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THE ROLE OF SIALIDASE NEU3 IN THE CARDIAC RESPONSE TO ISCHEMIA AND REPERFUSION INJURY

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INDEX

ABSTRACT

Acute myocardial infarction (AMI) is still one of the most common causes of death worldwide. Although reperfusion strategies represent the currently most used life-saving approaches to restore the blood flow in the cardiac tissue after AMI, they also come with the drawback that they inevitably induce the ischemia/reperfusion injury (IRI), ultimately resulting in increased cardiomyocytes damage and heart failure. Many efforts have been made to clarify the molecular mechanisms involved in IRI. In this context, the activation of pro-survival kinases, as well as the hypoxia inducible factor (HIF-1**α**), have been recognized as key steps in the cellular response to IRI. Along this line, our research group recently identified a novel mechanism of HIF-1**α** activation, PHDs independent and mediated by the sialidase NEU3. Interestingly, NEU3 is upregulated under chronic hypoxia in cyanotic congenital cardiac patients. Moreover, the induced activation of NEU3 increased myoblast resistance to hypoxic stress, maintaining their proliferation rate and counteracting apoptosis.

Thus, the aim of this study was to further investigate the possible role of NEU3 in protecting cardiac myoblasts during IRI, both in terms of increasing their resistance to the damage and reducing fibrosis. Initially, we set-up an *in-vitro* model of IRI on H9C2 rat cardiomyoblasts. Results showed a modulation of NEU3 during IRI, with a progressive down-regulation during the ischemic phase, followed by a reactivation during the reperfusion phase. Remarkably, overexpression of NEU3 significantly improved cardiomyoblasts resistance to IRI, both in terms of cell proliferation and resistance to apoptosis, as well as it induced an increased activation of the pro-survival kinases Akt and Erk and HIF-1**α**, as compared to controls. Interestingly, treatment with specific Akt and Erk inhibitors (LY294002 and PD98059), as well as with sialidase inhibitors (DANA and LR332), completely reverted the beneficial effects mediated by NEU3, thus supporting the hypothesis of a direct involvement of the sialidase in counteracting cardiomyocytes damage during IRI, through the activation of pro-survival kinases and HIF-1**α**. Moreover, we also investigated the possible involvement of NEU3 in regulating the process of cardiac fibrosis in response to tissue injury, which is characterized by the deposition of extracellular matrix proteins by activated myofibroblasts. Interestingly, we demonstrated that the overexpression of the sialidase NEU3 was sufficient to impair the fibroblasts/myofibroblasts conversion, decreasing the expression of the specific fibrosis markers **α**-smooth muscle actin and collagen type-1. In addition, to confirm that the observed effects were mainly mediated by the NEU3-induced GM3 depletion, we silenced the GM3 synthase, which is the enzyme responsible of the GM3 synthesis, to mimic sialidase NUE3 overexpression. Also in this case, the reduction of GM3 partially inhibited cardiac fibroblasts differentiation, finally diminishing the fibrosis markers expression.

In conclusion, taken together, the results of this thesis work show that NEU3 activation has a cardioprotective effect during IRI, calling for further studies to unveil its full potential as a therapeutic target to treat cardiac ischemia and reperfusion injury and to improve patients recover after AMI.

INTRODUCTION

1. Cardiovascular diseases: Acute Myocardial Infarction

Cardiovascular diseases (CVDs) still represent one of the major causes of morbidity and mortality worldwide *(Li et al., 2019)*. The latest data reported by the National Institutes of Health in 2017 revealed that 92.1 million US adults (36.6% of the total population) have at least one type of CVD and that the 30.87% of deaths are caused precisely by these types of diseases *(Benjamin et al., 2017)*. Among CVDs, Acute Myocardial Infarction (AMI) is one of the most dangerous, being a life-threatening condition that needs prompt and successful intervention. AMI is mostly caused by the rupture, ulceration, fissuring, erosion, or dissection of an atherosclerotic plaque, accompanied by intraluminal thrombus in one or more of the coronary arteries. This process induces a sudden decrease in the myocardial blood flow, consequently leading to the necrosis of the downstream cardiac tissue. Myocardium death becomes identifiable by the detection of an increased cardiac troponin T (cTnT) in plasma (Fig.1) *(Reddy, Khaliq, & Henning, 2015)*.

Myocardial ischemia produces a characteristic pattern of ultrastructural, cellular, molecular and metabolic alterations that lead to irreversible injury. It is possible to observe several changes in both myocardial interstitium and microvasculature. Moreover, damaged myocytes start a degenerative process that culminate in swelling of the cytoplasm, mitochondria and sarcoplasmic reticulum, accompanied by the margination and clustering of the nuclear chromatin. Therefore, cardiomyocytes necrosis and tissue alterations stimulate an inflammatory process associated to the activation of many signaling pathways that alter cellular metabolism, finally leading to irreversible cardiac damage *(Ibanez, Heusch, Ovize, & Van de Werf, 2015)*.

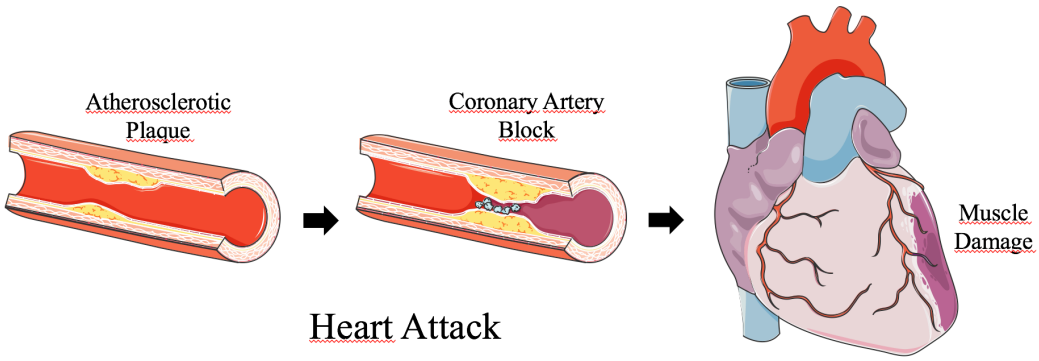

Fig. 1: *Acute myocardial infarction illustration. Cellular damage is caused by dissection of an atherosclerotic plaque that induces a thrombus in one or more of the coronary arteries.*

1.1 Cardiac Fibrosis

Destroyed myocardium is replaced by a collagen-based scar that, unfortunately, could not contribute to the myocardial contractile function, resulting in progressive chronic heart failure. This process is defined as cardiac fibrosis, whose major players are represented by cardiac fibroblasts. In particular, cardiac fibroblasts are an important component of the cardiac interstitium and play an important role in preserving the integrity of matrix network *(Banerjee, Fuseler, Price, Borg, & Baudino, 2007)*. In a young adult heart, cardiac fibroblasts remain quiescent and do not exhibit any significant proliferative or inflammatory activity. However, following an ischemic episode, cardiac fibroblasts differentiate into myofibroblasts, which are the main effector cells of the fibrotic process In particular, myofibroblasts are secretory and contractile cells that accumulate within sites of injury and presents ultrastructural and phenotypic characteristics similar to smooth muscle cells, such as an extensive endoplasmic reticulum and the expression of the **α**-smooth muscle actin (**α**-SMA) (Fig. 2). *(Hinz, 2010; Hinz et al., 2007)*.

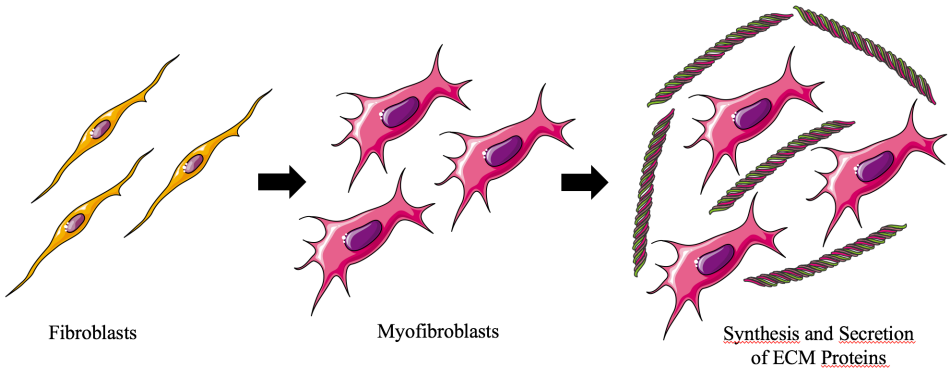

Fig. 2: *Schematic representation of the fibroblasts-myofibroblasts transition*

Myofibroblasts transdifferentiation is promoted by the activation of TGF-**β** in the cardiac interstitium, which induces the **α**-SMA transcription in fibroblasts through the activation of the Small Mother Against Decapentaplegic Homolog 3 (Smad3) signaling cascade. Moreover, also alterations in the composition and in the mechanical properties of the extracellular matrix facilitate the fibroblasts-myofibroblasts transition. Indeed, the induction of specialized matrix proteins increases deposition of non-fibrillar and fibrillar collagens, promoting fibrotic cardiac remodeling *(Dobaczewski, Gonzalez-Quesada, & Frangogiannis, 2010)*. Interestingly, it was observed that collagen type I and type III were increased in the remodeling fibrotic heart and, particularly, in models of myocardial infarction, collage type I is more and prolonged up-regulated as compared to collagen type III (*Mukherjee & Sen, 1993)*. However, non-fibrillar collagen, such as collagen type IV, also has a crucial role in fibroblast transdifferentiation, as demonstrated by an experimental study on myocardial infarction

(Naugle et al., 2006). Interestingly, collagen IV disruption reduced fibrosis and attenuated cardiomyocytes apoptosis in the infarcted tissue, confirming its important involvement in the fibrotic process *(Luther et al., 2012)*.

However, the group of fibrotic proteins released during cardiac remodeling also includes fibrinogen, plasma fibronectin and the so-called matricellular proteins. *(Frangogiannis, 2012)*

Fibrinogen and fibronectin are responsible of forming a temporary matrix network composed of fibrin and fibronectin, cooperating with TGF-**β** to stimulate fibroblasts proliferation and, subsequently, their migration and transdifferentiation *(Rybarczyk, Lawrence, & Simpson-Haidaris, 2003)*.

On the other hand, the matricellular proteins are a family of structurally unrelated extracellular macromolecules that are not components of a physiological tissue matrix. since they usually do not play a structural role. However, their function is principally to favor connections between cells and matrix proteins, transducing and modulating growth factors and cytokines responses *(Bornstein, 2009)*. This group of proteins includes tenascin-C, osteopontin (OPN), thrombospondins (TSPs) and periostin and their targets are represented by fibroblasts and inflammatory cells *(Bornstein, 2009)*.

In a normal heart, matrix proteins are finely regulated by a homeostatic control guided by fibroblasts. Alterations of the balance between collagen synthesis and degradation promotes heart abnormalities, related to coordination of the excitation-contraction coupling mechanism, both in diastole and in systole, inducing a diastolic or systolic impairment, respectively. Finally, cardiac fibrosis induces a ventricular remodeling in the heart trigger by left ventricular dilatation caused by a displacement of cardiomyocytes that induces a decrease in the number of molecular layers in the ventricular wall (Fig. 3) *(Kong, Christia, & Frangogiannis, 2014)*.

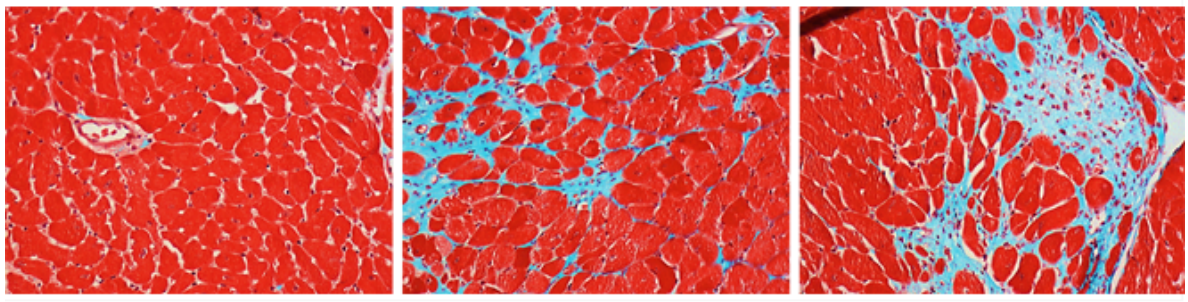

Fig. 3: *Histological images from normal and diseased hearts, blue staining indicates fibrosis.*

1.2 Sphingolipids and Cardiac Fibrosis

Bioactive sphingolipids regulate several cellular processes important for triggering cellular apoptosis, vascular leak and for TGF-**β** signaling and fibroblasts migration *(Hannun & Obeid, 2008; Shea &*

Tager, 2012). Many sphingolipids, in particular ceramides and sphingosine 1-phospate (S1P), are implicated in myofibroblasts differentiation and in the development and progression of cardiac fibrosis *in vitro (Watterson, Lanning, Diegelmann, & Spiegel, 2007)*. Moreover, also in several human diseases characterized by fibrosis, the levels of the circulating S1P resulted altered *(Ikeda et al., 2010; Shea et al., 2010)*. In particular, many evidences reported that mice overexpressing the S1P-producing enzyme and the sphingosine kinase 1, developed spontaneous cardiac fibrosis. Interestingly, fibrosis was attenuated when mice were crossed to $S1P_3$-deficient mice, supporting the important role of sphingolipids and their receptors in promoting the fibrotic process *(Takuwa et al., 2010)*. Moreover, it was also observed that alterations in the sphingolipid levels induced ROS production and subsequently TGF-**β** activation, myofibroblasts differentiation and increased collagen production in rat cardiac fibroblasts *(Gellings Lowe, Swaney, Moreno, & Sabbadini, 2009; Takuwa et al., 2010)*.

Therefore, since some of the negative consequences related to cardiac fibrosis are cardiac hypertrophy and ventricular dilatation, due mainly to pressure overload and vascular wall oxidative stress, sphingolipids could also have a key role in the mechanisms associated with these types of cardiac damage *(Frey & Olson, 2003; Levy, Garrison, Savage, Kannel, & Castelli, 1990)*. Indeed, it was reported that an accumulation of some glycosphingolipids (GSL) can induce oxidative stress, thus generating superoxides that interact with cardiomyocytes and vascular cells *(Alexander, 1995; Chatterjee, 1998)*. Particularly, among GSL, lactosylceramide exerted a concentration and time dependent increase in hypertrophy in H9C2 cells and in freshly cultured neonatal rat ventricle myocytes, precisely mediated by the generation of superoxides *(Mishra & Chatterjee, 2014)*. For this reason, it was supposed and demonstrated that hypertrophic mouse hearts treated with some inhibitors of the GSL glycosyltransferase showed a significant reduction of cardiac hypertrophy *(Mishra, Bedja, Amuzie, Avolio, & Chatterjee, 2015)*.

Thus, in conclusion, inhibiting glycosphingolipid synthesis or activity could represent a novel approach to mitigate cardiac dysfunctions associated with cardiac fibrosis.

1.3 Therapeutic strategies for AMI

The prompt diagnosis of AMI is fundamental to timely apply the so-called reperfusion strategies, which nowadays represent the gold standard life-saving approaches to treat the acute myocardial infarction. Among them, the most recent and effective are the fibrinolytic therapies (FT) and the primary percutaneous coronary intervention (P-IPC).

- Fibrinolytic and antithrombotic therapies are more effective in the very first hours from the beginning of the heart attack (<12 hours). These approaches are based on the administration of recombinant human tissue plasminogen activator, such as retaplase, alteplase and tenecteplase, which are responsible for the disruption of the clots that caused AMI. Moreover, Aspirin, Clopidogrel and Heparin should be given in addition to the administration of the fibrinolytic agents to prevent the formation of blood clots *(Reddy et al., 2015)*.
- P-IPC approaches are indicated for the treatment of patients after 12 hours from the onset of the heart failure symptoms. P-IPC is also known as Coronary Angioplasty, which is a technique based on the insertion of a balloon catheter to widen the occluded coronary artery, favoring the reoxygenation of the infarcted heart tissue. Indeed, balloon inflation inside the coronary is accompanied by the insertion of a bare metal or drug eluting stent to keep the blood vessel opened.

P-IPC, when performed in a timely manner, is more effective than FT. However, P-IPC is not universally available, because only specialized interventional cardiology centers could perform this type of interventions *(Reddy et al., 2015; Roule et al., 2016)*.

2. Ischemia and Reperfusion Injury

Reperfusion strategies are able to considerably reduce mortality in infarcted patients. However, they have some negative side effects that can induce cardiac injury and increase the extent of myocardial damage, reducing the beneficial effect of the intervention. This process has been named as Ischemia and Reperfusion Injury (IRI), which is defined as a myocardial damage caused by the restoration of coronary blood flow after an ischemic episode. Therefore, the identification of IRI existence may at least in part explain the discrepancy between the rate of death after an AMI, which is around 10%, and the incidence of cardiac failure following AMI, which instead is almost 25% *(Buja, 2005)*. In particular, four different cardiac dysfunctions have been associated to IRI: myocardial stunning and reperfusion-induced arrhythmias are a reversible form of damage, whereas microvascular obstruction and lethal myocardial reperfusion injury are the most dangerous complications which could irremediably compromise cardiac function *(Yellon & Hausenloy, 2007)*.

Myocardial stunning is a post-ischemic mechanical dysfunction that typically resolves between 48 and 72 hours after the ischemic episode *(Neri, Riezzo, Pascale, Pomara, & Turillazzi, 2017)*. On the other hand, reperfusion-induced arrhythmias are defined as disturbance of the cardiac rhythm that arise as a consequence of partial or total restoration of blood flow in the cardiac tissue that was previously ischemic. These arrhythmias are potentially harmful, but effective treatments are available (Hearse & Tosaki, 1987). The microvascular obstruction is characterized by the block of

the blood flow in the coronary artery with the absence of effective physical vessel obstruction (Ito, 2006).

Finally, we focused mainly on the last type of cardiac dysfunction, the lethal reperfusion injury, which is the component of cell death occurring as a consequence of reperfusion and it was demonstrated that mediates cardiomyocytes death in a different and independent manner from the ischemic injury *(Manning & Hearse, 1984)*.

2.1 Lethal Ischemia and Reperfusion Injury: molecular mechanisms

Lethal IRI is a complex phenomenon involving many players, all contributing to the final damage inflicted to the cardiac tissue. The deprivation of oxygen during the ischemic phase results in the inhibition of the myocardial contractile function, caused by mitochondrial membrane depolarization and ATP depletion. Moreover, the switch from fatty acids metabolism to glycolytic metabolism induces lactate accumulation, which reduces the intracellular pH lower than 7.0 and unbalances ion exchanger on the membrane, ultimately increasing intracellular Ca^{2+} levels *(Avkiran & Marber, 2002)*. Finally, acidosis during ischemia prevents the opening of the mitochondria permeability transition pore (MPTP), counteracting the effects of the influx of Ca^{2+} in the mitochondrion *(Garcia-Dorado, Ruiz-Meana, & Piper, 2009)*.

2.1.1 Mitochondrial Permeability Transition Pore

Reperfusion after ischemia induces a rapid correction of the pH level but conversely promotes the MPTP opening, leading to cardiac damage due to the uncoupling of the electron transport chain with the oxidative phosphorylation and due to an increase in the reactive oxygen species (ROS) production within the mitochondria *(Hausenloy & Yellon, 2013)*. In particular, MPTP is a high conductance nonselective channel that regulates the permeability of the inner mitochondrial membrane to solutes, being also involved in the physiological regulation of Ca^{2+} and the ROS homeostasis *(Garcia-Dorado et al., 2009; Xia, Li, & Irwin, 2016)*.

Reperfusion induces the mitochondrial permeability transition, increasing the inner membrane permeability and causing mitochondrial dysfunctions such as depolarization, cessation of ATP synthesis, inhibition of respiration, pyridine nucleotide depletion, Ca^{2+} release and matrix swelling. Moreover, this swelling stimulates the cytochrome-c release from the organelles and, consequently, the activation of pro-apoptotic signals inside the cells *(Bernardi & Di Lisa, 2015; Manning & Hearse, 1984; Xia et al., 2016)*.

2.1.2 Oxidative stress

MPTP opening, as previously reported, leads a massive burst of ROS production that include superoxide anion (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radicals, causing oxidative stress. Small amounts of ROS are physiological and positive in normal cell signaling but damaged mitochondria produce large quantities of ROS which finally results in Ca^{2+} overload, further increasing the opening of MPTP (Fig. 4) *(Hausenloy & Yellon, 2013; Xia et al., 2016)*.

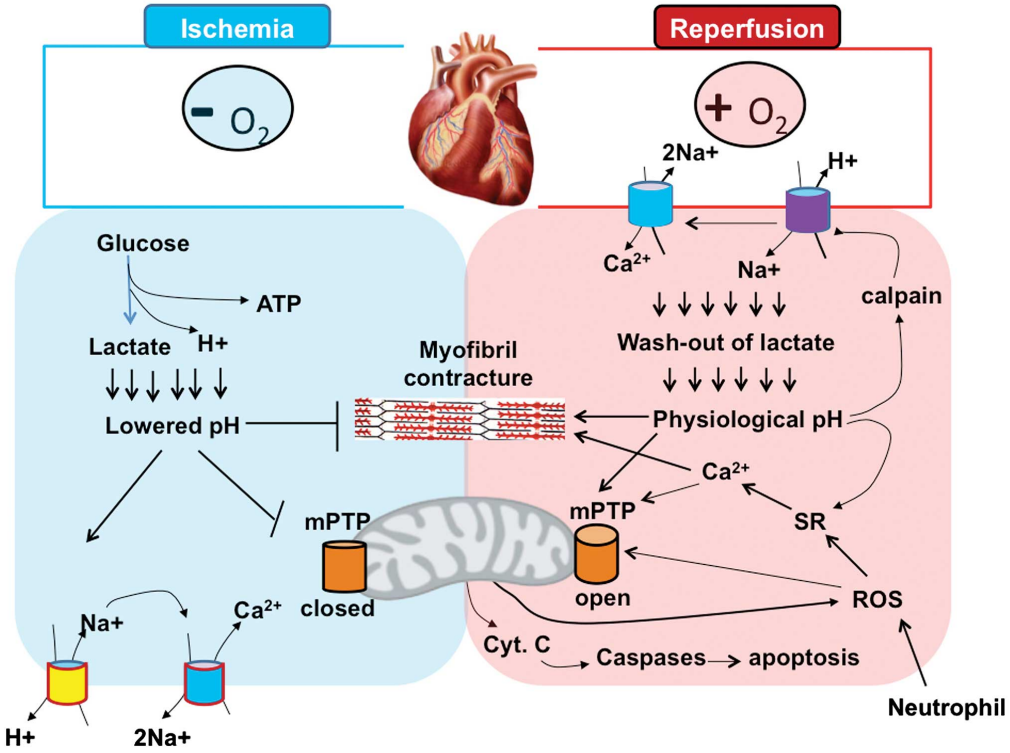

Fig. 4: *Schematic representation of the mechanism of myocardial ischemia reperfusion injury.*

Oxidative stress is also associated to the reduction of the bioavailable nitric oxide (NO) during ischemia and reperfusion injury. NO is produced by NO synthases, which exist in 3 different isoforms: endothelial (e-NOS), neuronal (n-NOS) and inducible (i-NOS). NO has been recognized cardioprotective against IRI. However, it was observed that prolonged ischemia reduces the activation and the activity of the NOS, thus exacerbating cardiac damage. In particular, NO mediates its protective role through its anti-inflammatory and anti-oxidative functions, promoting the activation of distinct mechanisms, including the activation of cGMP, the enhancement of the cyclooxygenase-2 and the inhibition of the mitochondrial Ca^{2+} influx *(Hausenloy & Yellon, 2013)*. Moreover, NO cardioprotective effects were demonstrated in eNOS knockout mice, which showed an increase of

post-ischemic myocardial injury following IRI, as compared to eNOS overexpressing mice, in which, on the contrary, the cardiac functional recovery was improved *(Kanno et al., 2000)*.

In parallel, oxidative stress can be produced also by the xanthine oxidase system and the NADPH oxidase system. Xanthine oxidoreductases are a complex group of enzymes that play a crucial role in purine catabolism. During the restoration of blood flow after ischemia, xanthine oxidase reacts with O_2, inducing the formation of xanthine and uric acid, using oxygen as an electron acceptor. During this reaction, O_2^- and H_2O_2 are released, inducing high levels of oxidative stress *(Brunner et al., 2003; Kanno et al., 2000)*.

The Nox/Duox family of NADPH oxidase also produces ROS during ischemia and reperfusion injury. Indeed, it was reported that Nox overexpressing enzymes increase their activity in IRI and rapidly generate O_2^- , which is then transferred to H_2O_2 that passes through the inner mitochondrial membrane and lead NO degeneration.

Moreover, during IRI, the cells release several chemical molecules that activate NADPH oxidase, such as the phospholipase A2, which triggers a cell signaling pathway, ultimately promoting local inflammation *(Wu et al., 2018)*.

2.1.3 Inflammation

Inflammation is the last player that contributes to the pathogenesis of lethal myocardial ischemia and reperfusion injury. Indeed, acute and chronic immune response is able to induce functional deterioration of the heart. It was also observed that IRI induces sterile inflammation in cardiac tissue, characterized by the activation of the complement, the innate and the adaptive immune system. In particular, neutrophils adhere and infiltrate the vascular tissue, thus activating platelets that can interact whit vascular endothelia, shifting from the low-affinity state to the high-affinity state. This activation induces the release of inorganic polyphosphate that, directly, activates several factors, which contribute to coagulation and inflammation. Moreover, **γδ** T-cells, $CD4^+$ and $CD8^+$ contribute to tissue injury by releasing pro-inflammatory interleukins and chemokines (Eltzschig & Eckle, 2011; Yang et al., 2005).

Moreover, recently, various endogenous ligands called Danger-Associated Molecular Patterns (DAMPs) have been associated to the inflammatory process induced by IRI, besides leukocytes. DAMPs act as “danger signals” during IRI and promote inflammation, finally accelerating apoptosis. Several Toll-Like Receptors (TLRs) present on leukocyte and parenchymal cells are able to bind and respond to these signals inducing damage to several cellular structures (Fig. 5) *(Eltzschig & Eckle, 2011)*.

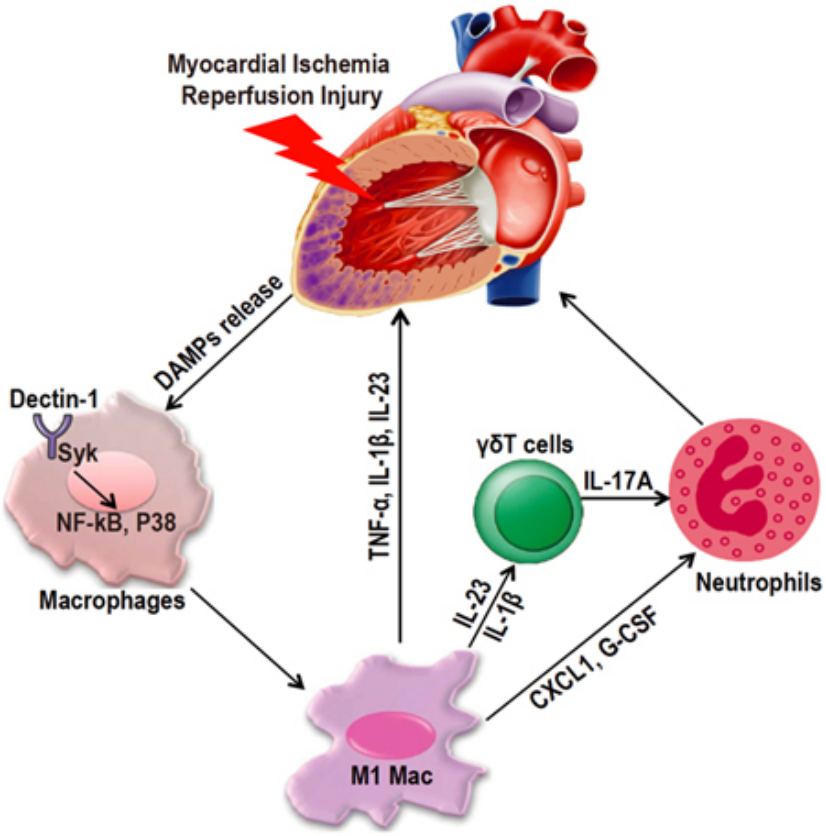

Fig. 5: *Schematic representation of the immunological mechanism of pattern recognition in myocardial ischemia/reperfusion*

The complex molecular scenario associated with IRI has prompted the researchers to investigate different possible approaches to counteract Ischemia and Reperfusion Injury. Among them, the most effective therapies for limiting the infarct size are represented by Ischemic Preconditioning (IPC), Ischemic Postconditioning (IPoC) and Remote Ischemic Conditioning (RIPC).

3. Ischemic Preconditioning

The ischemic preconditioning is obtained by the induction of several cycles of transient non-lethal ischemia that attenuate tissue injury during the subsequent ischemia and reperfusion, and is characterized by a biphasic protective effects.

The early phase occurs immediately after ischemia and lasts for 2 or 3 hours. During this phase, pro-survival proteins prevent harmful modification within the heart.

On the other side, the late phase occurs 12/24 hours and last till 48/72 hours after the initial preconditioning. During this period of time, cytoprotective proteins are produced, protecting cardiomyocytes from cell death *(Frank et al., 2012; Xia et al., 2016)*.

3.1 Pro-survival kinases and the RISK pathway in IPC

The cardioprotective effects of the IPC are mainly mediated by its capacity to stimulate the activation of numerous pro-survival protein kinases, such as the protein kinase C (PKC), the mitogen activated protein kinase (MAPK), the extracellular signal-regulated kinases 1/2 (ERK 1/2) and the protein kinase B or Akt, which could be grouped together in the so-called reperfusion injury salvage kinase (RISK) pathway *(Javadov, Jang, & Agostini, 2014)*. *I*n particular, several studies reported that PKC is activated during IPC by a signaling pathway triggered by the phosphatidylinositol 3-kinase (PI3K), a crucial enzyme, which regulates metabolism and cell survival. Indeed, PI3K activates Akt by phosphorylation, increasing the production of NO and, subsequently, inducing the activity of PKC, ultimately contributing to the beneficial effects of IPC *(Tong, Chen, Steenbergen, & Murphy, 2000; Ytrehus, Liu, & Downey, 1994)*.

PI3K regulates also cardiac cell metabolism during IPC because it promotes the increase of the glycogen synthesis whereas, at the meantime, it induces the inhibition of the glycogen breakdown through Akt phosphorylation, thus inactivating the glycogen synthase kinase (GSK) and protecting the cardiac tissue from the anaerobic metabolic shifting during IRI *(Tong et al., 2000; Ytrehus et al., 1994)*.

However, the most important target of IPC is the mitochondrion. Indeed, it was observed that preventing MPTP opening by the administration of Cyclosporin A at the beginning of the reperfusion phase, reduced infarct size by 40-50% in animal models and protected human atrial trabeculae during IR *(Hausenloy, Maddock, Baxter, & Yellon, 2002)*. Therefore, the inhibition of MPTP opening has been considered the final step of the IPC process since it is triggered by the activation of the RISK pathway. Indeed, the rapid activation of the pro-survival kinase signaling cascade, is sufficient to counteract the MPTP opening, protecting mitochondria from ATP depletion and rigor contracture. In particular, the stimulation of the PI3K and Akt could increase NO synthesis, inhibiting MPTP opening in rat cardiomyocytes *(Shanmuganathan, Hausenloy, Duchen, & Yellon, 2005)*. Conversely, the use of the inhibitors LY294002 and SH-6, specific for PI3K and Akt respectively, completely counteract their protective action against the MPTP opening, confirming the main involvement of the RISK pathway in this process *(Shanmuganathan et al., 2005)*. Furthermore, it was also observed that ERK1/2 phosphorylation levels are modulated during IPC. In particular, the mitochondrial ROS induced by precondition promote the activation of this kinase, contributing to the reduction of the infarct size in murine models (Fig. 6) *(Davidson, Hausenloy, Duchen, & Yellon, 2006; Samavati et al., 2002)*.

3.2 Hypoxia Inducible Factor-1 (HIF-1) in IPC

Recent studies reported that the inhibition of the MPTP opening could also be triggered by the transcriptional heterodimeric complex Hypoxia Inducible Factor-1 (HIF-1). HIF-1 is an oxygen-sense transcription factor which is considered the master regulator of the cellular hypoxic response, orchestrating a protective reaction mediated by the activation of about 200 genes, some involved in the regulation of the cell survival and the heart response to IRI *(Ong & Hausenloy, 2012)*.

HIF-1**α** enzymatic complex is composed by an oxygen-sensitive **α**-subunit (120 kDa) and a constitutively expressed **β**-subunit (91-94 kDa) whom stabilization is finely regulated by prolyl hydroxylases (PHDs). Indeed, the activity of HIF-1**α** is inhibited in normoxic conditions due to its hydroxylation on two specific proline residues in the Oxygen-dependent Degradation Domain (ODD) operated by PHDs, targeting HIF-1**α** to ubiquitination and proteasomal degradation *(Wang, Jiang, Rue, & Semenza, 1995)*. On the contrary, under hypoxic conditions, PHDs are inhibited, allowing the accumulation and translocation of HIF-1**α** to the nucleus where it dimerizes with HIF-1**β**, favoring its binding to hypoxia-responsive elements present and activating transcription. Three different PHDs isoforms have been identified which specifically regulate different isoforms of HIF-**α**. In particular, the most ubiquitous isoform is PHD2, which is specific for HIF-1**α** degradation and not for HIF-2**α**. All the three isoforms are present in the heart, but the most abundant are PHD2 and PHD3 *(Ong et al., 2014)*.

However, HIF-1**α** could also be regulated by protein kinase phosphorylation. Indeed, Akt and ERK1/2 stimulate a signaling cascade, which is important to induce HIF-1**α** activation in response to hypoxia. Interestingly, it was observed that the inhibition of these pro-survival kinases in rat cardiomyocytes was sufficient to abolish both HIF-1**α** activation and the cardioprotective effects of IPC *(Milano et al., 2013; Ong et al., 2014)*.

Thus, it is well accepted that HIF-1**α** stabilization plays a crucial role to improve myocardial tolerance to acute IRI and its up-regulation increases the synthesis of important target genes, such as erythropoietin (EPO), the vascular endothelial growth factor (VEGF), the hemeoxygenase-1 (HO-1) and i-NOS, which are all implicated in IPC-mediated cardioprotection *(Ong & Hausenloy, 2012; Ong et al., 2014)*.

However, also HIF-1**α** could exert its cardioprotective role by interacting with mitochondria. As mentioned before, the production of ROS during IRI guides the opening of the MPTP. Conversely, the metabolic switch from oxidative phosphorylation to anaerobic glycolysis induced by HIF-1**α**, counteracts ROS generation and prevents the MPTP opening. In particular, the glycolytic enzyme hexokinase II (HK-II), a downstream target gene of HIF-1**α**, is able to bind mitochondria during

ischemia and early reperfusion, considerably contributing to the protective effects of IPC in the heart *(Ong et al., 2014)*. The reprogramming of the basal metabolism induced by HIF-1 is the principal physiological mechanism that mediate the protective process against cardiac IRI. To support this evidence, it has been demonstrated that the stabilization of HIF-1**α** in normoxia by a novel specific PHD inhibitor (GSK360A) increased HK-II levels during IRI, stabilizing the cell metabolism and limiting the production of oxidative stress and preventing MPTP opening, ultimately maintaining the mitochondrial integrity *(Ong et al., 2014)*.

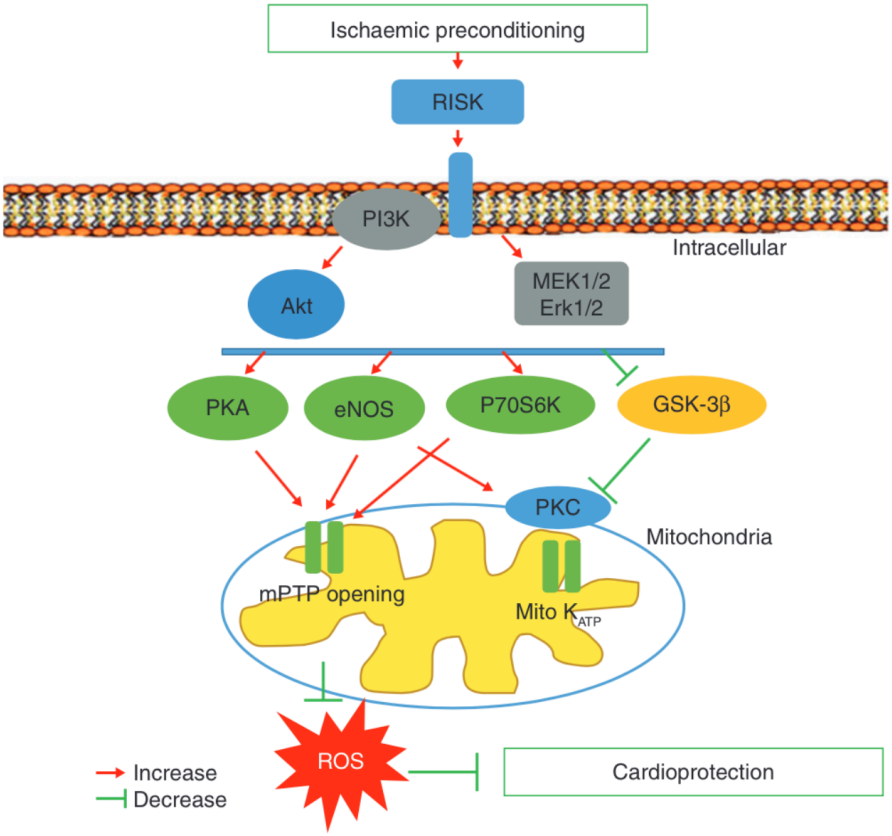

Fig. 6: *Schematic representation of the mechanism of ischemic preconditioning cardioprotection*

4. Ischemic Postconditioning

Although IPC offers some potential benefits to counteract myocardial ischemia, this approach is not really practicable due to the pre-requisite for any preconditioning intervention, which need to be applied prior to the onset of ischemic phase that, in the case of an acute myocardial infarction, is difficult to predict. On the contrary, intervening against IRI, following the onset of acute myocardial infarction, represent a more attractive and feasible prospective. Thus, ischemic postconditioning approaches could be considered the best strategies for cardioprotection *(Hausenloy, 2013)*.

IPoC was first described in 2003 when it was demonstrated that, after 60 minutes of coronary occlusion, 3 cycles of 30 seconds of reperfusion followed by 30 seconds of occlusion, were able to considerably reduce the post-ischemic myocardial infarct size in rabbit *(Zhao et al., 2003)*. Furthermore, also in some clinical settings and surgeries, the application of IPoC induced a significant reduction of the infarct size, also increasing functional recovery *(Hausenloy & Yellon, 2006)*. In particular, the cardioprotective effect of IPoC is mainly mediated by two molecular pathways: the Survival Activating Factor Enhancement pathway (SAFE) and the already mentioned RISK pathway.

4.1 The SAFE pathway in IPoC

The SAFE pathway is triggered at the onset of reperfusion by a moderate increase of the tumor necrosis factor-**α** (TNF-**α**), which binds its receptor TNFR2 and phosphorylates STAT3, promoting its translocation into the nucleus, ultimately inducing the transcription of several stress-response genes. Moreover, in murine models of IRI, phospho-STAT3 migrates also in the mitochondria, regulating the respiratory chain and preserving the MPTP opening *(Lacerda, Somers, Opie, & Lecour, 2009)*. Conversely, in humans, it seems that STAT5, instead of STAT3, plays a relevant role in cardioprotection *(Rossello & Yellon, 2018)*. Interestingly, it was also observed in mice that the STAT3 knockout not only counteracted the cardioprotective effects of IPoC, but also it compromised the phosphorylation of Akt, suggesting the hypothesis that Akt could be activated by STAT3 during IPoC, and supporting the existence of a cross-talk between the SAFE and the RISK pathway *(Xia et al., 2016)*.

4.2 The RISK pathway in IPoC

The RISK pathway has an important role in the setting of ischemic postconditioning. Indeed, it was observed that, in rat hearts, IPoC induced a significant increase of Akt levels, responsible for the activation of NOS and p70S6K *(Tsang, Hausenloy, Mocanu, & Yellon, 2004)*.

As already described for IPC, this protective signaling cascade converges on mitochondria. Specifically, the principal target is represented by the MPTP, whose opening is counteracted. In particular, IPoC induces the phosphorylation and the inactivation of several pro-apoptotic signals, such as Bcl-2 and BAD, which exert their apoptotic action exactly promoting the MPTP opening *(Rossello & Yellon, 2018)*. Furthermore, the activation of NOS, mediated by Akt, stimulates the increase in NO production, as well as the inhibition of GSK3**β**, thus counteracting the opening of mitochondrial pore *(Davidson et al., 2006; Hausenloy, Tsang, & Yellon, 2005)*. Finally, it was also observed that ERK1/2, stimulated by IPoC, forms functional complexes with an isoform of the

mitochondrial PKC, which importantly counteracts the MPTP opening, ultimately conferring cardioprotection *(Baines et al., 2003; Brookes et al., 2000)*.

Based on these premises, the components of the RISK pathway Akt and ERK1/2 seem to be a convergence point of both ischemic pre-and postconditioning, thus representing potential novel therapeutical targets to protect cardiac tissue against IRI. In this regard, the pharmacological up-regulation of pro-survival kinases during reperfusion through the administration of specific drugs could be pursued as a potential innovative cardioprotective approach in the clinical–setting (Fig. 7) *(Rossello & Yellon, 2018)*.

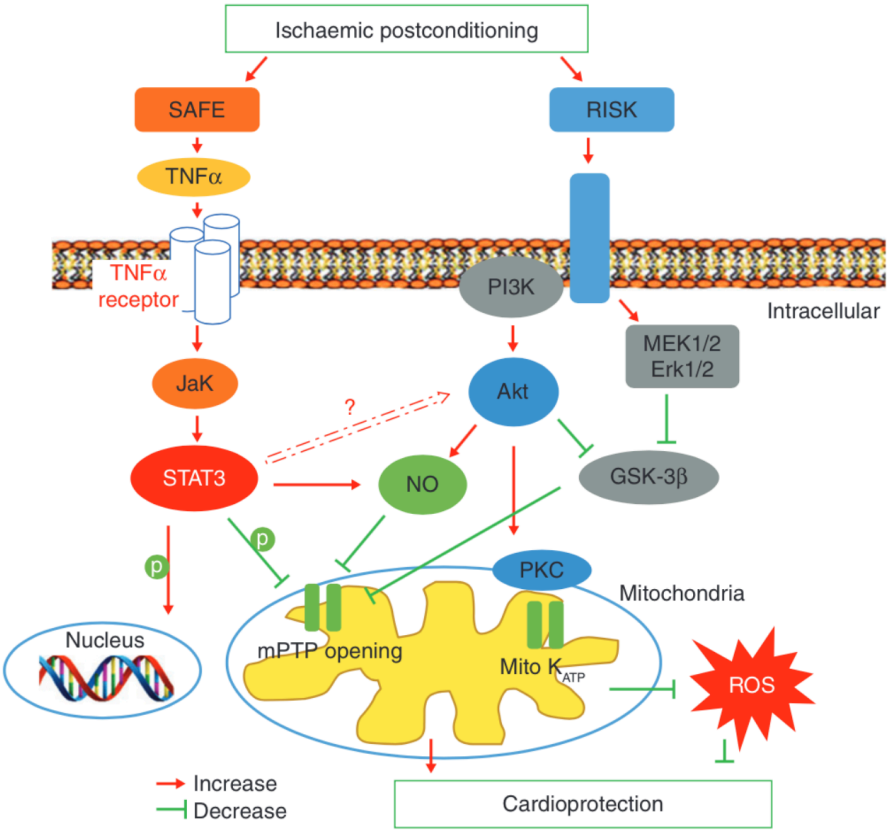

Fig. 7: *Schematic representation of the mechanism of myocardial ischemic postconditioning cardioprotection.*

5. Remote Ischemic Conditioning

Remote conditioning is defined as a technique characterized by one or more cycles of non-lethal ischemia reperfusion in a tissue or an organ distant from the heart, which could be applied both before ischemia (remote preconditioning) ore after the ischemic event (remote postconditioning, RIPC) *(Schmidt et al., 2007)*. This approach was discovered in 1997, when it was observed that repeated occlusion of rabbits' limb could reduce the infarct size after IRI *(Birnbaum, Hale, & Kloner, 1997)*. RIPC was subsequently applied also on humans during cardiac surgeries, resulting cardioprotective, as demonstrated by the reduction of serum troponin release *(Xia et al., 2016)*.

Interestingly, the remote stimuli could be different, such as chemical, pharmacological, electrical or mechanical. The signals are then transferred from the periphery to the target organ, where the final cellular response is activated *(Kleinbongard, Skyschally, & Heusch, 2017)*.

Unfortunately, the mechanism of RIPC still remain unclear, although several signaling mediator have been recognized, including NO, pMAPK, STAT3, ROS, PKC, Akt and ERK1/2, which are the same molecules involved in both IPC and IPoC *(Heusch, Botker, Przyklenk, Redington, & Yellon, 2015)*. Moreover, in patients undergoing heart surgery with a cardiopulmonary bypass it was also observed that HIF-1α expression was increased in the right atrial tissue by RIPC, supporting the possible involvement of HIF-1α in the regulation of the remote protective response *(Albrecht et al., 2013)*. In any case, as well as for IPC and IPoC, mitochondria have been identified as the major common intracellular targets during remote ischemic conditioning *(Kleinbongard et al., 2017)*.

Therefore, it is interesting to observe how all the ischemic conditioning strategies described (IPC, IPoC and RIPC) converge on the activation of pro-survival kinases, especially AKT and ERK1/2, and on the stimulation of the master regulator of the cell response to hypoxia, HIF-1α. Many efforts have been made to better clarify the molecular mechanisms regulated by these molecules, which nowadays represent the most important targets for the development of therapeutic strategies against IRI.

6. The sialidase NEU3

The sialidase NEU3 is a member of the mammalian sialidases family (neuroaminidases or NEUs), which catalyzes the removal of sialic acid residues from glycoproteins and glycolipids, especially gangliosides such as GD1a and GM3. In humans, four different isoforms of sialidases have been recognized, differing for kinetics properties, response to external stimuli and principally for their subcellular localization. Indeed, NEU1 is located in lysosomes; NEU2 within the cytosol; NEU3 is associated to the plasma membrane and NEU4 is known as the mitochondrial sialidase *(Monti et al., 2010)*. In particular, the membrane-bound sialidase NEU3 covers an important role in the regulation of the transmembrane signaling at the level of the cell surface. Moreover, NEU3 possess a trans-activity because it could interact with gangliosides present on the membrane of adjacent cells, thus modulating cell to cell interactions and many signaling cascades *(Papini et al., 2004)*. Along this line, the hydrolytic removal of neuraminic acids from gangliosides could influence many cellular processes, such as cell proliferation and differentiation (Fig. 8) *(Scaringi et al., 2013; Zamora, Ryan, d'Alarcao, & Kumar, 2015)*.

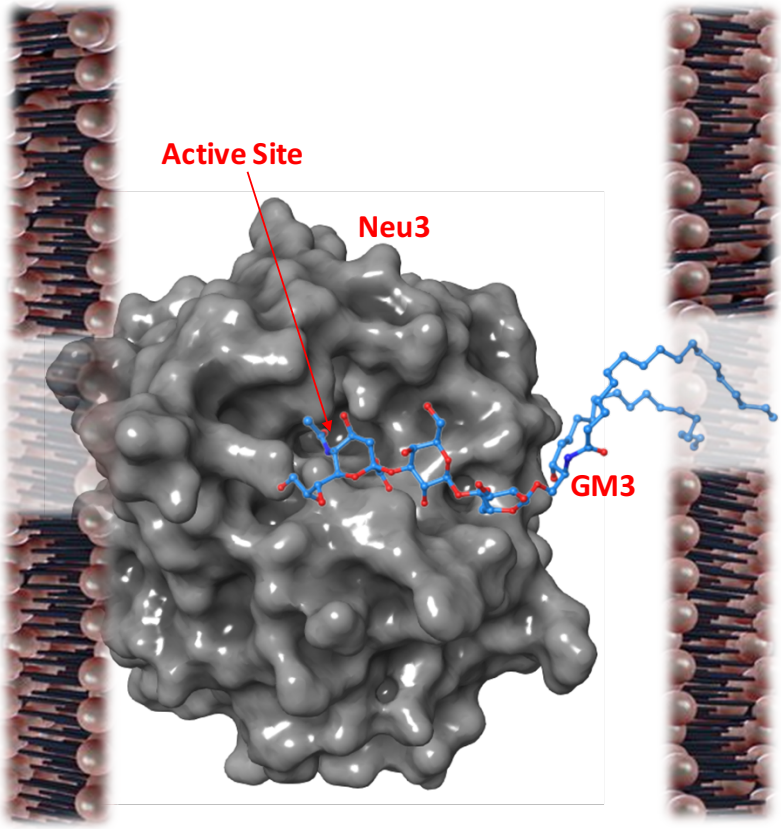

Fig. 8: *Schematic representation of sialidase NEU3. NEU3 removes sialic acid from gangliosides exposed on the outer membrane of adjacent cells.*

6.1 Pathological role of NEU3

Sialidase NEU3 has been studied mainly for its tumorigenic potential and its involvement in cancer progression. Particularly, it was demonstrated that altered sialylation is closely related to malignant properties, including metastatic potential and invasiveness *(Miyagi, 2008)*. Moreover, an aberrant expression of NEU3 has been linked to the carcinogenesis process, causing an alteration in the regulation of the transmembrane signaling at the cell surface *(Lau & Dennis, 2008; Scaringi et al., 2013)*. Furthermore, it was observed that an up-regulation of the membrane-bound sialidase NEU3 is associated to various human cancers, such as kidney, ovary, prostate and predominantly colorectal cancer *(Shiga et al., 2015; Takahashi et al., 2015)*. Indeed, it was reported, *in vivo*, in transgenic mice, that NEU3 participated to the formation of colonic aberrant crypt foci. In particular, NEU3 overexpression triggered the activation of the Wnt/**β**-catenin signaling pathway, participating in its crosstalk with the endothelial growth factor receptor (EGFR) pathway, whose activation is considered to be essential for the initiation and progression of colon cancer *(Takahashi et al., 2015)*. Therefore, the alteration of gangliosides content and composition on the cell membranes caused by NEU3 up-regulation, is related to the maintenance of the cellular self-renewal and the tumorigenic potential in transgenic mice *(Tringali et al., 2012)*.

Furthermore, there are evidences that also in human cells NEU3 is significantly up-regulated in colon cancers, altering the sialylation state of cell surface glycoproteins and their resistance to apoptosis. Indeed, in this study, it was observed that colon cancer HCT-116 cells showed increased expression of NEU3 in terms of both mRNA levels and enzymatic activity. Moreover, NEU3 up-regulation resulted in the inhibition of apoptosis, accompanied by the increase of the anti-apoptotic gene Bcl-2 and a decrease of the caspase-3 and caspase-9 expression. In particular, the NEU3-mediated inhibitory role of apoptosis could be also driven by the accumulation of lactosylceramide, which is the product of GM3 degradation, thus confirming that NEU3, through sphingolipids modulation, might influence several cellular processes, such as cell death *(Kakugawa et al., 2002)*.

6.2 Physiological role of NEU3

Although the sialidase NEU3 has been mostly characterized for its pathological role in tumors, this protein is highly conserved in all species and, basically, it is expressed in almost all tissues *(Miyagi & Yamaguchi, 2012)*. Thus, based on these premises, it is quite obvious that NEU3 should also play some physiological roles.

To this regard, few years ago, our research group focused its attention on the NEU3 involvement in physiological processes.

Interestingly, we previously demonstrated that the NEU3 localization on the plasma membrane is really dynamic and that this enzyme could be found also in endosomal vesicular structures *(Cirillo et al., 2016)*. Indeed, NEU3 could be present in two different cell districts, the plasma membrane and the cytosol, suggesting the possible existence of a dynamic equilibrium of the enzyme within the cell, and supporting its involvement in regulating several cellular processes. In particular, our study revealed that NEU3 has a different role on the cell membrane and in the endosome. Indeed, it was observed that the endosomal compartment functions as a storage for the inactive form of the enzyme, which is ready to be translocated to the outer plasma membrane leaflet, when required, such as in response to stress conditions *(Cirillo et al., 2016)*.

Regarding stress conditions, our interest moved in the direction of discovering the involvement of NEU3 in the cell response to the hypoxic stress.

To this purpose, the enzyme was both stably overexpressed and silenced in murine skeletal myoblasts C2C12, that were cultured under hypoxic conditions and compared with controls C2C12. This study showed that NEU3 overexpressing cells (L-NEU3) proliferated more than controls in normoxia, whereas silenced cells (i-NEU3) grew less than control cells. Interestingly, at 1% O_2, L-NEU3 had a proliferation reduction that was much lower than controls. Conversely, i-NEU3 cells underwent a

very big drop in cell proliferation. Moreover, L-NEU3 cells showed a marked reduction in caspase 3/7 activation, accompanied by a lower degree of cytotoxicity, as compared with wild-type C2C12. On the other hand, i-NEU3 were characterized by higher caspase activation and cytotoxicity degree than controls. Therefore, this study revealed that an increase of both sialidase expression and activity in skeletal myoblasts was protective against hypoxic conditions *(Scaringi et al., 2013)*. Moreover, to support our findings, NEU3 expression is regulated by Sp1/Sp3 transcription factors, which are also up-regulated in hypoxia *(Yamaguchi et al., 2010)*

However, the most interesting finding of our work was the identification of a novel molecular mechanism of HIF-1α activation, which is different from the "canonical" inhibition of prolyl hydroxylase 2 (PHD2) and is mediated by the sialidase NEU3 and the EGFR pathway activation. In particular, we demonstrated that NEU3 can activate the EGFR pro-survival signaling cascade by controlling ganglioside GM3 content, which is a known EGFR auto-phosphorylation inhibitor *(Scaringi et al., 2013)*. Therefore, NEU3 up-regulation causes a reduction of this ganglioside, promoting EGFR activation and inducing its downstream signaling pathway, which includes several pro-survival kinases, such as Akt, p70S6K and ERK1/2, ultimately leading to an increase of the HIF-1α levels. Finally, the increased expression of HIF-1α, together with the activation of several pro-survival kinases, were the responsible of the protective effects induced by the sialidase NEU3 against acute hypoxia (Fig. 9) *(Scaringi et al., 2013)*.

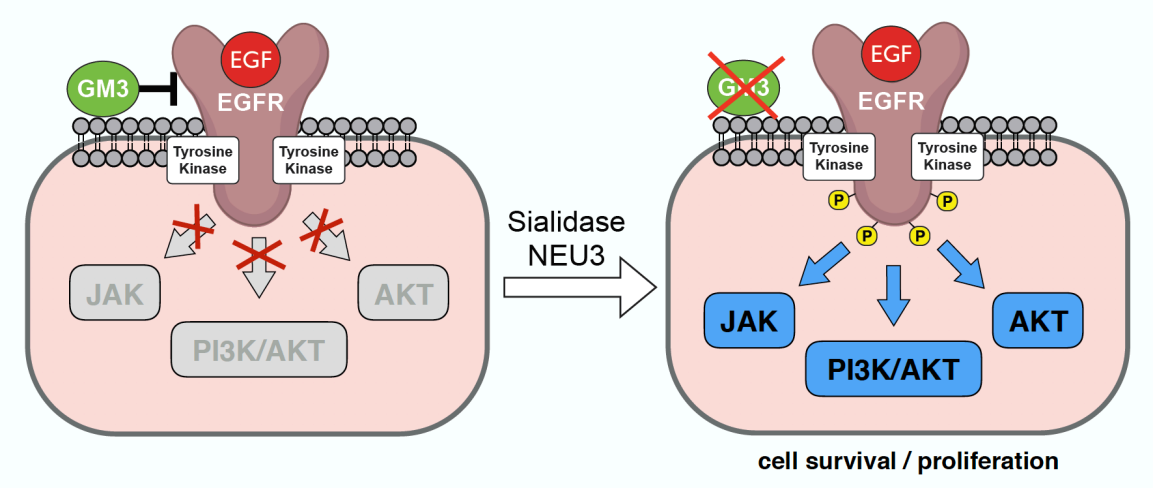

Fig. 9: *Scheme of EGFR signaling pathway activated by NEU3-induced GM3 reduction.*

More recently, our research group investigated the role of NEU3 in the response to hypoxic conditions also in humans. For this purpose, we performed our analysis on heart biopsies, from the right atrial appendage of cyanotic patients undergoing surgeries for congenital heart defects, whose hearts are exposed to chronic hypoxia, contributing to the disease pathophysiology *(Piccoli et al., 2017)*.

Obviously, HIF-1**α** expression of cyanotic patients was increased both in terms of mRNA and protein levels, as compared to acyanotic cardiac patients, who have been used as controls. Moreover, the expression of several HIF-1**α** downstream targets, involved in glucose metabolism, such as the glucose transporter protein type-1 (GLUT1), the pyruvate dehydrogenase kinase-1 (PDK1) and the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were more expressed in cyanotic samples, confirming the shift from the oxidative to the glycolytic metabolism *(Piccoli et al., 2017)*.

Interestingly, also the expression of the sialidase NEU3 was affected by chronic hypoxia. Indeed, both its mRNA levels and the protein associated to the plasma membrane were increased in cyanotic patients, as well as the expression of the transcription factors SP1 and SP3, as compared to control samples. Furthermore, cyanotic patients showed an increased activation of the EGFR, as well as of its downstream targets ERK1/2, AKT and p70S6K, confirming our hypothesis of a direct involvement of the sialidase NEU3 in regulating HIF-1**α** levels under chronic hypoxia conditions, through the stimulation of the EGFR signaling cascade (Fig. 10). Finally, to support our findings, sialidase NEU3 expression positively correlates with HIF-1**α** expression levels in both cyanotic and acyanotic patients *(Piccoli et al., 2017)*.

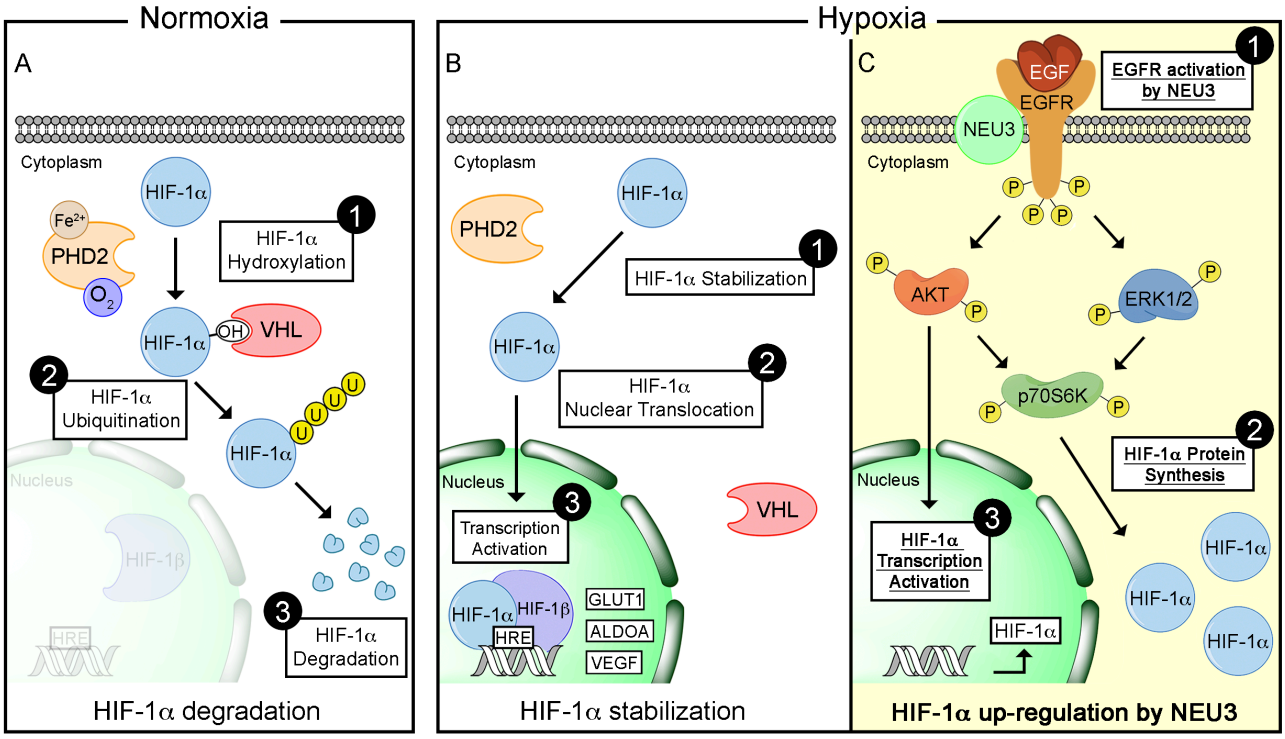

Fig. 10: *Representation of a novel HIF-1α mechanism of activation in congenital cyanotic patients mediated by NEU3.*

AIM OF THE STUDY

It is now clear that many mechanical, extracellular and intracellular processes are involved in the pathogenesis of ischemia and reperfusion injury, and several interconnected critical factors have been identified as responsible for cardiac tissue damages *(Ferdinandy, Schulz, & Baxter, 2007)*. Many efforts have been made seeking cardioprotective approaches to be applied as adjunctive tools to existing reperfusion interventions and, in particular, ischemic preconditioning and postconditioning strategies resulted as the most effective therapies for limiting the infarct size *(Yellon & Hausenloy, 2005)*. Both strategies converge on the activation of a molecular mechanism which involves a signaling cascade known as RISK pathway and on the activation of HIF-1α. HIF-1α plays a critical role in orchestrating the cell defense machinery against ischemia through the transcriptional activation of up to 200 genes, which are critical for cell survival and metabolic adaptation to low oxygen, which therefore may be important during IRI *(Ong et al., 2014; Tekin, Dursun, & Xi, 2010)*. As previously described, our research group discovered a new pathway of HIF-1α activation during hypoxia, which is different from the inhibition of prolyl hydroxylase 2 and is mediated by the sialidase Neu3 and the EGFR signaling cascade.

Therefore, based on our previously results in hypoxic condition, and well aware that hypoxia is a fundamental component of the ischemic phase, we wondered whether an up-regulation of NEU3 could also increase myocardial cell resistance to IRI.

To pursue this aim, we employed both molecular and cellular biology approaches. We developed an *in vitro* model of ischemia and reperfusion that was used to study how NEU3 expression and activity were modulated under IRI conditions. The same model has been employed to test whether NEU3 overexpression could also promote cardiac myoblast survival against ischemia and reperfusion.

Moreover, considering the involvement of the sphingolipid metabolism in the regulation of the cardiac fibrosis process, which is strictly correlated with IRI, the effects of NEU3 overexpression have been also analyzed in an *in vitro* model of fibrosis, using human cardiac fibroblasts.

Clearly, the possibility to demonstrate that the sialidase NEU3 is crucial during the cardiac cells response to IRI and that its up-regulation could increase cardiac resistance to the ischemia and reperfusion damage, both increasing cardiomyocytes survival and reducing the scar formation, arise new translational perspectives related to this protein. Therefore, sialidase NEU3 could represent a possible novel target for the development of more effective therapeutic strategies for CVDs treatment.

MATERIALS & METHODS

1. Cell culture and treatments

A. H9C2 Cardiomyoblasts

H9C2 rat cardiomyoblasts were obtained from Sigma-Aldrich and cultured in Dulbecco's modified Eagle's medium with low glucose (DMEM, Sigma-Aldrich), containing 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 U/ml penicillin and 100 mg/ml streptomycin at 37°C in 5% CO_2, 95% air-humidified atmosphere. To mimic ischemic conditions, cells were cultured for different time lengths in an hypoxic hood (SCI-tive, Baker Ruskinn) in the presence of DMEM without glucose, L-glutamine, phenol red, sodium pyruvate and sodium bicarbonate. Cells were brought back to 37°C in 5% CO_2, 21% O_2 and cultured in DMEM low glucose with supplements to simulate reperfusion.

B. Cardiac fibroblasts

Human cardiac fibroblasts were obtained from right atrial appendage biopsies of patients who underwent cardiac surgery correction with extracorporeal circulation at the Cardiac Surgery Division of the IRCCS Policlinico San Donato Hospital. All subjects, who respected the inclusion and exclusion criteria, gave their informed consent before being enrolled in the study, which was conducted in accordance with the Declaration of Helsinki, and the protocol was approved by the Ethics Committee.

Cardiac fibroblasts were cultured in Dulbecco's modified Eagle's medium with high glucose (DMEM, Sigma-Aldrich) containing 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 U/ml penicillin and 100 mg/ml streptomycin at 37°C in 5% CO_2, 95% air-humidified atmosphere.

2. Fibroblasts activation

Cardiac fibroblasts were plated at 80-90% confluency and serum-starved for 48 hours. Then, human recombinant TGF-**β** (Peprotech) was added to a final concentration of 10 ng/ml for 72 hours.

3. Stable overexpression of NEU3 in H9C2 cells and cardiac fibroblasts

One day before transfection, H9C2 were plated at a density of 1 x 10^5 cells in growth medium without antibiotics to reach 70-80% confluency at the time of transfection. Cells were divided in two groups: one transfected with a Neu3 Lentiviral Vector (Rat) (CMV) (pLenti-GIII-CMV-GFP-2A-Puro) (Applied Biological Materials) and one transfected with a scramble (SCR) plasmid, used as controls, according to the ViaFect™ Transfection Reagent manufacturer's protocol.

Transfected clones were isolated using 10 mg/ml puromycine (Invivogen) and were checked for both NEU3 expression and activity. The clone with the highest NEU3 expression and activity was selected for the further experiments.

Cardiac fibroblasts, instead, were infected with a lentiviral system containing the human sialidase NEU3 coding sequence. The stably overexpressing NEU3 cells were isolated after selection with 5 μg/ml blasticidin (Thermo Fisher Scientific), and used for the further experiments.

4. RNA extraction and gene expression by quantitative PCR (qPCR)

Total RNA was isolated with Reliaprep^{TM} RNA cell miniprep system (Promega), following the manufacturer's protocol.. Then, 1 μg of RNA was reverse transcribed to cDNA with the iScript cDNA synthesis kit (Bio-Rad), according to the manufacturer's instructions. Real time PCR was performed with 10 ng of cDNA template, 0.2 μm primers, and 1× GoTaq® qPCR Master Mix (Promega) in 20 μl of final volume, using a StepOnePlus® real time PCR system (Applied Biosystem). The amplification protocol was: 95°C for 2 min, 40 cycles of 5 seconds each at 95°C, 30 seconds at 57°C and 30 seconds at 72°C, and a final stage at 72°C for 2 minutes.

The relative quantification of the expression of target genes was calculated by the equation $2^{-\Delta\Delta Ct}$ using the RPLI gene as an housekeeper. Melting curves were monitored to guarantee the accuracy and the specificity of the amplicon. All reactions were performed in triplicate. The primers sequences are listed in Table 1.

Gene	Forward Primer	Reverse Primer
Rat NEU3	5'-ATGCCCTCTGATGGACAGAT-3'	5'-CATGTCCCTGATGGTGCTC-3'
Rat RPL13A	5'-TCTCCGAAAGCGGATGAACAC-3'	5'-CAACACCTTGAGGCGTTCCA-3'
Human NEU3	5'-TGGTCATCCCTGCGTATACC-3'	5'-TCACCTCTGCCACTTCACAT-3'
ACTA2	5'-CTGGACTCTGGAGATGGTG-3'	5′-GCAGTAGTAACGAAGGAATAGC-3′
Collagen 1	5'-CGACCTGGTGAGAGAGGAGTTG-3'	5'-AATCCATCCAGACCATTGTGTCC-3'
UBC	5'-CTGGAAGATGGTCGTACCCTG-3'	5'-GGTCTTGCCAGTGAGTGTCT-3'
S14	5'-GTGTGACTGGTGGGATGAAGG-3'	5'-TTGATGTGTAGGGCGGTGATAC-3'

Table 1*: qPCR primers sequences*

5. Sialidase Activity Assay

Sialidase activity assay was performed to investigate the enzymatic functionality of the sialidase NEU3. The activity of the enzyme was determined by an assay based on the 2′-(4-Methylumbelliferyl)-**α**-D-N-acetylneuraminic acid. The removal of the sialic acid by the neuraminidase results in the formation of a fluorescent product, which is directly proportional to the neuraminidase activity in the sample.

In particular, H9C2 cell samples were obtained by scraping and centrifugation and then resuspended in PBS with a protease inhibitor cocktail (SIGMA-Aldrich). Subsequently, they were lysed by two cycles of sonication and centrifuged at 800 x *g* for 10 min to eliminate all nuclear components and the residues of broken cells. The obtained supernatant was centrifuged at 30000 x *g* for 75 min with an Avanti^{TM} J-30I Centrifuge (Beckman) to isolate the membrane fraction. The sialidase activity associated to this fraction was assayed by incubating 30 μg of protein with the 4-MU-NeuAc at pH 3.8 for 1 hour at 37°C. At the end of the incubation, glycine 0.2 M was added to each sample to stop the entire reaction. The fluorescent signal was read by a multiplate reader (Varioskan Lux, Thermo Scientific) with an excitation wavelength of 365 nm and an emission filter of 448 nm.

6. Cell growth analysis

Cell count was performed by the trypan blue assay on H9C2 plated in 35 mm Petri dishes after 12 hours of ischemia and 12 – 24 – 36 – 48 hours of reperfusion. Cell number was determined by the automated cell counter Countess II FL™ (Life Technologies).

7. Cytotoxicity detection

CellTox^{TM} Green Cytotoxicity Assay (Promega), was used to investigate the cytotoxic effect of the ischemia-reperfusion treatment on H9C2 cells, according to the manufacturer's instructions.

The CellTox^{TM} Green Cytotoxicity Assay measures changes in the membrane integrity that occur as a result of cell death. This assay system uses a proprietary asymmetric cyanine dye that is excluded by viable cells but, preferentially, stains the dead cell's DNA. When the dye binds the DNA in cells with impaired membranes is activated and emits fluorescence. Viable cells do not produce any appreciable increases in fluorescence.

Thus, the fluorescent signal emitted by the dye bonded DNA is directly proportional to cytotoxicity. Briefly, 2.5 x 10^3 cells were plated in triplicate in 96 dark-walled plates, exposed to 12 hours of ischemia and 3 – 6 – 12 – 24 – 48 hours of reperfusion. Buffer containing a 1:1000 dilution of CellTox Green Dye was added to each well and incubated at room temperature in the dark for 15 minutes. The

fluorescent signal was detected by Varioskan Lux with an excitation wavelength of 480-500 nm and an emission filter of 520-530 nm.

8. Apoptosis assay

Apoptosis was evaluated by nuclear DAPI staining. 7 x 10^4 cells were plated in 35 mm Petri dishes and exposed to 12 hours of ischemia and 3 – 6 – 24 – 48 hours of reperfusion. At any time-point analyzed, cells were fixed in paraformaldehyde 4% for 15 min at room temperature (RT) and then washed 3 times with PBS. Blocking and permeabilization were performed in PBS with 5% BSA + 0.1% Triton-X100 for 15 min RT, followed by staining with Hoechst 33258 (1:500 dilution) for 15 min at RT in the dark. Apoptotic cells were analyzed under a fluorescent microscope (Olympus TH4-200) with magnification 20x. The % of cell death was obtained by counting the number of altered nuclei in 15 different fields for each sample, which was then normalized by the number of the total nuclei.

9. Caspase 3/7 activation assay

Caspase 3/7 activation were analyzed using a luminescent assay (Caspase-Glo® 3/7 Assay Kit, Promega). The Caspase-Glo® 3/7 assay provides a proluminescent caspase-3/7 substrate, which contains the tetrapeptide sequence DEVD. This substrate is cleaved by caspase to release aminoluciferin, a substrate of luciferase used in the production of light. Mixing the Caspase-Glo® 3/7® reagent to the samples results in cell lysis, followed by the caspase-mediated cleavage of the substrate and the generation of a luminescent signal. Thus, the luminescence detected is proportional to the caspase activity of the samples.

Briefly, H9C2 cells were seeded 7 x 10^4 in 35 mm Petri dishes and then exposed to 1 – 3 – 6 – 12 hours of ischemia and 3 – 6 – 12 - 24 – 48 hours of reperfusion. At the end of each time-point, 350 µl of Caspase-Glo® 3/7 Reagent were added to the samples and incubated 1 hour at RT in the dark. At the end of the incubation, 200 µl of each sample were transferred into a 96 white-walled plate in triplicate and the luminescent signal was measured using Varioskan Lux. In parallel, for each time-point, a cell count was performed by trypan blue assay as described in paragraph 5. The levels of Caspase 3/7 activation were calculated as the ratio of caspase activity detected, normalized by the number of cells.

10. Protein extraction and Western Blot analysis

For protein expression analysis, cells were lysed with RIPA buffer (1% Nonidet P-40 in 50 mm Tris-HCl, pH 7.5, 150 mm NaCl, 0.1% sodium deoxycholate, 1% protease inhibitor cocktails), incubated in ice for 30 minutes and then centrifuged at 13000 x *g* for 15 minutes at 4°C. The supernatant was collected and the Protein concentration was determined by the Pierce BCA Assay Kit (Thermo Scientific). Then, 30 µg of total protein were denatured for 5 min at 100°C in Sodium Dodecyl Sulfate (SDS) sample buffer and separated by SDS-Page, before being transferred to nitrocellulose membranes. To block non-specific binding sites, membranes were incubated with Tris-HCl buffer pH 7.5 (TBS) containing 5% bovine serum albumin (BSA) or 5% non-fat dried milk for 1 hour and then incubated with primary antibodies at 4°C overnight. The following primary antibodies were used: anti-**α**-SMA (1:5000, Sigma Aldrich), anti-collagen I (1:1000, Invitrogen), anti-GM3 synthase (1:1000, Santa Cruz), anti-phospho-Akt T308 (1:1000, Cell signaling), anti-Akt (1:1000, Cell signaling), anti-phospho-p44/42 MAPK (p-Erk1/2) T202/Y204 (1:1000, Cell signaling), anti-p44/42 MAPK (Erk1/2) (1:2000, Cell signaling), anti-HIF-1**α** (1:1000, Cell signaling), anti-Calnexin (1:10000, Abcam). The membranes were washed with TBS + 0.1% Tween20 4 times for 5 min, and incubated with the appropriate secondary antibodies HRP-conjugated for 1 hour at RT. Membranes were subsequently washed with TBS + 0.1% Tween20 and bands were identified using Luminata™ Forte Western HRP Substrate (Millipore Corporation) as reported in the relative protocol. Each experiment was performed in triplicate and the quantitative analysis of the bands intensity was performed using the Image Studio Lite software.

11. NEU3 chemical inhibition in H9C2

For the chemical inhibition of NEU3, the cells have been treated with both the general mammalian sialidase inhibitor 2,3-Dehydro-2-deoxy-N-acetylneuraminic acid (DANA) and the sialidase NEU3 specific inhibitor C9-modified Zanamivir analogue (LR332), used at 50 µM concentration.

The molecules have been both synthesized in collaboration with our chemistry laboratory at the University of Milan.

12. Dual- Luciferase Reporter Assay

The stability of HIF-1**α** in cells exposed to different time length of ischemia (1 – 3 – 6 – 12 hours) has been evaluated using the Dual-Glo® Luciferase Assay System Kit (Promega).

The Dual-Glo® Luciferase Assay allows a high-throughput analysis in mammalian cells containing the reporter genes Firefly Luciferase (ODD-Luciferase-pcDNA3) and Renilla luciferase (pRL-

CMV). This reagent, directly added to cell medium, induces cell lysis and acts as a substrate for the firefly luciferase. Addition of the Dual-Glo® Stop and Glo® Reagent quenches the luminescence from the firefly reaction by at least 10,000-fold and provides the substrate for Renilla luciferase in a reaction that can also be read within 2 hours (with a similar retention in signal).

In particular, H9C2 were seeded 5 x 10^3 in a 96-well plate and exposed to 1 – 3 – 6 – 12 hours of ischemia. Cells were transfected with an ODD-luciferase-pcDNA3 (80 ng) or an empty-luciferase-pcDNA3 (80 ng) and pRL-CMV (8 ng), used as an internal control, according to the ViaFect Transfection Reagent® protocol (Promega). At the end of the treatment, an equal volume of Dual-Glo® Luciferase Reagent to the volume of culture medium was added to the samples, mixed well and incubated for 30 minutes at RT in the dark. When the incubation was concluded, the solution was transferred to 96-well white plates and the luminescence emitted at 550 nm by the Firefly luciferase was measured using Varioskan Lux (Thermo Scientific). Then, to turn off the luminescence of the Firefly luciferase and provide the substrate for the Renilla luciferase, it was added an equal volume to the original culture medium of Dual-Glo® Stop and Glo Reagent. After 30 minutes of incubation, luminescence emitted was detected at 480 nm with Varioskan Lux (Thermo Scientific).

HIF-1**α** stability levels have been obtained by calculating the ratio of luminescence from the experimental reporter (ODD) to luminescence from the control reporter.

13. Immunofluorescence staining

H9C2 cells were plated in 6-well plates 3 x 10^5 for one day. Subsequently, cells were subjected to serum starvation for 48 hours and then treated with TGF-**β** 10 ng/ml for 72 hours. At the end of the treatment, cells were washed 3 times in PBS and fixed for 15 min in 4% paraformaldehyde at room temperature. For permeabilization and blocking, the cells were incubated for 1 hour in the presence of PBS 0.1% Triton X-100 (TX-100) and 5% fetal bovine serum (FBS) at RT. Then, cells were incubated with an anti-Smooth Muscle **α**-Actin antibody (Sigma-Aldrich) diluted 1:200 in PBS 0.1% Triton X-100 (TX-100) and 5% FBS for 2 hours at RT. Subsequently, cells were washed 3 times in PBS and incubated with an anti-mouse FITC-conjugated secondary antibody (Jackson ImmunoResearch) for 1 hour at RT. After 3 washing in PBS, cell nuclei were stained with Hoechst 33258 (1:500 dilution). At the end of the staining, cells were analyzed under a fluorescent microscope (Olympus TH4-200) equipped with an acquisition camera, with magnification 10x. Finally, the **α**-SMA positive cells were compared to the **α**-SMA negative controls.

14. GM3 synthase silencing

Specific siRNA duplexes targeting the GM3 synthase, siRNA transfection reagents, and reduced-serum transfection medium were purchased from Santa Cruz Biotechnology. The day before transfection, 7 x 10^5 cardiac fibroblasts were seeded in each well of a 12-well cell culture plate in DMEM low glucose, containing 10% FBS without antibiotics and incubated for 24 hours at 37°C and 5% CO_2. The next day, transfection complexes were prepared using 3 μg of GM3 synthase siRNA, siRNA transfection reagent, and transfection medium according to the manufacturer's protocol and were added to each well. A scrambled siRNA (Santa Cruz Biotechnology) was used as negative control.

15. Statistical Analysis

For all quantified data, mean ± SD values are presented. The Student's *t*-test was used to determine significance using Prism 8 Software. *P* values of less than 0.05 were considered to be significant. All *P* value were calculated from data obtained from at least of three independent experiments. All error bars represent the standard deviation of the mean.

All the results are expressed as Relative Quantity (R.Q), which allows to measure differences between different samples, and represented as fold increase or decrease in comparison to a reference sample control (CTRL=1).

RESULTS

The results obtained during my PhD, finalized to investigate the possible involvement of sialidase NEU3 in the cardiac response to ischemia and reperfusion injury, will be presented and analyzed in this chapter.

1. Ischemia and Reperfusion in vitro model

Before starting to perform any experiment, we optimized an *in vitro* model of ischemia and reperfusion injury (IRI), using rat cardiomyoblasts H9C2. To mimic ischemia, cells were cultured for 12 hours at 1% O_2 in a hypoxic chamber with pre-conditioned ischemic medium (DMEM no glucose, without any supplement). Cells were then switched to normoxic condition (21% O_2) in complete growth medium (DMEM low glucose, 10% FBS) up to 48 hours, to obtain reperfusion (Fig. 1). To validate our *in vitro* model, proliferation and toxicity have been evaluated after 12 hours of ischemia and at different time lengths of reperfusion. Results showed that IRI treatment caused a marked reduction in cell proliferation, together with a progressive increase in cell toxicity, that reach a peak right after the ischemic treatment (12 hours) and at 24 hours of reperfusion (Fig. 2A-B).

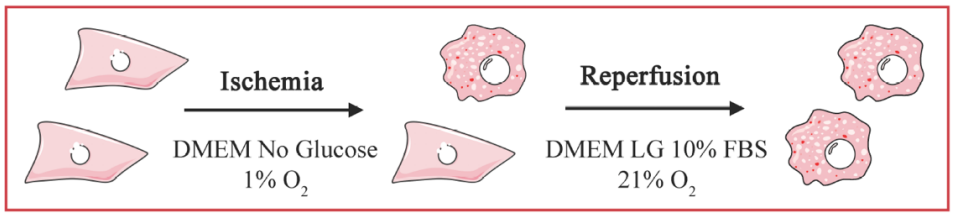

Fig 1: *Schematic representation of the ischemia and reperfusion in vitro model.*

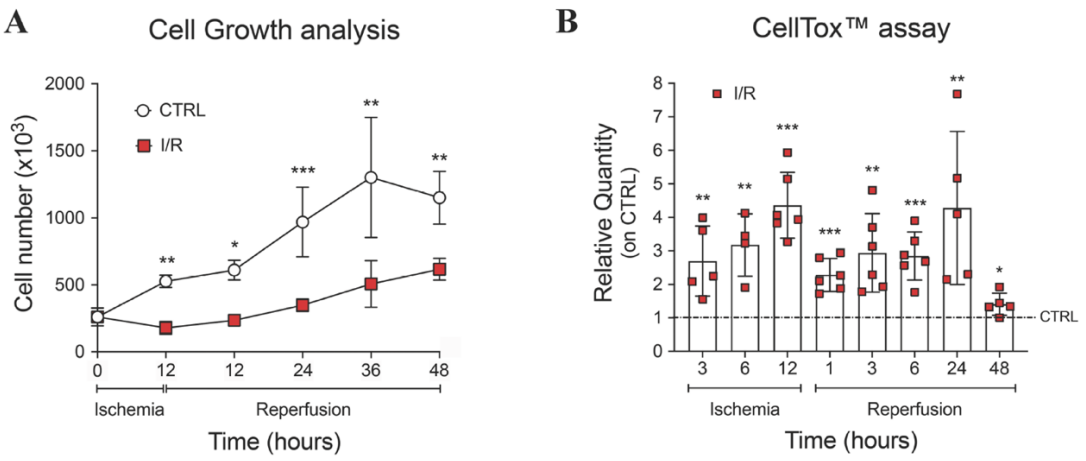

Fig 2: *Effects of IRI on H9C2 in terms of proliferation (***A***) and cytotoxicity (***B***). Data represent mean ± SD of 5 independent experiments. Statistical significance was determined by Student's t test. *P<0.05; **P<0.01; ***P<0.001.*

2. NEU3 modulation under ischemia and reperfusion

Subsequently, to investigate how ischemia and reperfusion could influence sialidase NEU3, we evaluated NEU3 mRNA levels and the sialidase activity during IRI. Interestingly, we observed an initial up-regulation of NEU3 in the early phase of ischemia, followed by a progressive decrease in the sialidase expression and activity that culminated after 12 hours of ischemia exposure. NEU3 was then reactivated during reperfusion (Fig. 3A-B).

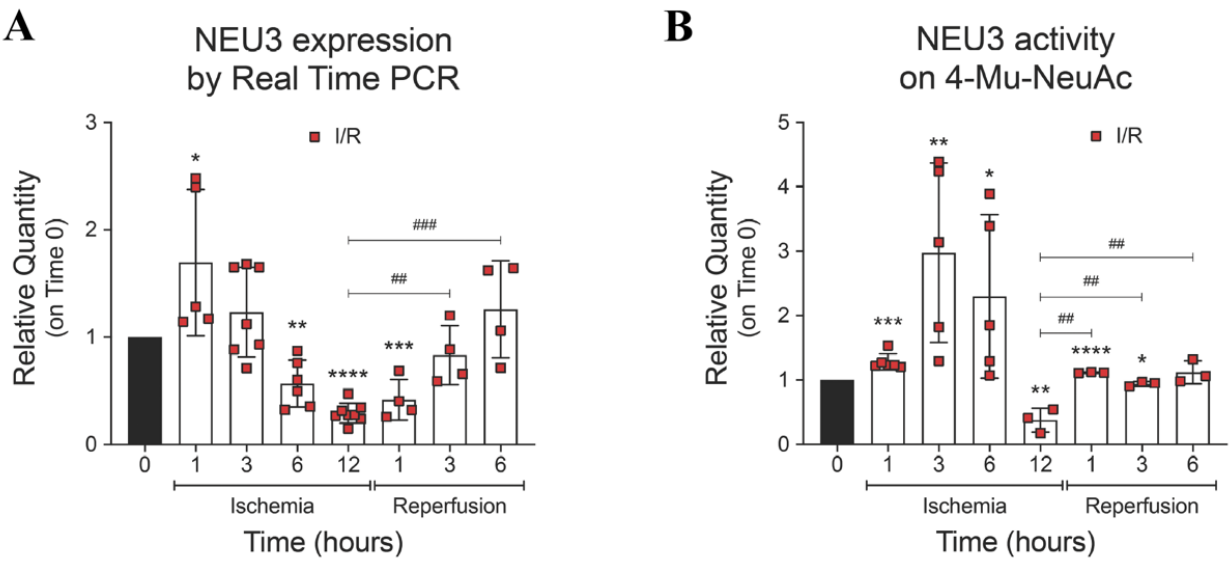

Fig. 3: *Effects of IRI on NEU3 expression (***A***) and activity (***B***). Data represent mean ± SD of 3-5 independent experiments. Statistical significance was determined by Student's t test. *P<0.05; **P<0.01; ***P<0.001; ****P<0.0001; ##P<0.01; ###P<0.001.*

3. NEU3 overexpression in cardiomyoblasts H9C2

To further investigate the role of NEU3 in IRI, we stably overexpressed the enzyme in H9C2 cells with a lentiviral vector containing the rat sialidase NEU3 coding sequence and a Green Fluorescent Protein (GFP) that allowed us to identify the transfected cells (Fig. 4A). Control cells were transfected with an empty scramble (SCR) vector. Several clones were selected and tested for NEU3 expression and activity and the clone which showed the highest sialidase activity (about 3-fold higher than controls) was used for the further experiments (Fig. 4B).

Then, SCR and NEU3-overexpressing cells (hereafter simply NEU3-cells) were subject to IRI, as described above, and a cell growth analysis was performed. As expected, both SCR and NEU3 treated cells showed a decrease in cell proliferation as compared to untreated controls, but comparing the two cell lines, we observed that NEU3 up-regulation was sufficient to maintain significant higher levels of cell proliferation than SCR controls after 12 hours of ischemia and during the entire reperfusion phase (Fig. 4C). Remarkably, NEU3 overexpression was also able to considerably reduce

apoptosis. Indeed, as revealed by the nuclear DAPI staining, the number of apoptotic cells, which are characterized by chromatin condensation and nuclear blebbing, was significantly lower after NEU3 up-regulation, as compared to SCR cells (Fig. 4D). Furthermore, we also evaluated caspase 3/7 activation during IRI. Caspase-Glo® analysis revealed a 3-fold reduction of caspase activation in NEU3 cells, as compared to SCR, mainly during the ischemic phase. However, a significant lower level of activation is maintained in NEU3 cells also during the entire reperfusion phase (Fig. 4E).

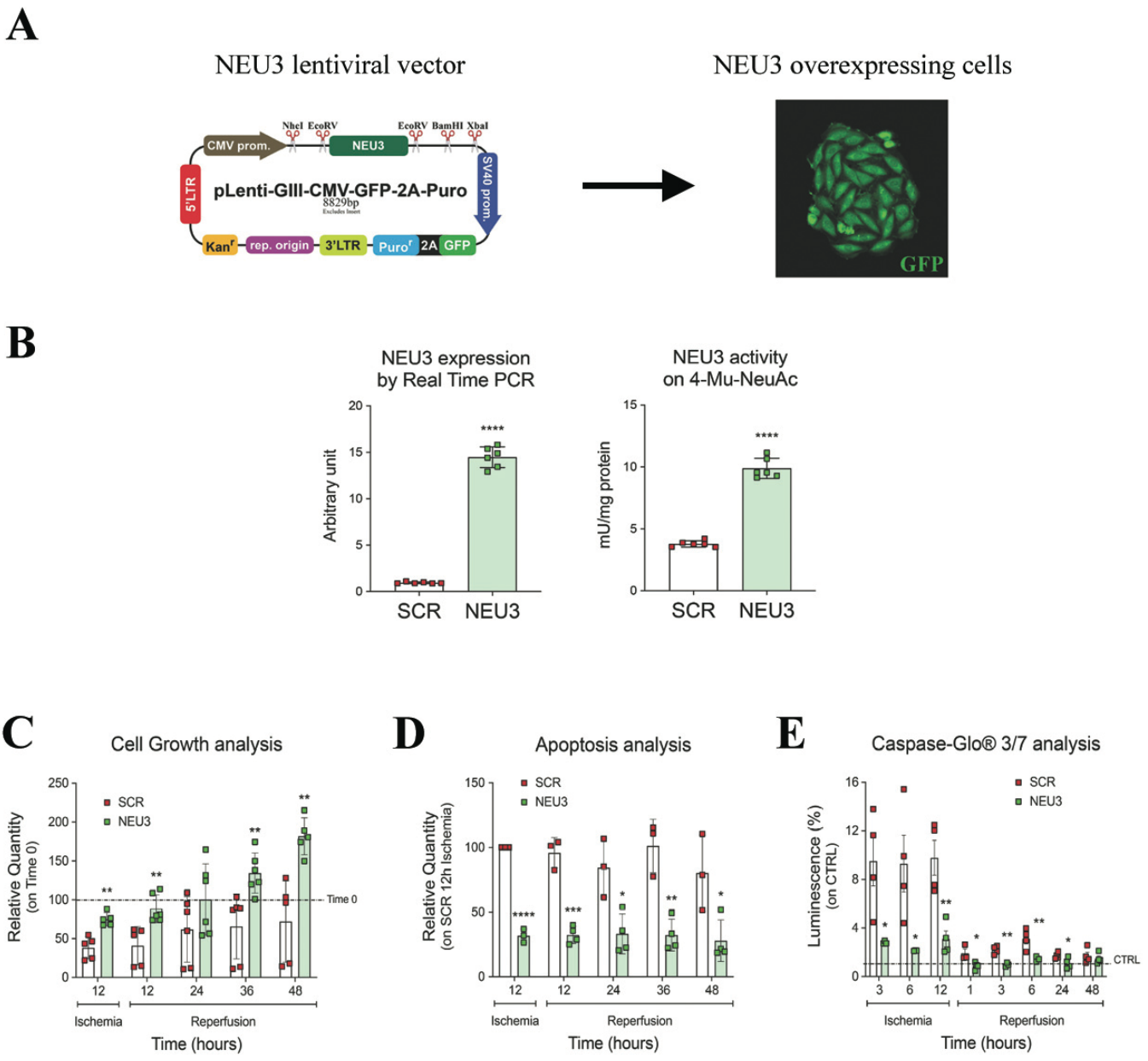

Fig. 4: *Effects of NEU3 overexpression in H9C2 exposed to IRI. Plasmid vector for NEU3-overexpression. The GFP marks the transfected cells (***A***). Analysis of NEU3 expression and activity of the selected H9C2 overexpressing clone (***B***). Analysis of the effects of the IRI exposure on SCR and NEU3 overexpressing cells in terms of cell proliferation (***C***), apoptosis (***D***) and Caspase 3/7 activation (***E***). Data represent mean ± SD of 4 independent experiments. Statistical significance was determined by Student's t test. *P<0.05; **P<0.01; ***P<0.001; ****P<0.0001.*

4. NEU3 overexpression and the RISK pathway

In order to investigate the molecular mechanism leading to NEU3-induced protective effects, we analyzed the activation of the pro-survival kinases Akt and Erk1/2, two of the main components of the RISK pathway. SCR and NEU3 cells were exposed to our model of IRI and Akt and Erk1/2 activation was evaluated by Western Blot, by comparing the ratio between the inactive and the active (phosphorylated) forms of both proteins. All the bands analyzed corresponded to the expected molecular weights and their intensity has been normalized by the intensity of the calnexin protein, which was used as housekeeper (data not shown). Results revealed that both kinases showed a 2-fold increase in the activation levels in NEU3 overexpressing cells at any time point analyzed, as compared to controls (Fig. 5).

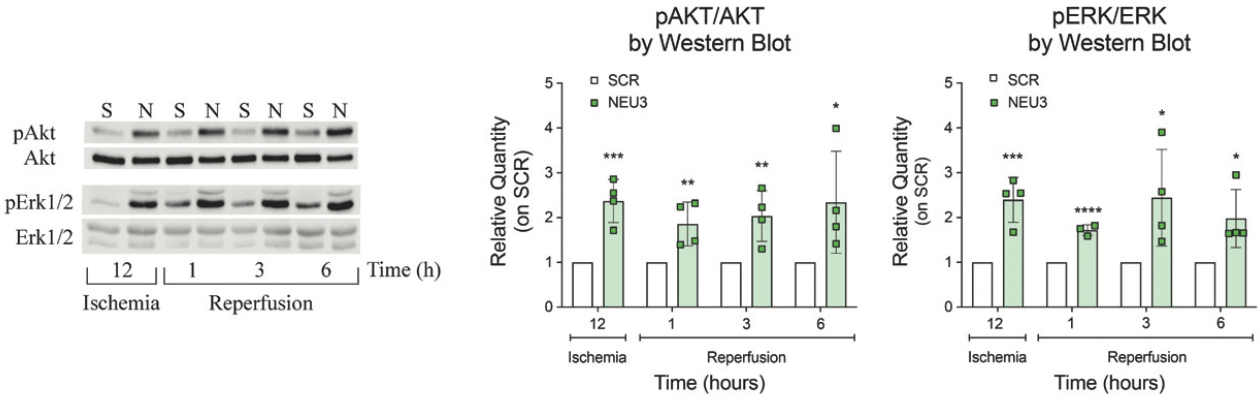

Fig. 5: *Western Blot analysis of Akt and Erk1/2 activation in SCR and NEU3 H9C2 during IRI. Data represent mean ± SD of 4 independent experiments. Statistical significance was determined by Student's t test. *P<0.05; **P<0.01; ***P<0.001; ****P<0.0001.*

Therefore, to confirm the real involvement of the RISK pathway activation in the beneficial effects mediated by NEU3, we inhibited Akt and Erk1/2 in H9C2 cells using two specific inhibitors. In particular, LY294002 (LY), which is a potent, cell permeable inhibitor of the phosphatidylinositol 3-kinase (PI3K), is able to block the PI3K-dependent Akt phosphorylation, and PD98059 (PD), which instead is a highly selective inhibitor of the MEK1 activation and of the MAP kinase cascade, was used to block ERK phosphorylation on both threonine and tyrosine residues (Fig. 6A). Initially, several inhibitor concentrations were tested on wild-type H9C2 cells and the activation of both Akt and Erk1/2 was evaluated by Western Blot, as already described before, following the stimulation with the epidermal growth factor (EGF), which is a known activator of the RISK pathway (Fig. 6B). Based on these data, the most suitable concentration of both inhibitors resulted the 50 μM, which has been selected for the further treatments

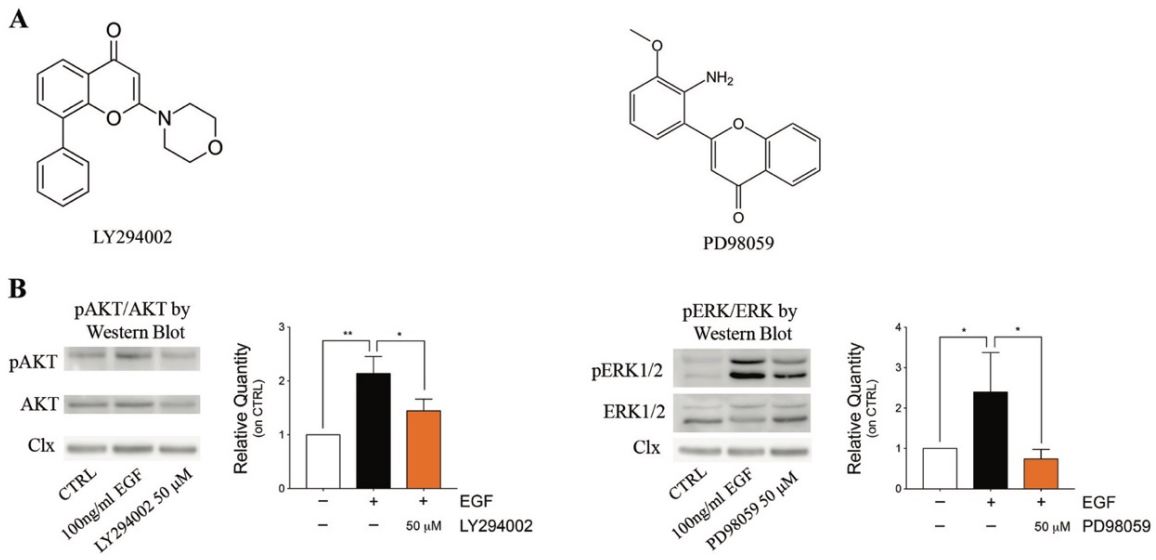

Fig. 6: *Specific inhibitors of Akt (LY294002) and Erk1/2 (PD98059) activation (***A***). Western blot analysis of Akt and Erk1/2 activation after treatment with both inhibitors, following the stimulation with EGF (100 ng/ml) (***B***). Data represent mean ± SD of 3 independent experiments. Statistical significance was determined by Student's t test. *P<0.05; **P<0.01.*

At this point, to test the consequences of the RISK pathway inhibition on the protective effects mediated by the sialidase NEU3, SCR and NEU3 cells were treated with both LY and PD (50 μM) and exposed to our IRI *in vitro* model.

Initially, the levels of Akt and Erk1/2 activation have been analyzed by Western Blot to confirm the effectiveness of the inhibitors treatment following the cell exposure to IRI. As expected, both cell lines, treated with LY, showed a progressive decrease of Akt activation, which is more evident during the entire reperfusion phase. Similarly, PD treatment induced a marked reduction of Erk1/2 activation on SCR and NEU3 cells at any time point analyzed (Fig. 7).

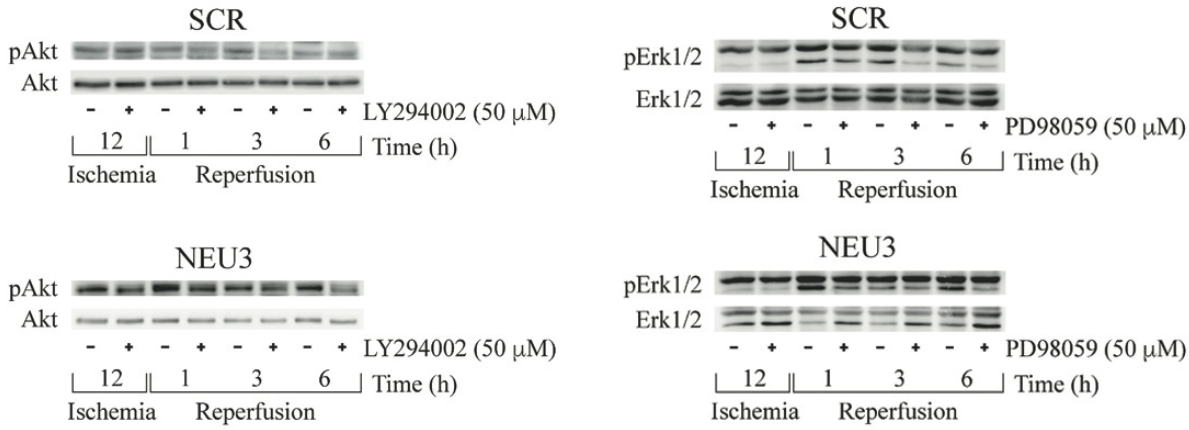

Fig. 7: *Representative images of the effects of the treatment with the specific inhibitors of Akt (LY294002) and Erk1/2 (PD98059) on SCR and NEU3 H9C2 cells exposed to IRI. Western blot analysis of Akt and Erk1/2 activation. The experiments have been repeated 4 times, independently.*

Then, in order to evaluate the inhibitors treatment effects on cell survival and death, we performed a cell growth analysis, as described in details in the material and methods section.

Interestingly, results revealed that LY and PD completely abolished the protective effects mediated by NEU3 up-regulation, rendering NEU3 overexpressing cells sensitive to IRI, as well as the control cells. In particular, the reduction in cell survival reached the peak at 48 hours of reperfusion, since NEU3 cells treated with both inhibitors showed an almost 3-folds lower cell growth levels as compared to untreated samples (Fig. 8).

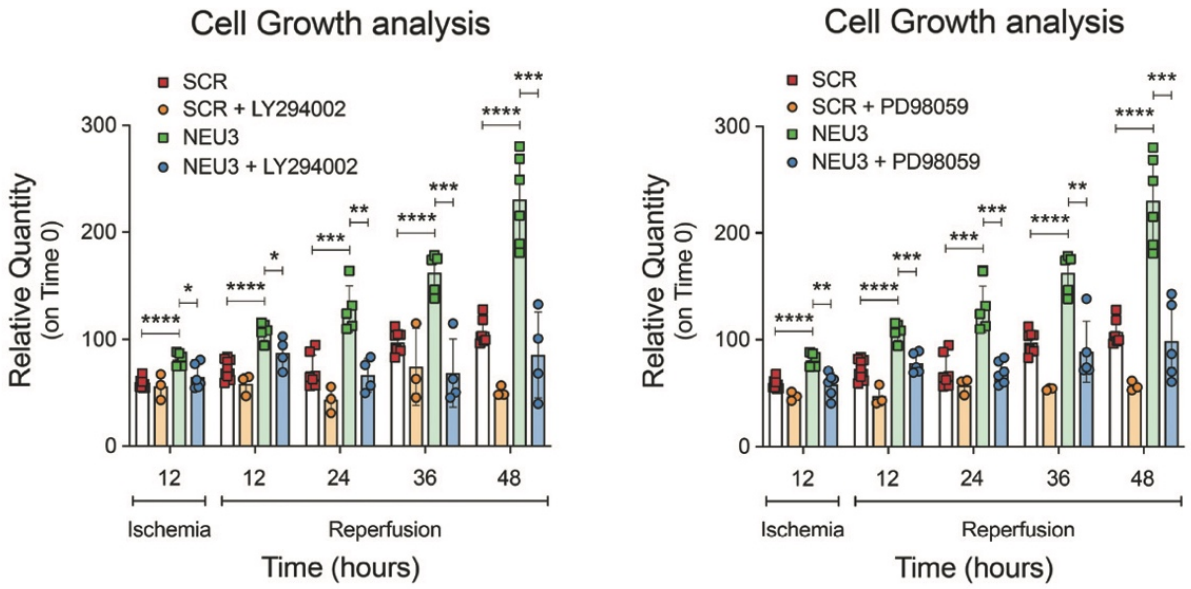

Fig. 8: *Cell growth analysis of SCR and NEU3 H9C2 cells exposed to IRI following the treatment with the specific inhibitors of Akt (LY294002) and Erk1/2 (PD98059). Data represent mean ± SD of 5 independent experiments. Statistical significance was determined by Student's t test. *P<0.05; **P<0.01; ***P<0.001; ****P<0.0001.*

Interestingly, the cell growth results were also confirmed by the apoptosis analysis.

As shown by the images and the graphs, NEU3 cells treated with LY and PD presented increased number of apoptotic nuclei, as compared to the untreated samples. In particular, after the inhibitors treatment, the percentage of NEU3 H9C2 apoptotic cells increased from 5% to around 25%, which is a level exactly comparable to SCR controls, during both ischemia and the entire reperfusion phase (Fig. 9).

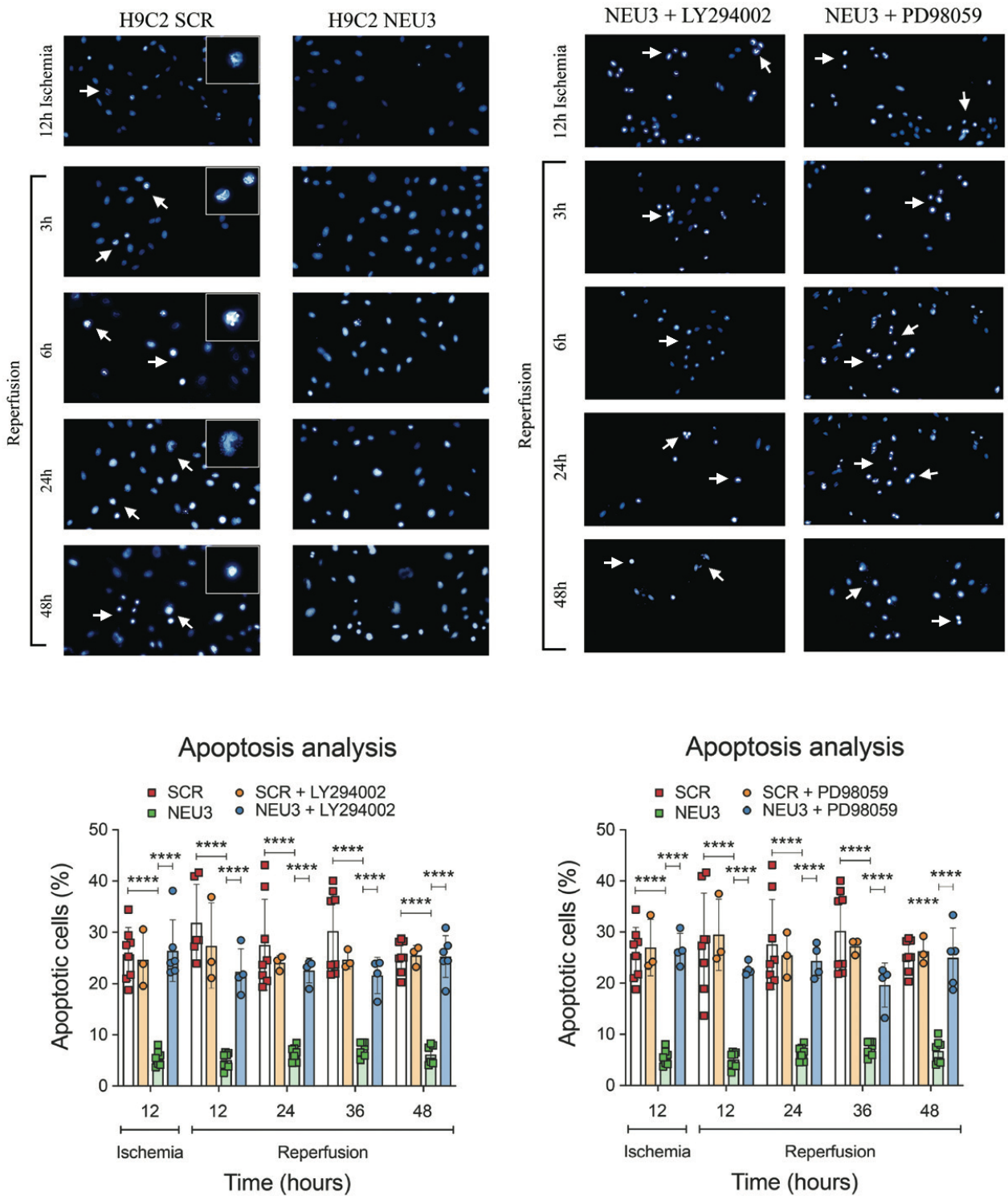

Fig. 9: *Apoptosis analysis of SCR and NEU3 H9C2 cells exposed to IRI following the treatment with the specific inhibitors of Akt (LY294002) and Erk1/2 (PD98059). White arrows identify apoptotic cells and chromatin condensation. Data represent mean ± SD of 5 independent experiments. Statistical significance was determined by Student's t test. ****P<0.0001.*

5. HIF-1α activation is regulated by NEU3 under ischemia and reperfusion

As previously described, HIF-1**α** is the major regulator of the cell response to hypoxia and has been identified as one the crucial mediators of cardioprotection during stress conditions. Since we already identified, in hypoxic skeletal and cardiac muscle, a novel mechanism of HIF-1**α** activation mediated by the sialidase NEU3, through the EGFR pathway, we decided to investigate whether the same sialidase could also promote HIF-1**α** triggering during IRI in H9C2. To this purpose, we evaluated the stability of the HIF-1**α** protein during the ischemic phase. In particular, we measured the activation of the HIF-1**α** oxygen-responsive domain (ODD) by a specific luciferase assay.

Results revealed that HIF-1**α** stability was already increased in NEU3 H9C2 after 1 hour of ischemia and was maintained higher than SCR cells at 3 and 6 hours of ischemia (Fig. 10A). Moreover, to confirm these evidences, the levels of HIF-1**α** were analyzed by Western Blot. As shown in figure 10B, NEU3 overexpression induced a significant increase of the protein, which was 2-folds higher than controls in all the time points analyzed (Fig. 10B).

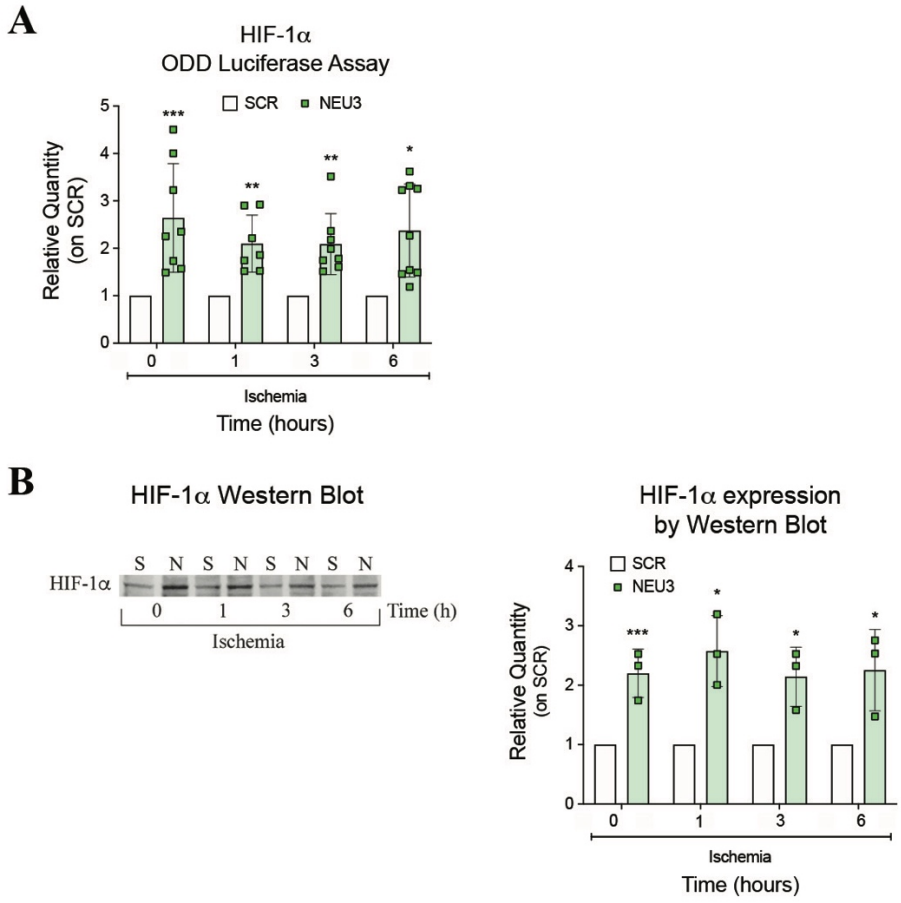

Fig. 10: *ODD luciferase assay (***A***) and western blot analyses (***B***) of HIF-1α during IRI. Data represent mean ± SD of 3 independent experiments. Statistical significance was determined by Student's t test. *P<0.05; **P<0.01; ***P<0.001.*

6. NEU3 inhibition in cardiomyoblasts H9C2

To further demonstrate the direct involvement of sialidase NEU3 in the cardioprotection against IRI, the enzyme was inhibited through the N-Acetyl-2,3-dehydro-2-deoxyneuraminic acid or DANA, which is a general inhibitor of the mammalian sialidases. Initially, several DANA concentrations were tested (10 - 50 - 100 μM) on NEU3 overexpressing cells to identify the best conditions for treatment. As expected, we observed a dose-dependent reduction of the sialidase activity, measured by an assay based on the synthetic substrate 2'-(4-Methylumbelliferyl)-α-D-N-acetylneuraminic acid (4-Mu-NeuAc), as described in the materials and methods section. The 50 μM concentration of DANA was able to reduce NEU3 activity to levels comparable to controls, therefore being selected for the further experiments (Fig. 11).

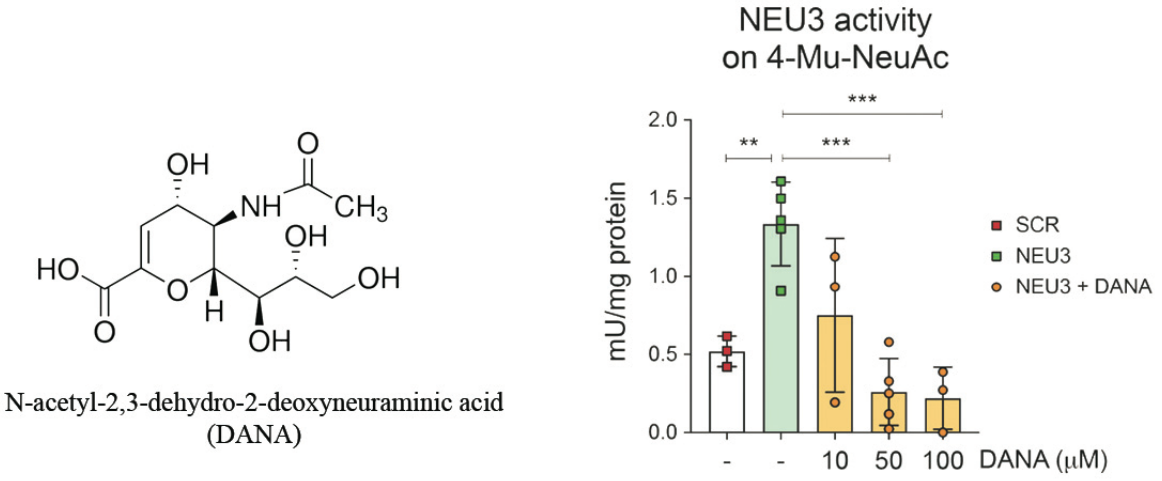

Fig. 11: *NEU3 activity analysis in NEU3 overexpressing treated with 10-50-100 μM DANA. Data represent mean ± SD of 3-5 independent experiments. Statistical significance was determined by Student's t test. **P<0.01; ***P<0.001.*

Thus, we exposed SCR and NEU3 H9C2, treated with 50 μM DANA, to our IRI model and we performed a cell growth analysis. The results showed that, while DANA did not modify the sensitivity of control cells to ischemia and reperfusion, the treatment of NEU3 overexpressing cells with the inhibitor completely counteracted the beneficial effects mediated by the sialidase up-regulation, significantly reducing their resistance to levels comparable to SCR cells (Fig. 12A).

These data were also confirmed by the apoptosis analysis. As shown by the images and the graphs, NEU3 cells treated with 50 μM DANA presented an increased number of apoptotic nuclei, as compared to the untreated NEU3 samples. In particular, after the inhibitor treatment, the percentage of NEU3 H9C2 apoptotic cells increased from 5% to around 20% at any time point analyzed, which is a level comparable to SCR controls. On the contrary, DANA treatment on SCR cells exposed to IRI did not modify the number of apoptotic cells (Fig. 12B).

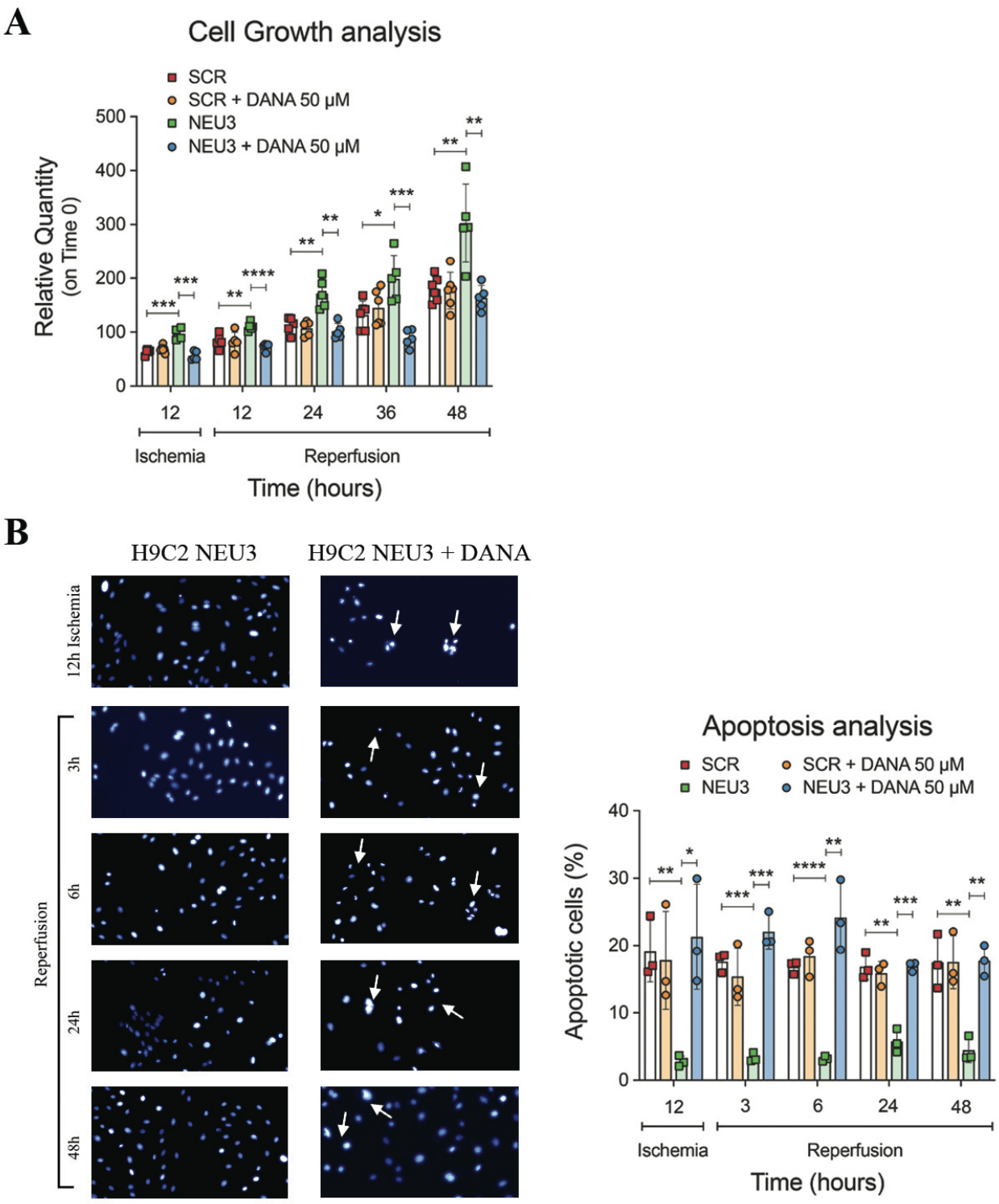

Fig. 12: *Cell growth (***A***) and apoptosis (***B***) analysis of SCR and NEU3 H9C2 cells exposed to IRI following the treatment with the sialidases inhibitor DANA (50 µM). Data represent mean ± SD of 3 independent experiments. Statistical significance was determined by Student's t test. *P<0.05; **P<0.01; ***P<0.001; ****P<0.0001.*

However, because it is well known that DANA affects all the four different isoforms of sialidases, we concomitantly used a recently identified inhibitor, which is a C9-modified Zanamivir analogue

(LR332), that has been demonstrated to be selective on the sialidase NEU3 *(Guo et al., 2018)*. Several LR332 concentrations were tested (10 - 50 - 100 μM) on NEU3 overexpressing cells to identify the best conditions for treatment. Also in this case, we observed a dose-dependent reduction of the sialidase activity, measured on 4-Mu-NeuAc. The 50 μM concentration of LR332 resulted the lower concentration able to reduce NEU3 activity to levels comparable to controls, therefore has been selected for the further experiments (Fig. 13).

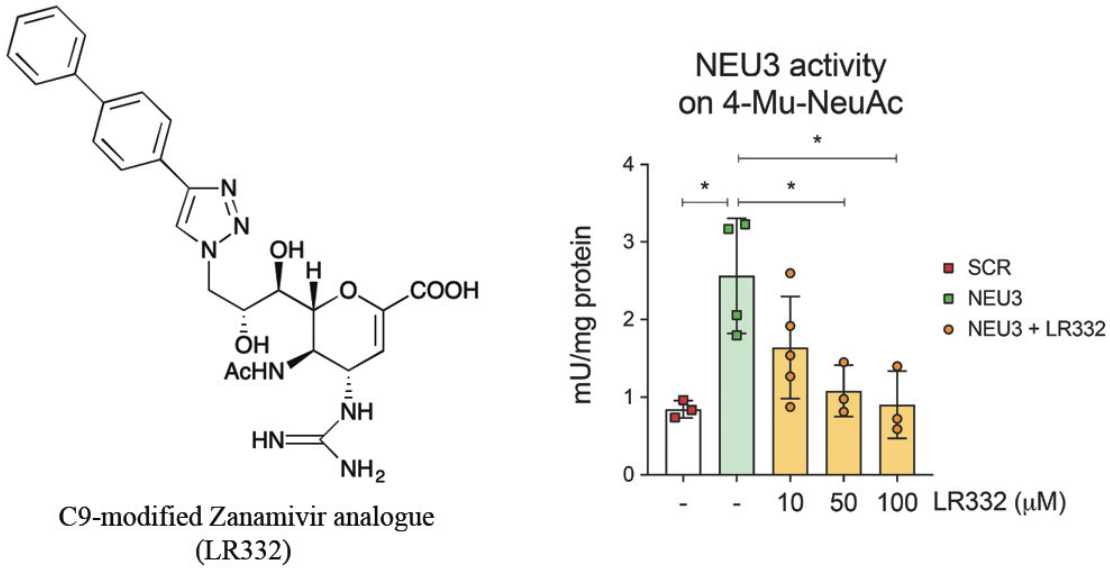

Fig. 13: *NEU3 activity analysis in NEU3 overexpressing treated with 10-50-100 μM LR332. Data represent mean ± SD of 3-5 independent experiments. Statistical significance was determined by Student's t test. *P<0.05.*

At this point, NEU3 overexpressing cells were treated with LR332 50 μM, exposed to IRI, and the cell growth analysis was performed. As expected, the inhibition of the sialidase activity was sufficient to counteract the beneficial effects mediated by NEU3 up-regulation, as already demonstrated by DANA treatment, reducing considerably cell proliferation in NEU3 overexpressing cells, which is now comparable to controls (Fig. 14A).

The reduction of the cardioprotective effects mediated by the sialidase NEU3 inhibition have been also confirmed through the analysis of apoptosis. As shown by the images and the graphs, NEU3 cells treated with 50 μM LR332 underwent a significant increase of apoptotic cells, as compared to the untreated ones. In particular, after the inhibitor treatment, the percentage of NEU3 H9C2 apoptotic nuclei shifted from 5% to around 30% during both the ischemia and the reperfusion phases, thus reaching levels similar to SCR cells. On the contrary, LR332 treatment on SCR cells exposed to IRI did not modify the number of apoptotic cells during the ischemic phase, whereas induced a slight increase of apoptosis after 24 – 48 hours of reperfusion (Fig. 14B).

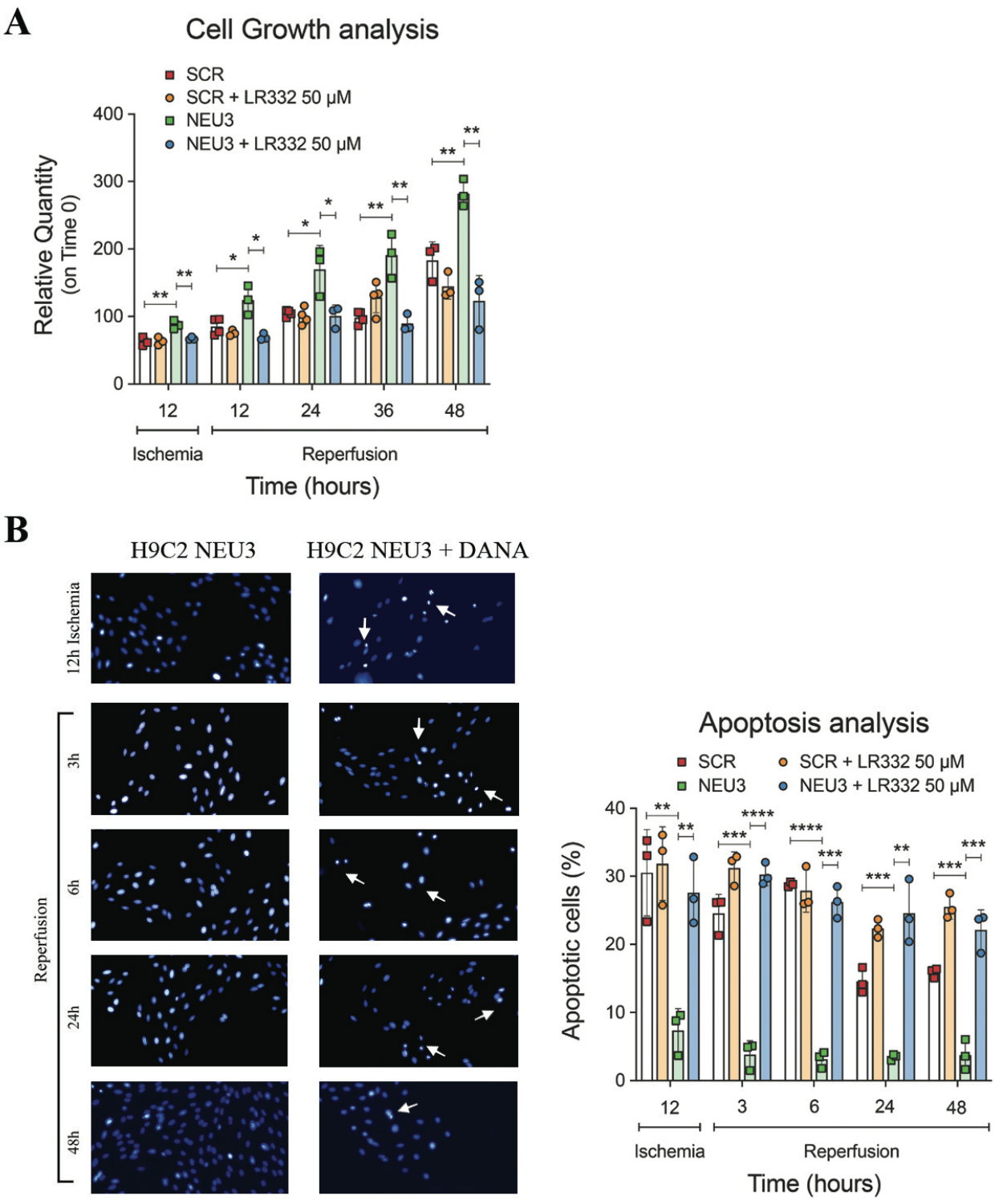

Fig. 14: *Cell growth (**A**) and apoptosis (**B**) analysis of SCR and NEU3 H9C2 cells exposed to IRI following the treatment with the sialidases inhibitor LR332 (50 µM). Data represent mean ± SD of 3 independent experiments. Statistical significance was determined by Student's t test. **P<0.01; ***P<0.001; ****P<0.0001.*

7. The role of NEU3 in Cardiac Fibrosis

Considering that high levels of sphingolipids increase TGF-**β** activation, promoting the myofibroblasts differentiation in cardiac tissue, and that NEU3 is responsible of the modulation of GM3 levels, we wondered if sialidase NEU3 could also be involved in the regulation of the cardiac fibrotic process. Thus, in order to verify whether NEU3 could directly influence the fibroblasts-myofibroblasts transition, we treated cardiac fibroblasts, which have been isolated by our research group from auricles of cardiac surgery patients, with TGF-**β** for 72 hours to induce their differentiation into active myofibroblasts. Results revealed that the treatment with TGF-**β** was sufficient to promote the expression of several specific markers of myofibroblasts such as the **α**-smooth muscle actin (**α**-SMA) and collagen type-1, both in terms of mRNA and protein expression (Fig. 15A). Moreover, immunofluorescence analysis further confirmed myofibroblasts differentiation since cardiac cells appeared positive for **α**-SMA, following TGF-**β** treatment, as compared to untreated controls (Fig. 15B).

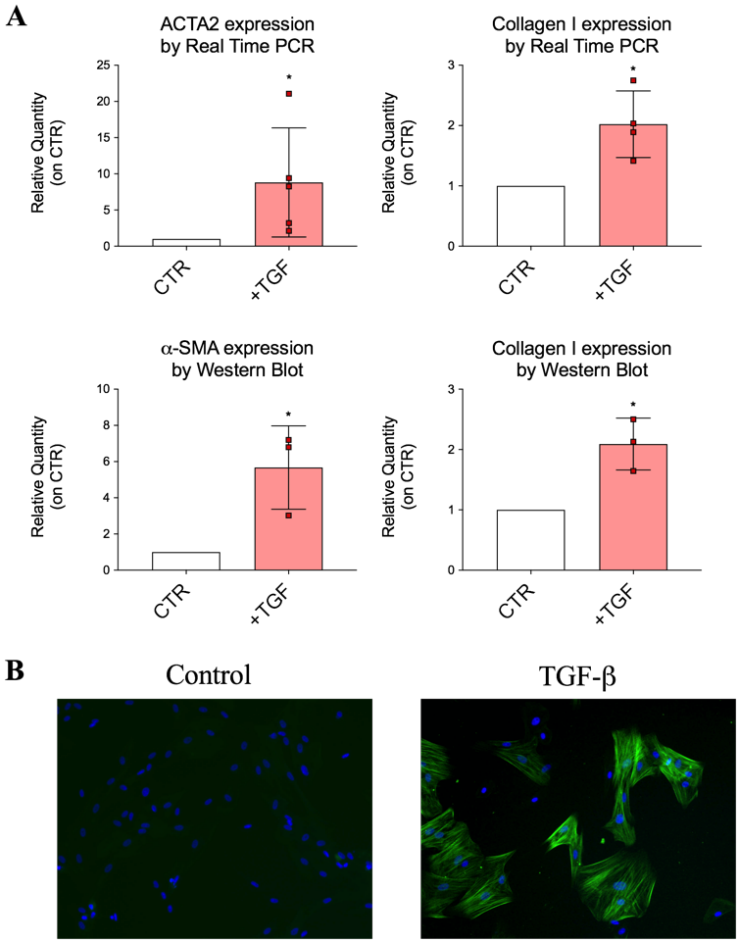

Fig. 15: *mRNA and protein expression analysis of the specific myofibroblasts markers α-SMA and collagen type-1 in cardiac fibroblasts treated with TGF-**β** (**A**). Immunofluorescence analysis of α-SMA positive cardiac fibroblasts treated with TGF-**β** (**B**). Data represent mean ± SD of 3 independent experiments. Statistical significance was determined by Student's t test. *P<0.05.*

At this point, we investigated whether the sialidase NEU3 was affected by the fibroblasts-myofibroblasts transition. Interestingly, we observed that TGF-**β** treatment induced an alteration in both NEU3 expression and activity. In particular, we detected a 25% reduction of the NEU3 mRNA level and a 50% decrease of its enzymatic activity (Fig. 16).

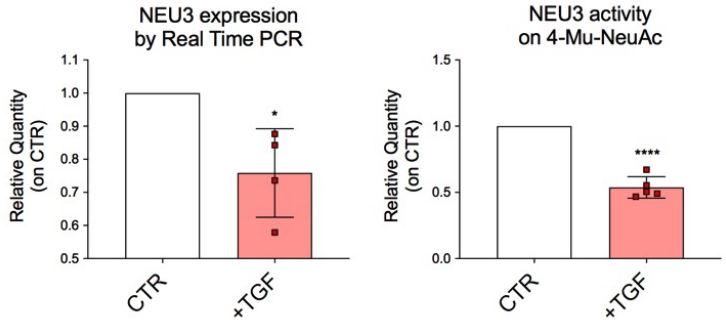

Fig. 16: *Effects of TGF-**β** treatment on NEU3 expression and activity. Data represent mean ± SD of 5 independent experiments. Statistical significance was determined by Student's t test. *P<0.05; ****P<0.0001.*

8. NEU3 overexpression in cardiac fibroblasts

Subsequently, to further investigate the role of NEU3 in the fibrosis process, we infected human cardiac fibroblasts with a lentiviral vector containing the human sialidase NEU3 coding sequence to stably overexpress the enzyme. Control cells were infected with an empty scramble (SCR) lentivirus. Cardiac fibroblasts were then tested for NEU3 expression and activity to confirm the effective NEU3 up-regulation.

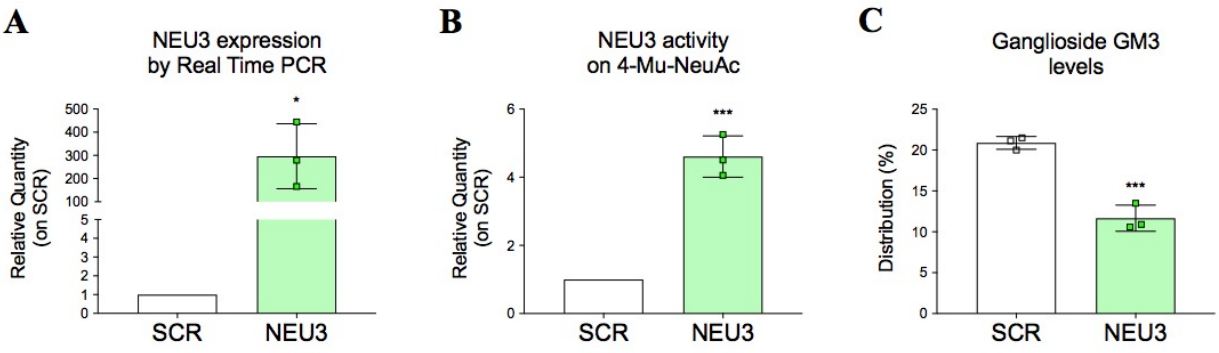

Fig. 17: *Analysis of NEU3 expression (**A**) and activity (**B**) of the overexpressing cardiac fibroblasts. Ganglioside GM3 levels analysis in NEU3 overexpressing cardiac fibroblasts (**C**). Data represent mean ± SD of 3 independent experiments. Statistical significance was determined by Student's t test. *P<0.05; ***P<0.001.*

As expected, the infected cells showed a significant increase of the mRNA expression of the sialidase NEU3 as well as a 5-folds up-regulation of its enzymatic activity (Fig. 17A-B). Moreover, the analysis of the ganglioside GM3, which is one of the preferred target of the sialidase, revealed a significant decrease in the overexpressing cells, as compared to SCR controls (Fig. 17C).

At this point, both SCR and NEU3 cardiac fibroblasts were treated with TGF-**β** to induce fibroblasts differentiation and activation, as mentioned in the previous paragraphs, and the expression of fibrosis markers was evaluated. Interestingly, the mRNA expression of the **α**-SMA and the collagen type-1 showed a statistical significant increase in both cell lines upon TGF-**β** treatment. However, this increase in NEU3 fibroblasts was appreciably lower than in SCR cells for both genes. Indeed, **α**-SMA and collagen type-1 expression was 20- and 7-folds higher in TGF-**β** treated SCR cells, as compared to untreated controls, whereas it reached only a 6-folds and 3.5-folds increase in NEU3 fibroblasts, respectively (Fig. 18A). Consistent results were obtained in the analysis of **α**-SMA and collagen type-1 protein expression (Fig. 18B).

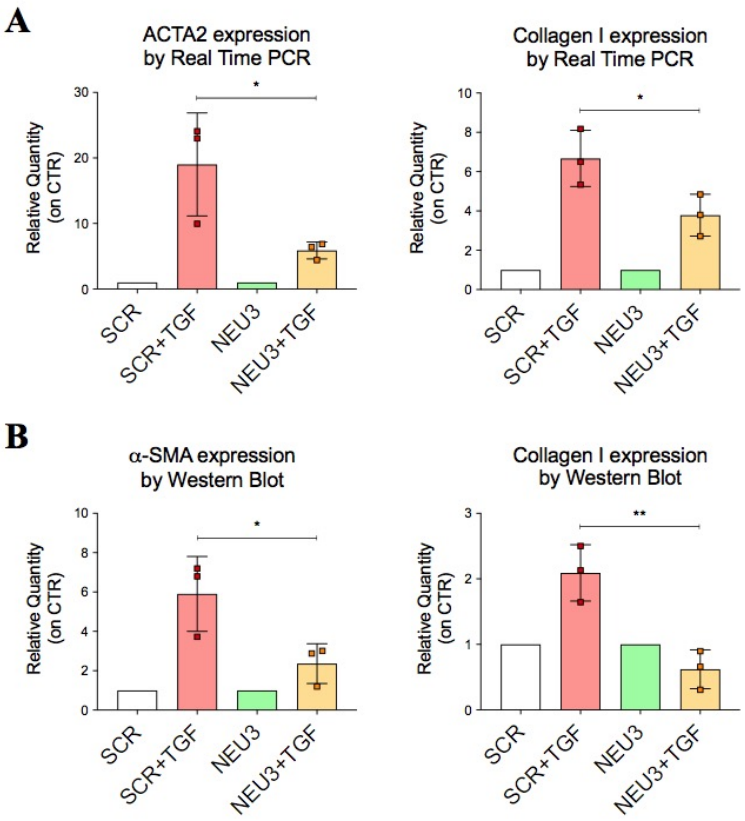

Fig. 18: *mRNA (***A***) and protein (***B***) expression analysis of the specific myofibroblasts markers α-SMA and collagen type-1 in SCR and NEU3 cardiac fibroblasts treated with TGF-**β**. Data represent mean ± SD of 3 independent experiments. Statistical significance was determined by Student's t test. *P<0.05; **P<0.01.*

The inhibitory effect on the fibroblasts/myofibroblasts transition mediated by the up-regulation of the sialidase NEU3 was also confirmed morphologically by the immunofluorescence analysis of the **α**-SMA. Therefore, as shown by the pictures below, there was a marked increase of **α**-SMA positive cells in SCR fibroblasts, upon TGF-**β** treatment, as compared to untreated cells. On the contrary, we did not observe any significant difference in the **α**-SMA expression between TGF-**β**-treated NEU3 overexpressing fibroblasts and the relative control cells (Fig. 19).

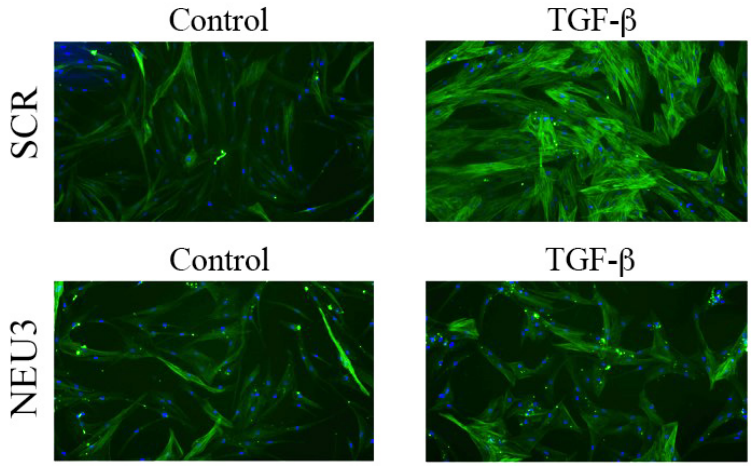

Fig. 19: *Immunofluorescence analysis of α-SMA positive SCR and NEU3 cardiac fibroblasts treated with TGF-β. Images are representative of 3 independent experiments.*

9. GM3 synthase silencing in cardiac fibroblasts

Finally, to confirm that the observed effects are mainly mediated by the NEU3-induced GM3 depletion and the consequent block of TGF-**β** pathway activation, we investigate whether the specific silencing of the GM3 synthase, which has opposite effects than NEU3, could mimic the sialidase overexpression. To this purpose, cardiac fibroblasts were transfected with specific siRNA duplexes targeting the GM3 synthase and their mRNA and protein expression was evaluated by Real-Time PCR and Western Blot. Results revealed a 70% reduction of the GM3 Synthase mRNA expression, which was accompanied by a 35% reduction also of the protein levels (Fig. 20).

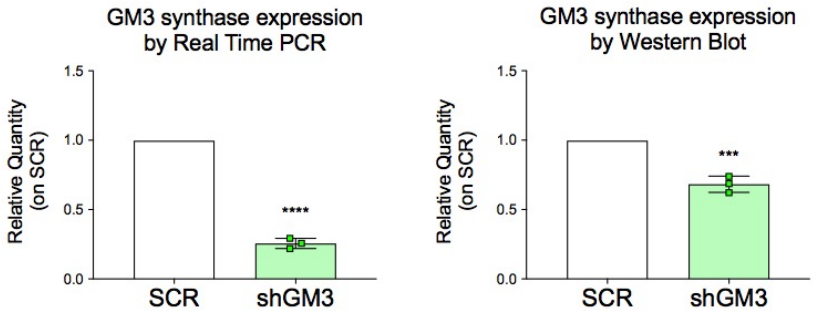

Fig. 20: *mRNA and protein expression analysis of GM3 synthase in cardiac fibroblasts treated with specific siRNA duplexes targeting the enzyme. Data represent mean ± SD of 3 independent experiments. Statistical significance was determined by Student's t test. *P<0.05; **P<0.01.*

Control and GM3 synthase-silenced cells (shGM3 cells) were then treated with TGF-**β** to induce fibroblasts differentiation and activation. The mRNA and protein expression analysis of the fibrosis markers showed results consistent with the data obtained in the NEU3 overexpressing cells. In particular, both **α**-SMA and collagen type-1 showed a statistical significant increase in both cell lines

upon TGF-**β** treatment. However, this increase in shGM3 fibroblasts was appreciably lower than in SCR cells for both genes (Fig. 21A). Moreover, same results were obtained in the analysis of **α**-SMA and collagen type-1 protein expression (Fig. 21B), as well as by the **α**-SMA immunofluorescence (Fig. 22).

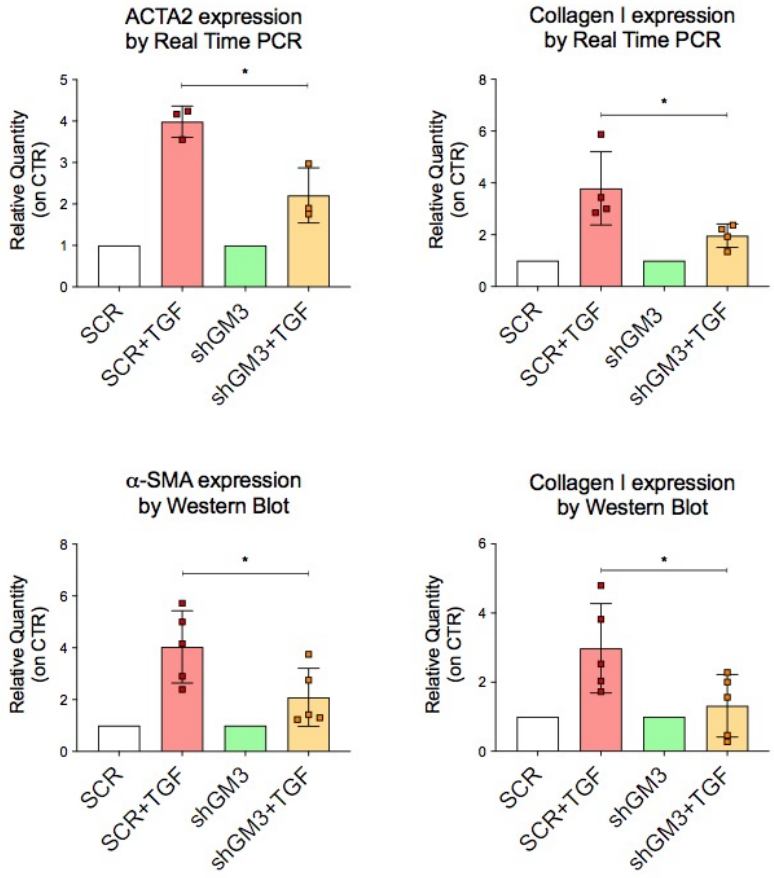

Fig. 21: *mRNA (***A***) and protein (***B***) expression analysis of the specific myofibroblasts markers α-SMA and collagen type-1 in SCR and shGM3 cardiac fibroblasts treated with TGF-**β**. Data represent mean ± SD of 5 independent experiments. Statistical significance was determined by Student's t test. *P<0.05.*

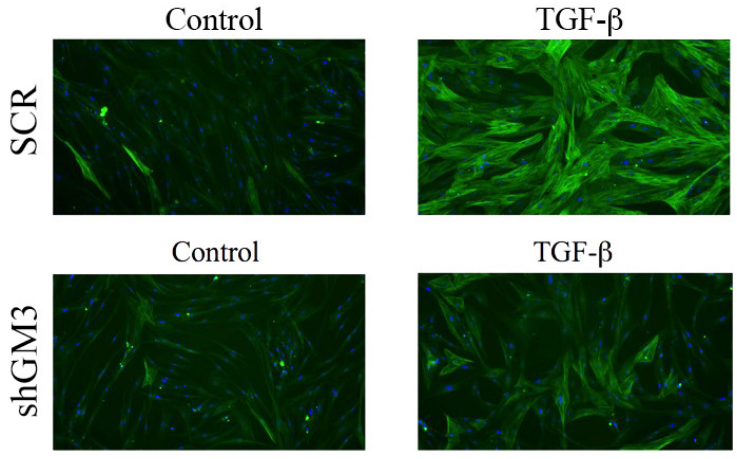

Fig. 22: *Immunofluorescence analysis of α-SMA positive SCR and NEU3 cardiac fibroblasts treated with TGF-**β**. Images are representative of 3 independent experiments.*

10. Ischemia and Reperfusion in vivo model

Recently, we developed also an *in vivo* murine model of IRI, which consist of the transient ligation of the left anterior descending coronary artery (LAD) for 30 minutes, followed by reoxygenation of the tissue. Briefly, as shown in the pictures below, the surgical procedure consisted in opening the mouse chest between the second and the third rib to expose the left ventricle. Once recognized and located the LAD, a silk suture was passed under the coronary vessel and a 5 mm long piece of tubing was placed. Then the knot was tightened around the artery and tubing simulating the ischemic phase. To confirm the occlusion of the LAD a paler color in the anterior wall of the LV appeared. After 30 minutes of ischemia, the tubing was removed to simulate reperfusion (Fig. 22A).

The significant reduction of the principal cardiac parameters such as the ejection fraction (Fig. 22B) and the fractional shortening (Fig. 22C) established the induction of cardiac damage. Furthermore, 4 weeks after surgery, the formation of an extensive scar tissue was identified by the specific Masson's trichrome staining, thus confirming the effectiveness of our *in vivo* model, which will be employed to fully characterize the role of the sialidase NEU3 in the cardiac response to IRI.

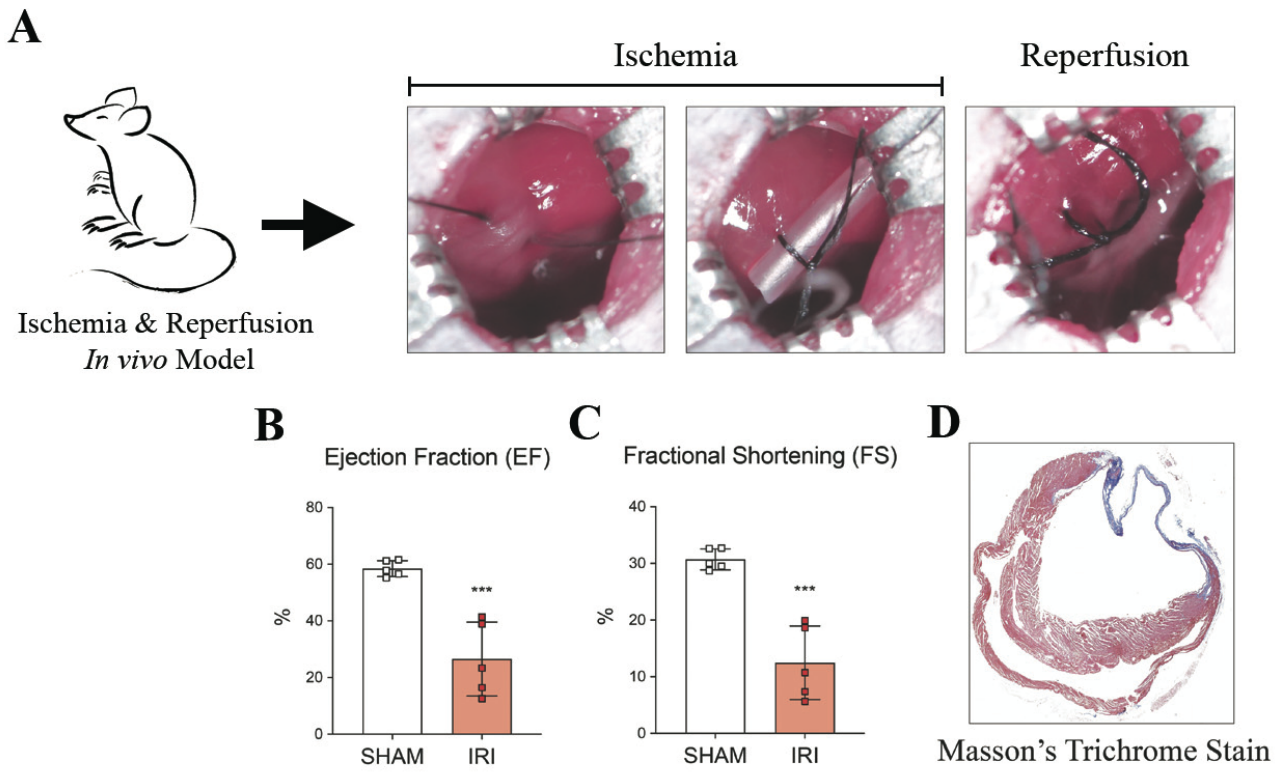

Fig. 22: *Ischemia and Reperfusion in vivo mouse model. Left anterior descending artery ligation was obtain using a small plastic tube, subsequently removed to reoxygenate the tissue (***A***). Ejection fraction (***B***) and fractional shortening (***C***) analyses in murine hearts. Scar tissue formation analysis by Masson's Trichrome staining (***D***). Data represent mean ± SD of 5 independent experiments. Statistical significance was determined by Student's t test. ***P<0.001.*

DISCUSSION & CONCLUSIONS

This thesis work is based on the recent discovery by our research group that HIF-1α can be activated by a novel mechanism, that is different for the canonical inhibition of PHD2, and is mediated by sialidase NEU3. In particular, our group reported that NEU3 was able to activate HIF-1α in skeletal and cardiac muscle during hypoxia, increasing cellular resistance to hypoxic stress through ganglioside GM3 inhibition *(Piccoli et al., 2017; Scaringi et al., 2013)*. Thus, main goal of this thesis was to test the hypothesis that sialidase NEU3 could play a role in cardiac cell response to IRI. Initially, we tested this hypothesis on an in-vitro model of IRI that we devised and optimized during this Ph.D. thesis. This allowed to observe that a 12-hours cycle of ischemia ($1\%O_2$) in nutrient-free medium and followed by a period of reperfusion under normal growing conditions ($21\%O_2$), has been effective to reduce cell proliferation and viability in H9C2 cell model, confirming the extremely sensitivity of rat cardiomyoblasts to IR. Moreover, we founded that sialidase NEU3 was modulated under these conditions. In particular, the downregulation observed at the end of the 12 hours of ischemia followed by a reactivation of the expression levels and enzymatic activity during reperfusion, suggested that probably H9C2 cells decreased NEU3-mediated pro-proliferating signals because of severe stress due to the absence of nutrients and oxygen; NEU3 was then reactivated in the surviving cells during reperfusion, as a recovery mechanism to counteract the reperfusion injury. Furthermore, based on our previously results, in which we revealed that an up-regulation of sialidase NEU3 increased the activation of cell survival mechanisms in skeletal muscle cells under hypoxia *(Scaringi et al., 2013)*, we stably overexpressed the enzyme in H9C2 to test the effect of NEU3 on cardiac cells during IR. Interestingly, we found that induced overexpression of NEU3 significantly increased cell resistance and reduced apoptosis in cardiomyoblasts exposed to IR; on the other hand, NEU3 chemical inhibition completely abolished the positive effects of the enzyme. In fact, both DANA and NEU3-specific inhibitor (LR332) treatment reduced cell viability and increased cell death during IR. Overall, these results seemed to support the hypothesis that NEU3 could play a cardioprotective role in counteracting IRI.

To further confirm this hypothesis, we investigated the molecular mechanism associated to NEU3 beneficial effects and, as anticipated, we analyzed the RISK pathway and HIF-1α activation. Initially, we focused our attention on pro-survival kinases Akt and ERK1/2, because many evidences reported that pro-survival kinases activation exerts cardioprotection against IRI. Indeed, it was observed that expression of constitutively active Akt was able to protect murine myocardium to reperfusion injury, minimizing myocyte apoptosis in the damaged region of the heart *(Fujio, Nguyen, Wencker, Kitsis, & Walsh, 2000)*. Moreover, it was observed that Akt and ERK1/2 appear to act as a point of

convergence between IPC and IPoC and their activation during ischemia, but mainly at time of reperfusion, exerts strong protection against IRI through several signaling pathway that include the inhibition of mPTP opening *(Hausenloy et al., 2005)*. long this line, we evaluated Akt and ERK1/2 activation in our model of IR, which let us observe that both kinases activation was higher in NEU3 overexpressing cells as compared to our controls, supporting the hypothesis of a possible involvement of pro-survival kinases in the molecular mechanism responsible of NEU3 positive effects. This was confirmed when we inhibited Akt and ERK1/2 using the specific inhibitors LY294002 and PD98059. Indeed, the significant increase in cell resistance observed in NEU3 overexpressing cells during IR was completely lost following the treatment with the inhibitors, confirming the real involvement of the RISK pathway activation in the cardioprotective mechanism.

Then, we investigated whether NEU3 could also influence the activation of HIF-1**α**, another important element in the cellular response to the ischemic stress. Actually, HIF-1**α** up-regulation was associated with improved myocardial tolerance to acute IRI due to the activation of its downstream target genes, including erythropoietin *(Cai et al., 2003),* hemeoxygenase-1 *(Ockaili et al., 2005)* and nitric oxide synthase *(Natarajan, Salloum, Fisher, Kukreja, & Fowler, 2006)*. Moreover, HIF-1**α** stabilization triggered the metabolic switch from oxidative phosphorylation to anerobic glycolysis, reducing mitochondrial ROS production during IRI and counteracting mPTP opening at the onset of myocardial reperfusion *(Ong & Hausenloy, 2012)*.

Our results revealed that NEU3 up-regulation stabilized HIF-1**α** during the ischemic phase of IRI and also considerably increased the expression levels of this transcription factor, confirming our previously results obtained under hypoxia *(Scaringi et al., 2013)* and supporting the hypothesis of NEU3 mediated HIF-1**α** activation as response also to the ischemic phase of IRI.

Then, we focused our attention on cardiac fibrosis, which is an integral components of most cardiac pathologic conditions *(Berk, Fujiwara, & Lehoux, 2007)*. Given the low regenerative capacity of the heart *(Bergmann et al., 2009)*, the repair process aims to remove the dead cardiomyocytes with a fibrotic scar produced by activated fibroblasts. This response is fundamental since it stabilizes ventricular walls, preventing their rupture *(Park, Nguyen, Pezhouman, & Ardehali, 2019)*. However, its uncontrolled progression provokes chamber dilatation, hypertrophy, increases stiffness, impaired electrical coupling, ultimately leading to heart failure *(Fan, Takawale, Lee, & Kassiri, 2012)*. The principal players of this mechanism are the cardiac fibroblasts that under appropriate stimuli could transdifferentiate toward their active form, becoming myofibroblasts *(Travers, Kamal, Robbins, Yutzey, & Blaxall, 2016)*. The director stimulator of fibroblasts-myofibroblasts transition is TGF-**β**, which induces the activation of specific markers of myofibroblasts, such as α-SMA and collagen type

1 *(Meng, Nikolic-Paterson, & Lan, 2016)*. TGF-**β** signaling is finely regulated and among its principal modulator there is ganglioside GM3, which was able to increase TGF-**β** response. Particularly, it has been demonstrated that ganglioside GM3 boosts the effects of TGF-**β** through the direct interaction with the TGF-**β** R1 in human lens epithelial cells, promoting the epithelial-to-mesenchymal transition *(Kim et al., 2013).*

In this PhD work, we found that both the expression and the activity of endogenous sialidase NEU3 were down-regulated in cardiac fibroblasts, upon fibrosis induction through TGF-**β** stimulus. As a consequence, the forced overexpression of the enzyme, significantly decreases the effects of TGF-**β** on cardiac fibroblasts, reducing their activation toward the myofibroblasts phenotype, as was demonstrated by lower expression levels of the fibrosis markers in NEU3 overexpressing cells. Thus, these data indicate that NEU3 up-regulation was able to reduce myofibroblasts activation and the fibrotic process.

Then, we hypothesized that this effect could be mediated by the reduced content of the ganglioside GM3 by NEU3. Indeed, we widely demonstrated the pivotal role of NEU3 in regulating the intracellular levels of GM3 *(Anastasia et al., 2008; Papini et al., 2012; Piccoli et al., 2017; Scaringi et al., 2013)*, even in adjacent cells *(Papini et al., 2004)*. To further support this hypothesis, we mimicked NEU3 overexpression effects on reducing myofibroblasts differentiation by silencing GM3 synthase. Remarkably, we observed the reduction of both the mRNA and protein expression of α-SMA and collagen type 1 in GM3 silenced cells had the same effects of NEU3 up-regulation on fibrosis induction, confirming that NEU3 effects are mediated by a decrease of GM3 levels.

In summary, our results revealed that endogenous sialidase NEU3 is modulated under ischemia and reperfusion in cardiomyoblasts. In particular, NEU3 up-regulation increased cardiac cells resistance to ischemia and reperfusion injury, ultimately maintaining cell proliferation and counteracting apoptosis. Our results support the notion that thee effects are due to a NEU3-mediated activation of the RISK pathway, and the modulation of HIF-1**α** during ischemia.

Finally, we also observed that sialidase NEU3 modulated the fibro-myofibroblasts transition, as NEU3 activation eventually reduced the shifting of fibroblast to myofibroblast phenotype through the decrease in GM3 cell content.

Clearly, further investigations are needed to fully elucidate NEU3 role in cardiac IR. In particular, we are investigating the effects of NEU3 on cardiac cell metabolism under ischemia and reperfusion, and we will analyze the oxidative metabolism and the mitochondrial functionality. Moreover, the development of an *in vivo* model of IR will be fundamental to implement our results. Indeed, we will be able to study NEU3 expression in the infarcted area and the risk area of rat hearts subjected to IRI.

Furthermore, we are going to generate a cardiac-specific transgenic mouse model overexpressing NEU3, in which we will be able to evaluate the effects of the sialidase upregulation on the cardiac tissue after IRI, analyzed both in terms of heart morphology and function. We will also be able to investigate the effect of inducible NEU3 overexpression on the fibrotic process, particularly we will measure the extension and the distribution of the scar, in order to confirm our *in vitro* results also in the animal model.

Overall, the results of this thesis work let us envision a possible role of NEU3 in developing novel therapies to counteract the detrimental effects of ischemia-reperfusion, including the development of cardiac fibrosis. However, we can't foresee a direct activation of NEU3 as a therapeutic strategy. In fact, a sustain upregulation of NEU3 has been reported in many solid tumors *(Kakugawa et al., 2002; Takahashi et al., 2015)*, and it could be very difficult to genetically fine-tune its activity. Therefore, a pharmacological approach, that would mimic NEU3 effects would be intrinsically safer. Along this line, in our laboratory, we are developing novel synthetic small molecules that, at least *in vitro*, showed encouraging results in mimicking NEU3 activation by inhibiting GM3 synthesis. Further studies in this direction are currently undergoing and will proceed beyond this thesis work.

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