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# Investigating cytoprotective molecular mechanisms against inflammation and oxidative stress stimuli by using newly developed multi-cistronic genetic tools

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*A voi,*
*a cui devo chi sono.*

*Grazie MP*

## *CHAPTER 1*

*CHAPTER 1*

# Introduction

# Ischemia – Reperfusion Injury (IRI)

Ischemia and reperfusion (I/R) is a pathological condition characterized by an initial restriction of blood supply to an organ followed by the subsequent restoration of perfusion and concomitant reoxygenation. It is well known that an impairment of blood flow (ischemia) can result in tissue injury and organ dysfunction, with duration and severity of the ischemic insult determining the reversibility of the injury response and ultimate survival of the tissue [1]. Ischemic tissue injury is generally attributed to a profound and lengthy period of tissue hypoxia and the consequent depletion of cellular ATP. It has long been appreciated that survival of ischemic tissue can be ensured by the timely restoration of blood flow (reperfusion), which should serve to minimize the magnitude of the hypoxic insult, allow for the replenishment of cellular ATP levels, re-establish ionic balance within the cell, and ultimately result in full restoration of organ function. However, the predictable beneficial influence of early reperfusion on tissue recovery following ischemia was challenged in the 1970s by reports describing a paradoxical enhancement of the injury response following reperfusion (or reoxygenation) of ischemic (or hypoxic) tissue [2]. This led to the proposal by Hearse *et al*. that the sudden reintroduction of molecular oxygen to energy- (and oxygen-) starved tissue results in a unique type of injury response that is not manifested during the period of hypoxic stress [3]. The discovery of this reoxygenation-dependent injury response, which is now commonly called “reperfusion injury”, opened a new field of scientific investigation that has grown rapidly and consistently to this day.

Ischemia and reperfusion injury (IRI) contributes to several pathological conditions (Table 1), as cardiac arrest and other forms of trauma that are associated with ischemia of multiple organs and subsequent reperfusion injury when blood flow is restored. Multiorgan failure may occur when a single organ, for instance the liver, is exposed to ischemia and reperfusion and subsequently cause inflammatory activation in other organs, as the intestine [4]. Indeed, it is now generally accepted that reperfusion injury has the potential to jeopardize the functional recovery of patients that experience transient disruption of blood perfusion to a single tissue or multiple organs, either as a consequence of a medical/surgical procedure (e.g., organ transplantation and thrombolytic therapy) or in response to a disease process (e.g., acute kidney injury, neonatal necrotizing enterocolitis) (Table 1) [1,5]. Conditions that are not traditionally considered to be associated with transient episodes of ischemia and/or hypoxia, such as sickle cell disease [6], osteoarthritis [7], or Alzheimer's disease [8], are now receiving attention as a clinical manifestation of I/R injury. The periodic episodes of painful vaso-occlusion and reperfusion that is characteristic of a sickle cell crisis have been likened to I/R injury [6]. Similarly, reperfusion injury has been implicated in the pathophysiology of obstructive sleep apnea, a condition associated with transient periods of airway obstruction (and hypoxia) followed by reoxygenation [9]. Finally, there is also growing evidence in animal models that hepatic I/R promotes the metastasis of cancerous cells to the liver from the pancreas [10] and colorectum [11]. While reperfusion injury is being assigned a role in the pathogenesis of a growing list of clinical conditions, it is important to point out that ischemic syndromes

are a heterogeneous group of conditions and the relative importance of reperfusion-dependent vs reperfusion-independent mechanisms in the overall morbidity and mortality of these conditions remains poorly defined.

A wide range of pathological processes contribute to ischemia and reperfusion associated tissue injury (Figure 1), especially hypoxia, as occurs during the ischemic period, which causes a reduction of adenylate cyclase activity and intracellular cAMP levels leading to the impairement of endothelial cell barrier function and, consequently, the increase in vascular permeability and leakage [12].

| *Single organ involvement* | |
|---|---|
| *Organ* | *Condition* |
| Central nervous system | Stroke<br>Perinatal hypoxic-ischemic encephalopathy<br>Alzheimer's disease |
| Heart | Acute coronary syndrome |
| Joints | Osteoarthritis |
| Gastrointestinal tract | Gastric ulcers<br>Neonatal necrotizing enterocolitis<br>Volvulus |
| Kidney | Acute kidney injury |
| *Multiple organ involvement* | |
| *Condition* | *Manifestations* |
| Hemorrhage & resuscitation | Multiple organ failure |
| Sickle cell disease | Acute chest syndrome, priapism |
| Sleep apnea | Oxidative damage, hypertension |
| *Medical or surgical procedure-related* | |
| *Condition* | *Manifestations* |
| Thrombolytic therapy | Stroke, myocardial infarction |
| Organ transplantation | Acute graft failure |
| Cardiopulmonary bypass | Lung injury, heart failure |
| Coronary angioplasty | Heart failure |
| Arterial tourniquet release | Local tissue injury |
| Thoracoabdominal aortic surgery | Spinal cord injury |
| Compartment syndrome decompression | Limb edema, muscle injury |
| Testicular or ovarian torsion/detorsion | Infertility |

**Table 1**. Clinical conditions associated with ischemia-reperfusion injury. (*D. Neil Granger and Peter R. Kvietys, 2015*)

As a consequence of attenuated vascular relaxation after reperfusion, a 'no reflow phenomenon', characterized by increased impedance of microvascular blood flow after reopening the occluded blood vessel, may occur [13], and in a clinical setting is associated with poor outcomes. Hypoxia also produces significant alterations in the transcriptional control of gene expression, leading, for instance, to an inhibition of oxygen-sensing prolylhydroxylase (PHD) enzymes because they require oxygen as a cofactor. Hypoxia-associated inhibition of PHD enzymes leads to the post-translational activation of hypoxia and inflammatory signaling cascades, which control the stability of the transcription factors hypoxia-inducible factor (HIF) and nuclear factor-κB (NF-κB), respectively [14]. Cell death programs, including necrosis, apoptosis and autophagy-associated cell death are also activated following I/R [15]. Furthermore, reperfusion injury is characterized by autoimmune responses, including natural antibody recognition of neoantigens and subsequent activation of the complement system (autoimmunity) [16].

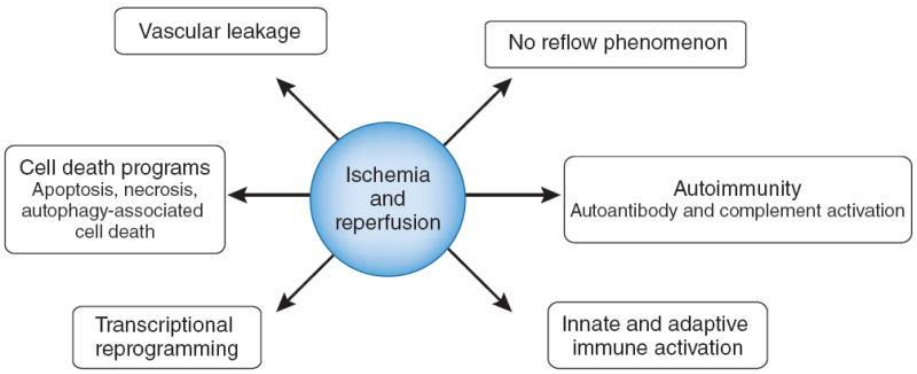


**Figure 1**. Biological processes implicated in ischemia and reperfusion. (*H.K. Eltzschig and T. Eckle, 2011*).

## Inflammation and Immunity in IRI

It is well known that inflammation is a complex response to both infection and tissue injury. It is necessary for the resolution of damage, but if uncontrolled, chronic inflammation can occur. The sustained recruitment of leucocytes and consequent oxidative stress can lead to worsening injury; tight regulation of inflammation is therefore crucial.

In IRI, the inflammatory process begins immediately after arterial occlusion; the ensuing hypoxia in the intravascular compartment changes in shear stress, and production of reactive oxygen species (ROS) leads to the activation of endothelial cells (EC), platelets and trigger coagulation and complement cascades.

Even though ischemia and reperfusion typically occurs in a sterile environment, innate and adaptive immune responses activate and contribute to injury, including signaling events through pattern-recognition molecules such as Toll-like receptors (TLRs). Ligand binding to TLRs leads to the activation of downstream signaling pathways, as NF-κB, mitogen-activated protein kinase (MAPK) and type I interferon pathways, resulting in the induction of pro-inflammatory cytokines and chemokines [17].

Zhao H. and colleagues proposed a molecular mechanism involved in renal graft IRI, in which TLR-4 has a central role in promoting the release of pro-inflammatory mediators, leukocytes migration and infiltration at the injury site, leading to innate and adaptive immune responses and finally to sustained tubular necrosis, renal fibrosis and graft rejection [18]. Indeed, TLR-4 mediates the release of pro-

inflammatory mediators by triggering the transcription of inflammatory genes, and this is accompanied by increased expression of macrophage inflammatory protein-2 (MIP-2) and monocyte chemoattractant protein-1 (MCP-1), chemokines involved in the recruitment of neutrophils and macrophages, respectively. Cytokine release, as IL-6, IL-1 and TNF-α, begins during the ischemic phase and is amplified upon reperfusion [18].

'Damage-associated molecular patterns' (DAMPs), are endogenous molecules that can activate these receptors in condition of cell damage or death, as they occur during ischemia and reperfusion [17]. Many of them, as the high-mobility group box 1 (HMGB1) protein and ATP, are released into the extracellular compartment upon tissue damage where they can activate an immune response [19]. Others, such as adenosine and the fibrinogen-derived peptide Bβ, are generated or released in the process of catabolism [17] and they can either activate an immune response or function as a safety signal to restrain potentially harmful immune responses and promote tissue integrity during ischemia and reperfusion [20,21].

Local TLR-4 activation by endogenous ligands leads to cytokine/chemokine release and subsequent activation of innate immunity [22]. Vascular endothelial activation is marked by the expression of adhesion molecules, which facilitate monocyte/macrophages and leukocyte migration and infiltration. Leukocyte rolling is mainly mediated by three transmembrane receptors: E-selectin, P-selectin, and L-selectin. Infiltrating monocyte/macrophages release proteolytic enzymes and inflammatory mediators including TNF-α, IL-1, and IFN-γ [23]. Additionally, the

innate immune system induces adaptive immune responses via antigen presentation and enhances neutrophil infiltration. Thus, it is naturally accepted that suppressing innate immunity through inhibiting TLR-4 might agitate the adaptive immune system to a less extent and promote immune tolerance.

Moreover, during IRI, innate recognition proteins can be self reactive and initiate inflammation against self, a process known as 'innate autoimmunity [16]. Several studies has linked reperfusion injury to the occurrence of 'natural' antibodies, produced in the absence of deliberate immunization, that lead to the activation of the complement system. Neo-epitopes have been identified which are expressed on ischemic tissues as targets for natural antibody binding during the reperfusion phase with subsequent complement activation, neutrophil recruitment and tissue injury [24,25].

## Microvascular dysfunction, complement and coagulation cascades

Increased vascular permeability, endothelial cell activation, an imbalance between vasodilating and vasoconstricting factors and the activation of coagulation and the complement system are all conditions related to the vascular phenotype associated with Ischemia and Reperfusion. Following IRI, microvascular disfunction can lead to respiratory failure that is caused by a disruption of the alveolar-capillary barrier function, leading to increased microvascular permeability. This phenomenon can occur, for instance, in patients with graft ischemia and reperfusion during solid organ transplantation [26]. During the ischemic period, hypoxia can cause increased vascular permeability. Similarly to

what happens in cultured endothelial cells which showed increased permeability after hypoxia exposure caused by lower cAMP levels [12], an increase in pulmonary edema, albumin leakage into multiple organs and elevated cytokine levels have been reported in mice exposed to ambient hypoxia [27,28]. ROS generation and damages, leukocyte endothelial cell adhesion, platelet- leukocyte aggregation and complement system activation further aggravate microvascular dysfunction after reperfusion [29].

A characteristic response of the vasculature to IRI is an enhanced recruitment of inflammatory cells, particularly neutrophils [30]. Moreover, the microvessel wall is the major source of oxygen and nitrogen radicals that cause the microvascular dysfunction associated with IRI. Xanthine oxidase (XO)-derived superoxide and/or secondary radical species were considered largely responsible for the endothelial barrier dysfunction. The ischemic insult results in depletion of the energy charge of the cell, accumulation of hypoxanthine from the catabolism of ATP, and a concomitant conversion of the XDH (xanthine dehydrogenase), the predominant form in normal healthy tissue, to XO. Upon reperfusion, the restored tissue $O_2$ reacts with hypoxanthine (or xanthine) and XO to generate both superoxide ($O_2^-$) and hydrogen peroxide ($H_2O_2$), which can consequently interact to yield more reactive secondary species (Figure 2) [31].

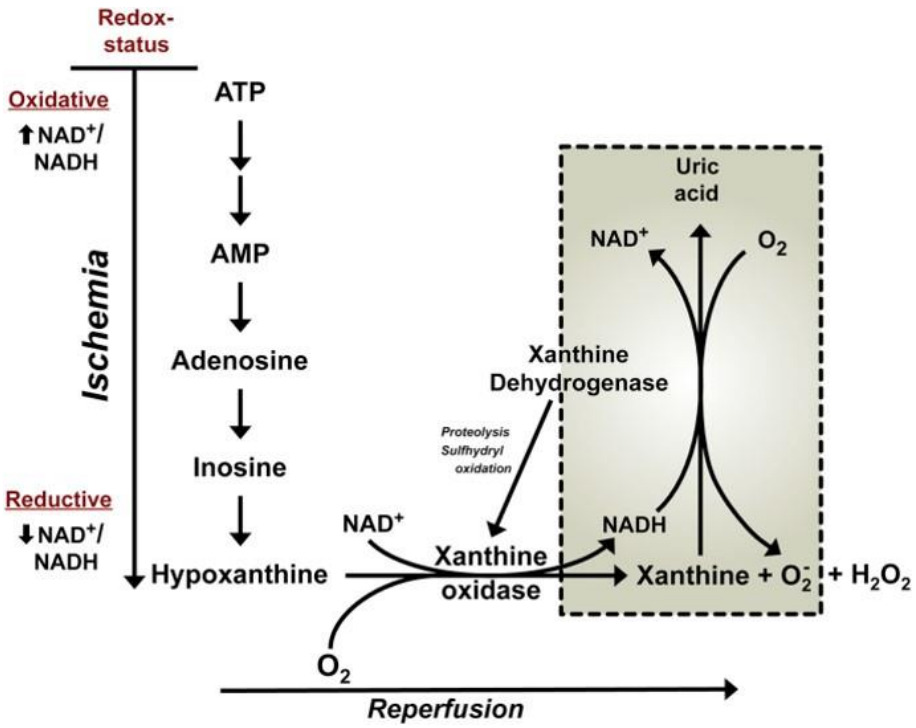


**Figure 2.** Potential mechanisms of ROS production by xanthine oxidoreductase (XOR) in tissues exposed to I/R. In the setting of ischemia, ATP is catabolized to hypoxanthine and the dehydrogenase form of XOR (XDH) is converted, via limited proteolysis and sulfhydryl oxidation, to the oxidase form (XO). Upon reperfusion, the restored tissue $O_2$ reacts with hypoxanthine (or xanthine) and XO to generate both superoxide ($O_2^-$) and hydrogen peroxide ($H_2O_2$), which can consequently interact to yield more reactive secondary species. During ischemia, the redox status of the tissue is altered from an oxidative state (higher level of NAD+ relative to NADH) to a reductive state (higher NADH relative to NAD+). This altered redox state has been shown to enhance the generation of O − from XDH in the presence of xanthine (*D. Neil Granger and Peter R. Kvietys, 2015*).

Xanthine oxidase (XO), as major enzymatic source of ROS, and ROS scavenging enzymes, such as Superoxide Dismutase (SOD) and Catalase (CAT), have been implicated in the leukocyte recruitment that occurs following IRI *in vivo* and Hypoxia/Reperfusion (HR) *in vitro*. The neutrophil recruitment observed in tissues exposed to IRI [32], the

enhanced leukocyte–endothelial cell adhesion in post-ischemicvenules [30], and the increased neutrophil adhesion to post-hypoxic endothelial cell monolayers [33] are all significantly attenuated by treatment with an XO inhibitor, SOD, or CAT.

Hydrogen peroxide, which is known to initiate the production of platelet activating factor (PAF) and increased surface expression of P-selectin [33], appears to play an important role in this regard. Endothelial cell monolayers exposed to HR exhibit a biphasic neutrophil adhesion response, with an early (30 min) enhancement of adhesion that is transcription-independent and inhibited by treatment with blocking antibodies for either P-selectin or ICAM-1. At 240 min following reoxygenation, a second phase of neutrophil hyperadhesivity is noted that is transcription-dependent and inhibited by treatment with either a PAF receptor antagonist, E-selectin blocking antibody, or antisense oligonucleotide directed against the oxidant-sensitive transcription factors, nuclear factor kappa-B (NFkB) or activator protein-1 (AP-1) [34]. Furthermore, a study of mouse models of sickle cell disease and lung injury has also implicated the so-called neutrophil ‘sandwiches’, in which neutrophil microdomains mediate heterotypic interactions with endothelial cells, red blood cells or platelet in microcirculation injury, further supporting that endothelial selectins influence neutrophil behavior [35].

The complement system acts as an immune surveillance system to discriminate among healthy host tissue, cellular debris, apoptotic cells and foreign intruders, varying its response accordingly. The complement system generates cleavage products that function as intermediaries, amplifying inflammation during IRI by complement-

mediated recognition of damaged cells and the recruitment of immune cells [36]. It has been reported that inhibition of the complement system in animal models might effectively treat ischemia and reperfusion injury, even if results from clinical studies have largely been disappointing [37]. A study of hepatic ischemia and reperfusion injury in mouse model showed that the complement system could have a dual effect: even though an excessive complement activation is detrimental, a threshold of complement activation is crucial for liver regeneration. Moreover, an inadequate complement activation can cause an impaired liver regeneration, leading to acute failure following hepatic resection or liver transplantation [38]. The complexity of the complement system and incomplete knowledge about the functional consequences of manipulating individual components of the cascade may contribute to difficulties in therapeutic targeting of complement pathways.

Furthermore, An excessive platelet aggregation and release of platelet-derived mediators can exacerbate tissue injury following IRI. Ischemia and reperfusion triggers coagulation by inflammatory mediators and platelet activation in many ways. Platelet activation can occur through integrin-mediated endothelial interactions [39], and they can be transported by inflammatory cells across epithelial barriers to sites of injury or inflammation [40]. Fortunatly, several natural anticoagulant mechanisms can inhibit clot formation following IRI, such as those mediated by antithrombin- heparin, tissue factor inhibitor and protein C [41].

## Oxidative stress-mediated injury in IRI

Since the inception of the concept of reperfusion injury, much attention has been devoted to defining the molecular and cellular basis of the unique injury response that results when ischemic tissues are reperfused [1]. In the early 1980s, oxygen-derived free radicals (now more commonly referred to as reactive oxygen species, ROS) were proposed as potential mediators of reperfusion injury. The premise that highly reactive and unstable molecules like ROS could account for reperfusion injury was quickly embraced, in large part because it was consistent with the observation that the injury response was dependent on the reintroduction of molecular oxygen. Furthermore, evidence was soon provided to support the notion that, with reperfusion of ischemic tissue, an imbalance is created between the rate of generation of ROS and the tissue's ability to detoxify these reactive species [42]. In the 35 years since ROS were first implicated in reperfusion injury, the molecular basis and pathophysiological significance of this ROS dependent response has been extensively studied, providing new insights into the enzymatic and cellular sources of the ROS, the magnitude of ROS production elicited by reperfusion/reoxygenation, and how ROS production ultimately leads to tissue injury.

The concept that ROS play a role in the injury response to I/R is largely based on three lines of evidence: (i) interventions that enhance ROS scavenging and/or detoxification protect against reperfusion injury, (ii) artificial generation of ROS in otherwise normal tissue recapitulates the injury response to I/R, and (iii) detection of enhanced ROS production and their characteristic cellular 'footprints' in post-ischemic tissues.

The early ROS scavenging studies employed native superoxide distmutase (SOD) in the presence or absence of catalase to demonstrate protection against injury in both *in vivo* and *ex vivo* models of I/R. Mutant mice that are either deficient in overexpressing ROS scavenging enzymes, such as SOD, catalase, and glutathione peroxidase, have also provided results that are consistent with a role for ROS as mediators of IRI in different organs [43-45].

Additional support for the involvement of ROS in IRI has come from studies describing phenotypic responses of cells or tissues exposed directly to ROS (or a ROS-generating enzyme) that recapitulate the responses elicited by IRI. Hydrogen peroxide, which derived from $O2^-$ dismutation in tissues exposed to IRI, gave rise to highly toxic hydroxyl radicals through the Haber-Weiss reaction, and it has been extensively used as a representative ROS to assess the response of cells to oxidative stress. At pathophysiologically relevant concentrations, $H_2O_2$ can elicit most of the phenotypic changes in endothelial function that are evidenced in post-ischemic (post-hypoxic) tissues (endothelial cell monolayers) including endothelial barrier dysfunction (increased vascular permeability) [46], increased expression of endothelial cell adhesion molecules and enhanced leukocyte-endothelial cell adhesion, increased production of inflammatory mediators (e.g., platelet activating factor), and the induction of a procoagulant, prothrombotic phenotype [47].

The premise that ROS are generated following IRI was initially based on the detection of chemical products generated by the reaction of ROS with cellular lipids, proteins, and other molecules, that have been widely used as ‘footprints’ of ROS generation in different models of

IRI [48,49]. Lipid peroxidation and oxidation of cellular sulfhydryl groups and the generation of oxidized glutathione (GSSG) have been offered as evidence of oxidative stress and redox imbalance following IRI in different tissues [50,51]. Electron spin resonance (ESR) spectroscopy and spin trapping has become the 'gold standard' for detection and identification of different ROS produced by tissues and cells in response to IRI. This approach, applied to several tissues and cultured cells, has revealed that the enhanced ROS production elicited by IRI is detected immediately (within 20s) following reperfusion and that superoxide is the parent radical that serves as a precursor for the hydroxyl radical, carbon-centered radicals and other secondary species [48].

## Cell death during IRI

Different programs of cell death activate following IRI [15]. Necrosis is a frequent outcome of ischemia and reperfusion. It consists in cell and organelle swelling with subsequent rupture of surface membranes and the spilling of their intracellular contents, leading to immune response stimulation, inflammatory-cell infiltration and cytokine production. Contrarily, apoptosis involves caspase signaling cascade that induces a self-contained program of cell death, characterized by the shrinkage of the cell and its nucleus, with plasma membrane integrity persisting until late in the process [15]. Recent studies have shown that also apoptosis could induce immunostimulation, since extracellular release of ATP from apoptotic cells acts as a 'find-me' signal that attracts phagocytes [52]. Inhibition of apoptosis may have promise as a

therapeutic strategy for IRI, for instance by preventing the activation of the transcription factor NF-κB. Indeed, limited oxygen availability during ischemic period is associated with activation of NF-κB through a mechanism involving hypoxia-dependent inhibition of oxygen sensors [53]. Mice with disruption of the gene encoding IKK-β, the catalytic subunit of IKK that is essential for NF-κB activation, offer an opportunity to study the consequences of preventing canonical NF-κB pathway activation. A selective ablation of IKK-β is necessary since this manipulation results in embryonic lethality due to massive apoptosis driven by tumor necrosis factor-α [54]. Study of intestinal ischemia and reperfusion revealed that although IKK-β deficiency in enterocytes is associated with reduced inflammation, severe apoptotic damage occurred in the reperfused mucosa [55]. Thus, the use of NF-κB inhibitors for treating intestinal IRI would require caution since it is associated simultaneously with the prevention of systemic inflammation and increased local injury.

## Protective pathways in IRI

Therapeutic approaches currently in use in clinical settings of Ischemia and Reperfusion mainly aim to induce ischemic tolerance in involved tissues and organs. Such therapies could be used both during a surgery associated with IRI, or after ischemic injury in patients during an intervention aimed at the restoration of blood flow and reperfusion. For instance, ischemic preconditioning is an experimental strategy in which exposure to short, non-lethal episodes of ischemia results in attenuated tissue injury during subsequent ischemia and reperfusion. In order to

find pharmacological approaches that would imitate ischemic preconditioning, several genetic and pharmacologic studies have investigated the underlying mechanisms, identifying oxygen-dependent signaling [56] and purinergic signaling [57,58], as relevant pathways in this context.

## Hipoxia-induced metabolic pathways

Hypoxia-Induced Factor (HIF) is responsible for the transcriptional induction of glycolytic enzymes when oxygen levels fall during an ischemic period. The switch in energy metabolism from fatty acid oxidation to more oxidation efficient glycolysis allows tissues to sustain cellular viability for a longer amount of time [14].

Among several therapeutic gases that have been used for the treatment of ischemia and reperfusion, endogenously produced carbon monoxide (CO) or the administration of CO-releasing molecules are well known to mediate anti-inflammatory and cytoprotective effects in injured tissues during I/R. This protection seemed to involve HIF stabilization and activation of a HIF-dependent transcriptional response [59].

Furthermore, the stability of HIF is regulated by the oxygen-sensing PHD enzymes. Indeed, it has been shown that the loss of Phd1 reduces oxygen consumption by reprogramming glucose metabolism to a more anaerobic route of ATP production in ischemic skeletal muscle [60]. Moreover, treatment with pharmacological PHD inhibitors results in increased ischemia tolerance of the kidneys [61] and in cardioprotection [56]. To date, PHD inhibitors seem to be well tolerated in humans [62], suggesting that they could be readily tested in larger clinical trials.

## Nucleotide and nucleoside signaling

Nucleotides, particularly in the form of ATP, have been strongly implicated in promoting tissue inflammation during ischemia and reperfusion (Figure 3). ATP is normally retained intracellullary at relatively high concentration (5-8 mM), but it is released into the extracellular compartment upon Ischemia and reperfusion. It can be spilled by necrotic cells [17] or released from both apoptotic cells [52] in a controlled way or activated inflammatory cells [63]. When it accumulates in the extracellular compartment, ATP acts as pro-inflammatory molecule to recruit phagocytes [52], activates the Nlrp3 inflammasome during ischemia and reperfusion [19] and promotes the chemotaxis of inflammatory cells [64]. Moreover, ATP-elicited activation of nucleotide receptors, such as P2Y and P2X, can enhance vascular inflammation [65,66]. Therefore, pharmacological strategies blocking ATP release or ATP receptor signaling may have promise for attenuating the inflammatory response during I/R. In the extracellular compartment, ATP and ADP are enzymatically converted to the nucleoside adenosine by the combined action of two ectonucleotidases [67]. The treatment of animal models of IRI with such nucleotidases has been shown to be effective in attenuating tissue injury and inflammation [68,69]. Notably, beyond alleviating the detrimental effects of ATP, the ATP conversion to adenosine may be desirable because of the beneficial effects of adenosine itself. Several pharmacological and genetic studies have shown that signaling through adenosine receptors is protective in mouse models of ischemia and reperfusion, by mediating the activation of the adenosine A2A receptor (Adora2a) on inflammatory cells [70] or Adora2b on vascular

endothelia, epithelia or myocytes [56, 57, 71]. The administration of agonists of adenosine receptors in animal models showed promising results for IRI treatment. The selective $A_{2B}$ agonist BAY 60-6583 allowed achieving protection against myocardial ischemia and reperfusion [56] and intestinal ischemic injury [71], while $A_{2A}$ agonist regadenoson (CVT-3146) was approved by the US Food and Drug Administration as a coronary vasodilator for patients requiring pharmacologically-induced stress echocardiography [72, 73,74].

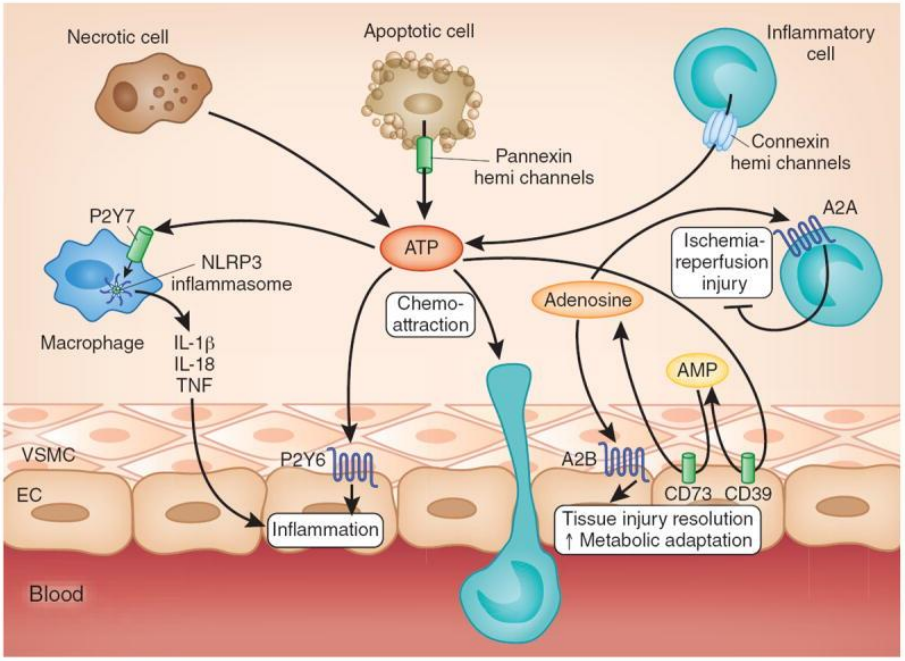


**Figure 3.** Nucleotide and nucleoside signaling during I/R. Multiple cell types release ATP during IRI, as necrotic cells or controlled release through pannexin hemichannels from apoptotic cells or connexin hemichannels from activated inflammatory cells. Subsequent binding of ATP to P2 receptors enhances pathological inflammation and tissue injury (e.g., P2X7-dependent Nlrp3 inflammasome activation and P2Y6-dependent enhancement of vascular inflammation). ATP can be rapidly converted to adenosine through the combined action of CD39/CD73. Adenosine signaling dampens sterile inflammation, enhances metabolic adaptation to hypoxia and promotes the resolution of injury through activation of $A_{2A}$ receptors expressed on inflammatory cells and activation of $A_{2B}$ receptors expressed on tissue-resident cells, as cardiac myocytes, vascular endothelia or intestinal epithelia. EC, endothelial cell; VSMC, vascular smooth muscle cell (*H.K. Eltzschig and T. Eckle, 2011*).

## Ischemia/Reperfusion in Allo- and Xeno-transplantation

All the advances in the understanding of the overall transplant process, including ischemia-reperfusion, organ preservation techniques and immunological mechanisms underlying rejection and graft function, together with a more individualized immunosuppressive therapy have been combined to progressively increase the success of the human allotransplantation. Unfortunately, the supply of human organs is insufficient to treat all the patients who present each year with organ failure, and who could benefit if a compatible graft were available. These considerations has led to the possibility of addressing the shortage of organs needed for today's aging population, by developing animal organs for human xenotransplantation [75,76].

Pigs are actually considered the preferred potential source animal for many reasons, among which breeding characteristics, the relatively short gestation time and large litter size, the availability of techniques for oocyte manipulation and artificial insemination and the ability of the genome of these animals to be modified by modern technologies of genetic engineering [77]. Xenotransplantation is not yet world wide a clinical reality because some obstacles still exist. It has now been determined that most of the difficulties inherent within xenotransplantation stem from the independent evolutionary background of pigs and primates. When a pig organ is transplanted into a non-human primate (or into a human) can be rejected through several reactions: hyperacute rejection, acute humoral vascular rejection, trombotic microangiopathy, and chronic rejection [78-79]. Hyperacute rejection is mainly mediated by natural preformed anti-pig antibodies,

which bind the vascular endothelium of the graft with consequently activation of the complement cascade. The most important target for human anti-pig antibodies is the galactose-α1,3-galactose (Gal) antigen [80], an oligosaccharide very similar in structure to the B blood group antigen. Hyperacute rejection have been significantly reduced via the production of α1,3-galactosyltransferase (GalT) gene knockout (KO) pigs [81]. When hyperacute rejection does not occur, as in closely-related primate species (e.g., chimpanzee to man), or if it is prevented through the use of α -gal KO pigs, xenografts still eventually fail as a consequence of acute vascular rejection (AVR). Several elements have been found to be involved in the pathogenesis of AVR and its pathology is primarily characterised by vascular thrombosis, blood extravasation and edema. Deposits of fibrin, immunoglobulins and complement in the graft do not differ substantially from those observed in allograft rejection. Cellular infiltrates include neutrophils, macrophages, CD8+ T cells and NK cells secreting TNF-α and amplifying graft inflammation [82]

Recent studies hypothesize that a state of systemic inflammation develops after pig organ xenotransplantation, which is generated by both adaptive and innate immune responses and suggested that inflammation can lead to activation of the coagulation system. Additionally, pro-coagulant proteins, e.g. thrombin, are considered as pro-inflammatory factors. In fact, a considerable crosstalk is deemed to exist between inflammation and coagulation, leading to escalation of each other [83]. Even if T cell-directed immunosuppression can control activation of coagulation induced by adaptive immune responses, pro-inflammatory signals induced by the innate immune system can still

promote activation of coagulation [84]. Together, these observations indicate that the immune response to a pig xenograft cannot be considered in isolation and that equal attention needs to be directed to the innate immune, coagulation, and inflammatory responses. Just as there may be a need for exogenous immunosuppression and/or antithrombotic therapy, there may also be a need for the administration of anti-inflammatory agents. In this respect, in addition to corticosteroids, there is evidence that high-dose statin therapy not only reduces the inflammatory response and platelet activation [85] but also down-regulates the primate cellular response to pig antigens [86]. Anyhow control or reduction of the inflammatory response is also most likely to be controlled by genetic manipulation of the pig. Expression of thrombomodulin, endothelial protein C receptor, and/or CD39 is anticipated to reduce the inflammatory response in addition to coagulation dysfunction [87]. Furthermore, pigs are now available that express anti-inflammatory hemeoxygenase-1 [88].

Before xenotransplantation can be introduced successfully into the clinic, the problems of the innate, coagulopathic, and inflammatory responses will have to be overcome, most likely by the transplantation of organs from specifically genetically engineered pigs.

# Protective genes against inflammatory and oxidative stress stimuli

Over the years, several genes, whose over-expression is capable to down-regulate the inflammatory response, counteract oxidative injuries and limit apoptosis were identified. A lot of evidences showed, among others, three genes that gained much interest in the context of ischemia reperfusion processes due to their involvement in the regulation of inflammatory, oxidative and vascular response: the human heme oxygenase-1 (HO-1), CD73 (Ecto- 5'-nucleotidase, E5'N) and CD39 (Ecto nucleoside triphosphate diphosphorylase 1, ENTPDase).

## Heme oxygenase-1

Heme oxygenase-1 (HO-1), which is encoded by the Hmox1 gene, is a stress-responsive enzyme (32kDa) that degrades free heme (iron protoporphyrin IX) to yield equimolar amounts of three products: the gas carbon monoxide (CO), iron (Fe2+) and biliverdin, which is converted to bilirubin by NAD(P)H biliverdin reductase (BVR). Conventionally HO-1 is known to be localized to microsomes and ER, anchored by a single transmembrane spanning region at the carboxy-terminal end. Recent studies have raised the possibility of the functional compartmentalization of HO1 in other subcellular domains beside the ER, including but not limited to the nucleus, plasma membrane and also caveolae [89,90]. Expression of HO1 is regulated essentially at the transcriptional level and is induced by a broad range of chemical and physical stress stimuli, such as ROS generated substances (heme, hemin, $H_2O_2$), thiol reactive substances, heavy metals, lipid

metabolites, nitric oxide and derivatives, LPS, endotoxins, $O_2$ tension, UV light, shear stress, cytokines (IL-1, IL-6, TNF-α) and growth factors [91]. One common feature of these inducers is their capacity to generate reactive oxygen species.

Presumably, the main biologic function of HO-1 is to avoid the accumulation of highly deleterious free heme that can catalyze the production of free radicals through Fenton reaction. It has been well known that HO-1 plays as a protective gene by preventing cells death because of its anti-apoptotic properties, via inhibition of caspase-3 [92] and attenuating TNF-α induced damage, by suppression of TNF/TNFR1-mediated apoptotic signaling likely via attenuation of DISC adaptor protein expression and their association with TNFR1 [93]. Through the years a wide variety of additional protective effects has been attributed to HO-1 and in particular to its reaction products (Figure 4).

The protective effects of CO were initially demonstrated in a model of acute lung injury [94] and endotoxic shock [95] and subsequently in a mouse cardiac xenotransplantation model [96]. HO-1 expression in the transplanted heart was essential to prevent rejection in this model [97]. If donor and recipient were both treated with CO, a heart that could not harbor HO-1 activity still survived indefinitely [96]. Therefore, CO appeared able to substitute for HO-1 and suppress the pro-inflammatory response that would otherwise lead to graft rejection. It was shown that CO suppresses the pro-inflammatory response and promotes the anti-inflammatory response of macrophages [95].

Three other actions of CO contribute to its anti-inflammatory effects. First, CO prevents platelet activation and aggregation, thereby

suppressing thrombosis and the pro-inflammatory response stimulated by activated platelets [98]. Second, CO down-modulates the expression in macrophages of plasminogen activator inhibitor type 1 (PAI-1); this action appears to be crucial for the ability of CO to exert a protective effect in a model of ischemia-reperfusion of the lung [99]. Third, CO prevents apoptosis in several cell types, including endothelial cells [100], fibroblasts, hepatocytes and β-cells of the pancreas [101].

Moreover CO suppresses the proliferative response of smooth muscle cells that contribute to neointimal proliferation associated with inflammatory lesions in vivo [102]. The cytoprotective effect of CO is mediated by the activation of several signal transduction pathways, among which the p38 MAPK signal transduction pathway [103]. CO triggers the proteolytic degradation of the proapoptotic p38α MAPK isoform favoring signaling via the antiapoptotic p38β MAPK isoform [104]. Activation of p38β MAPK by CO is involved in the mechanism by which HO-1 interacts functionally with c-IAP-2 and A1 to suppress TNF-mediated apoptosis, and also induces the expression of Bcl-xl via the phosphatidylinositol-3-kinase (PI3K/AKT) signal transduction pathway (Figure 4) [105].

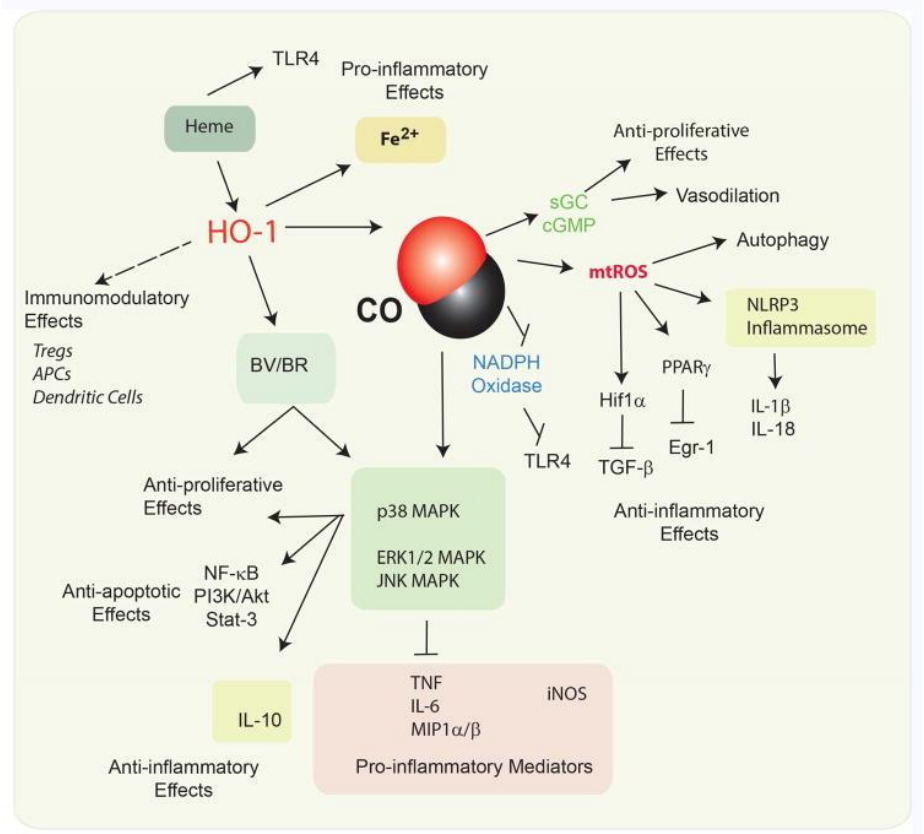


**Figure 4**. Pivotal functions of HO-1. HO-1 may have immunomodulatory effects with respect to regulating the functions of antigen presenting cells, dendritic cells, and regulatory T-cells. Heme may exert pro-inflammatory effects. HO-1 end products generated from heme degradation may modulate inflammation. Iron release from HO activity may be pro-inflammatory in the case of excess activation, and has been associated with neurodegenerative diseases. CO whether endogenously produced or applied as a pharmacological treatment, has been shown to modulate apoptotic, proliferative, and inflammatory cellular programs. In particular, CO can downregulate the production of pro-inflammatory cytokines (*e.g.*, IL-1β, IL-6, TNF-α, Mip1α/β, and upregulate the anti-inflammatory cytokines (IL-10). These effects were attributed to alterations of MAPK activities including p38 MAPK. CO can stimulate mitochondrial ROS production, which can promote the autophagy program, activate HIF-1α, and downregulate pro-inflammatory transcription factor Egr1. Recent evidence also suggests that CO can modulate the activation of the NLRP3 inflammasome, which regulates the production of IL-1β, and IL-18. BR, a product of heme degaradtion, also may exert anti-inflammatory and anti-proliferative effects.

Biliverdin and bilirubin probably exert their protective (including anti-apoptotic) effects largely because of their anti-oxidant properties [106]. Exogenous administration of biliverdin or bilirubin, mainly tested in rodents, provides beneficial effects in terms of disease occurrence and/or severity, including in ischemia reperfusion injury [107], graft rejection [108], endotoxic shock, neointima formation after balloon injury and the development of autoimmune neuroinflammation [109]. Moreover, bilirubin derived from heme degradation suppresses MHC class-II expression in endothelial cells [110].

Labile Fe produced by HO-1 upregulates the expression of H-ferritin, which associates with light-chain (L-) ferritin subunits to form a multimeric protein complex that has a high capacity for storing Fe (4500 mol of Fe per mol of ferritin). Expression of ferritin is cytoprotective under a variety of conditions, an effect that is in large measure attributable to the ferroxidase activity of the H-chain subunit [111], which catalyzes the oxidation of iron from the ferrous (Fe2+) to the ferric form ($Fe^{3+}$).

## Ectonucleoside triphosphate diphosphohydrolase-1 (CD39)

CD39 is a plasma membrane ectonucleotidase with molecular mass ~58kDa that belongs to the Nucleoside Triphosphate Diphosphohyrolase (NTPDase) family. It is now known that this family contains other seven NTPDases, three of which being expressed as cell surface- located enzymes: NTPDase1, which is present on Kupffer and vascular endothelial cells, NTPDase2, which is expressed by portal fibroblasts and activated hepatic stellate cells and NTPDase8 that seems to be the major ATPase of the hepatic canaliculus [112]. The vascular isoform of CD39 is abundantly expressed on vascular endothelial and smooth muscle cells [113] dendritic cells [114], neutrophils, monocytes and certain T- and B- cell subsets, but not on platelets and red blood cells [115].

The expression of this gene appears to be induced by several pro-inflammatory stimuli, oxidative stress and hypoxia [116]; in particular, some cloning studies on CD39 promoter region revealed a prominent role for the transcription factor Sp-1 in regulating CD39 transcription in response to hypoxic stimuli. Sp-1 is a member of the family of transcription factors Sp/XFLF, expressed ubiquitously, and it is involved in the transcription of hypoxic genes, such as VEGF and the same CD39 [117].

CD39 is $Ca^{2+}$ and $Mg^{2+}$ dependent and contains two predicted transmembrane domains at the N- and C-terminus with a large extracellular loop containing a more central hydrophobic region and six potential glycosylation sites, and a palmytoil group that targets the enzyme to caveolae [118].

This enzyme is involved in the modulation of vascular cell and platelets purinergic receptors activities, by the breakdown of extracellular adenine nucleotides, that is ATP being hydrolyzed to ADP and then to AMP. Subsequently, AMP is hydrolyzed to adenosine by CD73 (Ecto-5'-nucleotidase, E5'N) (Figure 5).

## Ecto-5'-nucleotidase (CD73)

CD73 is a membrane bound 70kDa enzyme that functions downstream of CD39 and catalyzes the hydrolysis of extracellular AMP to adenosine. It belongs to the 5'- nucleotidase family, which also contains other six isozymes, five of which are located in the cytosol and the last one in the mitochondrial matrix [119]. CD73 consists of two glycoprotein subunits that are tethered by non-covalent bonds, binds zinc and other divalent ions at the N-terminal domain and is anchored to the plasma membrane at the C-terminus by a glycosyl-phosphatidylinositol (GPI) [120,121].

CD73 is expressed to a variable extent in different tissues, with abundant expression in the colon, kidney, brain, liver, heart and lung [120]. In the vasculature, CD73 is predominantly associated with the vascular endothelium of large vessels such as the aorta, carotid and coronary artery [122]. In the case of circulating T- and B-lymphocytes, the enzyme expression is restricted by certain cell types and strongly correlates with cell maturity, while neutrophils, erythrocytes, platelets and other blood cells express little or no CD73 [123]. There are evidences that the expression and function of this enzyme are upregulated under hypoxic conditions [63,124], as well as by the presence of several proinflammatory mediators, such as transforming

growth factor (TGF)-β, interferons (IFNs), tumor necrosis factor (TNF)-α, interleukin (IL)-1β, and prostaglandin $E_2$ [125,126]. An increase in CD73 expression has also been reported in several neoplastic tissues [125], suggesting the involvement of this enzyme in the onset and progression of neoplasia.

CD73 is essential for the extracellular formation of adenosine, even if nowadays it is known that adenosine is also produced intracellularly and once reached high concentrations, is shunted into the extracellular space through specialized nucleoside transporters [127]. However this pathway is minor than the extracellular catabolism of precursor adenine nucleotides (ATP, ADP, AMP) to adenosine by the action of CD39-CD73 system [128] (Figure 5).

**Adenosine and protective mechanisms of CD39-CD73 axis**

Adenosine is a nucleoside reported to be cytoprotective, antithrombotic and immunosuppressive [128]. Adenosine exerts its protective effects binding to the G protein-coupled adenosine receptors (ARs), $A_1$, $A_{2A}$, $A_{2B}$ and $A_3$ (Figure 5). By this way, adenosine exerts cardioprotection by vasodilatation and protection against myocardial ischemia, antagonism of the chronotropic and ionotropic effects of catecholamines, promotion of endothelial barrier function, inhibition of platelet aggregation, protection against vascular inflammation and neointima formation, inhibition of TNF-α, reduction of the complement component C2 level and inhibition of neutrophil adhesion and free radical generation, as well reviewed by Antonioli et al. [116]. NK cells have long been shown to respond to adenosine that inhibits granule exocytosis [129] and attenuates tumour recognition and adhesion [130].

By terminating the prothrombotic and proinflammatory effects of ATP and ADP, CD39 exerts its hemostatic and vascular protective functions against platelets aggregation and vessels occlusion [131]. Thus, ADP is an important mediator of vascular thrombosis in inflammatory states and, consequently, ATP diphosphohydrolase (ATPDase) has a fundamental role in the inhibition of ATP-ADP signal transduction induced in platelets, leukocytes and vascular endothelium mediated by purinergic receptors. The ATPDase-mediated hydrolysis of ATP and ADP reduces these purinergic mediators from the extracellular space and generates adenosine, resulting in protective mechanism against inflammation process [132]. Data from mutant mice deficient in NTPDase1/CD39 or overexpressing human CD39 further confirmed an important role for this ectoenzyme in the control of hemostasis, platelet reactivity, thrombotic reactions and vascular growth in vivo and further support its therapeutic potential in clinical vascular diseases and during transplantation [133,134]. Recently, the protection against myocardial ischemic injury in hCD39 transgenic mice hearts has been reported [135]. The importance of CD73 in producing adenosine for AR signalling has been revealed through studies with CD73- deficient mice. For example, CD73-generated adenosine reduces inflammation and fibrosis in lungs of bleomycin-treated mice [136] and is tolerogenic for cardiac and airway allografts [137]. CD73-dependent $A_{2B}$ signaling protects mice during renal ischemia inhibits systemic vascular leakage during hypoxia [138], and is also required for cardioprotection as a result of ischemic preconditioning [139].

The purinergic pathway leads to adenosine formation that mediates anti-inflammatory and anti-thrombotic effects mainly by binding to $A_{2B}$

receptor. This molecular binding results in signaling pathways transduction involving Akt and Erk1/2 proteins that mediate cell survival [140]. Therefore, taking in account that CD39 and CD73 degrade ATP to adenosine, they can be viewed as 'immunological switches' that shift the ATP pro-inflammatory stimulus toward an anti-inflammatory state mediated by adenosine; thus, the protective effects of the CD39-CD73 axis are mainly related to the final extracellular product formed as a result of their combined action.

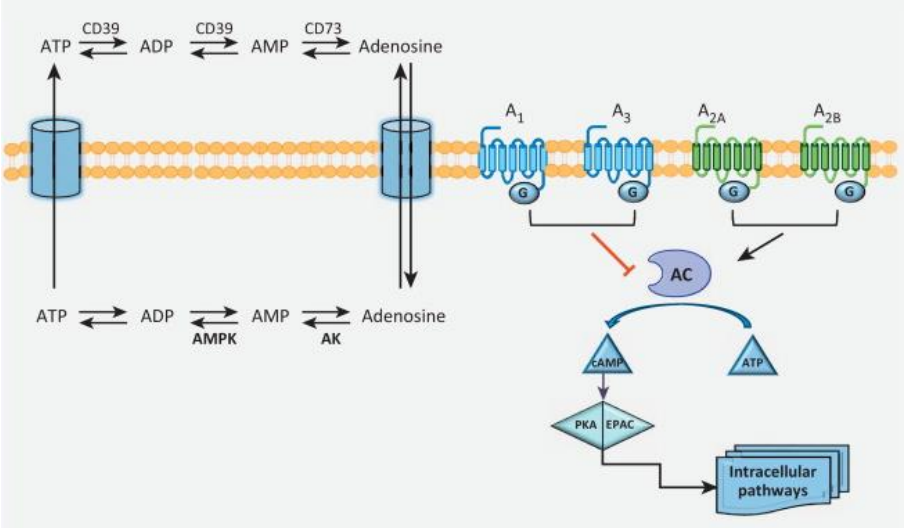


**Figure 5**. Adenosine synthesis and receptor activation in the cell. ATP is converted to adenosine via a series of dephosphorylation steps involving cytosolic ecto-5′-nucleotidases CD39 and CD73. Adenosine is converted to ATP via phosphorylation steps mediated by adenosine kinase (AK) and AMP kinase (AMPK). Both ATP and adenosine can be transported outside the cell via diffusion or active transport. Outside the cell, there is again ATP metabolic degradation to adenosine, which activates one of the four adenosine receptors, triggering a positive ($A_{2A}R$, $A_{2B}R$) or negative ($A_1R$ and $A_3R$) coupling to adenylyl cyclase (AC) with the corresponding activation of inhibition of cAMP, that will differentially activate protein kinase A (PKA), exchange protein activated by cAMP (EPAC), and several intracellular pathways (*A. Mediero and B.N. Cronstein 2013*).

# Polycistronic vectors

An increasing demand for polycistronic vectors has arisen in recent years to obtain complex multi- gene transfer/therapy effects. A broad range of biotechnological and biomedical applications, spacing from metabolome engineering to recombinant viruses production for vaccination purpose, from hetero-multimeric proteins expression to multi-gene therapy and production of multi-transgenic animals, has been achieved by the introduction of several strategies for co-expression of two or more genes in polycistronic vectors.

Multi-genes expressing strategies may be arranged in different groups - multiple internal promoters, internal ribosome entry sites (IRESes), messenger RNA splicing, fusion proteins, post-translational proteolysis - based upon the mechanism used to produce the several proteins involved [141]. However, several disadvantages are associated with each of these approaches. For instance, steric effects often lead to loss of function of fused proteins. In order to avoid such problems, proteinase cleavage sites can be incorporated but, in this case, both the polyprotein substrate and the processing enzyme must be co-expressed in the same subcellular site. Moreover, interference between promoters occurred by using multiple promoters strategy leading to promoter suppression and rearrangement [141]. The major issue associated with IRES elements, identified both in viral and cellular eukaryotic mRNAs, is their size than can cause problems in packaging within size-restricted vectors commonly used for biomedical applications such as adeno-associated and retroviral vectors. Furthermore, expression from IRESes is dependent on various cellular binding factors, which vary among

different cell types. Critically, the protein downstream of the IRES is only expressed to ~10% of that upstream [142,143].

## Self-processing peptides and 2A Translational Recording Sequences

Self-processing peptides are now widely used. Sequences are fused with a self-processing peptide or CHYSEL (cis-acting hydrolase element) inserted in frame that undergoes a co-translational "self-cleavage". There is not a termination of translation after the first gene and the ribosomes do not need to reinitiate their translating activity. Translation of the single ORF produces each component of the polyprotein as an individual, discrete, product. The main advantages of this strategy are the small size of the CHYSEL sequence compared with IRES sequences and, as happens when the two genes are fused, that co-expression of both genes is ensured [141].

This new co-translational activity was first discovered in the 2A peptide of a virus from the picornavirus group (foot-and-mouth disease virus, FMDV) [144,145]. From a biotechnological standpoint, all that is needed is to clone the coding sequence of 2A, followed by the codon encoding the first aminoacid of the next FMDV protein (2B), in frame between the two coding sequences to co-express (the first of which without the stop codon). This peptide has showed to be active in heterologous contexts in all different eukaryotic cell-types tested (mammals, insects, plants, fungi, yeast), but not in prokaryotic cells [146].

Analysis of recombinant polyproteins and artificial polyprotein systems in which 2A was inserted between two reporter proteins showed that the FMDV 2A oligopeptide co-translationally 'self-cleaved' at the glycyl-prolyl pair site corresponding to the 2A/2B junction (−LLNFDLLKLAGDVESNPG↓P-) [144].

Thus, this group of oligopeptide sequences, collectively known as 2A, mediate a translational recoding event known as "ribosome skipping," "StopGo" or "Stop Carry-on" translation [146-148]. Briefly, the model of this non-canonical form of translation proposes that when a ribosome translates an mRNA sequence encoding 2A, the nascent 2A oligopeptide interacts with the exit tunnel of the ribosome (through which the elongating polypeptide product leaves the structure) and "stalls" the progress of the ribosome. Although a stop codon has not been encountered, the nascent peptide is released (forming the C-terminus of 2A), but then translation may resume, synthesizing the polypeptide sequence downstream of 2A: the synthesis of the peptide bond at this specific point in the protein backbone is "skipped". Eukaryotic translation release (termination) factors 1 and 3 (eRF1/eRF3) are proposed to play a key role in the release of the nascent protein from the ribosome (Figure 6) [149].

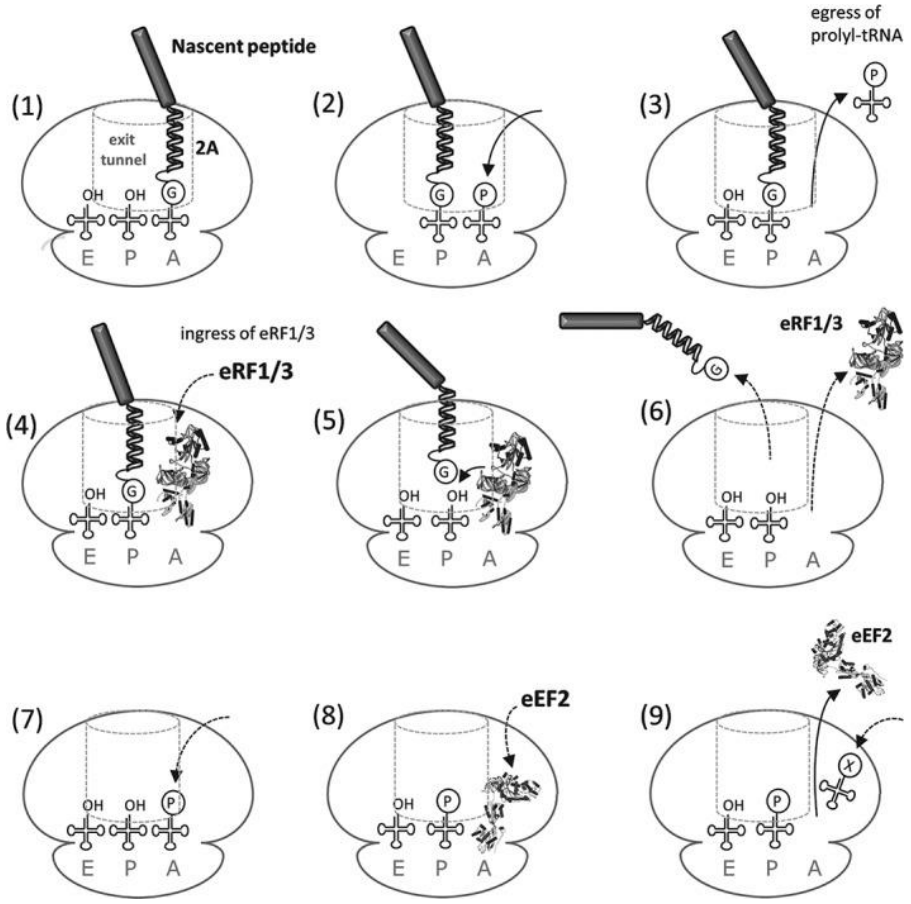


**Figure 6.** Model of 2A- mediated translational 'recoding'. (1) when the nascent peptide upstream of 2A is emerging from the ribosome, 2A is positioned in the ribosome exit tunnel. (2) the nascent peptide- 2A and glycyl- tRNA is translocated from the A- to P- site and prolyl- tRNA enters the A- site. (3,4) the nascent 2A peptide interacts with the exit tunnel of the ribosome such that the C- terminal portion (−ESNPG- ) is sterically constrained within the peptidyl- transferase centre (PTC) of the ribosome. Nucleophilic attack of the ester linkage between 2A and $tRNA^{gly}$ by prolyl- tRNA in the A- site is inhibited; effectively stalling, or pausing, translation. The failure to form a new peptide bond leads to dissociation of prolyl- tRNA from the A- site of the ribosome, required for entry of release factors into the A- site. (5,6) This block is relieved by the action of translation release factors eRF1 and eRF3, hydrolyzing the ester linkage and releasing the nascent protein. eRF1 leaves the complex, eRF3 being involved in this process. Two, mutually exclusive, outcomes may then arise: translation terminates at the C- terminus of 2A, or (7-9) prolyl- tRNA (re)enters the A- site, is translocated by eukaryotic elongation factor 2 (eEF2) from the A- to the P- site, allowing the next amino- acyl tRNA to enter the A- site to permit the synthesis of the sequences downstream of 2A (*Roulston C. et al., 2016*).

The advantages of this system as a co-expression strategy are: (i) co-expression of proteins linked by 2A is independent of the cell type, since cleavage activity is only dependent on eukaryotic ribosomes, structurally highly conserved amongst the eukaryota); (ii) multiple proteins are co-expressed at equimolar amounts from a single transcript mRNA (single ORF) under the control of only one promoter and, (iii) 2A is smaller (54-174 bp) compared to IRES elements. This makes this unique sequence an attractive substitute for previously used approaches for co-expression of multiple genes [150].

In recent years, 2A peptide has been used in several biomedicine and biotechnology applications. For example, it has been successfully used for the co-expression of multiple transcription factors, notably for the generation of induced Pluripotent Stem (iPS) cells starting from somatic cells in both viral and non- viral expression cassettes [151,152]. Moreover, the introduction of a desirable trait into an organism often requires the co-expression of multiple genes. 2A has been used in several examples of metabolome engineering and for the introduction of novel product traits in both plants and animals [153,154]. F2A technology has also been used in the construction of efficient multicistronic vectors containing three to four graft-protective genes, that are human CD55, thrombomodulin, CD39 and CTLA4-Ig [155]. 2A is actually considered particularly useful in the production of multi transgenic large animal models in which combining multiple independent genetic modifications by breeding is time consuming and expensive.

However, some limits of this technology have to be mentioned. First, it has been observed that after the cleavage, the upstream protein product retains a tail of 18 aa corresponding to 2A sequence translation and the downstream product retains a proline residue. While the N-terminal

addition to the downstream protein does not appear to be problematic [153] the longer C-terminal extension on the upstream protein might have unpredictable side effects, potentially interfering with post-translational modification, trafficking or function, or inducing an immune response that could limit biomedical applications [155]. To date, many proteins have been successfully expressed from the upstream position and no immunogenicity or toxicity of F2A peptides has been observed, at least in mice [156]. Any potential other problems could be abrogated by removal of 2A tail. This has been successfully achieved by the inclusion of a furin cleavage site (RXXR) upstream of 2A [156]. Furin is an ubiquitously expressed enzyme predominantly located in the trans Golgi network, and is implicated in the post-translational maturation of proteins that take the secretory pathway. In several cases, the addition of furin cleavage site upstream of 2A caused not only the removal of the 2A C-terminal extension, leaving only a dipeptide (RA) tail, but also a marked increase in proteins expression [156,157]. Moreover, it was shown that the addition of amminoacidic spacer sequences (GSG or SGSG) between the furin cleavage site and the 2A sequence mediated an increase of expression compared to the addition of furin cleavage site alone and the expression level became optimal when another sequence (V5 peptide tags) was added between the furin site and the spacer [157].

Another problem is the subcellular targeting of cleavage products. Signal sequences for post-translational targeting to the nucleus, chloroplast, mitochondria, membranes or cytosolic tubules have been included within 2A polyproteins, either up- or downstream of 2A and every time correct targeting of both products was achieved [153]. Co-translational targeting of cleavage products was investigated by the inclusion of a range of co-translational signal sequences into reporter polyprotein systems and it was

shown that correct localization of both products was achieved in yeast and plants but not always in mammalian cells [153]. While in constructs containing co-translational signal sequences inserted downstream of 2A both the products were localized correctly, (the first in the cytosol and the second in the ER, Golgi or plasma membrane, depending upon the signal used), in constructs where the first protein bore a co-translational signal sequence, but the protein downstream of 2A did not, it was observed that both proteins were translocated into the ER [158]. It was hypothesized that once a signal sequence is encountered and a translocon pore established, the ribosome remains attached to the translocon throughout the translation of remainder of the ORF. So proteins encoded downstream of a protein containing a signal sequence are susceptible to being transported through the established translocon complex: this phenomenon was called "slipstream translocation" [153,158]. New investigations showed that this phenomenon was due to the inhibition of the 2A cleavage by the C-terminal region (immediately upstream of 2A) of some proteins when translocated into the ER. It was concluded that the interaction between the nascent protein and the translocon may affect the conformation of 2A within the ribosome tunnel and, in consequence, abrogate the cleavage. As a result, a large proportion of the translation products are uncleaved, leading to translocation of the polyprotein into the exocytic pathway [154]. To overcome this issue, it was proposed to use a longer version of 2A either incorporating a 39aa tract from the C-terminus of FMDV protein 1D, that it was shown to increase cleavage efficiency [147] or adding a spacer, as the flexible Gly-Ser-Gly or Ser-Gly-Ser-Gly linker sequence separating the upstream protein from the 2A sequence, leading to an improvement in the 2A cleavage efficiency [157,159,160].

Together all these observations, establishes the 2A peptide as a viable and, being more reliable and easier to use, a superior alternative to the IRES in multiple genetic modifications. It fulfills all the functions IRES sequences are currently used for and, at the same time, it provides the advantage of reliable and reportedly almost stoichiometric levels of expression, a particularly useful feature when accurate proportion of the expression levels of two or more proteins is important.

# Scope of the thesis

**Chapter 2: Co-expression of functional human Heme Oxygenase 1 (HO-1), Ecto-5′-Nucleotidase (CD73/E5NT) and Ecto-nucleoside triphosphate diphosphohydrolase-1 (CD39/ENTPD1) by "self-cleaving" 2A peptide system.**

Over the years several genes, whose over-expression is capable to down-regulate the inflammatory response and limit apoptosis, were identified. Since has been estimated that at least 10 genetic modifications are required in order to achieve acceptable protection of a xenograft, we aimed to develop an effective system for the simultaneous expression of human CD39 and CD73 in combination with the overexpression of the human heme oxygenase 1, as important target for xenograft protection and inflammation regulation. To this purpose, we have produced a transgenic multicistronic construct by exploiting the features of 2A sequence from Foot and Mouth Disease Virus.

**Chapter 3: Simultaneous Overexpression of Functional Human HO-1, E5NT and ENTPD1 protects murine fibroblasts against TNF-α-induced injury *in vitro*.**

The aim of the present study was to evaluate comprehensively the effect of the combined activity of human CD39, CD73 and HO-1 on the down-regulation of the pro-inflammatory and pro-apoptotic stimuli represented by TNF-α and investigate whether this potential protection may be greater than that mediated by the single gene individually.

## Chapter 4: Simultaneous overexpression of human E5NT and ENTPD1 protects porcine endothelial cells against $H_2O_2$-induced oxidative stress and cytotoxicity *in vitro*.

Purinergic signaling is known to play an important role in inflammation, oxidative stress and ischemia reperfusion injury (IRI) that occur in transplantation settings. Extracellular pathway that converts ATP /ADP to AMP, and AMP to adenosine is mainly mediated by the two important vascular ectonucleotidases ecto-nucleoside triphosphate diphosphohydrolase 1, (ENTPD1 or CD39) and ecto-5'-nucleotidase (E5NT or CD73) respectively. To clarify feasibility of combined expression of human ENTPD1 and E5NT and to study its functional effect against oxidative stress stimuli we transfected an endothelial cell line with both genes together.

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# *CHAPTER 2*

# Co-expression of functional human Heme Oxygenase 1, Ecto-5′-Nucleotidase and ecto-nucleoside triphosphate diphosphohydrolase-1 by “self-cleaving” 2A peptide system

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## ABSTRACT

We developed an F2A-based multicistronic system to evaluate functional effects of co-expression of three proteins important for xenotransplantation: heme oxygenase 1 (HO1), ecto-5′-nucleotidase (E5NT) and ecto-nucleoside triphosphate diphosphohydrolase-1 (ENTPD1). The tricistronic p2A plasmid that we constructed was able to efficiently drive concurrent expression of HO1, E5NT and ENTPD1 in HEK293T cells. All three overexpressed proteins possessed relevant enzymatic activities, while addition of furin site interfered with protein expression and activity. We conclude that our tricistronic p2A construct is effective and optimal to test the combined protective effects of HO1, E5NT and ENTPD1 against xeno-rejection mechanisms.

## 1. INTRODUCTION

Clinical xenotransplantation, which involves the transplantation of cells or organs from pigs to humans, could resolve the issue of limited availability and complications derived by the use of human organs and cells currently available for allo-transplantation. However, all immunological and species-related barriers, as well as the multiple required genetic modifications of the donor pigs, are still major hurdles to overcome (Cooper et al., 2013). Several transgenic pigs overexpressing human genes have been created and their organs have shown to be protected against hyperacute (Cozzi et al., 1997) and acute (Petersen et al., 2011) xenograft rejection mechanisms. It has been

estimated that at least 10 genetic modifications are required in order to achieve acceptable protection of the xenograft (Gock et al., 2011) making it necessary to test different combinations of protective genes in relevant pre-clinical models. Many multigene expression strategies have been developed such as co-transfection with several vectors or coinfection with multiple lentiviruses and also the use of internal ribosomal entry sites (IRES) (Mansouri and Berger, 2014). The F2A sequence from the foot-and-mouth disease virus (FMDV) is a well-established method for co-expressing multiple proteins starting from a single open reading frame (de Felipe et al., 1999). Briefly, the peptide bond formation between the glycine and the proline at the F2A site is prevented, which causes the ribosome to skip and begin to translate the next protein from the next codon. The F2A sequence remains as a tail at the C-terminus of the upstream protein while the ribosome starts translating the following coding sequence (de Felipe et al., 2006). For the purpose of xenotransplantation, the F2A technology has been proposed as the tool to solve the issue of combining multiple genetic modifications in the pig genome instead of time consuming breeding strategies (Gock et al., 2011). Our aim was to test the feasibility of a new combination of three xenotransplatation relevant human genes, Heme Oxygenase 1 (HO1), Ecto 5′ Nucleotidase (E5NT) and Ecto Nucleoside Triphosphate Diphosphohydrolase 1 (ENTPD1) using F2A technology. HO1 is a stress inducible enzyme that catabolizes heme to carbon monoxide (CO), free iron and biliverdin, which have antioxidant, anti-inflammatory and anti-apoptotic effects (Soares and Bach, 2009). Among its known protective effects, HO1 was reported to inhibit the expression of pro-inflammatory genes associated with

endothelial cells activation (Soares et al., 2004) and protect the heart from transplant associated ischemia-reperfusion injury (Akamatsu et al., 2004). ENTPD1 and E5NT are ectonucleotidases involved in the modulation of purinergic signaling by the conversion of extracellular pro-inflammatory ATP/ADP to AMP and AMP to anti-inflammatory and immunosuppressive adenosine, respectively (Antonioli et al., 2013). ENTPD1 exerts a vascular protective function against platelet aggregation and vessel occlusion by terminating the pro-thrombotic and pro-inflammatory effects of ATP and ADP (Huttinger et al., 2012). E5NT-mediated adenosine production protects against vascular inflammation and neointima formation (Zernecke et al., 2006), diminishes trans-endothelial leukocyte trafficking and mitigates inflammatory and immune sequelae of cardiac transplantation (Hasegawa et al., 2008). The protective role for each of these genes alone has been previously demonstrated in different xenotransplantation models (Dwyer et al., 2004; Osborne et al., 2005; Petersen et al., 2011), moreover, the combined overexpression of E5NT and ENTPD1 has been recently suggested to promote the protective roles of E5NT-mediated adenosine pathway to the ENTPD1-based beneficial thromboregulatory effects (Cooper et al., 2012). However, the feasibility of expressing HO1, ENTPD1 and E5NT as a new combination of genes has not been investigated. In this work we developed two versions of F2A-based multicistronic constructs and we analyzed their functionality in an easily transfectable cell line. We demonstrated that p2A plasmid mediated the correct expression of the three exogenous proteins. Moreover, HO1, ENTPD1 and E5NT were functional as shown by appropriate enzymatic activity assays.

## 2. MATERIAL AND METHODS

*2.1 Construction of tricistronic expression cassettes*

Primers and oligonucleotides used for plasmids construction were purchased from Sigma-Aldrich. The 2A fragments were obtained by annealing the two complementary single strand DNA molecules of FMDV 2A sequences (Table S1 Supporting information). In a total volume of 50 µl 1mM EDTA), 2 µg of each complementary oligonucleotide was mixed; this solution was incubated for 3 minutes at 95 °C and then allowed to slowly cool down for 2 hours at 37 °C. After annealing, the first F2A sequence (F2A1 or FurF2A1) was ligated by directional cloning into pcDNA3.1 + (Invitrogen) digested with NheI/AflII. The second F2A sequence (F2A2 or FurF2A2) was ligated into pcDNA3.1 F2A1/FurF2A1 digested with BamHI/XhoI, obtaining pcDNA3.1-F2A1/FurF2A1-F2A2/FurF2A2. An Eppendorf Mastercycler EP silver block thermocycler was used for synthesizing DNA by PCR. Primers used for PCR are listed in Table S1 (Supporting information). Two rounds of recombinant PCR were performed to amplify human Heme Oxygenase 1 (HO1, NCBI: NM_002133). Firstly, HO1 was PCR-amplified from pcDNA3.1-HO1 plasmid (a gift of Prof. Fritz Bach lab, Harvard Medical School, Boston) without the stop codon using primers EcoRI Kozak HO1 fw/NheI HO1 rev and the corresponding amplicon was cloned into pGEM T-easy vector (Promega). This intermediate vector was used as template for the second PCR performed using primers NheI HO1 fw/HO1 rev and the corresponding product was cloned again into pGEM T-easy vector. The entire coding sequence was excised by NheI digestion and cloned into

NheI-linearized pcDNA3.1-F2A1/FurF2A1-F2A2/FurF2A2 upstream of F2A1/FurF2A1, obtaining pcDNA3.1-HO1-F2A1/ FurF2A1-F2A2/FurF2A2. Human Ecto-5′-Nucleotidase (E5NT or CD73, NCBI: NM_002526.3) was PCR-amplified from pcDNA3-E5NT plasmid (received from Dr. Jozef Spychala, University of Michigan, Ann Arbor) without the stop codon using primers AflII E5NT fw/BamHI E5NT rev and the product was cloned into pGEM T-easy vector. Then the entire coding sequence was excised by AflII/BamHI double digestion and ligated into AflII/BamHI-linearized pcDNA3.1-HO1-F2A1/ FurF2A1-F2A2/FurF2A2, in frame between F2A1/FurF2A1 and F2A2/FurF2A2, obtaining pcDNA3.1-HO1-F2A1/FurF2A1- E5NT-F2A2/FurF2A2. Two rounds of recombinant PCR were performed to amplify human ecto-nucleoside triphosphate diphosphohydrolase 1 (ENTPD1 or CD39, NCBI: NM_001776.5). Firstly, ENTPD1 was PCR-amplified from pcDNA3-ENTPD1 plasmid (received fromDr. Simon Robson, Harvard Medical School, Boston) including the stop codon using primers XhoI ENTPD1 fw/EcoRI ENTPD1 rev and the corresponding product was cloned into pGEM-T easy vector. This intermediate vector was used as a template for the second PCR performed using primers ENTPD1 fw/XbaI ENTPD1 rev and the corresponding product was cloned into pGEM-T easy vector. Then the entire coding sequence was excised by XhoI/XbaI double digestion and ligated into XhoI/ XbaI-linearized pcDNA3.1-HO1-F2A1/FurF2A1-E5NT-F2A2/ FurF2A2 downstream of F2A2/FurF2A2, obtaining the final constructs pcDNA3.1-HO1-F2A1-E5NT-F2A2-ENTPD1 and pcDNA3.1-HO1-FurF2A1-E5NT-FurF2A2-ENTPD1, which were named p2A and

pFur2A respectively. Restriction and sequencing analyses were performed on all the intermediate and final constructs (data not shown).

*2.2. Cell culture and transfections*

HEK293T cells were cultured in high glucose DMEM (EuroClone) supplemented with 10% heat inactivated fetal bovine serum (EuroClone) and 1X penicillin/streptomycin (EuroClone), at 37 °C and 5% CO2. Cellswere transiently transfected using TurboFect in vitro Transfection Reagent (Fermentas) following the manufacturer instructions and incubated for 48 hours prior to performing expression or activity assays.

*2.3. Immunoblotting analyses*

Thirty micrograms of total protein extracted in RIPA buffer was separated on 10% NuPAGE BT gel (Life Technologies) and then transferred onto nitrocellulose membranes using the iBlot system (Life Technologies). After blocking in TBST with 5% w/v nonfat dry milk, membranes were probed with rabbit anti-hHO1 (1:2000, EP1391Y, Epitomics), mouse anti-hE5NT (1:500, LS-C138754, LifeSpan BioSciences), mouse anti-hENTPD1 (1:500, HPA014067, Sigma Aldrich), and anti- β-actin (1:5000, AC-15, Sigma Aldrich) primary antibodies. Anti-mouse IgG (H + L) HRP-conjugate (Alpha Diagnostic Intl. Inc.) and ECL anti-rabbit IgG HRP linked (GE Healthcare) secondary antibodies were used at 1:5000 dilutions. Immunoreactive proteins were visualized by enhanced chemiluminescence (Super Signal West Dura, Thermo Scientific) and digitally acquired using G:BOX (Syngene) instrument.

*2.4. Immunofluorescence analyses*

HEK293T cells were washed with PBS and fixed with methanol–acetone (1:1) for 10 min at −20 °C. Following fixation, cells were blocked in 1% BSA (w/v) PBS for 30 min. Fixed cells were co-incubated with rabbit anti-hHO1 (1:200, EPR1390Y, Epitomics) and mouse anti-hE5NT (1:200, 4G4, Novus Biologicals) or mouse anti-hENTPD1 (1:200, BU61, Santa Cruz) primary antibodies for 1 h. All primary antibodies were diluted in 1% BSA (w/v) PBS. After three washes in PBS, cells were incubated for 30 minutes with the appropriate secondary antibodies diluted 1:5000 in 1% BSA (w/v) PBS (Alexa Fluor 488-conjugated anti rabbit and Alexa Fluor 594-conjugated anti mouse, Life Technologies). Cells were washed twice with PBS and counterstained with DAPI. The stained cells were mounted with mounting medium (Fluoromount; Sigma Aldrich) and analyzed by Eclipse 80i microscope (Nikon). Images were acquired by Genikon software (Nikon).

*2.5. E5NT and ENTPD1 activity assay*

Ectonucleotidases activity assay was performed as previously described (De Giorgi et al., 2014). Briefly, HEK293T cells were pre-incubated for 15 minutes in HBSS supplemented with glucose (1 mg/ml) and adenosine deaminase inhibitor, erythro-9-(2-hydroxy-3-nonyl) Adenine, EHNA (5 μM). Cells were incubated with 50 μM of AMP or ATP and supernatant samples were collected after 0, 5, 15, 30 min and analyzed for nucleotide metabolite content by reverse micrograms of crude lysatewas incubated with 15μM hemin and 10 U/ml recombinant biliverdin reductase A. Bilirubin fluorescence was detected every 2 min

for 2 h in a fluorescence reader (Infinite M200; Tecan) at 37 °C (excitation/emission wavelengths: 441/528 nm).

*2.7. Statistical analysis*

Values are presented as mean ± standard error of mean (SEM). Statistical analyses were performed using SPSS v.19 for Mac. One-way analysis of variance (ANOVA) with Tukey's post hoc test was used to compare between cell experimental groups. A p-value of $<0.05$ was considered as significant difference.

## 3. RESULTS AND DISCUSSION

*3.1. Plasmids construction*

In this study we developed two F2A-based plasmid systems and evaluated functionality of co-expression of three proteins that are important for xenotransplantation: heme oxygenase 1 (HO1), ecto-5′-nucleotidase (E5NT) and ectonucleoside triphosphate diphosphohydrolase-1 (ENTPD1). We produced p2A plasmid by cloning HO1 as the first coding sequence linked to the first F2A sequence (F2A1) in frame with the E5NT coding sequence, which was then followed by the second F2A sequence (F2A2) and finally the ENTPD1 coding sequence (Fig. 1). HO1 has a cytoplasmic localization (Gottlieb et al., 2012) while active E5NT and ENTPD1 are localized at the plasma membrane (Kaczmarek et al., 1996; Klemens et al., 1990). Moreover, E5NT encodes a N-terminal signal peptide, which directs it through the endoplasmic reticulum before reaching the plasma

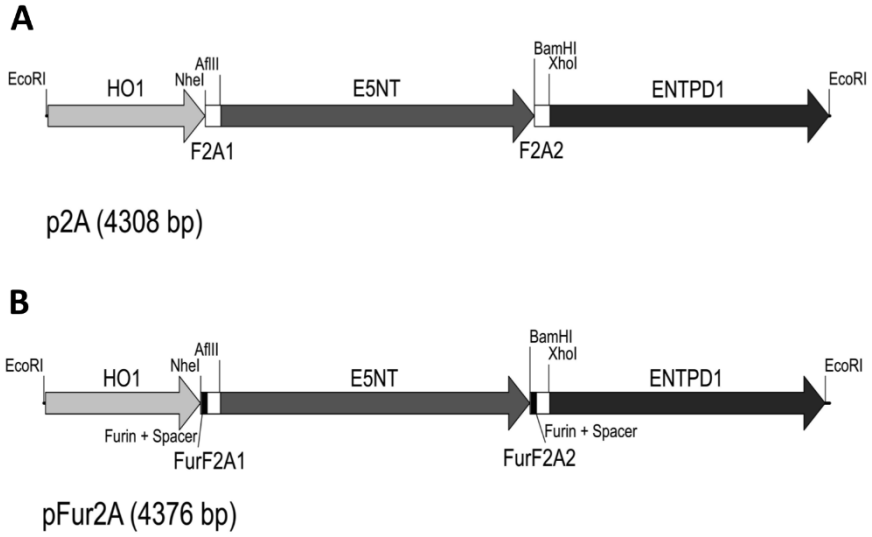


**Fig. 1.** Schematic representation of p2A (A) and pFur2A (B) constructs. Two F2A (87 bp) or FurF2A (111 bp) sequences have been cloned in pcDNA3.1 commercial vector (Life Technology). FurF2A sequences contain a furin recognition site and a spacer site upstream of the F2A sequences. PCRamplified HO1 (867 bp), E5NT (1725 bp) and ENTPD1 (1533 bp) have been respectively cloned as first, second and third coding sequences. Plasmid representations are not in scale.

membrane (Misumi et al., 1990). The selected gene order was chosen to maximize the likelihood of correct subcellular processing of each of the three proteins and to avoid HO1 (lacking any signal sequence) being "slipstreamed" through the translocon formed by the (signal-bearing) E5NT (de Felipe and Ryan, 2004). In a attempt to reduce the possible negative effects of the C-terminal F2A tail on HO1 and E5NT expression and activity (Rothwell et al., 2010), we generated a second F2A-based construct (named pFur2A (Fig. 1B)) by adding a furin recognition site (RAKR) followed by a spacer (SGSG) upstream of each F2A sequence (FurF2A1 and FurF2A2), according to a previously described strategy (Fang et al., 2005; Yang et al., 2008).

*3.2. Expression analyses*

We tested the functionality of the two tricistronic constructs (p2A and pFur2A) by transiently transfecting HEK293T cells for 48 h. Appropriate mock- and single gene transfected cells were used as controls. We investigated expression of each the three proteins by performing immunoblot analyses (Fig. 2). Immunodetection with the indicated antibodies in p2A-transfected cells clearly detected bands corresponding to the exogenously expressed HO1 (Fig. 2A), E5NT (Fig. 2B), and ENTPD1 (Fig. 2C). Lower expression levels of each exogenous protein were observed in pFur2A- as compared to p2A-transfected cells (Fig. 2A–C), suggesting that the furin/spacer sites had a negative impact on F2A-mediated cleavage. The molecular weight of HO1 exogenously expressed from either p2A- or pFur2A-transfected cells was larger than those cells transfected with HO1-alone (Fig. 2A), resulting from the presence of the F2A-derived or furin + spacer + F2A-derived C-terminal extension, respectively, indicating an absence of any furin mediated cleavage of 2A tail in pFur2A-transfected cells. No difference in size was observed between the exogenously expressed E5NT in either p2A- and pFur2A-transfected cells as compared to the corresponding protein in E5NT-transfected cells, suggesting the absence of F2A sequence at the C-terminus of E5NT expressed from both of the tricistronic plasmids (Fig. 3B). It was reported that E5NT undergoes proteolytic cleavage of its C-terminal signal and simultaneous replacement with glycosylphosphatidylinositol, which functions as the plasma membrane anchor of the mature protein (Misumi et al., 1990). We speculated that this cleavage may be responsible for the release of the F2A tail at the C-terminus of

exogenous E5NT. Transfection of each of the three genes alone resulted in very high protein expression levels (Fig. 2A–C) as compared to expression from the tricistronic plasmids, probably due to the reduction in transfection efficiency and consequently amount of translated proteins, which has been reported in larger size plasmids as compared to small plasmids (Yin et al., 2005). On the contrary, pFur2A was only 48 base pairs (bp) larger than p2A, not enough in our opinion to be cause of the observed protein expression differences. A slight band of 130 kDa uncleaved product, probably corresponding to E5NT- 2A-ENTPD1, has been observed both in p2A- and pFur2Atransfected cell lines of ENTPD1 immunoblot, however this band was more intense in pFur2A- as compared to p2Agroup (Fig. 2C). Additionally, the correct subcellular localization of HO1, E5NT and ENTPD1 was found to be the same in both p2A- and pFur2A-transfected cells, as detected by immunofluorescence. HO1 had a main perinuclear localization, while both E5NT and ENTPD1 showed a plasmamembrane staining pattern (Fig. 2D), accordingly with the literature (Gottlieb et al., 2012; Kaczmarek et al., 1996; Klemens et al., 1990). We observed higher protein expression in the p2Atransfected cells as compared to pFur2A-transfected cells (Fig. 2A–C) with more uncleaved product in the latter group (Fig. 2C), suggesting that the added furin cleavage sites and spacers in pFur2Amay interfere with the F2A-mediated cleavage. Taken together, these analyses indicated that p2A results in a more efficient expression of HO1, E5NT and ENTPD1 than pFur2A. Although an increase in expression levels derived by the addition of furin recognition site and spacers (Yang et al., 2008) as well as the versatile usage of the furin-2A system in the production of multi-

transgenic constructs for xenotransplantation purposes (Fisicaro et al., 2011) were reported, we did not obtain the same outcomes in our system. Verrier et al. showed that the addition of a furin recognition site upstream of 2A sequence in their GFP-2A-mCher plasmid interfered with 2A cleavage (Verrier et al., 2011) and suggested that the inclusion of an additional amino acid spacer between the furin and 2A siteswould improve cleavage efficiency of exogenous proteins (Verrier et al., 2011). Whereas the addition of both furin recognition site and spacer upstream of F2A sequences in our system resulted in an inefficient production of our transgenes. To improve the 2A efficiency, recent efforts are aimed to identify the optimal length of the F2A sequence itself for successful co-expression strategies (Minskaia et al., 2013). The presence of the F2A derived C-terminal extension could potentially induce an immune response in xenotransplantation settings. Several F2A-based multitransgenic pigs have already been produced (Deng et al., 2011; Park et al., 2014) but no 2A-mediated immune responses have been reported so far. Recently, 2A sequences have been successfully incorporated in vectors used in human gene therapy studies without eliciting discernible immune responses (Di Stasi et al., 2011). Moreover, the exposure of human PBMCs to viral-derived 2A sequences did not produce T cell responses in an ex-vivo model (Arber et al., 2013). These last reports strengthen the use of F2A technology even in xenotransplantation research.

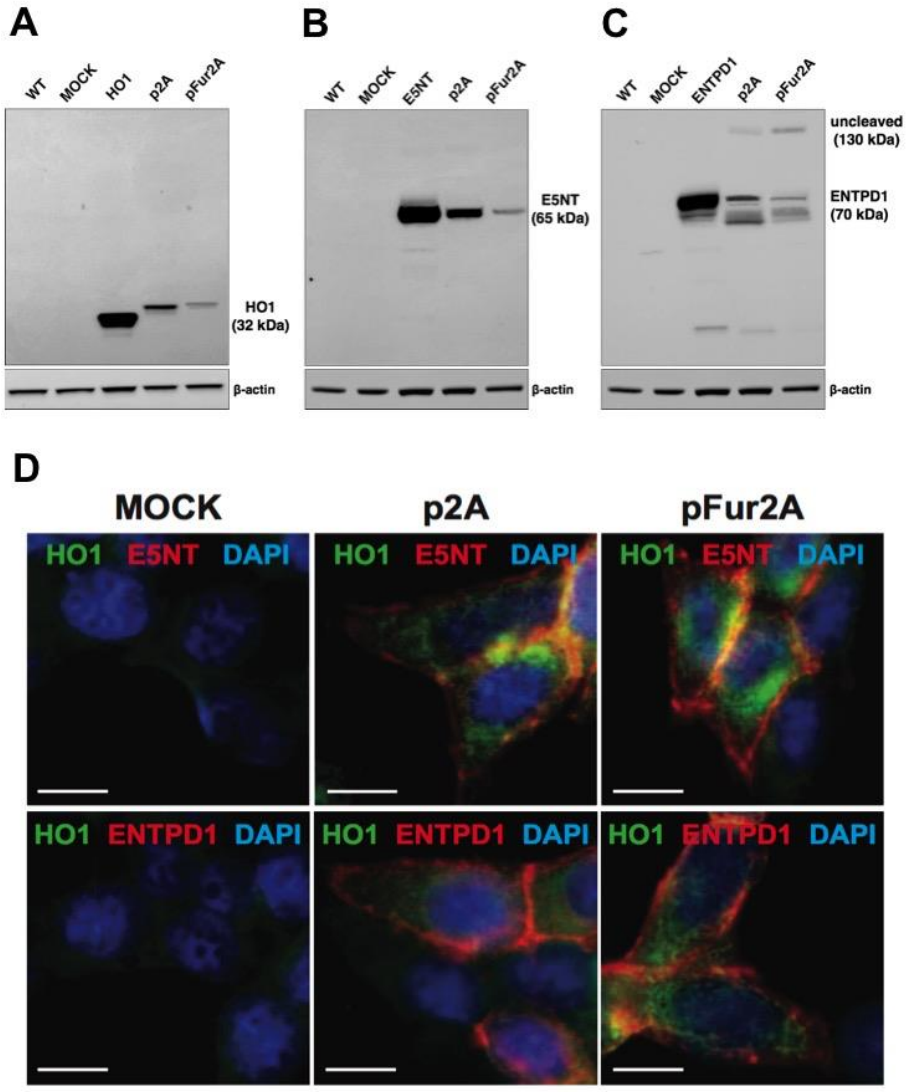


**Fig. 2.** Expression analyses in wt and transfected HEK293T cells. Immunoblotting analyses of HO1 (A), E5NT (B) and ENTPD1 (C) from wt, mock-, p2Aand pFur2A-transfected cells. HO1-, E5NT- and ENTPD1-transfected cells lysates have been used as positive controls in (A), (B) and (C) respectively. β-Actin has been used as loading control. HO1 band: ≈32 kDa; E5NT band: ≈63 kDa; ENTPD1 band: ≈70 kDa; β-actin band: 42 kDa. An uncleaved product of ≈130 kDa has been detected in p2A and pFur2A lanes. (D) Immunofluorescence analysis with double stainings of HO1 (green) and E5NT or ENTPD1 (red) in mock- (left), p2A- (middle) and pFur2A- (right) transfected cells. Nuclei were counterstained with DAPI (blue). Scale bar, 10 μm.

*3.3. Enzymatic activity analyses*

Next, we tested the enzymatic activity of ENTPD1 and E5NT produced from the two tricistronic plasmids using an extracellular nucleotide metabolism assay. Confluent untransfected (wt) and transiently transfected cells were incubated with ATP or AMP and supernatant samples were collected at different time points and analyzed for their nucleotide content by HPLC (De Giorgi et al., 2014; Smolenski et al., 1990). During incubation with ATP, extracellular ADP concentration in ENTPD1-transfected cells reached a maximal concentration after 5 min (4.9 ± 0.1 μM) after which ADP was completely catabolized (Fig. 3A). A similar but delayed trend was observed in p2A-transfected cells, where the ADP concentration decreased to 4.9 ± 1.6 μMafter reaching a maximal level at 15 min (5.8 ± 0.1 μM, Fig. 3A). In pFur2A-, E5NT-transfected cells and controls an increasing formation of ADP over time was observed with the highest concentration being observed in the pFur2Atransfected cells (8.3 ± 0.6 μM, Fig. 3A). Conversion of AMP in ENTPD1-transfected cells increased to 50.9 ± 0.4 μM while the concentration remained below 2 μM in untransfected mock- and E5NT-transfected cells ($p < 0.05$), suggesting a very low basal ATP hydrolyzing activity under these control conditions (Fig. 3B). In p2A- and pFur2A-transfected cells the extracellular AMP increased to 18.7 ± 1.9 and 19.3 ± 1.5 μM, respectively, with no statistical difference being observed between the two tricistronic plasmids (Fig. 3B). A substantial increase in adenosine formation from ATP was also observed only for the p2A- and pFur2A-transfected cells (34.7 ± 3.3 and 22.8 ± 2.9 μM respectively, Fig. 3C) with adenosine production being higher in p2A- as compared to pFur2A-transfected cells at all time points ($p < 0.05$,

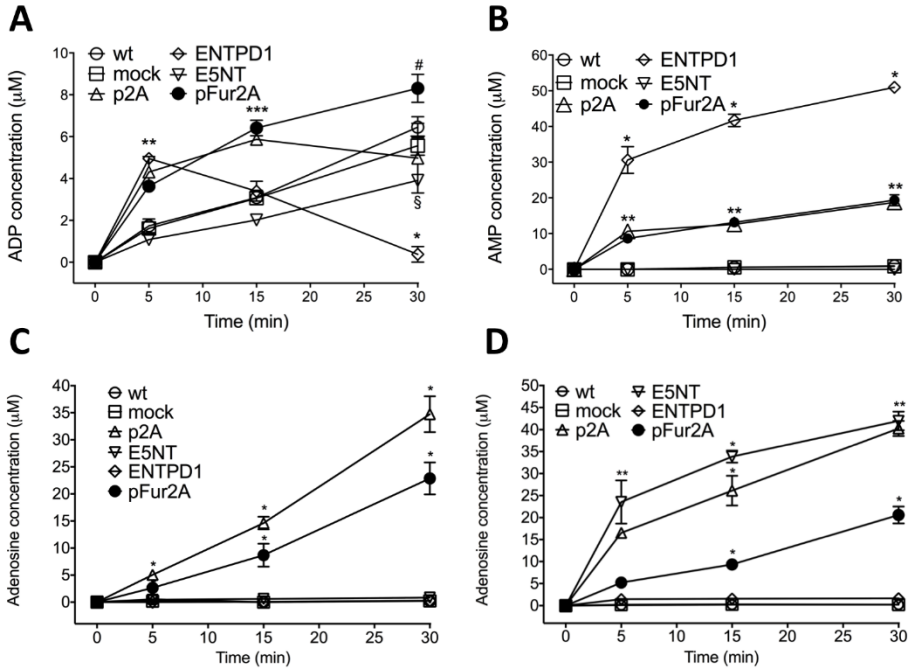


**Fig. 3.** ENTPD1 and E5NT activity assays in wt and transfected HEK293T cells. (A) ADP production during incubation with 50 µM ATP in wt, mock-, E5NT-, ENTPD1-, p2A- and pFur2A-tranfected cell groups. **p* < 0.05 ENTPD1- vs. all groups; ***p* < 0.05 pFur2A-, p2A-, ENTPD1- vs. wt, mock- and E5NT-groups; ****p* < 0.05 p2A- and pFur2A- vs. wt, mock-, E5NT- and ENTPD1-groups; #*p* < 0.05 pFur2A- vs. p2A-, E5NT- and ENTPD1-groups; §*p* < 0.05 p2A- and E5NTvs. pFur2A- and ENTPD1-groups. (B) AMP production during incubation with 50 µM ATP. **p* < 0.05 ENTPD1- vs. all groups; ***p* < 0.05 p2A- vs. all groups except pFur2A-group. (C) Adenosine production during incubation with 50 µM ATP. **p* < 0.05 vs. all groups. (D) Adenosine production during incubation with 50 µM AMP. **p* < 0.05 vs. all groups; ***p* < 0.05 E5NT vs. all groups except p2A-group. Values at each time points represent mean ± SEM (n = 3).

Fig. 3C). No adenosine production in the time of incubation has been observed in ENTPD1- and E5NT-transfected cells as well as in controls (Fig. 3C). During incubation with AMP, the concentration of adenosine in the medium of p2A-, pFur2A- and E5NTtransfected cells increased to 40.2 ± 1.7, 20.6 ± 1.9 and 41.9 ± 2.1 µM, respectively, while it remained below 2 µM in ENTPD1-transfected and controls cells (Fig. 3D). At every time points the concentration of adenosine in p2A

transfected cells was significantly higher than in pFur2Atransfected cells (Fig. 3D), correlating with a more efficient production of active E5NT in p2A-transfected cells (Fig. 2B). The adenosine levels were also similar between p2A- and E5NT-transfected cells at every measured interval (Fig. 3D). No evident adenosine production in the period of incubation has been observed in control cells as well as in ENTPD1-transfected cells suggesting a very low basal AMP hydrolyzing activity (Fig. 3D).

The activity of HO1 was investigated by measuring the increase of bilirubin fluorescence during the incubation of total cellular lysates with hemin (Klemz et al., 2009). As shown in Fig. 4, the activity of HO1 in p2Atransfected cells was 5-fold higher than the basal activity observed in untransfected (wt) and mock-transfected control cells (1.54 ± 0.12 nmol/h/mg vs. 0.31 ± 0.04 and 0.33 ± 0.07 nmol/h/mg, respectively, in wt and mocktransfected cells, $p < 0.05$). No difference in activity was observed between wt and mock-transfected cells ($p > 0.05$, Fig. 4). Moreover a slight activity increase was observed in pFur2A-transfected cells (0.74 ± 0.05 nmol/h/mg) as compared to untransfected (wt) and mock-transfected control cells ($p > 0.05$, Fig. 4). This result correlated with the lower protein expression of HO1 in pFur2A- with respect to p2Atransfected cells (Fig. 2A). A substantial increase in activity was observed in HO1-transfected cells (4.94 ± 0.4 nmol/h/mg, Fig. 4), which correlated with a markedly higher expression level of exogenous HO1 in HO1-transfected cells than in p2A-transfected cells (Fig. 2A). All together, our findings on enzymatic activity suggested a more efficient production of active enzymes driven by p2A than those driven by pFur2A.

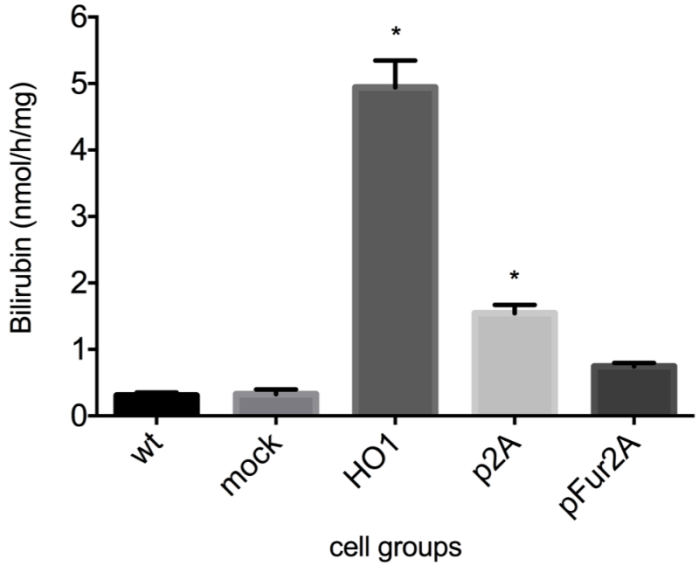


**Fig. 4.** Heme oxygenase activity assay on wild type and transfected HEK293T cells. Bilirubin production during a 2 h incubation of wt, mock-, HO1-, p2A- and pFur2A-transfected cells with 15 μM hemin in reaction buffer containing 300 μM BSA and 10 U/ml of recombinant bilverdin reductase. Bilirubin production is expressed as nanomoles of bilirubin produced per hour and per milligram of proteins. *$p < 0.05$ vs. all groups. Values represent mean ± SEM (n = 3).

The significantly higher production of adenosine in p2A-transfected cells following incubation with ATP (Fig. 3C) suggested a better coordination of both ectonucleotidases activity in p2Atransfected cells. We hypothesize that the lower ENTPD1- AMP production following ATP incubation in both p2A- and pFur2A-transfected cells as compared to ENTPD1-transfected cells was due to the simultaneous E5NT-mediated AMP catabolism to adenosine (Fig. 3B). Although the ENTPD1- mediated AMP production in p2A- and pFur2A-transfected cells was apparently similar (Fig. 3B), we observed a more efficient production of adenosine in the former group (Fig. 3C) which let us hypothesize a higher ENTPD1-mediated production of AMP substrate

for E5NT in p2A- as compared to pFur2A-transfected cells. However, an initial increase in ADP concentration during incubation with ATP in p2A-transfected cells (Fig. 3A) highlights the need for careful and complex analysis of consequences of such manipulations on coagulation and inflammation. A higher HO1 activity in p2A- as compared to pFur2A-transfected cells has been also shown (Fig. 4). We reported that F2A sequences alone gave an efficient expression of the transgenes with no interference with their enzymatic activity regardless a slight amount of uncleaved E5NT/ENTPD1 dimer (Fig. 2C).

*4. Conclusions*

To translate xenotransplantation to the clinic, major immunologic and biochemical barriers need to be overcome (Cooper et al., 2013). Multiple genetic modifications in donor pigs are necessary and different combinations of these will need to be tested in relevant pre-clinical models. The 2A technology has been proposed as an important tool with which to resolve the issue of combining genetic modifications (Gock et al., 2011).We previously demonstrated that F2A-mediated co-expression was functional for expression of E5NT and ENTPD1 in an appropriate endothelial cell line (De Giorgi et al, 2014) and here we now demonstrated the correct and functional co-expression of three proteins, HO1, E5NT and ENTPD1. We showed that p2A allowed the correct expression of three proteins that were differentially processed (Fig. 2). More importantly, these proteins were found to be functional as demonstrated by the increase of Heme Oxygenase 1 and both Ectonucletotidases products in their respective activity assays (Fig. 3 and 4). Therefore the catabolism of pro-inflammatory heme and

ATP/ADP may be enhanced together with the production of anti-inflammatory products.We observed less efficient protein expression levels together with more uncleaved product (Fig. 2) and decreased activities (Figs. 3 and 4) in pFur2A-transfected cells as compared to p2Atranfected cells. In conclusion, having demonstrated its correct functionality, we propose p2A construct to be tested in a relevant pre-clinical model of xenotransplantation to investigate if the combining protective effects of HO1, E5NT and ENTPD1 may be able to attenuate a larger spectrum of xeno-rejection mechanisms. The p2A plasmid could be used to produce human HO1, E5NT and ENTPD1-triple transgenic pigs by a single round of Somatic Cell Nuclear Transfer (SCNT) (Deng et al., 2011) or by Sperm Mediated Gene Transfer (SMGT) (Lavitrano et al., 2002).

**Keywords**: 2A peptide; Heme oxygenase 1; Ecto 5′ nucleotidase; Ecto nucleoside triphosphate diphosphohydrolase 1; Multicistronic vector; Xenotransplantation.

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## Supporting Information

Table 1: List of F2A/FurF2A oligonucleotides and primers used in the construction of multi-gene cassettes

| Name | Sequence (5'-3') |
|---|---|
| F2A1-*1* | CTAGCGTGAAACAGACTTTGAATTTTGACCTTCTCA AGTTGGCGGGAGACGTGGAGTCCAACCCAGGGCCC GGCAGCGGCC |
| F2A1-*2* | TTAAGGCCGCTGCCGGGCCCTGGGTTGGACTCCAC GTCTCCCGCCAACTTGAGAAGGTCAAAATTCAAAG TCTGTTTCACG |
| F2A2-*1* | GATCCGTGAAACAGACTTTGAATTTTGACCTTCTCA AGTTGGCGGGAGACGTGGAGTCCAACCCAGGGCCC GGCAGCGGCC |
| F2A2-*2* | TCGAGGCCGCTGCCGGGCCCTGGGTTGGACTCCAC GTCTCCCGCCAACTTGAGAAGGTCAAAATTCAAAG TCTGTTTCACG |
| FurF2A1-*1* | CTAGCCGTGCCAAGCGATCTGGATCTGGCGTGAAA CAGACTTTGAATTTTGACCTTCTCAAGTTGGCGGGA GACGTGGAGTCCAACCCAGGGCCCGGCAGCGGCC |
| FurF2A1-*2* | TTAAGGCCGCTGCCGGGCCCTGGGTTGGACTCCAC GTCTCCCGCCAACTTGAGAAGGTCAAAATTCAAAG TCTGTTTCACGCCAGATCCAGATCGCTTGGCACGG |
| FurF2A2-*1* | GATCCCGTGCCAAGCGATCTGGATCTGGCGTGAAA CAGACTTTGAATTTTGACCTTCTCAAGTTGGCGGGA GACGTGGAGTCCAACCCAGGGCCCGGCAGCGGCC |

| | |
|---|---|
| FurF2A2-*2* | TCGAGGCCGCTGCCGGGCCCTGGGTTGGACTCCAC GTCTCCCGCCAACTTGAGAAGGTCAAAATTCAAAG TCTGTTTCACGCCAGATCCAGATCGCTTGGCACGG |
| *EcoRI* Kozak HO1 fw | GAATTCCGGATGGAGCGTCCGCAA |
| *NheI* HO1 rev | GCTAGCCATGGCATAAAGCCCTACAGCAA |
| *NheI* HO1 fw | GCTAGCGAATTCCGGATGGAGCGT |
| HO1 rev | GCTAGCCATGGCATAAAGC |
| *AflII* E5NT fw | CTTAAGATGTGTCCCCGAGCCGC |
| *BamHI* E5NT rev | GGATCCTTGGTATAAAACAAAGATCACTGC |
| *XhoI* ENTPD1 fw | CTCGAGATGGAAGATACAAAGGAGTCTAACG |
| *EcoRI* ENTPD1 rev | GAATTCCTATACCATATCTTTCCAGAAATATGAAG |
| ENTPD1 fw | CTCGAGATGGAAGATACAAAGG |
| *XbaI* ENTPD1 rev | TCTAGAGAATTCCTATACCATATCTTTCCAG |

# *CHAPTER 3*

# Simultaneous Overexpression of Functional Human HO-1, E5NT and ENTPD1 protects murine fibroblasts against TNF-α-induced injury *in vitro*

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**ABSTRACT**

Several biomedical applications, such as xenotransplantation, require multiple genes simultaneously expressed in eukaryotic cells. Advances in genetic engineering technologies have led to the development of efficient polycistronic vectors based on the use of the 2A self-processing oligopeptide. The aim of this work was to evaluate the protective effects of the simultaneous expression of a novel combination of anti-inflammatory human genes, ENTPD1, E5NT and HO-1, in eukaryotic cells. We produced an F2A system-based multicistronic construct to express three human proteins in NIH3T3 cells exposed to an inflammatory stimulus represented by tumor necrosis factor alpha (TNF-α), a pro-inflammatory cytokine which plays an important role during inflammation, cell proliferation, differentiation and apoptosis and in the inflammatory response during ischemia/reperfusion injury in several organ transplantation settings. The protective effects against TNF-α-induced cytotoxicity and cell death, mediated by HO-1, ENTPD1 and E5NT genes were better observed in cells expressing the combination of genes as compared to cells expressing each single gene and the effect was further improved by administrating enzymatic substrates of the human genes to the cells. Moreover, a gene expression analyses demonstrated that the expression of the three genes has a role in modulating key downstream regulators of TNF-α signalling pathway, as *Nemo*, that promoted pro-survival phenotype in TNF-α injured cells. These results could provide new insights in the research of protective mechanisms in transplantation settings.

## 1. INTRODUCTION

The expression of multiple proteins in eukaryotic cells has become crucial in many biomedical applications of contemporary cell biology [1]. Advances in genetic engineering technologies have led to the production and development of efficient polycistronic vectors essentially based on two strategies: the use of internal ribosome entry site (IRES) sequence [1] or the 2A self-processing oligopeptide [2,3]. The 2A coexpression system works in all eukaryotic expression systems and in all cell types [2] and it is based on the co-translationally self-processing activity of 2A sequences, such that each constituent protein encoded by a single mRNA is generated as a discrete translation product [4]. The 2A and '2A-like' sequences have been successfully used to express several proteins in lentivirus-mediated gene therapy approaches [5] and in the production of monoclonal antibodies in transgenic mice [6].

Another biomedical application requiring multiple genetic modifications in eukaryotic cells is xenotransplantation, where the complexity of immunological barriers to be overcome for a successful experiment requires several human genes to be overexpressed in the cells of the potential donor species [7,8]. In pig to non-human primates models two main processes, hyperacute rejection (HAR) and acute vascular rejection (AVR), rapidly attack vascularized organs [9]. Transplanted organs are also subjected to several antigen-independent injuries, such as ischemia/reperfusion injury (IRI) [10,11] and free radicals production [12].

Over the years several genes, whose induction or over-expression is able to modulate the inflammatory response and preserve the metabolism of transplanted organs were identified.

Heme oxygenase 1 (HO-1) plays a protective role by preventing oxidative stress because of its antioxidant and antiapoptotic properties and via suppression of the immune response through anti-inflammatory mechanisms [13-15]. In a model of cardiopulmonary by-pass, we previously demonstrated the protective effects of HO-1/CO against ischemia reperfusion injury [16]. Anyhow transgenic expression of hHO-1 is promising to prolong survival of xenografts but should be part of multiple transgenic modification for xenotransplantation.

Vascular endothelium within the transplanted organ is the primary target of rejection for all the mechanisms described in xenotransplantation. The nucleotide metabolism of endothelial cells may contribute significantly to the vascular diseases in acute humoral rejection [17]. Nucleosides and catabolites of adenosine in mammals are of particular interest in the field of xenotransplantation due to their combined cytoprotective, immunosuppressive and anti-inflammatory effects [18-20]. Extracellular adenosine is produced by a pathway mediated mainly by ectonucleotidases ecto-nucleoside triphosphate diphosphohydrolase 1 (ENTPD1 or CD39), and ecto-5'-nucleotidase (E5NT or CD73) [21]. Several *in-vitro* or *in-vivo* models have been produced with genetic defects or overexpression of ENTPD1 or E5NT [22-25] to investigate the role of these proteins in modulating inflammation. The aim of this work was to evaluate the protective effects of the simultaneous expression of a novel combination of anti-inflammatory human genes, ENTPD1, E5NT and HO-1, in eukaryotic

cells. We produced an F2A system-based multicistronic construct to express three human proteins in murine NIH3T3 cells exposed to an inflammatory stimulus represented by human tumor necrosis factor alpha (TNF-α), a pro-inflammatory cytokine which plays an important role in the immune system during inflammation, cell proliferation, differentiation and apoptosis [26] and in the inflammatory response during ischemia/reperfusion injury in several organ transplantation settings [8,27-30] .

This study demonstrated, for the first time, the protection against inflammatory stimuli of a novel combination of human genes, when they are simultaneously expressed in murine NIH3T3 cells.

## 2. MATERIAL AND METHODS

### 2.1. Reagents and antibodies

Recombinant human TNF-α, hemin and ATP were purchased from Sigma Aldrich. NIH3T3 cells were treated with reagents diluted in complete medium at concentrations determined by preliminary experiments and detailed below. Anti-hE5NT (4G4, Novus Biologicals), anti-hHO-1 (EP1391Y, Epitomics) and anti-hENTPD1 (BU61, Santa Cruz) primary antibodies, Alexa Fluor 488-conjugated anti-mouse and Alexa Fluor 555-conjugated anti-rabbit (Life Technologies) secondary antibodies were used for immunofluorescence analysis. Anti-hHO-1 (EP1391Y, Epitomics), anti-hE5NT (EPR6115, LifeSpan BioSciences), anti-hENTPD1 (HPA014067, Sigma Aldrich), and anti-β-actin (AC-15, Sigma Aldrich) primary antibodies were used

for immunoblotting analysis. Phycoerythrin (PE)-conjugated anti-hE5NT (BD Biosciences) and Alexa Fluor 647-conjugated anti-hENTPD1 (Life Technologies) antibodies were used for FACS analysis and cell sorting.

### 2.2. Triple cistronic vector construction

The triple cistronic vector was prepared following a strategy similar to those previously reported by Ryan *et al*. [31] and by our group [32]. Briefly, the coding sequence of the plasmid pcDNA3.1-hHO1-F2A1-hE5NT-F2A2-hENPTD1 [32] was excised and ligated into pCX-C1 plasmid (a pCX-EGFP plasmid [33] to which a neomycin resistance cassette has been added) to form the final pCX-hHO1-F2A1-hE5NT-F2A2-hENTPD1-C1, which was called pCX-TRI-2A. Control plasmids, expressing only one of the three human proteins at once, were prepared by ligation of the PCR-amplified coding sequence into pCX-C1 plasmid, to obtain pCX-hHO1, pCX-hE5NT and pCX-hENTPD1 respectively. To facilitate cell sorting of pCX-hHO1-transfected cells, EGFP gene was cloned in frame downstream the HO1/F2A sequence. Restriction and sequencing analyses were performed on all the intermediate and in the final construct. Empty pCX-C1 plasmid was used for mock transfections.

### 2.3. Cell culture and electroporation

NIH3T3 cells were grown in Dulbecco's minimum essential medium (DMEM) (EuroClone) supplemented with 10% fetal calf serum (Sigma Aldrich), at 37°C and 5% $CO_2$.

Cells were split and plated to reach 80-90% confluence on the day of transfection. pCX-TRI-2A, pCX-HO1, pCX-hE5NT, pCX-hENTPD1 and empty vector plasmids were introduced into NIH3T3 cells by electroporation using Neon Transfection System (Life Technologies) according to the manufacturer's instructions for NIH3T3 cell type. Cells were immediately re-suspended in growth medium with serum without antibiotics and plated. After 24 hours, cells were transferred in standard medium plus 0.5 mg/ml of G418 (Sigma Aldrich) and selected for 7 days.

**2.4. Flow Cytometry analysis and cell sorting**

WT and pCX-TRI-2A transfected cells were detached with trypsin/EDTA and washed once with PFN buffer (serum 3%, $NaN_3$ 0,01% in PBS). Cells were then incubated for 30 min in the dark with fluorophore-conjugated anti-hE5NT and anti-hENTPD1 antibodies. The excess and non-specifically bound antibodies were removed by washing with PFN buffer. Flow cytometric analysis of stained cells was performed with a FACSAria flow cytometer (Becton Dickinson). Lymphocytes were used as a positive control, wild type and mock-transfected NIH3T3 cells were used as a negative controls and the not specific cross-reaction of antibodies was excluded by incubating cells with isotype-matched immunoglobulins. pCX-hE5NT and pCX-hENTPD1 transfected cells were stained only with the corresponding antibody. pCX-hHO1 transfected cells were sorted and analyzed on the basis of EGFP expression.

### 2.5. Immunofluorescence and Confocal Microscopy

Mock- and pCX-TRI-2A-transfected cells were seeded at $4x10^4$cells/well in 8-well chamber slides for 24 hours (LabTek Chamber slides, Thermo Fisher Scientific). The next day, cells were washed with PBS and fixed with methanol-acetone 1:1 for 10 min at -20°C. After fixation, cells were blocked with 1% BSA for 30 min. Fixed cells were incubated for 1 hour with primary antibodies, and for 30 minutes with the appropriate secondary antibodies diluted in 1% BSA (w/v) in PBS. Cells were then washed and counterstained with DAPI. The stained cells were mounted with mounting medium (Fluoromount; Sigma Aldrich) and analyzed by LSM 710 confocal microscope (Zeiss). Images were acquired by ZEN 2009 software (Zeiss).

### 2.6. Immunoblotting

Transfected cell lines were lysed in RIPA buffer and whole protein concentration was quantified by Bradford assay (Sigma Aldrich). 20 μg of total protein extracts were separated in a 10% NuPAGE BT gel (Life Technologies) and then transferred onto nitrocellulose membranes using the iBlot system (Life Technologies). The membranes were probed with anti-hHO-1, anti-hE5NT, anti-hENTPD1 and anti-β-actin primary antibodies. Immunoreactive proteins were visualized by enhanced chemiluminescence (SuperSignal West Dura, Thermo Scientific) and digitally acquired using G:BOX (Syngene) instrument.

### 2.7. Heme Oxigenase activity assay

Heme oxygenase activity assay was performed as previously described [32,34]. Briefly, cells were lysed in lysis buffer (100 mM Tris-HCl, 150

mM NaCl, 1% Triton X-100, pH 7.4, supplemented with 2% protease inhibitor cocktail) and protein concentration was quantified by Bradford assay. 300 μg of crude lysate were with 15 μM hemin and 10U/ml recombinant biliverdin reductase A. The fluorescence of bilirubin was detected every 2 minutes in a fluorescence reader (Infinite M200; Tecan) at 37°C (excitation/emission wavelengths: 441/528nm). As a positive control of specific detection of heme oxygenase 1 activity, WT and mock transfected NIH3T3 cells were stimulated with 50 μM Cobalt-Protoporphyrin for 24 hours before the assay.

### 2.8. E5NT and ENTDP1 activity assay

Ectonucleotidases activity assay was performed as previously described [35]. Briefly, NIH3T3 cells were washed with Hank balanced salt solution (HBSS) and pre-incubated for 15 minutes in HBSS supplemented with glucose (1mg/ml) and Adenosine Deaminase inhibitor, erythro-9-(2-Hydroxy-3-Nonyl) adenine, EHNA (5 μM). Cells were then incubated with 50 nmol/ml of AMP or ATP and supernatant samples were collected after 0, 5, 15, 30 minutes, frozen at -80°C and then analyzed by reverse phase HPLC [36] on Agilent 1100 HPLC instrument with a diode array detector.

### 2.9. Cytotoxicity assay

WT and transfected cell lines were plated in quadruplicate in 96-well plate at $12x10^3$ cells per well. The day after plating, cells were incubated in culture medium with of without different combination of drugs (TNF-α 50 ng/ml, hemin 20 μM and ATP 200 μM) for 24h and 48h.

Cell toxicity was quantified by measurement of lactate dehydrogenase (LDH) release into the medium by using the LDH assay kit (Roche Diagnostics) following manufacturer's instructions.

### 2.10. Caspase activity assay

The Caspase-Glo 3/7 (Promega) assay was performed on WT and transfected cell lines grown in a white 96-well plate to reach 80% confluence, following manufacturer's instructions. Briefly, after 16h and 24h of treatments with TNF-α 50 ng/ml, hemin 20 μM and ATP 200 μM, lyophilized Caspase-Glo 3/7 substrate was resuspended and added into each well. The contents of the wells were mixed gently and incubated at room temperature for 1 hour. Luminescent signal was measured with a 96 multi-well plate reader (Infinite M200; Tecan).

### 2.11. Real time PCR analysis of TNF-α signaling genes

The expression of 84 TNF-α pathway-related genes in mouse were examined using the $RT^2$ Profiler PCR array (PAMM-063C, SuperArray Bioscience). WT and transfected cell lines were treated with TNF-α 50ng/ml alone or in combination with hemin 20 μM and ATP 200 μM for 16h. Untreated cells for each cell line were used as a control. Total RNA was isolated from treated and control cells by using the RNeasy Mini kit (Qiagen) according to manufacturer's instructions. RNA samples were treated with DNase to ensure elimination of genomic DNA, and the extracted RNA was converted to cDNA using the $RT^2$ First Strand Kit from SuperArray Bioscience (Qiagen) following manufacturer's protocol. PCR was performed with the $RT^2$ Profiler PCR array system according to the manufacturer's instructions using

Step One Plus instrument (Applied Biosystems). The mRNA expression levels of each gene in each cell treatment were normalized using the expression of the housekeeping genes *B2m, Gapdh, Gusb, Hsp90ab1*, and *Actb*. The results were confirmed by quantitative reverse transcriptase-PCR performed using individual RNA samples from the cells in each group by Step One Plus instrument (Applied Biosystems). The primers used for real-time PCR are listed in S1 Table.

### 2.12. Statistical Analysis

Statistical analyses were performed using SPSS v.19 for Mac and values of $p \leq 0.05$ were considered to be statistically significant. LDH assay, caspase 3/7 assay and real-time PCR were independently performed 3 times. The results are represented as mean ± standard deviation (SD). Analysis of variance (one-way ANOVA) with Tukey *post hoc* test was used for multiple comparisons.

## 3. RESULTS

### 3.1. Transgenic constructs design and generation of stable transfectants

We previously reported that the F2A technology can be used to link in frame three coding sequences obtaining a single open reading frame of 4.3 Kbp that can be expressed in eukaryotic cells as three discrete protein products [32]. In order to obtain a stable and unsilenced expression in eukaryotic cells we moved the sequence encoding for hHO1, hE5NT and hENTPD1 proteins under the control of the CAGGS

promoter (Fig. 1), to give the tricistronic pCX-TRI-2A plasmid. Single gene-expressing vectors have been produced as controls and cells transfected to investigate the contribution of each gene in the downregulation of the inflammatory response. hHO-1, hE5NT and hENTPD1 coding sequences were cloned into the same vector backbone used to produce pCX-TRI-2A plasmid (Fig. 1).

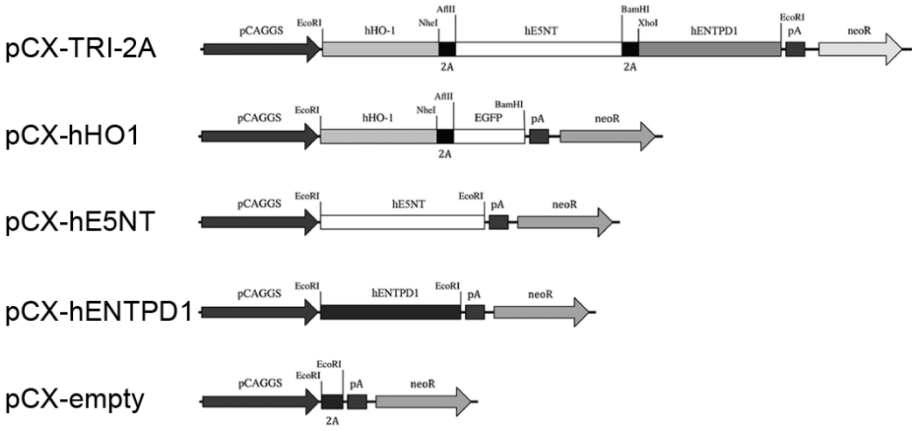


**Figure 1.** Schematic maps of tricistronic pCX-TRI-2A vector and all the constructs used in the work. The transgenic plasmid is composed of the CAGGS promoter, followed by the coding sequence of human HO-1 gene without the stop codon, fused in frame to the first F2A sequence (F2A), then the coding sequence of human E5NT gene without the stop codon, the second F2A sequence and the coding sequence of human ENTPD1 gene followed by a polyadenilation signal (pA).

Murine NIH3T3 cells were electroporated with pCX-TRI-2A and control plasmids, and selected for neomycin resistance for one week. In order to verify the presence and the functionality of pCX-TRI-2A vector, genomic DNA and total RNA were extracted from transfected cells and analyzed for the presence of the exogenous molecules. PCR analysis on genomic DNA using transgene-specific oligonucleotides confirmed the genetic modification of the cells (S1 Fig.). RT-PCR analyses on total RNA, using oligonucleotides specific for transgenic

transcript, also confirmed the correct transcription of the tricistronic cassette (S2 Fig.). In order to enrich the population of transfected cells, the expression of hENTPD1, hE5NT or EGFP was firstly assessed by flow cytometry (data not shown), and then those cells expressing the human proteins were sorted and expanded. pCX-TRI-2A transfected cells were FACS-sorted on the basis of high hE5NT and hENTPD1 expression. Sorted cells were expanded in culture for ten days and then analyzed for the expression of both hENTPD1 and hE5NT: approximately 93% of cells were expressing both human proteins (Fig. 2A-B). pCX-hHO1, pCX-hE5NT and pCX-hENTPD1 transfected cells were sorted and analyzed for EGFP, hE5NT and hENTPD1 expression respectively. After sorting >98% of cells expressed the exogenous protein, conversely no signal was detected in WT and mock-trasnfected cells (S3 Fig.).

To verify if the enriched cells overexpressed all the three human proteins, controls and transfected cell lysates were analyzed by immunoblotting. As expected, all the three human proteins were found to be strongly expressed in pCX-TRI-2A cells and to have the correct molecular weight. Single gene transfected cell groups showed in each case higher expression levels (Fig. 2C) as compared to pCX-TRI-2A cells.

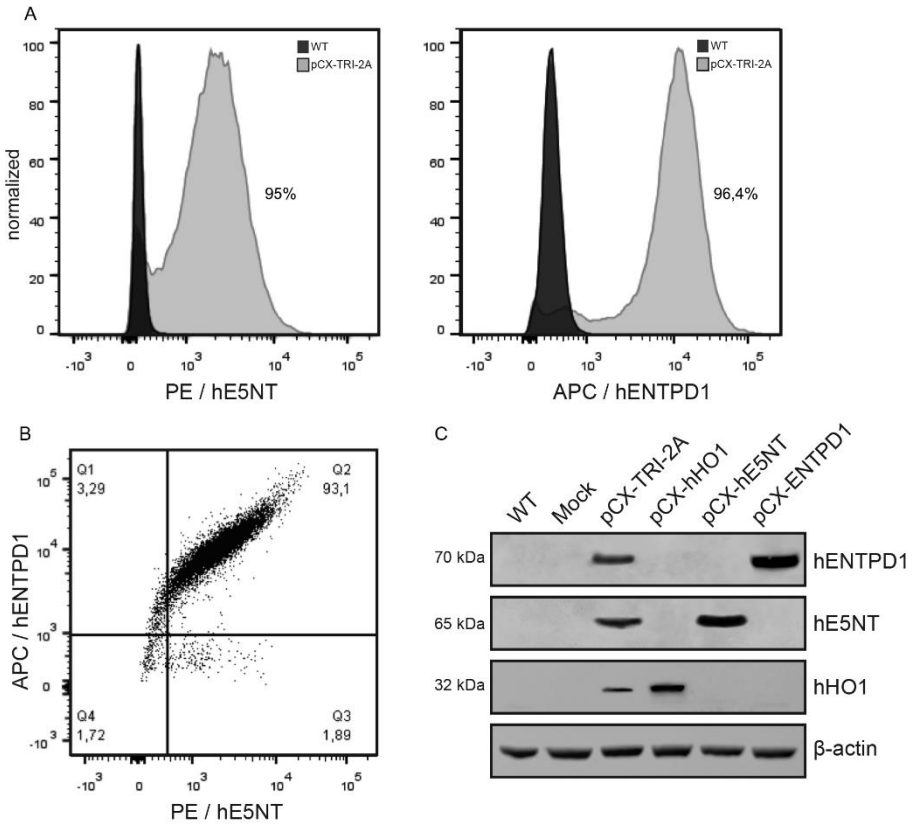


**Figure 2.** pCX-TRI-2A-transfected cells were enriched via FACS for the expression of hE5NT and hENTPD1. Expression analysis on sorted transfectants showed that E5NT and ENTPD1 were expressed by more than 95% of the pCX-TRI-2A-transfected cells (**A**) and that more than 93% of those cells expressed both the two ecto-enzyme simultaneously (**B**). (**C**) The three human proteins were found strongly expressed into the transfected cells after sorting, and expression levels were unaffected by the number of genes in the construct, as there was no evidence of incomplete separation of individual proteins.

## 3.2. The tricistronic transgene encodes for all the three human proteins with a correct subcellular localization

Since it has been demonstrated that different subcellular localization might influence the expression pattern of target genes coupled to the 2A peptide [37], we investigated if the human proteins encoded by the muticistronic 2A-based transgene had a correct subcellular localization

by immunofluorescence and confocal analysis. Co-staining analysis for hE5NT and hHO1 or for hENTPD1 and hHO1 indicated that in pCX-TRI-2A transfected cells both the hENTPD1 and hE5NT signals had, as expected, a distribution pattern similar to that of plasma membrane proteins, whereas hHO1 signal was detected mainly in the perinuclear area suggesting, for this protein, a cytoplasmic localization related to the ER (Fig. 3A-B). Pixel intensity analysis further confirmed the correct localization of the exogenous proteins (Fig. 3C-D).

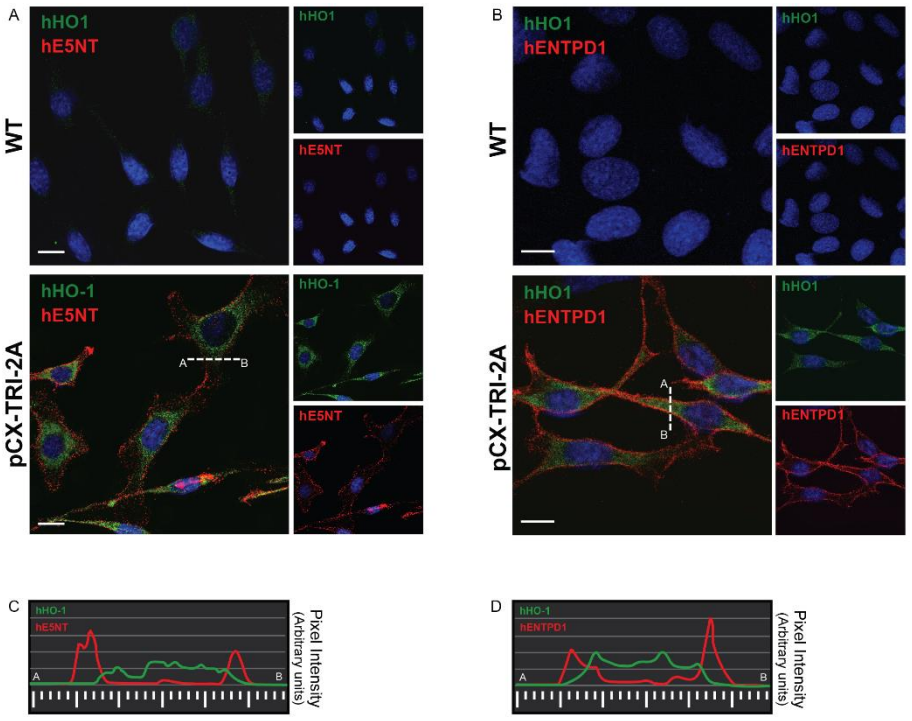


**Figure 3.** All the three exogenous proteins were correctly localized in pCX TRI 2A-transfected cells. WT and pCX-TRI-2A-transfected cells were co-stained with anti-hHO1 and anti-hE5NT antibodies (**A**) or with anti-hHO1and anti-hENTPD1antibodies (**B**). Transfected cells positive to hE5NT or hENTPD1 (red) were also positive to hHO1 (green). hE5NT and hENTPD1 localized on the cell surface, while hHO1 had a perinuclear and/or ER membranes cytoplasmic localization. (**C**-**D**) Plot of the signal intensity for hE5NT and hENTPD1 (red) and hHO1 (green) along the line drawn in A and B indicated that the most intense hE5NT and hENTPD1 signals are not colocalized with hHO1 signal.

### 3.3. pCX-TRI-2A mediates the increase of HO-1 and ENTPD1/E5NT activity

Since it has been reported that the function of the upstream protein in 2A-based constructs could be disrupted by the residual 2A peptide fused to its C-terminus [6], we carefully investigated the functionality of all the three exogenous proteins.

The enzymatic activity of HO-1 in pCX-TRI-2A-transfected cells was evaluated by measuring the fluorescence of bilirubin during the incubation of lysates from WT, mock and pCX-TRI-2A-transfected cells with hemin. As shown in figure 4A, the HO-1 activity in pCX-TRI-2A-transfected cells was about 2.5 fold higher than the basal activity seen in controls ($1.43 \pm 0.08$ nmol/h/mg versus $0.54 \pm 0.08$ and $0.58 \pm 0.1$ nmol/h/mg respectively in WT and mock transfected-cells, $p<0.05$). Moreover, no significant differences were observed between pCX-TRI-2A and control cell lines (WT and mock-transfected cells) previously treated with CoPP. These data showed that the exogenous expression of hHO-1, encoded by pCX-TRI-2A plasmid, was comparable to the CoPP induced expression levels of endogenous HO-1.

The enzymatic activity of the ENTPD1/E5NT system encoded by the tricistronic plasmid was evaluated by an extracellular nucleotide metabolism assay. Confluent control and transfected cells were incubated with ATP or AMP and the nucleotide content of supernatant samples collected at 0, 5, 15 and 30 min was analysed by HPLC [32,35]. As shown in figure 4B, in pCX-TRI-2A-transfected cells the supernatant content of AMP, which is the product of ENTPD1 enzymatic activity, was significantly higher than in controls (WT and

mock-transfected cells) and pCX-hE5NT-transfected cells at every time points ($p<0.05$). As expected, the production of AMP in the supernatant from the pCX-hENTPD1-transfected cells was even higher at every time point ($p<0.05$ versus all the other experimental groups).

On the other hand, a significant increase of adenosine concentration has been detected in medium from pCX-TRI-2A-transfected cells ($4.8 \pm 0.3$ μM after 30 min, Fig. 4C, $p<0.05$ vs. all the other experimental groups). A slight increase has been observed in pCX-hE5NT-transfected cells ($0.7 \pm 0.05$ μM after 30 min, Fig. 4C, $p<0.05$) while no adenosine formation has been detected in WT, mock and pCX-hENTPD1-transfected cells, suggesting a very low AMPasic activity in these latter cell groups (Fig. 4C). As control of hE5NT activity, confluent cells were incubated with 50 μM AMP for 30 min. As shown in figure 4D, formation of adenosine in pCX-hE5NT-transfected cells increased significantly to $34.6 \pm 0.1$ μM. A significant increase of adenosine production has been observed also in pCX-TRI-2A-transfected cells ($8.2 \pm 0.3$ μM) as compared to WT, mock and pCX-hENTPD1-transfected cells in which no detectable levels have been observed in all the time of incubation (Fig. 4D).

These data suggested that the overexpression of both ectonucleotidases is efficient in the removal of the pro-inflammatory ATP and AMP molecules and, at the same time, to increase the production of the anti-inflammatory adenosine.

In conclusion, these data suggested that the simultaneous expression of the three genes does not alter the enzymatic activity of each of the pCX-TRI-2A encoded genes and that they were able to mediate the

production of anti-inflammatory molecules in the pCX-TRI-2A transfected cells.

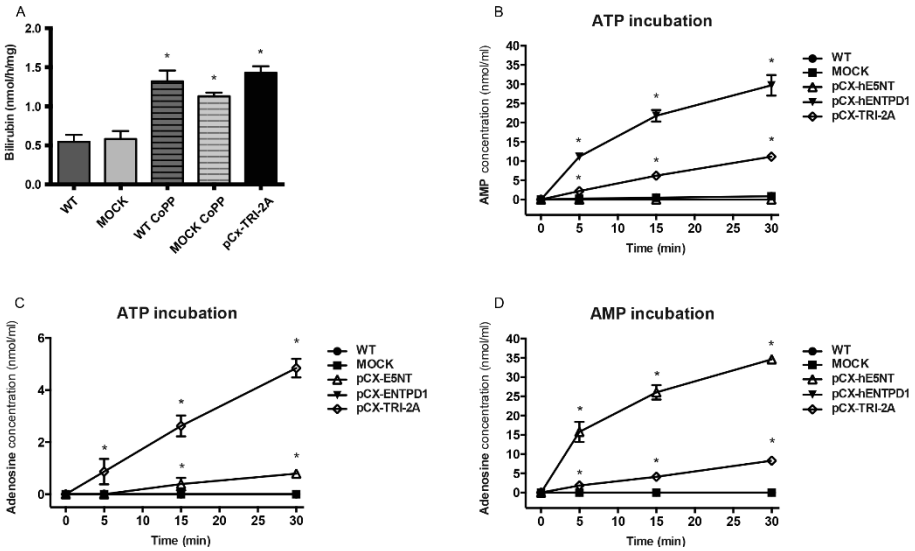


**Figure 4.** Heme oxygenase-1 and ectonucleotidases functional assays. (A) Heme Oxygenase 1 activity assay on NIH3T3 cells. Lysates from WT, mock- and pCX-TRI-2A-transfected cells were incubated for 2 hours with Hemin, BSA, Biliverdin Reductase A in reaction buffer as described in Materials and Methods. As positive control of the assay, wt or mock-transfected cells were pre-stimulated with 50 μM of HO1 inducer, Cobalt Protoporphyrin for 24 hours (wt CoPP, mock CoPP). Enzymatic activity is reported as nanomoles of bilirubin per hours per milligrams of protein extract. Data are expressed as mean ± SEM of 3-4 independent experiments. *$p$<0.05 versus wt and mock-transfected cells. (B) ENTPD1-mediated AMP production and (C) E5NT-mediated adenosine production by wild type, mock and pCX-TRI-2A transfected-cells. Cells were incubated with 50 μM ATP for 30 min. The nucleotide content of supernatants collected at 0, 5, 15, 30 min time points was measured by reverse phase-HPLC as detailed in Material and Methods. Data shown are mean ± S.D. (n=3). *$p$<0.05 versus all groups. (D) E5NT-mediated adenosine production by wild type, mock and transfected-cells. Cells were incubated with 50 μM AMP for 30 min. The nucleotide content of supernatants collected at 0, 5, 15, 30 min time points was measured by reverse phase-HPLC ad detailed in Material and Methods. Data shown are mean ± S.D. (n=3). *$p$<0.05 versus all groups.

### 3.4. The expression of hHO1, hCD73 and hCD39 protects cells from TNF-α-mediated cytotoxicity and apoptosis

We next evaluated whether the simultaneous expression and activity of this new combination of human proteins confers protection against

inflammatory stimuli and if this protection would be higher than the protective effect given by each single human protein. To this extent, controls and transfected cells were exposed to 50 ng/ml of TNF-α alone or in combination with appropriate molecules that served as a substrate for the enzymatic activity of exogenous proteins, hemin (20 μM) and/or ATP (200 μM), for up to 48 hours and the cytotoxicity was measured by LDH assay.

As shown in figure 5, the percentage of dead cells in pCX-TRI-2A transfected cells was 26.2 ± 3.8% after 24 hours (Fig. 5A) and 48 ± 3.9% after 48 hours (Fig. 5B) of treatment with TNF-α alone, which was significantly lower as compared to all other cell lines at 48 hours of treatment ($p<0.05$), even in absence of enzymatic substrate of human genes, and to all the other cell lines ($p<0.05$) except to pCX-hHO1 transfected cells at 24 hours of treatment. On the other hand, the administration of enzymatic substrates, hemin, ATP or both, to pCX-TRI-2A transfected cells treated with TNF-α, induced a further reduction of cytotoxicity (17.6 ± 3.8%, 15 ± 3.1% and 9.5 ± 1.6% respectively) as compared to the same cells treated with TNF-α alone at 24 hours (26.2 ± 3.8%, $p<0.05$). Similarly, the addition of both hemin and ATP to TNF-α treatment induced a 24.6 ± 2.2% of cell death in pCX-TRI-2A cells at 48 hours (Fig. 4B), which was significantly lower than cytotoxicity of same cells treated with TNF-α alone (48 ± 3.9%, $p<0.05$).

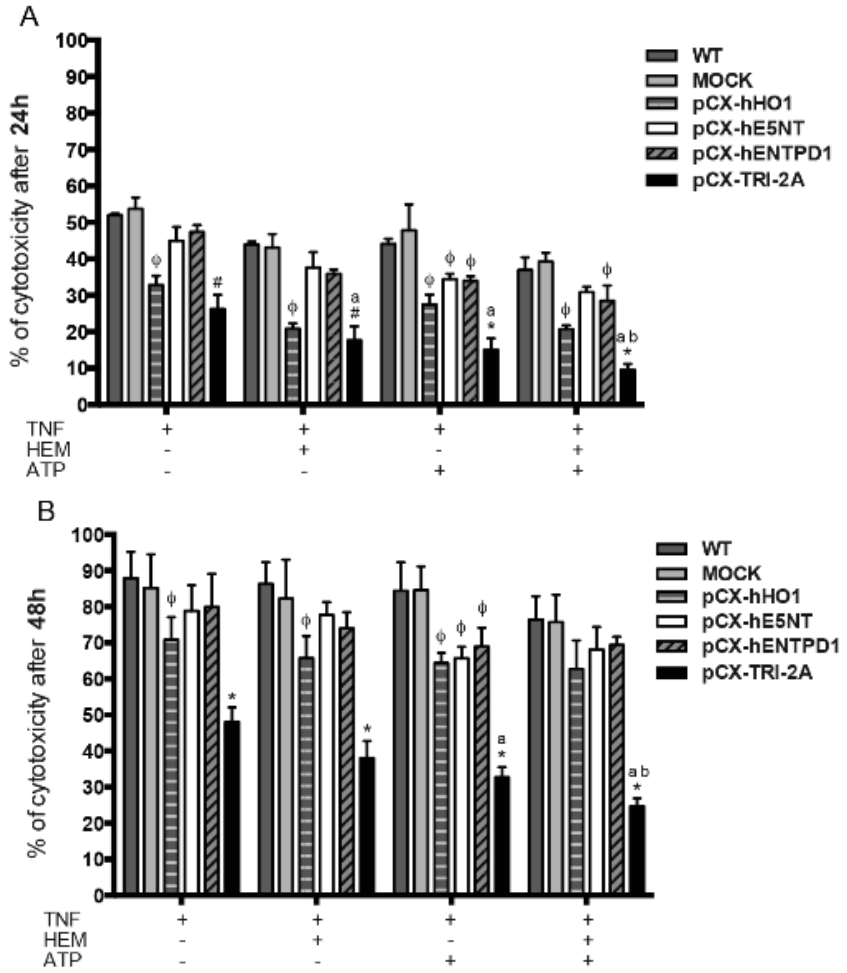


**Figure 5. pCX-TRI-2A transfected cells are protected against TNF-α-induced cytotoxicity.** WT cells and transfected cell lines were incubated with 50 ng/ml TNF-α for 24 h (**A**) and 48 h (**B**) alone or in combination with 20 μM hemin and/or 200 μM ATP. Cytotoxicity was assessed by lactate dehydrogenase (LDH) release and expressed as follows: relative cytotoxicity (%) = [(*A* e − *A* c) / (*A* b − *A* c)] × 100 (%), where '*A* e' is the experimental absorbance, '*A* b' is the absorbance of lysed controls and '*A* c' is the absorbance of untreated controls. The data are expressed as mean ± SD of three independent experiments. [#] indicates significant difference between pCX-TRI-2A and all the other groups, except for pCX-hHO1, within the same treatment (ANOVA, $p<0.05$). [∗] indicates significant difference between pCX-TRI-2A and all the other groups within the same treatment (ANOVA, $p<0.05$); [ϕ] indicates a significant difference between single gene-transfected cells and WT/mock within the same treatment (ANOVA, $p<0.05$); [a] indicates significant difference as compared to TNF-α treatment alone within the same cell type (ANOVA, $p<0.05$); [b] indicates a significant difference as compared to TNF-α + hemin treatment within the same cell type (ANOVA, $p<0.05$).

Since soluble TNF-α can lead to cell apoptosis [38,39], and considering that the activity of the three human proteins has been reported to exert anti-apoptotic effects [40,41], we investigated their potential role in protection against cell death induced by TNF-α via a caspase 3/7 activity assay. As shown in figure 6, pCX-TRI-2A-transfected cells were protected against apoptotic death after both 16 (Fig 6A) and 24 (Fig. 6B) hours of TNF-α challenging as compared to WT and mock cells ($p$<0,05). Furthermore, pCX-TRI-2A-transfected cells resulted better protected from TNF-α-induced caspase activation as compared to pCX-hE5NT, pCX-hENTPD1 and pCX-HO1 after 24 hours of treatment ($p$<0.05). On the other hand, the better protection was also observed at 16 hours of treatment as compared to pCX-hENTPD1 ($p$<0.05) and pCX-hE5NT ($p$<0.05) but not as compared to pCX-HO1. The addition of hemin (20 μM) to pCX-TRI-2A-transfected cells treated with TNF-α further reduced caspase activation after 16 hours (Fig. 6A) and this anti-apoptotic effect was still observed at 24 hours (Fig. 6B) as compared to pCX-TRI-2A-transfected cells treated with TNF-α alone. This reduced TNF-α-dependent caspase activation was observed also in presence of ATP (200 μM) to pCX-TRI-2A transfected cells after 24 hours of TNF-α treatment as compared to pCX-TRI-2A-transfected cells treated with TNF-α alone (Fig. 6B). The combined treatment with TNF-α, hemin and ATP inhibited apoptosis at 16 hours in pCX-TRI-2A transfected cells (3.97 ± 0.81; fold change as compared to TNF-α treatment alone, $p$ <0,05), similarly to the observed anti-apoptotic effect of hemin (Fig. 6A). Interestingly, in pCX-TRI-2A transfected cells treated with TNF-α together with both hemin and ATP

after 24 hours, it was observed an anti-apoptotic effect significantly greater than in all the other treatment groups ($p$ <0,05, Fig. 6B).

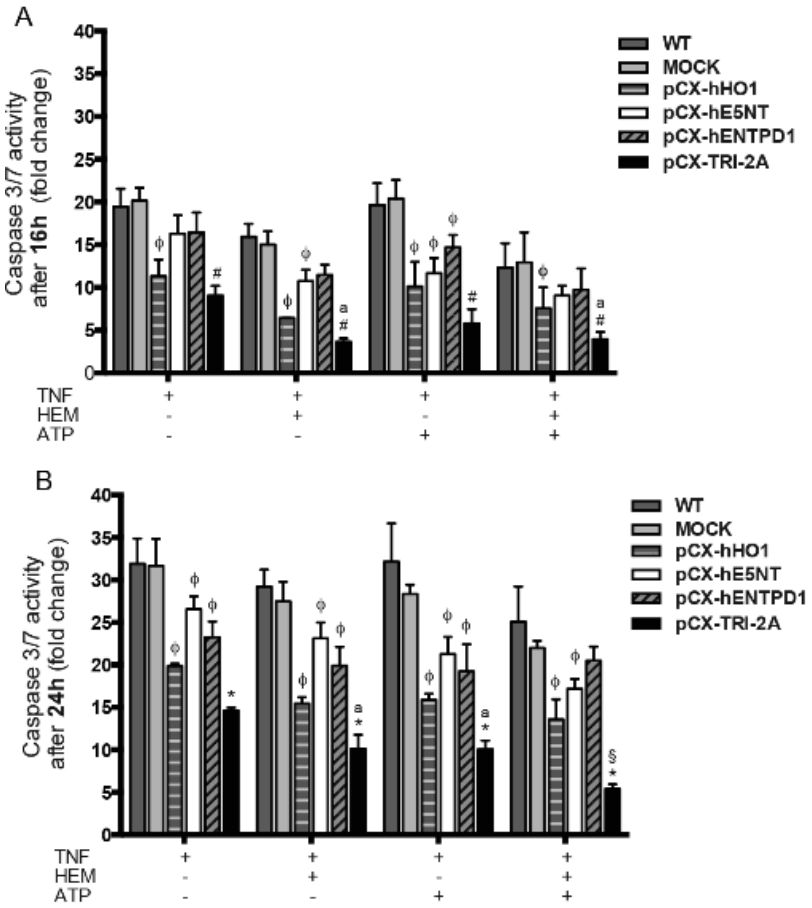


**Figure 6.** pCX-TRI-2A transfected-cells are protected against TNF-α-induced apoptosis. Caspase 3/7 activities were determined in all cell groups after 16h (A) or 24h (B) of incubation with 50 ng/ml TNF-α alone or in combination with 20 μM hemin and/or 200 μM ATP. Expression of the three human genes in TG cells significantly reduced the activation of effector caspases 3/7. The data are expressed as mean ± SD of three independent experiments. [#] indicates significant difference between pCX-TRI-2A and all the other groups, except for pCX-hHO1, within the same treatment (ANOVA, $p$<0.05). [∗] indicates significant difference between pCX-TRI-2A and all the other groups within the same treatment (ANOVA, $p$<0.05); [ϕ] indicates a significant difference between single gene-transfected cells and WT/mock within the same treatment (ANOVA, $p$<0.05); [a] indicates significant difference as compared to TNF-α treatment alone within the same cell type (ANOVA, $p$<0.05); [§] indicates a significant difference as compared to all the other treatments within the same cell type (ANOVA, $p$<0.05).

To further evaluate the protective response to the TNF-α injury after a persistent exposition to the pro-inflammatory cytokine, a propidium iodide incorporation assay was performed. Consistently with the results of cytotoxicity and caspase assays, the pCX-TRI-2A-transfected cells were protected against TNF-α injury as compared to WT cells up to 72 hours (S4 Fig.).

Taken together, these data suggest that the expression of the three genes is protective against TNF-α-induced cytotoxicity and apoptosis and that the protection is more effective when all the three genes, hHO1, hENTPD1 and hE5NT are simultaneously present and exerting their enzymatic activity.

### 3.5. Molecular characterization of anti-inflammatory response mediated by the combination of the human genes

Trying to unravel the molecular mechanism of the anti-inflammatory response mediated by the combination of the three human genes in pCX-TRI-2A transfected cells, the expression of 84 TNF-α pathway-related genes was analyzed by quantitative $RT^2$–PCR Profiler Array (Qiagen). By this screening, *Ikbkg (Nemo)*, a gene encoding for an anti-apoptotic protein involved in the modulation of Nf-kB response to cytokine exposure, was selected and then validated for its differential modulation as comparing the control and pCX-TRI-2A transfected cells and, within the same cell type, the treatments. A significant up-regulation of *Ikbkg* was observed only in pCX-TRI-2A-transfected cells treated with ATP plus TNF-α or Hemin or both (Fig. 7), as compared to the respective untreated cells ($p<0.05$) or to TNF-α treated cells ($p<0.05$). Taken together, these data suggest that pCX-TRI-2A-

transfected cells exposed to TNF-α injury up-regulated the anti-apoptotic *Ikbkg* gene.

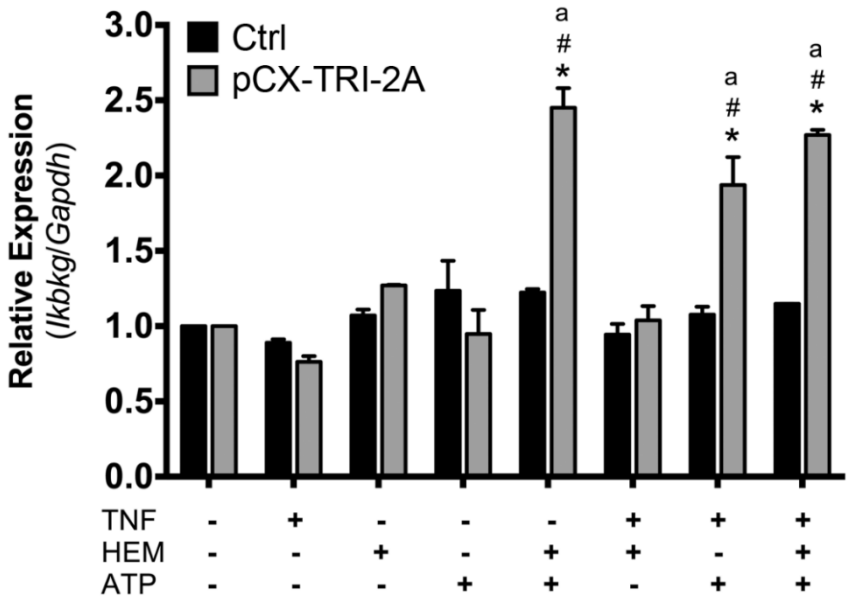


**Figure 7. Changes in *Ikgkb* (*Nemo*) mRNA expression in control (Ctrl) and pCX-TRI-2A-transfected cells.** Cells were incubated with 50 ng/ml TNF-α for 16h, alone or in combination with 20 μM hemin and/or 200 μM ATP. Murine *Ikgkb* mRNA was quantified by real-time PCR. The data (mean±SD of three independent experiments), normalized for *Gapdh* gene, and expressed as fold change respect to the untreated cells. [*] indicates a significant difference between pCX-TRI-2A-transfected cells and Ctrl cells within the same treatment (t Student, $p<0.05$); [#] indicates a significant difference as compared to untreated cells within the same cell type (ANOVA, $p<0.05$) [a] indicates a significant difference as compared to TNF-α treatment alone within the same cell type (ANOVA, $p<0.05$).

## 4. DISCUSSION

The results shown in the present paper contribute to the understanding of the protective role of a novel combination of human genes, HO-1, ENTPD1 and E5NT, against inflammatory stimuli such as TNF-α. Each human gene used in this study has been reported to have anti-

inflammatory and anti-apoptotic properties when overexpressed or induced in the cells or organisms [21,40,42-45]. For the first time we report here the protective effects given by the simultaneous expression and enzymatic activities of the combination of these genes as compared to their use as a single agent.

The coding sequences of the three human genes were included in an expression cassette that allowed the simultaneous translation of three proteins starting from a single mRNA by using the F2A technology [4,46]. The western blotting analyses, performed on protein extracts of pCX-TRI-2A and control plasmids transfected cells, confirmed the enrichment of cells expressing exogenous proteins obtained by FACS and no evidence of incomplete separation of individual proteins was found. The cells transfected with plasmids expressing each of the three genes alone were found to have higher levels of exogenous protein as compared to the pCX-TRI-2A transfected cells, and this is probably due to the smaller size of the single gene expressing plasmids as compared to the pCX-TRI-2A plasmid as previously reported [47]. The order of genes encoded by the expression cassette was designed to maximize the likelihood of the correct processing and maturation of each protein product [32] and we found the expected subcellular localization for hHO1, hE5NT and hENTPD1 in pCX-TRI-2A transfected cells.

Taken together, the expression analyses data confirmed that the application of the F2A system and the design of the multi-cistronic construct allowed the expression of three exogenous proteins that were correctly processed and localized within the cells.

Next, to verify the absence of possible interference on protein function in F2A-based encoded peptides [6], we focused on the investigation of

enzymatic activity of each gene encoded by the multi-cistronic plasmid. The activity of HO-1, evaluated by means of measuring bilirubin's fluorescence during the incubation with hemin [48], was found to be higher in pCX-TRI-2A transfected cells as compared to mock-transfected or untransfected cells (Fig. 4A). Furthermore, the HO-1 activity of pCX-TRI-2A transfected cells was similar to the activity observed in control cells previously treated with a known HO-1 inducer, CoPP, suggesting that the pCX-TRI-2A transfected cells constitutively express HO-1 at amounts comparable to those induced in stress conditions within the cells. The enzymatic activities of ENTPD1 and E5NT was measured by an extracellular nucleotide metabolism assay [32,36]. The production of AMP following incubation with ATP was significantly higher in pCX-hENTPD1 and pCX-TRI-2A transfected cells as compared to all the other experimental groups, which is consistent with the overexpression of ENTPD1 protein only in these two cell types. The pCX-hENTPD1 transfected cells had a significantly higher content in AMP as compared to pCX-TRI-2A transfected cells, suggesting that in the latter the AMP is produced in less amount or enzymatically converted in adenosine. In fact, the analysis of adenosine production after incubation with ATP revealed a significantly higher production in pCX-TRI-2A transfected cells as compared to all the other cell types, suggesting that, only in pCX-TRI-2A cells, the ATP could be converted in ADP and AMP by ENTPD1 and AMP converted in adenosine by E5NT. In order to evaluate the specific enzymatic activity of E5NT, the cells were incubated with AMP and adenosine production in the supernatant was measured. Only in the supernatant of hE5NT expressing cells (pCX-hE5NT and pCX-TRI-2A transfected

cells) adenosine was detected, with significantly higher levels in pCX-hE5NT as compared to pCX-TRI-2A transfected cells (Fig. 4D), and this finding is consistent with the higher amount of E5NT protein detected in pCX-hE5NT transfected cells (Fig. 3B). The enzymatic assays demonstrated that the three proteins encoded by the multi-cistronic cassette were fully functional and allowed the increased production of their enzymatic products.

In order to evaluate the protective effects of the combination of human genes in the cells against inflammatory stimuli, pCX-TRI-2A and control cells were exposed to TNF-α alone or in combination with appropriate molecules that served as a substrate for the enzymatic activity of exogenous proteins and cytotoxicity and caspase assays were performed. TNF-α was chosen to mimic an inflammatory settings because it plays one of the most important roles in inflammation and in inflammatory conditions [26,49,50]. The expression of the combination of the three genes, better than the expression of each single gene, protected the cells against TNF-α induced cytotoxicity even in absence of enzymatic substrate of human genes. The expression of single genes appeared to be somehow protective against TNF-α-induced cytotoxicity as compared to WT or mock-transfected cells only if appropriate enzymatic substrate was administered to cells and the protective effect was observed exclusively when TNF-α-treated cells were treated with only one substrate (hemin or ATP). Taking into account that caspases play an important role in TNF-α-induced apoptotic cell death [38], we determined caspase activity in pCX-TRI-2A transfected and control cell types at 16 and 24 hours post-incubation with inflammatory stimuli (TNF-α) alone or in combination with enzymatic substrates of human

genes (hemin and/or ATP). After 16 hours of treatment, cells overexpressing HO-1 (pCX-hHO1 and pCX-TRI-2A transfected cells) were protected from TNF-α-induced apoptosis, suggesting that the anti-apoptotic effect was mediated mainly by HO-1. On the other hands, the expression of the combination of the genes was better protective, as compared to the expression of each single gene, at 24 hours of TNF-α treatment and it was more effective in presence of enzymatic substrates of the three human proteins.

Taken together, these findings suggest that the simultaneous presence and activity of the three genes is necessary to further improve a persistent protection against TNF-α injury as compared to the effects induced by the expression of each single gene.

In order to better understand how the combined activity of the two systems represented by hHO-1 and hENTPD1/hE5NT were able to protect pCX-TRI-2A transfected cells against TNF-α-mediated injury we investigated the molecular mechanisms involved in TNF-α pathway. To this extent, TNF-α pathway-related genes were analysed by $RT^2$ array and, among the several genes resulted to be differentially modulated between control and pCX-TRI-2A transfected cells, we focused our attention on *Ikbkg* (*Nemo*). It has been demonstrated that, upon TNF-α binding, the TNFR1 forms two different and consecutive complexes. The complex I controls the expression of anti-apoptotic proteins and the complex II triggers cell death process [26,38,39,51]. The complex I is responsible for the downstream activation of the transcriptional activation of NF-kB through the regulatory subunit of the IKK complex, *Nemo* [38]. In this context, Nf-kb promotes pro-survival signaling within the cells. The gene expression analysis of

*Nemo* showed a significant up-regulation of this gene in pCX-TRI-2A transfected cells treated with ATP plus TNF-α or Hemin or both at 16 hours post-treatment (Fig. 7), as compared to untreated or to TNF-α treated pCX-TRI-2A-transfected cells. This suggested that *Nemo* modulation could be dependent to ATP administration to cells. Moreover, expression of *Nemo* was markedly lower in WT cells compared to pCX-TRI-2A-transfected cells within these three treatments (Fig. 7). On the other hand, TNF-α plus hemin administration to pCX-TRI-2A-transfected cells did not induce *Nemo* expression. This behaviour of the *Nemo* regulation in presence of hemin needs further experiments to be explained, although it can be hypothesized a protective effect of the HO-1 activity that could have abrogated the cell's need of *Nemo*'s up-regulation. In summary, pCX-TRI-2A-transfected cells up-regulated *Nemo*, which promotes pro-survival effects of Nf-kB, in response to TNF-α injury when ATP is added to the medium, resulting in a protection against TNF-α induced cell death.

In conclusion, this study demonstrated, for the first time, the anti-inflammatory and anti-apoptotic effects of a combination of three human genes simultaneously expressed in murine cells via an F2A system-based multicistronic approach. The protective effects against TNF-α-induced cytotoxicity and cell death, mediated by HO-1, ENTPD1 and E5NT genes were better observed in cells expressing the combination of genes as compared to cells expressing each single gene and the effect was further improved by administrating enzymatic substrates of the human genes to the cells. Moreover, a gene expression analyses suggested that the expression of the three genes has a role in

modulating key downstream regulators of TNF-α signalling pathway, as *Nemo*, that promoted pro-survival phenotype in TNF-α injured cells.

**Keywords**

Multicistronic vector; 2A peptide; Inflammation; Heme Oxygenase-1; Ectonucleotidases; Cytoprotection

**Abbreviations list**

F2A: Foot and Mouth Disease Virus 2A sequence; hHO-1: human heme oxygenase 1; hE5NT: human ecto-5'-nucleotidase; hENTPD1: human ecto-nucleoside triphosphate diphosphohydrolases; NF-kB: nuclear factor kB; TNF-α: tumor necrosis factor-alpha; TNFR1: TNF receptor 1; TNFR2: TNF receptor 2.

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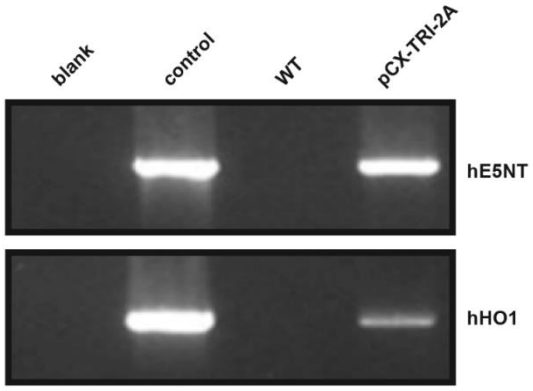
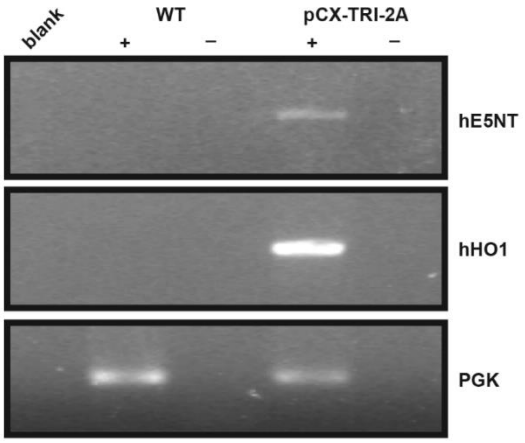
## SUPPORTING INFORMATION

**S1 Fig.** PCR analysis on genomic DNA. PCR were performed on 75ng of genomic DNA extracted from WT and pCX-TRI-2A-transfected cells. Two primer pairs were used: 5'HO1 fw (CTGGAGGAGGAGATTGAGCG) / 2A rev (CGCCAACTTGAGAAGGTCAAAA) pair that covers the region from 5' of hHO1 CDS to the first 2A sequence; intern E5NT fw (TGTTGGTGATGAAGTTGTGG) / 2A rev (CGCCAACTTGAGAAGGTCAAAA) pair that covers the region from hE5NT CDS to the second 2A sequence. Results show the presence of amplicons with expected size, respectively 753bp for hHO-1 and 1297bp for hCD73. As positive control 75ng of gDNA from WT cells mixed with $10^2$ copies of pCX-TRI-2A plasmid were used.

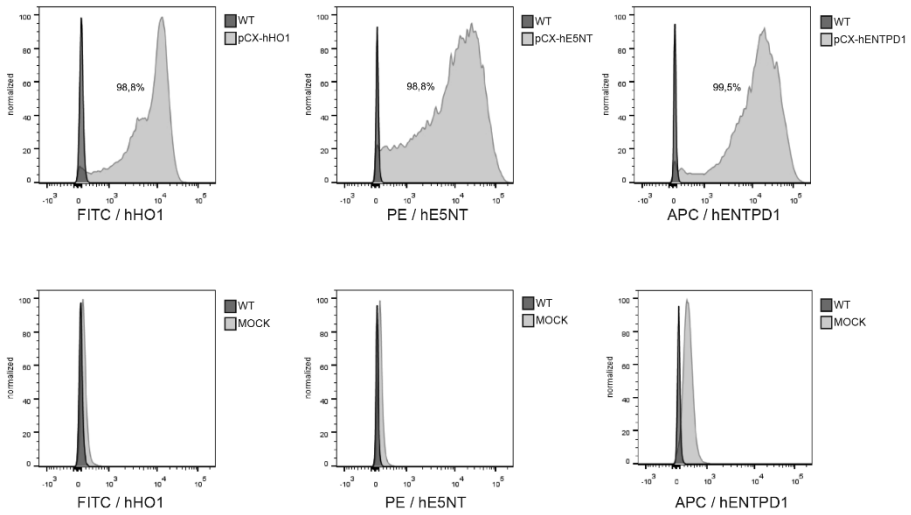


**S2 Fig.** PCR analysis on RNA. End-point PCR were performed on 25ng of cDNA retrotranscribed (+) from total RNA extracted from WT and pCX-TRI-2A-transfected cells. Two primer pairs were used: 5'HO1 fw (CTGGAGGAGGAGATTGAGCG) / 2A rev (CGCCAACTTGAGAAGGTCAAAA) pair that covers the region from 5' of hHO1 CDS to the first 2A sequence; intern E5NT fw (TGTTGGTGATGAAGTTGTGG) / 2A rev (CGCCAACTTGAGAAGGTCAAAA) pair that covers the region from hE5NT CDS to the second 2A sequence. Results show the presence of amplicons with expected size, respectively 753bp for hHO-1 and 1297bp for hCD73. $10^3$ copies of plasmids diluted into 25ng of WT cDNA were amplified as positive controls of PCR reaction. Phosphoglycerate kinase (PGK) housekeeping end-point PCR were performed using PGK1-HK-fw (GTATCCCTATGCCTGACAAGT) / PGK1-HK-rev (TTCCCTTCTTCCTCCACAT) primers pair, on 25ng of cDNA from WT and TG cells. Expected size band, 187bp, is visible in RT+ of each type of cells.

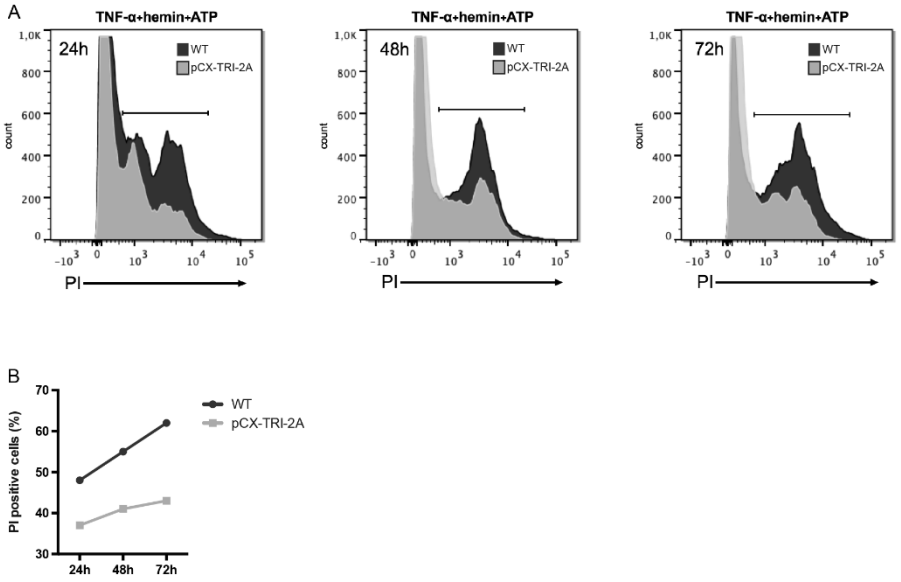



**S3 Fig.** Single-gene transfected cells expression analysis. Appropriate single gene-vectors have been produced as control of transfection as well as to investigate the contribution of each gene in the downregulation of the inflammatory response. pCX-E5NT and pCX-hENTPD1 transfected cells were sorted and analyzed for hE5NT and hENTPD1 expression respectively. pCX-HO1 transfected cells were sorted and analyzed on the basis of EGFP expression. After sorting each population count at least 98% of cells expressing the exogenous protein.

WT and mock-transfected cells showed no expression of any of the three human proteins.




**S4 Fig.** Propidium Iodide incorporation assay. 1×106 cells were seeded in 10 ml culture petri and treated with medium containing TNF-α (50 ng/ml) alone or with TNF-α (50 ng/ml), hemin (20 μM) and ATP (200 μM) for 24, 48 and 72 hours. Untreated cells were also cultured as a control of basal level of cell death. Cell death was detected, at each time point, using propidium iodide (PI, Sigma Aldrich) influx evaluation. At the end of treatment, the cells were harvested by centrifugation and suspended in PBS. Subsequently, the cells were incubated with 2 μg/mL of propidium iodide (PI) in the dark for 15 min at room temperature immediately before cytometric evaluation on FACSARIA flow cytometer (Becton Dickinson, San Jose, CA). PI incorporation was detected by red fluorescence on a log scale and cell death percentages were calculated on PI+cells combined with the scatter (FSC) by subtracting the % of untreated cells at each condition. Data were collected (at least 50,000 events) and analyzed using DIVA software (Becton Dickinson) and FlowJo software.




**S1 Table.** Oligonucleotides used for real time PCR experiments. The primers name and sequences are reported. The melting temperature (Tm) is indicated in Celsius grade. Primers for *Ikbkg* genes were designed by using Primer3 software (Untergasser A, *et al*. Primer3Plus, an enhanced web interface to Primer3. *Nucl. Acids Res.* 2007 35: W71-4). Primer sequences for *Gapdh* gene were recovered from PrimerBank repository (Spandidos A, *et* al. PrimerBank: a resource of human and mouse PCR primer pairs for gene expression detection and quantification. *Nucl. Acids Res*. 2010 38: D792-9).

| **Primer Name** | **Sequence** | **Tm** |
|---|---|---|
| ***Ikbkg fw2*** | **5'-GAG GCC CTG GTA GCC AAA C-3'** | **60** |
| ***Ikbkg rev2*** | **5'-ATG GCA GCC AAC TTT CAG CTT-3'** | |
| ***Gapdh PB1 fw*** | **5'-AGG TCG GTG TGA ACG GAT TTG-3'** | **60** |
| ***Gapdh PB1 rev*** | **5'-TGT AGA CCA TGT AGT TGA GGT CA-3'** | |

# *CHAPTER 4*

# Simultaneous overexpression of human E5NT and ENTPD1 protects porcine endothelial cells against $H_2O_2$-induced oxidative stress and cytotoxicity *in vitro*

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**ABSTRACT**

Ischemia-reperfusion injury (IRI) and oxidative stress still limit the survival of cells and organs in xenotransplantation models. Ectonucleotidases play an important role in inflammation and IRI in transplantation settings. We tested the potential protective effects derived by the co-expression of the two main vascular ectonucleotidases, ecto-5'-nucleotidase (E5NT) and ecto nucleoside triphosphate diphosphohydrolase 1 (ENTPD1), in an *in vitro* model of $H_2O_2$-induced oxidative stress and cytotoxicity. We produced a dicistronic plasmid (named pCX-DI-2A) for the co-expression of human E5NT and ENTPD1 by using the F2A technology. pCX-DI-2A-transfected porcine endothelial cells simultaneously overexpressed hE5NT and hENTPD1, which were correctly processed and localized on the plasma membrane. Furthermore, such co-expression system led to the synergistic enzymatic activity of hE5NT and hENTPD1 as shown by the efficient catabolism of pro-inflammatory and pro-thrombotic extracellular adenine nucleotides along with the enhanced production of the anti-inflammatory molecule adenosine. Interestingly, we found that the hE5NT/hENTPD1 co-expression system conferred protection to cells against $H_2O_2$-induced oxidative stress and cytotoxicity. pCX-DI-2A-transfected cells showed reduced activation of caspase 3/7 and cytotoxicity than mock-, hE5NT- and hENTPD1-transfected cells. Furthermore, pCX-DI-2A-transfected cells showed decreased $H_2O_2$-induced production of ROS as compared to the other control cell lines. The cytoprotective phenotype observed in pCX-DI-2A-transfected cells was associated with higher detoxifying activity of catalase as well as increased activation of the survival signaling molecules Akt,

extracellular signal-regulated kinases 1/2 (ERK1/2) and p38 mitogen-activated protein kinase (MAPK).

Our data add new insights to the protective effects of the combination of hE5NT and hENTPD1 against oxidative stress and constitute a proof of concept for testing this new genetic combination in pig-to-non-human primates xenotransplantation models.

## 1. INTRODUCTION

Purinergic signaling plays an important role in conditions of inflammation and ischemia reperfusion injury (IRI) that occur in transplantation settings [1,2]. Extracellular ATP and ADP accumulate in the sites of injury leading to pro-inflammatory and pro-thrombotic responses by signaling through P2 receptors (P2X and P2Y) on vascular and immune cells [1]. The extracellular levels of adenine nucleotides are mainly modulated by ectonucleotidases: ecto nucleoside triphosphate diphosphohydrolase 1 (ENTPD1, also known as CD39) converts ATP and ADP to AMP [3]; AMP is further dephosphorylated to adenosine by ecto-5'-nucleotidase (E5NT, also known as CD73) [3]. Extracellular adenosine exerts anti-inflammatory and anti-thrombotic effects by means of P1 receptors (A1, A2A, A2B, A3) signaling [1].

During the ischemia-reperfusion phenomenon occurring in allo- and xenotransplantation, there is a concomitant increasing production of reactive oxygen species (ROS) that cause injury via several mechanisms [4]. If not adequately scavenged, ROS can directly injure cells through membrane lipid peroxidation, enzymatic function impairment and DNA damage, eventually leading to cell death [5]. ROS

can also induce up-regulation of leukocyte adhesion molecules, pro-inflammatory cytokines and chemokines in vascular endothelium and pancreatic islets [6,7]. That leads to increased leukocyte recruitment to the site of injury and subsequent immune cells activation, therefore exacerbation of inflammation and oxidative stress [4]. Leukocyte-derived free radicals contribute to injury, rejection and dysfunction of xenotransplanted heart and liver [8,9].

It has been reported that oxidative stress significantly impairs the activity of ectonucleotidases with the consequent loss of their protective functions. For instance, ROS inhibit the ectonucleotidase activity of ENTPD1 leading to loss of platelet inhibitory properties [10]. Moreover, histopathologic analyses have revealed the loss of ENTPD1 expression in the vasculature of rejected cardiac xenografts [11]. Similarly, E5NT activity in porcine hearts or endothelial cells decreases following exposure to human blood [12]. Therefore, the loss of ectonucleotidases activity along with the consequent impaired extracellular adenine nucleotide metabolism may critically contribute to the coagulation dysregulation and exacerbated inflammation observed in xenotransplanted organs. Moreover, it has been reported that porcine endothelial cells show much lower activity of ectonucleotidases as compared to human [13,14]. Additionally, such altered extracellular nucleotide metabolism may constitute a feedback mechanism of increased ROS production because of the ATP-induced oxidative stress response in leukocytes [15]. Several evidences showed the protective effects exerted by ENTPD1 and E5NT against IRI in allo- and xenotransplantation models. It has been reported that the transgenic overexpression of ENTPD1 protects against renal, hepatic and cardiac

ischemia-reperfusion and transplant vascular injury [16-19]. E5NT protects against renal, cardiac and intestinal ischemic damage via adenosine production [20-22]. Moreover, the overexpression of human E5NT in porcine endothelial cells inhibits Natural Killer-mediated cytotoxicity via adenosine production [13].

Given the reported protective effects of ENTPD1 and E5NT, we aimed to overexpress both genes by using the F2A technology [23-25] and to inquire into their combined action in a condition of ROS production, mimicking what happens in xenografted tissues during IRI. We already showed the feasibility of the F2A-mediated co-expression of ENTPD1 and E5NT in terms of their synergic enzymatic activities [26] and here, we investigated the protective role of ENTPD1/E5NT combination against oxidative stress. This study demonstrated, for the first time, the protection, mediated by activation of Akt, extracellular signal-regulated kinases 1/2 (ERK1/2) and p38 Mitogen-Activated Protein Kinase (MAPK) against $H_2O_2$-induced apoptosis and cytotoxicity along with an increased catalase-mediated $H_2O_2$ scavenging activity and decreased production of ROS in endothelial cells simultaneously overexpressing ENTPD1 and E5NT.

## 2. MATERIAL AND METHODS

### 2.1. Reagents and antibodies

Restriction enzymes (New England Biolabs) were purchased from Euroclone, hydrogen peroxide ($H_2O_2$) and oligonucleotides (listed in Supplementary Table 1) were purchased from Sigma Aldrich. The

following antibodies were used in FACS experiments: phycoerythrin (PE)-conjugated anti-human E5NT ([AD2], mouse monoclonal, BD Pharmigen, 10 µl per $5\times10^5$ cells in a 100µl staining volume); Alexa Fluor 647-conjugated anti-human ENTPD1 ([A1], mouse monoclonal, Life Technologies, 25µg/ml per $5\times10^5$ cells in a 100µl staining volume). The following primary antibodies were used in immunoblotting experiments: anti-human E5NT ([EPR6115], rabbit monoclonal, LifeSpan BioSciences, 1:500); anti-human ENTPD1 ([HPA014067], rabbit polyclonal, Sigma-Aldrich, 1:250); anti-beta-actin ([AC-15], mouse monoclonal, Sigma-Aldrich, 1:5000); anti-Akt (rabbit polyclonal, Cell Signaling Technology [#9272], 1:1000); anti-phosphoSer473-Akt (rabbit polyclonal, Cell Signaling Technology [#9271], 1:1000); anti-p44/p42 ERK1/2 ([137F5], rabbit monoclonal, Cell Signaling Technology [#4695], 1:1000); anti-phospho-p44/p42 ERK1/2 (Thr202/Tyr204, [20G11], rabbit monoclonal, Cell Signaling Technology [#4376], 1:1000); anti-p38 MAPK (rabbit polyclonal, Cell Signaling Technology [#9212], 1:1000); anti-phospho-p38 MAPK (Thr180/Tyr182, [28B10], mouse monoclonal, Cell Signaling Technology [#9216], 1:2000); anti-Vinculin ([hVIN-1], mouse monoclonal, Sigma Aldrich, 1:5000). The following primary antibodies were used in immunofluorescence experiments: anti-human E5NT ([4G4], mouse monoclonal, Novus Biologicals, 1:200); anti-human ENTPD1 ([BU61], mouse monoclonal, Santa Cruz Biotechnology, 1:200).

## 2.2. Dicistronic plasmid construction

The F2A sequence was obtained as previously described [27] and ligated by directional cloning into BamHI/XhoI-digested pcDNA3.1+ (Invitrogen) to produce the pcDNA3.1-F2A plasmid. hE5NT (NCBI: NM_002526.3) and hENTPD1 (NCBI: NM_001776.5) coding sequence (CDS) were sequentially cloned into the pcDNA3.1-F2A plasmid, as detailed in Supplementary Material. Briefly, with two rounds of recombinant PCR, hE5NT CDS was amplified without the stop codon and ligated upstream the F2A sequence into the pcDNA3.1-F2A plasmid. hENTPD1 CDS was PCR-amplified and ligated downstream the F2A into the pcDNA3.1-hE5NT-F2A plasmid. The dicistronic cassette hE5NT-F2A-hENTPD1 was excised by EcoRI digestion and ligated in EcoRI-linearized pCX-C1 [28] plasmid acceptor obtaining pCX-hE5NT-F2A-hENTPD1, which was named pCX-DI-2A.

pCX-hE5NT, pCX-hENTPD1 and pCX-empty plasmids, used as controls were obtained as previously described [29]. All the intermediate and final constructs were verified by restriction digestion analysis and by sequencing analyses performed on both strands using the BigDye Terminator Cycle Sequencing kit v1.1 and an automated ABI-3130 DNA sequencer (Applied Biosystems, Foster City, CA).

## 2.3. Cell culture, transfection, sorting and treatments

Porcine iliac artery endothelial cells (PIEC) were cultured in RPMI 1640 medium (EuroClone) supplemented with 10% FBS (Life Technologies) and 1x Penicillin-Streptomycin (EuroClone) at 37 °C

and 5% $CO_2$. Cells were detached by using 1x trypisin/EDTA (EuroClone) when at 80-90% of confluence. PIEC cells were transfected using 10 µl of Lipofectamine 2000 (Invitrogen) with 4 µg of pCX-DI-2A, pCX-hE5NT, pCX-hENTPD1 or pCX-empty plasmid following manufacturer's protocol. Transfected cells were selected by Neomycin (400 µg/ml, Sigma Aldrich) treatment for 2 weeks before being enriched by cell sorting.

For cell sorting experiments, transfected cells were detached by trypsin/EDTA and washed with FACS buffer (3% FBS and 0.01% $NaN_3$ in PBS, EuroClone). $5*10^5$ cells were then incubated for 30 min at 4°C in the dark with anti-human E5NT ([AD2], PE-conjugated) for pCX-hE5NT-transfected cells, or anti-human ENTPD1 ([A1], Alexa Fluor 647-conjugated) for pCX-hENTPD1-transfected cells or both antibodies for pCX-DI-2A-transfected cells. The excess and non-specifically bound antibodies were removed by washing with FACS buffer. Sorting was performed by using a FACSAria flow cytometer (Becton Dickinson). Post sorting FACS analysis was performed to check the purity of enriched cells. Isotype- matched control antibody staining did not reveal any signals. Mock-transfected cells were used as negative control of human E5NT and ENTPD1 staining (data not shown).

Apoptosis cell death was assessed on PIEC transfected cell lines after treatment with 100 µM $H_2O_2$ for 4, 6 and 8 hours or after treatment with 10 ng/ml human TNF-α (Sigma Aldrich) for 8, 16 and 24 hours. Immunoblotting analysis and cytotoxicity assay in response to hydrogen peroxide were performed on cells treated with 400 µM $H_2O_2$ for 2, 4 and 6 hours. ROS generation was measured after 30, 60 and 90

minutes of 100 µM and 400 µM $H_2O_2$ exposure, while the GSH/GSSG ratio was measured after 30, 60 and 90 minutes of 400 µM $H_2O_2$ exposure. Catalase (CAT) and Glutathione Peroxidase (GPx) enzymatic assays were performed on cells treated with 400 µM $H_2O_2$ for 30 minutes and 2, 4 and 6 hours.

### 2.4. Immunoblotting

Immunoblotting analyses to detect the expression of human proteins in transfected cells were performed as previously described [27,29]. Immunoblotting analyses to evaluate the molecular response of PIEC cell lines exposed to $H_2O_2$ toxic insult, were performed as follows: at the end of treatments, cells were lysed with 1X RIPA lysis buffer (50mM Tris-HCl pH 7.4, 150mM NaCl, 1% Triton X-100, 0.1 % SDS) added with 1mM DTT, 1mM EDTA and EGTA, and 1.5% Protease Inhibitor Cocktail and Phosphatase Inhibitor Cocktail, and protein extracts were quantified by Bradford assay (Sigma Aldrich), following manufacturer's instructions. Protein extracts were then loaded on NuPAGE Bis-Tris pre-casted mini gels (Life Technologies) following manufacturer instructions. Blotting onto nitrocellulose membrane (Life Technologies) was performed using iBlot System 2 (Life Technologies). After blocking, membranes were incubated with the selected primary antibody and then with the appropriate secondary antibody: anti-mouse IgG (H+L) HRP-conjugate (Alpha Diagnostic Intl. Inc.) and ECL anti-rabbit IgG HRP linked (GE Healthcare) secondary antibodies (1:5000). Super Signal West Dura Extended duration substrate (Thermo Scientific) was added to the membranes and

chemiluminescent signal was digitally acquired by GBox (Syngene). Densitometric analysis of Western blot bands was performed by using "Gel analyzer" function of ImageJ software[30].

### 2.5. Immunofluorescence and confocal microscopy

pCX-DI-2A- and mock-transfected cells were seeded at $4\times10^4$ cells/well in 8-well chamber slides (LabTek Chamber slides, Thermo Fisher Scientific) for 24 hours. The next day, cells were washed with PBS and fixed with methanol-acetone 1:1 for 10 min at -20 °C. After fixation, cells were blocked with 1% BSA for 30 min. Fixed cells were co-incubated for 1 hour with either mouse anti-human E5NT or mouse anti-human ENTPD1 antibodies and plasma membrane marker Alexa Fluor 488 conjugated-wheat germ agglutinin (WGA, Thermo Fisher Scientific, 1:200). Alexa Fluor 594-conjugated anti mouse IgG (Life Technologies) was used as secondary antibody for 30 min (1:5000 in 1% BSA (w/v) PBS). Cells were then washed and counterstained with DAPI. The stained cells were mounted with mounting medium (Fluoromount; Sigma-Aldrich) and analyzed by LSM 710 confocal microscope (Zeiss). Images were acquired by ZEN 2009 software (Zeiss).

### 2.6. hE5NT and hENTPD1 activity assay and extracellular adenine nucleotides metabolism determination

hE5NT and hENTPD1 activity assay was performed as previously described [26,27]. Briefly, PIEC cells were pre-incubated for 15

minutes in HBSS supplemented with glucose (1 mg/ml; Sigma-Aldrich) and adenosine deaminase inhibitor, erythro-9-(2-hydroxy-3-nonyl) Adenine, EHNA (5 μM; Sigma-Aldrich). Then, cells were incubated with 50 μM AMP, or ADP, or ATP (Sigma-Aldrich) and supernatant samples were collected after 0, 5, 15, 30 minutes.

Extracellular adenine nucleotides and their metabolites were measured in supernatant samples of PIEC cells incubated with 400 μM $H_2O_2$ in HBSS supplemented with glucose (1 mg/mL) for 0, 5, 15, 30, 60 minutes. Samples were analyzed for nucleotide metabolite content by reversed-phase high performance liquid chromatography (RP-HPLC) as previously described [31].

### 2.7. Caspase 3/7 activity assay

Caspase 3/7 activity was measured by using Caspase-Glo 3/7 assay (Promega) as previously described [29]. Briefly, $10^4$ PIEC cells/well were seeded in a white 96-well plate (Corning). The day after seeding, cells were treated with $H_2O_2$ or human TNF-α and then, lyophilized Caspase-Glo 3/7 substrate was resuspended and added to cells according to the manufacturer's protocol. Luminescent signal was measured by using a 96 multi-well plate reader (Infinite M200; Tecan).

### 2.8. Cytotoxicity assay

Cytotoxicity was analyzed by using SYTOX® Green Nucleic Acid Stain (Life Technologies) following the manufacturer's instructions. Briefly, $2\times10^6$ PIEC cells were seeded in 100mm Petri dish (Euroclone)

and exposed to $H_2O_2$ the next day. Then, $5\times10^5$ cells were collected, washed with 1X TBS (50 mM Tris-HCl, pH 7.4 and 150 mM NaCl) and incubated with 100 nM SYTOX® Green Nucleic Acid Stain for 20 minutes at 4°C in the dark. After two washes with 1X TBS, cells were resuspended in 100 µl 1X TBS and analyzed for dye incorporation by Tali® Image Cytometer (Life Technologies). Data were plotted using FlowJo Single Cell Analysis Software (http://ww.flowjo.com).

### 2.9. Oxidative stress measurement

For measuring ROS production, the fluorogenic probe CellROX® Orange Reagent (Life Technologies) was used. Briefly, $2\times10^6$ PIEC cells were seeded in 100 mm Petri dish and exposed to $H_2O_2$ the next day. After one wash with PBS, cells were incubated with 100 nM CellROX® Orange Reagent (Life Technologies) for 30 minutes at 37°C in the dark. After two washes with PBS, $5\times10^5$ cells were resuspended in 100 µl PBS and analyzed for ROS production by Tali® Image Cytometer.

Alternatively, $4\times10^4$ PIEC cells/well were seeded in 8-well chamber slides for fluorescent *in situ* detection. After treatment with $H_2O_2$, cells were incubated with 100 nM CellROX® Orange Reagent Cells for 30 minutes in the dark. Then cells were fixed with methanol-acetone 1:1 for 10 minutes at -20°C, following by one wash with PBS and nuclear staining with DAPI. Stained cells were analyzed as described above.

For measuring the GSH/GSSG ratio, the GSH/GSSG-Glo assay (Promega) was used following the manufacturer instructions. This kit allows the measurement of both oxidized and total glutathione levels in

order to compute the ratios of reduced to oxidized glutathione. Briefly, $10^4$ PIEC cells/well were seeded in white 96-well plate and exposed to $H_2O_2$ the next day. After treatment, cells were lysed with Total Gluthatione Lysis or Oxidixed Gluthatione Lysis Reagent directly in the culture plate and immediately exposed to the luciferin reaction buffer for 30 minutes at room temperature in the dark. Then, luciferin detection reagent was added to the lysates and luminescence was measured after 15 minutes of incubation by using a 96 multi-well plate reader (Infinite M200; Tecan) according to the manufacturer's protocol. Background readouts were subtracted from all measurements and standard curve was generated using Glutathione (GSH) included in the kit. Three wells per experimental group were used.

### 2.10. Antioxidant enzymes activity assays

CAT activity was measured by using Catalase Assay Kit (Sigma Aldrich) following the manufacturer instructions. Briefly, $2\times10^6$ PIEC cells were seeded in 100 mm Petri dish and exposed to $H_2O_2$ the next day. Cells were collected at the indicated time points and proteins were extracted as described above. 3 µl of a 2 mg/ml dilution of each protein sample were used to start the reaction in 100 µl of reaction buffer containing 50 mM $H_2O_2$. After 5 minutes of incubation, an aliquot of the catalase enzymatic reaction mixture was added to the Color Reagent (150 mM potassium phosphate buffer, pH 7.0, containing 0.25 mM 4-aminoantipyrine, 2 mM 3,5-dichloro-2-hydroxybenzenesulfonic acid and 0.5-0.8 U/ml peroxidase) followed by 15 minutes of incubation at room temperature in the dark. Absorbance at 520 nm was measured by

using a 96 multi-well plate reader (Infinite M200; Tecan) according to the manufacturer's protocol. A standard curve was generated using a series of standard solution of $H_2O_2$ included in the kit.

GPx activity was measured by using Glutathione peroxidase cellular activity assay kit (Sigma Aldrich) following the manufacturer instructions with some modifications. Briefly, cell treatments and protein extraction were carried out as described above for catalase assay. 40 µg of proteins were used for measuring the GPx activity in 100 µl of reaction buffer containing 0.25 mM NADPH, 2.1 mM GSH, 0.5 U/ml glutathione reductase and 300 µM t-Bu-OOH. A standard curve was generated using Glutathione peroxidase standard (Sigma Aldrich). The kinetic decrease in absorbance at 340 nm was measured by using a 96 multi-well plate reader (Infinite M200; Tecan) according to the manufacturer's protocol.

### 2.11. Statistical analysis

Statistical analyses were performed with SPSS v.19 software (IBM). Data are expressed as mean ± standard error of the mean (S.E.M.). Caspase 3/7, Sytox, oxidative stress measurements and activity assays were independently performed at least 3 times. One-way ANOVA followed by Tukey's *post hoc* was used for comparing multiple experimental groups. Data were considered statistically significant when $p<0.05$.

## 3. RESULTS

### 3.1. pCX-DI-2A-transfected cells simultaneously expressed human E5NT and ENTPD1

In order to investigate the protective effects of the simultaneous expression of human E5NT and ENTPD1 against oxidative stress, we engineered an F2A-based dicistronic plasmid encoding for both proteins and we used this plasmid for transfecting porcine iliac endothelial cells (PIECs).

The dicistronic plasmid was designed and produced with a similar strategy used for previously reported F2A-based constructs [27,29]. Briefly, the hE5NT CDS was cloned without the stop codon upstream of the F2A sequence. The hENTPD1 CDS was cloned in frame downstream of the F2A sequence and the resulting dicistronic CDS was then moved in pCX-plasmid under the control of the Citomegalovirus early enhancer/chicken beta actin promoter (pCAGGS) (Figure 1A).

PIECs were transfected with pCX-DI-2A plasmid and enriched for the expression of both hE5NT and hENTPD1 by cell sorting. Post-sorting FACS analysis showed a population of around 95% of cells double positive for hE5NT and hENTPD1 (Figure 1B). The size of the exogenously expressed hE5NT and hENTPD1 in pCX-DI-2A-transfected cells was verified by western blot analyses (Figures 1C-D). Then, we checked the subcellular localization of hE5NT and hENTPD1, which both correctly co-localized with the plasma-membrane marker, wheat germ agglutinin (WGA) (Figures 1E-F). Single-gene expressing vectors [29] were used for transfection of PIECs as control of the effects derived by the separate expression of

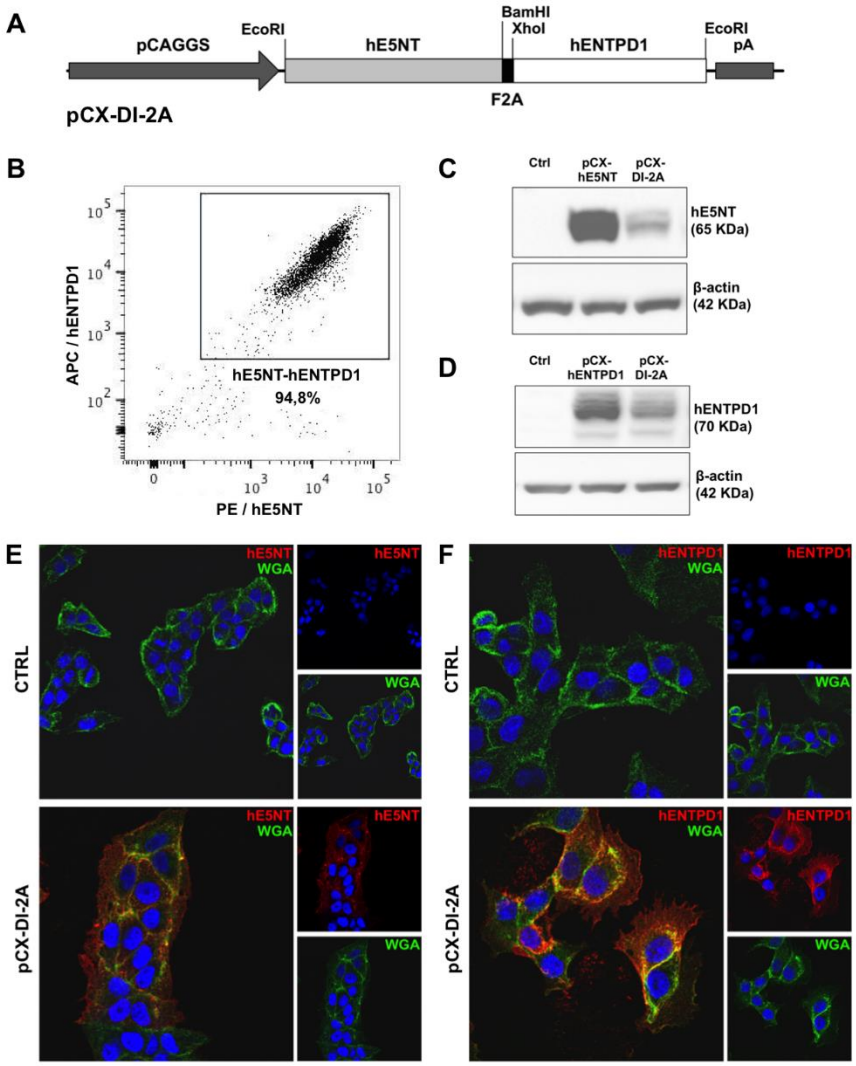



**Figure 1.** pCX-DI-2A-transfected porcine endothelial cells efficiently express both hE5NT and hENTPD1. (A) Schematic map of the dicistronic pCX-DI-2A plasmid used to simultaneously express hE5NT and hENTPD1 in PIEC cells; (B) FACS analysis revealed that hE5NT (PE) and hENTPD1 (APC) were both expressed at high levels in pCX-DI-2A-transfected cells after cell sorting; (C-D) Western blotting analysis of hE5NT (C) or hENTPD1 (D) showing the expression of the human enzymes only in transfected cells; (E-F) Immunofluorescence analysis of hE5NT (red in E) or hENTPD1 (red in F) expression co-stained with plasma membrane marker WGA (green), showing the correct membrane-localization of human genes in pCX-DI-2A-transfected cells. Mock-transfected cells (ctrl) did not express human genes. DAPI (blue) was used for nuclear staining. Images in (B-F) are representative of at least 3 independent experiments.

hE5NT and hENTPD1. After cell sorting, the purity of cells positive for either hE5NT or hENTPD1 was up to 95% (Supplementary Figure 1). Taken together, these findings demonstrated that pCX-DI-2A plasmid mediates the simultaneous expression and the correct processing of human E5NT and ENTPD1 in porcine endothelial cells.

### 3.2. Synergic enzymatic activity of hE5NT and hENTPD1 in pCX-DI-2A-transfected cells

With the aim to evaluate the enzymatic activity of the simultaneously expressed hE5NT and hENTPD1, we incubated pCX-DI-2A-transfected and control cell lines with ATP, ADP or AMP and we measured the extracellular nucleotide metabolism over time by HPLC (Figure 2).

Following ATP incubation, ADP production was similar between pCX-DI-2A- and pCX-hENTPD1-transfected cells but significantly higher as compared to pCX-hE5NT- and mock-transfected cells at early time points (5 minutes, $p<0.05$, and 15 minutes, $p<0.05$; Figure 2A). pCX-hENTPD1-transfected cells showed an almost total conversion of ATP to AMP after 30 minutes of incubation (44.4±5 nmol/ml) whereas the extracellular AMP produced by pCX-DI-2A-transfected cells increased up to 15.3±3.3 nmol/ml after 15 minutes of incubation (Figure 2B). Nevertheless, pCX-DI-2A- and pCX-hENTPD1-transfected cells showed a similar rate of ATP degradation (Supplementary Figure 2A). No AMP production was revealed in pCX-hE5NT- and mock-transfected cells during the time of incubation suggesting a very low endogenous ATPase activity (Figure 2B). A significant production of adenosine (up to 28.3±2.5 nmol/ml following 30 minutes of incubation)

was observed only in pCX-DI-2A-transfected cells suggesting the synergic hE5NT-mediated catabolism of the AMP produced by hENTPD1 (Figure 2C).

Following ADP incubation, pCX-hENTPD1-transfected cells showed again the highest production of AMP (44.7±1.8 nmol/ml after 30 minutes; Figure 2D), but we did not reveal the simultaneous catabolism of AMP to adenosine in this cell line (Figure 2E). Despite a similar ADP degradation rate in pCX-DI-2A- and pCX-hENTPD1-transfected cells (Supplementary Figure 2B), pCX-DI-2A-transfected cells showed a lower accumulation of extracellular AMP as compared to pCX-hENTPD1-transfected cells (14.6±1.5 nmol/ml after 30 minutes, $p<0.05$). In pCX-DI-2A-transfected cells, AMP was indeed simultaneously converted to adenosine by hE5NT over the time of incubation (Figure 2E). Interestingly, we observed a slight production of AMP and adenosine also by pCX-hE5NT-transfected cells (Figures 2D-E).

Following AMP incubation, the production of adenosine was detected only in pCX-DI-2A- and pCX-hE5NT-transfected cells (Figure 2F). Consistently, the AMP degradation rate was similar in pCX-DI-2A- and pCX-hE5NT-transfected cells and almost absent in pCX-hENTPD1- and mock-transfected cells (Supplementary Figure 2C).

All together, these data showed that pCX-DI-2A-transfected cells not only efficiently catabolize the extracellular adenine nucleotides but also enhance the production of adenosine.

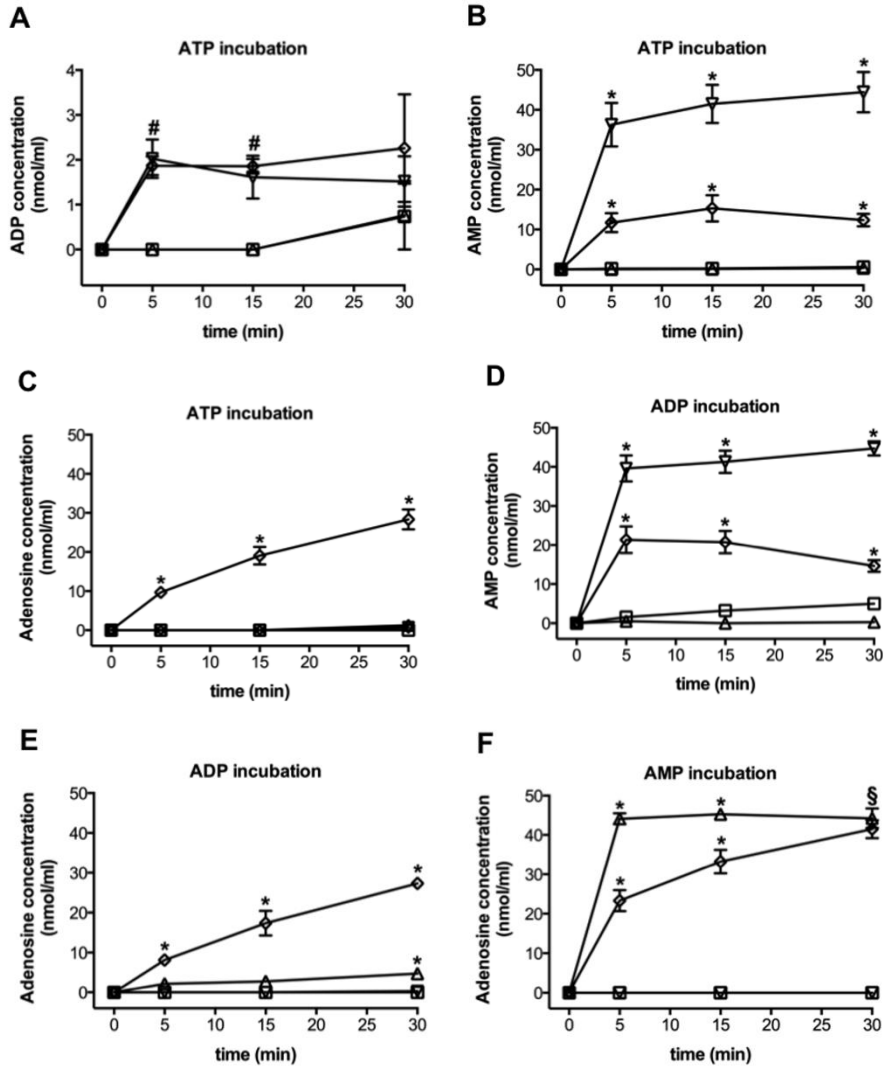



**Figure 2.** Enhanced extracellular adenine nucleotide metabolism in pCX-DI-2A-transfected cells. pCX-DI-2A- (◊), pCX-hE5NT- (Δ), pCX-hENTPD1- (∇) and mock- (•) transfected cells were incubated with 50 μM ATP, or ADP, or AMP and the respective enzymatic products were measured over different time points by reverse phase HPLC. (A-C) Production of ADP (A), AMP (B) and adenosine (C) following incubation with ATP. (D-E) Production of AMP (D) and adenosine (E) following incubation with ADP. (F) Production of adenosine following incubation with AMP. Error bars represent SEM (n=3). * $p<0.05$ versus all groups; # $p<0.05$ pCX-DI-2A-transfected cells versus all groups except pCX-hENTPD1-transfected cells; § $p<0.05$ pCX-DI-2A-transfected cells versus all groups except pCX-hE5NT-transfected cells.

### 3.3. The combined expression of hE5NT and hENTPD1 proteins reduced caspase activation induced by pro-apoptotic stimuli in pCX-DI-2A-transfected cells

It has been reported that the inhibition of apoptosis can reduce the IRI [32,33]. Since during IRI, E5NT and ENTPD1 reduce free radicals [34] and exert anti-apoptotic effects [19], respectively, we investigated the potential protective role of their combination against $H_2O_2$-induced apoptosis by caspase 3/7 activity assay (Figure 3). pCX-DI-2A-transfected cells did not show increase of caspase 3/7 activity over time (1.08±0.001, 1.02±0.003 and 1.02±0.07 after 4, 6 and 8 hours of exposure to $H_2O_2$, respectively), contrarily to what observed in the other PIEC cell lines (Figure 3). Moreover, pCX-DI-2A-transfected cells showed a significant lower level of caspases activation after 6 and 8 hours of treatment (1.02±0.003 and 1.02±0.07) as compared to both pCX-hENTPD1- (2.17±0.23 and 2.17±0.19; $p<0.05$) and mock-transfected cells (2.17±0.43 and 2.3±0.35; $p<0.05$). pCX-hE5NT-transfected cells showed only a trend of lower caspase 3/7 activity as compared to mock-transfected cells at 6 and 8 hours of $H_2O_2$ treatment (1.73±0.09 and 1.79±0.34; Figure 3).

To investigate whether hE5NT/hENTPD1 combination could afford protection also against other pro-inflammatory stimuli relevant in IRI, we incubated PIEC cells with 10 ng/ml human TNF-α. We observed that pCX-DI-2A-transfected cells were significantly protected against human TNF-α-induced apoptosis at 8, 16 and 24 hours of exposure as compared to mock-transfected cells (Supplementary Figure 3).

Moreover, pCX-DI-2A-transfected cells showed better protection as compared to pCX-hE5NT-transfected cells at 16 and 24 hours but not

as compared to pCX-hENTPD1-transfected cells (Supplementary Figures 3B-C).

These data suggest that the simultaneous expression of the two exogenous proteins better protected endothelial cells by inhibiting apoptosis induced by $H_2O_2$ and TNF-α.

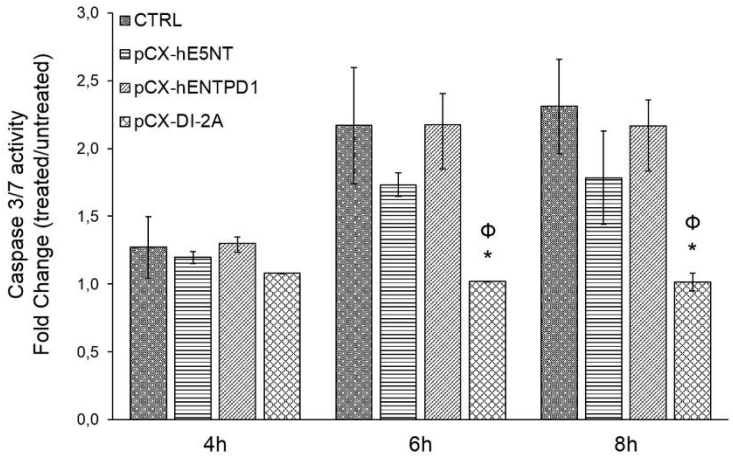



**Figure 3.** Protection against $H_2O_2$-induced apoptosis in pCX-DI-2A-transfected cells. pCX-DI-2A-, pCX-hE5NT-, pCX-hENTPD1- and mock-transfected (ctrl) cells were treated with 100 μM $H_2O_2$ for 4, 6 and 8 hours and apoptosis was measured by caspase 3/7 assay. Caspase activation in each treated cell line is expressed as a fold change value of the corresponding untreated cells. Error bars represent SEM (n≥3). * $p<0.05$ versus ctrl cells; Φ $p<0.05$ versus pCX-hENTPD1-transfected cells.

### 3.4. Cytoprotection mediated by the combination hE5NT/hENTPD1 in pCX-DI-2A-transfected cells

Since during IRI, abundant ROS production and release can lead to cell toxicity and death [33], we investigated if the combined expression of hE5NT and hENTPD1 in pCX-DI-2A-transfected cells would confer protection against oxidative injury mediated by $H_2O_2$. To this extent, controls and transfected PIEC cells were exposed to 400μM of $H_2O_2$

for 2, 4 and 6 hours and the cytotoxicity was measured by Sytox incorporation assay (Figure 4).

The percentage of dead cells was notably lower in pCX-DI-2A-transfected cells than in mock-transfected cells at 2, 4 and 6 hours of treatment (9±1.73% vs. 31±1%, $p<0.05$, Figure 4D; 15.5±0.5% vs. 51±2%, $p<0.05$, Figure 4E; 23±0.58% vs. 62.5±4.5%, $p<0.05$, Figure 4F). After 2 hours of treatment, the level of cytotoxicity in pCX-hENTPD1-transfected cells (6.33±3.84%) was significantly lower than in mock-transfected cells ($p<0.05$, Figure 4D), however it underwent a strong increase after 4 and 6 hours of $H_2O_2$ exposure (85±1%, $p<0.05$, Figure 4E; 85.5±5.5%, $p<0.05$, Figure 4F). pCX-hE5NT-transfected cells did not show a strong increase in percentage of dead cells after 2 and 4 hours of treatment (20.67±6.44%, Figure 4D; 30±5%, Figure 4E) but this percentage was doubled after 6 hours (42±2.83%, Figure 4F) as compared to the earliest time point. However, the cytotoxicity in pCX-hE5NT-transfected cells was significantly lower as compared to mock-transfected cells after both 4 and 6 hours of $H_2O_2$ exposure ($p<0.05$, Figures 4E and F). Interestingly, at the latest time point, the percentage of dead cells in pCX-DI-2A-transfected cells (23±0.58%) was significantly decreased as compared to both pCX-hE5NT- and pCX-hENTPD1-transfected cells (42±2.83% and 85.5±5.5% respectively, $p<0.05$, Figure 4F).

All together, these findings highlighted that the simultaneous expression of hE5NT and hENTPD1 had a protective effect against $H_2O_2$-induced cytotoxicity and that this action was more effective than the separate expression of each ectonucleotidase.

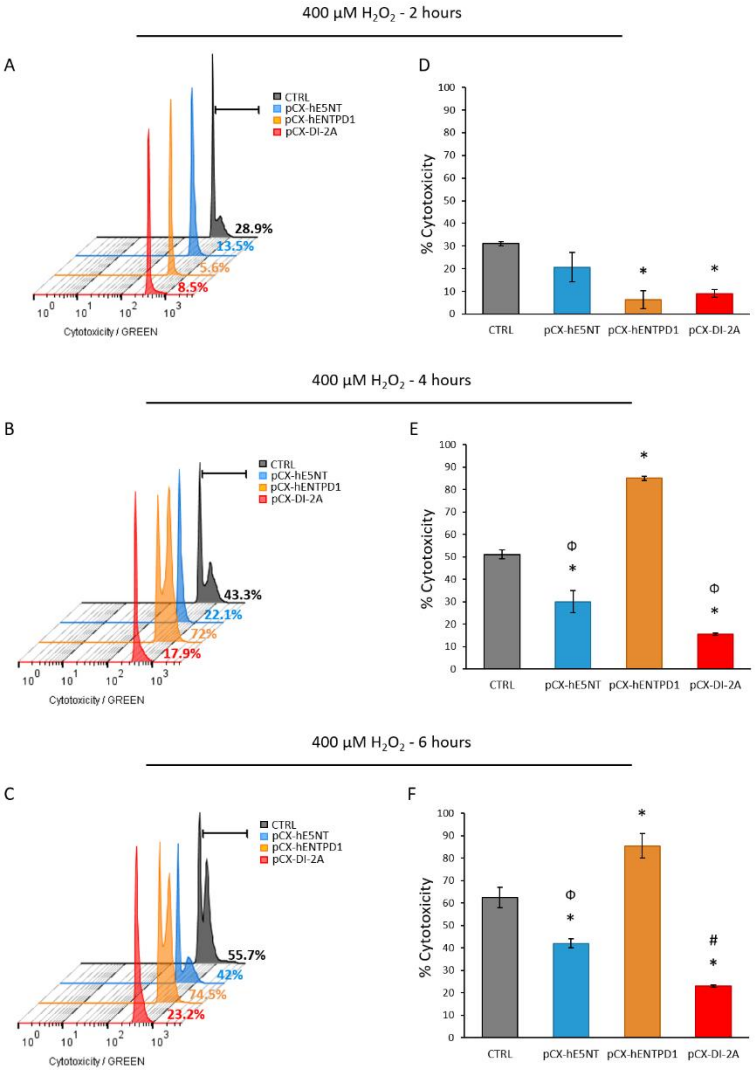



**Figure 4.** Protection against $H_2O_2$-induced cytotoxicity in pCX-DI-2A-transfected cells. pCX-DI-2A- (red), pCX-hENTPD1- (orange), pCX-hE5NT- (blue) and mock- (grey) transfected cells were treated with 400 µM $H_2O_2$ for 2 (A), 4 (B) and 6 (C) hours and cytotoxicity was measured by incorporation of 100 nM SYTOX Green Nucleic Acid Stain as described in Materials and Methods. Data shown in A, B and C are representative of one of three independent experiments. (D-F) Quantification of cytotoxicity after 2 (D), 4 (E) and 6 (F) hours of treatment with 400 µM $H_2O_2$. Cytotoxicity is expressed as the percentage of dead cells compared to the untreated sample for each cell line. Error bars in D, E and F represent SEM (n=3). * $p$<0.05 versus ctrl cells; # $p$<0.05 pCX-DI-2A-transfected cells versus single gene-transfected cells; Φ $p$<0.05 versus pCX-hENTPD1-transfected cells. CTRL, mock-transfected cells.

### 3.5. The combined activity of hE5NT and hENTPD1 resulted in catabolism of pro-inflammatory nucleotides and simultaneous production of protective adenosine in pCX-DI-2A-transfected cells during exposure to $H_2O_2$

To investigate the activity of the overexpressed ectonucleotidases during oxidative injury, we incubated PIEC cells with $H_2O_2$ and we measured the metabolism of pro-inflammatory adenine nucleotides and the production of adenosine.

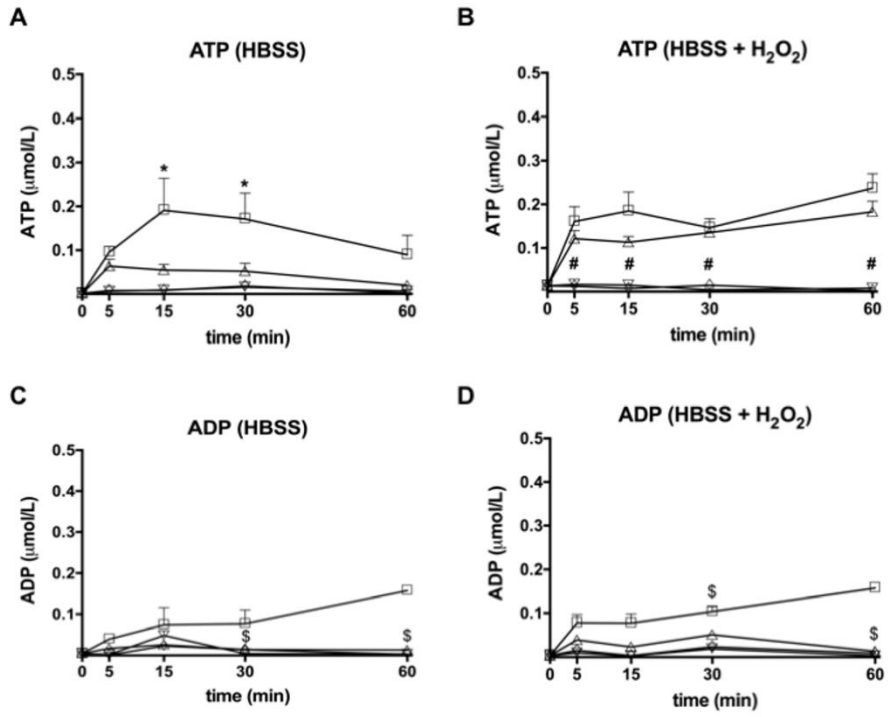
Under basal conditions, we observed accumulation of extracellular ATP, ADP and AMP in mock-transfected cells (Figure 5A, C, E). pCX-hENTPD1-transfected cells showed also accumulation of AMP (Figure 5E). We detected a slight production of adenosine in pCX-hE5NT- and pCX-DI-2A-transfected cells, but not significantly different from mock- and pCX-hENTPD1-transfected cells (Figure 5G).

During $H_2O_2$ treatment, pCX-DI-2A-transfected cells showed efficient removal of extracellular ATP (Figure 5B) and ADP (Figure 5D), similarly to pCX-hENTPD1-transfected cells. On the contrary, we observed accumulation of ATP in medium from mock- and pCX-hE5NT-transfected cells (Figure 5B). Mock-transfected cells also showed accumulation of ADP (Figure 5D). These data suggest very low endogenous ATPase and ADPase activity in PIEC cells that did not overexpress human ENTPD1.

Extracellular AMP accumulated in medium from mock- and pCX-hENTPD1-transfected cells whereas it was not detectable in medium from pCX-DI-2A- and pCX-hE5NT-transfected cells (Figure 5F), suggesting an efficient hE5NT-mediated AMP removal in these latter cell lines.

Interestingly, we observed significantly higher production of extracellular adenosine in medium from pCX-DI-2A-transfected cells (0.15±0.05 µmol/l after 60 minutes of incubation; Figure 5H) as compared to mock- and pCX-hENTPD1-transfected cells. pCX-hE5NT-transfected cells showed the highest production of adenosine.

Taken together, these data suggest that in conditions of $H_2O_2$-induced oxidative stress and cytotoxicity, pCX-DI-2A-transfected cells showed the efficient hENTPD1-mediated removal of pro-inflammatory and pro-thrombotic adenine nucleotides along with the simultaneous hE5NT-mediated production of anti-inflammatory and protective adenosine.



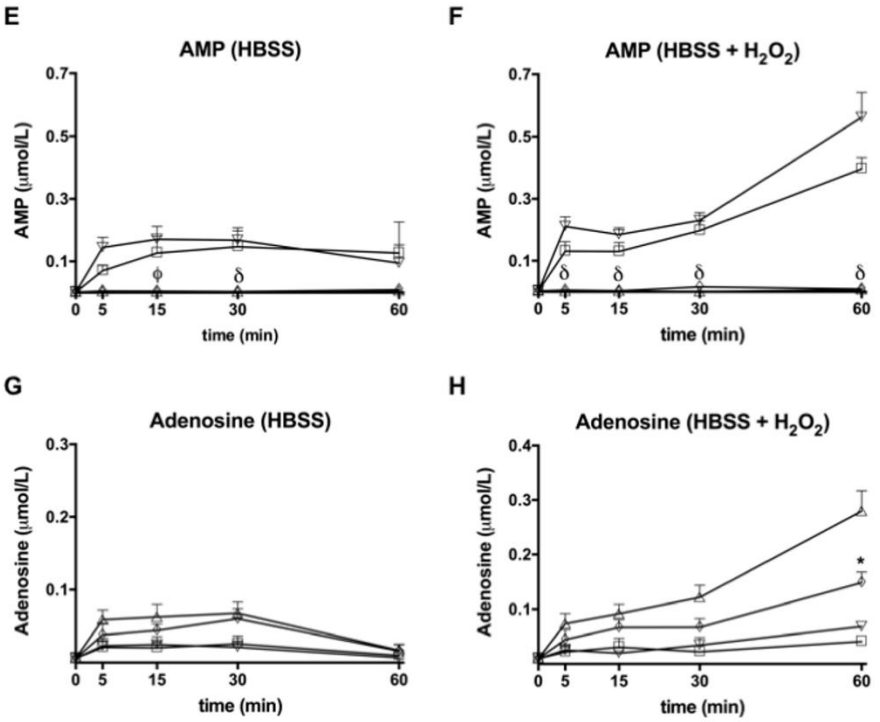



**Figure 5.** pCX-DI-2A-transfected cells showed efficient adenine nucleotides removal and simultaneous production of adenosine during exposure to $H_2O_2$. pCX-DI-2A- (◊), pCX-hE5NT- (Δ), pCX-hENTPD1- (∇) and mock- (**∇**)transfected cells were treated with 400 µM $H_2O_2$ (HBSS + $H_2O_2$) for 60 minutes and supernatant samples were collected at 0, 5, 15, 30 and 60 minutes time points. The extracellular nucleotides content was measured by RP-HPLC. As control of the basal nucleotides and nucleosides concentration, we incubated cells with basal medium (HBSS) and we collected supernatant samples at the indicated time points. Extracellular ATP, ADP, AMP and adenosine concentration was measured in supernatant samples from PIEC cells incubated with basal medium (A, C, E, G, respectively) or medium containing $H_2O_2$ (B, D, F, H, respectively). Error bars represent standard deviation (n=3). * $p<0.05$ pCX-DI-2A-transfected cells versus all groups; # $p<0.05$ pCX-DI-2A- versus mock- and pCX-hE5NT-transfected cells; $ $p<0.05$ pCX-DI-2A- versus mock-transfected cells; Φ $p<0.05$ pCX-DI-2A- versus pCX-hENTPD1-transfected cells; δ $p<0.05$ pCX-DI-2A- versus mock- and pCX-hENTPD1-transfected cells.

### 3.6. The combined expression of hE5NT and hENTPD1 reduced $H_2O_2$-induced ROS formation in pCX-DI-2A-transfected cells

Having demonstrated that pCX-DI-2A-transfected showed an increased conversion of pro-inflammatory nucleotides to adenosine even in presence of $H_2O_2$ and, at the same time, were protected against $H_2O_2$-mediated cell toxicity, we further investigated whether this effect was due to a lower ROS production within the cells. Cells were exposed to 100 µM $H_2O_2$ and cellular ROS were detected *in situ* by CellROX® Orange Reagent (Supplementary Figure 4). Only mock-transfected cells showed a well-defined staining already after 30 minutes of treatment (Supplementary figure 4A), whereas a marked signal was observed in both single gene-transfected cell lines only after 60 and 90 minutes of incubation (Supplementary figures 4B and C). pCX-DI-2A-transfected showed less stained cells with slight intensity as compared to all the other cell lines at every time points (Supplementary Figures 4).

Then, we assessed ROS generation following treatment with a cytotoxic concentration of $H_2O_2$ (400 µM). pCX-DI-2A-transfected cells were still protected from ROS formation as shown by the fewer and less stained cells as compared to all the other cell lines after 90 minutes of incubation (Figure 6). As expected, the generation of ROS was lower in all the PIEC cell lines after 30 and 60 minutes exposure to $H_2O_2$ (Supplementary Figures 5A and B) as compared to 90 minutes of treatment (Figure 6) and an increase in signal intensity was observed over time, prompting a progressive formation of ROS within cells.

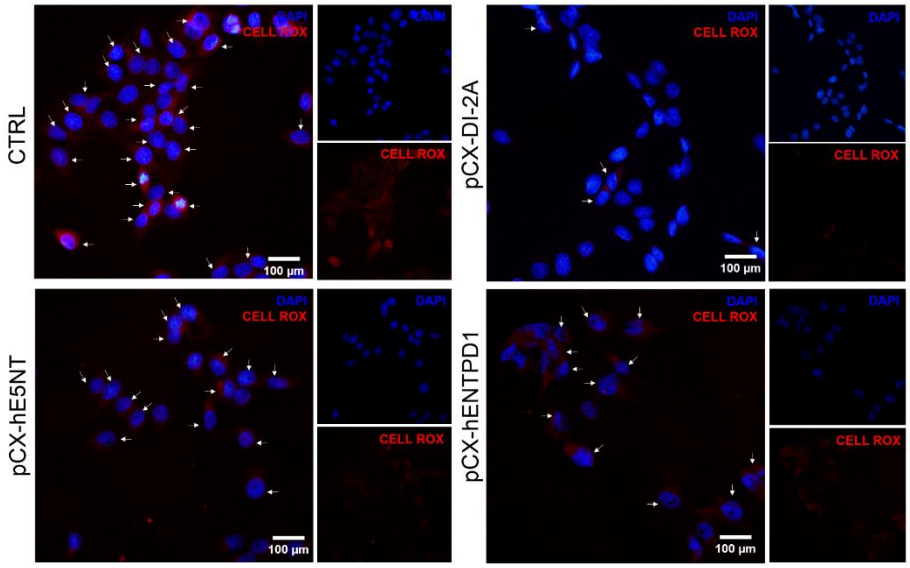



**Figure 6.** pCX-DI-2A-transfected cells showed lower ROS production than control cell lines after 90 minutes exposure to $H_2O_2$. pCX-DI-2A-, pCX-hE5NT, pCX-hENTPD1- and mock-transfected cells were treated with 400 µM $H_2O_2$ for 90 minutes and ROS were detected by fluorescence staining with CellROX® Orange Reagent as described in Materials and Methods. White arrows indicate cells positive to the staining (red). Scale bar represents 100 µm. DAPI (blue) was used for nuclear staining. Images are representative of at least 3 independent experiments. CTRL, mock-transfected cells.

In order to accurately quantify the production of ROS into the cells, PIEC cell lines were treated for 30, 60 or 90 minutes with 400 µM of $H_2O_2$ and the CellROX® Orange Reagent-positive cells were measured by Tali® Image Cytometer. As shown in Figure 7, ROS formation was significantly lower in pCX-DI-2A-transfected cells than in mock-transfected cells at 30, 60 and 90 minutes of treatment (5.63±2.22% vs. 99.8±0.17%, $p<0.05$, Figure 7A; 31.2±9.48% vs. 98.9±0.95%, $p<0.05$, Figure 7B; 31.4±9.55% vs. 99.7±0.27%, $p<0.05$, Figure 7C). Similarly, after 30 minutes of $H_2O_2$ exposure, pCX-hE5NT- and pCX-hENTPD1-transfected cells showed a significantly reduced ROS formation (3.47±2.03% and 0.33±0.24%, respectively) as compared to mock-

transfected cells ($p$<0.05, Figure 7A). However, the levels of cellular ROS underwent a remarkable increase after 60 and 90 minutes of treatment in both the pCX-hE5NT- (73.4±18.16%, Figure 7B; 81.4±14.35%, Figure 7C) and pCX-hENTPD1-transfected cell lines (58.1±17.07%, Figure 7B; 77.45±1.25%, Figure 7C) as compared to the earliest time point ($p$<0.05 for each cell line). Interestingly, despite the high ROS formation in pCX-hENTPD1-transfected cells at 90 minutes of treatment, it was still significantly lower than the level of ROS in mock-transfected cells (Figure 7C, $p$<0.05).

Noteworthy, after 60 and 90 minutes of $H_2O_2$ treatment, pCX-DI-2A-transfected cells produced a reduced level of ROS as compared to all the other cell lines ($p$<0.05, Figures 7B and C). Additionally, we measured the GSH/GSSG ratio in PIEC cells as an indicator of oxidative stress following exposure to $H_2O_2$ (Supplementary figure 6). However, we did not observe differences in the GSH/GSSG ratio modulation between the cell lines at every time points of treatment. (Supplementary figure 6).

Taken together, these data showed the involvement of hE5NT and hENTPD1 in the process of $H_2O_2$-induced cellular ROS generation, and suggest that their combined action protects pCX-DI-2A-transfected cells against the toxic formation of ROS.

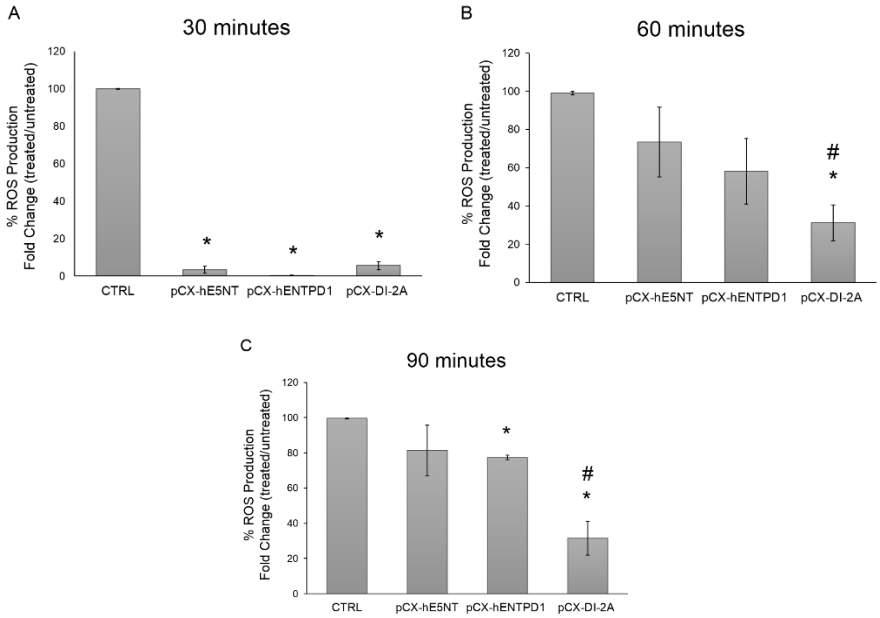



**Figure 7.** pCX-DI-2A-transfected cells were protected against $H_2O_2$-induced oxidative stress. pCX-DI-2A-, pCX-hE5NT-, pCX-hENTPD1- and mock-transfected cells were treated with 400 μM $H_2O_2$ for 30 (A), 60 (B) and 90 (C) minutes and the production of ROS levels was quantified following incubation with CellROX® Orange Reagent as described in Materials and Methods. ROS production in each treated cell line is expressed as percentage of the positive cells with respect to the corresponding untreated cells. Error bars represent SEM (n=3). * $p$<0.05 versus ctrl cells; # $p$<0.05 pCX-DI-2A-transfected cells versus single gene-transfected cells. CTRL, mock-transfected cells.

## 3.7. pCX-DI-2A-transfected cells showed enhanced $H_2O_2$ scavenging activity mediated by catalase

In order to unravel the hE5NT/hENTPD1-mediated activation of downstream pathways accounting for the protection against $H_2O_2$–induced toxicity and ROS generation, we first focused our attention on $H_2O_2$ metabolism. Cellular antioxidant systems, such as CAT and GPx, protect cells from $H_2O_2$-induced oxidative stress. Therefore, we evaluated the enzymatic activity of CAT and GPx in PIEC cell lines

treated with 400 µM $H_2O_2$ for 30, 120, 240 and 360 minutes (Figure 8 and Supplementary figure 7).

As shown in Figure 8, CAT activity was two fold higher in pCX-DI-2A-transfected cells as compared to mock-transfected cells in basal condition (102±4.3 U/ml/µg vs.51.6±3.4 U/ml/µg, $p<0.05$).

Under $H_2O_2$ exposure, pCX-DI-2A-transfected cells showed a significantly higher CAT activity than mock-transfected cells at every time points (89.1±3.3 U/ml/µg vs. 45.8±8.8 U/ml/µg at 30 minutes; 126.8 ±1.5 U/ml/µg vs. 51.4±17.8 U/ml/µg at 120 minutes; 132.5±9.2 U/ml/µg vs. 29.6±2.5 U/ml/µg at 240 minutes; 135.5±18.6 U/ml/µg vs. 2.5±0.3 U/ml/µg at 360 minutes; $p<0.05$; Figure 8).

Interestingly, pCX-DI-2A-transfected cells showed also a significantly higher CAT activity than pCX-hE5NT-transfected cells (69.1±17.6 U/ml/µg for untreated cells; 61.3±2.4 U/ml/µg at 30 minutes; 69.1±4.1 U/ml/µg at 120 minutes; 84.1±8.2 U/ml/µg at 240 minutes; 60.5±5.3 U/ml/µg at 360 minutes; $p<0.05$; Figure 8) and pCX-hENTPD1-transfected cells (49.4±12.6 U/ml/µg for untreated cells; 52.0±1.6 U/ml/µg at 30 minutes; 46.5±2.6 U/ml/µg at 120 minutes; 43.1±4.3 U/ml/µg at 240 minutes; 3.6±1.0 U/ml/µg at 360 minutes; $p<0.05$; Figure 8). Moreover, pCX-hE5NT-transfected cells showed a greater CAT activity than both mock- and pCX-hENTPD1-transfected cells after 240 and 360 minutes of exposure to $H_2O_2$ (84.1±8.2 U/ml/µg vs 29.6±2.5 U/ml/µg and 60.5±5.3 U/ml/µg vs. 2.5±0.3 U/ml/µg, respectively, $p<0.05$; Figure 8). No relevant differences were observed between mock- and pCX-hENTPD1-transfected cells at every condition tested (Figure 8).

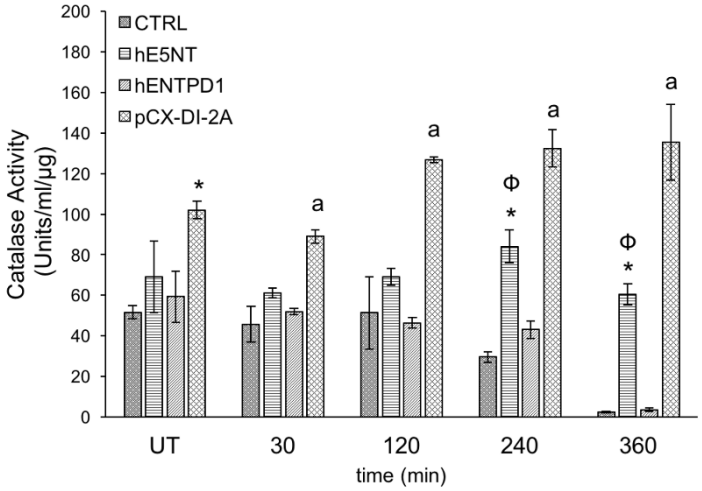



**Figure 8.** pCX-DI-2A-transfected cells showed higher activity of the antioxidant enzyme catalase. CAT activity was measured in untreated (UT) PIEC cells and cells treated with 400µM $H_2O_2$ for 30, 120, 240 and 360 minutes. Activity is expressed as enzymatic units per volume of reaction and µg of protein used for the assay. Data are presented as mean ± SEM (n=3). * $p<0.05$ versus ctrl cells; Φ $p<0.05$ versus pCX-hENTPD1-transfected cells. a $p<0.05$ pCX-DI-2A-transfected cells versus all the other cell lines; CTRL, mock-transfected cells.

Then, we measured the enzymatic activity of GPx in PIEC cells treated with $H_2O_2$. However, we did not detect relevant activity at every condition tested and no differences between all the cell lines (Supplementary figure 7).

Overall, these findings indicated that the protective effect mediated by the combined overexpression of hE5NT and hENTPD1 against $H_2O_2$-induced oxidative stress may be due to a high catalase activity, which remained constant over time.

### 3.8. Overexpression of hE5NT and hENTPD1 modulated MAPKs molecular pathway in cells exposed to $H_2O_2$-induced oxidative injury

Given the role of MAPKs in the regulation of cell proliferation, survival and metabolism in response to $H_2O_2$ injury, we evaluated the modulation of MAPKs in cells overexpressing hE5NT and hENTPD1. To this extent, the expression of total and phosphorylated Akt, ERK1/2, p38 proteins was assessed by western blot analyses on PIEC cell lines treated with 400 µM $H_2O_2$ for 2, 4 and 6 hours (Figure 9).

Phosphorylated Akt (p-Akt) showed a remarkable induction in pCX-DI-2A-transfected cells as compared to mock-transfected cells (CTRL) at 2, 4 and 6 hours of treatment (1.99±0.47 vs. 0.84±0.14, $p<0.05$; 2.03±0.14 vs. 0.89±0.15, $p<0.05$; 1.65±0.40 vs. 0.43±0.03, $p<0.05$), suggesting a higher amount of activated Akt in these cells (Figures 9A and D). Furthermore, after 2 and 4 hours of $H_2O_2$ exposure, the p-Akt expression level was also significantly greater as compared to both pCX-hE5NT- (0.73±0.004, $p<0.05$; 0.71±0.14, $p<0.05$) and pCX-hENTPD1-transfected cells (0.80±0.07, $p<0.05$; 0.78±0.09, $p<0.05$, Figures 9A and D). No significant differences in total protein expression levels were observed in each cell line over time.

Phosphorylated ERK1/2 (p-ERK1/2) showed a significant decrease in $H_2O_2$ treated mock-transfected cells (CTRL) as compared to untreated cells (UT, Figure 9B $p<0.05$); p-ERK1/2 expression levels in mock-transfected cells were significantly lower than those of all other PIEC cell lines in each condition tested (Figures 9B and E, $p<0.05$). After 2, 4 and 6 hours of $H_2O_2$ treatment, a great induction of p-ERK1/2 was observed in pCX-hE5NT- (1.58±0.007; 2.01±0.17; 2.43±0.13) and in pCX-hENTPD1-transfected cells (1.35±0.06; 1.51±0.03; 1.86±0.24).

Moreover, levels of p-ERK1/2 increased over time in both the single gene-transfected cell lines (Figures 9B and E, $p<0.05$). On the other hand, a weaker induction of ERK1/2 phosphorylation was noticed in pCX-DI-2A-transfected cells after 2 and 4 hours of $H_2O_2$ exposure (0.94±0.06 and 0.91±0.08, $p<0.05$) as compared to both pCX-hE5NT- and pCX-hENTPD1-transfected cells (Figures 9B and E). Total ERK1/2 protein amount was not different among the cell lines at each time point or between treated and untreated cells in each cell line (Figure 9B).

Phosphorylated form of p38 MAPK (p-p38) was almost totally abolished in $H_2O_2$-treated mock-transfected cells as compared to the untreated cells ($p<0.05$). Conversely, p-p38 expression levels underwent a significant increase in pCX-DI-2A-transfected cells as compared to mock-transfected cells after 2, 4 and 6 hours of $H_2O_2$ treatment (1.23±0.15 vs. 0.43±0.07, $p<0.05$; 1.21±0.08 vs. 0.50±0.02, $p<0.05$; 1.90±0.14 vs. 0.39±0.09, $p<0.05$; Figure 9F). Furthermore, after 6 hours of $H_2O_2$-exposure pCX-DI-2A-transfected cells showed a significant increase of p-p38 expression level as compared to both pCX-hE5NT- and pCX-hENTPD1-transfected cells (0.57±0.13, *$p<0.05$;* 0.80±0.08, *$p<0.05$;* respectively). Considering the change in p-p38 levels over time, pCX-DI-2A-transfected cells showed a significant increase by comparing 6 hours and 2 hours of $H_2O_2$ treatment (Figure 9F, $p<0.05$). On the other hand, p38 phosphorylation did not show relevant changes in pCX-hE5NT- and pCX-hENTPD1-transfected cells over time (0.90±0.10, 1.21±0.24, 0.57±0.13 and 0.96±0.09, 0.84±0.02, 0.80±0.08 respectively, Figure 9F). Total p38 protein did not show any

changes of expression level in each condition tested, as expected (Figure 9C).

Overall, these data showed that the MAPKs signaling pathway was induced in response to $H_2O_2$ in PIEC cells overexpressing at least one of the two human genes (ERK1/2 and p38) or both hE5NT and hENTPD1 (Akt), promoting beneficial effects that could counteract oxidative stress injury.

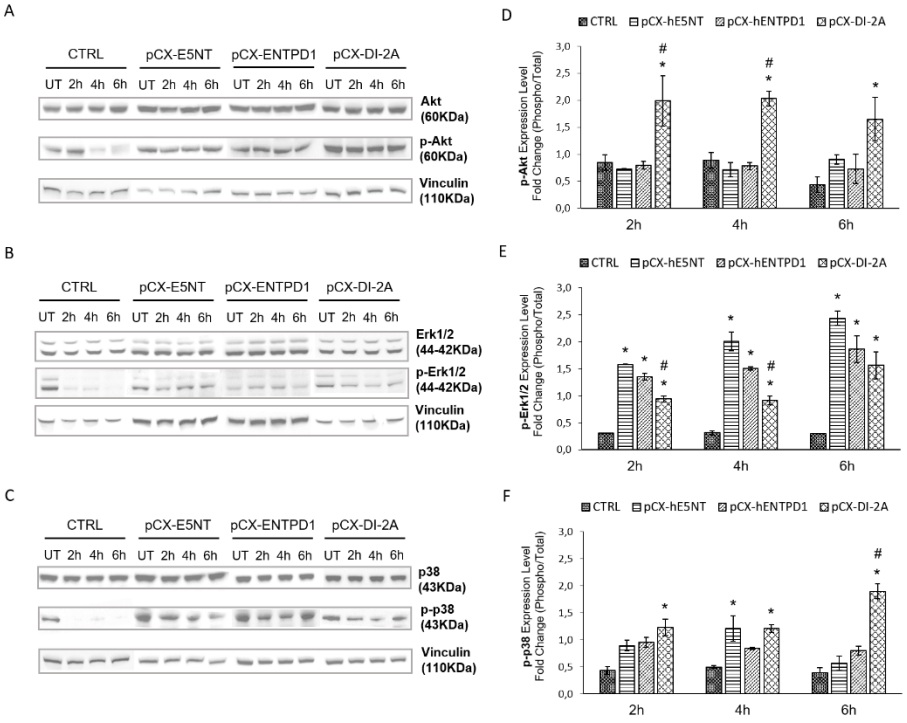



**Figure 9.** Mitogenic signaling pathways modulation in single gene- and pCX-DI-2A-transfected cells. (A-C) Western blot analysis of lysates from pCX-DI-2A-, pCX-hE5NT-, pCX-hENTPD1 and mock-transfected cells after 2, 4, 6 hours exposure to 400 μM $H_2O_2$: Akt and p-Akt (A), Erk1/2 and p-Erk1/2 (B), p38 and p-p38 (C). Immunoblots are representative of 3 independent experiments. (D-F) Densitometric analysis of protein expression levels in PIEC cell lines. Data are expressed as Phospho-/Total-protein expression fold change ratio: pAkt/Akt (D), pErk1-2/Erk1-2 (E), p-p38/p38 (F). Error bars represent SEM (n=3). * $p<0.05$ versus ctrl cells; # $p<0.05$ pCX-DI-2A-transfected cells versus single gene-transfected cells. CTRL, mock-transfected cells.

## 4. DISCUSSION

Xenotransplantation may solve the problem of shortage of organs available for allotransplantation [35]. Despite great advances in preventing the immediate mechanisms of xenorejection [36], there are remaining immune and inflammatory barriers that still need to be overcome, such as IRI, oxidative stress and hyper-activation of coagulation [9,37,38]. Therefore, multiple additional genetic modifications are required in pig donors in order to achieve a broader protection of xenografts.

E5NT and ENTPD1 are ectonucleotidases that regulate the extracellular concentration and signaling of adenine nucleotides and adenosine [3]. Several reports showed the anti-thrombotic and anti-inflammatory effects of ENTPD1, mainly by catabolizing ATP and ADP [39,40]. On the other hand, E5NT has been demonstrated to exert anti-inflammatory and cytoprotective action through its reaction product, adenosine [39]. We report here the effects of the simultaneous expression of hE5NT and hENTPD1 (Figure 1) in porcine endothelial cells exposed to oxidative stress. We used the F2A technology to link in frame the coding sequences of human E5NT and ENTPD1 (pCX-DI-2A, Figure 1A) because of the well-documented equal expression of genes provided by the F2A sequence [23]. As expected, we observed that pCX-DI-2A-transfected cells showed a simultaneous and comparable expression of both hE5NT and hENTPD1 proteins (Figures 1B-D) that were also correctly localized into plasma membrane (Figures 1E-F). Furthermore, the hE5NT/hENTPD1 co-expression system resulted in the synergic enzymatic activities of hE5NT and hENTPD1. That meant the simultaneous degradation of pro-inflammatory and pro-thrombotic

extracellular ATP and ADP along with the enhanced production of anti-inflammatory and immunosuppressive adenosine (Figure 2 and Supplementary Figure 2). Following incubation with ATP or ADP, only pCX-DI-2A-transfected cells showed a significant production of adenosine (Figures 2C, E). On the contrary, pCX-hENTPD1-transfected cells showed the highest accumulation of AMP, yet without a detectable production of adenosine (Figures 2B-E). The lower accumulation of AMP in pCX-DI-2A-transfected cells was associated with the observed production of adenosine (Figures 2B-E), as a result of the coordinated activity of hE5NT in catabolizing the hENTPD1-product AMP in adenosine. Following incubation with AMP, pCX-DI-2A-transfected cells showed a significant production of adenosine similarly to pCX-hE5NT-transfected cells (Figure 2F). Moreover, as we previously reported, we observed that mock-transfected cells showed very low endogenous enzymatic activity of both ectonucleotidases [13,14]. Taken together, these findings showed that pCX-DI-2A plasmid allows the efficient co-expression, correct subcellular localization and enzymatic activity of hE5NT and hENTPD1 in porcine endothelial cells.

Reactive oxygen species, such as hydroxyl radicals and superoxide anion ($O_2^{\bullet -}$) rapidly converted to hydrogen peroxide ($H_2O_2$) by superoxide dismutase (SOD), are generated during normal metabolic processes [41,42], and play crucial roles in cellular signaling cascades [43]. Since an imbalance between ROS production and antioxidant defense mechanisms (such as in case of IRI) induces oxidative stress and exacerbation of inflammation [33,44-46], we evaluated whether the co-expression of hE5NT and hENTPD1 could protect endothelial cells

in a model of $H_2O_2$-induced oxidative stress and cytotoxicity. A low concentration of ROS induces apoptotic cell death if not adequately scavenged [47]. In particular, $H_2O_2$-treatment results in the formation of mitochondrial permeability transition pores, a rapid decrease of the mitochondrial transmembrane potential and the release of cytochrome c, which leads in turn to the activation of effector caspase 3, therefore apoptosis [48]. The combined expression of the two ectonucleotidases, hE5NT and hENTPD1, protected pCX-DI-2A-transfected cells against $H_2O_2$-induced apoptosis (Figure 3). Conversely, the expression of single genes did not significantly reduce caspase 3/7 activation in pCX-hE5NT and pCX-hENTPD1-transfected cells, as compared to mock-transfected cells (Figure 3). Interestingly, pCX-hE5NT-transfected cells showed a trend of decreased activation of caspase 3/7 following 6 and 8 h of treatment with $H_2O_2$ as compared to mock-transfected cells (Figure 3).

On the other hand, high concentrations of $H_2O_2$ induce cell death mainly by necrosis [49]. We therefore evaluated the effect of the combined expression of hE5NT and hENTPD1 against the cellular cytotoxicity induced by a higher concentration of $H_2O_2$. Cytotoxicity results from Sytox incorporation assay pointed out a protective effect against oxidative cell injury in pCX-DI-2A- and pCX- hE5NT-transfected cells, but not in cells expressing only hENTPD1, after 4 and 6 hours of treatment (Figures 4B and E, 4C and F). Noteworthy, in pCX-DI-2A-transfected cells the beneficial effect was significantly increased as compared to both pCX-hE5NT- and pCX-hENTPD1-transfected cells (Figures 4B and E, 4C and F).

Overall, in apoptosis and cytotoxicity assays, pCX-DI-2A-transfected cells showed the highest level of protection as compared to all the other cell lines, whereas pCX-hE5NT-transfected cells showed only a partial protection. These findings suggest that hE5NT is necessary but not sufficient to confer a significant protection against the $H_2O_2$-induced cytotoxicity. In the attempt to investigate whether the combined enzymatic activity of hE5NT and hENPTD1 could be responsible for the observed protection in pCX-DI-2A-transfected cells, we measured the extracellular adenine nucleotides metabolism following exposure of cells to $H_2O_2$ (Figure 5). It is well documented that in conditions of inflammation or stress, ATP molecules are indeed actively released by cells in the extracellular milieu, where they act as paracrine danger signals [50,51]. The extracellular ATP is mainly modulated by the combined activities of ENTPD1 and E5NT. In our experimental settings, we observed that pCX-DI-2A-transfected cells efficiently catabolized toxic and pro-inflammatory ATP and ADP and simultaneously produced protective adenosine (Figures 5B, D and H). On the contrary, pCX-hE5NT-transfected cells showed the highest production of adenosine as compared to all the other cell lines (Figure 5H), but they also showed very low ATPase activity, leading to accumulation of ATP (Figure 5B) during $H_2O_2$ exposure. Nonetheless, we observed that the inhibition of adenosinergic receptors by pre-treatment with caffeine did not reverse the protective responses observed in pCX-DI-2A-transfected cells against the $H_2O_2$-mediated damage (data not shown). Therefore, despite the observed efficient combined activity of hE5NT and hENTPD1 during $H_2O_2$ treatment, the adenosine receptors activation seemed not to be primarily responsible

for the protection shown by pCX-DI-2A-transfected cells at least at the time points analyzed and so other protective mechanisms might be involved.

In order to elucidate these potential protective mechanisms, we then focused our attention on ROS formation. To this extent, we investigated the effects of the combination of hE5NT and hENTPD1 on the production of ROS following exposure to 100 and 400 µM $H_2O_2$. *In situ* analysis of PIEC cells exposed to hydrogen peroxide and stained with CellROX® Orange Reagent showed less production of ROS in pCX-DI-2A-transfected cells as compared to all the other cell lines (Figure 6 and Supplementary Figures 4-5). These data were then confirmed by the quantitative analysis of ROS production (Figure 7). On the other hand, the single expression of either hE5NT or hENTPD1 conferred significant protection only at the earliest time point (30 minutes, Figure 7A and Supplementary Figures 4 and 5), but neither of them was sufficiently protective at longer incubation times (Figure 7B-C).

Since we observed decreased ROS production in pCX-DI-2A-transfected cells following $H_2O_2$ treatment (Figures 6-7 and Supplementary Figures 4-5), we then evaluated the activities of $H_2O_2$ scavenging enzymes. The primary cellular enzymatic systems against hydrogen peroxide are the glutathione redox cycle and catalase [52]. Glutathione peroxidase converts $H_2O_2$ to $H_2O$ by oxidizing GSH to GSSG in both the cytosol and mitochondria [53]. On the other hand, catalase protects cells from the toxicity of $H_2O_2$ by converting it to $H_2O$ and $O_2$ and it is primarily present in the peroxisome fraction [53]. We measured the GPx activity in PIEC cells treated with $H_2O_2$ (Supplementary Figure 7). We did not detect relevant GPx activity and

any differences between all the cell lines at every time point of analysis (Supplementary Figure 7). In agreement with such findings, we also did not observe significant differences in the GSH/GSSG ratio among the cell lines following treatment with $H_2O_2$ (Supplementary Figure 6). Despite pCX-hENTPD1-transfected cells showed high basal GSH/GSSG ratio, we did not observe any further change following $H_2O_2$ exposure (Supplementary Figure 6). Further studies would be required to elucidate such behavior. Anyhow, these data suggest that the glutathione redox cycle was not primarily involved in the detoxification from $H_2O_2$ in our model.

On the other hand, we measured the enzymatic activity of catalase in PIEC cells exposed to $H_2O_2$ (Figure 8). Interestingly, we observed that pCX-DI-2A-transfected cells showed a higher catalase activity than all the other cell lines (Figure 8). We also detected that pCX-hE5NT-transfected cells showed a significantly higher catalase activity than mock- and pCX-hENTPD1-transfected cells at 240 and 360 minutes of $H_2O_2$ treatment (Figure 8). However, such activity was significantly lower than that one observed in pCX-DI-2A-transfected cells at each time point (Figure 8).

In accordance with such findings, the higher catalase activity correlated with the decreased formation of ROS observed in pCX-DI-2A-transfected cells (Figures 6-7 and Supplementary Figures 4-5).

It has been reported that the overexpression of human catalase protects hepatocytes from $H_2O_2$-induced cytotoxicity and apoptosis [52]. Similarly, the overexpression of catalase protects beta cells from the hydrogen peroxide- and streptozocin-mediated damage [54]. In agreement with such reports, pCX-DI-2A-transfected cells showed

higher activity of catalase (Figure 8) along with better protection from the $H_2O_2$-induced apoptosis and cytotoxicity (Figures 3-4) as compared to all the other cell lines. Furthermore, only the combined expression of hE5NT and hENTPD1 conferred enough and lasting protection to the cells against the oxidative cell damage. Moreover, these results suggest that the main intracellular ROS formation site would be the cytoplasm, where the catalase, which acts mainly at peroxisomes, is activated to mitigate the effects of hydrogen peroxide [53].

We also showed that the combination of hE5NT and hENTPD1 conferred protection against TNF-α-induced apoptosis (Supplementary Figure 3). During IRI, TNF-α is a very important mediator of the inflammatory and immune responses [55]. TNF-α is also a strong pro-apoptotic stimulus[56,57]. We previously demonstrated that the combination of hE5NT and hENTPD1 along with human heme oxygenase 1 protected fibroblasts against the TNF-α-mediated apoptosis and cytotoxicity[29]. Here, we reported that pCX-DI-2A-transfected cells showed significantly less caspase 3/7 activation as compared to mock- and pCX-hE5NT-transfected cells following TNF-α exposure (Supplementary Figure 3). Interestingly, we did not observe different caspase 3/7 activation between pCX-DI-2A- and pCX-hENTPD1-transfected cells, suggesting that the protection observed in pCX-DI-2A-transfected cells was mainly mediated by ENTPD1. Taken together, these data indicate that the combination of the two human ectonucleotidases might protect against multiple pro-apoptotic and oxidant effectors, which play a key role in the context of IRI.

We then aimed to investigate the molecular pathways of protection in pCX-DI-2A-transfected cells following exposure to $H_2O_2$. We

examined the effect of the co-expression of hE5NT and hENTPD1 on the activation of Akt, ERK1/2 and p38 kinases (Figure 9), which are known to exert protective roles against the oxidative stress and IRI [58-60]. As shown in Figures 9A and 9D, the increase of Akt phosphorylation was significant in pCX-DI-2A-transfected cells as compared to mock-transfected cells at every time points of $H_2O_2$ treatment and to single gene-transfected cells after 2 and 4 hours. Martin *et al* reported a connection between $H_2O_2$-induced cell death and down-regulation of p-Akt by mechanisms that require the generation of ROS [61]. Consistently with this, data from Cell Rox assay showed a great ROS production in mock-transfected cells treated with $H_2O_2$ and in pCX-hE5NT- and pCX-hENTPD1-transfected cells at late time points of treatment (Figures 6 and 7). The same cell lines underwent caspase 3/7 activation and cell death after 6 hours of $H_2O_2$ exposure (Figure 3). This effect may be due to the abundant formation of ROS, which may be involved in the downregulation of p-Akt, hence higher levels of apoptosis. On the other hand, pCX-DI-2A-transfected cells showed significantly enhanced enzymatic activity of catalase (Figure 8), which could efficiently counteract ROS generation (Figures 6, 7 and Supplementary Figures 4 and 5) as well as p-Akt down-regulation, thus resulting in the observed protection against apoptosis (Figure 3) and cytotoxicity (Figure 4).

A significant increase of phosphorylated ERK1/2 proteins was observed in pCX-E5NT-, pCX-hENTPD1- and pCX-DI-2A-transfected cells at every time point of $H_2O_2$ treatment as compared to mock-transfected cells (Figures 9B and 9E). However, pERK1/2 expression level was significantly lower in pCX-DI-2A-transfected

cells than in pCX-E5NT- and pCX-hENTPD1-transfected cells after 2 and 4 hours of $H_2O_2$ exposure (Figure 9E). Recently, it has been shown that $H_2O_2$ exerts its effect by acting on phosphorylation-dependent activation of ERK1/2 MAP kinases, without affecting its total expression [62-64]. Moreover, although ERK1/2 activity is generally associated with cell survival, a growing body of evidence suggests that it also mediates apoptosis cell death depending on the stimulus, the cell type and the subcellular localization. For instance, Song and colleagues showed that a sustained ERK1/2 activation causes its nuclear translocation and contributes to cell death via transcriptional regulation of pro-apoptotic proteins in neural cells [63]. In this perspective, the persistence of $H_2O_2$ stimulus may have induced a sustained activation of ERK1/2 protein that became "toxic" over time. This effect may cause an imbalance between pro- and anti-apoptotic signals transmitted by ERK1/2, leading cells towards injurious outcomes in single gene expressing cell lines. The observed enhanced detoxifying activity of catalase in pCX-DI-2A-transfected cells (Figure 8) may prevent an excessive $H_2O_2$-mediated ERK1/2 phosphorylation and activation (Figure 9E), leading to resistance against apoptosis and cytotoxicity (Figures 3-4). In fact, it has been documented that the injection of catalase mediated protection against brain IRI by abolishing ERK1/2 phosphorylation and promoting Akt phosphorylation [65].

Similarly to ERK1/2, $H_2O_2$ has been reported to induce phosphorylation of p38 MAPK and JNK [64], which have essential roles in the regulation of cellular responses, including cell survival and apoptosis [66]. Furthermore, activation of p38 has been reported to be involved in inducing the expression of antioxidant enzymes in human aortic

endothelial cells [67]. We therefore investigated if the phosphorylation of p38 MAPK (p-p38) could have been modulated in pCX-DI-2A-transfected cells (Figures 9C and 9F). p-p38 expression levels were significantly higher in pCX-DI-2A-transfected cells at every time points of treatment as compared to mock-transfected cells. No significant increase in p-p38 was observed in single gene-transfected cells, except for pCX-hE5NT-transfected cells after 4 hours of $H_2O_2$ exposure (Figure 9F). Furthermore, after 6 hours, pCX-DI-2A-transfected cells showed almost a 2-fold increase in p-p38/total p38 ratio, which was significantly higher than all the other cell types (Figure 9F). It has been reported that p38 MAPK activation upregulates catalase levels during $H_2O_2$ treatment [68]. Consistently with this, we observed both increased p38 MAPK phosphorylation (Figure 9F) and enhanced catalase activity in pCX-DI-2A-transfected cells (Figure 8), suggesting a correlation between these two mechanisms.

## 5. CONCLUSION

The co-expression of hE5NT and hENTPD1 in pigs has been suggested to mitigate the hyperactivation of coagulation and inflammation that are observed in xenotransplantation models [69]. Here we showed for the first time that this new combination of genes conferred protection against the $H_2O_2$-mediated oxidative stress and cytotoxicity in porcine endothelial cells.

Furthermore, our findings further clarified the connection between the combined activity of hE5NT/hENTPD1, catalase detoxifying activity and the MAPKs signaling pathways, suggesting possible mechanisms

involved in $H_2O_2$-mediated oxidative stress response in endothelial cells. In conclusion, our data add new insights to the protective effects of hE5NT and hENTPD1 against IRI and constitute a proof of concept for testing this new genetic combination in pig-to-non-human primates xenotransplantation models.

**Keywords**

ecto-5'-nucleotidase, ecto nucleoside triphosphate diphosphohydrolase 1**,** oxidative stress**,** xenotransplantation

**Highlights**

- human E5NT and ENTPD1 genes were correctly co-expressed in porcine endothelial cells;
- hE5NT/hENTPD1 co-expression led to enhanced production of adenosine;
- hE5NT/hENTPD1 co-expression protected cells against $H_2O_2$-induced cytotoxicity;
- hE5NT/hENTPD1 co-expression resulted in decreased ROS production;
- hE5NT/hENTPD1-transfected cells showed more activation of Akt, ERK1/2 and p38 kinases.

**Abbreviations**

E5NT or CD73, ecto-5'-nucleotidase; ENTPD1 or CD39, ecto nucleoside triphosphate diphosphohydrolase 1; ROS, reactive oxygen species; IRI, ischemia reperfusion injury; PE, phycoerythrin; PIECs, porcine iliac endothelial cells; WGA, wheat germ agglutinin; ERK1/2, extracellular signal-regulated kinases 1/2; MAPK, mitogen-activated protein kinase; CDS, coding sequence; EHNA, erythro-9-(2-hydroxy-3-nonyl) Adenine; CTRL, control.

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## Supplementary Material and Methods

### Dicistronic plasmid construction

An Eppendorf Mastercycler EP silver block thermocycler was used for synthesizing DNA by PCR reaction. Two rounds of recombinant PCR were performed to amplify hE5NT (NCBI: NM_002526.3) coding sequence (CDS). Firstly, hE5NT CDS was PCR-amplified from pCX-hE5NT (previously produced in our lab) without the stop codon by using EcoRI Kozak hE5NT forward and BamHI hE5NT reverse primers and the corresponding amplicon was cloned into pGEM T-easy vector (Promega). This intermediate vector was used as template for the second PCR, which was performed using AflII hE5NT forward and BamHI hE5NT reverse primers. The corresponding product was cloned into pGEM T-easy vector. Then, the entire CDS was excised by AflII/BamHI double digestion and cloned into AflII/BamHI-digested pcDNA3.1-F2A upstream of F2A sequence, obtaining pcDNA3.1-hE5NT-F2A. hENTPD1 (NCBI: NM_001776.5) CDS was PCR-amplified and then ligated in XhoI/XbaI-digested pcDNA3.1-hE5NT-F2A as previously described[1], obtaining pcDNA3.1-hE5NT-F2A-hENTPD1. The dicistronic cassette hE5NT-F2A-hENTPD1 was excised by EcoRI digestion and ligated in EcoRI-linearized pCX-C1 plasmid [2] acceptor obtaining pCX-hE5NT-F2A-hENTPD1, which was named pCX-DI-2A.

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## Appendix A. Supplementary material

**Fig. S1.** Expression analysis of single gene-transfected cells. (A) FACS analysis of hE5NT (PE) in pCX-hE5NT-transfected cells (black histogram); (B) FACS staining of hENTPD1 (APC) in pCX-hENTPD1-transfected cells (black histogram). Mock-transfected cells were used as negative control (grey histograms in A and B).

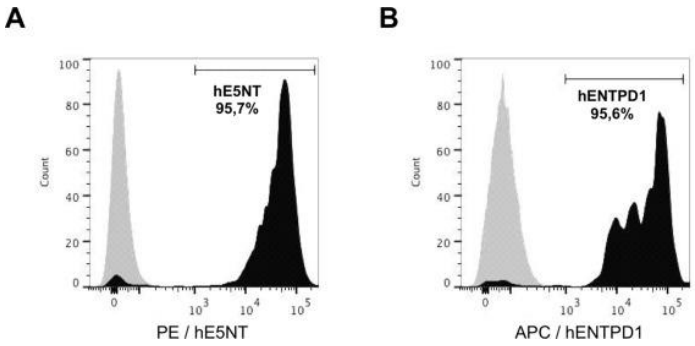



**Fig. S2.** Efficient catabolism of extracellular adenine nucleotides in pCX-DI-2A-transfected cells. pCX-DI-2A- (◊), pCX-hE5NT- (Δ), pCX-hENTPD1- (∇) and mock- (•) transfected cells were incubated with 50 μM ATP, or ADP, or AMP. The enzymatic degradation of ATP (A), ADP (B) and AMP (C) was measured over different time points by reverse phase HPLC. Error bars represent SEM (n=3). * $p<0.05$ versus all groups; # $p<0.05$ pCX-DI-2A-transfected cells versus all groups except pCX-hENTPD1-transfected cells; § $p<0.05$ pCX-DI-2A-transfected cells versus all groups except pCX-hE5NT-transfected cells.

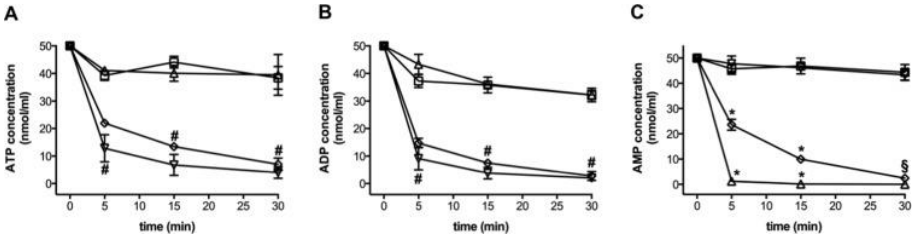



**Fig. S3.** Protection against TNF-α-induced apoptosis in pCX-DI-2A-transfected cells. pCX-DI-2A-, pCX-hE5NT-, pCX-hENTPD1- and mock-transfected (CTRL) cells were treated with 10 ng/ml human TNF-α for 8, 16 and 24 h and apoptosis was measured by caspase 3/7 assay. Caspase activation in each treated cell line was expressed as a fold change value of the corresponding untreated cells. Error bars represent SEM (n≥3). * $p<0.05$ versus CTRL cells; Φ $p<0.05$ versus pCX-hE5NT-transfected cells.

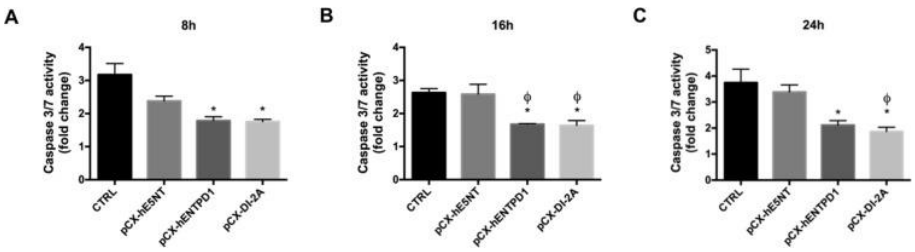


**Fig. S4.** ROS production following 100 µM $H_2O_2$ exposure. pCX-DI-2A-, pCX-hE5NT-, pCX-hENTPD1- and mock-transfected (CTRL) cells were treated with 100 µM $H_2O_2$ for 30 (A), 60 (B) and 90 (C) minutes and ROS levels were detected by immunofluorescence staining with CellROX® Orange Reagent. White arrows indicate cells positive to the staining (red). The bar represents 100 µm. DAPI (blue) was used for nuclear staining. Representative images are shown.

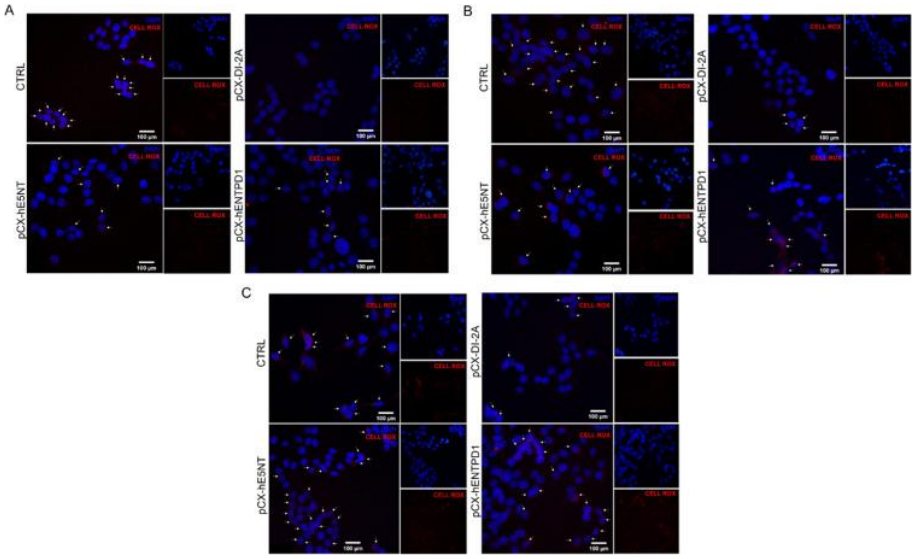



**Fig. S5.** ROS production following 400 µM $H_2O_2$ exposure. pCX-DI-2A-, pCX-hE5NT-, pCX-hENTPD1- and mock-transfected (CTRL) cells were treated with 400 µM $H_2O_2$ for 30 (A) and 60 (B) minutes and ROS levels were detected by immunofluorescence staining with CellROX® Orange Reagent. White arrows indicate cells positive to the staining (red). The bar represents 100 µm. DAPI (blue) was used for nuclear staining. Representative images are shown.

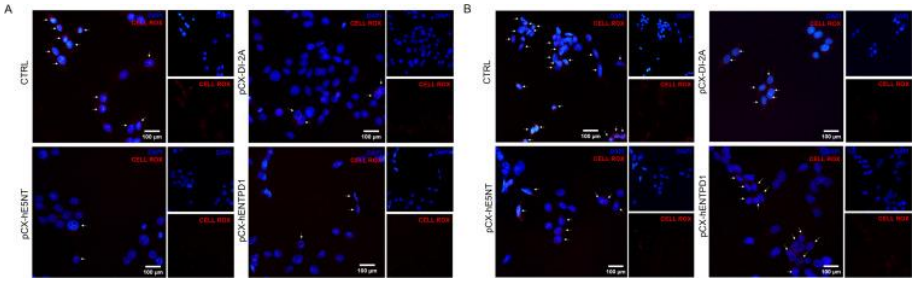


**Fig. S6.** Evaluation of GSH/GSSG ratio in PIEC cells treated with $H_2O_2$. pCX-DI-2A-, pCX-hE5NT-, pCX-hENTPD1- and mock-transfected (CTRL) cells were treated with 400 μM $H_2O_2$ for 30, 60 and 90 min and GSH/GSSG ratio was determined as described in Materials and Methods. Data are presented as mean ±SEM (n=3). * $p$<0.05 pCX-hENTPD1-transfected cells vs. all the other untreated cell lines. UT, untreated cells.

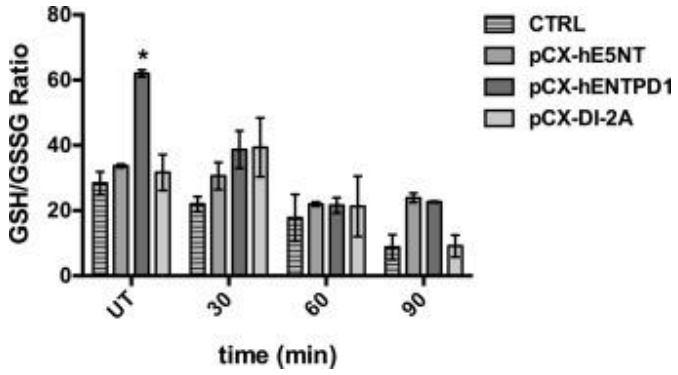
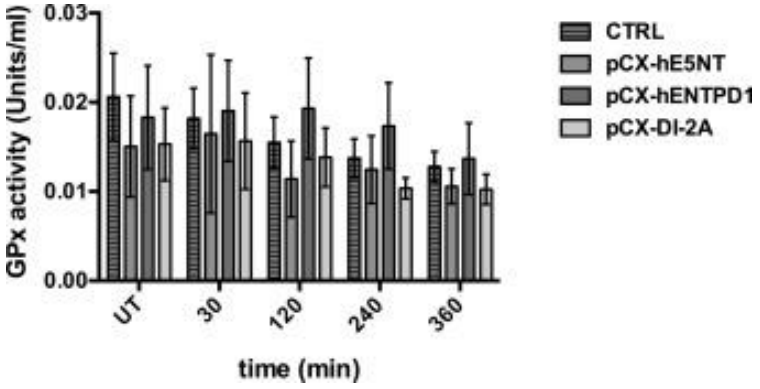



**Fig. S7.** Enzymatic activity of GPx in PIEC cells treated with $H_2O_2$. pCX-DI-2A-, pCX-hE5NT-, pCX-hENTPD1- and mock-transfected (CTRL) cells were treated with 400 μM $H_2O_2$ for 30, 120, 240 and 360 min and the GPx enzymatic activity was measured as described in Materials and Methods. The activity is expressed as units of enzyme per ml of reaction. Data are presented as mean ±SEM (n≥3). UT, untreated cells.



**Supplementary Table 1**

List of F2A sequence and primers used for recombinant PCR

| Name | Sequence (5'-3') | Tm |
|---|---|---|
| F2A-*sense* | GATCCGTGAAACAGACTTTGAATTTT GACCTTCTCAAGTTGGCGGGAGACG TGGAGTCCAACCCAGGGCCCGGCAG CGGCC | |
| F2A-*antisense* | TCGAGGCCGCTGCCGGGCCCTGGGT TGGACTCCACGTCTCCCGCCAACTTG AGAAGGTCAAAATTCAAAGTCTGTT TCACG | |
| EcoRI Kozak hE5NT fw<br>BamHI hE5NT rev | GAATTCAGGATGTGTCCCCGAGCCG C<br>GGATCCTTGGTATAAAACAAAGATC | 60°C |
| AflII hE5NT fw<br>BamHI hE5NT rev | CTTAAGGAATTCAGGATGTGTCCCC G<br>GGATCCTTGGTATAAAACAAAGATC | 60°C |
| XhoI hENTPD1 fw<br>EcoRI hENTPD1 rev | CTCGAGATGGAAGATACAAAGGAGT CTAACG<br>GAATTCCTATACCATATCTTTCCAGA AATATGAAG | 60°C |
| hENTPD1 fw<br>XbaI hENTPD1 rev | CTCGAGATGGAAGATACAAAGG<br>TCTAGAGAATTCCTATACCATATCTT TCCAG | 59°C |

*CHAPTER 5*

# Conclusion and Future Perspectives

## Summary

Oxidative stress is a major and recurring cause of damage during inflammation, as following ischemia-reperfusion injury (IRI) in organ transplantation. Increasing evidences report purinergic signaling as a key pathway in inflammation and IRI settings. Indeed, following IRI, extracellular ATP and ADP accumulate in the sites of injury leading to pro-inflammatory and pro-thrombotic responses on vascular and immune cells. The extracellular levels of adenine nucleotides are mainly modulated by the ectonucleoside triphosphate diphosphohydrolase 1 (ENTPD1) and ecto-5-nucleotidase (E5NT), whose combined enzymatic activities convert ATP into adenosine, an anti-inflammatory molecule. On the other hand, heme oxygenase-1 (HO-1) gained much attention in the context of IRI and transplantation because of its role in down-regulating inflammatory response and apoptosis and the well-known vasodilatory, anti-oxidant, and anti-inflammatory properties of its catabolites, carbon monoxide and bilirubin.Several biomedical applications, such as xenotransplantation, require multiple genetic modifications in eukaryotic cells to ensure a successful outcome. Advances in genetic engineering technologies have led to the development of efficient polycistronic vectors based on the use of the 2A self-processing oligopeptide.

We developed an F2A-based multicistronic system to evaluate functional effects of co-expression of the three anti-inflammatory proteins, HO-1, E5NT and ENTPD1. The novel tricistronic p2A plasmid we designed and produced was able to efficiently drive simultaneous expression of the three genes in HEK293T cells. All the exogenously overexpressed proteins possessed relevant enzymatic

activities and the tricistronic p2A construct resulted to be effective and optimal to test the combined protective effects of HO1, E5NT and ENTPD1 against inflammation and oxidative stress injury (De Giorgi M., *et al*., Plasmid, 2015). Thus, we evaluated the potential protection mediated by this new combination of genes in NIH3T3 cells exposed to tumor necrosis factor alpha (TNF-α), as a key pro-inflammatory cytokine involved in the inflammatory response during IRI. NIH3T3 cells stably transfected with an updated tricistronic plasmid (pCX-TRI-2A) showed a synergic cytoprotective effect against TNF-α-induced injury and cell death and this protection may be at least in part explained by the induction of a pro-survival phenotype in these cells (Cinti A., *et al*., PLoS ONE, 2015). Since encouraging results were obtained in our *in vitro* model, the triple cistronic cassette was used for the production of a multi-gene transgenic mouse model for the over-expression of hHO1, hE5NT and hENTPD1. Three novel transgenic mouse strains were produced and the proper enzymatic activity of the two ectonucleotidases was observed in different tissues of at least two of the three mouse lines. We furthermore investigated whether the co-expression of the two main vascular ectonucleotidases, might protect against $H_2O_2$-induced oxidative stress *in vitro*. To this purpose, we assembled a dicistronic plasmid (pCX-DI-2A) for the co-expression of hE5NT and hENTPD1. pCX-DI-2A-transfected porcine iliac endothelial cells (PIEC) simultaneously overexpressed and correctly processed the two human proteins and such co-expression system led to the synergistic enzymatic activity of hE5NT and hENTPD1 and a concomitant reduction of $H_2O_2$-induced ROS production, cytotoxicity and apoptosis. Interestingly, we showed that the cytoprotective

phenotype observed in pCX-DI-2A-transfected cells was associated with higher detoxifying activity of catalase as well as increased activation of the survival signaling molecules Akt, ERK1/2 and p38 MAPKs (Chisci E., *et al*., Free Rad Biol Med, 2017).

Overall, our work add new insights to the understanding of the anti-inflammatory effect and the ability to counteract oxidative stress mediated by a novel combination of the three human genes and constitute a proof of concept for testing this new genetic combination in pre-clinical models relevant for the study of Ischemia-Reperfusion Injury and inflammatory-based diseases, as transplantation and atherosclerosis.

## Conclusion

**Development of multicistronic constructs to simultaneous over-expressed HO-1, CD39 and CD73.**

To date, polycistronic constructs are employed extensively as multi-gene co-expression strategy in eukaryotic systems both *in vitro* and *in vivo*. The applications in the biomedical field are many, such as the co-expression of multiple transcription factors, in order to generate induced Pluripotent Stem cells (iPSCs) starting from somatic cells [1,2], produce relevant experimental models of diseases or induce a protective effect against a specific pathological state mediated by the simultaneous over-expression of relevant enzymes. For instance, construction of efficient multicistronic vectors containing three to four graft-protective genes, as human CD55, thrombomodulin, CD39 and CTLA4-Ig have been used to

test whether this combination might have a beneficial effect against xenotransplantation rejection mechanisms [3].

For the purpose of xenotransplantation, it has been estimated that at least 10 genetic modifications are required in order to achieve acceptable protection and clinical success of the xenograft [4] making it necessary to test different combinations of protective genes in relevant pre-clinical models. Production of genetically modified pigs for xenotransplantation might adequately represent the key step to solve the chronic gap between demand and availability of organs for transplantation. Several transgenic pigs overexpressing a combination of human genes have been produced and their organs have shown to be protected against hyperacute [5] and acute [6] xenograft rejection mechanisms.

Among different multi-gene expression strategies developed, F2A sequence from the foot-and-mouth disease virus (FMDV) is a well-established method for co-expressing multiple proteins starting from a single open reading frame [7]. It has been proposed as the tool to solve the issue of combining multiple genetic modifications in animal genome, to be used as model of disease, substituting the canonical breeding strategies, which are time consuming and expensive [4]. Indeed, 2A peptide provide a viable and more reliable and easier to use alternative to the IRES in achieving multiple genetic modifications. It fulfills all the functions IRES sequences are used for but, at the same time, it provides the advantage of reliable and almost stoichiometric levels of expression, a particularly useful feature when an accurate proportion of the expression levels of two or more proteins is fundamental.

Ischemia and reperfusion injury (IRI) contributes to pathology in a wide range of conditions, compromising the functional recovery of patients that experience transient disruption of blood perfusion to a single tissue or multiple organs, either as a consequence of a medical/surgical procedure (e.g., organ transplantation) or in response to a disease process (e.g., acute kidney injury) [8,9]. Moreover, exposure of a single organ to ischemia and reperfusion may subsequently cause inflammatory activation in other organs, eventually leading to multiorgan failure [4].

The fact that a wide range of pathological processes contribute to ischemia and reperfusion-associated tissue injury makes this condition much more complex to be both understood and treated with effective therapies. Indeed, limited oxygen availability (hypoxia), as occurs during the ischemic period, is associated with both impaired endothelial cell barrier function, consisting in an increase in vascular permeability and leakage [10], and significant alterations in the transcriptional control of gene expression, leading to the post-translational activation of hypoxia and inflammatory signaling cascades [11]. Cell death programs, including necrosis, apoptosis and autophagy-associated cell death are also activated following I/R [12], as well as innate, adaptive and autoimmune responses and subsequent activation of the complement cascade [13].

Therapeutic approaches to induce ischemic tolerance in order to make organs more resistant to ischemia could be used both in a preventive manner during organ transplantation or other types of major surgery associated with IRI, and after ischemic injury. Oxygen-dependent signaling pathways [14] and purinergic signaling [15,16] have been

studied as molecular pathways with a crucial role in down-regulating inflammatory and vascular response associated with IRI, in order to develop pharmacological approaches that would mimic ischemic preconditioning. Among several proteins involved in these signaling pathways, Heme Oxygenase-1 (HO-1), CD73 (Ecto- 5'-nucleotidase, E5'N) and CD39 (Ecto nucleoside triphosphate diphosphorylase 1, ENTPDase) gained much interest in the context of ischemia reperfusion processes. Indeed, numerous studies report that they are involved in the down-regulation of all the inflammatory events associated with vascular ischemic injury and xenograft rejection [6,15,17-20], such as ROS accumulation, endothelial cells activation, up-regulated expression of adhesion molecules, platelet adhesion, and ultimately thrombosis, extended intravascular coagulation followed by apoptotic cells death, suggesting these molecules as potentially protective against different pathological inflammatory-based conditions, as I/R injury. Although the protective role for each of these genes has been demonstrated *in vitro*, *ex-vivo* or in animal models [6,19,20], no one has investigated the combination of their beneficial effects in the attempt to reduce the inflammatory response.

The aim of this thesis work was to test the novel combined over-expression of the two ectonucleotidases, CD39 and CD73, with HO-1 enzyme, against the detrimental effects mediated by pro-inflammatory and oxidative stress stimuli, by mean of multicistronic constructs based on F2A technology.

HO1 is a stress inducible enzyme that catabolizes heme group to carbon monoxide (CO), free iron and biliverdin, which have antioxidant, anti-

inflammatory and anti-apoptotic effects [21]. HO-1 have been reported to have several beneficial effects, among which the inhibition of pro-inflammatory genes expression associated with endothelial cells activation [22]. Moreover, carbon monoxide generated from HO-1 enzymatic activity was proposed by Akamatsu *et al* to mediate protection against ischemia-reperfusion injury associated to heart transplant [23]. On the other hand, ENTPD1 and E5NT are ectonucleotidases involved in the modulation of purinergic signaling by the conversion of extracellular pro-inflammatory ATP/ADP to AMP, and AMP to anti-inflammatory and immunosuppressive adenosine, respectively [24]. The protective role for each of these genes alone has been previously demonstrated in different xenotransplantation [6,22,25] and ischemia reperfusion models [26-28].

In order to test the feasibility of co-expressing HO1, ENTPD1 and E5NT we first developed two versions of F2A-based multicistronic constructs and we analyzed their functionality in an easily transfectable cell line, HEK293T. Both the triple cistronic constructs contained the three genes linked in frame by two 2A sequences in a single Open Reading Frame (ORF). It has to be considered that we paid particular attention to the order of the genes CDS to maximize the likelihood of the correct processing and maturation of each protein product.

HO1 has a cytoplasmic localization [29] while active E5NT and ENTPD1 are localized at the plasma membrane [30,31]. Moreover, E5NT encodes a N-terminal signal peptide, which directs it through the endoplasmic reticulum before reaching the plasma membrane [32]. Thus, in order to avoid the phenomenon of “slipstream translocation”, in which an intracellular protein encoded downstream of a protein

containing a signal sequence can be translocated into the ER [33] and to maximize the likelihood that each single protein product would be able to correctly reach its functional subcellular compartment, we decided to place HO1 as a first gene, followed by CD73 and CD39, respectively as second and third gene of multi-gene coding sequence. We speculated that HO1 should be translated by free polysomes and then released in the cytosol by means of 2A mechanism of action, while ribosomes should proceed through the polycistronic mRNA and translate the N-terminal signal peptide of CD73 allowing its recognition and the formation of a traslocon, by which both CD73 and CD39 should be directed to the secretory pathway.

Moreover, a furin recognition site (RAKR) followed by a spacer (SGSG) was added upstream of each F2A sequence (FurF2A1 and FurF2A2) of one of the two triple cistronic constructs, in an attempt to reduce the potential negative effect of the C-terminal F2A tail on HO1 and E5NT expression and activity [34].

We showed that p2A not only allowed the correct expression of the three proteins that were differentially processed and properly subcellular localized but, more importantly, these proteins were found to be functional as demonstrated by the increase of Heme Oxygenase 1 and both Ectonucletotidases products in their respective activity assays. Therefore, by inducing the combined action of these three enzymes, the catabolism of pro-inflammatory heme and ATP/ADP may be enhanced together with the production of anti-inflammatory products. Overall, we observed less efficient protein expression levels together with more uncleaved product and decreased activities in pFur2A-transfected cells as compared to p2A-tranfected cells, suggesting that the addition of

furin cleavage sites and spacers in pFur2A may interfere with the F2A-mediated cleavage.

Even though an increase in expression levels derived by the addition of furin recognition site and spacers was reported [35], we did not obtain the same outcomes in our system. Verrier *et al*. showed that the addition of a furin recognition site upstream of 2A sequence in their GFP-2A-mCher plasmid interfered with 2A cleavage and suggested that the inclusion of an additional amino acid spacer between the furin and 2A siteswould improve cleavage efficiency of exogenous proteins [36]. However, the addition of both furin recognition site and spacer upstream of F2A sequences in our system resulted again in an inefficient production of our transgenes.

Taken together, our analyses indicated that p2A results in a more efficient expression of HO1, E5NT and ENTPD1 than pFur2A.

**Co-expression of at least two or three human genes protects against pro-inflammatory and oxidative stress stimuli *in vitro*.**

The novel tricistronic p2A plasmid we designed and produced was able to efficiently drive simultaneous expression of the three genes in HEK293T cells, thus resulting to be effective and optimal to test the combined protective effects of HO1, E5NT and ENTPD1 in the down-regulation of pro-inflammatory response.

First, in order to obtain a stable and unsilenced expression in eukaryotic cells, we moved the sequence encoding for hHO1, hE5NT and hENTPD1 proteins under the control of the CAGGS promoter, producing the tricistronic pCX-TRI-2A plasmid. WT, empty- (mock-) and single gene-expressing vectors have been produced as controls and

NIH3T3 cells have been transfected to investigate the contribution of each gene in the downregulation of the inflammatory response. The enzymatic assays demonstrated that the three proteins encoded by all the single-gene and the multi-cistronic constructs were fully functional and allowed the increased production of their enzymatic products. Furthermore, the functional assays performed to verify the correct enzymatic activity of protein products, as a result of their correct processing, and test their supposed protective roles against inflammation stimuli, demonstrated the relevant reduction of cell cytotoxicity, apoptotic signaling and death occurring after exposure to tumor necrosis factor alpha (TNF-α) in cells simultaneously expressing hHO-1, hCD73 and hCD39.

Among all the known physiological inducers of inflammation processes and apoptosis in mammalian cells, TNF-α is perhaps the most potent and well studied [37]. TNF-α elicits its pro-inflammatory signals by initially binding to receptors, TNFR1 and TNFR2, on the cell surface. TNFR1 and TNFR2 stimulate the cellular response to TNF-α via three principal and distinct signalling pathways, leading to the activation of AP-1 and NFκB transcription factors or the activation of caspases and cell death. This means that by activating the same set of receptors, biological effects as diverse as proliferation, cell survival or cell death can be obtained depending on the quantitative balance between the pro- and anti-apoptotic signals. To better evaluate the enzymatic activity of the three human proteins on cell cytotoxicity in response to TNF-α pro-inflammatory stimuli, all the NIH3T3 transfected cell lines were treated with appropriate molecules that served as substrate for HO-1 or the CD39/CD73 system. Hemin was used as HO-1 enzymatic activity

substrate, since it is a porphyrin containing iron which is catabolized by HO-1, as it occurs for the heme in an *in vivo* context [38]. On the other hand, ATP was administered as a substrate of CD39 and CD73 proteins, to induce their combined enzymatic activity that consists in converting ATP to adenosine as a final product [39]. Considering that caspases play an important role in TNF-α-induced apoptotic cell death [38] and that TNF-α-mediated apoptotic signalling finally results in effector caspase-3/7 activation and cell death execution [40], we investigated caspase-3/7 activity in pCX-TRI-2A transfected and control cell types after exposure to inflammatory stimuli (TNF-α) alone or in combination with enzymatic substrates of human genes (hemin and/or ATP). The combined expression of the three genes, better than the expression of each single gene, protected NIH3T3 cells against TNF-α induced cytotoxicity and apoptosis even in absence of enzymatic substrate of human genes but it was more effective when substrates were administered. Interestingly, the expression of single genes appeared to be somehow protective against TNF-α-induced cytotoxicity only if appropriate enzymatic substrate was administered to cells. Moreover, we found that only cells overexpressing HO-1 were protected from TNF-α-induced apoptosis, suggesting that the anti-apoptotic effect was mediated mainly by HO-1. Overall, these findings suggest that the simultaneous presence and activity of the three enzymes is necessary to further improve a persistent protection against TNF-α injury as compared to the effects induced by the expression of each gene individually.

Most is known about the mechanism by which TNF-α activates NF-kB. NF-kB pathway involves the interaction of TNF-α with its receptor at

the cell surface (TNFR), which then recruits a cascade of proteins, resulting in the translocation of NF-kB to the nucleus. In the nucleus it binds to its consensus sequence (5-GGGACTTTC-3) and positively regulates the transcription of genes involved in immune and inflammatory responses, cell growth control and apoptosis [41]. Thus, we decided to investigated the molecular mechanisms involved in TNF-α pathway, in order to better understand how the combined activity of the two systems represented by hHO-1 and hCD39/hCD73 axis was able to protect pCX-TRI-2A-transfected cells against TNF-α-mediated injury. To this extent, TNF-α pathway-related genes were analyzed by $RT^2$ array. Among the several genes resulted to be differentially modulated between WT and pCX-TRI-2A-transfected cells, we focused our attention on *Ikbkg/Nemo* and the modulation of this gene was then further investigated by single real-time PCR experiments.

Upon TNFR1 activation by TNF-α, the so-called complex I is formed and it is responsible for the downstream activation of the transcriptional activation of NF-kB through the regulatory subunit of the IKK complex, Nemo. In this context, Nf-kB promotes pro-survival signaling within the cells. Only if the injurious stimulus becomes persistent, prosurvival signaling pathways switch to pro-death signaling by evolution of complex I into the so-called complex II [40].

The gene expression analysis of *Nemo* showed that its expression was upregulated only in pCX-TRI-2A-transfected cells and that its modulation could be dependent on ATP, but not hemin, administration to cells. This behaviour of the *Nemo* regulation in presence of hemin needs further investigations, although it can be speculated a protective effect mediated by HO-1 activity that resulted in abrogating the cell's

need of *Nemo*'s up-regulation. In summary, pCX-TRI-2A-transfected cells up-regulated *Nemo*, which in turn promotes pro-survival effects of Nf-kB, in response to TNF-α injury when ATP is added to the medium, resulting in a protection against TNF-α induced cell death. Thus, the gene expression analyses suggested that the three over-expressed genes have a role in modulating key downstream regulators of TNF-α signalling pathway, such as *Nemo*, that promoted a pro-survival phenotype in TNF-α injured cells.

Reactive oxygen species, such as hydroxyl radicals and superoxide anion ($O_2^{\bullet-}$) rapidly converted to hydrogen peroxide ($H_2O_2$) by superoxide dismutase (SOD), are generated during normal metabolic processes [42,43], and play crucial roles in cellular signaling cascades [44]. An imbalance between ROS production and antioxidant defense mechanisms, such as in case of IRI, induces oxidative stress and exacerbation of inflammation [9,45,46], It has been reported that oxidative stress significantly impairs the activity of ectonucleotidases with the consequent loss of their protective functions [47,48] and that such altered extracellular nucleotide metabolism may constitute a feedback mechanism of increased ROS production because of the ATP-induced oxidative stress response in leukocytes [49].

Given the reported important role of purinergic signaling in conditions of inflammation and ischemia reperfusion injury (IRI) that occur in transplantation settings [50,51], and the reported protection mediated by CD39 and CD73 in this context [18,20,26], we aimed to evaluate whether the co-expression of the two ectonucleotidases could protect endothelial cells in a model of $H_2O_2$-induced oxidative stress and

cytotoxicity, mimicking what happens during IRI. A previous work in our laboratory showed the feasibility of the F2A-mediated co-expression of CD39 and CD73 in terms of their synergic enzymatic activities [52] and here, we used the F2A technology to link in frame the coding sequences of human CD73 and CD39 and expressed such dicistronic cassette under the control of CAGGS in a vector named pCX-DI-2A. As expected, we observed that pCX-DI-2A-transfected cells showed a simultaneous and comparable expression of both hCD73 and hCD39 proteins that were also correctly localized into plasma membrane where they exert their synergic enzymatic activities by converting pro-inflammatory and pro-thrombotic extracellular ATP and ADP to anti-inflammatory and immunosuppressive adenosine. In addition, the functionality of single gene-expressing plasmids was confirmed, since the accumulation of the corresponding enzymatic products were observed (AMP and adenosine in pCX-hENTPD1- and pCX-hE5NT-transfected cells, respectively).

A low concentration of ROS induces apoptotic cell death if not adequately scavenged [53]. In particular, $H_2O_2$-treatment results in the formation of mitochondrial permeability transition pores, a rapid decrease of the mitochondrial transmembrane potential and the release of cytochrome c, which leads in turn to the activation of effector caspase 3, therefore apoptosis [54]. On the other hand, high concentrations of $H_2O_2$ induce cell death mainly by necrosis [55]. Thus, the effect of the combined expression of hCD73 and hCD39 against the cellular apoptosis and cytotoxicity induced by lower or higher, respectively, concentrations of $H_2O_2$. Overall, in apoptosis and cytotoxicity assays, pCX-DI-2A-transfected cells showed the highest level of protection as

compared to all the other cell lines, whereas pCX-hE5NT-transfected cells showed only a partial protection against $H_2O_2$-induced cytotoxicity, suggesting that this molecule is necessary but not sufficient to confer a significant protection.

Even though we demonstrated the synergic activity of hCD39 and hCD73, we measured the extracellular adenine nucleotides metabolism also following exposure of cells to $H_2O_2$, since it is well documented that in conditions of inflammation or stress, ATP molecules are indeed actively released by cells in the extracellular milieu, where they act as paracrine danger signals [56,57]. We observed that pCX-DI-2A-transfected cells efficiently catabolized toxic and pro-inflammatory ATP and ADP and simultaneously produced protective adenosine, while even if pCX-hE5NT-transfected cells showed the highest production of adenosine as compared to all the other cell lines, they also showed very low ATPase activity, leading to accumulation of ATP during $H_2O_2$ exposure.

Specific assays of oxidative stress, targeted to elucidate which protective mechanisms might be responsible for the protection shown by pCX-DI-2A-transfected cells, were performed after exposure to $H_2O_2$. Overall, we found that pCX-DI-2A-transfected cells showed less production of ROS as compared to all the other cell lines, and that this decreased formation of ROS correlated with the higher catalase activity. It has been reported that the overexpression of human catalase protects hepatocytes from $H_2O_2$-induced cytotoxicity and apoptosis [58]. Similarly, the overexpression of catalase protects beta cells from the hydrogen peroxide- and streptozocin-mediated damage [59]. In agreement with such reports, pCX-DI-2A-transfected cells showed

higher activity of catalase along with better protection from the $H_2O_2$-induced apoptosis and cytotoxicity as compared to all the other cell lines. Furthermore, only the combined expression of hCD73 and hCD39 conferred enough and lasting protection to the cells against the oxidative cell damage. Moreover, these results suggest that the main intracellular ROS formation site would be the cytoplasm, where the catalase, which acts mainly at peroxisomes, is activated to mitigate the effects of hydrogen peroxide [60].

Finally, activation of molecular pathways, such as Akt, ERK1/2 and p38 kinases, which are known to exert protective roles against the oxidative stress and IRI [61-63], were also investigated the in pCX-DI-2A-transfected cells following exposure to $H_2O_2$. First, we observed an increase in Akt phosphorylation in pCX-DI-2A-transfected cells as compared to all the other cell lines at almost every time points of $H_2O_2$ treatment. A connection between $H_2O_2$-induced cell death and down-regulation of p-Akt by mechanisms that require the generation of ROS has been reported [64]. Consistently with such finding, data from Cell Rox assay showed a great ROS production in mock-transfected cells treated with $H_2O_2$ and in single gene-transfected cells at late time points of treatment and the same cell lines underwent caspase 3/7 activation and cell death after $H_2O_2$ exposure. Thus, we can speculate that this effect may be due to the abundant formation of ROS, which may be involved in the downregulation of p-Akt, hence higher levels of apoptosis. On contrast, pCX-DI-2A-transfected cells showed significantly enhanced enzymatic activity of catalase, which could efficiently counteract ROS generation, as well as p-Akt down-

regulation, thus resulting in the observed protection against apoptosis and cytotoxicity.

Recently, it has been shown that $H_2O_2$ exerts its effect by acting on phosphorylation-dependent activation of ERK1/2 MAP kinases, without affecting its total expression [65-67]. Moreover, although ERK1/2 activity is generally associated with cell survival, a growing body of evidence suggests that it also mediates apoptosis cell death depending on the stimulus, the cell type and the subcellular localization. For instance, Song and colleagues showed that a sustained ERK1/2 activation causes its nuclear translocation and contributes to cell death via transcriptional regulation of pro-apoptotic proteins in neural cells [66]. Based on these findings and our results, we speculated that the persistence of $H_2O_2$ stimulus may have induced a sustained activation of ERK1/2 protein that became "toxic" over time. This effect may cause an imbalance between pro- and anti-apoptotic signals transmitted by ERK1/2, leading cells towards injurious outcomes in single gene expressing cell lines. On contrast, the observed enhanced detoxifying activity of catalase in pCX-DI-2A-transfected cells may prevent an excessive $H_2O_2$-mediated ERK1/2 phosphorylation and activation, leading to resistance against apoptosis and cytotoxicity. In fact, it has been documented that the injection of catalase mediated protection against brain IRI by abolishing ERK1/2 phosphorylation and promoting Akt phosphorylation [68].

Similarly to ERK1/2, $H_2O_2$ has been reported to induce phosphorylation of p38 MAPK and JNK [64], which have essential roles in the regulation of cellular responses, including cell survival and apoptosis [69]. Furthermore, activation of p38 has been reported to be involved

in inducing the expression of antioxidant enzymes in human aortic endothelial cells [70]. The higher p-p38 expression levels in pCX-DI-2A-transfected cells than all the other cell types, showed after 6 hours of $H_2O_2$ treatment, is consistent with the report that p38 MAPK activation upregulates catalase levels during $H_2O_2$ treatment [71]. Indeed, we observed both increased p38 MAPK phosphorylation and enhanced catalase activity in these cells, suggesting a correlation between these two mechanisms.

In summary, this study demonstrated the protection against $H_2O_2$-induced apoptosis and cytotoxicity, mediated by activation of Akt, extracellular signal-regulated kinases 1/2 (ERK1/2) and p38 Mitogen-Activated Protein Kinase (MAPK) along with an increased catalase-mediated $H_2O_2$ scavenging activity and decreased production of ROS in endothelial cells simultaneously overexpressing CD39 and CD73.

## Future perspectives and translational relevance

Our study demonstrated, for the first time, the protection against inflammatory and oxidative stress stimuli of a combination of three human genes simultaneously expressed via F2A peptide-based multicistronic constructs in *in vitro* models. Since encouraging results were obtained in our *in vitro* model, the triple cistronic cassette was used for the production of a multi-gene transgenic mouse model for the over-expression of hHO1, hCD73 and hCD39. Three novel transgenic mouse strains were produced by applying the DNA pronuclear microinjection strategy in a single experiment of transgenesis, thus with the advantage to save time, resources and animals number. These

mouse strains showed a proper enzymatic activity of the two ectonucleotidases in different tissues, especially heart tissue and liver, as *ex vivo* nucleotides analyses on these tissues pointed out a significant catabolism of ATP substrate into adenosine mediated by CD39/CD73 enzymatic axis. Future plans will include the genetic characterization of the three mouse strains and the evaluation of the transgene expression at RNA and protein levels. Afterwards, we will evaluate the beneficial effect of the combination of human genes against inflammation and oxidative stress processes in an *in vivo* setting of I/R injury, aiming to prove their synergistic protective effect, confirming our previous *in vitro* results.

Furthermore, to exploit the potential of multi-cistronic transgenes for the genetic modification of cell and mammalian animal genome, we also designed and developed a conditional construct based on site-specific gene editing of the murine *Rosa26* locus by mean of a novel and powerful genome editing technique, as the CRISPR/Cas9 system (Figure 7). This conditional cassette achieves not only a simultaneous but also a conditional, Cre/loxP-mediated, expression of the three human genes (Figure 7A). To this purpose, different 20-bp target sequences have been selected, synthetized and cloned into a plasmid encoding for both a nickase form of Cas9 nuclease and the sgRNA scaffold (pSpCas9(BB), Addgene; figure 7B). Moreover, a plasmid-based donor repair template has been obtained by subcloning the triple cistronic cassette into a vector containing the homology arms flanking the target integration site into the first intron of murine *Rosa26* locus, since this specific region has been chosen as site of integration of the transgene (pGEM-floxedEGFP-TRI2A, figure 7C).

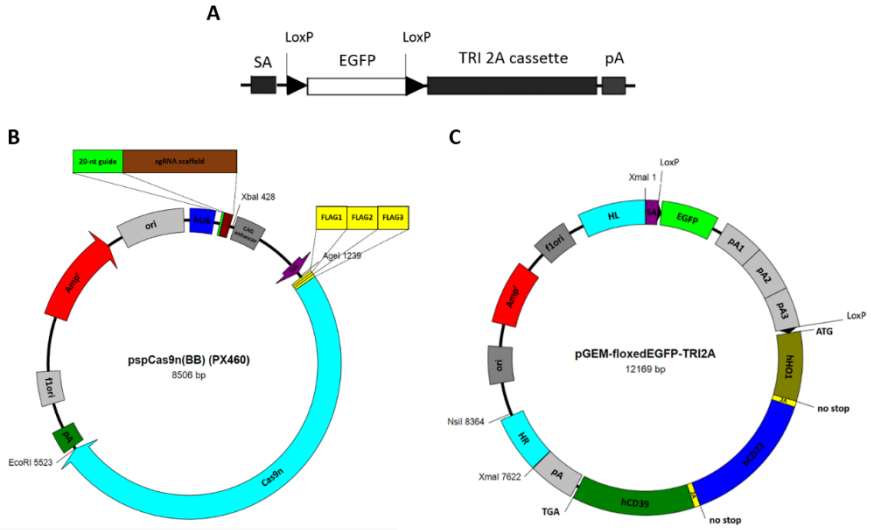



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**Figure 7.** Crispr/Cas9-mediated site-specific *knock in* of the triple-cistronic cassette into the first intron of the murine *Rosa26* locus**.** (**A**) Schematic map of the conditional triple-cistronic construct: splice acceptor (SA); LoxP/EGFP: floxed EGFP coding sequence; TRI 2A cassette: F2A-based tricistronic cassette for the simultaneous expression of hHO1, hCD73 and hCD39; pA: poly-adenine signal. (**B**) Scheme of pspCas9n (BB) plasmid encoding for both the sgRNA and Cas9n nuclease. (**C**) Scheme of pGEM-floxedEGFP-TRI2A plasmid as donor repair template for homologous recombination.

The functionality of this construct has been tested, at least in part, in NIH3T3 cells, in term of EGFP expression and Cre-induced expression of the tricistronic cassette. Further genetic and protein expression analyses have to be performed.

An inducible system for the strong and simultaneous over-expression of the genes would be more suitable to the purposes of our study, and the CRISPR/Cas9 system-mediated gene targeting constructs can now be used in mouse embryos for the generation of conditional, Cre-dependent, multi-gene transgenic mice. Crossing these mice with tissue-specific Cre-expressing transgenic mice, would be useful to deepen how these genes are able to counteract the strong inflammatory

and oxidative stress response which occur in vascular endothelium following ischemic events and that, in transplantation settings, finally result in a severe cellular injury and chronic graft failure. The clarification of these complex mechanisms may bring a further step forward closer to the development of potential drug treatments for human Ischemic Vascular Disease (IVD) based on these molecules mode of action.

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