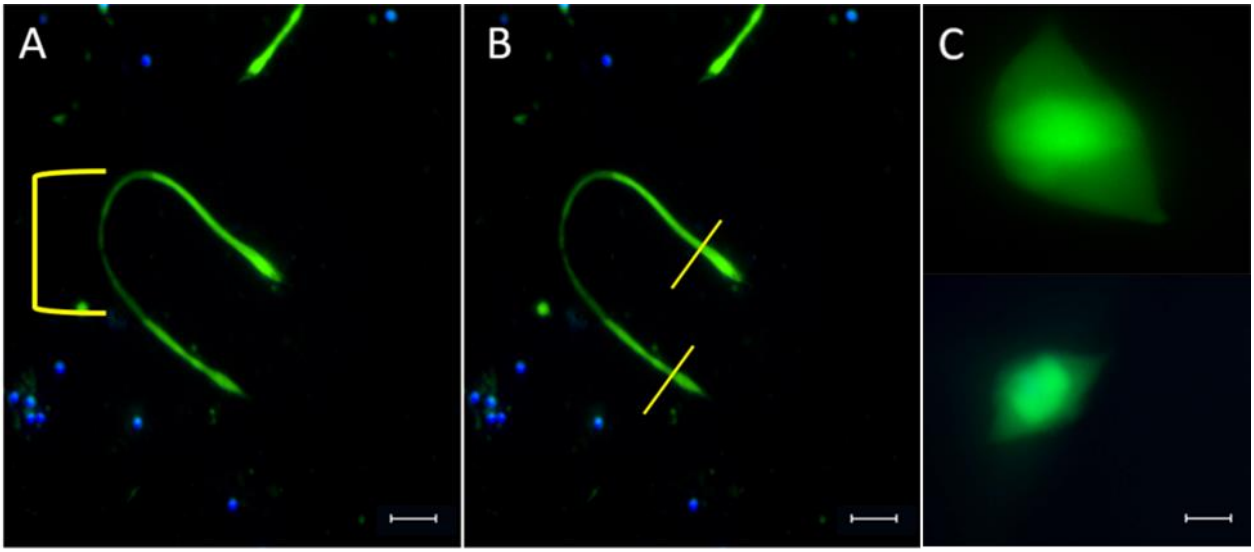


Figure 16 *Two SLS's are released into the extracellular medium from a muscle fiber.* Cellular components found in our cultures were assembled into a chain of events that best represented how SLS's are formed from dedifferentiating muscle fibers. **A.** Dedifferentiation process consists in the loss of polymerized actin that starts at the center of the muscle fiber and extends to each end (yellow line). **B.** Actin is condensed at each end of the muscle fibers, separating each end (yellow lines) into **C.** two SLS's that are released from a single muscle fiber. Cultures were labeled with phalloidin-FITC (green) and DAPI (blue). Scale bar = 50µM **(A, B)** and 100µM **(C)**.

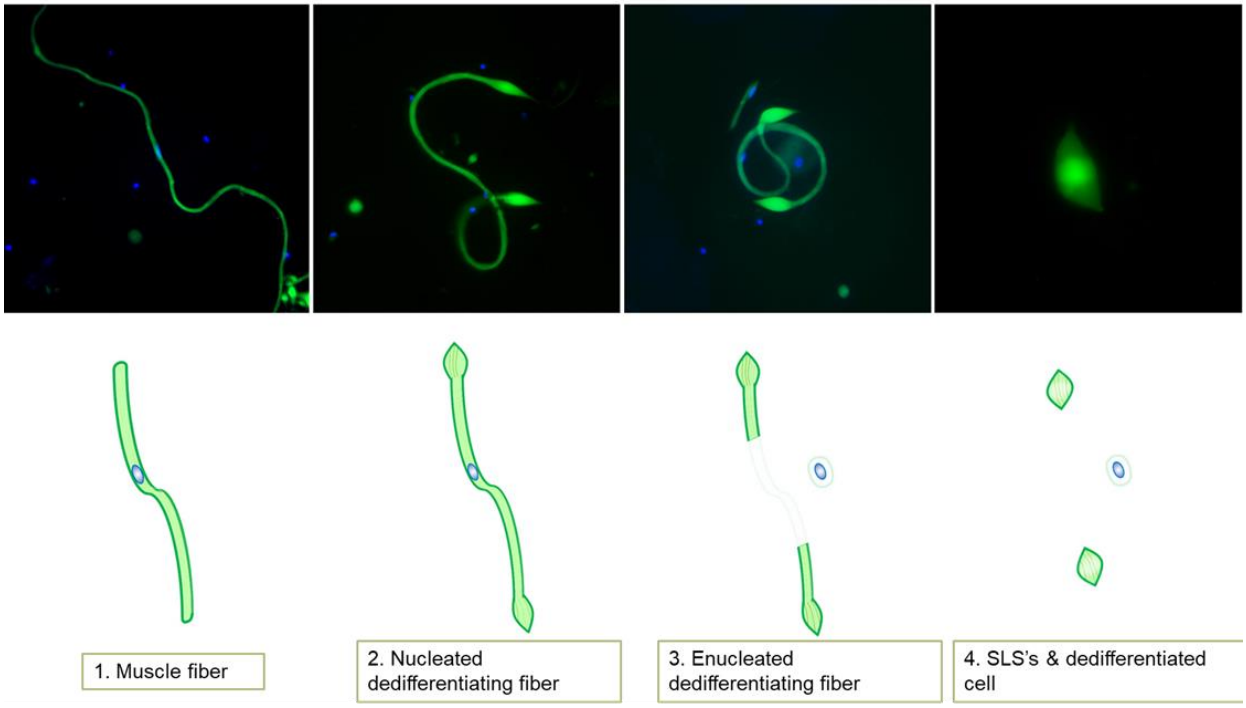


Figure 17 *Schematics of the order of events that represent the dedifferentiation of a muscle fiber.* We followed repeated structures and ordered them in a sequence that seemed to best reflect the conformational changes that lead to the creation of SLS's and dedifferentiated fibers. We begin with **1**. a nucleated muscle fiber which **2.** starts to depolarize actin along its axis, **3.** expels its nucleus into the extracellular medium and **4.** forms a dedifferentiated cell and two SLS's. Cultures were labeled with phalloidin-FITC (green) and DAPI (blue).

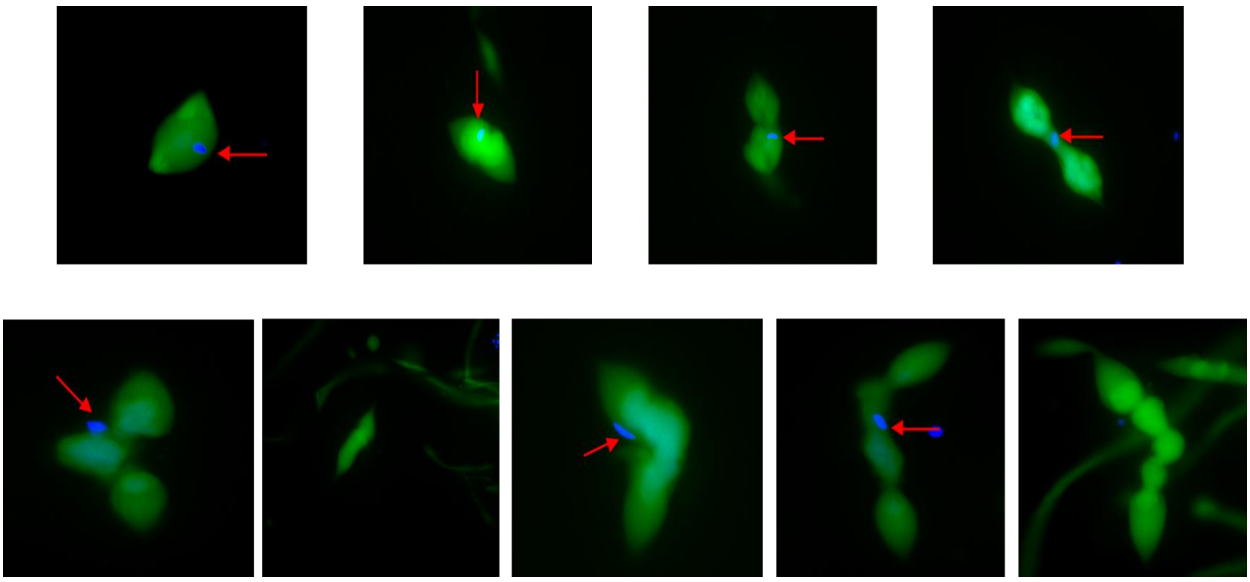


Figure 18 *Cellular components, with and without a nucleus, were constantly present in cultures from dissociated muscle tissue.* Other structures may form part of the dedifferentiation process or may not complete it. Alternatively, the presence of a nucleus (red arrow), may suggest the process of myogenesis from dedifferentiated cells. Cultures were labeled with phalloidin-FITC (green) and DAPI (blue)

Pharmacological treatments on *H. glaberrima* primary muscle culture

Our observations of the cultured cells suggest that muscle cell dedifferentiation is taking place in our cultures. This raised the possibility of using these cultures to study the factors that might be involved in the dedifferentiation process. As mentioned in our introduction, by searching in the scientific literature we found possible candidates' drugs that may have an effect on muscle tissue dedifferentiation. The pathways affected by these drugs have been found to play crucial roles in the process of dedifferentiation, migration, proliferation and/or apoptosis, each part of the process of regeneration. In this section, we focus on the results obtained from manipulating these pathways.

Manipulations of the pathways consisted of adding pharmacological activators and inhibitor to the cell's culture medium, exposing cells for 72 hours and fixing them to conduct our quantitative analyses. These analyses included: The number of nuclei/area, number of muscle fibers/area, number of nucleated fibers/area, number of SLS's/area, where the area was the field of view (FOV) and analyses were done determining the percentages of muscle fibers containing a nucleus, the ratio SLS's to nuclei, and others. Thus, we expected to determine if a pharmacological agent was involved in possible dedifferentiation processes. In few additional experiments we assessed the effect of the drugs by adding it to the dissociation media for 6 hours, prior to culturing the cells.

Initially we focused on the Wnt pathway, since the GSK3 component of this pathway has been shown to play a role on the dedifferentiation process of the mesenterial muscle (Bello *et al.*, 2019). Thus, the use of GSK3 modulating drugs could serve as a case study for studying the modulation of muscle dedifferentiation.

Wnt/GSK3 pathway- As previously stated by Bello S. and colleagues (2019), putative pharmacological agents of the *Wnt* pathway, LiCl (20,000µM) and iCRT14 (50µM), can be used to elucidate the role of *Wnt/GSK3* as a cellular mechanism for the process of dedifferentiation. Thus, we cultured our disassociated longitudinal muscle bands with the pharmacological agents at the same concentrations used previously for intestinal regeneration studies by Bello and colleagues (2019). Administration of a putative activator for Wnt signaling pathway increased dedifferentiation markers in our cultures. In cultures treated with LiCl we can clearly see an induction of events associated with the dedifferentiation process such as an increase in the presence of SLS's and in the number of dedifferentiating fibers (Figure 19A-B). When iCRT14-

treated cultures were observed and compared for qualitative purposes, we saw a decrease of the dedifferentiation process when compared with LiCl (Figure 19). As seen in figure 20A, no differences in the percentages of fibers can be observed for any of the treatment and control groups. Quantitative analyses reaffirmed our previous observation, there's a significant increase of about 200% in the formation of dedifferentiating fibers when we applied LiCl to cell cultures (Figure 20B). Although a reduction in the number of dedifferentiating fibers was observed, no significant difference was obtained between the Wnt inhibitor and the control, leading us to believe that inhibition of Wnt signaling decreases the induction of dedifferentiation in muscle fibers (Figure 20B). There was no difference in the number of SLS's between the treatments and the vehicle (Figure 20C).

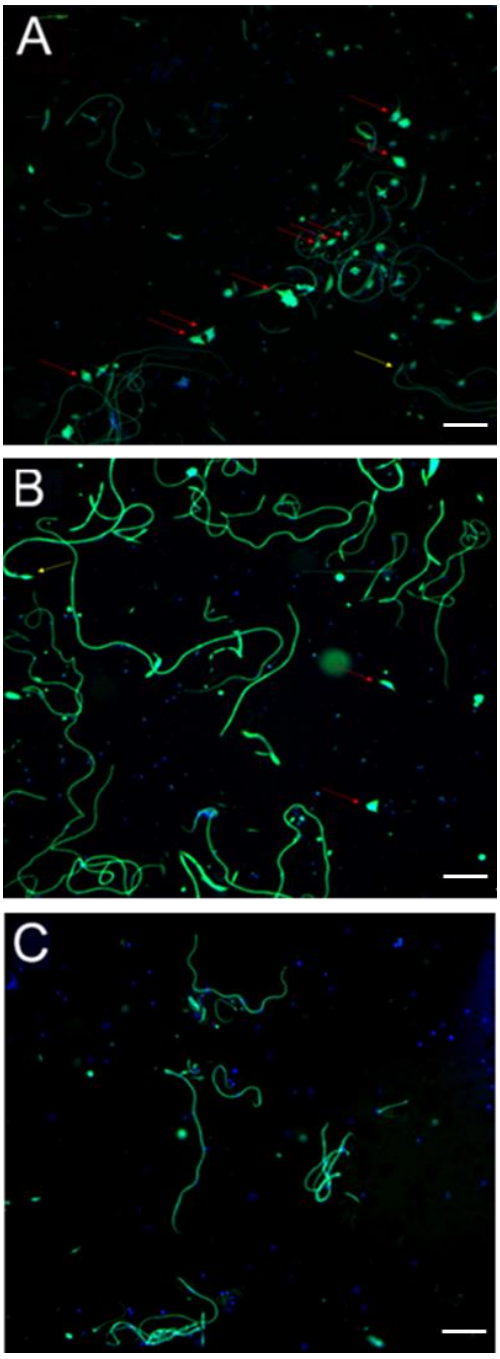


Figure 19 *In vitro visualization of phalloidin-labeled myocytes after pharmacological treatments for Wnt signaling.* **A.** Putative activator of Wnt signaling pathway, LiCl, led to higher presence of SLS's (red arrows) and dedifferentiating fibers (yellow arrows) when compared to **B.** the inhibitory effects of iCRT14 on Wnt signaling. **C.** Staining cultures that have been treated with the vehicle showed little to no structures associated with the process of dedifferentiation. Difference on the number of dedifferentiating fibers were more prominent when statistically analyzed. Cultures were labeled with phalloidin-FITC (green) and DAPI (blue). Scale bar = 1µM **(A)** and 10µM **(B, C)**.

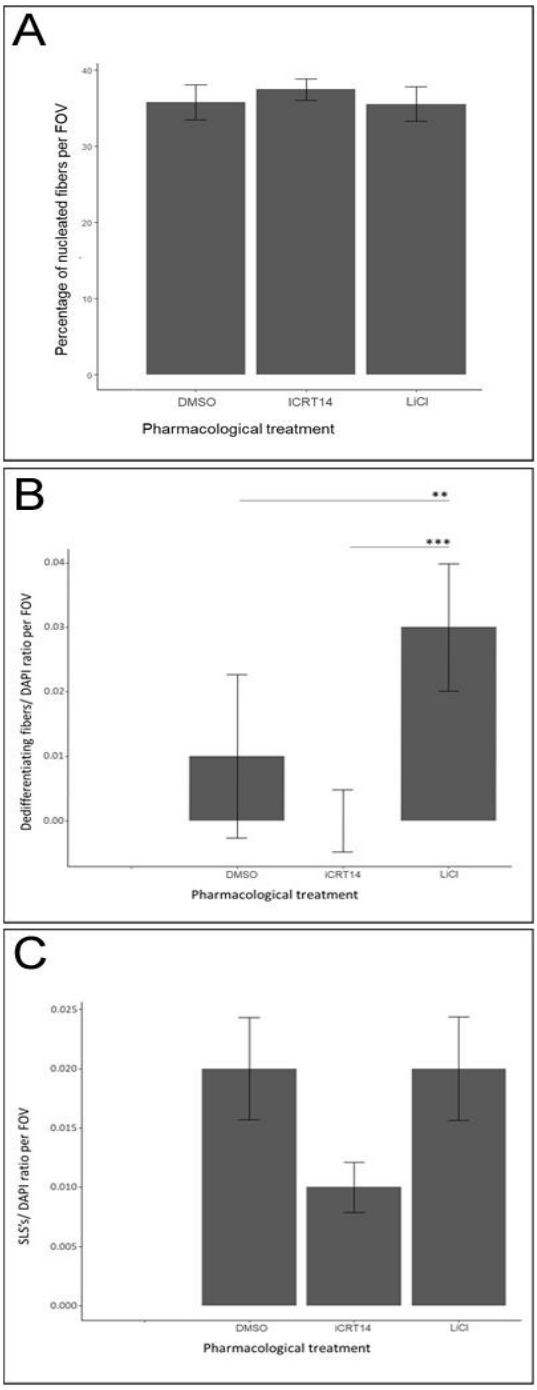

A
Percentage of nucleated fibers per FOV
40
30
20
10
0
DMSO
ICRT14
LiCl
Pharmacological treatment
B
**
***
Dedifferentiating fibers/ DAPI ratio per FOV
0.04
0.03
0.02
0.01
0.00
DMSO
iCRT14
LiCl
Pharmacological treatment
C
SLS's/ DAPI ratio per FOV
0.025
0.020
0.015
0.010
0.005
0.000
DMSO
iCRT14
LiCl
Pharmacological treatment

Figure 20 *In vitro trends in cultures treated for Wnt/GSK3 signaling.* **A.** No difference was found in the percentages of nucleated fibers present in our cultures **B.** Statistical increase was found in the number of dedifferentiating fibers for LiCl, when comparing with iCRT14 and the vehicle **C.** The presence of SLS's was not significantly increased, although a trend can be seen forming in cultures with LiCl. Approximately, 10^6 cells were seeded per well. Field of view from 20X magnification. Bars are Mean ± S. E. N= 8. *P < 0.05, **P < 0.01, ***P < 0.001.

To further test the effects of activating or inhibiting Wnt-associated pathways, we performed another experiment where we added the pharmacological agents in the dissociation media and were removed prior to culturing the cells. The rationale for this change is that the disassociation process may be exerting cellular stress, as it has been seen previously (Selvin D. et al, 2015). First observations on phalloidin- stained cultures showed a higher increased in muscle tissue debris (Figure 21). No significant value was observed for either of the markers used to determine dedifferentiation. It is worth noting that SLS's/cell ratio followed the same trend seen as in our previous experiments using Wnt/GSK3 modulators (Figure 22). The expected trend for dedifferentiating fibers, which had significant differences between inhibitor and activator was not seen in these experiments (Data not shown). Due to the increase in cellular debris, as well as the decrease of muscle fibers, this methodology was excluded from our experiments.

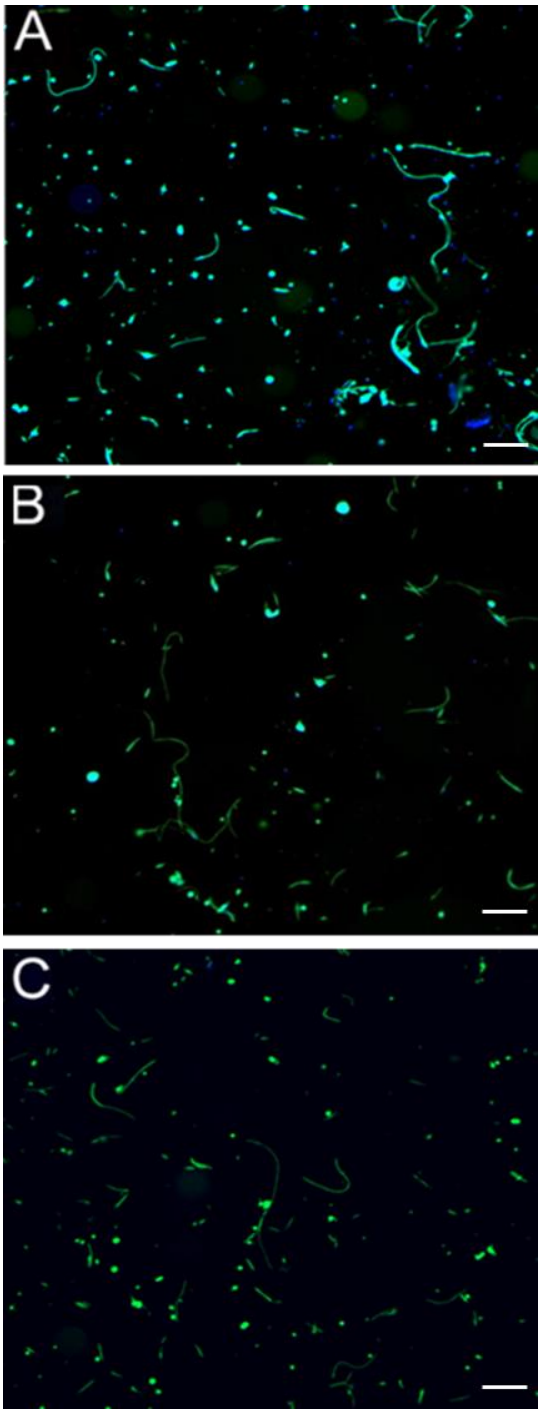


Figure 21 *In vitro visualization of phalloidin labeled myocytes after pharmacological treatments for Wnt signaling are added to collagenase type 1A treatment prior to seeding.* Both pharmacological treatments, **A.** iCRT14 and **B.** LiCl, yielded a high degree of cellular debris when stained with phalloidin. **C.** Similar results were obtained when the vehicle was added. Long fibers were observed for treatments and control, but fragments from these fibers were mostly present in our cultures. Cultures were labeled with phalloidin-FITC (green) and DAPI (blue). Scale bar = 10µM.

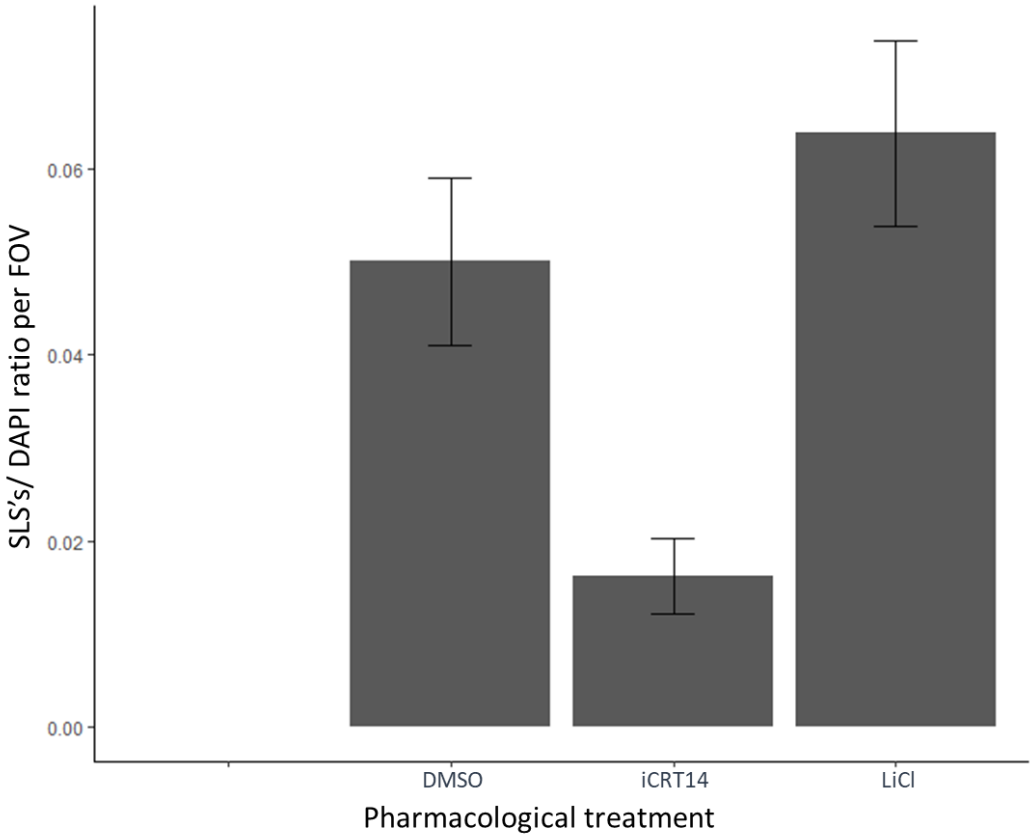


Figure 22 *In vitro trends in SLS's/ DAPI ratio increase during Wnt pathway activation.* Pharmacological induction of Wnt signaling pathway leads to an increase in the presence of SLS's per FOV in cells treated with LiCl during dissociation protocol. Inhibition of Wnt signaling pathway through iCRT14 diminished the presence of SLS's/ FOV ratio to levels compared with the control group. Approximately, 10^6 were seeded. Field of view from 20X magnification. Bars are Mean ± S. E. N= 8.

ROS pathway- Assessing the involvement of ROS- activated pathways included the addition of an exogenous source of ROS, H_2O_2 (2µM), and flavoenzyme inhibitors, DPI (25µM) and Vas2870 (25µM). Qualitative analyses showed less dedifferentiation markers on cultures treated with hydrogen peroxide when compared to those treated with inhibitors, as shown in figure 23. We proceeded to calculate the percentages of muscle fibers presenting a nucleus. No significant difference was obtained between treatments and controls, with results ranging from 32% to

39% (Figure 24A). We followed by quantifying the ratio of the dedifferentiation markers with the number of nuclei per FOV. Our initial observation was asserted by quantifiable assessment, with a significant increase of 455% in the number of SLS's/cell ratio per FOV in cultures treated with DPI, when compared with control and hydrogen peroxide treatment, as shown in figure 24C. Another interesting finding was the similarity between the control vs. hydrogen peroxide treatment. These two showed almost the same average number of SLS's/cell per FOV, with no significant difference, leading us to conclude that hydrogen peroxide may be inhibiting cells from undergoing the dedifferentiation process. We also looked for the number of dedifferentiating fibers/cell ratios, and although we didn't find any significant difference, the trend for each treatment seemed to indicate similar to the SLS's/cell ratio findings; inhibition of the proteins that induced the released of ROS in cells lead to induction of dedifferentiation, while adding exogenous ROS to cell culture inhibited this process (Figure 24B).

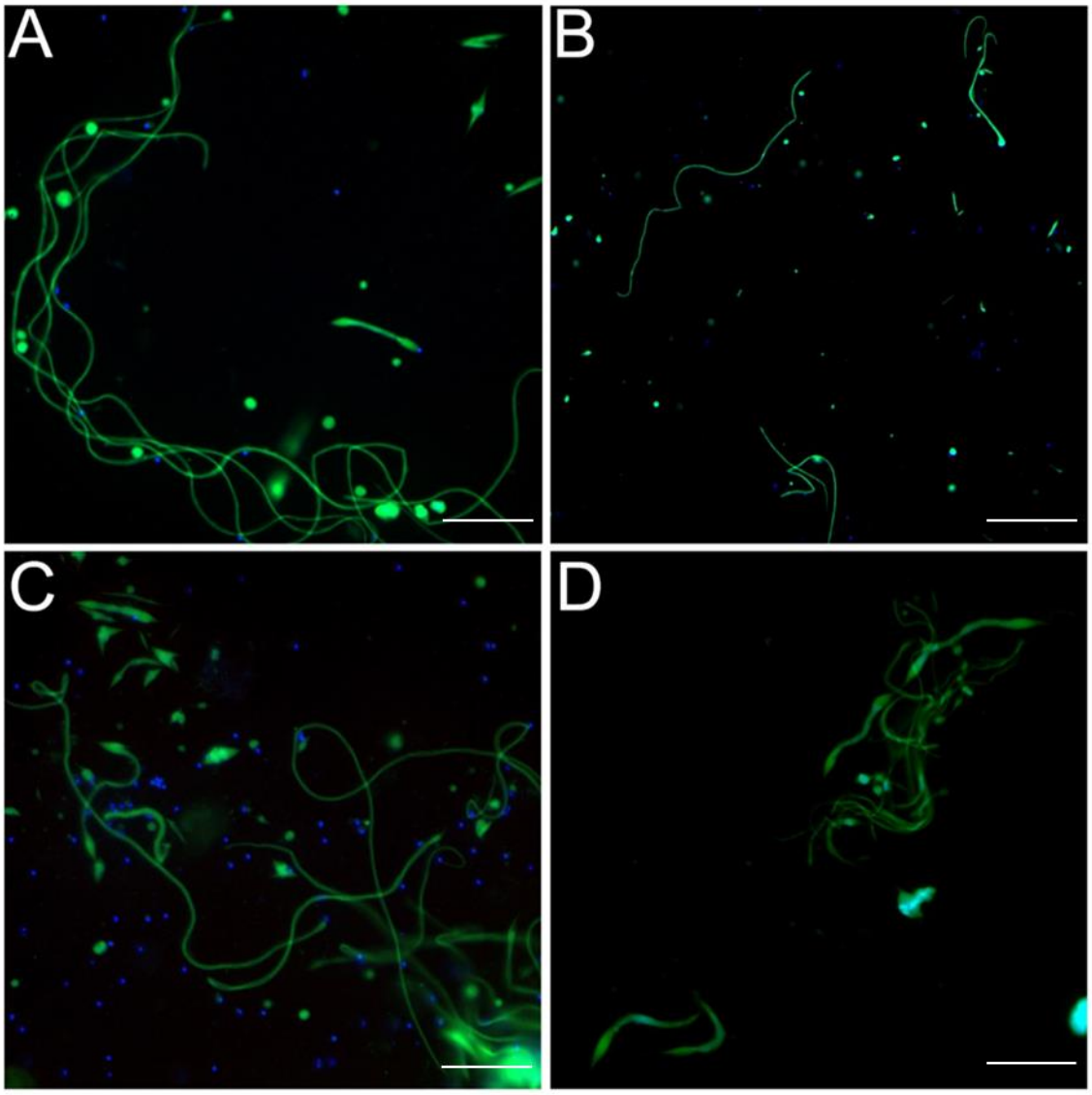


Figure 23 *Phalloidin staining of cultures treated with pharmacological agents that target ROS pathway.* Qualitative comparison of the dedifferentiating fibers and SLS's of three different treatments for inhibiting or stimulating ROS pathway. **A.** Vehicle and **B.** hydrogen peroxide treatment produced cellular cultures with low levels of structures associated with dedifferentiation. **C.** Cultures with DPI and **D.** VAS- 2870 presented an increased number in SLS's and dedifferentiating fibers. Cultures were labeled with phalloidin-FITC (green) and DAPI (blue). Scale bar = 1µM **(B)** and 10µM **(A,**

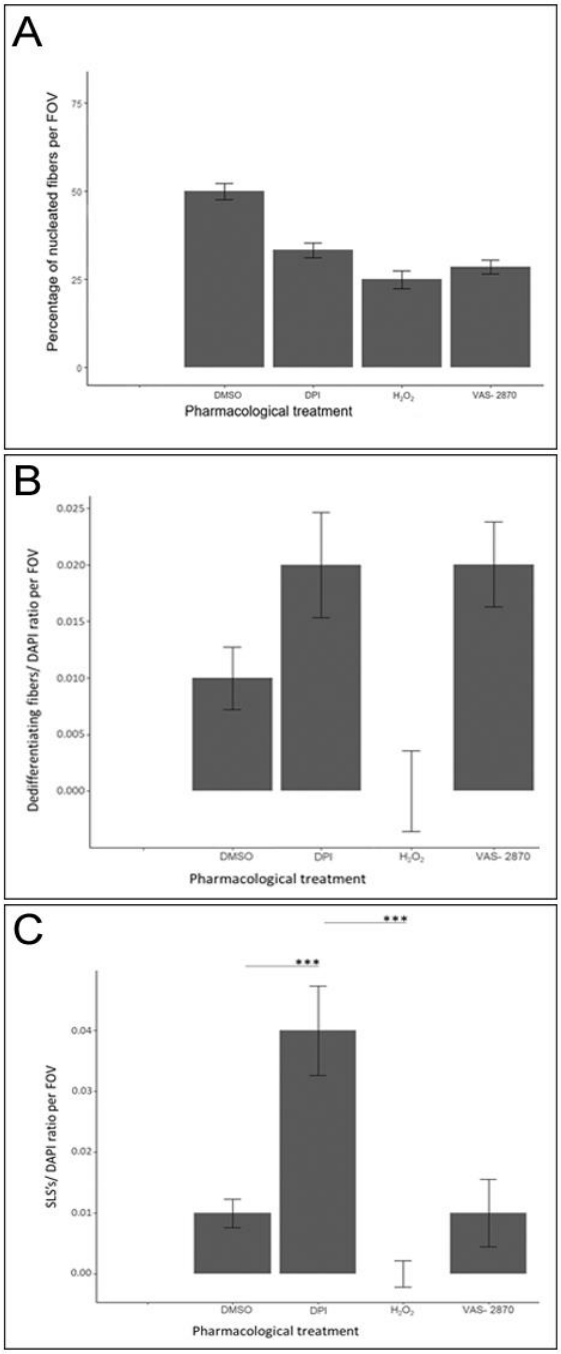

A
Percentage of nucleated fibers per FOV
75
50
25
0
DMSO
DPI
H_2O_2
VAS- 2870
Pharmacological treatment
B
Dedifferentiating fibers/ DAPI ratio per FOV
0.025
0.020
0.015
0.010
0.005
0.000
DMSO
DPI
H_2O_2
VAS- 2870
Pharmacological treatment
C
***
***
SLS's/ DAPI ratio per FOV
0.04
0.03
0.02
0.01
0.00
DMSO
DPI
H_2O_2
VAS- 2870
Pharmacological treatment

Figure 24 *In vitro trends in cultures treated for ROS signaling.* **A**. No difference was obtained in the percentages of nucleated fibers with the treatments for ROS signaling **B.** An increase in dedifferentiating fibers can be observed between ROS production inhibitors when compared to hydrogen peroxide and control group, but no significant value was obtained. **C.** A significant increase was calculated for the presence of SLS's upon inhibition of ROS production by DPI when compared with control and hydrogen peroxide group Approximately, 10^6 cells were seeded per well. Field of view from 20X magnification. Bars are Mean ± S. E. N= 8. *P < 0.05, **P < 0.01, ***P < 0.001.

We proceeded to test the effects of the pharmacological agent when added to the enzyme disassociation media, prior to culturing. As with the previous experiments where we added Wnt/GSK3 modulating drugs to the enzyme dissociation media, extensive degree of cellular debris was observed in our cultures (Figure 25A). Similar to the experiments where the pharmacological agent was added to the culturing medium, quantitative analyses showed a significant difference between DPI and hydrogen peroxide in terms of SLS's/ cell ratio as shown in figure 25C. The structure for dedifferentiating fibers had no significant value. The trend for hydrogen peroxide is an increase in dedifferentiating fibers, while the inhibitor decreases the number of dedifferentiating structures. Also, there's a difference between both inhibitors, with DPI decreasing the number of dedifferentiating fibers while VAS2870 increases the presence of these structure (Figure 25B). Since adding the pharmacological agent to the enzyme dissociation media seemed to induce more cellular stress than adding it to the culturing media, we decided to refrain from using this experiment in further analysis of other pharmacological agents.

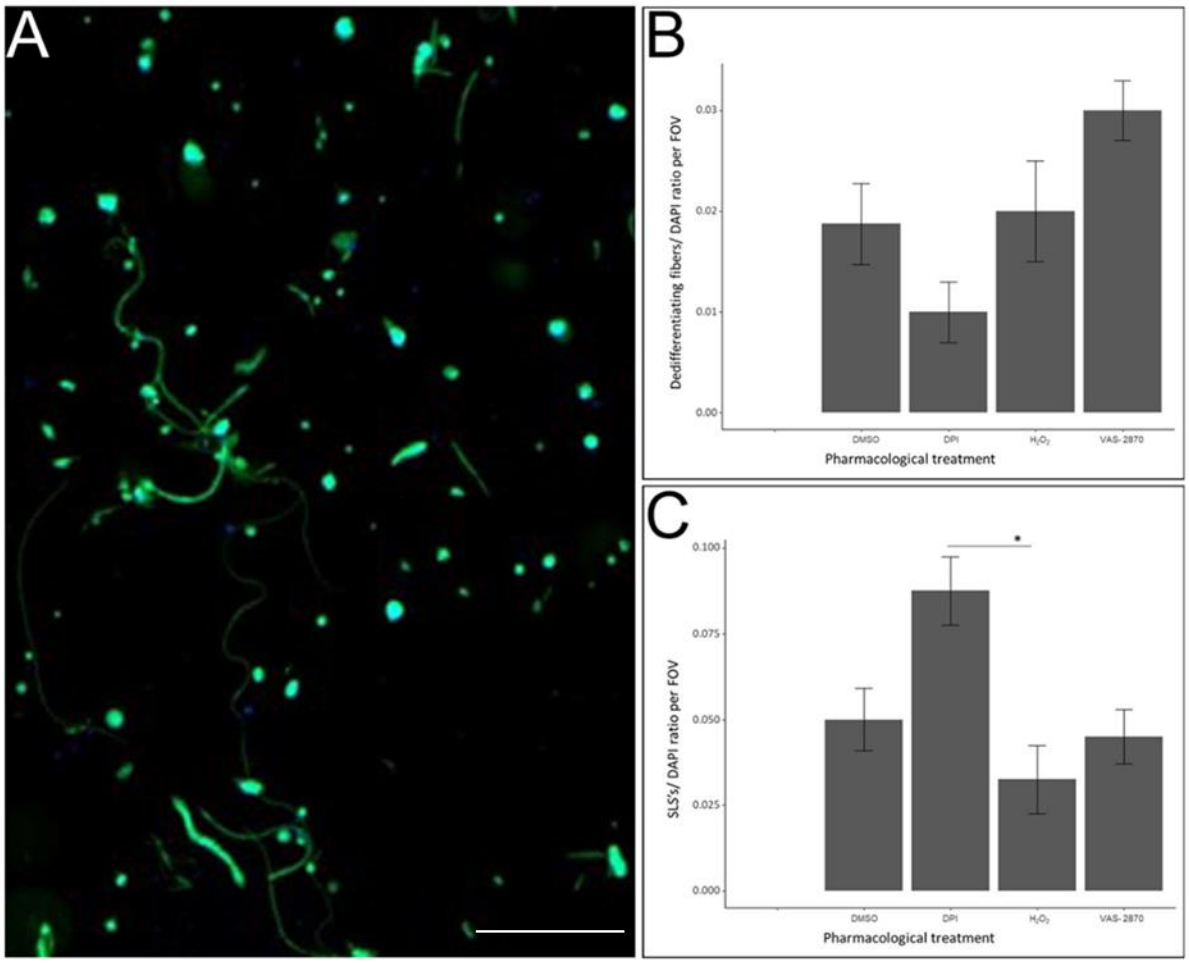


Figure 25 *In vitro trends in cultures treated for ROS signaling during dissociation protocol*. **A**. Extensive levels of cellular debris were found in culture that were treated with pharmacological agents pertaining to ROS signaling. We saw a reduced presence of muscle fibers and structures associated with dedifferentiation **B.** Previous trends for the increase of dedifferentiating fibers were reversed. We can see an increase of dedifferentiating fibers when exogenous sources of ROS were applied and contrasting results between the two inhibitors of ROS production **C.** Similar results were obtained in the presence of SLS's, but an increase in SLS's per cell was observed in both control and hydrogen peroxide treated cells when compared with our previous pharmacological assay protocol. Approximately, 10^6 cells were seeded per well. Field of view from 20X magnification. Bars are Mean ± S. E. N= 8. *P < 0.05, **P < 0.01, ***P < 0.001. Cultures were labeled with phalloidin-FITC (green) and DAPI (blue). Scale bar = 10µM.

Calcium pathway- Since calcium ions were already present in our culture media, elucidating the effects of calcium ions in our cultures consisted of a mobile ion-carrier, Calcium Ionophore

A23187, and a chelating agent, EGTA. We applied concentrations of 0.07mM, 1mM and 10mM for EGTA and 0.01µM, 0.05µM and 10µM for the ionophore. No difference was found in the percentages of nucleated muscle fiber in any of the treatments for calcium (26A; 28A). A trend in the increasing number of dedifferentiating fibers was observed for EGTA in each concentration, but no significance was obtained from this analysis (Figure 26B). A dose-dependent increase in SLS's can be observed in cultures treated with EGTA (Figure 26C). The 0.07mM concentration of EGTA didn't increase SLS's when compared to the vehicle. We observed an increase of 250% in SLS's in cultures treated with 1mM of EGTA, but it wasn't significant. We only obtained a significant change in SLS's for 10mM of EGTA, with an increase of 450% when compared with the control. With 10µM of the ionophore we found extensive levels of debris as well as fibers with protuberances coming from the axis of the fiber on our cultures (Figure 27A). We lowered the concentration to 0.05µM and obtained similar fibers to those found at 10µM (Figure 27B). With this concentration we observed an increased in the ratio of dedifferentiating fibers, but no significance was obtained (Figure 28B). It's worth mentioning that we couldn't see an increase in SLS's using this concentration of the ionophore. With 0.01µM of the ionophore we managed to produce healthy looking muscle fibers (Figure 27C). Except for 0.05µM of the ionophore, which produced an increase in dedifferentiating fibers, we didn't observe an increase in either the ratio of dedifferentiating fibers or of SLS's (Figure 28C).

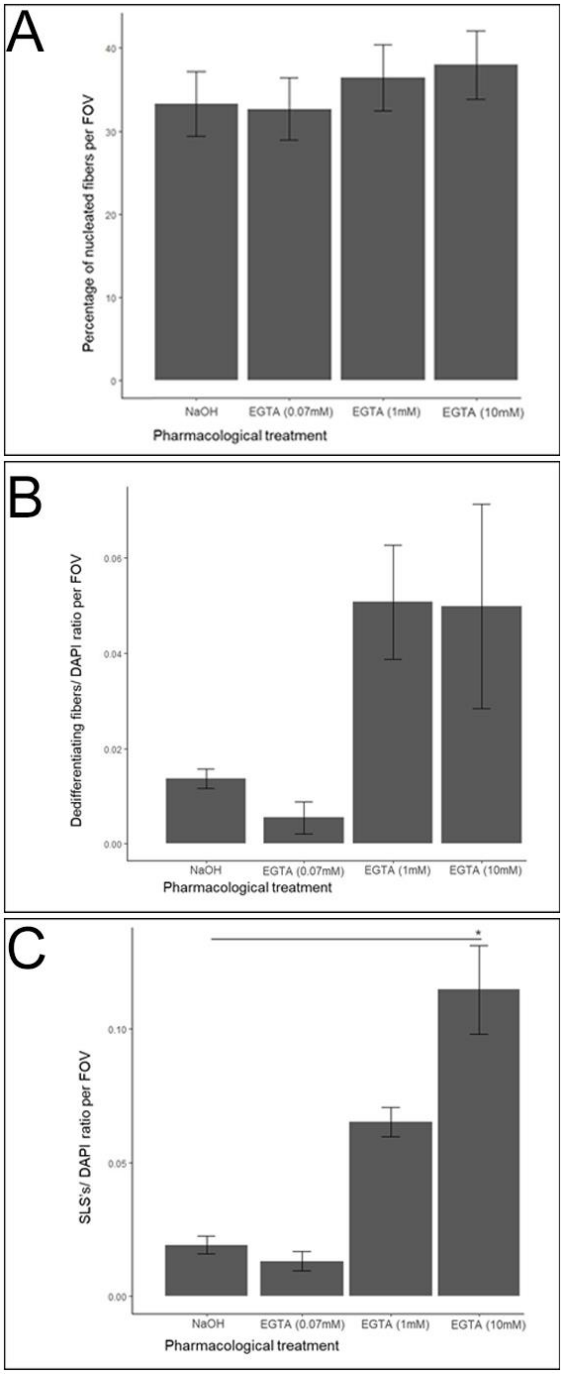


Figure 26 *In vitro trends in cultures treated with a Ca^{2+} chelator.* **A.** No difference in the percentages of nucleated fibers were obtained **B.** An increase in dedifferentiating fibers can be observed for 1mM and 10mM for the cultures treated with EGTA **C.** A dose-dependent gradual increase was obtained for the three concentrations of the chelator. Only a significance was obtained for the higher concentration. This increase was reverted at 0.07mM of the pharmacological agent. Approximately, 10^6 cells were seeded per well. Field of view from 20X magnification. Bars are Mean ± S. E. N= 8. *P < 0.05, **P < 0.01, ***P < 0.001.

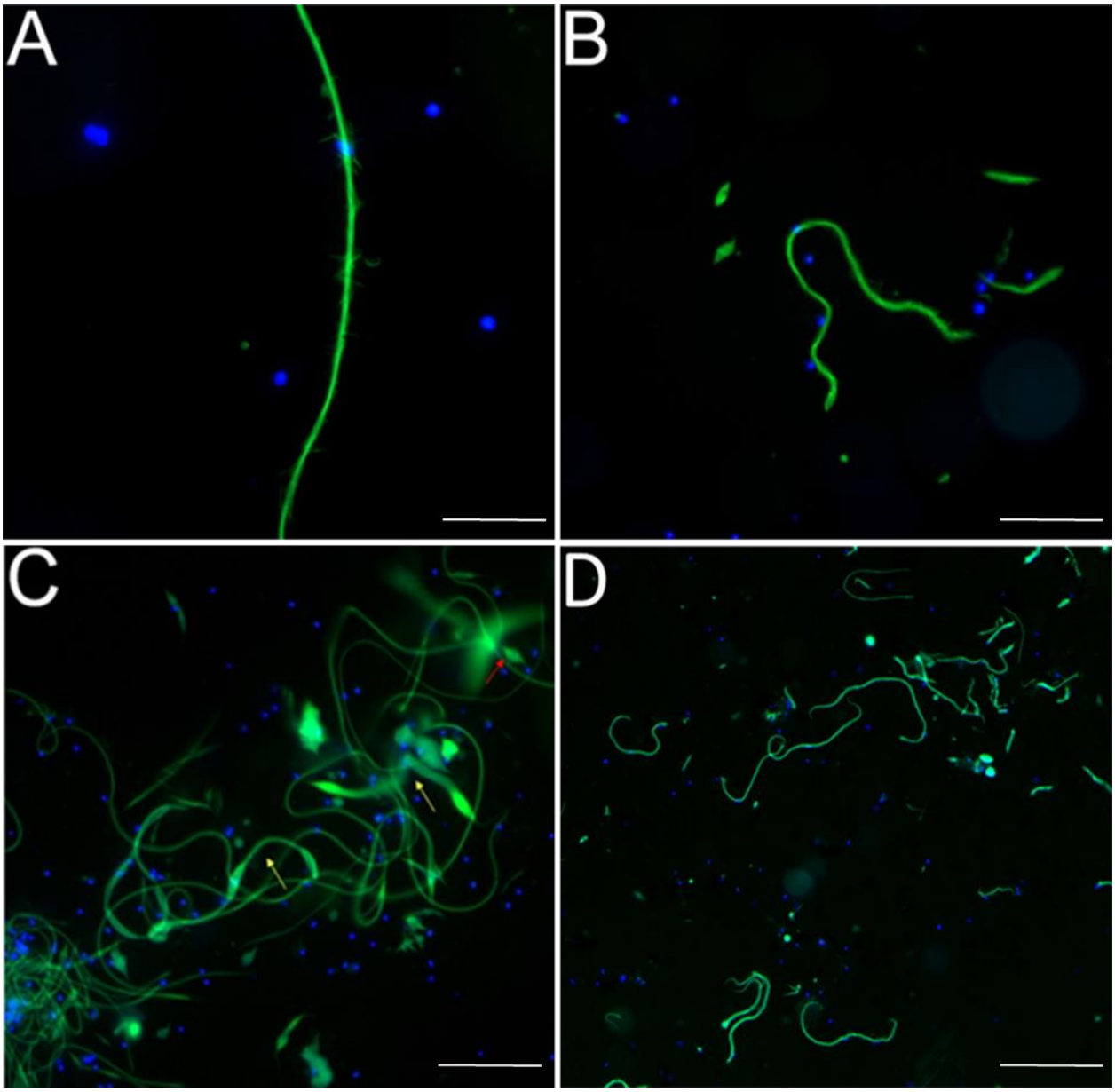


Figure 27 *Cellular damage is seen when increasing Ca^{2+} concentration using the ion-carrier Calcium Ionophore A23187* **A**. Cellular damage was observed in muscle fibers at 10µM of the ionophore. Damage was characterized by the appearance of protuberances along muscle fiber's axis **B.** Lowering the concentration to 1µM also produced damaged muscle fibers, and we can see an increase in the presence of SLS's **C.** Healthy looking fibers were produced at the lowest concentration of the ionophore, when compared with **D.** the vehicle. In the field of view, it was also observed dedifferentiating fibers. Approximately, 10^6 cells were seeded per well. Cultures were labeled with phalloidin-FITC (green) and DAPI (blue). Scale bar = 50µM **(A, B, C)** and 10µm **(D)**.

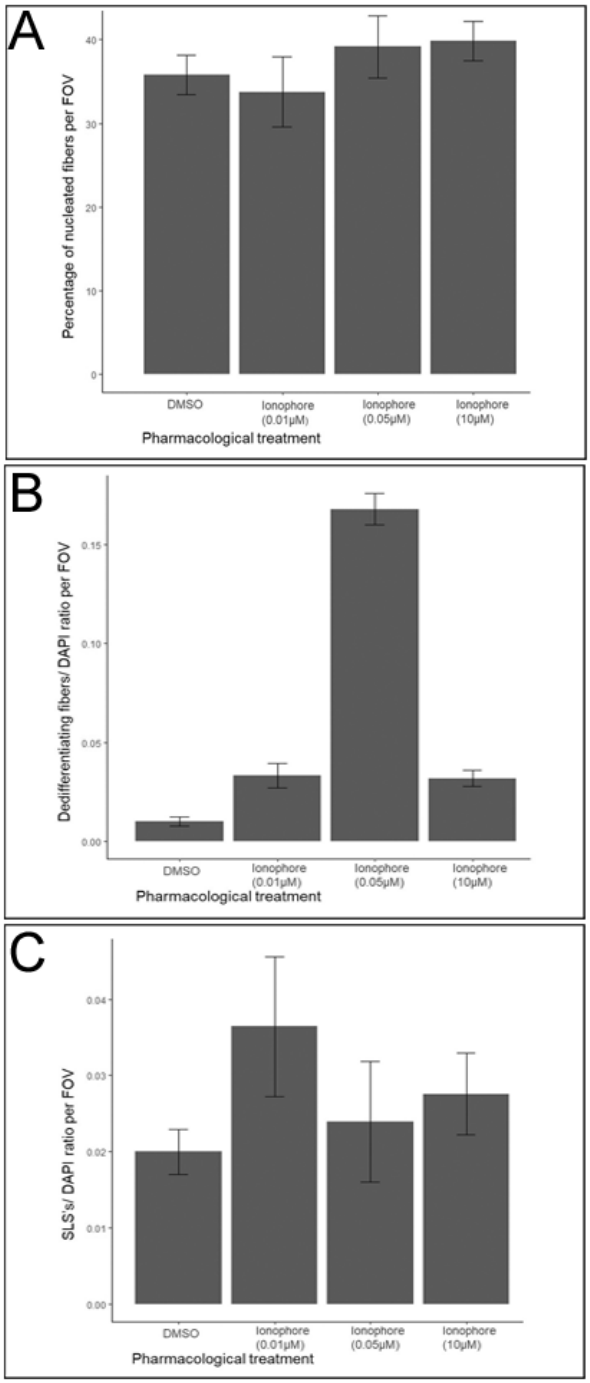


Figure 28 *In vitro trends in cultures treated with a Ca^{2+} ion- carrier.* **A.** No difference in the percentages of nucleated fibers was obtained **B.** An increase in dedifferentiating fibers could only be observed at 1µM, with no significant value. Other concentration presented results like the control group. **C.** Variable results were obtained for the presence of SLS's, with a visible increase at the lowest concentration. No significant value was obtained. Approximately, 10^6 cells were seeded per well. Field of view from 20X magnification. Bars are Mean ± S. E. N= 8. *P < 0.05, **P < 0.01, ***P <

PI3K pathway- Kinase activity has been found to regulate processes of proliferation, cell growth, cell survival and migration. They have also been found to be upregulated through developmental and regeneration processes. To study the possible effects of protein kinases in our culture, we used the pharmacological inhibitor LY 294,002 hydrochloride (1mM), a kinase inhibitor of PI3K that also acts on other kinases like mTOR, CK2, PLK1, PIM1 and PIM3. We sought to analyze the effects of this pharmacological agent on the percentage of nucleated muscle fibers and the ratio from dedifferentiating fibers and SLS's per cell. The percentage of muscle fibers presenting a nucleus didn't change between cultures treated with the drug or the vehicle (Figure 29A). Dedifferentiating fibers increased while SLS's decreased (Figure 29B-C). Neither of these values were significant.

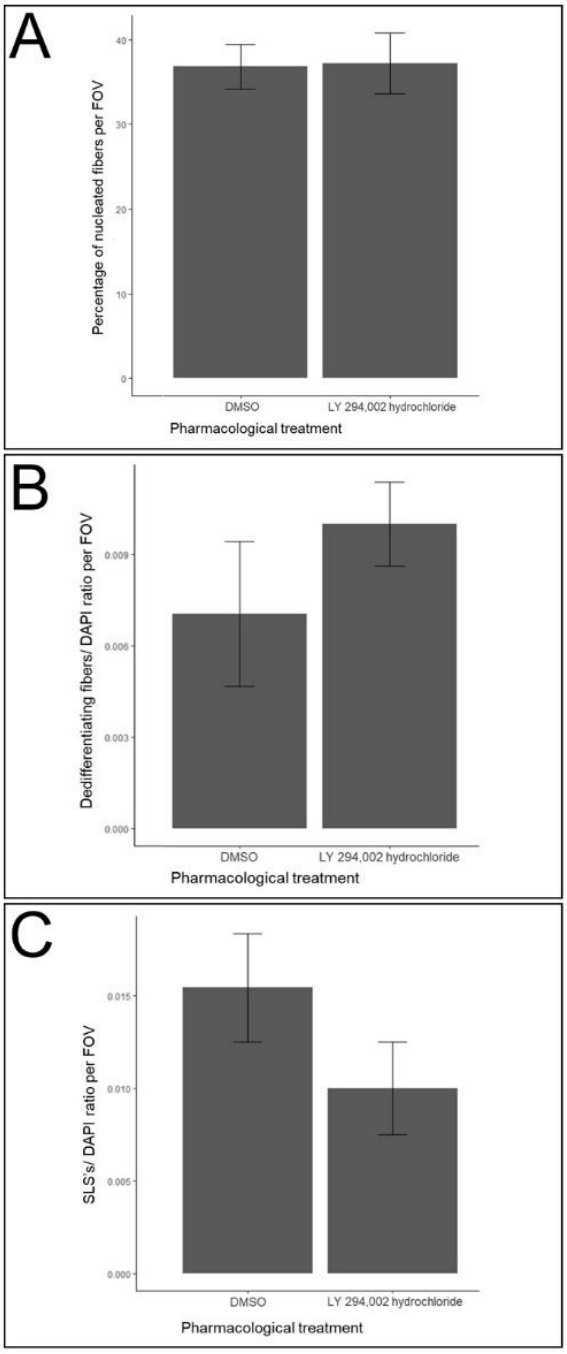


Figure 29 *In vitro trends in cultures treated with a PI3K inhibitor*. **A.** No difference in the percentages of nucleated fibers was obtained **B.** No difference could be obtained in the number of dedifferentiation fibers **C.** or SLS's per cell. Approximately, 10^6 cells were seeded per well. Field of view from 20X magnification. Bars are Mean ± S. E. N= 8. *P < 0.05, **P < 0.01, ***P < 0.001.

BMP-1- We sought to investigate the effects that the inhibitor for BMP-1, UK-383,367, might have on our cell cultures and in the process of dedifferentiation. Observations into the state of each culture showed a slight increase in the number of SLS's (Figure 30). In terms of the percentages of nucleated fibers, no statistical difference was observed when we compared the cultures treated with the vehicle and those treated with each of the agent's concentration (Figure 31A). A trend can be observed in the number of fibers in the process of dedifferentiation as compared with its vehicle, but no statistical significance was found (Figure 31B). Lastly, we found an increase of 350% in SLS's with 5µM of the pharmacological agent and proceeded to increase the concentration to deduce if the inhibitor worked in a dose- dependent manner (Figure 31C). All three concentrations clearly presented a higher number of SLS's, with 10µM increasing the ratio of SLS's by 450% and 30µM to 300% (Figure 30). No signs of cellular stress were observed in the first two concentration of UK-383,367, 5µM and 10µM (Figure 30A-B). Extensive levels of cellular debris were found on 30µM of the pharmacological agent (Figure 30C). One- way ANOVA analysis showed a marked significance for each of the concentration.

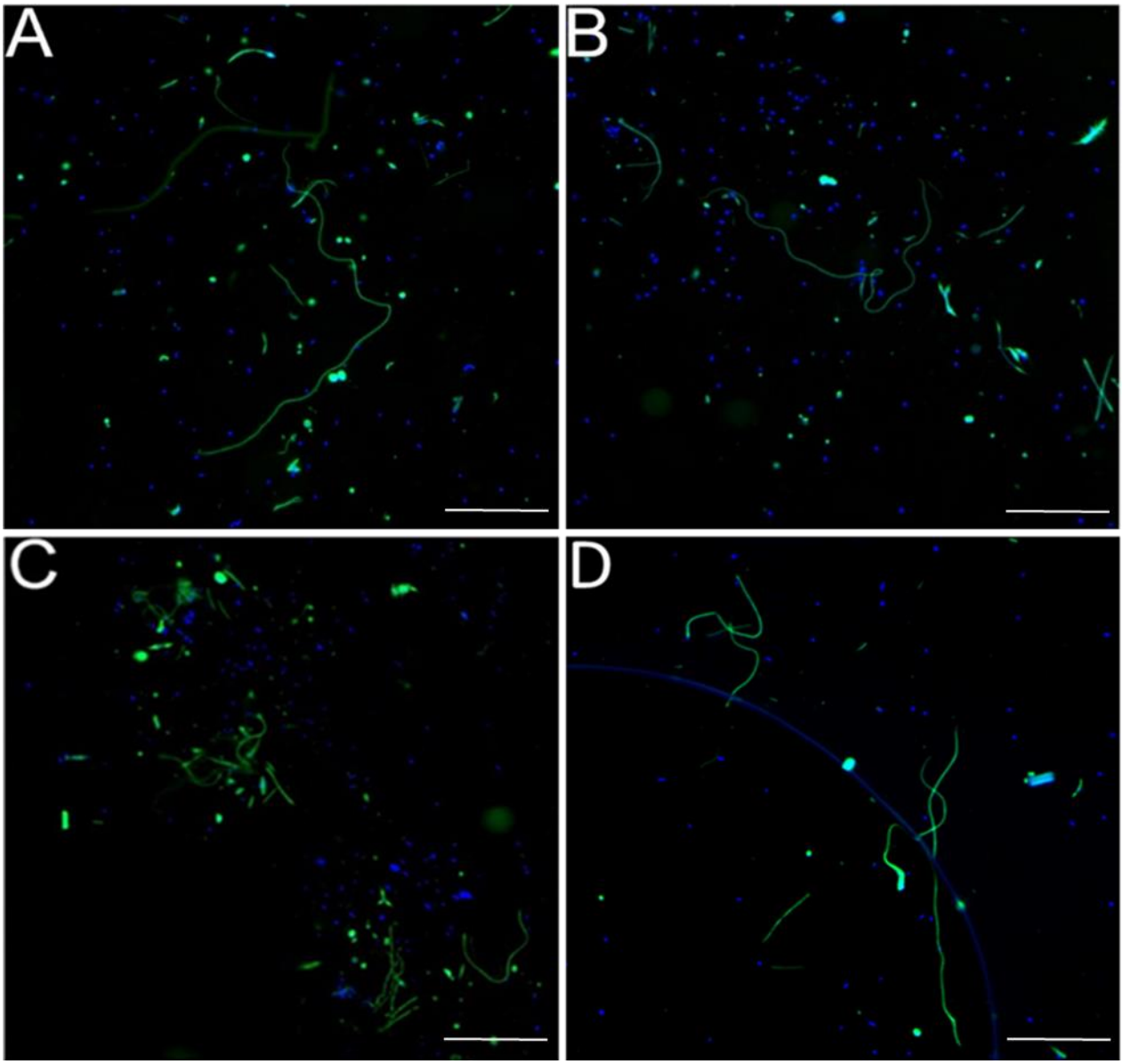


Figure 30 *In vitro phalloidin-stained muscle cell cultures treated with a BMP-1 inhibitor*. **A.** An increase in the presence of structures associated with dedifferentiation can be observed at the lowest concentration, 5µM, of UK 383,367 **B.** Dedifferentiating fibers and SLS's tended to increase in the field of view at 10µM of UK 383,367 **C**. At 30µM, the highest concentration of the BMP-1 inhibitor, muscle fibers presented a discontinuous structure associated with cellular stress. **D.** Vehicle produced muscle fibers that resemble does found at the lowest concentration of the pharmacological agent. Approximately, 10^6 cells were seeded per well. Cultures were labeled with phalloidin-FITC (green) and DAPI (blue). Scale bar = 10µM.

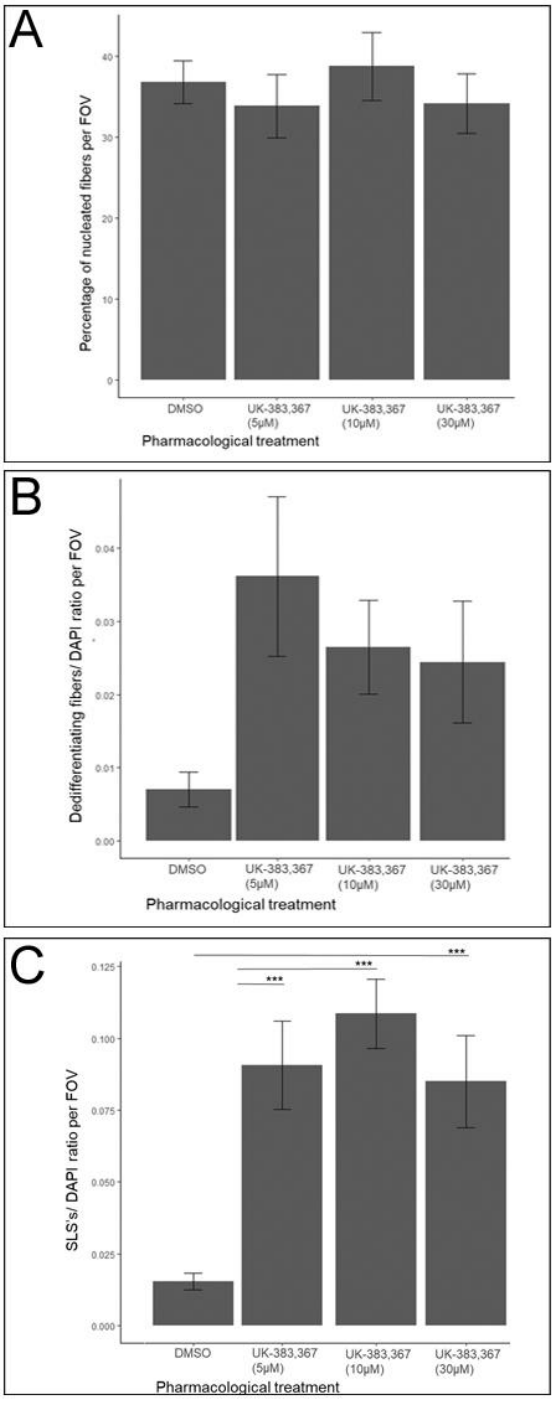

A
Percentage of nucleated fibers per FOV
0
10
20
30
40
DMSO
UK-383,367 (5μM)
UK-383,367 (10μM)
UK-383,367 (30μM)
Pharmacological treatment
B
Dedifferentiating fibers/ DAPI ratio per FOV
0.00
0.01
0.02
0.03
0.04
DMSO
UK-383,367 (5μM)
UK-383,367 (10μM)
UK-383,367 (30μM)
Pharmacological treatment
C
SLSs/ DAPI ratio per FOV
0.000
0.025
0.050
0.075
0.100
0.125
***
***
***
DMSO
UK-383,367 (5μM)
UK-383,367 (10μM)
UK-383,367 (30μM)
Pharmacological treatment

Figure 31 *In vitro trends in cultures treated with a BMP-1 inhibitor.* **A.** No difference in the percentages of nucleated fibers was obtained **B.** A trend for the increase in the presence of dedifferentiating fibers was obtained, without a significance **C.** A significant increase was observed for the presence of SLS's. The presented increase seemed to be dose-dependent, except for the concentration of 30µM, where the fibers presented cellular stress. Approximately, 10^6 cells were seeded per well. Field of view from 20X magnification. Bars are Mean ± S. E. N= 8. *P < 0.05, **P <

Autophagy pathway- Autophagy can be a useful cellular mechanism for the expulsion and degradation of the contractile apparatus (Salabei J. *et al.*, 2013). We proceeded to investigate the possible role of autophagy in the process of dedifferentiation in our cell cultures. For this, we used Chloroquine diphosphate (1mM), a classical lysomotropic agent known to inhibit autophagy. As with other treatments, we didn't see any significant difference in the percentages of muscle fibers containing a nucleus (Figure 32A). The number of dedifferentiating fibers seemed to increase when comparing the cultures treated with the drug with those treated with the vehicle but didn't show a significant difference (Figure 32B). As with dedifferentiating fibers, an increase can be observed in the number of SLS's present in our cultures (Figure 32C). We can almost see twice the ratio SLS's/cells when we compared the experimental group with our control group, but the effect was not significant.

SIGNALING PATHWAY	DRUG	AVERAGE NUMBER OF FIBERS	PERCENTAGE OF FIBERS PRESENTING A NUCLEI	FIBERS IN THE PROCESS OF DEDIFFERENTIATION/ NUCLEI RATIO	SPINDLE- LIKE STRUCTURES (SLS'S)/ NUCLEI RATIO

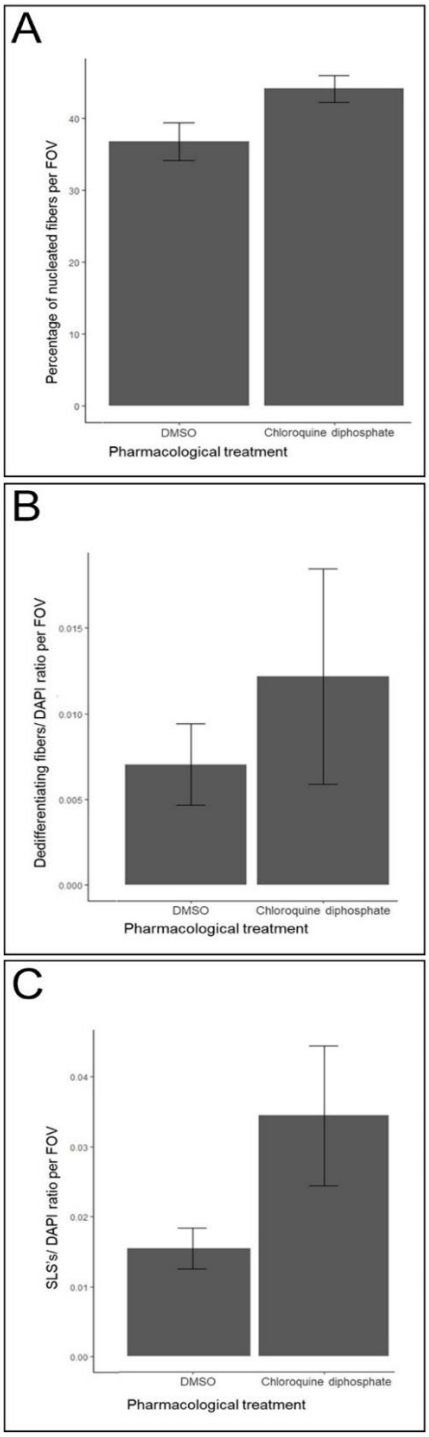


Figure 32 *In vitro trends in cultures treated with an inhibitor for autophagy*. **A.** No difference in the percentages of nucleated fibers was obtained **B.** A slight increase can be observed for the number of dedifferentiating fibers and **C.** SLS's without a statistical significance. Approximately, 10^6 cells were seeded per well. Field of view from 20X magnification. Bars are Mean ± S. E. N= 8. *P < 0.05, **P < 0.01, ***P < 0.001.

WNT/B-CATENIN AND GSK-3 PATHWAYS					
	Lithium Chloride (LiCl)	⌄	›	↑	›
	iCRT14				
ROS PATHWAY					
	Hydrogen peroxide (H_2O_2)				
	DPI	⌄	›	^	↑
	VAS2870 *				
CALCIUM PATHWAY					
	Calcium Ionophore A23187				
	EGTA	⌄	›	^	↑
BMP-1 PATHWAY					
	UK-383,367	›	^	^	↑
AUTOPHAGY PATHWAY					
	Chloroquine diphosphate	^	⌄	^	^
PI3K PATHWAY					
	LY-294,002 hydrochloride	⌄	⌄	^	⌄

Table 3. Summary of the effects of pharmacological agents on dissociated muscle cultures

Means that the drug increased the ratio when compared to the control

Means that the drug didn't change the ratio when compared to the control

Means that the increased value was not significant

Means that the reduced value was not significant

Discussion

Optimization of cellular cultures techniques and identification of cellular components

In these experiments we manipulated different variables for an established culture protocol that could be used to test pharmacological agents and elucidate the processes involved in dedifferentiation. We improved upon a previous protocol that appeared to be acidifying the cultures and making a toxic environment for our cells. We tested another protocol previously used for human neural stem cell (Yang K. *et al.*, 2012) and found an increase in the number of cells that we could adhere as well as an increase in the presence of muscle cells. We were able to propose a schematic representation of the dedifferentiation process that could be followed *in vitro*. From there on, we were able to test different pharmacological drugs to determine a possible effect on the dedifferentiation process. Notable results were found for the pathways involving Wnt/GSK-3, ROS, Ca^{2+} and BMP-1. A summary of the results obtained from our experimental procedures can be found in table 3.

Adhesion protocols

Acetic acid was previously proposed as an easy to manage cost–effective method for adhering muscle cells from *H. glaberrima* muscle tissue. The rationale was that the 1% of water that acetic acid contained traces of positively charged ions that interact with the cells'

membrane, making them able to adhere to the chambers. Instances have shown how cellular cultures like fibroblasts from chicken embryonic sclera require bivalent cations to enhance attachment to negatively charged substrates (Takeichi M. & Okada T., 1972). Indeed, bivalent ions enhances membrane bound proteins to create cell- cell and cell-substrate linking (Alberts B. *et al.*, 2002). Takeichi M. & Okada T. research showed that in the presence of these ions, adherence is enhanced even when pH was in an acidic or alkaline range. It is well known that acidic conditions might affect cellular interactions such as proliferation and apoptosis (Mikos A. *et al.*, 1998). Extracellular pH can also influence NO and hydroxyl radical (OH) production, and the induction of oxidative stress has been shown to manipulate fate switches in cells and induce the process of a degradation pathway in cardiomyocytes from atrial myocardial tissue (Shu Z. *et al.*, 1997; Sovboda L. *et al.*, 2012).

We opted to try a different adherence protocol known for the polymerization of dopamine into polydopamine. Dopamine, a compound of the catecholamine family, consist of both 3,4-dihydroxy-L-phenylalanine (DOPA) and lysine amino acids, both structures known to play a crucial role in the adhesion of mussels to a variety of surfaces. The catechol is known to react and induce adhesion through cross-linking (Figure 2), and polymerization with other catechol's producing binding moieties by functionalizing organic and inorganic materials such as metals, metal oxides, silica, ceramics, and polymers. Adhesion of cells have been suggested to be induced by the chemical nature of the catechol and its interaction with cell's serum proteins (Nielsen S. *et al.*, 2013; Yang K. *et al.*, 2012). Polymerization of dopamine monomers, resulting in the generation of a polydopamine layer, is easy to accomplish by the application of alkaline conditions and the presence of oxygen in solution (Figure 5) (Deng Z. *et al.*,2021). The

treatment of materials with polydopamine (pDA) has already paved way for stem cell technology, permitting the adherence of, for example, human neural stem cells (NSC), and other types of cells (Nielsen S. *et al.*, 2013) but its application on primary cell culture of echinoderm tissue for the study of regenerative processes has yet to be explored. This technique creates opportunities for pharmacological studies that require the adhesion of cells in cultures. Our results suggest a significant increase of almost 2- fold in the number of fibers, showing that this method is more efficient at adhering our muscle fibers as compared with acetic acid adherence protocol. Cellular adhesion seems to be time dependent. The number of cells and muscle fibers increased gradually withing the first three days of culture. Although the increase could be due to an increase in the rate of proliferation, we do not believe this to be the case since PD alone doesn't enhance proliferation but does increase the number of adhere cells when compared to other methods (Yang K. *et al.*, 2012; Nielsen S. *et al.*, 2013).

Dissociation protocol

We sought to further improve our cell cultures, so they can better reflect the effects of the pharmacological agents on the dedifferentiation process. For this, we needed to maintain muscle fiber integrity by maintaining a healthy morphology in our muscle fibers and reducing the levels of dedifferentiation that might result from the dissection or dissociation protocol. We used artificial sea water without magnesium or calcium as a dilutant for collagenase, opposed to the previously used sea cucumber culture medium. Excluding calcium and magnesium from the dissociation media may help by reducing cell-cell interaction due to the bivalent ion's ability to interact with negatively charged amino acids on another cell, inducing the cross- linkage between on cell and another (Malenkov A., Melikyants A). However,

we obtained the opposite result, where there was little muscle dissociation when the enzyme was used in this medium. We believe that this can be explained by the fact that there's a specific domain in in the enzyme that needs one of the two bivalent ions as a co- factor for its function. This explanation may account for the lack of muscle fibers present in our culture when no ions were present in the dissociation protocol.

Next, we tried to include fetal bovine serum in our dissociation media as collagenase has been known to induce the degradation and change the conformation of muscle fibers from mouse's muscle explants (Selvin D. *et al.*, 2015). Our results present a variability in the number of fibers present in cultures with FBS vs. those without it that indicated a gradual increase that was not associated with the presence of FBS. No significant value was determined for the presence of FBS, indicating that our culturing protocol do not need the serum.

Finally, we sought to try different concentrations of collagenase to deduce its effects on our cultures. We found no difference between the number of cells in our cultures between the concentration of 0.15%, 0.20% or 0.30%. We also didn't find any difference in the total number of fibers, but we did find a significant increase in the number of fibers presenting a nucleus at higher concentrations of collagenase. The relevance of this may be the pathway of cellular dedifferentiation taken by muscle fibers in the presence of different concentrations of collagenase. As mentioned in this work, muscle cell dedifferentiation due to muscle tissue damage may take one of two pathways depending on the extent of the damage inflicted in the tissue. If wounding of muscle tissue is major, then the muscle fiber separates its cytoplasm from its nucleus without the formation of a SLS (García-Arrarás J. & Dolmatov I., 2010). The correlation between the increase of nucleated muscle fibers with the increase in collagenase

percentage, may indicate that collagenase is inflicting cellular stress like dedifferentiation in major wounding of muscle tissue.

Alternatively, we found a 2-3-fold increase of SLS's in cultures with 0.20% and 0.30%. This reflects the pathway of greater stress or damage and creates a conflict in our interest of using these concentrations for dedifferentiation processes. The lower concentration of collagenase was sufficient to effectively dissociate *H. glaberrima's* muscle tissue without significantly inducing the process of dedifferentiation in our cultures. This data led us to believe that collagenase may be inflicting some cellular stress on muscle fibers, leading to the induction of dedifferentiation, as seen both in the fluorescent images and the analytical data.

During wounding, collagen is constantly degraded and deposited to maintain the structure of the extracellular matrix. The extracellular matrix is known to serve as a reservoir for growth factors, inhibiting their proteolytic degradation or inducing their activation (Vlodavsky I. *et al.*, 1991; Ge G. & Greenspan D., 2006). The rate of collagen degradation has been implicated in the repair of muscle tissue following injury for both skeletal and smooth muscle tissue (Chen W. *et al.*, 2021; Chen W. *et al.*, 2010). Loss in the ability to remodel an extracellular matrix is enough to impair the regeneration of muscle tissue (Chen W. *et al.*, 2021). Changes in the composition of the extracellular matrix can also be observed during *H. glaberrima* intestine regeneration. In *H. glaberrima,* these changes follow a dedifferentiation gradient that is observed during gut rudiment formation and can be correlated with the loss of muscle fibers (Quinones et al. 2002). Our data suggest that increasing the time of enzymatic dissociation or the concentration of the collagenase that composes it, increases the presence of structures associated with the process of dedifferentiation. We believed that collagenase may

be imparting cellular stress in our muscle fibers, inducing the disappearance of muscle fibers from our cultures, possibly by degradation. Following the relevance of the extracellular matrix in the process of regeneration, and the correlation between loss of collagen fibrils and muscle fibers in *H. glaberrima*, an alternative explanation in the loss of muscle fibers in our cultures can be proposed. Enzymatic degradation of collagen during the dissociation period may be releasing molecules, presumably growth factors, responsible for inducting the process of dedifferentiation. Moreover, metalloproteases can induce signals that contribute to regeneration and development by proteolysis of cell-surface proteins (Werb Z., 1991; Chang C. & Werb Z., 2009). In our experiments, we have seen that inhibition of BMP-1, a metalloprotease, can regulate the formation of SLS's and dedifferentiating fibers. Finally, separation of muscle fibers may be enough to induce the dedifferentiation of muscle fibers due to the loss in signals obtained from cell-extracellular matrix attachments or cell-cell interactions. An example of the latter is provided below in pharmacological assay for calcium signaling.

Cell's morphology

Our morphological analyses revealed two muscle-derived, phalloidin-labeled structures that were present in our cultures. Their abundance or frequency depended on the treatment being applied to the culture. Most of them didn't present a nucleus, thus they are thought to be the remnants of dedifferentiated cells. The first we were able to identify were SLS's, which have been previously documented as markers for the process of dedifferentiation and characterized as a disorganization of the contractile apparatus (Dolmatov I., 1992; Di Bennedeto C. *et al.*, 2014). The second structure was documented in our work as a

dedifferentiating fiber and characterized by the loss of phalloidin staining at the center of the muscle fiber and the formation of a rhombus shape at each end of the fiber. These rhombus-shaped structures have been previously reported by Dolmatov I. (1993) and had been associated with the process of myogenesis. They were described as a cubical form, and their appearance was accompanied by a reduced activity of proliferation and the formation of a new contractile apparatus, suggesting that these structures were in the process of re-differentiation. Since the appearance of these structure were correlated with the appearance of SLS's in our work, and since they were present at higher number with LiCl treatment, which have been documented by Bello S. and colleagues (2019) to increase levels of structures associated with the process of dedifferentiation *in vitro*, we argue that the presence of these structures most likely represent the process of dedifferentiation. Also, we saw loss of polymerized actin as seen by a decreased in phalloidin staining at the center of the muscle fiber. In some cases, such as chondrocyte dedifferentiation into myofibroblast, the transition into muscle-like phenotype requires the polymerization of actin into filaments, suggesting that the depolarization observed in our cultures is due to the loss of a contractile phenotype (Kumar D. & Lassar A., 2009).

Our ongoing hypothesis is that these structures represent different stages of dedifferentiating muscle cells. In support of this, we managed to order the structures to create a visual schematic of the process of dedifferentiation, leading from a nucleated muscle fiber to the release of SLS's from each end of the fiber. The process starts with a nucleated muscle fiber, as a fully differentiated cell, then, as the molecular machinery start the process of dedifferentiation, the two ends of a muscle take the morphology of a rhombus. As the ends form, the nucleus goes through evagination and is expulsed into the extracellular space. The

next step results in a loss of polymerized actin at the center of the muscle fiber axis, and continue until it reaches the rhombus shape, as explained by a disorganization of its contractile apparatus. Lastly, each end is separated as a single SLS and released into the extracellular space. Therefore, the final products of a single dedifferentiated muscle fiber are a small cell with little cytoplasm, known as the dedifferentiated cell, and two SLS's. Other abnormal structures could also be observed, which could be part of the events leading to dedifferentiation, the malformation of cells or possible myogenesis of cells in our cultures.

Elucidating molecular pathways through pharmacological treatments on our cell cultures

Optimization of cell cultures led to a platform in which pharmacological treatments could be applied to cellular cultures to determine their effects on the dedifferentiation process. We quantified three variables which we thought could give us information on the molecular pathways being activated for dedifferentiation: the percentages of fibers presenting a nucleus, dedifferentiating fibers and SLS's. The first served to indicate if the pharmacological agent modulates the pathways associated with minor and major wounding, as described by García-Arrarás J. & Dolmatov I., 2010. The quantification of dedifferentiating fibers and SLS's indicate which signaling pathways is controlling the process of dedifferentiation. We applied pharmacological agents targeting candidate molecules that have been found to play a role in either regeneration as general or in the process of dedifferentiation. Here, we discuss the data obtained from each of the pharmacological treatments applied to the muscle cultures.

Wnt/β-catenin and GSK-3 signaling

As previous data indicated that putative activator or disruptor for Wnt signaling were influencing *in vitro* cultures from *Holothuria glaberrimas* gut explants, we decided to replicate the conditions in our muscle tissue to see if it could be used as a positive control for our studies. In our cultures, dedifferentiating fibers increased 3- fold when compared to both control and the putative inhibitor of Wnt signaling. No significant increase could be seen between the control and the inhibitor, leading us to believe that inhibition of Wnt signaling may be needed to maintain cellular phenotypes. It is true that Wnt signaling influence dedifferentiation and regeneration in different species. For example, zebrafish cardiomyocytes need Wnt signaling inhibition to dedifferentiate (Zhao *et al.*, 2019; Peng X. *et al.*, 2020), while in adipocytes Wnt signaling is known to induce proliferation, migration and more importantly, dedifferentiation. Previous studies in our laboratory showed that the expression of Wnt- 7b, Wnt- 9a and Wnt- 6 are up-regulated in gut rudiment and mesentery from *Holothuria glaberrima* following 1-3 days post-evisceration (Ortiz-Pineda P. *et al.*, 2009; Quispe-Parra D. *et al.*,2021). Other works with *Apostichopus japonicus* and *Eupentacta fraudatrix* have shown the up-regulation of Wnt molecule variants in the process of gut regeneration (Sun *et al.*, 2013; Girich *et al.*, 2017). The effects of Wnt-signaling putative pharmacological manipulators, along with the information already known for Wnt- signaling properties in *Holothuria sp.* signaling may point to Wnt-signaling pathway to be involved in muscle cell dedifferentiation in vitro, but it's worth noting that the effects seen in our results may be because of GSK-3 signaling.

As previously mentioned, LiCl inhibits the effects of GSK-3, leading to a higher presence of cytoplasmic β-catenin, protein synthesis and muscle-related proteins (Cormier R. & Woodgett J., 2017; Bertsch S. *et al.*, 2013). As expected, when disrupting Wnt pathway directly,

GSK-3 inhibition leads to dephosphorylation and stabilization of β-catenin which stimulates the transcriptional processes control by β-catenin, which include embryonic development, and presumably regeneration processes (Sancho E. *et al.*, 2004; Logan C. and Nusse R., 2004). Expression of GSK-3 doesn't seem to be up-regulated during regeneration, but it may be inhibited by phosphorylation by growth factors or other kinases presented in the pathway, downregulating its activity without interfering with its expression (Quispe-Parra D. *et al.*,2021; Rayasam G. *et al.*, 2009). Conversely, inhibition of β-catenin with DsiRNA on gut rudiment didn't have an effect in dedifferentiation or apoptosis, only in proliferation. Only direct inhibition of GSK-3 had any effects on dedifferentiation on *Holothuria glaberrima*, while inhibition of upstream factors from Wnt/β-catenin pathway did not (Alicea-Delgado M. & García-Arraras J., 2021). Our laboratory has hypothesized the possibility of GSK-3 exerting its role on dedifferentiation through another regulated pathway that doesn't involve Wnt/β-catenin. For example, GSK-3 can modulate another kinase of interest in our studies, PI3K, which in turn regulate the activity of GSK-3 by phosphorylation (Maurer U. *et al.*, 2014). Activity of PI3K and GSK-3 are also modulated by growth factors over expressed during developmental and regeneration processes (Kipryushina Y. *et al.*, 2015; Suzuki M. *et al.*, 2007).

As research shows, the effects of LiCl on our cultures may not be directly ligated with the manipulation of the Wnt/β-catenin pathway. But its possible role on dedifferentiation cannot be completely dismissed. As with Bello S. and colleagues (2019), iCRT14 was able to maintain low levels of both dedifferentiation makers, having a significant difference with LiCl of about 3 times the number of dedifferentiating fibers and almost 2 times the amount of SLS's. In Bello S. et al. 2019, the amount of SLS's reduced by 42% compared to the control. iCRT14 works

by disrupting the interaction between β-catenin and the TCF/LEF complex, inhibiting the effects of gene transcription by Wnt pathway. Even if the effects of LiCl were not mediated by β-catenin, the effects of iCRT14 cannot be dismissed. Therefore, although the pathway in which LiCl induces the increase in SLS's and dedifferentiating fibers is unclear, we propose that this treatment be used as positive control for future dedifferentiation assays. Finally, using specific inhibitors and activator for both GSK-3 and Wnt/β-catenin could be a reliable method for assigning a role each pathway.

ROS signaling

Reactive oxygen species were used as possible candidate for the dedifferentiation process for their well-documented activity on regenerative and developmental processes on organisms ranging from planarians to fruit flies (Pirrote N. *et al.*, 2015; Fogarte C. *et al.*, 2016). The molecular pathways involved in regenerative organisms share a common event, the outburst of ROS at the site where an excision has been performed, that when inhibited leads to a malformation in the regenerative site. It has been stated that high amounts of ROS production are toxic to cell types, but regulated amounts serve as an intracellular signaling second messenger involved in processes of growth and differentiation in different cell types (Cheng J. *et al.*, 2012; Clempus R. & Griendling K., 2006). It can be argued that *H. glaberrima* is inducing the production of ROS following evisceration, as gene expression profiles from the mesentery indicate that NADPH oxidases are differentially upregulated from 1 to 12 days post-evisceration (Quispe-Parra D. *et al.*,2021). However, our studies indicated a trend showing both dedifferentiation structures to decrease in the presence of hydrogen peroxide. An increase in the ratio of SLS's per nuclei was observed when comparing DPI with its control. An increase was

also observed with the dedifferentiating fibers, but no significance was acquired. As with the other pharmacological treatments, no difference in the quantity of fibers with or without a nucleus was observed in these studies.

These results are indeed surprising since there are multiple instances in which ROS production leads to dedifferentiation in various cell types; in rabbits' chondrocytes through Erk and PI3K pathways (Yu S. & Kim S., 2014); in mouse primary hepatocytes and adipocytes the reduction of ROS led to less dedifferentiation and loss of function from these cells and was concluded to be mediated by Erk and GSK-3β signaling (Guo R. *et al.*, 2017; Liu L. *et al.*, 2015).

Although evidence seem to pinpoint ROS as an inducer of dedifferentiation, the downstream effects in different cell types seem to be diverse. For example, in mammals, smooth muscle cells dedifferentiation can be measured by the presence of expression markers such as SM22α, α- SMA and SM-MHC, and by their low rate of proliferation and migration (Rensen S. *et al.*, 2007). Oxidative stress by ROS can activate mitogen-activated protein kinase module (MAP kinase module), which in turn activates JNK pathway, leading to proliferation, differentiation, inflammatory responses, and apoptosis (Gauron C. *et al.*, 2013; Son Y. *et al.*, 2011). Reduction of p38 MAP kinase by ROS, or other mechanisms, reduces the expression of markers associated with a differentiated phenotype in vascular smooth muscle cell line A7r5 (Dan J. *et al.*, 2014). This suggest that ROS has a role in maintaining cell culture phenotypic identity for smooth muscle cells (Dan J. *et al.*, 2014). The effects of MAPK in cellular differentiation have been widely studied, and its effect on differentiation established (Rodriguez- Carballo E. *et al.*, 2016; Lim J. *et al.*, 2008; Kornasio R. *et al.*,2009). For example, in PAC-1 VSMC's, TGF-β1 activates MAPK to induce the expression of smooth muscle genes (Tang

Y. *et al.*, 2011). Conversely, other studies using VSMC's have found that a ROS-mediated phenotypic transition into a dedifferentiated state can be blocked by inhibiting one or more of the signaling molecules in a ROS/NFκB/mTOR/P70S6K signaling cascade (Lu Q. *et al.*, 2018). Thus, the effects of ROS on regeneration seem to be dependent on the organism, the cell type and the molecular pathway activated by this intracellular signaling molecule, which means that other molecular events, possibly those downstream, are required for the path chosen by cells leading to the regeneration process. We propose that in holothurian longitudinal muscle regeneration, ROS maintains cell's identity, probably through mechanisms that resembles those in smooth muscle cell differentiation. Its noteworthy to mention in this section that somatic musculature cells in *H. glaberrima* mostly resemble vertebrate smooth muscle tissue as opposed to the other two muscle tissues, skeletal and cardiac muscle (García-Arrarás J. & Dolmatov I., 2010).

It is worth mentioning that the possible effects of ROS during dedifferentiation processes may be affected by the application of polydopamine. Polydopamine has a hydroquinone moiety, a free radical scavenger, which makes it an antioxidant, much like ascorbic acid, possibly exerting the same functions as our pharmacological approaches to reducing ROS in culture medium (Ju K. *et al.*, 2011). Therefore, polydopamine may induce biofunctional effects as a ROS scavenger, as showed by Deng Z. 2021 published paper on the properties of polydopamine on scavenging extracellular ROS on proliferating human mesenchymal stem cells (MSC). Oxidation processes are required for polymerization of dopamine, a process that may be inhibited by the application of antioxidants or ROS scavenging molecule or stimulated by experimental application of ROS (Du X *et al.*, 2014; Feng- Gao *et*

al.,2019). Taking this into consideration, additional functional assays, comparing dedifferentiation scores on both culturing techniques, acetic acid and polydopamine, are needed to further assert our pharmacological studies' results using hydrogen peroxide.

Finally, since modulation of the ROS pathway provided clear results for qualitative and quantitative analyses, we propose that it should be prioritized for further dedifferentiation assays. As explained in this work, ROS have been known to crosstalk with other molecular pathways. Combining ROS-modulating drugs with activators and inhibitors for other pathways which have been known to crosstalk with ROS, could provide insight into the complete pathway involved in dedifferentiation. Also, since VAS-2870 didn't provide the same significant results as DPI, using different concentration of VAS-2870 or other known inhibitor would further imply ROS absence as a modulator for dedifferentiation induction.

BMP-1

Our results also suggest a possible role for BMP-1 in the process of muscle dedifferentiation. BMP-1 homologs are known to be differentially expressed in regenerating organisms from 1 to 12 days post-evisceration (Quispe-Parra D. *et al.*, 2021). Now we showed that applying the pharmacological agent UK-383,367, resulted in a significant increase of SLS's for each of the concentration, although the increase seen in 30µM can be attributed to the cellular stress exerted by the drug.

As previously mentioned, BMP-1, BMP-1 is a metalloprotease involved in the assembly of the extracellular matrix, that plays an important role in developmental processes such as in Drosophila melanogaster's dorso-ventral axis patterning and mouse's ventral body

wall formation (Finelli A. *et al.*, 1995; Suzuki N. *et al.*, 1996). BMP-1has the capacity to regulate the expression of growth factor including TGF-β1, BMP-2/-4, GDF-8/-11 and IGFs (Vadon-Le Goff S. *et al.*, 2015). These growth factors have been implicated in embryonic development and some have been studied for their capacity to regulate the regeneration. Functional roles have been already characterized in echinoderms for TGF-β1; embryonic skeletogenesis in the echinoidea class (Zito F. *et al.*, 2003); regenerating arms of the crinoidea and ophiuroidea classes (Bannister R. *et al.*, 2005; Patruno M. *et al.*, 2003). BMP-1 proteolytic activity is enough to activate latent TGF-1, which in turn increases the activity of the SMAD canonical pathway, a pathway responsible for epithelial dedifferentiation and endothelial to mesenchymal transition in kidney fibrosis (Gwon Mi. *et al.*, 2019; Anastasi C. *et al.*, 2020). Here, we propose that the effects of BMP-1 on modulating growth factors is inducing dedifferentiation of muscle fibers *in vitro*. A stimulator of BMP-1 should be used to induce an opposite effect. One way could be the activation of one of its upstream activators, PI3K (Voloshenyuk T. *et al.*, 2012). We could also cross-reference with ongoing experiments with *H. glaberrima* to see if inhibition of BMP-1 leads to dedifferentiation in other tissues. We believe that studies analyzing the role of BMP-1 on dedifferentiation should be prioritized as with the other molecular pathways that showed positive results in our assays

Ca^{2+} signaling

Calcium ions can modulate a wide variety of cellular processes, some associated with tissue regeneration. Calcium's versatility led us to investigate it in the context of dedifferentiation. To investigate the effects of calcium, we used a chelator to remove the ions from the culture medium and an ion-carrier to increase intracellular levels. We didn't observe a

difference between the percentages of fibers containing a nucleus when compared with the control group. We did find that higher concentrations of the ionophore produced malformed muscle fibers with some containing protuberances emanating from its axis. These protuberances were similar to those found at higher concentration of collagenase. We believe that these malformation in our muscle cells are the consequence of an over-induction of calcium-activated genes that have been previously found to induce cell's hypertrophy (Wamhoff B. *et al.*, 2004). We observed that there was a correlation between the high levels of malformations and the presence of dedifferentiating fibers and SLS's. The lowest concentration of the ionophore produced muscle fibers that resembled those found in the control group. This concentration did not change the presence of structures associated with dedifferentiation. On the other hand, EGTA significantly increased the presence of SLS's in our cultures. This increase was ablated by gradually reducing the concentration of the chelator. In terms of percentages of nucleated muscle fibers, we observed similar number to those found in both the control group and the experimental group with the ionophore.

There could be countless ways in which calcium ion's presence may be modulating regeneration. The release of calcium ions has been known to induce the activation of molecular pathways, like ERK1/2 and PI3K/Akt, that are associated with regeneration (Liu D. *et al.*, 2008; Danciu T. *et al.*, 2005). For example, these pathways, along with MAP kinases, are responsible for the response of bone marrow-derived mesenchymal stem cells (MSCs) in bone regeneration (Riddle R. *et al.*, 2006; Jaiswal R. *et al.*, 2000; Lai C. *et al.*, 2001). In vascular smooth muscle cells, the loss in function or under-expression of L-type calcium channels is enough to induce dedifferentiation of VSMC into a synthetic phenotype (Clunn G. *et al.*, 2010). The increased

expression of L-type calcium channel receptor is thus hypothesized to increase the pressure on phenotype modulation (Albinsson S. *et al.*, 2014). The same type of receptor is significantly down regulated in the mesentery from 1 to 12 days post evisceration (Quispe-Parra D. *et al.*,2021). Also, it has been stated that intracellular calcium ions are needed to maintain contractile gene expression in muscle cells (Wamhoff B. *et al.*, 2004). Finally, calcium has been found to directly promote dedifferentiation and redifferentiation of cardiomyocytes. Mouse cardiomyocytes dedifferentiate during dissociation of cardiac tissue, and the process has been linked to downregulation of calcium regulatory genes (Porrello E. *et al.*, 2011; Szibor M. *et al.*, 2014). These cardiomyocytes re-differentiated by relaying intercellular calcium signals via gap junctions. Impairment of connexin 43, a major mediator of the propagation of intercellular calcium, was sufficient to inhibit redifferentiation of cardiomyocytes (Wang W. *et al.*, 2017).

There is vast evidence on calcium's involvement in the regeneration process. Its involvement in smooth muscle cells differentiation and dedifferentiation led us to expect a shift in the number of dedifferentiating fibers and SLS's when calcium concentrations were modulated. Given that EGTA significantly increased the number of SLS's and showed a similar pattern for dedifferentiating fibers in our cultures and that the presence of both structures was lowered by reducing its concentration, we propose that in *H. glaberrima's* muscle system calcium is needed to maintain a differentiated state of myocytes by inducing the expression of muscle genes. We also based this statement in the absence of both structures in the working concentration for the ionophore. We believe that calcium plays a vital role in the regeneration of muscle tissue, although more studies on the effects of gene expression due to calcium application may be needed to corroborate these statements. Finally, we think that calcium

should be further studied by combining the chelator or the ionophore with treatments for other pathways known to be modulated or to modulate calcium outbursts during dedifferentiation to further describe a complete molecular cascade.

Autophagy

It has been previously stated that autophagy regulates the process of cytoplasmic remodeling (Folmes C. *et al.*, 2011). In zebrafish muscle injury, autophagy regulates the process of dedifferentiation by remodeling the cellular architecture and eliminating specialized protein complexes that provide muscle cells with their identity (Saera-Vila A. *et al.*, 2016). Also, there's increasing evidence of autophagy's role in the phenotypic switching that vascular smooth muscle cells undergo to proliferate and migrate, a process that leads to vascular restenosis (Saera-Vila A. *et al.*, 2016). This evidence suggest that autophagy may increase the formation of SLS's in our cellular cultures. Since we didn't apply an activator of autophagy, such as rapamycin, it was unlikely to see a significant increase of the dedifferentiating structures in our cultures. Although our data showed a slight increase in the number of SLS's and dedifferentiating fibers when compared to the drug's vehicle, the standard error and the lack of significance in our analyses invalidates any difference obtained in our cultures. We expected similar results between the drug and the vehicle for both dedifferentiation markers, since inhibition of autophagy using chloroquine diphosphate should be maintaining basal levels of the dedifferentiating structures. Further analyses are still needed to deduce if autophagy is promoting cellular dedifferentiation. For example, we could use atg5 and LC3 antibodies to

deduce if autophagy is being differentially activated in experimental groups that have been known to increase dedifferentiation, or we could use an activator of autophagy to see if we can increase the ratio of dedifferentiating fibers and SLS's.

We believe that autophagy is a prime candidate for dedifferentiation, and that elucidating the effects *in vitro* and *in vivo* is necessary to further understand regeneration of tissue in our model organism. Therefore, we propose that autophagy, as a process of structural remodeling, should be further analyzed to understand the mechanisms of dedifferentiation. For this, we have suggested different methods that would further clarify its involvement on muscle cell dedifferentiation.

PI3K/Akt pathways

The PI3K/ Akt pathway seems to be activated not only with growth factors, but also by the other molecular pathways also associated with our experiments, for example; GSK-3β is one of the major targets of PI3K/AKT, and it has been found that PI3K modulates smooth muscle cells dedifferentiation via an Akt/GSK-3β pathway (Frismantiene A. *et al.*, 2016); Reactive oxygen species bursts induced by growth factor are mediated through activation of PI3K (Park H. *et al.*, 2004); PI3K inhibits autophagy activity by repression of Atg1 (ULK) complex (Salabei J & Hill B., 2013); It regulates the increase in cytosolic calcium and is responsible for differentiation of skeletal muscle cells through insulin growth factors (Valdes J. *et al.*, 2012); BMP-1 expression is regulated by TGF-β1 in a PI3K dependent mechanism in both osteoblast and cardiac fibroblast (Voloshenyuk T. *et al.*, 2011; Ghosh-Choudhury N. *et al.*, 2003). Dedifferentiation markers, the presence of SLS's and dedifferentiating fibers, showed no

significant difference between the pharmacological treatment and its vehicle. Since we didn't apply an activator for this pathway, we were unable to assign a specific role for PI3K/Akt in the process.

The results obtained in this work would have been complemented with a growth factor or a pharmacological activator of PI3K/Akt. Inducing PI3K/Akt could show an increase in dedifferentiation marker, leading to an inverse correlation when compare with its inhibitor. More so, cross-referencing with the pharmacological agents presented in this work may elucidate the exact network in which a crosstalk between an upstream or downstream factor may be influencing muscle tissue regeneration through dedifferentiation. Conversely, an increase in the dose provided to cell cultures may also be needed to show an opposing effect by this pathway. Therefore, we propose that PI3K/Akt pathway maintains it candidacy for an important modulator of dedifferentiating muscle fibers.

Minor and major wounding mechanisms

We calculated the percentages of nucleated muscle fibers to standardize the dissociation protocol and the effects that each treatment may have in our cultures. We also used this number to see if we could identify a difference between each treatment that we could attribute to the process of either minor or major wounding, as described in our introduction and by García-Arrarás J. & Dolmatov I. (2010). If any of our treatment presented a disparity in the percentages of nucleated fiber, we could deduce which pathway modulates either type of wounding. For example, if major wounding would have been activated by any of the drugs, then a significant reduction in the percentage of nucleated fibers would likely be

seen when compared to the control. Alternatively, if minor wounding mechanisms were to be activated, we would see an increase in dedifferentiating structures and a statistical decrease in the number of total muscle fibers found in treated groups when compared to the control groups. Although we did find an increase of dedifferentiating structures in the cultures treated for the Wnt/β-catenin/GSK3, ROS, Ca^{2+} and BMP-1 pathways, we didn't find any difference in the total number of fibers or nucleated fibers. Our results suggest that minor wounding is the process most likely being activated. Alternatively, our in vitro model may not be suitable to associate pharmacological treatments with a specific type of wounding.

Conclusions

We optimized dissociated muscle tissue culture protocols that can be utilized for determining cellular mechanisms involved in the process of dedifferentiation. Furthermore, this protocol can be also used for immunohistochemical studies, migration assays, or other studies that may require cell adherence. Our *in vitro* model provided a better understanding of the morphological changes that a muscle fiber undergoes to provide a dedifferentiated cell, the precursor of a regenerated tissue. This model also provided a new marker that could be used for statistical analyses to indicate the induction of the dedifferentiation process. Using this model, we were able to identify possible mechanisms involved in inducing the dedifferentiation of muscle fibers. Our results suggest that modulation of the pathways involving Wnt/β- catenin and/or GSK- 3, reactive oxygen species, Ca^{2+} and BMP-1 play a role in controlling the re-programming of muscle fibers into a morphological distinctive cell that has been known to change its gene expression profile. To further understand our results, we have correlated them with expression profiles previously analyzed from intestinal tissue in the process of

regeneration. We were able to find changes in the expression of the molecules that increased the presence of structures associated with dedifferentiation in our cultures. Finally, our model and results could be further used to decipher the functional roles of upstream or downstream effector molecules and proteins that affect dedifferentiation of muscle fibers in *H. glaberrima*, creating the opportunity of defining complete molecular pathways that help us understand the events that occur during regeneration.

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