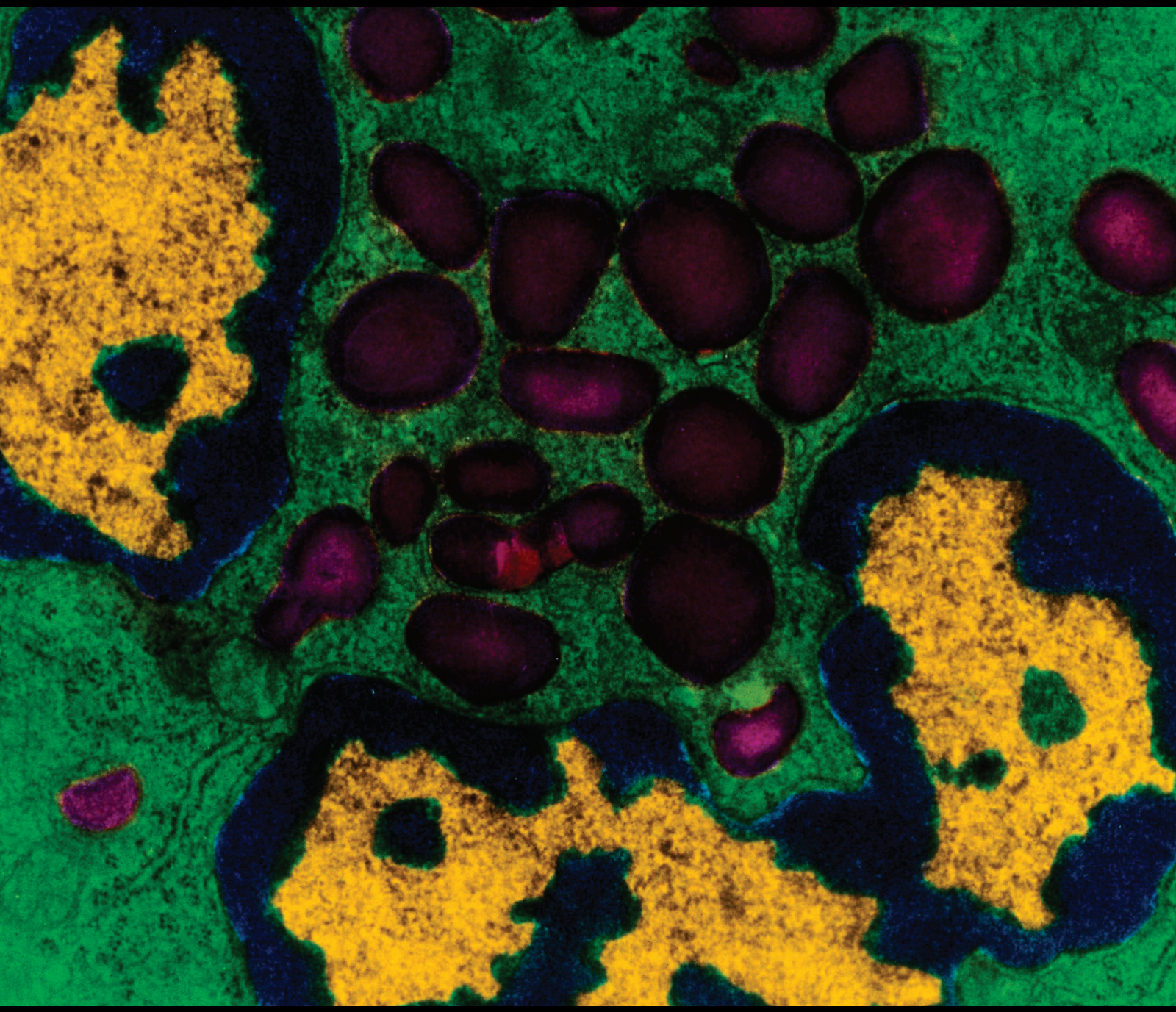
Mediators of Inflammation

Live or Die: Choice Mechanisms in Stressed Cells

Guest Editors: Elio Ziparo, Francesco Cecconi, Laura Soucek, and Dennis D. Taub



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Editorial Board

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Editorial

Live or Die: Choice Mechanisms in Stressed Cells

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Cells respond actively to the environmental changes, constantly adjusting their structure and function in order to maintain cellular homeostasis. Such changes consequent to both physiological stresses and/or pathological stimuli may be modulated in terms of type, degree, and duration and induce multiple cellular responses. Several different signaling pathways can in fact lead to repair, adaptation, or, in some cases, cell death. A prolonged inflammatory response may ultimately lead to several pathological conditions, including cancer. The specific cell response under stressful conditions has therefore crucial effects not only on the single cell fate but also more generally on tissue homeostasis and ultimately on organism health. This special issue, analyzing different experimental models, aims at highlighting some of the key mechanisms involved in cellular stress response.

Topics of this issue include different stressful conditions such as *oxidative*, *hypoxic*, *endoplasmic reticulum (ER)* and *inflammatory stress* and consequent cell responses such as *apoptosis*, *necroptosis*, *cell cycle arrest*, *mitotic cell death*, *pyroptosis*, and *survival*.

Among different types of stress, *oxidative stress* is causally implicated in degenerative diseases onset. In particular nitrooxidative stress has been reported to be implicated in the loss of specific neuronal populations in Parkinson's disease and amyotrophic lateral sclerosis. In the present special issue, the manuscript entitled "S-Nitrosoglutathione Reductase Plays Opposite Roles in SH-SY5Y Models of Parkinson's Disease and Amyotrophic Lateral Sclerosis" by *S. Rizza* and collaborators demonstrates that denitrosylating enzyme S-nitrosoglutathione reductase (GSNOR) modulation has different effects on neuronal viability depending on the applied stimulus and highlights GSNOR as a crucial molecular player of neuronal homeostasis. The review entitled "Redox Signaling in Diabetic Nephropathy: Hypertrophy versus Death Choices in Mesangial Cells and Podocytes" by *G. Manda* and collaborators emphasizes the role of the *oxidative stress* in diabetic nephropathy, which may act as trigger, modulator, and linker within the complex network of pathologic events. This review particularly addresses the molecular switches deciding on the renal cells fate in diabetic nephropathy leading to hypertrophy versus death choices in mesangial cells and podocytes.

On the other hand, amyotrophic lateral sclerosis is a multifactorial disease caused by motor neuron degeneration and is often associated with mutation of the *superoxide dismutase 1 (SOD1)* gene (encoding an enzyme that uses copper and zinc to break down toxic, charged oxygen molecules called superoxide radicals), thus affecting SOD1 antioxidant activity. The most studied *SOD1* mutation is the G93A substitution. The manuscript entitled "Postmitotic Expression of $SOD1^{G93A}$ Gene Affects the Identity of Myogenic Cells and Inhibits Myoblasts Differentiation" by *M. Martini* and collaborators

demonstrates that *oxidative stress* associated with $SOD1^{G93A}$ expression in C2C12 myogenic cells inhibits their myogenic program and promotes a fibroadipogenic phenotype.

Hypoxic stress plays a key role in heart disease, cancer, stroke, and other causes of mortality. The review entitled "Hypoxia Inducible Factor Pathway and Physiological Adaptation: A Cell Survival Pathway?" by *H. Kumar* and D.-K. Choi describes the role of the Hypoxia Inducible Factors (HIFs) in the *survival pathways* activated to regulate cellular adaptation to hypoxia.

Hypoxia and other environmental factors such as nutrient deprivation, pH changes, and reduced vascularization contribute instead to endoplasmic reticulum stress (*ER stress*) and activate the Unfolded Protein Response (UPR) in tumor cells. The review "Cancer Microenvironment and Endoplasmic Reticulum Stress Response" by *C. Giampietri* and collaborators describes the molecular mechanisms of UPR and their role in promoting cancer cells survival and proliferation.

Drug Induced Liver Injury (DILI) represents an adverse drug reaction leading to severe liver damage. Kupffer cells sense hepatic stress and produce cytokines thus stimulating an immune response. In the manuscript entitled "Subtoxic Concentrations of Hepatotoxic Drugs Lead to Kupffer Cell Activation in a Human *In Vitro* Liver Model: An Approach to Study DILI" *V. Kegel* and collaborators describe a human Kupffer cell culture model *in vitro* for the investigation of immune-mediated signaling in hepatic *inflammatory stress* involved in the pathogenesis of DILI.

Long-standing *inflammatory stress* may also promote tumor development, growth, and progression. In the manuscript entitled "Anti-Inflammatory Effects of a Methanol Extract from the Marine Sponge *Geodia cydonium* on the Human Breast Cancer MCF-7 Cell Line" *S. Costantini* and collaborators demonstrate that a methanol extract from this marine sponge reduces levels of VEGF and exerts a dose-dependent anti-inflammatory effect.

In the manuscript entitled "Multidrug Resistance Protein-4 Influences Aspirin Toxicity in Human Cell Line," *I. Massimi* and collaborators investigate aspirin effect on the efflux transporter protein Multidrug Resistance Protein-4 (MRP4) expression in HEK-293 cells. The authors demonstrate that low nontoxic aspirin dosages may lead to MRP-4 overexpression, thus increasing cellular detoxification of aspirin. Since overexpression of efflux transporters in human cells is a mechanism of resistance to drugs, the manuscript by *I. Massimi* et al. contributes to a better comprehension of molecular mechanisms adopted by cancer cells for *survival* in stressful conditions during chemotherapy.

Various pathological processes such as ischemic brain injury, myocardial infarction, organ transplantation, and virus replication are accompanied by strong inflammatory stress and *necroptosis*. The manuscript entitled "Necroptotic Cell Death Signaling and Execution Pathway: Lessons from Knockout Mice" by *J. Belizário* and collaborators describes recent discoveries regarding necroptosis execution pathway. The authors outline and discuss important phenotypes of knockout mice models that serve to define the role of *CASPASE 8*, *FLIP*, and *FADD* genes and other major components of necroptotic signaling.

In parallel, *pyroptosis* is a recently identified type of regulated cell death associated with inflammatory response and with features distinct from both apoptosis and necroptosis. Its role in cardiovascular diseases is addressed in the manuscript "Looking for Pyroptosis-Modulating miRNAs as a Therapeutic Target for Improving Myocardium Survival" by *S. Lee* et al. The authors aim at elucidating the possible role of miRNAs as therapeutic targets for preventing excessive myocardial pyroptosis in cardiovascular diseases.

Progression through the cell cycle is one of the most important decisions during the life of a cell and several kinds of stress are able to influence this choice. The manuscript "Cellular Response upon Stress: p57 Contribution to the Final Outcome" by *M. N. Rossi* and *F. Antonangeli* focuses on the contribution of cyclin-dependent kinase inhibitor p57 in regulating *cell cycle arrest* and *apoptosis* after cellular stress with particular attention to cancer cells.

Finally, the review entitled "Targeting the mitotic catastrophe signaling pathway in cancer" by *M. M. Mc Gee* describes the molecular mechanism of *mitotic cell death*, a mechanism to remove defective and genomically unstable cells. Mitotic catastrophe is a regulated antiproliferative process that occurs as a consequence of defective mitosis and may precede apoptosis, thus representing a further response under stressful conditions.

The readers will find in this special issue not only interesting data and updated reviews on possible mechanisms of cellular stress response, but also relevant questions waiting to be answered. The study of multiple pathways activated under stressful conditions has become increasingly complex, requiring expertise from all fields of biology. A better comprehension of such pathways might help elucidation of disease pathogenesis and may be of crucial interest in order to develop new therapeutic strategies.

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Research Article

Subtoxic Concentrations of Hepatotoxic Drugs Lead to Kupffer Cell Activation in a Human *In Vitro* Liver Model: An Approach to Study DILI

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Drug induced liver injury (DILI) is an idiosyncratic adverse drug reaction leading to severe liver damage. Kupffer cells (KC) sense hepatic tissue stress/damage and therefore could be a tool for the estimation of consequent effects associated with DILI. Aim of the present study was to establish a human *in vitro* liver model for the investigation of immune-mediated signaling in the pathogenesis of DILI. Hepatocytes and KC were isolated from human liver specimens. The isolated KC yield was $1.2 \pm 0.9 \times 10^6$ cells/g liver tissue with a purity of >80%. KC activation was investigated by the measurement of reactive oxygen intermediates (ROI, DCF assay) and cell activity (XTT assay). The initial KC activation levels showed broad donor variability. Additional activation of KC using supernatants of hepatocytes treated with hepatotoxic drugs increased KC activity and led to donor-dependent changes in the formation of ROI compared to KC incubated with supernatants from untreated hepatocytes. Additionally, a compound- and donor-dependent increase in proinflammatory cytokines or in anti-inflammatory cytokines was detected. In conclusion, KC related immune signaling in hepatotoxicity was successfully determined in a newly established *in vitro* liver model. KC were able to detect hepatocyte stress/damage and to transmit a donor- and compound-dependent immune response via cytokine production.

1. Introduction

Drug induced liver injury (DILI) represents an idiosyncratic adverse drug reaction responsible for severe patient morbidity and mortality and in consequence for the withdrawal of about 20% of new drugs from the market [1, 2]. In the USA, about 60% of all cases of acute liver failure and 10–20% of fulminant or subfulminant hepatitis originate from drug toxicity [3, 4]. Milder forms of DILI are assumed to occur in a high number of unknown cases. Therefore, the incidence and prevalence of DILI are only partially known [5]. The idiosyncratic origin of DILI and its unspecific reactions are the reason why it is still difficult to predict the potential risk of DILI in preclinical drug testing [6]. *In vivo* animal studies are not suitable for reflecting the idiosyncratic nature of DILI and its low frequency would require very high numbers of animals to detect DILI events [2, 7].

Additionally, the occurrence of immune tolerance reactions in the liver can influence DILI consequences *in vivo* [8]. *In vitro* studies using human cells could bypass systemic tolerance reactions and thus better reflect the human situation. However, it is known that many *in vivo* hepatotoxic effects are not detected in primary human hepatocytes (PHH) monocultures, which are however considered to be the gold standard of *in vitro* liver models. The lack of a physiological 3D environment and the absence of nonparenchymal cells are

discussed as possible reasons for an insufficient reflection of DILI mechanisms in conventional 2D hepatocyte cultures [9–11].

The mechanisms of DILI are not yet sufficiently clarified. According to different hypotheses, an immune-mediated mechanism is considered to be a major factor in its pathogenesis [2, 12, 13]. This mechanism of action starts with the hepatic biotransformation of drugs, which can lead to the production of reactive metabolites [14]. Hydroxylation by cytochrome P450 enzymes especially can produce hydroquinone, benzoquinoneimine, and catechol structures, which are of electrophilic nature. Such compounds disturb the redox balance and induce the generation of reactive oxygen species (ROS) leading to oxidative stress. Additionally, electrophilic metabolites can react with endogenous nucleophilic groups of DNA and proteins. The reaction with proteins leads to the formation of haptens. If released, these haptens can be identified by immune cells due to their antigenic character [12, 13], causing sensitizing reactions or, at worst, the induction of autoimmune diseases [15].

Kupffer cells (KC) are the primary macrophage population of the liver. They are on the one hand part of the scavenger system, which is responsible for systemic blood clearance and on the other hand responsible for detection of local tissue damage. In this function, KC are active in phagocytosis of cell debris, soluble macromolecules, and colloids as well as endogenous and foreign proteins [16]. Besides the recognition of cellular stress and cell death in hepatotoxic events, KC also fulfill a transmitter role in the communication to the immune system by antigen presentation and cytokine secretion [17]. KC activation by lipopolysaccharides (LPS), cell debris, haptens, or cytokines is accompanied by intracellular activation of the NF-κB signaling pathway mediated by reactive oxygen intermediates (ROI) [18]. Once activated, KC can differentiate into M1 type and M2 type macrophages depending on the signals received and on the genetic background. M1-KC play an important role in innate immunity and proinflammatory reactions. This inflammatory cell type is supported by TH1 cells. The alternative M2 response is depending on TH2 cells and ends in tissue-protective reactions. M2-KC promote maturation and activation of other KC, enhance tissue repair, and have a beneficial effect on vascular growth and nutrient homeostasis [19, 20]. Each KC class is associated with specific cytokines. M1-KC produce the proinflammatory cytokines IL-6, IL-8, and TNF-α, while M2-KC are associated with the anti-inflammatory cytokines IL-4, IL-5, and IL-10 [21–23]. Additionally, prostaglandin E_2 (PGE-2) can be released, which inhibits TNF-α and IL-6 production by KC in an autocrine feedback loop and attenuates the induction of acute-phase proteins [24]. PGE-2 is therefore associated with the M2-KC response rather than with the M1-KC response.

Two well-known hepatotoxic compounds responsible for the induction of DILI are acetaminophen (APAP) and diclofenac (DIC) [3]. Both compounds are nonsteroidal anti-inflammatory drugs (NSAID). APAP is transformed by cytochrome P450 (CYP) 2E1 and CYP1A1 to the reactive metabolite N-acetyl-p-benzoquinone imine [23]. APAP is known for the induction of hepatic oxidative stress and in consequence glutathione depletion leading to acute liver failure. Protein adduct formation has also been described but plays a minor role in APAP hepatotoxicity [25]. DIC is metabolized by CYP2C9 and CYP3A4 to two hydroxylated metabolites consequently transformed in secondary reactions into metabolites with benzoquinone imine structure [26]. Both metabolites have been shown to react with proteins. Hapten formation correlates with the occurrence of sensitization reactions towards DIC. Beside hapten formation, the generation of ROS is also described but is of minor clinic relevance compared to APAP [23].

Aim of the present study was the establishment of a human *in vitro* KC culture model for the investigation of immune-mediated signaling in hepatic pro- and anti-inflammatory reactions involved in the pathogenesis of DILI.

For the present study, PHH and KC were isolated from human liver resectates using a two-step collagenase perfusion technique followed by selective separation steps to get purified PHH and KC fractions. KC were identified and characterized by morphological und functional investigations. Optimization of KC culture conditions allowed for a cultivation for up to 5 d. The known hepatotoxic drugs APAP and DIC were used at subtoxic concentrations to simulate a DILI-like event in PHH cultures. Supernatants of drug-treated PHH were then used to stimulate KC cultures. While most liver models for hepatotoxicity testing usually need high concentrations leading to definite toxic effects, this new model allows for detecting hepatotoxic cell stress also in a subtoxic concentration range.

2. Material and Methods

2.1. Chemicals. The hepatocyte culture medium was based on Williams' Medium E with GlutaMAX (Gibco, Paisley, UK), supplemented with 10% FCS (Gibco), 32 mU/mL Insulin (Sanofi Aventis, Frankfurt am Main, Germany), 15 mM HEPES, 0.1 mM MEM NEAA (100×), 1 mM pyruvate (all by Gibco), and 1 mg/L dexamethasone (Fortecortin, Merck, Darmstadt, Germany).

KC culture medium was based on RPMI low glucose (GE Healthcare, Pasching, Austria) supplemented with 10% FCS, 1% L-glutamine, and 6.3 mM N-acetyl-L-cysteine (all by Gibco). KC starvation medium was based on RPMI low glucose supplemented with 1% L-glutamine. All media were supplemented with 100 U/100 μM penicillin/streptomycin (Gibco) prior to use.

PBS was purchased from Gibco. Percoll, Trypan Blue, and Hanks Balanced Salt Solution (HBSS) were provided by Biochrom (Berlin, Germany). All other chemicals were purchased from Sigma (Munich, Germany), if not stated differently.

2.2. Isolation and Culture of Primary Human Hepatocytes and Kupffer Cells. For mimicking immune-mediated reactions in DILI, a human *in vitro* liver model based on primary human liver cells was established. PHH and KC were isolated in parallel from the same donor tissue to avoid immune reactions due to incompatibility.

PHH and KC were isolated from nontumorous human liver tissue, which remained after partial liver resection in patients with primary or secondary liver tumors. Additionally, corresponding human blood samples were obtained and used for production of autologous serum. Informed consent of the patients was obtained according to the ethical guidelines of the Charité-Universitätsmedizin Berlin.

PHH were isolated by a two-step collagenase perfusion technique according to Nüssler et al. [27]. PHH contained in the gained cell suspension were enriched by double centrifugation at 50 ×g, 5 min, 4°C. The pellet was suspended in hepatocyte culture medium and seeded at a density of 145,000/cm^2 in cell culture plates. Culture medium exchange was performed 12 h after seeding and afterwards every 24 h. Prior to starting APAP or DIC treatment, the culture medium was exchanged against hepatocyte starvation medium.

The supernatant of the initial centrifugation of the cell suspension was used for KC isolation [28]. To eliminate remaining erythrocytes, the supernatant was centrifuged at 72 ×g, 5 min, 4°C. The supernatant, which contained the nonparenchymal liver cells (NPC), was centrifuged at 650 ×g, 7 min, 4°C. The pellet consisting of KC, hepatic stellate cells, and liver endothelial cells was resuspended in 20 mL HBSS. For enrichment of KC, the cell suspension was subjected to a Percoll density gradient centrifugation. A two-level gradient consisting of a 25% Percoll solution on top of a 50% Percoll solution was prepared. The cell suspension was carefully placed on top of the 25% Percoll gradient and centrifuged at 1800 ×g, 15 min, 4°C, without brake. The cells in the interphase between 25% and 50% Percoll were collected, washed once with HBSS, and resuspended in KC starvation medium. The cell number and viability of the contained KC were determined by using the Trypan blue exclusion technique. To remove remaining NPC, the selective adherence capacity of KC on cell culture plastics was used. KC were seeded at a density of 0.5×10^6 cells/cm^2 on a 24-tissue culture plate (Falcon BD, Heidelberg, Germany) and cultured for 25 min at 37°C, 5% CO_2 in a humidified incubator. Not adhered NPC were removed by washing the culture plate with HBSS. KC were then maintained in KC culture medium for at least 12 h overnight. The medium was replaced by KC starvation medium on the next day and KC were cultured at least for 4 h in that medium before the cells were used for experiments.

2.3. Optimization of Culture Conditions. Previous studies had shown that KC isolated from human liver tissue show a donor-dependent initial activation [29]. Due to potentially varying initial KC activation levels, we performed experiments designed to reduce or stabilize the KC activation. KC are activated by different factors like endogenous or foreign proteins, LPS, or environmental changes.

To exclude additional KC activation by xenogenous proteins contained in fetal calf serum, cultivation in KC culture medium with autologous serum or without any serum was tested. Autologous serum was generated by centrifuging 10 mL blood from the patient at 1000 ×g, 10 min and 4°C.

Additionally, a potential reduction of the initial KC activation by addition of antioxidants to KC cultures was investigated. Therefore, KC were cultured for up to 108 h by using KC culture medium supplemented with or without 10 mM *n*-acetylcysteine or 10 mM ascorbic acid.

2.4. Characterization of KC

2.4.1. Immunofluorescence Staining. The purity of the isolated KC was determined by immunofluorescence staining of CD68, which is a surface protein of the macrophage lineage, including monocytes, histiocytes, giant cells, KC, and osteoclasts. An antibody against CD68 (R&D Systems, Minneapolis, USA) and a secondary antibody coupled with phycoerythrin (PE) (Santa Cruz Biotechnology Inc., Heidelberg, Germany) were used for staining.

Additionally, the ability for phagocytosis was evaluated by using FITS coupled latex beads (FluorisBite plain YG3.0 microspheres, Polyscience). Cell nuclei were stained with Hoechst 33342 (Sigma-Aldrich GmbH, St. Louis, US). Images were taken with a fluorescence microscope (Zeiss, Jena, Germany).

2.4.2. Cell Viability/Cell Activity. In order to evaluate the cell viability as well as changes in energy metabolism, the cell activity was determined using the XTT assay (Roche Diagnostics GmbH, Mannheim, Germany). The test was performed according to the manufacturer's protocol. After 2 h incubation time, the supernatants were transferred into a 96-well plate and the absorbance was measured at 492 nm in a microplate reader (FLUOstar OPTIMA, BMG Labtech, Ortenberg, Germany).

2.4.3. Measurement of Intracellular Reactive Oxygen Intermediates (ROI). ROI play an essential role in signaling pathways of inflammatory reactions. Therefore, the formation of intracellular ROI was measured by using the fluorogenic substance dichlorodihydrofluorescein diacetate (DCF-DA) according to [30] with minor modifications. The cell-permeable DCF-DA diffuses into cells and is deacetylated by cellular esterases and oxidized by ROI to dichlorodihydrofluorescein (DCF). For ROS measurement, the culture medium was replaced with RPMI medium without serum and phenol red, but containing 20 μM DCF-DA (Santa Cruz Biotechnology, Inc., Heidelberg, Germany) followed by incubation for 30 min at 37°C, 5% CO_2 in a humidified incubator. Subsequently, the supernatants were aspirated and the cells were incubated with fresh medium without serum and phenol red for 1 h. Fluorescence was measured in a microplate reader at an excitation wavelength of 492 nm and an emission detection of 520 nm.

2.5. Kupffer Cell Stimulation. Selective activation of KC was performed by incubation with hepatotoxic drugs (APAP, DIC) or using lipopolysaccharide (LPS) stimulation. For equilibration of the KC, cells were cultured in starvation medium for 24 h. Subsequently, KC were incubated with 100 μM APAP, 100 μM DIC, or with different LPS concentrations in starvation medium for further 24 h. KC activation was determined by the measurement of ROI formation and cell viability as described above.

Table 1: Anamnesis data of investigated donors.

	Diagnosis	Sex	Age	BMI	Notes
Healthy	Adenoma	F	48	29	—
	Cholangiocellular carcinoma	F	50	20	—
Multimorbidity	Klatskin tumor	M	80	31	Hypercholesterolemia
	Colorectal liver metastasis	M	72	23	Coronary heart disease
	Cholangiocellular carcinoma	M	74	29	Diabetes, hypertension, and in situ split
	Gall bladder carcinoma	F	57	28	Portal vein embolisation, hypertension, and cholestasis
	Cholangiocellular carcinoma	F	75	24	Hypercholesterolemia
	Cholangiocellular carcinoma	M	61	31	Hypertension, hypercholesterolemia
	Hemangioma	F	43	28.4	Diabetes
	Cholangiocellular carcinoma	M	47	23	Terminal renal failure, hypercholesterolemia
Chronic inflammation	Klatskin tumor	M	72	24	Chronic inflammation, diabetes, and hypertension
Portal vein embolisation	Colorectal liver metastasis	F	60	—	Portal vein embolisation
	Colorectal liver metastasis	M	57	28	Portal vein embolisation, chemotherapy
Cholestasis	Cholangiocellular carcinoma	F	72	22	Cholestasis
	Cholangiocellular carcinoma	F	77	22	Cholestasis
Chemotherapy	Colorectal liver metastasis	F	71	—	Chemotherapy
Hepatic steatosis	Hepatocellular carcinoma	M	75	—	Resection area, steatohepatitis
	Adenoma	F	32	—	Diabetes, steatohepatitis
Resection area	Hemangioma	M	47	27	Resection area
	Cholangiocellular carcinoma	M	62	25	Resection area, chemotherapy
Donor A	Focal nodular hyperplasia	F	19	22	—
Donor B	Colorectal liver metastasis	M	50	23	Diabetes, smoker
Donor C	Klatskin tumor	M	52	25	Diabetes, portal vein embolisation

To investigate the activation of KC following hepatocyte damage, KC were incubated with the supernatants of drug-treated PHH from the same donor. Therefore, the PHH were cultured for 4 h and KC for 5 h in starvation medium for equilibration of the cells. Subsequently, the PHH were stimulated with 100 μM APAP or 100 μM DIC in starvation medium for 1 h, respectively. Then, the supernatants of the compound-treated PHH were transferred onto the KC. After 2 h incubation time, ROI formation and cell activity were measured as described above. Additionally, the supernatants were collected and stored at −80°C after freezing in liquid nitrogen for subsequent measurement of pro- and anti-inflammatory cytokine formation as marker for KC response. These experiments were performed with cells from three independent donors and evaluated individually for each donor (Table 1).

2.6. Cytokine ELISA. In order to evaluate inflammatory reactions of KC, the formation of pro- and anti-inflammatory cytokines was investigated. Tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), and interleukin-10 (IL-10) ELISA Kits (PeproTech GmbH, Hamburg, Germany) as well as a prostaglandin E2 (PGE-2) ELISA Kit (Thermo Fischer Scientific, Waltham, USA) were used for the measurement of cytokine concentrations in cell culture supernatants. Cytokine formation was measured following the manufacturer's instruction.

2.7. Statistical Analysis. Data were analyzed by one-way or two-way ANOVA, with a t-test or a Mann-Whitney test using Graph Pad Prism 5 software. Results are given as means ± SEM or as median including the interquartile range, minimum and maximum values presented as box plots. Differences were considered as significant at $P < 0.05$. Only data from experiments performed at least three times with cells from different donors were subjected to statistical analysis.

3. Results

3.1. Isolation and Characterization of PHH and KC. KC were successfully isolated from 37 different donors using the supernatants remaining from PHH isolation. Trypan blue staining showed that >90% of KC were viable. A yield of $1.2 \pm 0.9 \times 10^6$ KC per gram of liver tissue was obtained. KC were identified by immunostaining of the macrophage-specific surface protein CD68 and by their ability for phagocytosis of fluorescent latex beads (Figure 1(a)). CD68-positive and phagocytosis-positive cells were counted in relation to total cells stained with Hoechst dye (Figure 1(b)). A purity of 60% was obtained in the KC-rich cell suspension. The performance of the adherence separation step increased the purity to >80% (Figure 1(c)).

The determination of KC activity was performed directly after the isolation procedure. Intracellular ROI levels as

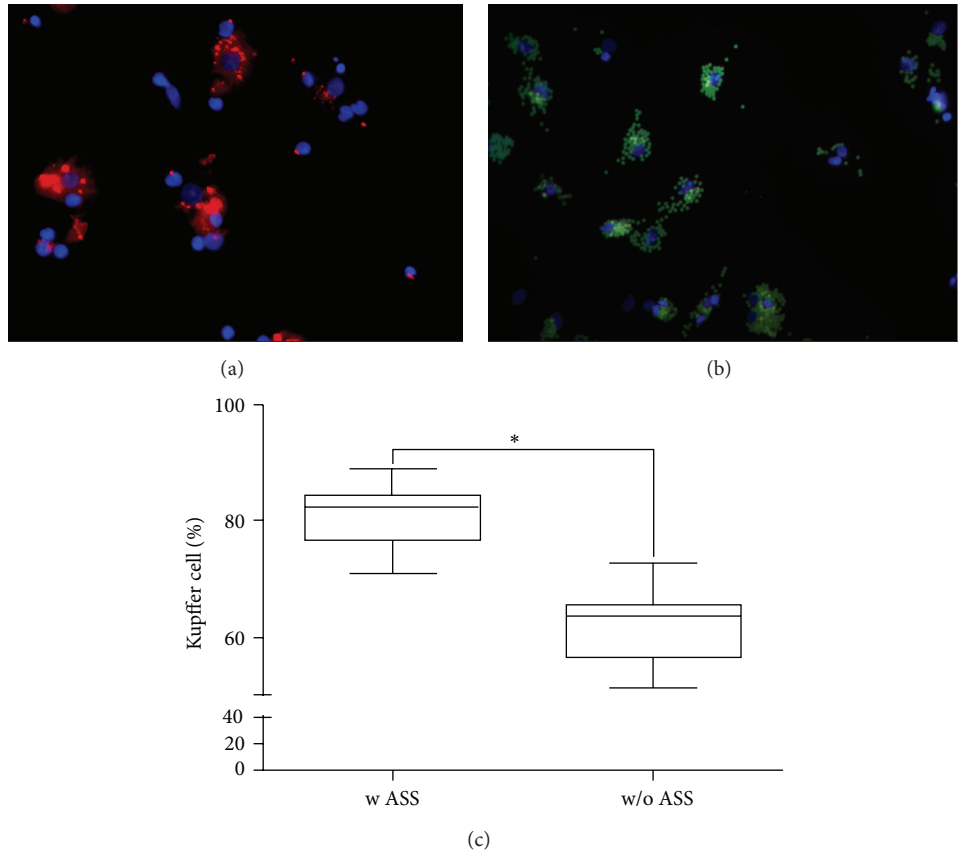

Figure 1: *Identification of KC and determination of the purity of KC cultures.* KC were identified by IF staining for CD68 (a) and the phagocytosis of fluorescent latex beads (b). The percentages of CD68-positive and phagocytosis-positive cells isolated with an adherence separation step (w ASS) or without an adherence separation step (w/o ASS) are shown in (c). Data are shown as box plots, representing the median, the interquartile range, and minimum and maximum values. ∗ At least $P \leq 0.0001$ (unpaired t-test), $N = 3$, $n = 9$.

mediator in the NF-κB signaling pathway were measured by the DCF assay. The measurement of the initial KC activation revealed variable intracellular ROI concentrations in cultures from donors with different donor anamnesis and tissue quality (Figure 2, Table 1). The lowest KC activity was detected in healthy patients with low BMI and early tumor stages (considered as healthy tissue). Patients with multimorbidity or chronic inflammation showed preexisting moderate KC activation as an indicator of chronic cell stress/damage. In contrast, the KC from livers with tissue damage caused by portal vein embolization, cholestasis, or recently performed chemotherapy revealed the highest KC activation levels. The same was true for steatotic liver or liver tissue close to the resection margin with direct injury by cauterization, which led to the highest KC activation levels. The comparison between ROI levels and donor anamnesis indicates that donor conditions and liver tissue quality influence the initial KC activation, although no definitive statement can be made due to the low numbers of donors in each group.

3.2. Experimental Setup and Evaluation of Optimal Culture Conditions. In general, cultured KC showed a loss in cell viability associated with a decrease of ROI over 5 d regardless of the type of serum used. The cultivation with FCS had a slightly positive effect on cell viability, while a slight increase of KC activation was observed compared to serum-free cultivation or cultivation with autologous serum (see Supplementary Figure 1 available online at http://dx.doi.org/10.1155/2015/640631).

Cultivation of KC in the presence of the antioxidants *n*-acetyl cysteine or ascorbic acid also showed an increase in the ROI level. However, a beneficial effect on cell viability in comparison to the control was observed during the first 12 h after seeding. N-acetyl cysteine had a stronger effect than ascorbic acid (Supplementary Figure 2).

3.3. Compound-Dependent KC Activation. KC can be activated by a variety of stimuli. For determination of the sensitivity of KC towards cell stress, KC were exposed to hepatotoxic drugs (APAP, DIC) or to the known KC activating agent LPS [18]. Of special interest in this experiment was the question, if an additional activation on top of the initial activation is detectable. Therefore, KC cultures were stimulated with varying concentrations of APAP, DIC, and LPS for 24 h. At the end of the activation period, the intracellular ROI levels were measured and normalized to the cell viability.

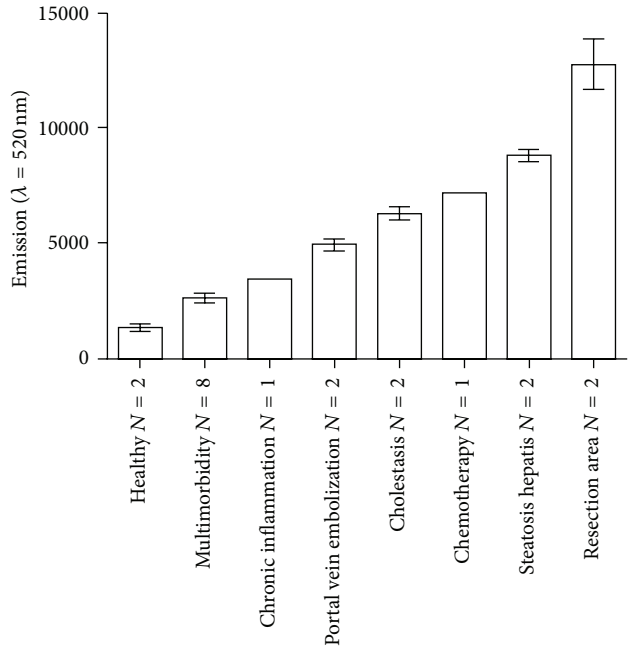

Figure 2: *Initial KC activation*. Initial ROI levels correlated with tissue quality and donor anamnesis. Initial ROI levels were measured in KC cultures by means of the DCF assay performed directly after KC isolation. Data show means ± SEM. *N*: shown in the figure.

Treatment of KC with APAP for 24 h showed for both concentrations (0.1 and 1 mM) a slight increase in the relative oxidative stress levels by trend (Figure 3(a)). The treatment with DIC for 24 h showed no effect for 0.1 mM DIC and a slight increase in the relative oxidative stress level for 1 mM DIC (Figure 3(b)).

The stimulation with LPS showed a tendency towards a concentration-dependent increase in the relative ROI levels compared to the untreated control (Figure 3(c)). The results showed large variations due to different initial ROI values in activation of KC from individual donors. Taken together, for various concentrations of APAP and DIC, no statistically significant activation was detectable. Furthermore, the positive control LPS did not show any statistically significant KC activation. However, a clear trend for a concentration-dependent activation was observable, which suggests that an additional activation of preactivated KC is possible.

3.4. Response of Kupffer Cells to Hepatocyte Stress/Damage

3.4.1. Hepatocyte Stress/Damage after Stimulation with Hepatotoxic Drugs. For the simulation of hepatocyte stress/damage, we treated isolated hepatocytes from 3 different donors (Donor A, Donor B, and Donor C) with the hepatotoxic drugs APAP or DIC (100 μM) for 1 h, respectively. Donor characteristics are detailed in Table 1.

At the end of drug incubation, the DCF assay was performed for the evaluation of potential oxidative stress induction and the XTT assay was performed for assessment of the cell viability. Untreated PHH from the same donor served as control, since they reflect the basal oxidative stress level of the hepatocytes. Due to large variances between the reactions of PHH cultures from individual donors towards the drugs, the results were not merged and are presented on a case by case basis instead.

The investigation of drug-mediated oxidative stress induction in PHH revealed that the basal oxidative stress level varied donor dependently. While donor A and donor B showed similar ROS concentrations in control cultures (Figures 4(a1) and 4(b1)), donor C showed a 1.5 times higher oxidative stress level (Figure 4(c1)). However, all donors showed a low (donor A and B) to moderate (donor C) increase of oxidative stress for APAP, but not for DIC incubation. Investigation of cell viability by means of the XTT assay revealed that stimulation with APAP did not influence the cell viability, while application of DIC resulted in a clear increase in cell activity in donor A (Figure 4(a2)) and donor B (Figure 4(b2)). Donor C (Figure 4(c2)) showed a constant level in cell activity after drug treatment. All three donors showed no decrease in cell activity and therefore no loss in cell viability could be detected.

3.4.2. Kupffer Cell Response to Hepatocyte Stress/Damage. To evaluate the immunological response of KC after hepatocyte stress/damage, the isolated KC were stimulated with the supernatants of PHH having been pretreated with hepatotoxic drugs (see Section 3.4.1). To evaluate the KC activation, the cell activity and intracellular ROI formation, the KC response and the cytokine secretion were measured, respectively. The cell activity increased compound dependently in KC from all three tested donors (Donor A, Donor B, and Donor C) after stimulation with supernatants from drug-treated PHH. The corresponding ROI formation and the cytokine release as markers for KC activation and response, respectively, showed donor- and compound-dependent signals (Figures 5 and 6). Due to these very individual reactions, the donors were investigated on a case by case basis.

Donor A was a young healthy woman with a benign liver tumor (Table 1). The activation of KC increased slightly after stimulation with supernatants from APAP-treated PHH, compared to the untreated control. In contrast, there was no increase in cell activity. KC stimulation with DIC did not lead to any changes neither in the activation measured by intracellular ROI formation nor in cell activity (Figure 5(a)).

Cytokine release of KC after stimulation with APAP-treated PHH showed a slight increase in cytokines IL-6 and TNF-α but no change in the IL-10 level compared to the KC stimulation with control hepatocytes. Supernatants from DIC-treated PHH evoked a notable decrease of the secretion of IL-6 and TNF-α in KC cultures. The PGE-2 release in donor A was comparable to the release in donor B. In both donors, PGE-2 release decreased drug dependently, whereas the effect of KC stimulated with DIC-treated PHH was higher than after APAP treatment (Figures 6(a) and 6(b)).

Donor B was a 50-year-old man who suffered from a colorectal liver metastasis (Table 1). The stimulation of KC with the supernatants of DIC- or APAP-exposed PHH led to an elevated KC activation quantified by an increase in the ROI level and in cell activity (Figure 5(b)).

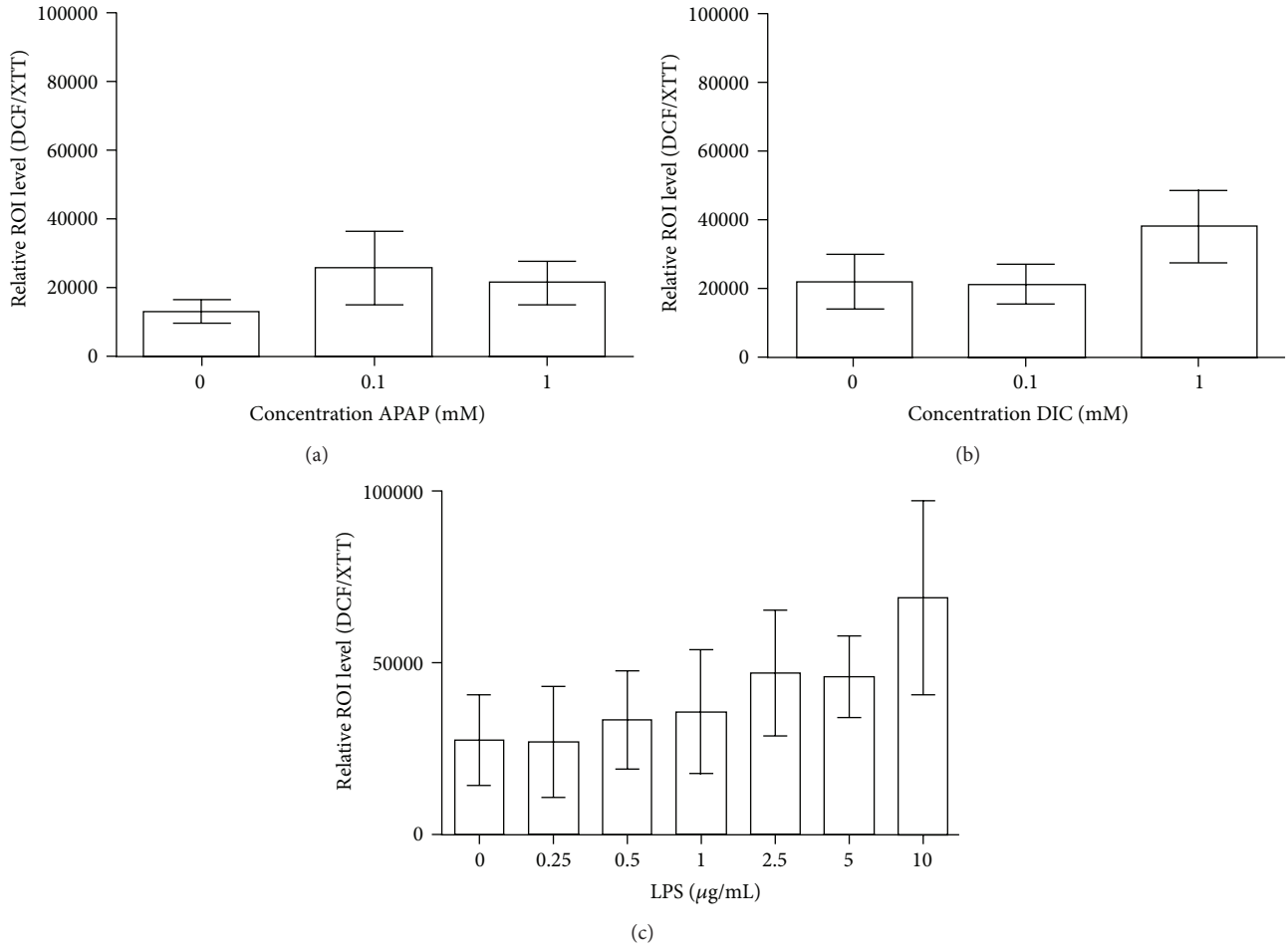

Figure 3: *APAP-, DIC- or LPS-induced stimulation of KC.* APAP (a), DIC (b), or LPS (c) in different concentrations were used for the stimulation of KC for 24 h. The intracellular ROI formation was investigated by the DCF assay. The detected ROI levels were normalized to the cell viability measured by means of the XTT assay. Data show means ± SEM. $N_{LPS} = 3$, $N_{APAP/DIC} = 5$, $n_{LPS} = 6$, and $n_{APAP/DIC} = 2$.

Regarding the KC reaction, the stimulation with APAP-treated PHH showed a slight increase in the release of TNF-α comparable to donor A but no effect on the level of IL-6. After KC stimulation with supernatant from DIC-treated PHH beside an increase of TNF-α, an increase of the IL-6 concentration was measured (Figure 6(b)). In terms of IL-10 secretion, no differences were detected between KC incubated with supernatant from compound-treated PHH and those from control hepatocytes (Figure 6(b)).

Donor C was a 57-year-old man with diabetes suffering from a Klatskin tumor. This rare form of cholangiocellular carcinoma is closely connected to cholestasis and therefore to a stress/damage to the affected liver tissue. Additionally, the patient had previously undergone portal vein embolization (Table 1). The results of this donor were different from those of the other two investigated donors. The KC activity measured by changes in intracellular ROI levels was clearly decreased after stimulation with supernatant from drug-treated PHH compared to the stimulation with that of control hepatocytes. This decrease in KC activation was more intense after the stimulation with DIC-treated PHH compared to the stimulation with APAP-treated PHH (Figure 5(c)). The effect on the KC activity was inverse. Here, a strong increase in cellular activity was detected after stimulation of KC with both compounds.

The KC reaction after stimulation with supernatant from APAP-treated PHH showed no changes in TNF-α, IL-6, and IL-10 secretion compared to stimulation with untreated PHH. In contrast, the PGE-2 secretion increased noticeably after treatment with APAP-stimulated PHH. DIC-treated PHH induced an increase in the release of IL-10 and TNF-α in KC. After stimulation of the KC with supernatant from DIC-treated PHH, the PGE-2 levels decreased comparably in comparison to the other investigated donors. The IL-6 level remained unchanged (Figure 6(c)).

4. Discussion

DILI is responsible for severe patient morbidity and mortality [31] and, in consequence, causes massive economic losses in pharmaceutical industry [2]. While different mechanisms of action are described, there is evidence that the involvement

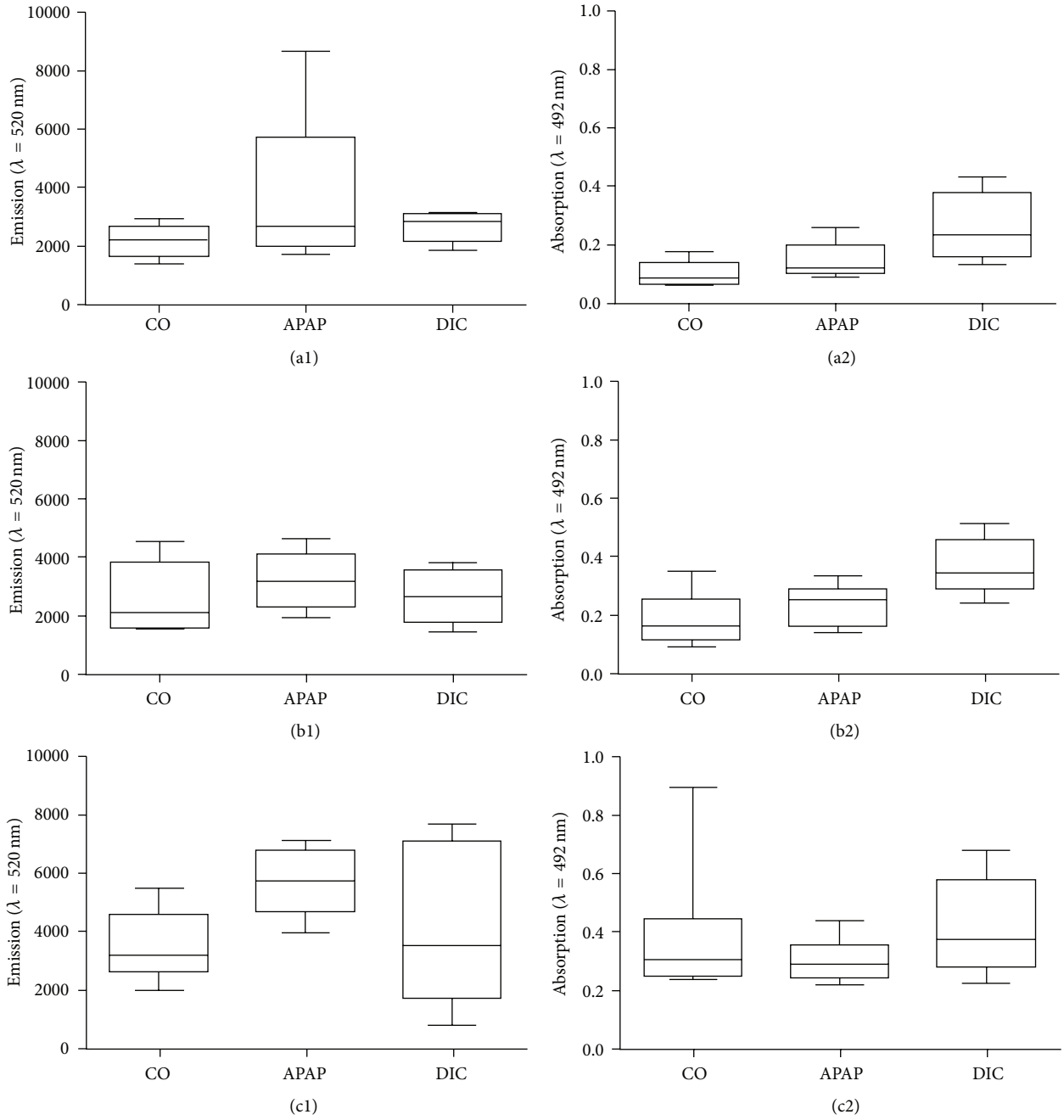

Figure 4: *Induction of oxidative stress in PHH*. PHH from three individual donors (A, B, and C, see Table 1) were treated with 100 μM acetaminophen (APAP) or 100 μM diclofenac (DIC) for 1 h. Intracellular ROI formation ((a1), (b1), and (c1)) was investigated by the DCF assay and the cell activity ((a2), (b2), and (c2)) was determined by the XTT assay. Data are shown as box plots, representing the median, the interquartile range, and minimum and maximum values, $n = 4$.

of immunologic reactions leading to sensitization reactions and autoimmune diseases might play a major role [32]. Previous *in vivo* preclinical testing strategies failed due to the idiosyncratic nature of DILI causing its low frequencies. The idiosyncrasy is a result of immunologic reactions in hepatic inflammation leading in most cases to immune tolerance towards drug-mediated hepatotoxicity. To bypass systemic tolerance reactions, we hypothesize that a DILI risk could be detectable in an *in vitro* liver model, which enables the investigation of immunologic cell-cell communication at an early stage of the hepatotoxic event. Aim of the present study was the establishment of a human *in vitro* model for the simulation of hepatic tissue stress/damage, which allows for the investigation of immune-mediated signaling in hepatic inflammatory reactions.

KC are the first cells confronted with a hepatic tissue damage. These tissue-resident macrophages sense tissue damage and cell stress, process incoming signals, and communicate

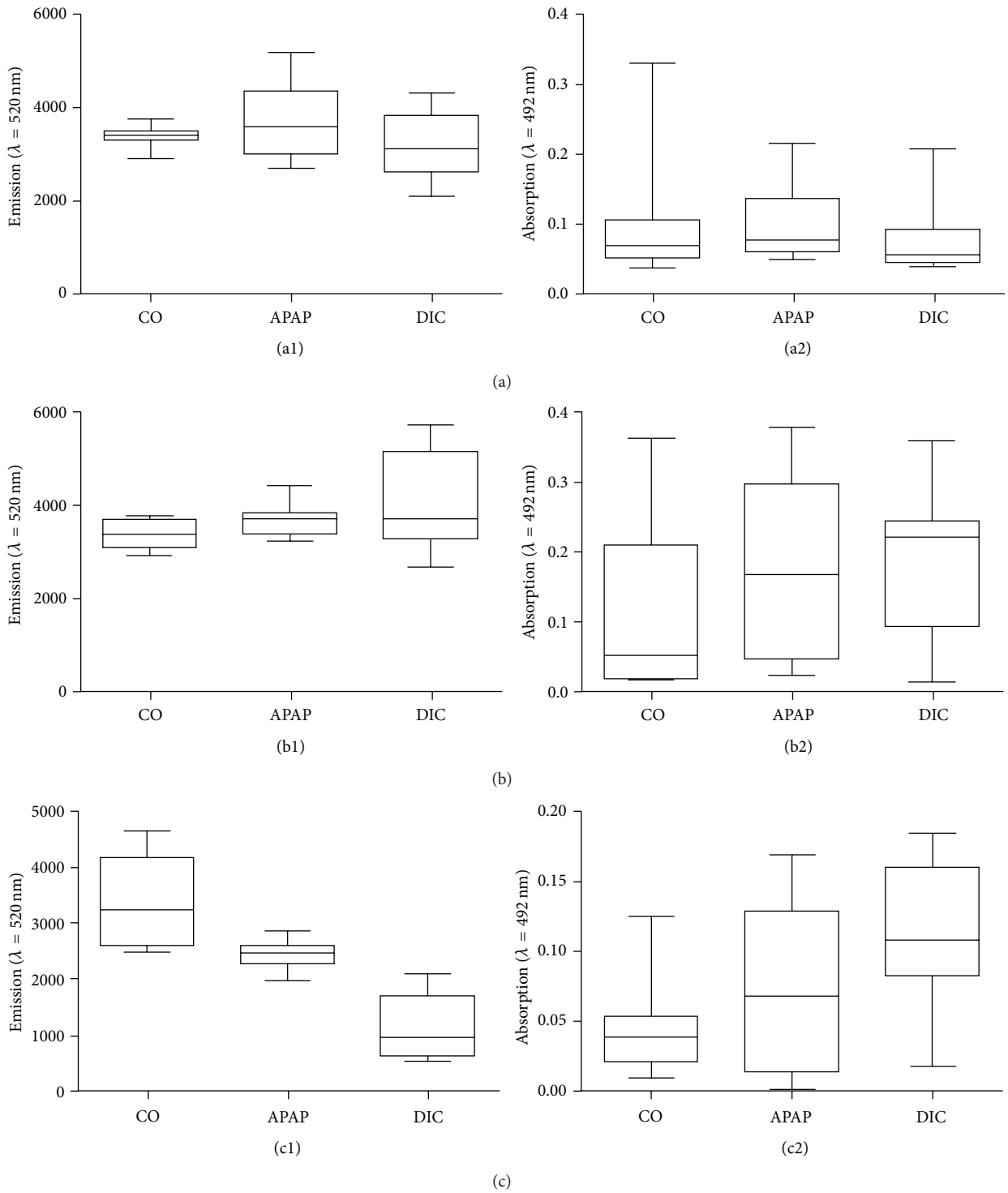

Figure 5: *KC activation with supernatants of PHH previously incubated with subtoxic concentrations of hepatotoxic drugs*. PHH of three donors (A, B, and C, see Table 1) were treated with 100 μM acetaminophen (APAP) or 100 μM diclofenac (DIC) for 1 h. The supernatants were used to stimulate KC from the same donor for 2 h. The intracellular ROI formation ((a1), (b1), and (c1)) was investigated by the DCF assay and the KC activity ((a2), (b2), and (c2)) was determined by the XTT assay. Data are shown as box plots, representing the median, the interquartile range, and minimum and maximum values, $n = 4$.

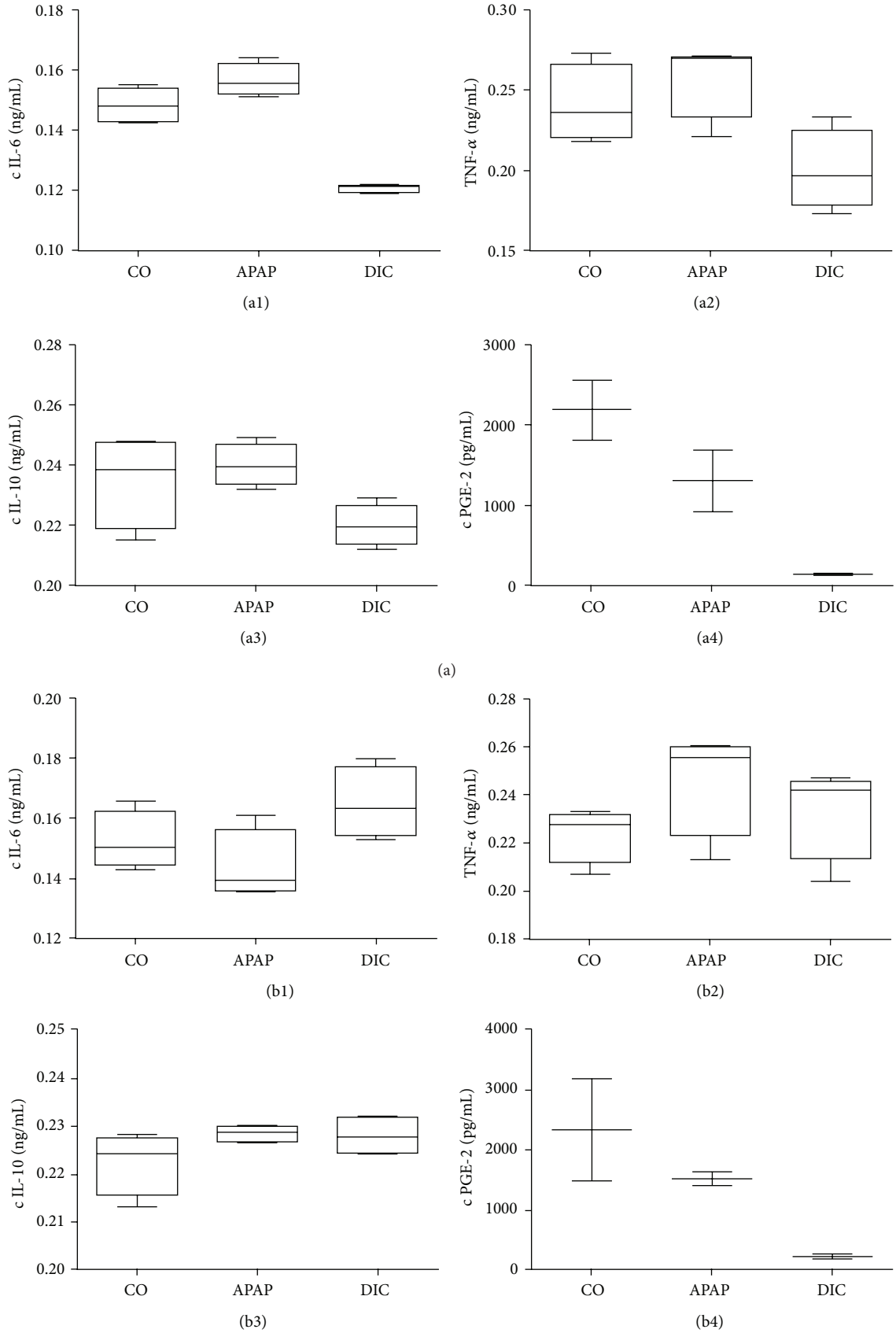

Figure 6: Continued.

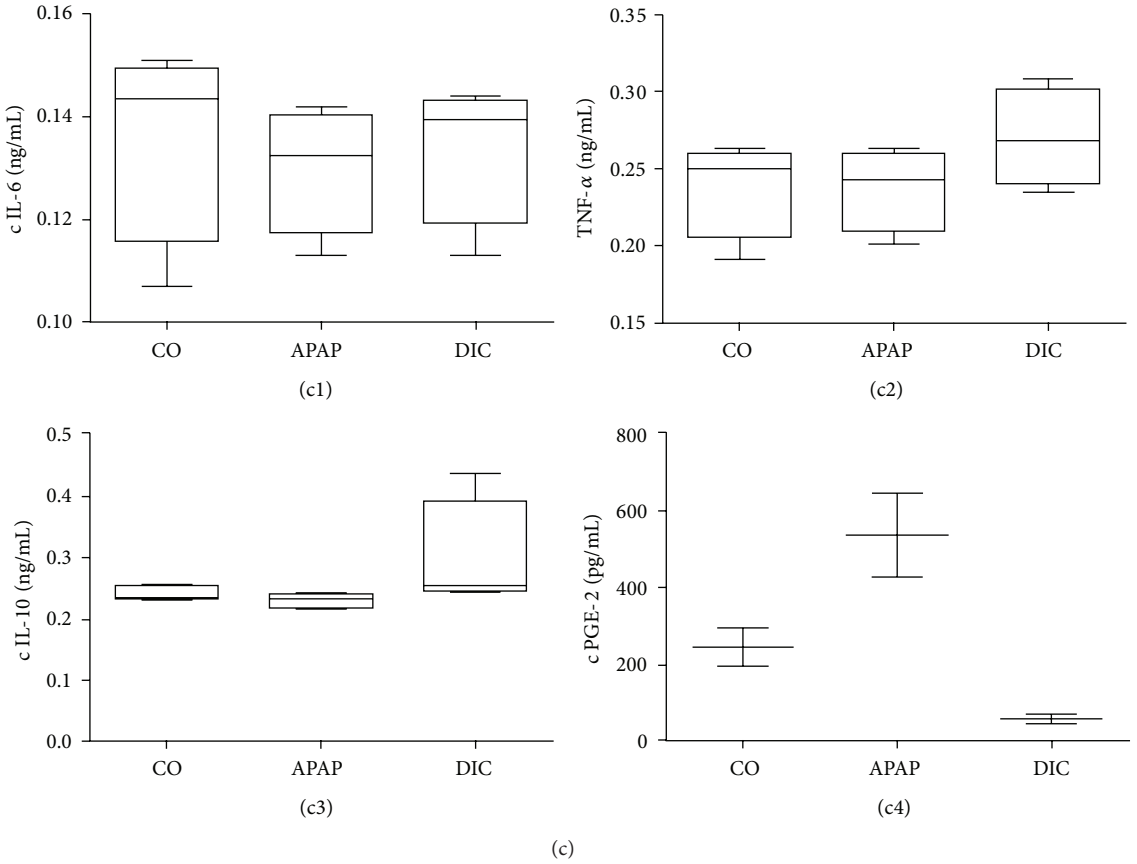

Figure 6: *Secreted cytokine profiles of KC activated with supernatants of PHH previously incubated with subtoxic concentrations of hepatotoxic drugs*. PHH from three donors (A, B, and C; see Table 1) were treated with 100 μM acetaminophen (APAP) or 100 μM diclofenac (DIC) for 1 h. The supernatants were used to stimulate KC from the same donor for 2 h. Inflammatory reactions of KC due to hepatocyte stress/damage were determined by cytokine analysis in KC supernatants. IL-6 ((a1), (b1), and (c1)), TNF-alpha ((a2), (b2), and (c2)), IL-10 ((a3), (b3), and (c3)), and PGE-2 ((a4), (b4), and (c4)) were investigated using cytokine ELISAs. Data are shown as box plots, representing the median, the interquartile range, and minimum and maximum values, $n_{(\text{IL-6,TNF-alpha,IL-10})} = 4$ $n_{(\text{PGE-2})} = 2$.

a reaction to other NPC and to the systemic immune system [20, 24, 33].

Therefore, we established a liver model consisting of PHH being responsible for displaying the hepatotoxic event and KC for mediating immunologic cell-cell communication. In the present study, PHH and KC were successfully isolated from resected human liver tissue samples. An optimized separation procedure using an adherence separation step allowed for the isolation of KC in a high quantity and purity. KC were clearly identified by detection of the macrophage-specific surface protein CD68 and by their ability for phagocytosis.

The determination of intracellular ROI formation revealed that KC are already partially activated after isolation (initial KC activation). Activated KC produce ROI as part of the NF-κB signaling pathway, which can be used as a marker for the determination of the KC activation [34]. Lowest KC activation levels were measured in healthy liver tissue from young donors with benign tumors and no secondary diseases or interventions. KC can be activated by various endogenous sources arising from tissue damage, like intracellular components, cell debris released by necrosis, apoptotic bodies [35], and inflammatory cytokines [36]. This was reflected in KC isolated from liver tissue containing a resection border with freshly damaged and destroyed liver tissue. Diseases and medical interventions leading to hepatic tissue damage may consequently increase the initial KC activation. We suggest that the correlation of donor anamnesis to the KC activation level is proportional to the time span since induction and intensity of the tissue damage, respectively. The testing of different media supplements to reduce the initial activation showed that KC activation is not reversible under the conditions used, although the use of FCS or of FCS in combination with *n*-acetyl cysteine showed both beneficial effects on KC viability.

However, the results from the additional KC activation by LPS confirmed that KC can be used for the detection of inflammatory events when compared to untreated control cultures. Therefore, an additional activation of KC is possible and can be quantified by using ROI as a marker for Kupffer cell activation. Using ROI as a marker for KC activation was demonstrated previously by Uchikura et al. and by Hosomura et al. [18, 30]. In consequence, KC can be used for the experiments on drug-mediated hepatocyte damage and its effect on KC.

Table 2: Summary of KC activation and reaction.

Donor	Drug tested	ROI formation	Cell activity	IL-6	TNF-a	IL-10	PGF-2
A	APAP	↑	—	↑	—	—	↓
	DIC	—	—	↓↓	↓	—	↓↓
B	APAP	↑	↑	—	↑	—	↓
	DIC	↑↑	↑	↑	↑	—	↓↓
C	APAP	↓	↑	—	—	—	↑
	DIC	↓↓	↑↑	—	↑	↑	↓↓

↑: slight increase, ↑↑: increase, ↓: slight decrease, ↓↓: decrease, and —: no change.
All data were compared to the control.

The evaluation of the response of KC to drug-mediated stress/damage in hepatocytes was tested in cultures from three different donors in two steps: in the first step, PHH were incubated with hepatotoxic compounds (APAP or DIC). The investigation of drug-mediated oxidative stress revealed APAP but not DIC-induced oxidative stress in all three donors. These results are in accordance with those from other studies showing higher ROS induction for APAP compared to DIC [26, 37]. However, the evaluation of cell viability evaluated by measurement of the cell activity showed no decrease in energy metabolism in PHH cultures after compound treatment. Therefore, the short incubation time and low concentration represent subtoxic conditions, which can induce cell stress, but did not lead to irreversible toxic effects. Moreover, we observed that the amplitude of cell stress was donor-dependent with age and preexistence of a liver damage.

In contrast, incubations of APAP and DIC in KC cultures from three different donors for 24 h showed no statistically significant KC activation. These results were plausible due to the requirement of CYP450 isoenzymes for the hepatotoxic mode of action of APAP and DIC, which were less expressed in KC [38]. However, a KC activation for 100 μM of APAP and for both compounds for 1 mM concentration was observable, though not statistically significant. Accordingly, we used 100 μM in our main study. The drug is first metabolized by the PHH before coming in contact with KC. Therefore, far lower concentrations of remaining parent compounds are expected in the transferred supernatants. Additionally, we used much lower incubation times. Therefore, a direct effect of hepatotoxic compounds on KC from remaining compound in the supernatants of drug-treated hepatocytes is unlikely.

In the second step, transfer of the supernatant of drug-treated PHH cultures to corresponding KC cultures revealed a donor- and compound-dependent activation of KC (see overview in Table 2). In general, we observed an increased KC activity correlating with the vulnerability of donors to hepatotoxicity as seen by APAP-mediated ROS induction. The KC activation measured on the signaling level demonstrated a change in ROI formation interpreted as pro- or anti-inflammatory signaling.

The readout for ROS measurement in PHH and KC following activation was partly very low and showed an obvious change only for one donor. Choosing subtoxic conditions (100 μM) to induce cell stress rather than cell death required short incubation times and low drug concentrations [26]. Hepatotoxic effects in this range of drug concentration are rather mild as shown in 3D coculture liver models [11]. However, the observed donor- and compound-dependent activation was confirmed by the measurement of specific pro- and anti-inflammatory cytokines (see overview in Table 2). We observed a proinflammatory KC reaction when supernatant from stressed/damaged hepatocytes from healthy donor tissue was used. In contrast, an increased concentration of anti-inflammatory cytokines was detected if the supernatant was obtained from liver tissue with a preexisting liver damage. These results indicate that PHH from older and diseased donors are more vulnerable to toxic compounds than PHH from younger donors. This is in accordance with observations demonstrating that detoxification capabilities decrease with ongoing age [39]. While healthy donors showed tendencies towards proinflammatory reactions a clear anti-inflammatory reaction was observed in a donor with preliminary tissue damage due to cholestasis as a result from his tumor. This finding is in accordance with studies showing that, in chronic or preexisting liver damage, KC silence an additional inflammatory signal to avoid overreaction [33, 40, 41]. The observed compound-specific effects could be due to hapten formation in case of DIC-mediated hepatotoxicity, which has a stronger impact on KC activation and reaction than cell stress mediated by ROS formation from APAP. In this context, KC are capable of detecting drug-mediated cell stress at an early stage even before cell damage by hepatotoxicity occurs. In our experimental setup, only soluble mediators from the PHH supernatant can be responsible for the induction of the immunologic reactions in KC, like, for example, cytokines, endogenous proteins, and haptens. Experiments using hepatotoxic compounds and their corresponding drug-protein adducts have revealed a regulating role of KC [33]. Other studies showed KC activation by cytokines released as a response to different compounds, like, for example, oncostatin [24], LPS [18, 20], or HCV-related proteins [30]. These data confirm that KC allow for detection of immunological signals as a first reaction to drug-mediated hepatocyte stress/damage.

The low number of cases of this study is a major limitation even though the results were evaluated by means of different correlating readout parameters. Additionally the use of primary human cells led to large variations in some experiments,

for example, the LPS stimulations. Even if we observed clear trends in our results, statistical significance is missing in some cases. We conclude that KC related immunologic reactions are donor-specific and that the complex *in vitro* model consisting of primary human cells is influenced by many patient-related factors. Therefore, the results have to be considered on a case by case basis until further donors are investigated to validate these data.

DILI is described as an idiosyncratic reaction towards specific drugs and its prediction is difficult [6]. The results from this study suggest that using KC as detector cells a hepatotoxic risk can be estimated and reflects compound- and donor-specific effects. Moreover, this hepatotoxic stress is also measurable when subtoxic concentrations of hepatotoxic drugs are investigated. This is the first study using human KC for detection of hepatotoxic stress/damage induced by DILI compounds. It is known that existing *in vitro* models for the investigation of hepatotoxicity using PHH monocultures are not capable of reflecting the *in vivo* toxicity. The models suffer in general from the need of much higher concentrations of toxic drugs to induce hepatotoxicity compared to the *in vivo* situation. The effect on ROS induction leading to cell stress and KC activation observed for APAP was less pronounced in comparison to DIC. We suggest that DIC tends to formation of protein adducts rather than ROS induction. Classical hepatotoxicity testing measuring cell viability and oxidative stress does not capture a DILI risk in this case. Therefore, cocultures of PHH and KC could be used as a tool for evaluation of a DILI risk based on different mechanisms of actions. Using liver cells from different donor groups would also allow for the investigation of donor-specific effects. Thus, our established liver model is a useful tool for the investigation of hepatotoxic effects of DILI compounds and could contribute to an improved drug safety in drug development.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

Postmitotic Expression of SOD1^{G93A} Gene Affects the Identity of Myogenic Cells and Inhibits Myoblasts Differentiation

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To determine the role of mutant SOD1 gene (SOD1^{G93A}) on muscle cell differentiation, we derived C2C12 muscle cell lines carrying a stably transfected SOD1^{G93A} gene under the control of a myosin light chain (MLC) promoter-enhancer cassette. Expression of MLC/SOD1^{G93A} in C2C12 cells resulted in dramatic inhibition of myoblast differentiation. Transfected SOD1^{G93A} gene expression in postmitotic skeletal myocytes downregulated the expression of relevant markers of committed and differentiated myoblasts such as MyoD, Myogenin, MRF4, and the muscle specific miRNA expression. The inhibitory effects of SOD1^{G93A} gene on myogenic program perturbed Akt/p70 and MAPK signaling pathways which promote differentiation cascade. Of note, the inhibition of the myogenic program, by transfected SOD1^{G93A} gene expression, impinged also the identity of myogenic cells. Expression of MLC/SOD1^{G93A} in C2C12 myogenic cells promoted a fibro-adipogenic progenitors (FAPs) phenotype, upregulating HDAC4 protein and preventing the myogenic commitment complex BAF60C-SWI/SNF. We thus identified potential molecular mediators of the inhibitory effects of SOD1^{G93A} on myogenic program and disclosed potential signaling, activated by SOD1^{G93A}, that affect the identity of the myogenic cell population.

1. Introduction

The function of the metalloenzyme SOD1 is to convert superoxide, a toxic by-product of mitochondrial oxidative phosphorylation, to water or hydrogen peroxide. However, alteration in wild type SOD1 expression or mutations in the gene have been held responsible for the activation of catabolic pathways associated with degenerative diseases, including amyotrophic lateral sclerosis (ALS) [1]. ALS is a disorder involving the degeneration of motor neurons, muscle atrophy, and paralysis [1]. In few familiar forms of ALS, mutations in SOD1 gene have been associated with the pathogenesis of the disease [1]. Initially it has been suggested that mutation in SOD1 gene led to a decrease in the protein enzymatic activity (loss of function hypothesis). However, subsequent studies have clarified that mutant SOD1 possesses a neurotoxic property (gain of function hypothesis) responsible for the pathogenic mechanism of the disease [2]. Indeed, the finding that overexpression of mutant SOD1 in transgenic mice recapitulates several clinical features of ALS disease even in the presence of endogenous mouse SOD1 has led to the conclusion that the disease results from a toxic gain of function [3]. Mutations in SOD1 that impair its functions may lead to increased oxidative damage, promoting the activation of apoptotic pathways.

Oxidative stress plays an important role in the physiopathology of tissues. The effects of the reaction oxidative species (ROS) are dose-dependent, and low ROS concentration is necessary to guarantee cellular homeostasis while high ROS dose exerts toxic effects on the cells and may contribute to cellular dysfunction. Indeed, oxidative stress is a hallmark of aging and several chronic diseases such as Alzheimer's disease, Duchenne dystrophy, and ALS [4]. How such an oxidative insult plays a direct role in the disease-related decrease of muscle performance and mass remains

largely unknown. In addition, the discrepancy among different studies has further complicated the achievement of a conclusive link between altered balance of ROS generation and altered homeostasis-associated diseases.

In a previous work we demonstrated that muscle specific expression of the mutant isoform of SOD1 gene ($SOD1^{G93A}$) induces muscle atrophy associated with a significant reduction in muscle strength and alterations in the contractile apparatus [5]. We provided evidences that muscle-restricted expression of $SOD1^{G93A}$ gene is sufficient to increase oxidative stress and to induce a reduction in protein synthesis and the activation of proteolytic pathway [6].

It has been demonstrated that lactate-induced oxidative stress delays C2C12 differentiation [7] while treatment of the same cell line with resveratrol, that confers resistance against oxidative stress, promotes myogenesis and hypertrophy [8]. Interestingly, high glucose-induced oxidative stress has been correlated with lipid deposition in muscle derived stem cells leading to their adipogenic differentiation [9].

In this study, we address the role of the toxic effect of mutant SOD1 gene ($SOD1^{G93A}$) on *in vitro* myogenic program and we demonstrate that $SOD1^{G93A}$ expression prevents myoblasts differentiation and retains C2C12 cells in an undifferentiated state that show features common to fibro/adipogenic cells.

2. Materials and Methods

2.1. Generation of C2C12 MLC/SOD1^{G93A}. C2C12 cells were stably transfected with pPURO and pMexMLC/$SOD1^{G93A}$ plasmids (ratio 1 : 10) by using SuperFect Transfection Reagent (Qiagen) according to the manufacturer's instructions, as control C2C12 cells were also transfected with pPURO and pMex empty vector. After 1 day from transfection the medium was replaced with fresh medium containing puromycin 3 μg/mL (Sigma Aldrich). After 48 hours the cells were split 1 : 10 into the selective medium. The medium was changed every 2 days for 8 days. Single clones were picked, plated in 60 mm dishes, and expanded.

2.2. Cell Culture. C2C12 and C2C12 MLC/$SOD1^{G93A}$ cells were maintained in Dulbecco's modified Eagle's medium (DMEM) with 4.5 g/L glucose, L-glutamine (Sigma), supplemented with 15% fetal bovine serum (Gibco), 100 U/mL penicillin (Sigma Aldrich), 100 μg/mL streptomycin (Sigma Aldrich), and 2,5 μg/mL of puromycin (Sigma Aldrich) for C2C12 MLC/$SOD1^{G93A}$ cells. To induce differentiation, cells were shifted to differentiation medium (DM), DMEM with 2% horse serum (Gibco). Cells were harvested at D0, D2 (48 h after shift in DM), and D5 (120 h after shift in DM).

2.3. Protein Extraction and Western Blot Analysis. C2C12 and C2C12 MLC/$SOD1^{G93A}$ cells grown in 60 mm culture dishes were washed twice with cold phosphate-buffered saline, pelleted, resuspended in 100 uL of modified lysis buffer (Tris-HCl, ph 7.5/20 mM, EDTA/2 mM, EGTA/2 mM, sucrose/250 mM, DTT/5 mM, Triton-X/0.1%, PMSF/1 mM, NaF/10 mM, SOV4/0.2 mM, and cocktail protease inhibitors/1X (Sigma Aldrich)), and processed for western blot analysis. Filters were blotted with antibodies against hSOD1 (Santa Cruz), gp91phox (BD) anti-Perilipin 2 (Lifespan Biosciences), phospho-p42/p44 MAPK (Millipore), p42/p44 MAPK (Cell Signaling) phospho-Akt (Thr 308) (Sigma Aldrich), Akt (Cell Signaling), phospho-P70 (Thr389) (Cell Signaling), p70 (Cell Signaling), and HDAC4 (Cell Signaling). Protein levels of α-tubulin were used as control for equal protein loading. Signals were acquired by ChemiDoc-It Imaging System (UVP, LLC) and the analysis was performed using VisionWorks LS Image Acquisition analysis software.

2.4. RNA Extraction and Quantitative RT-PCR. Total RNA extraction was performed using TRIzol reagent method (Sigma Aldrich) as described by the manufacturers. MicroRNA (miRNA) was reverse-transcribed using the TaqMan MicroRNA Reverse Transcription Kit (Life Technologies), and mRNA was reverse-transcribed using QuantiTect Reverse Transcription Kit (Qiagen). The reverse-transcription reactions were performed according to the manufacturers' instructions. Quantitative PCR was performed on an ABI PRISM 7500 SDS (Life Technologies), using premade 6-carboxyfluorescein- (FAM-) labeled TaqMan assays for beta actin, Pax7, Pax3 MyoD, Myogenin, MRF4, and Smarcd3 (Life Technologies). FAM-labeled TaqMan MicroRNA Assays for miR1, miR133a, miR206, and U6 snRNA (Applied Biosystems, USA) were performed as described. Quantitative RT-PCR sample values were normalized to the expression of beta-actin or U6 snRNA for mRNA and microRNA, respectively. The relative level for each gene and miRNA was calculated using the 2-DDCt method [10] and reported as mean fold change in gene expression.

2.5. Lipid Staining. Lipid accumulation was visualized by Oil Red O staining. Cells were fixed in 4% paraformaldehyde for 1 h. After being washed with ddH2O, cells were treated with 100% propylene glycol for 5 min and stained with a filtered Oil Red O solution (0.5% Oil Red O in propylene glycol) for 8 min at 60°C. The cells were treated with 85% propylene glycol solution for 5 min, washed twice with ddH2O, and mounted with glycerol. All reagents for this staining were from Sigma Aldrich. Samples were visualized using an inverted microscope (Axioskop 2 plus; Carl Zeiss MicroImaging Inc.).

2.6. Trichostatin Treatment. C2C12 and C2C12 MLC/$SOD1^{G93A}$ cells were exposed to 100 nm (Sigma Aldrich) Trichostatin A (TSA) in GM (growth medium) for 24 h. TSA was removed and the cells were analyzed for myosin expression (at day 5 in DM) or by cytofluorimetric profile (at day 2 in DM).

2.7. Morphometric Analysis. Cells were fixed in 4% paraformaldehyde and incubated overnight at 4°C with primary antibody against MHC (MF-20 Hybridoma Bank); nuclei were visualized using Hoechst staining. Samples were viewed under an inverted microscope (Axioskop 2 plus; Carl

Zeiss MicroImaging Inc.). To quantify the differentiation and fusion of control and TSA treated we calculated the differentiation index as the percentage of MHC-positive cells above total nuclei and the fusion index as the average number of nuclei in MHC-positive cells with at least three nuclei above total number of nuclei, respectively. The images were analyzed using ImageJ software.

2.8. Flow Cytometry. C2C12 and C2C12 MLC/SOD1^{G93A} were detached from culture with 0.25% trypsin, 2 mM EDTA (Sigma Aldrich) at indicated times. The isolated cells were then filtered through a 40 μm cell strainer (Falcon) and incubated with the following antibodies (10 ng/mL): CD31-PECy7, CD45-eFluor 450 (eBioscience), Sca-1-FITC (Macs), and α7 integrin-PE (R&D Systems Inc.). A subsequent incubation with 7-aminoactinomycin D (1/1000; Sigma) was performed to stain nonviable cells. For each analysis, data were collected from 10,000 cells and analyzed on CyAN ADP (Dako) flow cytometer using the Summit 4.3 software (Dako).

2.9. Statistical Analysis. Statistical analysis was performed with GraphPad Prism Software. All data are expressed as mean ± SEM. Groups were compared using nonparametric tests (Mann Whitney Rank Sum test) and Student's *t*-test. A *P* value of <0.05 was considered statistically significant.

3. Results

3.1. Muscle Specific Expression of Mutant SOD1 Gene Prevents Differentiation of C2C12 Cells. To investigate the role of mutant SOD1^{G93A} gene in myoblast differentiation we stably transfected the C2C12 cells line with the MLC/SOD1^{G93A} expression cassette (Figure 1(a)) that allows the expression of the mutated isoform of SOD1 gene under the control of the myosin light chain promoter [11]. As expected, SOD1^{G93A} transgene expression was accumulated in C2C12 MLC/SOD1^{G93A} cells induced to differentiate (Figure 1(b)). The postmitotic expression of SOD1^{G93A} transgene induced the accumulation of Gp91 protein, a marker of mitochondria oxidative damage (Figure 1(c)) that might mediate the toxic properties of mutant SOD1 on muscle differentiation and homeostasis. To explore if SOD1^{G93A} transgene expression directly interferes with muscle differentiation, we stimulated differentiation by shifting the cells from growth to the differentiation medium (DM) (Figure 2(a)). Morphological and immunofluorescence analysis revealed a dramatic inhibition of muscle differentiation in C2C12 MLC/SOD1^{G93A} cells, with significant reduction in the number and size of the myosin positive cells, compared with control C2C12 myotubes (Figures 2(a)-2(b)). Morphometric analysis revealed a complete inhibition of fusion index, quantified as the percentage of Hoechst-stained nuclei located within multinucleated cells, positive to sarcomeric myosin (Figure 2(b)). The altered differentiated muscle phenotype was also confirmed by western blot analysis, revealing a drastic downmodulation of the sarcomeric myosin heavy chain expression, a specific marker of myogenic differentiation (Figure 2(c)). Of note, C2C12 cells stably transfected with wild type SOD1 cassette

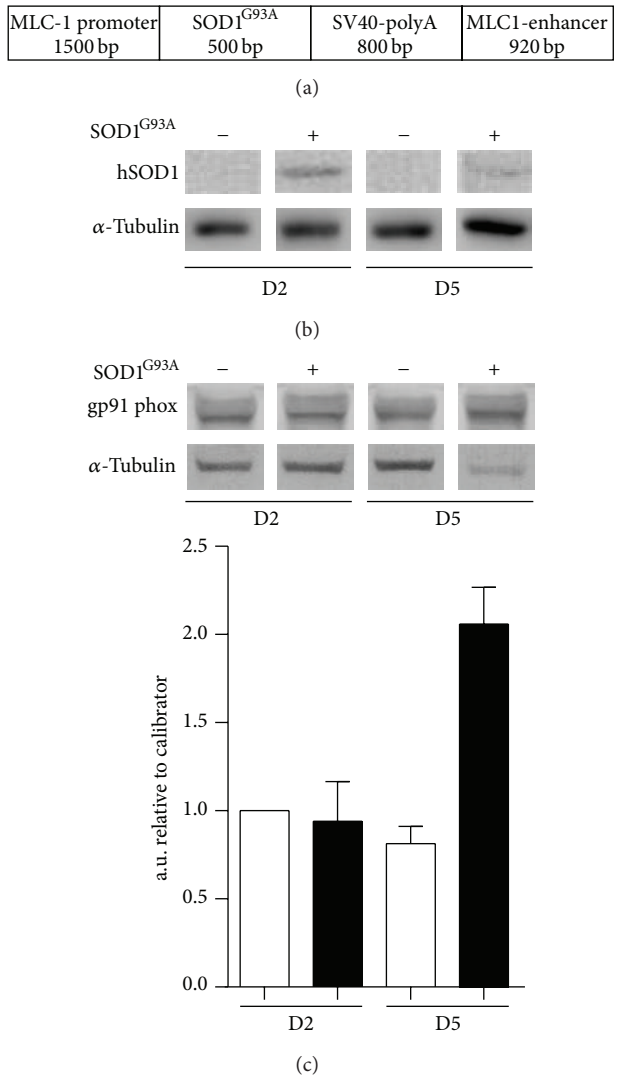


Figure 1: Characterization of C2C12 MLC/SOD1^{G93A}. (a) Schematic representation of MLC/SOD1^{G93A} construct. (b) Western blot analysis for mutant human SOD1^{G93A} protein in C2C12 and C2C12 MLC/SOD1^{G93A} at different time points during differentiation. (c) Upper panel shows representative western blot analysis of gp91phox expression in C2C12 and C2C12 MLC/SOD1^{G93A} cells. Lower panel shows the relative densitometric analysis of C2C12 (white bars) and C2C12 MLC/SOD1^{G93A} (black bars). D2 and D5 referred to days of culture in differentiation condition. Data are represented as mean ± SEM.

(MLC/SOD1^{Wt}) did not show any morphological differences compared to control C2C12 cell lines (data not shown).

A key myogenic factor that triggers myoblast differentiation is MyoD [12, 13], which resulted in significant downregulation throughout the time course of differentiation in C2C12 MLC/SOD1^{G93A} cells (Figure 3(a)). Myogenin is the myogenic factor that functions downstream of MyoD and plays a critical role in triggering terminal differentiation process of myoblasts [12, 14]. Myogenin expression resulted in significant downregulation in C2C12 MLC/SOD1^{G93A} cells during differentiation (Figure 3(b)). The final stage of

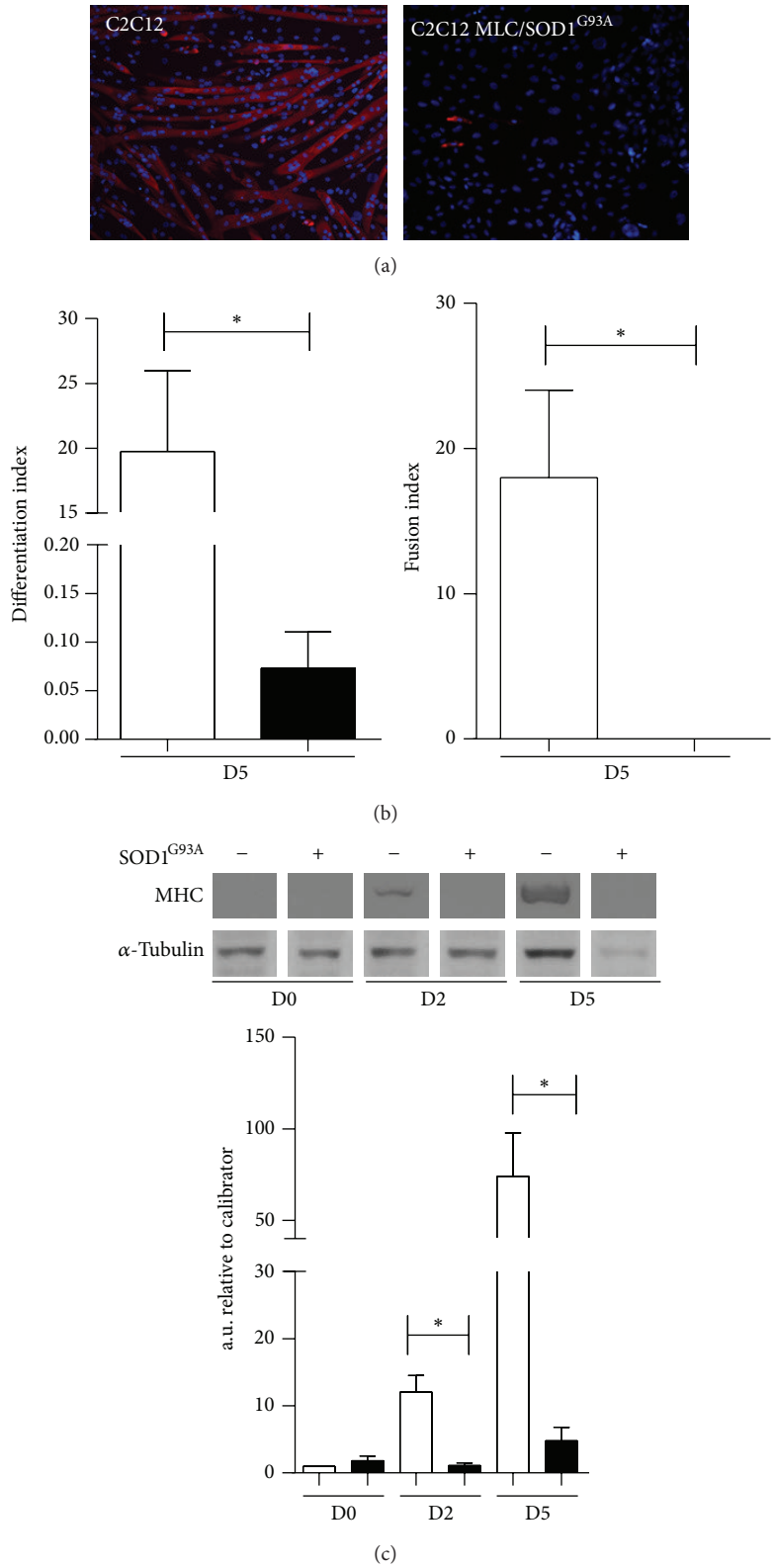


Figure 2: Postmitotic expression of mutant SOD1 gene inhibits C2C12 cells differentiation. (a) Representative immunofluorescence images of C2C12 and C2C12 MLC/SOD1^{G93A} cells stained with anti-myosin heavy chain (MHC) antibody after 5 days in differentiation medium. (b) The histograms represent the differentiation index (left panel) and fusion index (right panel) in control (white bars) and transfected cells (black bars). (c) Upper panel shows representative western blot analysis of MHC expression in C2C12 and C2C12 MLC/SOD1^{G93A} cells. Lower panel shows densitometric analysis for MHC expression in C2C12 (white bars) and C2C12 MLC/SOD1^{G93A} (black bars). D0, D2, and D5 referred to days of culture in differentiation condition. Data are represented as mean ± SEM ($^{*}P < 0.05$).

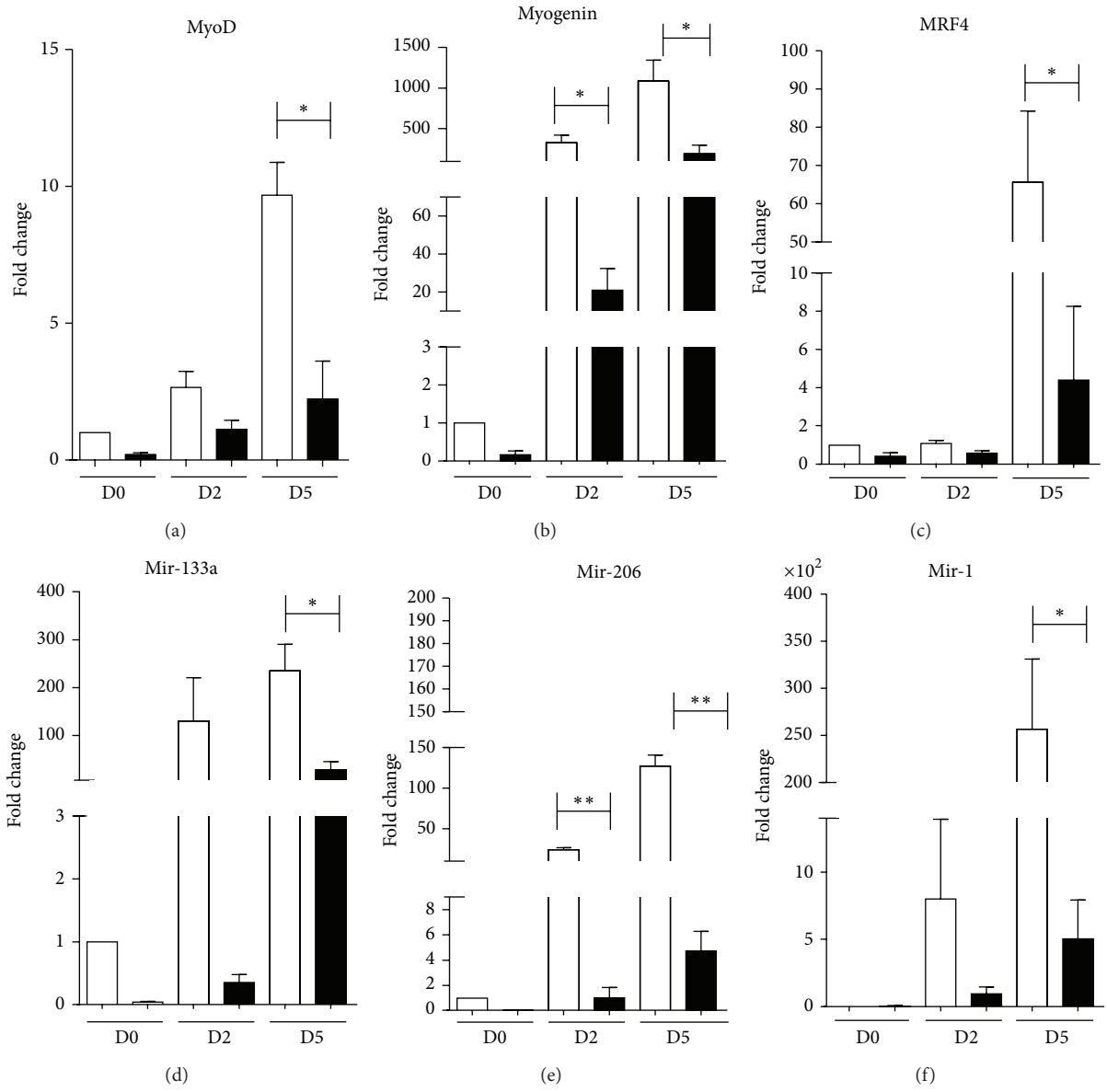


Figure 3: Mutant SOD1 gene downmodulates the players involved in C2C12 differentiation. Real time PCR for MyoD (a), Myogenin (b), MRF4 (c), miR133a (d), miR206 (e), and miR1 (f) in both control (white bars) and transfected cells (black bars). D0, D2, and D5 referred to days of culture in differentiation condition. Data are represented as mean ± SEM ($^{*}P < 0.05$; $^{**}P < 0.005$).

skeletal muscle differentiation and maturation program is also dependent on the concerted action of another myogenic factors, namely, MRF4, which promotes the activation of myosin heavy chain expression [12]. Real time PCR analysis revealed a significant reduction of MRF4 transcripts in C2C12 MLC/SOD1^{G93A} cells during differentiation, compared to control C2C12 myotubes (Figure 3(c)).

Recent works have shown that among genes which are important for proper muscle differentiation and function, microRNAs (miRNAs) play a crucial role [15–17]. Among them miR133, miR206, and miR1 are abundantly expressed in muscle tissue and specifically induced during myogenesis and C2C12 differentiation [18]. It has been reported that miR1 and miR133 are involved in a complex molecular mechanism by which miR1 induces the downmodulation of an inhibitor of muscle differentiation, namely, histone deacetylase (HDAC) 4 [19] and miR133, which is clustered on the same chromosomal loci of miR1 and enhances myoblast proliferation inhibiting the serum response factor (SRF). In addition, miR206 facilitates satellite cell differentiation [20, 21] by restricting its proliferative potential through the repression of Pax-7 expression [22]. These findings implicate these myomiRNAs in a complex regulatory loop to control cell proliferation, commitment, and differentiation. Real time PCR analysis revealed a significant downmodulation of miR133, miR206, and miR1 during the differentiation time course of C2C12 MLC/SOD1^{G93A} cells compared to control C2C12 cells (Figures 3(d), 3(e), and 3(f)).

3.2. Muscle Specific Expression of Mutant SOD1 Gene Perturbs Signaling Pathways of Muscle Differentiation. The activation of a specific developmental program requires the integration of multiple extrinsic signals from the cell membrane that culminate in changes of nuclear gene expression patterns. Among the known signal transduction intermediates in muscle cells, the serine/threonine kinase AKT and the mitogen-activated protein kinases (MAPK) have been shown to modulate myogenic differentiation [23]. In a previous study [6], we have demonstrated that muscle specific expression of $SOD1^{G93A}$ gene in transgenic animals promotes a reduction of the phosphatidylinositol 3-kinase (PI3K)/Akt pathway and leads to muscle atrophy.

In this study, we explored whether the inhibitory effects of mutant SOD1 gene on myogenic program perturb the relevant signaling pathways of the myogenic program. Figure 4 shows that the absolute ratio of pAkt/Akt was negatively regulated (Figure 4(a)) and pP70/P70, the downstream effector of AKT, was significantly reduced in C2C12 MLC/$SOD1^{G93A}$ cultures compared with control C2C12 cells (Figure 4(b)). In addition we observed a significant downmodulation of the phosphorylated active form of a factor associated with MAPK differentiation cascade, namely, ERK1/2 (Figure 4(c)).

Overall these results demonstrate that the postmitotic expression of SOD1 mutant gene prevents C2C12 differentiation, affecting the activation of the muscle regulatory factors, muscle miRNA, and the signal transduction cascades responsible for myogenic differentiation, and might impinge the maintenance of the muscle phenotype.

3.3. Muscle Specific Expression of $SOD1^{G93A}$ Impinges the Identity of Muscle Cells and Promotes a FAPs Phenotype in C2C12 Myogenic Cells. To validate this hypothesis, we analyzed the expression of Pax-7, which is a key factor that triggers the specification of uncommitted skeletal muscle progenitors to myogenic cells [24]. Of note, we observed a complete inhibition of Pax-7 expression in C2C12 MLC/$SOD1^{G93A}$ cells compared to C2C12 myoblasts, suggesting that $SOD1^{G93A}$ expression not only inhibits muscle differentiation but also confers to transfected cells an immature state (Figure 5(a)).

Recently, it has been demonstrated that satellite cells in adult muscle are bipotential stem cells that can give rise to brown adipogenic as well as myogenic progenitors [25]. The lineage switch between myogenic and brown adipogenic commitment is controlled by the muscle specific miR133a, which is highly expressed in satellite cells and can repress the expression of adipogenic markers to enforce myogenic commitment in satellite cells [25].

Further, recent evidences revealed that Pax-3 transcription factor, whose ectopic expression in C2C12 myoblasts efficiently inhibits myogenic specification [26], plays a pivotal role during differentiation into adipocytes cells of human-induced pluripotent stem cells [27]. In addition, the exclusive expression of Pax-3 or MyoD gene in stem cells allows a clear and distinct choice between myogenic and fibro/adipogenic potential cell lineage [28]. Based on these evidences, we performed real time PCR and histochemical analysis in both C2C12 and C2C12 MLC/$SOD1^{G93A}$ cells to evaluate the potential adipogenic features in C2C12 MLC/$SOD1^{G93A}$ transfected cell lines. As shown in Figure 5(b) the levels of Pax-3 transcript were significantly upregulated in C2C12 MLC/$SOD1^{G93A}$ during all stages in culture, compared to C2C12. Moreover Oil Red O staining revealed the accumulation of intracellular lipid droplets in C2C12 MLC/$SOD1^{G93A}$ cells (Figure 5(c)). These data were corroborated by western blot analysis for Perilipin 2 (Plin2), a marker of fatty acids uptake and storage [29]. Western blot analysis revealed that Plin2 protein was accumulated in C2C12 MLC/$SOD1^{G93A}$ during the differentiation process compared to control cell line (Figure 5(d)). All these results together with the data of miR133a and MRFs downmodulation in C2C12 MLC/$SOD1^{G93A}$ (Figure 3) suggest that muscle specific expression of SOD1 mutant gene inhibits myoblasts differentiation and promotes adipogenic features in C2C12 cells through a miR133a and Pax-3 dependent mechanism.

Fibro-adipogenic progenitors (FAPs) are multipotent mesenchymal cells residing in skeletal muscle interstitium [30–32]. These cells are negative for CD31, CD45, and $\alpha 7$ integrin surface antigens and are characterized by the expression of the stem cell antigen 1 (Sca1) [30]. FAPs convert environmental cues into signals that modulate muscle regeneration or turn themselves into fibro-adipocytes, inducing fat deposition and fibrosis under pathologic conditions, such as dystrophic muscles [33].

To verify whether postmitotic expression of SOD1 mutant gene promotes a FAPs phenotype in C2C12 cells, we performed FACS analysis (Figure 6(a)) for Sca1, CD31, CD45, and $\alpha 7$ integrin. C2C12 and C2C12 MLC/$SOD1^{G93A}$ cells were negative for CD31 and CD45 antigens (data not shown). C2C12 were mainly $\alpha 7$ integrin$^+$ and a low percentage of them was Sca1$^+$ and $\alpha 7$ integrin$^-$ (Figures 6(a)–6(c)). In contrast, C2C12 MLC/$SOD1^{G93A}$ were mainly Sca1$^+$ and $\alpha 7$ integrin$^-$, with a significant reduction in the number of $\alpha 7$ integrin$^+$ cells (Figures 6(a)–6(c)). These results clearly evidenced the presence of fibro/adipogenic features in C2C12 MLC/$SOD1^{G93A}$. The molecular mechanism that, in concert with environmental cues, controls the identity and activity of FAP cells involves a HDAC-regulated network; this network consists of muscle specific miRNAs that target two alternative variants of the SWI/SNF chromatin remodeling complex, BAF60A and BAF60B, and favor the formation of a BAF60C-based SWI/SNF complex able to confer on MYOD the ability to activate the myogenic program [34].

We analyzed the protein and transcript levels, respectively, of HDAC4 and BAF60C in both C2C12 and C2C12 MLC/$SOD1^{G93A}$ cells. We observed that HDAC4 was significantly upregulated in cultures of C2C12 MLC/$SOD1^{G93A}$ cells compared to control differentiated (day 5 in DM) C2C12 cells (Figure 7(a)). In contrast, BAF60C was significantly downregulated in C2C12 MLC/$SOD1^{G93A}$ cells (Figure 7(b)).

To better correlate the induction of FAPs phenotype and HDAC activity with $SOD1^{G93A}$ expression and toxic properties, we treated C2C12 MLC/$SOD1^{G93A}$ cells with the HDAC class II inhibitor Trichostatin (TSA) and analyzed the signature profile of both FAPs and myogenic cells.

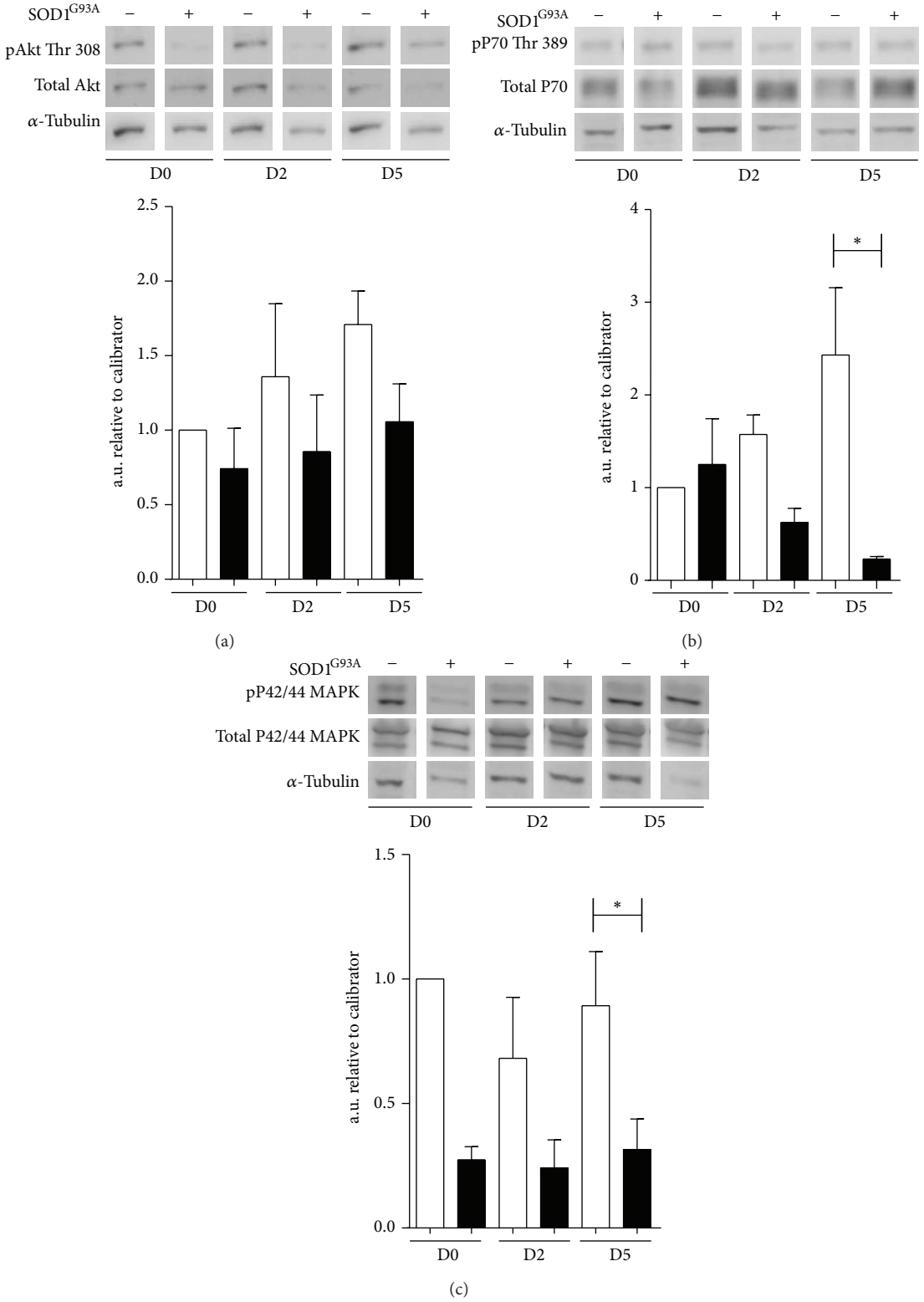


Figure 4: Muscle specific expression of human mutant SOD1 gene alters signaling pathways in muscle differentiation. (a) Representative western blot analysis for phospho-Akt (Thr 308) and total Akt expression (upper panel) and densitometric analysis (lower panel) of the ratio between phosphorylated Akt and the total form (lower panel) in C2C12 (white bars) and C2C12 MLC/SOD1^{G93A} (black bars). (b) Representative western blot analysis for phospho-P70 (Thr 389) and total P70 expression (upper panel) and densitometric analysis (lower panel) of the ratio between phospho-P70 and total P70 (lower panel) in C2C12 (white bars) and C2C12 MLC/SOD1^{G93A} (black bars). (c) Representative western blot analysis of phospho-p42/44 MAPK and p42/44 MAPK expression (upper panel) and densitometric analysis (lower panel) of the ratio between phospho-p42-44 MAPK and total p42/44 MAPK in C2C12 (white bars) and C2C12 MLC/SOD1^{G93A} (black bars). D0, D2, and D5 referred to days of culture in differentiation condition. Data are represented as mean ± SEM ($^{*}P < 0.05$).

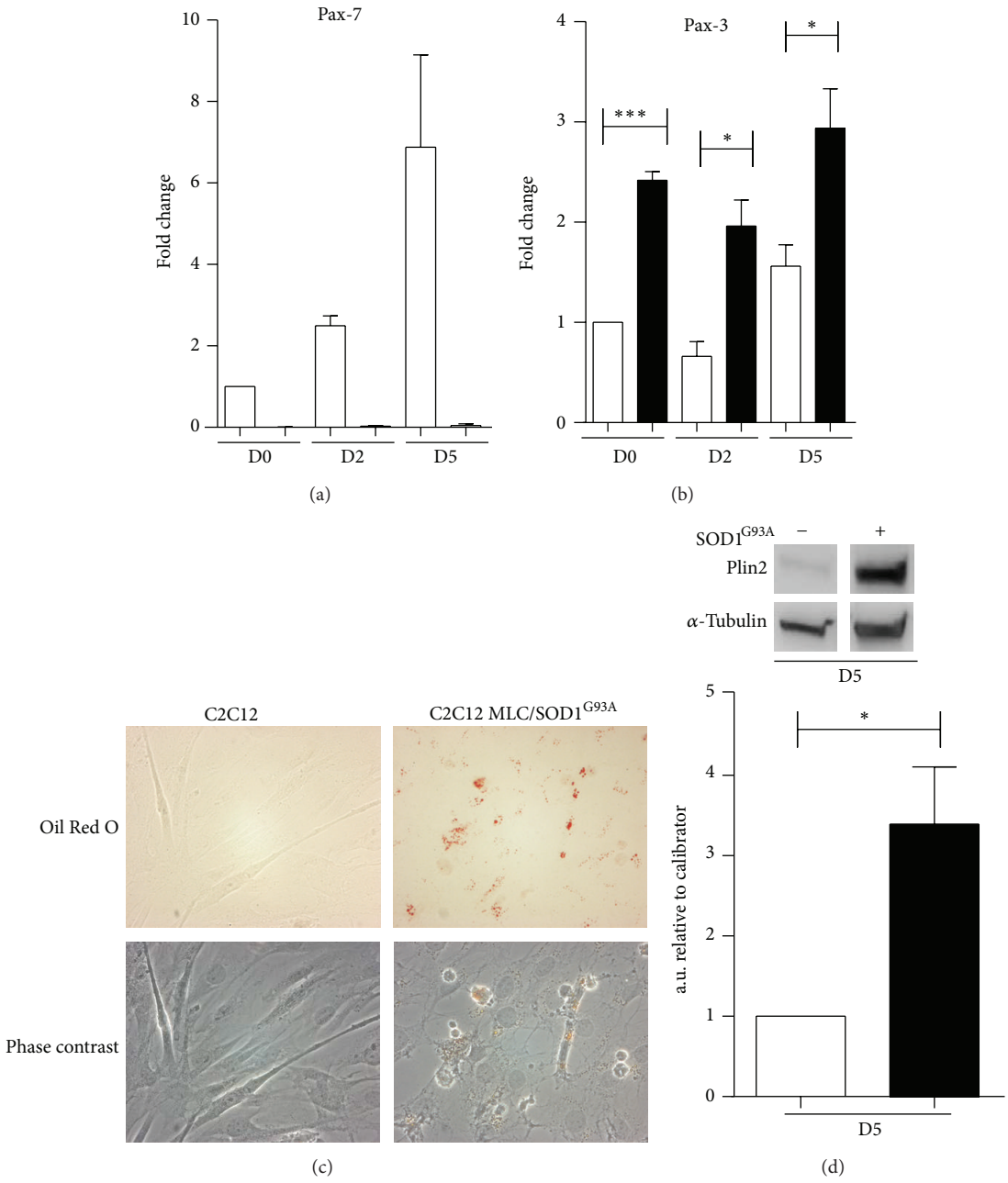


FIGURE 5: C2C12 MLC/SOD1^{G93A} cells exhibit adipogenic features. Real time PCR analysis for Pax7 (a) and Pax3 (b). (c) Upper panel shows Oil Red O staining and phase contrast of control C2C12 and C2C12 MLC/SOD1^{G93A} transfected cells at day 5 in culture. (d) Upper panel shows representative western blot analysis of Plin2 and relative densitometric analysis (lower panel) of the ratio between Plin2 and α-tubulin in C2C12 (white bars) and C2C12 MLC/SOD1^{G93A} (black bars). D0, D2, and D5 referred to days of culture in differentiation condition. Data are represented as mean ± SEM ($^{*}P < 0.05$; $^{***}P < 0.0005$).

Cytofluorimetric profile revealed that TSA treatment induced a significant downmodulation of Sca1^{+} and α7 integrin^{-} cells (Figure 7(c)) and an increased percentage of double positive Sca1^{+} and α7 integrin^{+} cells (Figure 7(d)). Of note we did not observe any significant difference in the number of α7 integrin^{+} Sca1^{-} cells after treatment (data not shown) suggesting that TSA treatment is able to counteract the establishment of a FAP phenotype but it only partially rescued the muscle phenotype. To support this evidence, we induced muscle differentiation in C2C12 MLC/SOD1^{G93A} cells treated with TSA and we revealed that inhibition of HDAC activity partially rescued myogenic differentiation, promoting myoblast fusion and differentiation (Figures 7(e)-7(f)).

Overall these data suggest that the postmitotic expression SOD1^{G93A} mutant gene promotes a FAPS phenotype in C2C12 cells, by upregulating HDAC4 protein and preventing the BAF60C-SWI/SNF complex myogenic commitment.

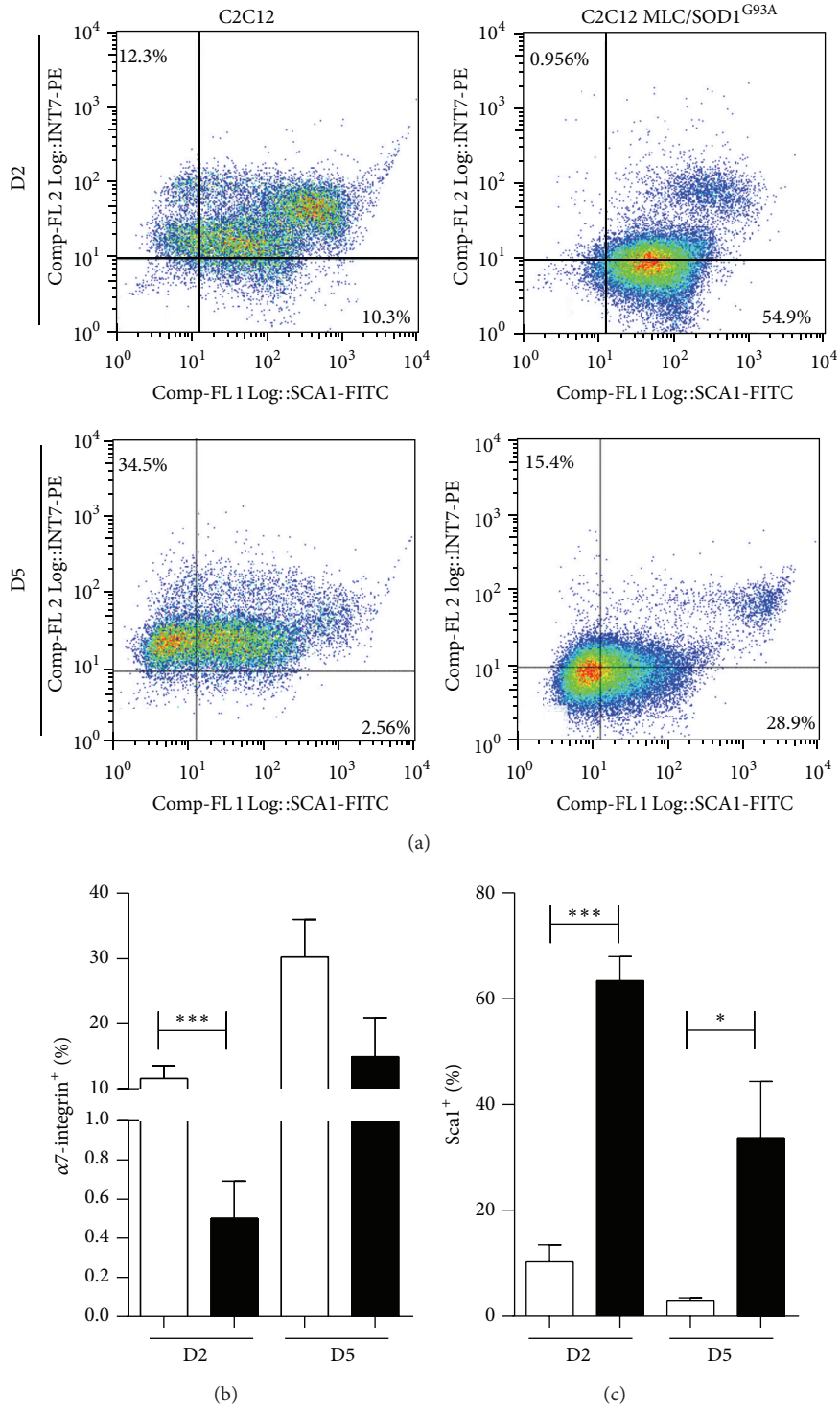


Figure 6: Expression of mutant SOD1 promotes a FAPS phenotype in C2C12 cells. (a) Flow cytometry profile for $\alpha 7$ integrin and Sca1 expression from control C2C12 and transfected C2C12 MLC/SOD1^{G93A} cells at days 2 and 5 in differentiation medium. (b, c) Histograms of the percentage of $\alpha 7$ integrin (b) and Sca1 (c) positive cells of C2C12 (white bars) and C2C12 MLC/SOD1^{G93A} (black bars) cells. D2 and D5 referred to days of culture in differentiation condition. Data are represented as mean ± SEM ($^{*}P < 0.05$; $^{***}P < 0.0005$).

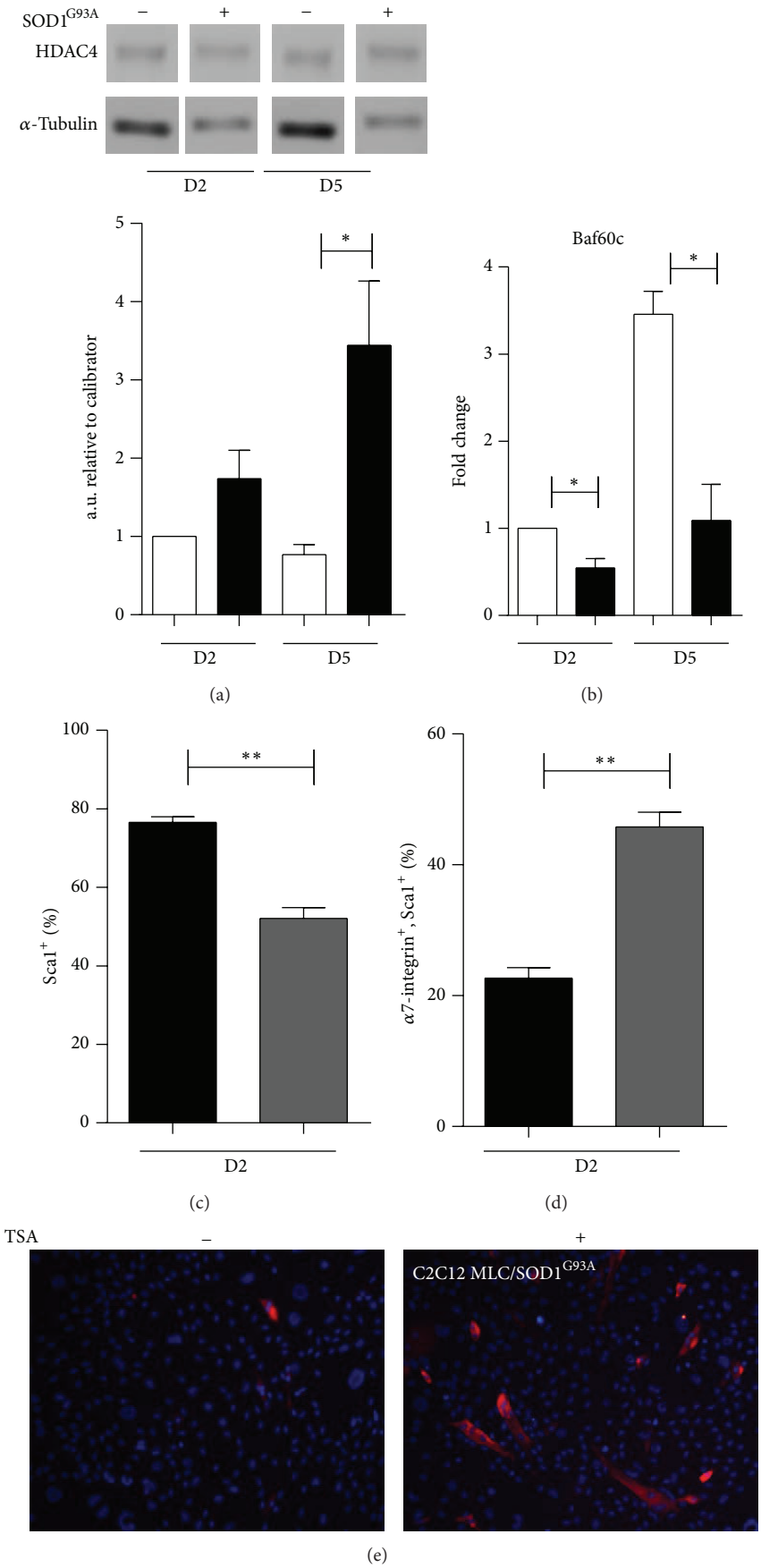
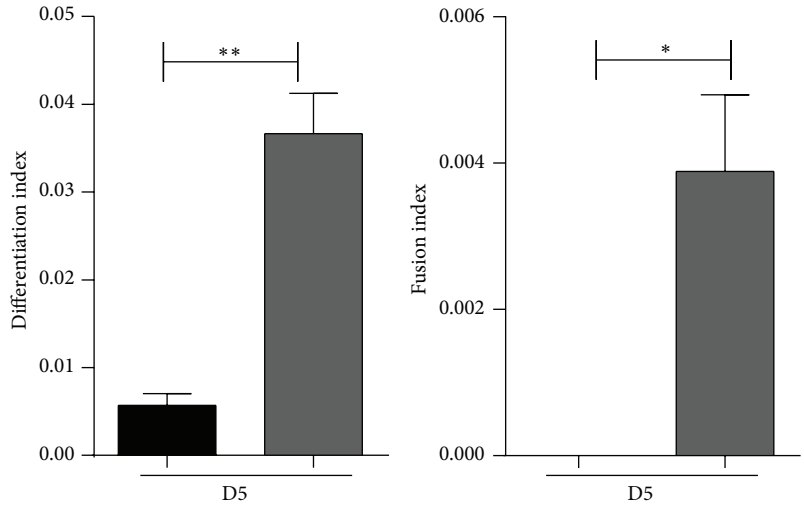


Figure 7: Continued.



(f)

Figure 7: The acquisition of fibro-adipogenic features involves a HDAC-regulated network. (a) Upper panel shows western blot analysis of HDAC4 expression in C2C12 and C2C12 MLC/SOD1^{G93A} cells and densitometric analysis (lower panel) of the ratio between HDAC4 and α-tubulin. (b) Real time PCR for BAF60C in C2C12 (white bars) and C2C12 MLC/SOD1^{G93A} (black bars). (c, d) Histograms of the percentage of Sca1 (c) and positive α7 integrin cells (d) of control C2C12 MLC/SOD1^{G93A} (black bars) cells and TSA treated C2C12 MLC/SOD1^{G93A} cells (grey bars). D2 referred to days of culture in differentiation condition. (e) Representative immunofluorescence analysis for MHC after 5 days in differentiation medium in untreated (left panel) and TSA treated (right panel) C2C12 MLC/SOD1^{G93A} cells. (f) Histograms of the differentiation index (left panel) and fusion index (right panel) in control (black bars) and treated cells (grey bars). D2 and D5 referred to days of culture in differentiation condition. Data are represented as mean ± SEM ($^{*}P < 0.05$; $^{**}P < 0.005$).

4. Discussion

In this work we defined the specific toxic effects of postmitotic expression of mutant SOD1^{G93A} gene on the myogenic program, demonstrating that mutant SOD1^{G93A} alters the identity of muscle cells, affects cell homeostasis, and inhibits muscle differentiation.

The physiological activity of the metalloenzyme SOD1 is to detoxify the cells from the accumulation of free radicals, converting superoxide, a toxic by-product of mitochondrial oxidative phosphorylation, to water or hydrogen peroxide. In contrast, the mutant SOD1 possesses a toxic property that is responsible for the pathogenic mechanism of ALS, a neurodegenerative disease associated with the degeneration of motor neurons, muscle atrophy, and paralysis.

Different studies also support the evidence that skeletal muscle is a primary target of mutant SOD1 toxicity in mice [5, 35], indicating that dysfunctions of affected muscle cells are not only a marginal consequence of denervation associated with motor neurons loss, but a direct consequence of cell muscle toxicity of mutant SOD1 [36]. The understanding of the mechanisms involved in mutant SOD1 toxicity in muscle may facilitate the design of treatments directed toward this specific tissue to treat ALS or at least to delay disease progression.

The aim of our work was to define the responses of myogenic cells to the toxic effects of SOD1^{G93A} and the signaling pathways that mediated the toxic properties of mutant SOD1 product. To this purpose, we generated a stable transfected C2C12 cell line, overexpressing the mutated isoform of the SOD1 gene under the control of the MLC promoter [11, 37].

Postmitotic expression of SOD1^{G93A} gene induced an excess of oxidative stress, as evidenced by the increased expression of gp91 protein, and impaired muscle differentiation and fusion of C2C12 cells, inducing a significant downregulation in the expression of molecular markers of myogenic differentiation, like MyoD, Myogenin, MRF4, and sarcomeric myosin heavy chain.

Epigenetic factors, including miRNAs expression, play important role in muscle homeostasis and represent good molecular markers to define the stage of myogenic program and to monitor the effects of toxic factors on the modulation of muscle phenotype. miR206, miR133a, and miR1 [18, 20, 38] contribute to the proper development of the myogenic program, and their alteration can impair the myogenic differentiation. Based on this evidence, we investigated their expression levels in C2C12 MLC/SOD1^{G93A} cells and demonstrated that transfected MLC/SOD1^{G93A} induced a significant downmodulation of all muscle microRNAs analyzed.

The myogenic program is governed by specific pathways of signal transduction. Here we have demonstrated that the inhibitory effect of the toxic SOD1^{G93A} protein on myogenic differentiation acts negatively on two major signaling pathways involved in muscle differentiation, such as the AKT/p70 and MAPK pathways.

These results suggest that muscle expression of SOD1^{G93A} impinges muscle differentiation and might alter the identity of muscle cells in line with previous studies that demonstrate

the impairment of myofiber-associated skeletal muscle satellite cells function in SOD1-G93A mice [39] and the altered expression of myogenic regulatory factors in the mouse model of amyotrophic lateral sclerosis [40].

Recent evidences indicate that ROS levels correlate with a high concentration of glucose. Aguiari and colleagues [9] have shown that high glucose growth medium induces an increase in ROS production and promotes the adipocyte differentiation of muscle derived stem cells [9].

Since C2C12 MLC/SOD1^{G93A} cells show higher levels of oxidative stress and impaired myogenic differentiation process, modulating relevant genes and myomiRNA of the maintenance of the muscle phenotype, we supposed a link between toxic properties of SOD1^{G93A} and activation of adipogenic differentiation. In particular, we demonstrated that transfected MLC/SOD1^{G93A} cells show low levels of miR133a, Pax-7, and MyoD expression and high levels of Pax-3 and Perilipin 2, a marker of lipid droplets.

Our data support the evidences by Yin and colleagues [25] who showed that miR133a controls satellite cells commitment to the adipocyte lineage; in this work the authors demonstrated that miR133a inhibition promotes proadipogenic differentiation of satellite cells and that the new-formed preadipocytes completely lose the expression of both Pax-7 and MyoD [41]. Moreover, Mohsen-Kanson et al. [27] demonstrated that Pax-3 plays a pivotal role during stem cell differentiation into adipocytes and that the exclusive expression of Pax-3 or MyoD allows cells to choose between myogenic and fibro/adipogenic cell lineage.

Our data indicate that muscle expression of SOD1^{G93A} induced deregulation of myogenic process, led to the impairment of myoblast differentiation, and promoted adipogenic commitment.

The fibro-adipogenic precursors or FAPs cells are bipotent cells positive for Sca1 and negative for $\alpha 7$ integrin, CD31, and CD45, markers of satellite cells, endothelial cells, and hematopoietic cells, respectively. FAPs are mesenchymal cells residing in skeletal muscle interstitium and quiescent in physiological conditions and are efficiently activated to proliferate after muscle injury [30]. FAPs convert environmental factors into specific signals that can modulate muscle regeneration [34]. It has been shown that FAPs may exacerbate the dystrophic phenotype turning into fibro-adipocytes, mediating fat deposition and fibrosis [33] and thereby disrupting the environment conducive for muscle regeneration.

The trigger that controls FAP lineage commitment and activity is currently unknown.

Interestingly, it has been demonstrated that, during ischemia-induced regeneration, oxidative stress negatively modulates myogenic differentiation [42] and it is well known that oxidative stress is a hallmark of chronic disease including the Duchenne muscle dystrophy where FAPs have been deeply studied.

Here we demonstrate, by histochemical assay and FACS analysis, the presence of lipid droplets in C2C12 MLC/SOD1^{G93A} cells and their significant and exclusive positivity for Sca1, demonstrating that C2C12 MLC/SOD1^{G93A} cells share common features with FAPs.

A recent study has revealed a link between HDACs, myomiRs, and chromatin remodeling that underlies FAPs commitment to the "promyogenic" phenotype [34]. The authors demonstrated that the signaling pathway that mediates the transition from myogenic to fibro/adipogenic phenotype requires the action of miR1, miR206, and miR133a, which favor the incorporation of specific core-protein, BAF60C, into the chromatin remodeling SWI/SNF complex. Moreover, the authors also demonstrated that in a murine model of Duchenne dystrophy treatments with the HDACs inhibitor (TSA) address FAPs to a proper myogenic fate, blocking the fibro-adipogenic one [34].

Since HDAC4 regulates FAPs fate and since our transfected cell line shares common features with FAPs, including higher levels of HDAC4 protein, lower levels of BAF60c, and deregulation of myomiRNA expression, we hypothesized that HDAC4 is responsible for C2C12 MLC/SOD1^{G93A} adipogenic choice. To prove our hypothesis we treated C2C12 MLC/SOD1^{G93A} cells with TSA and interestingly we observed a partial rescue of the myogenic phenotype. This suggests that inhibition of HDAC activity interferes with the establishment of a FAP phenotype and partially rescues muscle differentiation.

5. Conclusions

Three main conclusions can be drawn from the present work; the postmitotic expression of SOD1 mutant gene (1) induces the impairment of the myogenic differentiation process of C2C12 cells; (2) triggers the myoblast towards an adipogenic phenotype; (3) promotes FAPs features in C2C12 cells by epigenetic changes that involve HDACs proteins.

Further studies will clarify the different molecular mechanisms that are modulated by multiple toxic effects of mutant SOD1 protein in skeletal muscle and whether oxidative stress can represent a determinant for myoblasts choice toward a fibro/adipogenic fate.

Conflict of Interests

The authors do not have financial interest in relation to this paper.

Authors' Contribution

Martina Martini and Gabriella Dobrowolny contributed equally to this study.

Acknowledgments

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Research Article

Multidrug Resistance Protein-4 Influences Aspirin Toxicity in Human Cell Line

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Overexpression of efflux transporters, in human cells, is a mechanism of resistance to drug and also to chemotherapy. We found that multidrug resistance protein-4 (MRP4) overexpression has a role in reducing aspirin action in patients after bypass surgery and, very recently, we found that aspirin enhances platelet MRP4 levels through peroxisome proliferator activated receptor-α (PPARα). In the present paper, we verified whether exposure of human embryonic kidney-293 cells (Hek-293) to aspirin modifies MRP4 gene expression and its correlation with drug elimination and cell toxicity. We first investigated the effect of high-dose aspirin in Hek-293 and we showed that aspirin is able to increase cell toxicity dose-dependently. Furthermore, aspirin effects, induced at low dose, already enhance MRP4 gene expression. Based on these findings, we compared cell viability in Hek-293, after high-dose aspirin treatment, in MRP4 overexpressing cells, either after aspirin pretreatment or in MRP4 transfected cells; in both cases, a decrease of selective aspirin cell growth inhibition was observed, in comparison with the control cultures. Altogether, these data suggest that exposing cells to low nontoxic aspirin dosages can induce gene expression alterations that may lead to the efflux transporter protein overexpression, thus increasing cellular detoxification of aspirin.

Dedicated to the memory of Alberto Gulino, an extraordinary and unforgettable mentor, who very recently passed away

1. Introduction

Exposure of eukaryotic cells to drugs can trigger modifications in the expression of mechanisms susceptible to favour their elimination. In hepatocytes, this is most often related to the transient induction of the transcriptional regulation of genes by nuclear receptors [1–4].

Members of the ATP-binding cassette (ABC) transporter superfamily are widely recognized as major contributors to controlling drug distribution and pharmacokinetics and the acquisition of anticancer drug resistance [5].

In cancer cells, efflux transporters' overexpression is one mechanism of resistance to chemotherapy [6, 7]. *In vitro* exposure of cells for a prolonged period of time to anticancer agents shows a correlation between increasing concentrations of drugs and overexpression of transporters [3, 8].

This approach can be applied to many other drugs to identify the transporter responsible for their efflux and responsible for resistance mechanism.

A member of the MRP/ABCC subfamily of ATP-binding cassette transporters, which are capable of pumping a wide variety of endogenous (including cyclic nucleotides) and xenobiotic organic anionic compounds out of the cell, is MRP4, which can be upregulated to reduce intracellular organic anion toxicity or cholestasis [9].

We previously demonstrated that aspirin is a substrate for MRP4 in human platelets [10], and it was confirmed that both

aspirin and its salicylic acid (ASA) are substrates of mouse ABCC4 (MRP4) [11].

One of our recent studies showed aspirin ability to influence megakaryocytic gene expression, leading to upregulation of MRP4 in human platelets through the activation of the nuclear receptor PPARα [12], suggesting that even aspirin can activate mechanisms that favour its elimination and consequently reduce its toxic effect.

Acute salicylate poisoning is a common medical emergency, which carries a high mortality [13–15]. Salicylate poisoning remains a clinically hazardous therapeutically acquired intoxication at any age [16].

Aspirin poisoning is clearly dose related to increase toxicity in human subjects [17]. Daily aspirin use, whether regular strength or low dose, results in reduction in cancer incidence and mortality, although potential side effect profiles must be considered. It was suggested that one of the mechanisms by which aspirin is chemopreventive for cancer is its capability of inhibiting tumor cell proliferation [18]. In fact, aspirin toxicity results from the perturbation of the cell cycle and ultimately causes necrosis [19].

In this paper we showed that aspirin-dependent MRP4 overexpression effectively reduces the cytosolic concentration of aspirin in cells exposed to increasing concentrations of this drug, providing a simple resistance mechanism.

We have now examined how human cells would respond to stepwise exposure to increasing concentrations of drug either in basal or in enhanced efflux protein transporter expression (in the absence or in the presence of a detectable efflux transporter). Indeed, we observed reduced aspirin toxicity when the expression of MRP4 transporter is higher.

2. Material and Methods

2.1. Cell Line and Culture Conditions. Human embryonic kidney-293 cells, Hek-293 cell line, were maintained in DMEM supplemented with 10% heat-inactivated fetal bovine serum, 20 mM L-glutamine, 100 units/mL of penicillin G sodium, and 100 μg/mL streptomycin sulfate in a humidified atmosphere containing 5% CO_2 at 37°C. Cells were treated with aspirin, either to induce MRP4 expression (50 μM for 48 h) or to induce cell death (5 mM for 24 h) (SIGMA Chemicals Company, St. Louis, MO) to study either gene expression changes or aspirin toxicity, respectively. Control cells were treated with vehicle.

2.2. DNA Constructs and Transfection. The PCDNA 3.1-MRP4 vector expressing the human MRP4 protein was from Dr. Rius et al. [20]. Cell transfection was performed as previously described [21]; briefly, Hek-293 (1×10^6 cells plated in 60 mm dishes) cells were transfected with the indicated amount (0.5 μg) of MRP4 expression vectors by TransFectin Lipid Reagent (Bio-Rad Laboratories, Italy). An equivalent amount of transfection reagent (TransFectin Lipid Reagent, Bio-Rad), with PCDNA 3.1, was added as mock control. 24 hours after transfection, cells were treated with a high concentration of aspirin.

2.3. Cell Death Assay. In order to evaluate aspirin-dependent toxicity, trypan blue assay and 7-AAD staining were performed.

At the end of the treatment, cells were detached by trypsinization and incubated with trypan blue and counted for stained and unstained cells.

For FACS analysis, under different experiment conditions, 1×10^6 cultured Hek-293 cells were freshly harvested and incubated for 10 minutes in the dark with 5 μL/test of 7-AAD (BD Pharmingen) [21]. Within 1 h after dye incubation, the cells were analysed on FACSscan (Becton Dickinson, Mountain View, CA) using Cell Quest software (Becton Dickinson).

2.4. High-Performance Liquid Chromatography Analysis. Quantitative analysis of plasma concentrations of aspirin was performed using HPLC according to Cerletti et al., 2003 [22]. Cells were washed twice with cold PBS, collected, and centrifuged at 400 g for 10 min. After extraction with hexane (SIGMA Chemicals Company, St. Louis, MO), aspirin was quantified by high-performance liquid chromatography (HPLC) with ultraviolet detection at 229 nm. Aspirin concentration and peak-height ratios were linearly related up to 20 μg/mL. The lower limit of sensitivity was 0.1 μg/mL. The average recovery of aspirin was 27%.

2.5. Protein Extraction and Western Blot. In order to analyze MRP4 protein, cells were washed twice with cold PBS, collected, and centrifuged at 400 g for 10 min. Cell pellets were then resuspended in lysis buffer (RIPA buffer: 10 mM Tris-HCl (pH 7.6), 160 mM NaCl, 1 mM EGTA, 1% deoxycholic acid, 1% Triton, and 0.1% SDS) with protease inhibitors, incubated on ice for 30 min, and centrifuged at 12,000 g for 30 min; the supernatant was then collected.

Protein extracts (30 μg) were incubated at 37°C for 30 min [20] and separated on 4–12% SDS-PAGE gel, blotted onto PVDF membrane (GE Healthcare, Milano, Italy), and probed with rat anti-MRP4 (Alexis, Plymouth Meeting, Pennsylvania) and mouse anti-actin (Santa Cruz Biotechnology, Santa Cruz, CA) monoclonal antibodies. Immunoreactive bands were visualized by enhanced chemoluminescence (PerkinElmer, Waltham, MA, USA).

2.6. RNA Preparation and Real-Time PCR Analysis. Total RNA from human cell lines was extracted using TRIzol reagent (Invitrogen, San Diego, CA).

For mRNA detection 1 μg of total RNA was transcribed using the GeneAmp Gold RNA PCR Reagent Kit pAW109 (Applied Biosystems, Warrington, UK) according to the manufacturer's suggestion.

The analysis of gene expression was carried out with Q-RT-PCR using TaqMan Master Mix and TaqMan Assay Reagents (Applied Biosystems, Warrington, UK).

The amplification, monitored using ABI Prism 7900 Sequencer Detector (Applied Biosystems, Foster City, CA), was as follows: 50°C for 2 min, 95°C for 10 min, 95°C for 15 sec, and 60°C for 1 min; the latter two temperatures were repeated for 40 cycles.

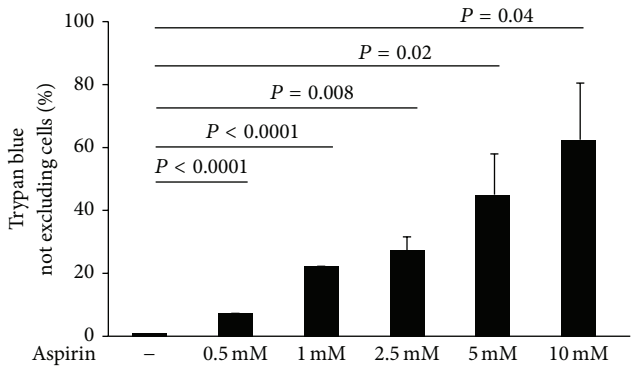


Figure 1: Cells survival depends on the dose of aspirin. Cell survival of either untreated or aspirin treated (from 0.5 mM to 10 mM) Hek-293 cells for 24 hours. Trypan blue exclusion test analysis was used to analyze cell death ("trypan blue-not excluding"). "Trypan blue-not excluding" cell analysis is reported as percentage of dead cells, in aspirin treated compared to untreated cells. Data are reported as mean ± SD of 3 experiments performed.

All amplification reactions were performed in duplicate using 25 ng of cDNA.

Changes in MRP4 and β-actin mRNA amounts were quantified by using $\Delta\Delta$Ct method for relative quantization of gene expression using SDS software version 2.3 (Applied Biosystems, Warrington, UK).

2.7. Statistical Analysis. Data are presented as mean ± SD. The level of significance was determined by paired, 2-tailed Student's t-test (KaleidaGraph software 3.6). Results were considered statistically significant if a P value of less than 0.05 was reached.

3. Results

3.1. Influence of Aspirin on Cell Viability. As a preliminary step in this work, we examined to what extent cells gone through the process of selection by aspirin showed a distinct pattern of viability compared to untreated cells (control). Human embryonic kidney-293 cells (Hek-293) were incubated with high concentrations of aspirin (from 0.5 mM to 10 mM, for 24 h) and then counted and subjected to trypan blue assays (Figure 1). A dose-response analysis through cell numeration showed that aspirin markedly reduced cell viability, suggesting that high concentrations (0.5–10 mM) of aspirin have a toxic effect. This was demonstrated by trypan blue assays; in fact, aspirin causes cell death in a dose-dependent manner: 24 h treatment with high concentrations of this drug was cytotoxic for a large proportion of Hek-293 cells, while low concentrations were less effective (Figure 1).

3.2. MRP4 Expression in Aspirin Treated Hek-293 Cells. We recently demonstrated that aspirin is a target for MRP4 and, in *in vitro* treatment, it increases MRP4 expression [10, 12]. These studies suggest that MRP4 might be important for aspirin outward transport by cells and for aspirin cell detoxification.

To confirm whether aspirin modulates MRP4 expression in Hek-293 cells, we analyzed mRNA and protein expression both in control and in aspirin treated cells.

Hek-293 cells treated with low-dose aspirin (50 μM for 48 h) showed higher MRP4 mRNA levels compared to mock culture (Figure 2(a)) suggesting a positive transcriptional control.

In the same experimental conditions, western blot analysis revealed an increase of MRP4 protein expression in cells treated with aspirin (50 μM for 48 h) (Figure 2(b)).

Densitometry analysis showed a statistically significant higher induction of MRP4 protein expression, which is enhanced more than 30% in comparison with untreated cells (Figure 2(c)).

Aspirin-dependent MRP4 overexpression may be important to reduce drug cytosolic concentration, leading to reduced aspirin toxicity.

3.3. Influence of High Concentrations of Aspirin on Control and Aspirin-Pretreated Cells. Recent studies showed that eukaryotic cells drug exposure modulates expression of mechanisms favouring their elimination.

To study whether this mechanism is evident also after aspirin treatment, Hek-293 cells were preincubated with aspirin (50 μM) and after 48 h they were treated with high concentrations of aspirin (5 mM for 24 h). After these incubations, both control and pretreated cells were counted and subjected to trypan blue dye exclusion and flow cytometry (FACS) analysis with 7-AAD.

Trypan blue assay showed that, in cells directly treated with 5 mM aspirin for 24 h, cell viability was markedly reduced in control cells, while it was almost unchanged in comparison with 48 h aspirin pretreated cells (Figure 3(a)).

Interestingly, since staining with 7-AAD is more sensitive to cellular damage, and to evaluate the effect, we performed FACS analysis using 7-AAD, measuring the percentage of dead cells with compromised membranes gating on the whole cell population (Figure 3(b)). As shown in the upper panels, no differences in 7-AAD staining, before treatment, were observed between control (6.8%) and pretreated cells (5.5%). Instead, the lower panels of Figure 3(b) show an increase of dead cells in response to high-dose aspirin (7-AAD$^+$ cells) in control cells (5-fold increase). On the other hand, in aspirin pretreated cells (50 μM for 48 h) still overexpressing MRP4 (as shown in Figure 2(b)), a lower percentage of dead cells (control 34.2% versus pretreated 13.0%) was observed in response to high-dose aspirin. Moreover, we analyzed the absolute number of 7-AAD positive over total cells, in both control and pretreated cell samples. The results show that the ratio of total numbers of 7-AAD positive cells in aspirin (5 mM) treated samples versus matched untreated cells was reduced in a statistically significant way, when cells were pretreated with low doses of aspirin (50 μM for 48 h) (Figure 3(c)).

The data could suggest that aspirin is able to induce gene expression changes, enhancing MRP4 expression in aspirin pretreated cells, which might favour aspirin transport outside the cells, leading to reduction of drug induced cell toxicity.

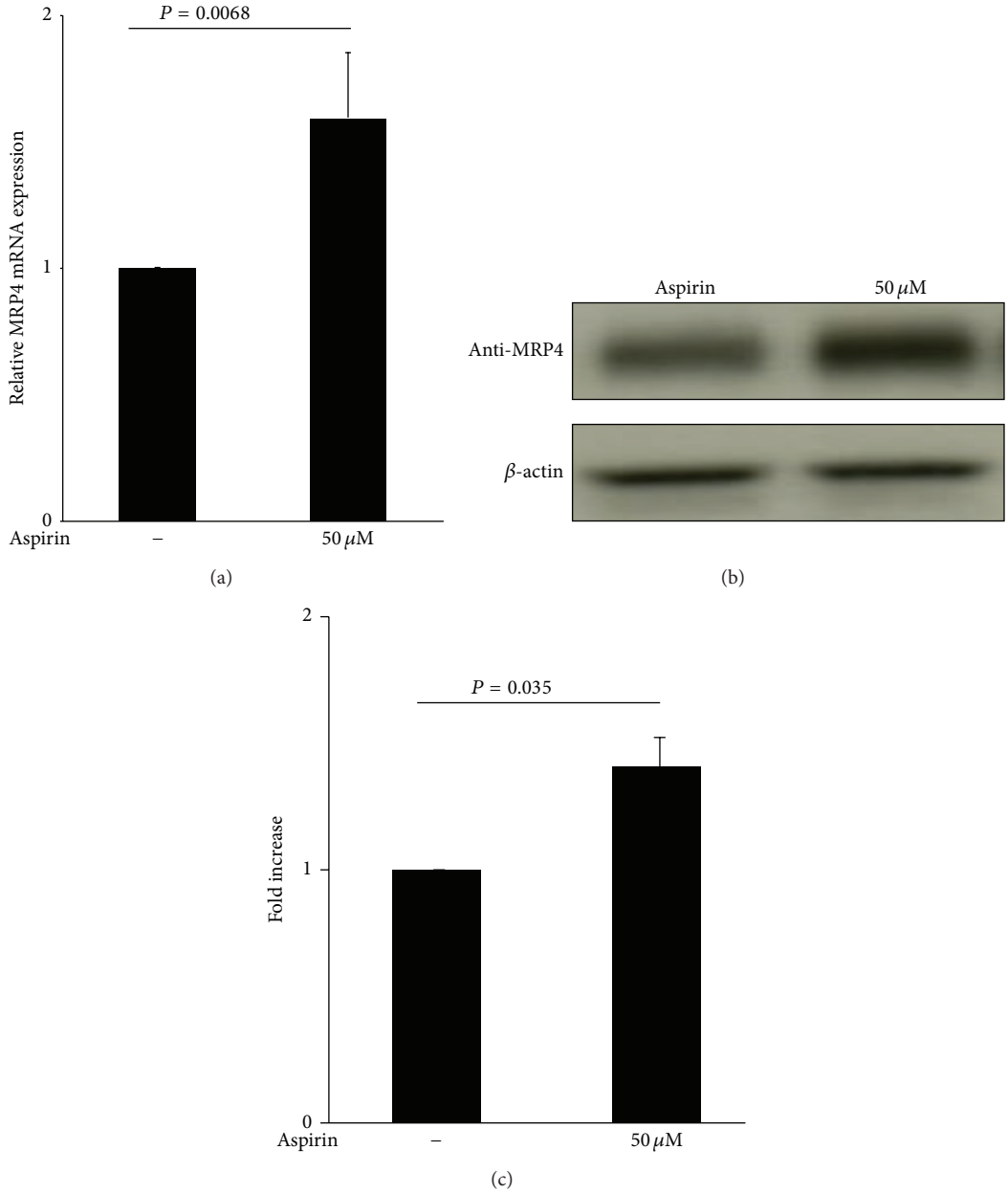


Figure 2: Aspirin stimulates endogenous MRP4 mRNA and protein expression in Hek-293 cells. (a) Q-RT-PCR analysis of endogenous MRP4 α expression after 48 h of treatment with 50 μM aspirin. Data were normalized with β-actin expression and reported as mean ± SD of increasing fold, compared to control ($n = 3$). (b) Representative western blot, of 3 experiments performed, of endogenous MRP4 expression after 48 h treatment with 50 μM aspirin. Aspirin untreated cells were used as control cells. (c) Densitometric analysis of western blot is reported as mean ± SD of increasing fold, compared to control ($n = 3$).

3.4. MRP4 Protects Hek-293 from Aspirin-Dependent Cytotoxicity. To confirm that MRP4 upregulation is responsible for the reduced aspirin toxicity, we explored the effects of exogenous MRP4 overexpression in Hek-293 cells. These cells were transfected either with pCDNA 3.1 (pCDNA 3.1), used as a control, or with MRP4 expression vector (MRP4) and, further, incubated for 24 h with high concentration of aspirin (5 mM). Afterwards, a comparative study of transfected cells, with trypan blue dye assay and FACS analysis using 7-AAD staining, was performed. The efficiency of the transfection was monitored by western blot analysis confirming MRP4 protein overexpression in transfected cells (Figure 4(a)).

Trypan blue assay showed that cell viability was markedly reduced in control cells when exposed to 5 mM aspirin for 24 h, while it remains unchanged in MRP4 transfected cells subjected to the same treatment (Figure 4(b)).

A comparative analysis by FACS of pCDNA 3.1 versus MRP4 transfected cells was performed (Figure 4(c)). In aspirin treated cells (5 mM), the percentage of 7-AAD stained cells was increased in pCDNA 3.1 transfected cells, rising

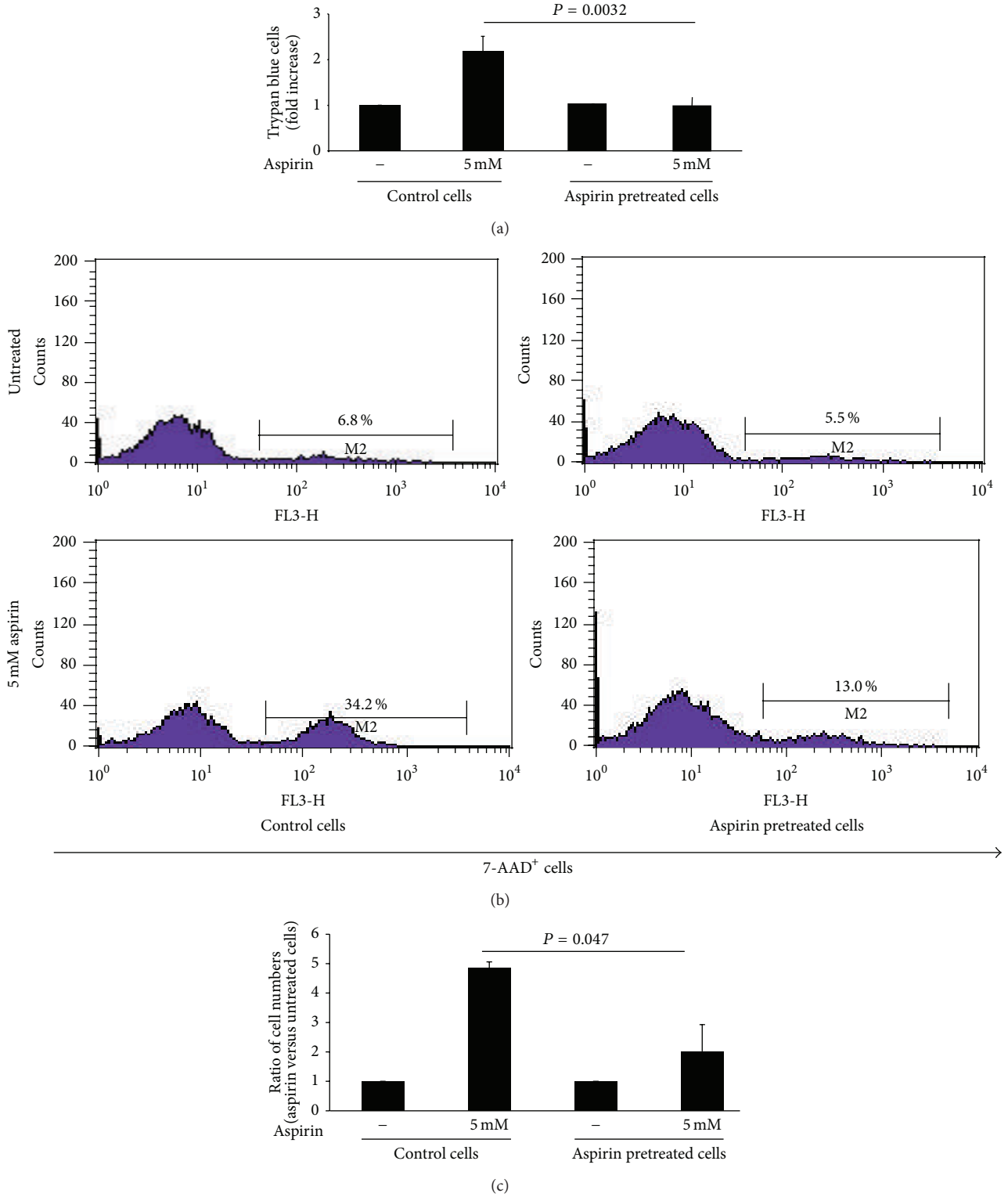


Figure 3: Aspirin pretreatment is important for reduction of its toxicity. Cell death in Hek-293 cells, pretreated with 50 μM of aspirin (aspirin pretreated cells) or with vehicle (control cells) for 48 h. In both conditions, toxicity was evaluated in untreated and aspirin treated (5 mM) cells 24 h later. (a) Expression of "trypan blue-not excluding" cells is evaluated as percentage of dead cells in aspirin (5 mM) treated cells compared with control cells. Data were reported as mean ± SD of 3 experiments (NS: not significant). (b) Dead cell profile, representative flow cytometry histograms of Hek-293 cells (1×10^6) stained with 7-AAD, out of three experiments performed. Left and right panels represent control and aspirin pretreated cells, respectively. Percentages of 7-AAD$^+$ cells are indicated in each panel. (c) Ratio of the total numbers of 7-AAD$^+$ cells in 5 mM aspirin treated versus matched untreated cells, both in control and in aspirin pretreated cells. Data were reported as mean ± SD of 3 experiments performed; $P = 0.046$ indicates significant differences in aspirin samples of control versus pretreated cells.

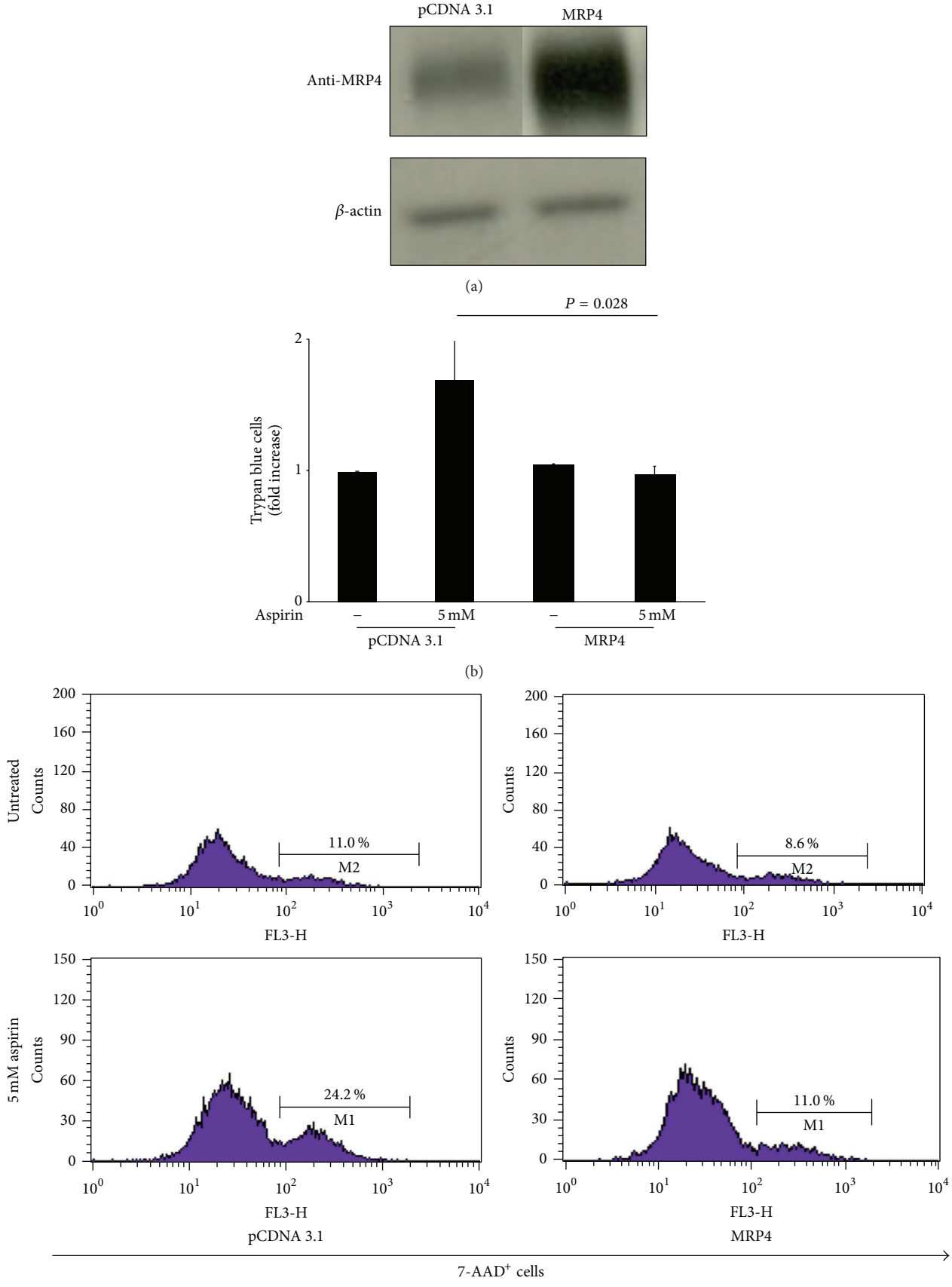
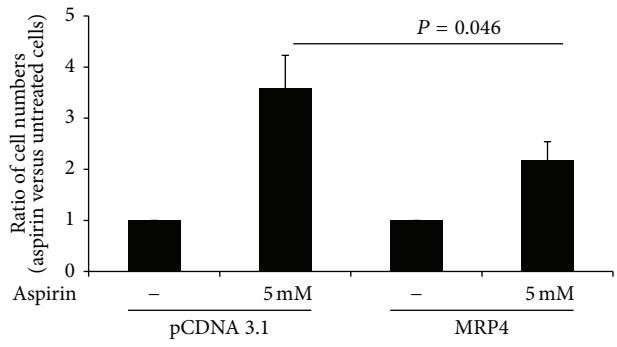


Figure 4: Continued.



(d)

Figure 4: MRP4 expression is relevant to reduce aspirin toxicity. Cell death in Hek-293 transfected cells (MRP4 cells). pCDNA3.1 transfected cells were used as control (pCDNA3.1). Aspirin toxicity was evaluated after 24 h aspirin (5 mM) treatment, with trypan blue exclusion test. (a) Multidrug resistance protein-4 (anti-MRP4) expression in Hek-293 cell lines is shown in the first lane (PCDNA 3.1); MRP4 transfected cultures are shown in the second lane (MRP4). A representative experiment, of 3 performed, is presented. (b) Expression of "trypan blue-not excluding" cells is evaluated as percentage of cell death in aspirin treated cells (5 mM) compared with control cells. Data were reported as mean ± SD of 3 experiments performed (NS: not significant). (c) Dead cell profiles, representative flow cytometry histograms of Hek-293 cells stained with 7-AAD, out of three performed experiments. The panel represents pCDNA3.1 and MRP4 transfected cells. Numbers in histograms indicate percentage of 7-AAD$^+$ cells. (d) Ratio of the total numbers of 7-AAD$^+$ cells in 5 mM aspirin treated versus matched untreated cells, both in pCDNA3.1 and in MRP4 transfected cells. Data were reported as mean ± SD of 3 experiments performed; $P = 0.046$ indicates significant differences in aspirin samples of pCDNA3.1 versus MRP4.

from 11.0% to 24.2%, while in MRP4 overexpressing cells the percentage of 7-AAD stained cells (7-AAD$^+$ cells) was slightly enhanced (from 8.6% to 11.0%).

Under aspirin treatment (5 mM), the ratio of total numbers of 7-AAD positive cells in MRP4 versus matched pCDNA3.1 transfected cells was decreased, as observed in three experiments performed (Figure 4(d)).

Altogether, these data support the hypothesis that MRP4 overexpression is important to reduce aspirin-selective cell death. The protective effect may depend on the MRP4-induced aspirin extrusion from the cell.

3.5. Accumulation of Aspirin in Control and MRP4 Transfected Cells. To analyze whether MRP4 is able to modulate aspirin transport outside the cell, we compared intracellular accumulation of aspirin in MRP4 versus pCDNA 3.1 transfected cells both treated with 5 mM aspirin for 24 h.

After such treatment, we analyzed cytosolic aspirin accumulation using a selective and sensitive method. As shown in Figure 5, aspirin accumulation is reduced, about 2 times less, in MRP4 transfected compared to pCDNA 3.1 cells (Figure 5). These data demonstrate an inverse correlation between transporter and intracellular substrate level, since very high MRP4 expression is directly related to a reduced intracellular aspirin concentration.

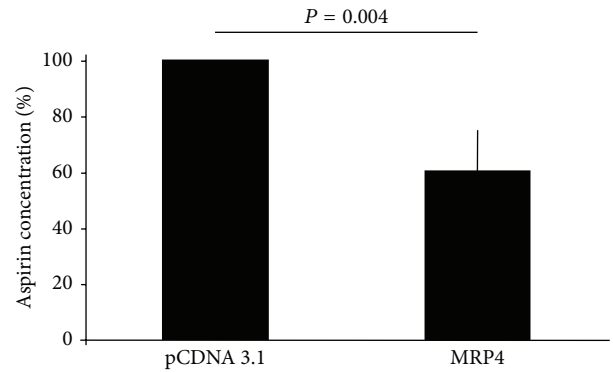


Figure 5: A reduced aspirin cytoplasmatic concentration in MRP4 transfected Hek-293 cells. Percentage of aspirin cytoplasmatic concentration in MRP4 transfected cells (MRP4) after aspirin treatment (5 mM for 24 h) in comparison with those found in control cells (pCDNA 3.1). The results are expressed as mean ± SD of 3 experiments performed.

4. Discussion

Many reports outlined a close correlation between increased MRP transporters expression and impairment of pharmacological treatment.

In the present study, we have shown that exposure of eukaryotic cells to aspirin, an MRP4 target [10, 11], can deeply modify the expression of this efflux transport protein, in a human cell line, Hek-293. Our results suggest that such modifications can result in enhanced aspirin transport outside the cells, leading to a reduction of the toxic effect of this drug.

Such relationship between drug toxicity and MRP4 upregulation is already reported in mouse. In fact, after *in vivo* perfluorooctanoic acid and perfluorodecanoic acid administration, liver mounts a compensatory hepatoprotective response, leading to a marked increase of MRP4 expression [23], in order to reduce drug toxicity.

As aspirin toxicity results in the perturbation of the cell cycle and ultimately causes necrosis [19], we investigated, first, through a dose-response curve, the effect of high-dose aspirin treatment, and we showed that aspirin is able to

increase cell death in Hek-293 cell line, at similar concentrations to those found by others [19].

In our recent study, we showed that also aspirin is able to modulate MRP4 expression in human platelets, and we speculated that the limited drug capacity in reducing platelet function, observed in aspirin long-term treated patients [24], could be due to a drug-dependent MRP4 upregulation [12]. Aspirin influences megakaryocytic gene expression leading to upregulation of MRP4 in human platelets, suggesting that even aspirin can activate mechanisms that favour its elimination, thus consequently reducing its toxic effect [12].

In agreement with this previous report, aspirin-dependent MRP4 upregulation was confirmed also in Hek-293 cells, widening the spectrum of the cells subjected to this mechanism. In fact, aspirin treatment increases MRP4 mRNA and protein expression levels for 48 hours in Hek-293 cells. This upregulation induced also by low nontoxic aspirin dosage can be important to regulate aspirin effect. Indeed, pretreatment with low doses of aspirin reduces its toxicity by decreasing the percentage and absolute numbers of nonviable cells, suggesting the induction of a protective mechanism. We cannot exclude the contribution of a mechanism of cell selection operated by aspirin. This may affect drug intended and drug side effects in many tissue districts since MRP4 is ubiquitously expressed, including hematopoietic cells [25]. Furthermore, modulation of aspirin effects may be important in long-term daily aspirin use.

Several studies show that acute salicylate poisoning is a common medical emergency which leads to a high mortality [13–15] and aspirin poisoning is clearly dose related in order to increase toxicity in human subjects [17].

The direct connection between aspirin-induced MRP4 expression and cell toxicity was further addressed by overexpressing MRP4 extruding protein in our cell line. Indeed, MRP4 high levels are well correlated with reduced numbers of 7-AAD positive cells. In this study we demonstrate that aspirin-dependent MRP4 upregulation is important to reduce intracellular aspirin concentration, by enhancing its transport. In fact, MRP4 overexpression leads to cells drug detoxification in a more efficient manner in human Hek-293.

To confirm that MRP4 is the aspirin transporter involved in such phenomenon, we compared cellular accumulation of aspirin in MRP4 transfected cells versus control cells treated with aspirin (5 mM for 24 h). Indeed, aspirin showed a reduced cytosolic accumulation in MRP4 transfected compared to control cells.

Our data support the evidence that aspirin-dependent overexpression of MRP4 is a cell adaptation to what seems to be a major toxic stress [26].

With our results, we can also suggest that, in the future, it should be important to study a possible correlation between the treatment with aspirin and that with chemotherapeutic agents, in order to verify drug efficiency.

5. Conclusion

In conclusion, this study demonstrates that aspirin, at low nontoxic drug dosage, is able to activate a positive transcriptional control of the MRP4/ABCC4 transporter gene, in human cells, thus enhancing the activity of the mechanism susceptible to increase aspirin efflux.

We speculate that our data are suggestive of the ability of low-dose aspirin to confer clinical drug resistance, particularly in long-term treated patients.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgments

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Research Article

Anti-Inflammatory Effects of a Methanol Extract from the Marine Sponge *Geodia cydonium* on the Human Breast Cancer MCF-7 Cell Line

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Many research groups are working to find new possible anti-inflammatory molecules, and marine sponges represent a rich source of biologically active compounds with pharmacological applications. In the present study, we tested different concentrations of the methanol extract from the marine sponge, *Geodia cydonium*, on normal human breast epithelial cells (MCF-10A) and human breast cancer cells (MCF-7). Our results show that this extract has no cytotoxic effects on both cell lines whereas it induces a decrease in levels of VEGF and five proinflammatory cytokines (CCL2, CXCL8, CXCL10, IFN-γ, and TNF-α) only in MCF-7 cells in a dose-dependent manner, thereby indicating an anti-inflammatory effect. Moreover, interactomic analysis suggests that all six cytokines are involved in a network and are connected with some HUB nodes such as NF-kB subunits and ESR1 (estrogen receptor 1). We also report a decrease in the expression of two NFKB1 and c-Rel subunits by RT-qPCR experiments only in MCF-7 cells after extract treatment, confirming NF-kB inactivation. These data highlight the potential of *G. cydonium* for future drug discovery against major diseases, such as breast cancer.

1. Introduction

Inflammation is a physiological process in response to acute tissue damage resulting from physical and ischemic injury, infection, exposure to toxins, chemical irritation, and/or other types of trauma. Many authors have suggested a correlation between chronic inflammation and cancer [1]. In fact, while inflammatory diseases increase the risk of developing many types of cancer, some nonsteroidal anti-inflammatory drugs reduce this risk for certain cancers (e.g., breast cancer) [2]. Interestingly, inflammation is involved in all three stages of tumor development, initiation, progression, and metastasis, where cytokines, chemokines, and growth factors play an important role in their evolution [3]. These are proteins that are expressed before and during the inflammatory process and play a key role during various disease stages so as to be considered as specific cancer markers as well as markers for various stages of the disease [2]. In general, the cytokinome is defined as the totality of these proteins and their interactions in and around cells [4]. Understanding the complex interaction network of cytokines in cancer patients should be very useful both to follow the evolution of cancer from its first steps and to define therapeutic strategies using innovative systems biology approaches. Several research groups are working to find new possible anti-inflammatory molecules [5]. Indeed, our laboratory has also recently evaluated the putative anti-inflammatory effects of different molecules such as sodium selenite, lipoic acid, and caffeic acid on cancer cell

lines [6, 7] as well as of natural extracts from pomegranate (*Punica granatum* L.) seed oil [8] and *Juniperus oxycedrus* ssp. *oxycedrus* berries [9].

The marine environment, with its impressive biodiversity, is a rich natural resource of many biologically active compounds such as antioxidants, polyunsaturated fatty acids (PUFAs), sterols, proteins, polysaccharides, and pigments. In fact, many marine organisms live in complex habitats and are exposed to extreme conditions and produce a variety of biologically active secondary metabolites, which often have no terrestrial counterparts. Sponges seem to be particularly rich sources of bioactive compounds which show antiviral [10], antibacterial [11], and anti-inflammatory activities [12–15]. They are also promising candidates as anticancer drugs considering that 3 of the 4 commercial anticancer drugs available have been derived from marine sponges, including Ara-C from the marine sponge *Tectitethya crypta* [16] that is currently used in the routine treatment of patients with lymphoma, Halaven (eribulin) from the sponge *Halichondria okadai* used for the treatment of breast cancer [17], and Yondelis from the marine tunicate *Ecteinascidia turbinata* for the treatment of soft tissue carcinoma [18]. For example, several anti-inflammatory metabolites have been isolated from sponges belonging to the class of the Demospongiae, including cavernolide from *Fasciospongia cavernosa* [19], contignasterol from *Petrosia contignata* [20], and cyclolinteinone from *Cacospongia linteiformis* [21]. Therefore, the purpose of the present study was to investigate the potential effects of a methanol extract from the marine demosponge, *Geodia cydonium*, which has not been studied previously for drug discovery, on normal human mammary epithelial cells (MCF-10A) and cells of human breast cancer (MCF-7).

2. Materials and Methods

2.1. Sample Collection and Preparation of the Methanol Extract. The marine sponge *G. cydonium* (Porifera, Demospongiae, Astrophorida, Geodidae) was sampled in the "Parco Sommerso di Baia" (Gulf of Naples, Italy). The sample was collected in October 2013 at a depth of 20 metres. Individual specimens were placed separately into plastic bags and kept in seawater basins of the Marine Resources for Research Service at the Stazione Zoologica Anton Dohrn of Naples at a temperature of 15–20°C for one month.

Sponges were cut into small pieces and 10 g of tissue was ground into liquid nitrogen and then soaked in 200 mL of methanol for 2 hours under stirring. The solvent was removed and filtered through Whatman filter paper No. 1; the sponge tissue was squeezed in cheesecloth. The solvent was evaporated at low pressure using a rotavapor (Büchi Rotavapor R114) at 40°C and the residual aqueous extract was partitioned three times against ethyl acetate (2 : 1 v/v). To accelerate the phase separation, we centrifuged the extract at 2750 rcf for 10 minutes at 4°C and the organic phase was collected. The organic phase of various preparations was pooled and treated with ammonium sulfate to remove any aqueous residues. Nonpolar components and phenolic molecules were separated by this extraction procedure [22]. The final extract was dried and stored at −20°C until use.

2.2. Cell Culture. MCF-7 human breast cancer cells and MCF-10A normal human breast epithelial cells were cultured and expanded at 37°C in a humidified atmosphere of 5% CO_2 in DMEM culture medium (Dulbecco's Modified Eagle's Medium, Lonza, Verviers, Belgium), supplemented with FBS (Invitrogen, Camarillo, CA, USA) at 10%, penicillin/streptomycin 1× (Euroclone, Devon, UK), and Glutamax 1× (Invitrogen). Moreover, in the case of MCF-10A the DMEM was supplemented also with human insulin (10 μg/mL), epidermal growth factor (20 ng/mL), and hydrocortisone (0.5 μg/mL) according to the procedure reported in [23].

2.3. Colorimetric Assay with Sulforhodamine B. Cell proliferation was assessed in the presence and absence of the methanol extract from *G. cydonium* by colorimetric assay with sulforhodamine B (SRB, Sigma Aldrich). The cells (3.5×10^{-4}–4×10^{-4}) were seeded in 96-multiwell plates in 200 μL of culture medium and left to grow for 24 h at 37°C to allow adhesion. Cells were then treated with varying concentrations of the extract: 2 μg, 50 μg, and 200 μg and incubated for 24 h. These concentrations were selected based on previous results on the effect of a methanol extract of this sponge on embryonic development in the sea urchin *Paracentrotus lividus*.

Dissolution of the extract was improved with 100 mM dimethyl sulfoxide (DMSO, Sigma Aldrich). From this stock solution, dilutions were made to obtain the different amounts of extracts with a final concentration of 0.05% DMSO. Control cultured cells were incubated with the same volume of the solvent. Cells were fixed by adding 10% trichloroacetic acid (Sigma Aldrich) for at least 1 h at 4°C. Subsequently, cells were washed with distilled water and air-dried. SRB (100 μL) was added to each well and the plate was incubated for 30 min at room temperature in the dark. To remove excess dye, cells were washed with 1% acetic acid. The number of viable cells was directly proportional to the protein bound-dye formation which was then solubilized with 100 μL of 10 mM Tris base solution pH 10.5, shaking the plates for at least 15 min on an orbital shaker to homogenize the dye solution. Optical density measurements were performed by using an automated 96-well plate reader (Microplate Reader, Bio-Rad, Hercules, CA, USA) at 540 nm. All experiments were performed in triplicate and were repeated for three times. Cellular viability was estimated as % compared to untreated cells.

2.4. Bio-Plex Assay. Levels of cytokines, chemokines, and growth factors were evaluated at the same time with the Bio-Plex assay. The multiplex biometric ELISA-based immunoassay, containing dyed microspheres conjugated with a monoclonal antibody highly specific for a target protein, was used, according to the manufacturer's instructions (Bio-Plex Bio-Rad), to evaluate the levels of different cytokines by Human Cytokine 27-Plex in MCF-10A and MCF-7 supernatants after

treatment with increasing concentrations of sponge extract. In particular, the following cytokines were evaluated: IL-1β, IL-1ra, IL-2, IL-4, IL-5, IL-6, IL-7, CCL2, CCL11, CXCL10, CXCL8, IFN-γ, IL-9, IL-10, IL-12 (p70), IL-13, IL-15, IL-17, basic FGF, G-CSF, GM-CSF, MIP-1α, MIP-1β, PDGF-$\beta\beta$, RANTES, TNF-α, and VEGF. Protein levels were determined using a Bio-Plex array reader (Luminex, Austin, TX, USA) that quantifies multiplex immunoassays in a 96-well format with very small fluid volumes. The analyte level was calculated using a standard curve, with software provided by the manufacturer (Bio-Plex Manager Software).

2.5. Bioinformatics Analysis. The expression levels of cytokines evaluated in MCF-10A and MCF-7 supernatants were compared by t-test. Values of $P < 0.05$ were considered to be statistically significant. The statistical program Prism 4 (GraphPad Software, San Diego, CA, USA) was used. The PANTHER (Protein ANalysis THrough Evolutionary Relationships) Classification System was designed to classify proteins according to their biological processes [24] as well as the metabolic pathways in which they are involved. Moreover, a network analysis was performed between the most significant proteins by ingenuity pathway analysis (IPA).

2.6. RNA Preparation and Reverse Transcription-qPCR (RT-qPCR) Analysis. Total RNA was extracted from MCF-7 cells that were either untreated (control) or exposed to increasing concentrations of sponge extract (2 μg, 50 μg, and 200 μg) using the RNAeasy Mini Kit (Qiagen Inc., Valencia, CA, USA) according to the manufacturer's instructions. The extracted RNA was dissolved in diethylpyrocarbonate treated water, and its concentration and purity were assessed by measurement of optical density at 260/280 nm. RNA samples were quantified using a NanoDrop 2000 spectrophotometer (Thermo Scientific, Wilmington, DE). Two micrograms of total RNA of each sample was reverse-transcribed with SuperScript VILO cDNA Synthesis Kit (Life Technologies-Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions and subsequently diluted with nuclease-free water (Life Technologies-Ambion).

Sequences for mRNAs from the nucleotide data bank (National Center for Biotechnology Information, USA) were used to design primer pairs for RT-qPCR (Primer Express, Applied Biosystems, CA, USA). Oligonucleotides were obtained from Sigma Aldrich. The primer sequences of 5 NF-κB subunits' mRNAs are provided in Table 1. An appropriate region of 18S rRNA was used as control. RT-qPCR assays were run on a Step-One Real Time PCR System (Applied Biosystems). 2 μL of cDNA was amplified in a total volume of 25 μL containing 2X SYBR Green PCR Master Mix (Applied Biosystems) and 300 nM of forward and reverse primers. The thermal cycling conditions were as follows: 5 min of denaturation at 95°C followed by 44 cycles of a two-step program (denaturation at 95°C for 30 sec and annealing/extension at 60°C for 1 min). For each target, the primer sequences and the melting temperature are reported in Table 1. Dilutions of standards and test samples were run in duplicate. Each reaction was repeated at least three times. Expression levels of each target gene in MCF-7 cells treated with different concentrations of the sponge extract were compared with those in untreated cells using the REST tool (Relative Expression Software Tool, Weihenstephan, Germany) [25]. The REST mathematical model is based on the correction for exact PCR efficiencies and the mean crossing point deviation between sample group(s) and control group(s). Subsequently the expression ratio results of the investigated transcripts are tested for significance by a Pair Wise Fixed Reallocation Randomisation Test © and plotted using standard error (SE) estimation via a complex Taylor algorithm (http://www.gene-quantification.de/rest.html). Data were normalized using the 18S rRNA as housekeeping gene already used in our recent paper [26]. A 1 x-fold expression level was chosen as the threshold for significance of target genes.

Table 1: Parameters for RT-qPCR analysis.

Gene	Tm [°C]	Sequence (5′ → 3′)
NFKB1	59	CCTCTGTGTTTGTCCAGCT (19)
		CCGAAAAATTGGGCATGAGC (20)
NFKB2	58	GCTTCTCTGCCTTCCTTAG (19)
		CACAGAGCCTGCTGTCTTG (19)
RELA	58	CACGAGCTTGTAGGAAAGG (19)
		GCGCTGACTGATAGCCTG (18)
RELB	60	TGCTTCGGTCTGGGCCAG (18)
		CAATTCATCTGTGCTCCTGG (20)
C-Rel	60	TCCTGACTGACTGACTGCG (19)
		CTAAAACGCATTCCCCTCTG (20)
18S	60	CTGCCCTATCAACTTTCGTG (20)
		GTAGTTTCTCAGGCTCCCTCTC (22)

3. Results and Discussion

3.1. Preparation and Viability Assay of the Methanol Extract. Sponges, due to the enormous diversity of associated microorganism communities, have always been considered an explicit source of pharmaceutical products. Our recent results showed that a diverse assemblage of bacteria resided in the marine sponge *G. cydonium* [27], including *Pseudoalteromonas* spp. that is reported to produce prodigiosin, a well-known tripyrrolic red pigment with immunosuppressive and anticancer activities, mainly in human breast cancer [28]. However, to our knowledge, this is the first study testing the biological activity of extracts of this sponge.

A methanol extract of *G. cydonium* was prepared as reported for other marine sponges [14, 15]. To identify the IC_{50} concentration, corresponding to the extract amount that causes 50% inhibition of cell growth, the cell viability of MCF-10A and MCF-7 was determined after 24 h stimulation with sponge extract by colorimetric assay with sulforhodamine B (Figure 1). We chose this incubation time because it has already been used in previous studies [14, 15]. Moreover, we tested the effect of methanol alone on two cell lines verifying that cell viability remained equal to 100%. Therefore, the cellular viability of untreated cells was used as control.

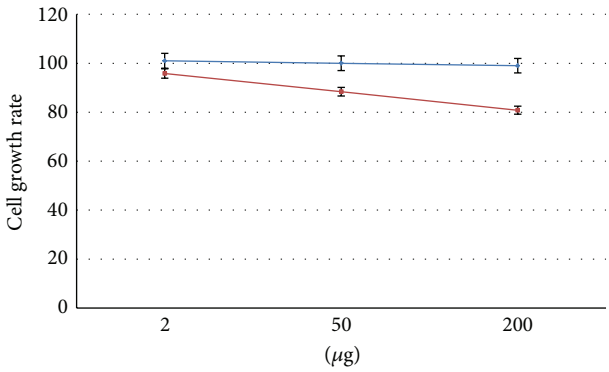

Figure 1: Cell growth rate after 24 h of treatment with different amount of sponge extracts in normal human breast epithelial cells, MCF-10A (blue), and human breast cancer cells, MCF-7 (red).

After 24 h of incubation, MCF-10A and MCF-7 cells retained a relatively constant viability with increasing concentrations of sponge extract, as demonstrated by the overlapping of growth curves in Figure 1. This suggests that our sponge extract had no antiproliferative or cytotoxic activities compared to other methanol extracts [14]. Interestingly, marine sponges of the genus *Geodia* (Demospongiae class, Tetractinellida order, Geodidae family) have been poorly explored for their chemical and biologically active components. Studies on the Mediterranean giant siliceous sponge *Geodia gigas* have led to the isolation of herbipoline (a biogenic amine) [29] and several steroidal ketones [30]. A series of cholestane derivatives have been isolated from another species, *G. megastrella* [31], and crude extracts of *G. corticostylifera* from the Brazilian coast have been reported as having antibacterial, antifungal, cytotoxic, haemolytic, and neurotoxic activities [32]. Some cyclic peptides geodiamolides A, B, H, and I were isolated from *G. corticostylifera* and their antiproliferative effects were demonstrated against sea urchin eggs and human breast cancer cell lines T47D and MCF7 by disorganizing actin filaments of these cells [33]. Furthermore, sponge depsipeptide, geodiamolide H, from *G. corticostylifera* increased gap junction length in hepatocarcinoma cells, affecting mainly the delivery pathway of connexin 43 that is membrane proteins that form gap junction channels between adjacent cells [34].

3.2. Evaluation of Cytokine Levels. Since some studies have reported that methanol extracts from marine sponges show anti-inflammatory effects [14, 15], we evaluated the concentrations of 27 cytokines (expressed in pg/mL) in MCF-10A and MCF-7 supernatants after treatment with *G. cydonium* sponge extract by multiplex biometric ELISA-based immunoassay. This simultaneous quantitative determination of a large cytokine panel enables us to correctly report ratios and dynamics between highly and poorly represented molecules. It represents an accurate, simple, specific, noninvasive, reproducible, and inexpensive method [6–8].

Results were compared with untreated cells used as controls. Experiments revealed that levels of VEGF and five proinflammatory cytokines (CXCL8, CXCL10, IFN-γ, CCL2, and TNF-α) decreased in a dose-dependent manner with increasing levels of sponge extract in breast cancer MCF-7 cells (Figure 2) but not in healthy breast MCF-10A cells. Some studies have already demonstrated that CXCL8 and VEGF promoters contain different recognition sites for nuclear factor- (NF-) kappa B [35]. For example, CCL2 expression is activated by NF-κB [36], TNF-α induces the activation of antiapoptotic transcription factor NF-κB [37], and IFN-γ requires NF-κB to induce expression of the CXCL10 gene [38]. These data suggest a specific anti-inflammatory effect of the sponge extract on cancer cells.

Anti-inflammatory effects have already been reported in marine sponges, including cavernolide from *Fasciospongia cavernosa* [19], contignasterol from *Petrosia contignata* [20], and cyclolinteinone from *Cacospongia linteiformis* [21]. These effects can be explained by the inhibition of enzymatic activities such as inhibition of inducible nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2) gene expression, and plasma exudation *in vivo* in response to ovalbumin and prostaglandin E2. Another compound halipeptin A was isolated from the marine sponge *Haliclona* sp., which has shown anti-inflammatory activity on mouse paw edema assay [39]. This metabolite seems to be more potent compared to standard anti-inflammatory drugs like indomethacin and naproxen for anti-inflammatory effects. Another metabolite petrosaspongiolide M, isolated from the Caledonian marine sponge *Petrosaspongia nigra*, is a potent inhibitor *in vivo* and *in vitro* of phospholipase A2 (PLA2), demonstrating anti-inflammatory activity in models of acute and chronic inflammation [40].

However, there are few reports of sponge-derived compounds acting on specific cytokines. For example, barettin isolated from the marine sponge *Geodia barretti* was able to inhibit the secretion of two proinflammatory cytokines, IL-1β and TNFα, from LPS-stimulated THP-1 cells, and the combination of its anti-inflammatory and antioxidant activities suggests that this compound could have an atheroprotective effect [41]. Moreover, Pfeifer et al. (1992) [42] identified a tumor necrosis-like factor in the sponge *G. cydonium*, an endotoxin that mediates necrosis formation in xenografts.

Our study provides a complete evaluation of the anti-inflammatory effects of a methanol extract from the marine sponge *G. cydonium*, using a cytokinome approach where the cytokinome is defined as the totality of the cytokines and their interactions in and around biological cells as in [4]. Understanding the complex interaction network of cytokines should be very useful both to follow the evolution of cancer from its early steps and to define innovative therapeutic strategies by using systems biology approaches [2, 4]. However, a correct and comprehensive understanding of cytokine functions can be obtained only from simultaneous and coherent measurements of the serum concentrations of cytokines. This point raises the inherent difficulty of a simultaneous measurement of the cytokine concentrations to obtain correct internal ratios among the various molecules present in the same biological fluid or cellular supernatants due to the often large difference in concentrations spanning several orders of magnitude. At present, it is possible to

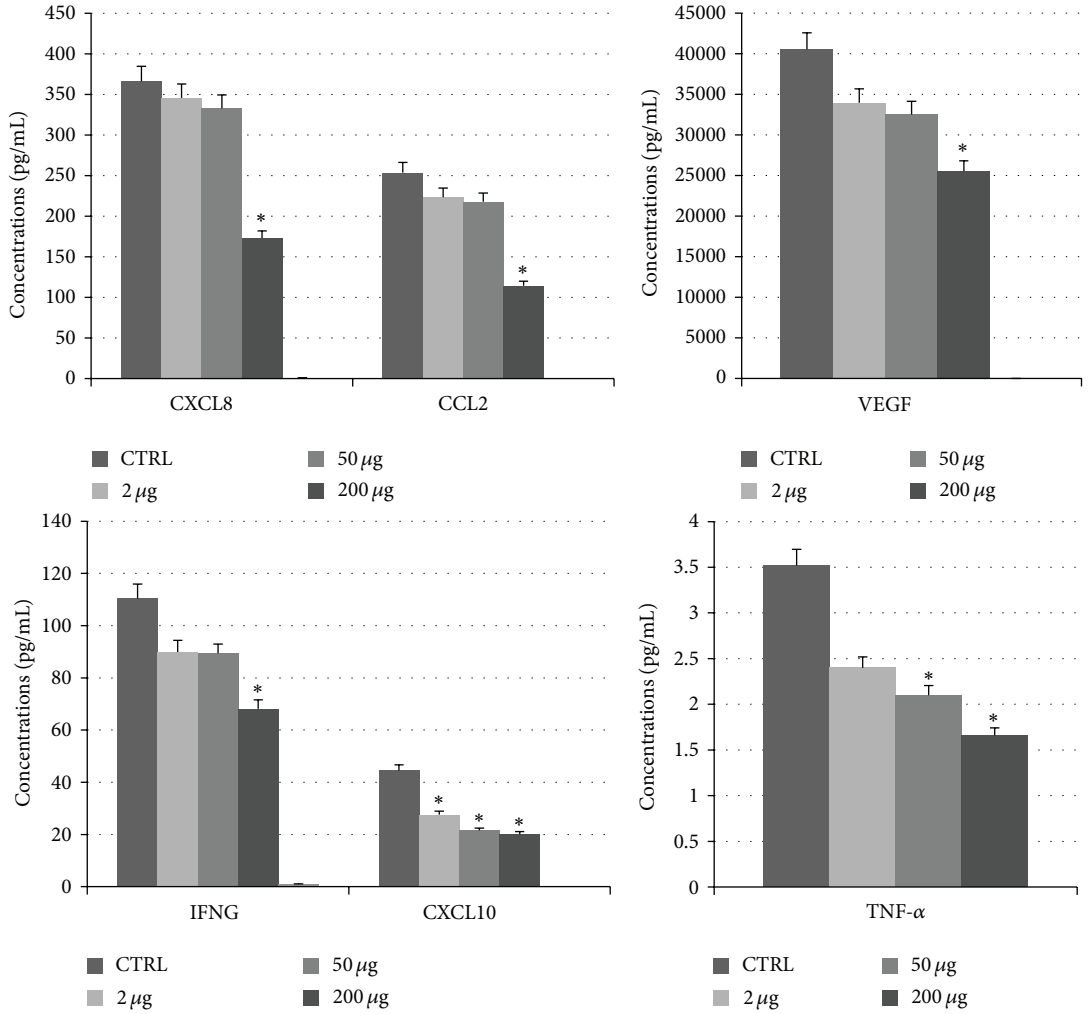

Figure 2: Statistically significant cytokine levels (expressed in pg/mL) evaluated in MCF-7 cells before and after treatment with different concentrations of sponge extract. Asterisks $*$ indicate the statistically significant differences between levels of untreated (used as controls and indicated with CTRL) and treated cells with $P < 0.05$ using the t-test. In the legend, we report the meaning of the bars by using different colors for untreated cells (CTRL) and three different treatments. In detail, we report CXCL8 (C-X-C motif ligand 8), CCL2 (C-C motif ligand 2), VEGF (vascular endothelial growth factor), IFNG (interferon gamma), CXCL10 (C-X-C motif ligand 10), and TNF-α (tumor necrosis factor alpha).

effectively characterize cytokine levels only using a broad-spectrum bead based multiplex immunoassay that was used in [4] and the present study.

3.3. Functional and Network Analysis. To verify if these proteins are correlated with one another through NF-κB, we performed a functional analysis on CCL2, CXCL8, CXCL10, IFN-γ, TNF-α, and VEGF using the PANTHER tool [24] and then conducted a network study by ingenuity pathway analysis (IPA). Figure 3 shows that these six proteins are involved in 7 metabolic pathways: CCL2, CXCL8, CXCL10, and IFN-γ in the inflammation signaling pathway mediated by chemokines and cytokines, VEGF in angiogenesis and the VEGF signaling pathway, TNF-α in apoptosis and the Wnt signaling pathways, CXCL8 in the interleukin signaling pathway, and IFN-γ in the interferon-gamma signaling pathway.

Moreover, interactomic analysis shows that all of the six analyzed significant cytokines are involved in a network named "*Cellular Movement, Immune Cell Trafficking, Hematological System Development and Function*" on the basis of associated functions and data mining from experimental studies reported in the literature (Figure 4). This network reveals that these proteins are connected with some HUB nodes such as NF-κB subunits (NFκB complex and RELA) and ESR1 (estrogen receptor 1), which is a commonly used clinical breast cancer marker [43]. More specifically, (i) CCL2, CXCL8, CXCL10, IFN-γ, TNF-α, and VEGF are connected with NF-κB subunits (NFκB complex and RELA) and (ii) CCL2, CXCL8, TNF-α, and VEGF with ESR1.

Therefore, our interactomic studies confirmed that these six significant cytokines were mutually interconnected by the NF-κB complex and suggested that a decrease in the levels

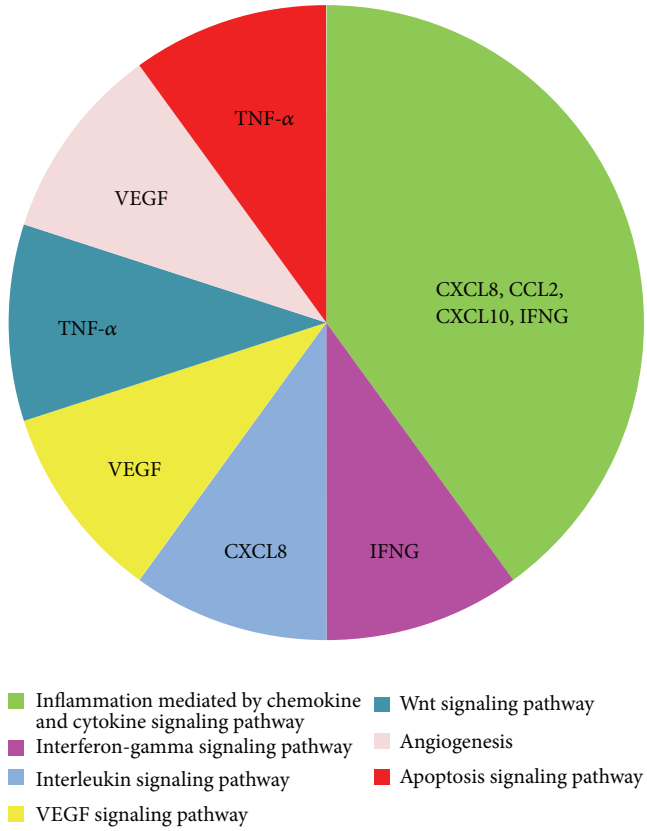

Figure 3: PANTHER pathway analysis in which we show the pathways related to the following six significant proteins: CXCL8 (C-X-C motif ligand 8), CCL2 (C-C motif ligand 2), VEGF (vascular endothelial growth factor), IFNG (interferon gamma), CXCL10 (C-X-C motif ligand 10), and TNF-α (tumor necrosis factor alpha).

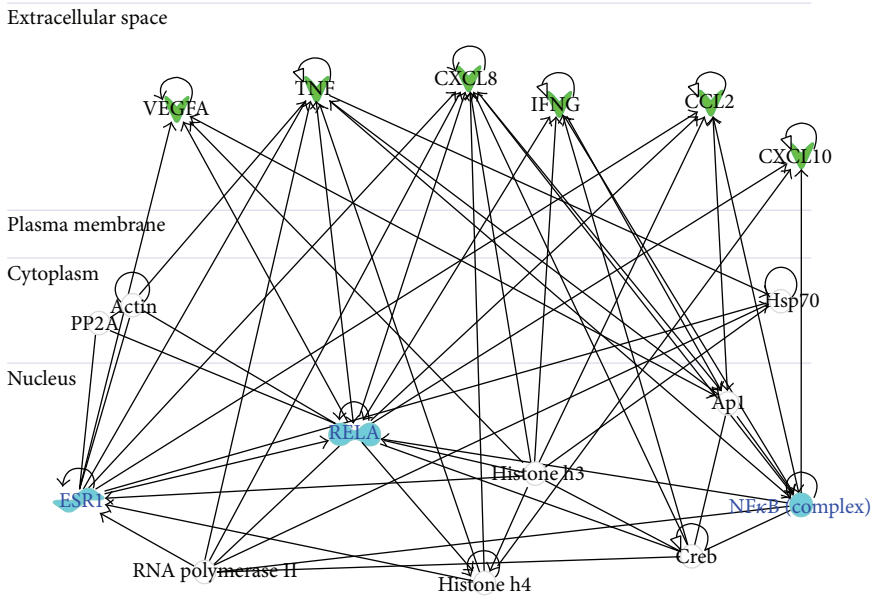

Figure 4: Interactomic analysis by ingenuity pathway analysis (IPA) of significant molecules. The interactome shows the close functional association between significant cytokines (indicated with green symbols) as well as the paths in which other functionally relevant molecules are also involved (indicated with white symbols). In detail, the cytokines reported are CXCL8 (C-X-C motif ligand 8), CCL2 (C-C motif ligand 2), VEGF (vascular endothelial growth factor), IFNG (interferon gamma), CXCL10 (C-X-C motif ligand 10), and TNF (tumor necrosis factor alpha). Moreover, three HUB nodes such as ESR1 (estrogen receptor 1), NF-κB (nuclear factor kappa B), and RELA (v-rel avian reticuloendotheliosis viral oncogene homolog A) subunits are indicated by cyan symbols.

of these cytokines can inhibit the activation of NF-κB by blocking tumor growth.

3.4. Expression Levels of NF-κB Subunits in MCF-7 Cells. It is known that the NF-κB family shares a Rel homology domain in their N-terminus. In particular, a subfamily of NF-κB proteins, including RELA, RELB, and c-Rel, has a transactivation domain in their C-terminal regions. On the other hand, NFKB1 and NFKB2 proteins are synthesized as large precursors (p105 and p100), which are processed to generate the mature NF-κB subunits (p50 and p52), respectively [44].

Therefore, to verify if NF-κB was modulated by the sponge extract, the gene expression levels of the five NF-κB subunits, NFKB1, NFKB2, RELA, RELB, and c-Rel, in MCF-7 cells, treated with increasing concentrations (2 μg, 50 μg, and 200 μg) of sponge extract, were evaluated by RT-qPCR compared to those in untreated cells (Table 1). No significant changes in expression levels were observed for NFKB2, RELA, and RELB, whereas the two genes, NFKB1 and c-Rel, showed a statistically significant downregulation after treatment with sponge extract (Figure 5), thus confirming the downregulation of NF-κB. We are currently performing Western blot analysis to validate these results by the same protocol recently used in our group [45]. Preliminary results indicate no significant changes for RELA expression and a significant downregulation for NFKB1 in MCF-7 cells after treatment with 200 μg of sponge extract compared to those in untreated cells (see Figure 1S in Supplementary Material available online at http://dx.doi.org/10.1155/2015/204975).

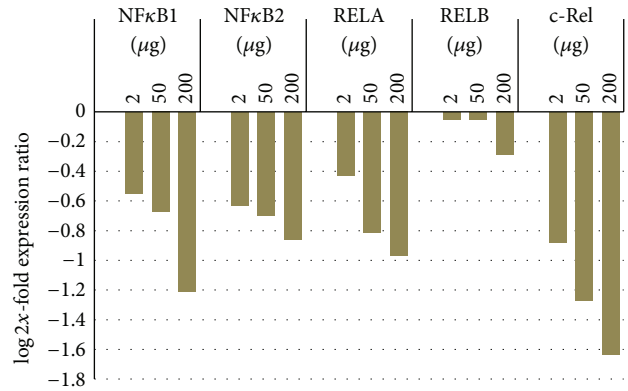

Figure 5: Changes in the expression levels of five NF-κB subunits, NFKB1 (nuclear factor kappa B1), NFKB2 (nuclear factor kappa B2), RELA (v-rel avian reticuloendotheliosis viral oncogene homolog A), RELB (v-rel avian reticuloendotheliosis viral oncogene homolog B), and c-Rel (cellular counterpart of the v-Rel oncogene) in breast cancer MCF-7 cells treated with increasing concentrations (2 μg, 50 μg, and 200 μg) of sponge extract compared to those of untreated cells.

4. Conclusions

Cell response to stress includes a wide range of molecular processes that are activated through the modulation of signaling pathways that lead to repair, adaption, or cell death. Moreover, stress may induce a prolonged inflammation leading to several pathological conditions, which include cancer [3]. For this reason, we are working to discover novel anti-inflammatory molecules [6–8].

In the past decades, several studies have shown that marine sponges contain compounds with anti-inflammatory activities [14, 15]. Our results show that *G. cydonium* sponge extract (i) does not have a cytotoxic effect on both MCF-10A and MCF-7 cell lines, (ii) induces a decrease in VEGF levels and of five proinflammatory cytokines (CCL2, CXCL8, CXCL10, IFN-γ, and TNF-α) in a dose-dependent manner only in MCF-7 cells, and (iii) induces the downregulation of two NF-κB subunits, NFKB1 and c-Rel.

In conclusion, our findings reveal for the first time that a methanol extract from the marine sponge *G. cydonium* had no antiproliferative or cytotoxic activities but anti-inflammatory effects by cytokinome approach on the human breast cancer MCF-7 cell line.

Studies are currently underway to chemically characterize the compounds produced by *G. cydonium* that are responsible for the effects on breast cancer cell lines for future drug development. Extension of this approach may help elucidate the function of individual molecular species and prioritize them as drug targets.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors' Contribution

Susan Costantini and Giovanna Romano contributed equally to this work.

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Review Article

Redox Signaling in Diabetic Nephropathy: Hypertrophy versus Death Choices in Mesangial Cells and Podocytes

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This review emphasizes the role of oxidative stress in diabetic nephropathy, acting as trigger, modulator, and linker within the complex network of pathologic events. It highlights key molecular pathways and new hypothesis in diabetic nephropathy, related to the interferences of metabolic, oxidative, and inflammatory stresses. Main topics this review is addressing are biomarkers of oxidative stress in diabetic nephropathy, the sources of reactive oxygen species (mitochondria, NADPH-oxidases, hyperglycemia, and inflammation), and the redox-sensitive signaling networks (protein kinases, transcription factors, and epigenetic regulators). Molecular switches deciding on the renal cells fate in diabetic nephropathy are presented, such as hypertrophy *versus* death choices in mesangial cells and podocytes. Finally, the antioxidant response of renal cells in diabetic nephropathy is tackled, with emphasis on targeted therapy. An integrative approach is needed for identifying key molecular networks which control cellular responses triggered by the array of stressors in diabetic nephropathy. This will foster the discovery of reliable biomarkers for early diagnosis and prognosis, and will guide the discovery of new therapeutic approaches for personalized medicine in diabetic nephropathy.

1. Introduction

Diabetes is a major concern of public health, affecting more than 371 million people [1], with an expected doubling of diabetes cases by 2030 [2]. Diabetic patients might experience life-threatening macrovascular (atherosclerosis, cardiovascular disease) and microvascular complications (microangiopathy) of the retina, nervous system, and kidney [3]. Neuropathy and peripheral ischemia result in foot ulcers, often leading to amputation and severe infections [4]. All diabetes complications cause severe morbidity and raise substantial economic and societal costs.

Development of diabetic nephropathy (DN) is a major medical concern, as it greatly increases the risk of premature death by end stage renal disease and is associated with increased cardiovascular mortality. Therefore, huge research efforts are focused on deciphering pathologic molecular mechanisms in DN, which may provide valuable tools for early diagnosis and prevention of DN onset and evolution.

DN is clinically characterized by albuminuria, proteinuria, elevated creatinine levels, and abnormal glomerular filtration rates. The key pathological features of DN include glomerular hypertrophy, mesangial matrix expansion, diffuse glomerular basement membrane thickening, podocyte loss and foot process effacement, nodular glomerulosclerosis, mesangiolysis and glomerular microaneurysms, interstitial fibrosis, and tubular atrophy. Inflammation and endothelial dysfunction play important roles in DN pathogenesis. Albuminuria and afterwards proteinuria associated to glomerular changes, and interstitial fibrosis are hallmarks of DN [5].

These complex and progressive pathologic changes are mainly induced by (a) hyperglycemia and enhanced formation of advanced glycation end products (AGE); (b) increased activity of angiotensin II (Ang II) within the renin-angiotensin system; (c) excessive TGFβ-signaling; (d) chronic inflammation associated to enhanced recruitment of leukocytes and release of proinflammatory cytokines, chemokines and growth factors [6, 7].

This review emphasizes the role of the oxidative stress in DN, which may act as trigger, modulator, and linker within the complex network of pathologic events. We highlight several molecular events underlining and connecting the metabolic, oxidative, and inflammatory stresses in DN. The aim was to bring forward key molecular networks and new hypothesis in the pathophysiology of DN, related to oxidative stress. We point out that an integrative approach is needed for identifying key molecular pathways controlling cellular responses to the complex array of pathologic signals in DN. This approach is required in order to find reliable biomarkers for early diagnosis and prognosis, and to guide the discovery of new therapeutic strategies for personalized medicine in DN.

2. Disease-Related Oxidative Stress in DN

Oxidative stress is linking most of the molecular events underlining the pathological process in DN, related to hyperglycemia and AGE, the renin-angiotensin system, TGFβ signaling, and chronic inflammation. Glomerular and tubular hypertrophy, mainly due to mesangial cells accumulation, extracellular matrix deposition, thickening of glomerular and tubular basement membranes, podocyte dysfunction, and apoptosis, all are redox-induced alterations leading to albuminuria, proteinuria, glomerulosclerosis, and tubulointerstitial fibrosis.

Reactive oxygen species (ROS) are both friend and foe of aerobic organisms. They adapted to oxidative aggression by developing potent antioxidant mechanisms, and learned how to use ROS in their favor, as signaling molecules which sustain vital redox-sensitive processes. Besides phosphorylation, subtle and reversible changes of the redox status can propagate and fine-tune signals from the membrane to the nucleus.

When the tightly controlled redox balance is even slightly altered either by increased and prolonged ROS production, or by inefficient antioxidant mechanisms, pathologic processes may arise. Above a physiological limit, ROS may induce significant conformational changes of lipids, proteins, glucides and nucleic acids, leading to distorted interactions and altered cellular functions. These biologic targets practically detoxify ROS, thus interrupting the oxidative cascade. Being more stable than ROS, they are potent propagators of the deleterious action of ROS, long after ROS disappeared.

Chronic oxidative stress is a constant and ubiquitous presence in DN, accompanying and interfering with hyperglycemia and inflammation. Conventional markers of oxidative stress in serum, urine, and various organs were evidenced in DN, ranging from markers of lipid peroxidation (malondialdehyde, 4-hydroxynonenal), protein carbonyls, and oxidized DNA [8]. These few validated biomarkers of oxidative stress are insufficient for early diagnosis and prognosis in DN, and therefore huge efforts are focused on biomarker identification by deciphering the molecular basis of oxidative stress in DN and other pathologies. For instance, oxidative and glycoxidative changes of proteins, reflecting the metabolic and oxidative stresses in diabetes, are mediators of multiple distorted signaling pathways [9]. AGE are risk factors for diabetes complications, that are formed through nonenzymatic aminocarbonyl interactions between reducing sugars and oxidized lipids, proteins, amino phospholipids, or nucleic acids [10]. Oxidative stress is not only involved in AGE formation, but AGE themselves amplify oxidative stress, as described in the following sections. Hemoglobin A1c (HbA1c), a glycosylated nonpathogenic form of hemoglobin, was added to the standards of care by the American Diabetes Association, as biomarker of the presence and severity of hyperglycemia in diabetes. It exhibits less biologic variability than glucose levels and responds to diet and treatment [11]. The glycation of skin collagen and the accumulation of AGE were shown to be consistently correlated with diabetes complications. Therefore, skin fluorescence due to AGE accumulation might be considered a useful noninvasive marker of cumulative tissue damage in diabetes [12]. New AGE, like 3-dioxiglucasone, methylglyoxal, methionine sulfoxide, and 2-aminoadipic acid, were recently demonstrated to have some prognostic power regarding DN progression [13]. However, due to the sophisticated methods required for their detection, they are still far from being translated into the practice.

Several other candidate biomarkers of oxidative stress in diabetes are under study [14] and, if validated, may impact on the management of patients with diabetic complications. Moreover, the development of new therapeutic strategies definitely needs a panel of reliable, readily accessible, and easy to monitor parameters for patients follow-up.

2.1. Sources of ROS in DN. The ROS cascade [15] is initiated from molecular oxygen by formation of superoxide anion, either intentionally or as byproduct of other reactions. It arises from enzymatic reactions mediated by NADPH oxidases, the oxidative phosphorylation chain, xanthine oxidases, uncoupled nitric oxide synthase, and so forth. Hydrogen peroxide is then formed from superoxide anion through a reaction catalyzed by superoxide dismutases. Hydrogen peroxide may further evolve towards a highly toxic reactant, hypochlorous acid, by the action of the myeloperoxidase system. The chain involving superoxide anion and hydrogen peroxide is then continued by radical-radical reactions, to generate hydroxyl radical and singlet oxygen.

The initial steps of this cascade are under a tight enzymatic control mediated by NADPH oxidases and superoxide dismutases. (a) NADPH oxidases, having multiple components scattered in the cytosol and membranes in resting cells, are activated and translocated to the membrane for assembling a functional enzyme only when cells must respond to a particular challenge by generating superoxide anion. Numerous homologue-specific mechanisms are controlling NADPH oxidases, including calcium ions, free fatty acids, protein-protein interactions, and posttranslational modifications (phosphorylation, acetylation, or sumoylation) [16]. (b) Superoxide dismutases, are located in critical cellular compartments where superoxide anion might be generated, for avoiding unwanted oxidative damage in mitochondria and cytosol. (c) Catalase, peroxidases, and peroxiredoxins promptly detoxify hydrogen peroxide. Cells are less protected against the generation of more advanced ROS, like the hydroxyl radical, as only the availability of Fe^{2+} is limiting

the Fenton reaction. Therefore, interrupting the chain of ROS generation in early phases is essential for limiting the oxidative stress damage.

2.1.1. Mitochondrial ROS. A defect in the mitochondrial electron transport chain, resulting in overproduction of superoxide anion, was considered the main mechanism underlining high glucose-induced oxidative stress and subsequent DN complications [17].

In vivo ROS imaging evidenced that mitochondrial superoxide anion production is reduced in diabetes (the Crabtree effect) and is accompanied by diminished mitochondria biogenesis [18]. This might be an adaptive mechanism for preserving renal glomerular function during hyperglycemia-associated oxidative stress, which is mediated by AMP-activated protein kinase (AMPK), pyruvate dehydrogenase (PDH), and the peroxisome proliferator-activated receptor coactivator 1 (PGC1α). AMPK is the main energy sensor in the organism, normally activated for overcoming temporary caloric restriction. AMPK works in tandem with the transcriptional coactivator PGC1α which sustains mitochondria biogenesis and their oxidative metabolism [19]. In diabetes, AMPK is inhibited and consumes fewer electrons and oxygen, whilst carbon skeletons are released to the cytosol for use as building blocks for cell growth (cataplerosis). The reduction of carbon flow into mitochondria under conditions of caloric excess is regulated in part by the inhibition of PDH through hyperphosphorylation, leading to reduced pyruvate entry into mitochondria [20]. The decreased ROS generation in mitochondria due to low AMPK activity is sustained by concomitant PGC1α inhibition, leading to a limited biogenesis of mitochondria. Recovery of mitochondria (in number, structure, and function), achieved by activating AMPK with AICAR (5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside), was shown to restore renal podocyte function [18].

The old therapeutic strategy, aiming to interrupt mitochondrial production of superoxide anion, is questionable, as the underlining hypothesis of elevated mitochondrial ROS in diabetes might not be always true. We should be precautious with these new findings, as long as the tempo-spatial ROS alterations in diabetes are largely unknown. There is a lack of reliable *in vivo* observations, and the available experimental models have important drawbacks.

Reduced mitochondrial ROS generation in DN is uncoupled from the substantial damage of mitochondrial DNA (mtDNA), suggesting that either some other sources of ROS might be active, or non-ROS-mediated mechanisms underline DNA deletions.

Induction of DNA deletions is an early "danger response" to metabolic stress, necessary for upholding energy metabolism to rescue the cell. Above a threshold, ROS cause significant oxidative damage to mitochondria and mtDNA, and elicit apoptosis/mitophagy. Mitochondria move within the cell and frequently undergo fission and fusion. Chronic high-glucose exposure and consequent oxidative stress can induce mitochondrial morphologic changes by the activation of fission signals mediated by ERK1/2-mediated phosphorylation of dynamine-like protein-1 [21, 22]. Mitochondria fragmentation further triggers a ROS response, thus amplifying mitochondria dysfunction and cell apoptosis [23]. Fragmented and dysfunctional mitochondria are selectively targeted for degradation to autolysosomes through the multistep autophagy pathway. It is regulated by the mTOR complex-1 and AMPK sensors of the nutrient status. The elimination of damaged mitochondria is also mediated by the PTEN-induced putative protein kinase 1 and the E3 ubiquitin ligase Parkin [24]. The accumulation of fragmented mitochondria in the renal cortex in DN proves that biogenesis and clearance of mitochondria may be impaired [25, 26]. Reduced activity of AMPK, accompanied by increased signaling *via* the mTOR pathway, accounts for defective autophagy in DN. AMPK plays a central role in regulating mitochondriopathy by limiting both mitochondrial superoxide generation and autophagy of damaged mitochondria.

The evidence of reduced mitochondrial superoxide generation in DN is intriguing, considering the overwhelming evidence of advanced oxidative stress in DN. As proposed by Towler (2013), the inactivation of AMPK may shift ROS formation from the unintentional production of superoxide anion in mitochondria towards other sources, such as the Nox family of NADPH oxidases [27, 28].

2.1.2. NOX Enzymes. The discovery of new members in the NADPH-oxidase family (Nox family), besides the Nox2 prototype expressed by phagocytes, which generates superoxide anion as nonspecific antimicrobial defense mechanism, changed the picture [28]. Nox family consists of 7 members, Nox1 to Nox5, Duox1, and Duox2, all of them transporting electrons across biological membranes for reducing oxygen to superoxide. They are transmembrane proteins with a certain degree of structural homology but different in their cell/tissue distribution, mechanisms of activation, and functional involvement in local redox homeostasis. The discovery of the Nox family was a breakthrough in ROS biology, emphasizing that ROS are more than toxic weapons of the innate immune response, as they are involved in redox-based signaling networks in almost all cell types [29].

The presence of various Nox isoforms, Nox4 and Nox2, was documented in renal cells and synergistically contributes to ROS generation in DN [30].

Nox4 (Renox), the most abundant Nox isoform in kidney, was found in glomeruli and proximal and distal convolute tubules. Nox4 is located in mitochondria and in the plasma membrane [31]. Nox4 exhibits a particular functional pattern among Nox members, as it is constitutively active and its contribution to oxidative stress is regulated only through expression.

Nox2 is expressed by phagocytes recruited in kidney by the macrophage chemotactic protein 1 (MCP-1) during inflammation, but also by podocytes, mesangial cells, and renal endothelial cells [31]. Unlike Nox4, Nox2 activation is tightly regulated for avoiding unintentional production of superoxide anion: its constituents are scattered in the cytoplasm and plasma membrane, and get assembled into an active membrane enzyme in response to various activation signals, through a sophisticated network of intracellular events [15].

Several studies showed that Nox4 is the main source of superoxide anion and hydrogen peroxide in DN, and that its pharmacologic inhibition or siRNA-mediated knockdown almost completely abrogates diabetic complications and the associated intracellular signaling networks [32, 33]. Inhibition of Nox4 using competitive pyrazolo pyridines inhibitors resembling NADPH may thus hold promise as therapeutic approach to prevent renal injury. As Nox4 has also a role in cardiovascular disorders [34], its pharmacologic inhibition may be a valuable tool to control DN complications more generally.

Other studies invalidated the theory stating that Nox4 is solely a major driver of renal disease; it was shown that, under particular conditions, Nox4 may limit injury and disease progression [35]. This is not surprising, knowing that Nox4 is expressed in normal renal cells in an active form, indicating that low levels of ROS, continuously produced by the constitutively active Nox4, maintain kidney homeostasis [36]. The type of experimental model and the specific experimental stressor used in these studies might explain contradictory results [37]. One may observe that Nox4 inhibitors could exert also nontargeted effects. The hypothesis that Nox4 might have a dual role, as stressor and protector of renal cells, depending on the microenvironment and the kidney pathology, has to be further explored [38].

We point out that all NADPH oxidases can act as double-edged swords triggering feedback defense against excessive ROS generation. The ROS-elicited activation of receptor tyrosine kinases and of the redox-sensitive Nrf2-Keap1 signaling pathway induces the transcription of potent antioxidants to combat the deleterious effects of chronic oxidative stress [39].

2.1.3. Hyperglycemia-Induced ROS. The mechanisms of glucose metabolism involved in DN complications include glucose autooxidation, the shunt of glucose to the polyol pathway, formation of AGE, and elevated hexosamine pathway activity [40, 41].

An increased production of ROS triggered by high glucose has been suggested as a unifying process that links the pathways of hyperglycemia-induced damage: (a) the influx of glucose through the polyol pathway increases AGE formation, which, by themselves or consequent to AGE receptors signaling, cause a sustained oxidative stress and the release of inflammatory cytokines; (b) due to NADPH consumption, aldose reductase activity limits the antioxidant response to elevated ROS [40].

Although most experimental studies on DN are using high glucose as triggering stress, clinical data show that not all patients with poor glycemic control develop nephropathy, and conversely, renal complications develop sometimes even when glucose control is achieved. This evidence highlights a "metabolic memory" in diabetes, supported by a feedforward mechanism even at normal glycemia. Possibly, a vicious cycle involving AGE, oxidative stress, and epigenetic changes propagates the signals delivered initially by hyperglycemia [42].

In an oxidative environment, the polyol pathway promotes the generation of AGE, such as N-carboxymethyl lysine, N-carboxyethyl lysine, and pentosidine, and orchestrates changes leading to diabetic complications [43]. AGE propagate metabolic signals through interaction with several specific receptors, such as RAGE, macrophage scavenger receptor, and galectin-3, and induce proliferation, apoptosis, autophagy, or cell migration, depending on the target cell and the context [44]. Intracellular ROS production is triggered by AGE-RAGE interaction [45] *via* the peroxisome proliferator-activated receptor-γ [46]. AGE enhance the formation of cytosolic ROS which accelerate mitochondrial superoxide production. AGE-mediated signaling pathway involves functional NADPH oxidases, $p21^{ras}$, protein kinase C, and p38 and Erk1/2 MAP kinases. Downstream transcription factors like NFκB, AP-1, and SP-1 are further activated by redox-sensitive signaling pathways, triggering a plethora of pro-inflammatory and pro-fibrotic responses. AGE and even RAGE itself induce increased expression of RAGE, thus amplifying renal dysfunction. This in turn increases AGE concentrations due to reduced clearance. The cascades triggered by AGE suggest that prevention and treatment must focus not only on early glycemic control, but also on limiting the factors related to oxidative stress and AGE formation [47].

2.1.4. Inflammation-Related ROS. Hyperglycemia and the associated oxidative stress promote inflammation through endothelial cell damage, increased microvascular permeability, elevated expression of chemokines, adhesion molecules, along with recruitment of inflammatory cells into the diseased kidney.

Endothelial dysfunction is an important source of oxidative stress and inflammation in DN [48]. Injured endothelial cells are active signal transducers of metabolic, hemodynamic and inflammatory factors that modify the function of the vessel wall, interact with adjacent cells, and elicit inflammatory, proliferative, and profibrotic responses. A critical mechanism of endothelial dysfunction is the impaired activity of endothelial nitric oxide synthase (eNOS), produced by posttranslational modification of the enzyme through the hexosamine pathway, S-nitrosylation, and downregulation of its expression [49]. AGE, alterations in the cellular redox state, deregulation of protein kinase C, all may contribute to eNOS impairment [50] and subsequent reduced nitric oxide (NO) bioavailability. This has important pathologic consequences, as NO opposes the effects of endothelium-derived vasoconstrictors, such as Ang II and endothelin, and protects against the damage induced by proinflammatory cytokines (TNFα). Oxidative stress can interfere with the production and activity of NO [50]. Superoxide anion rapidly inactivates NO and destroys tetrahydrobiopterin (BH_4), a cofactor required for NO synthesis. At low levels of BH_4, eNOS has been shown to generate superoxide anion instead of NO, in a NADPH-dependent manner (uncoupling of NOS activity) [51]. Increased production of superoxide anion further decreases the tissue bioavailability of NO by formation of peroxynitrite through a radical/radical reaction which takes place faster than the interaction with superoxide dismutases [52].

Neutrophils, followed by T cells, and finally macrophages are recruited from circulation, accumulate in the glomeruli

and interstitium even in the early stages of DN, and inflict damage to renal and endothelial cells through cytokines, matrix degrading enzymes, and ROS [53, 54].

Neutrophils and macrophages express toll-like receptors (TLR) and consequently respond to AGE and other mediators by generating superoxide anion *via* Nox2, thus amplifying the local oxidative stress. Activated phagocytes release matrix-metalloproteinases which degrade the extracellular matrix. This in turn challenges resident and newly recruited cells to produce ROS and proinflammatory cytokines. In particular oxidative conditions, matrix-metalloproteinases might be inactivated, probably as rescue mechanism to limit oxidative stress-induced tissue destruction during inflammation [55].

CCR2-expressing monocytes are recruited in the DN kidney by MCP-1. In response to high-glucose, AGE, and oxidative stress, MCP-1 is secreted by mesangial and kidney epithelial cells, including glomerular podocytes and tubular cells. The process is mediated by NFκB, at least in mesangial cells. In contrast to serum, the urine levels of MCP-1 mirror chemokine production in the kidney and correlate with disease stage and progression [56]. Pharmacologic blocking of MCP-1 seems to have beneficial effects in DN. Noxxon Pharma developed Emapticap pegol, a Spiegelmer that binds and neutralizes CCL2/MCP-1. The phase IIa proof-of-concept data showed a relevant decrease in urinary albumin excretion and better glycemic control, which persisted after treatment cessation (http://www.noxxon.com/).

In the DN kidney, blood monocytes and tissue macrophages are functionally polarized towards a proinflammatory M1 phenotype [57] and release considerable amounts of proinflammatory, profibrotic, and antiangiogenic factors (TNFα, IL-1, IL-6, plasminogen activator inhibitor-1, matrix metalloproteinases, TGFβ, platelet-derived growth factor, Ang II, and endothelin) [53]. This plethora of soluble factors triggers and sustains inflammation-based pathological events in DN.

In DN, the alarmin HMGB1 (high mobility group box 1) is functioning as endogenous redox-sensitive promoter of the immune response to injury [58, 59]. HMGB1 is a nuclear nonhistone DNA-binding protein which, consequent to inflammation-induced acetylation in monocytes, translocates from the nucleus to cytoplasmic secretory lysosomes and thereafter is released [60]. Alternatively, cell necrosis, but not apoptosis, allows passive HMGB1 release from damaged or dying cells. Extracellular HMGB1 binds to various receptors, such as RAGE and TLR to which AGE also bind, and signals damage to the neighboring cells. HMGB1 is a danger associated molecular pattern [61]. Extracellular HMGB1 can act as an inflammatory mediator through MyD88, MAPK, PI3K/Akt, and NFκB. Finally, HMGB1 induces recruited and resident leukocytes to release proinflammatory cytokines.

HMGB1 responds to ROS and induces ROS formation. HMGB1 is a redox-sensitive protein containing three critical cysteines which, under various oxidative settings, are oxidized or reduced and distinctively regulate HMGB1 activity. HMGB1 not only reacts to oxidative stress, but also triggers ROS generation in phagocytes by receptor-mediated activation of Nox2 [62]. The expression of HMGB1 is upregulated in the kidney of diabetic rats, suggesting that the release of hyperglycemia-triggered HMGB1 may induce renal inflammatory injury. The pathogenic role of HMGB1 is dependent on RAGE or TLR4 expression and on NFκB activation, and may be related to tubulointerstitial inflammation in DN [63, 64].

The proinflammatory signals delivered by monocytes/macrophages in DN go beyond soluble factors. Recently, it was shown that communication between renal cells and monocytes/macrophages might be mediated by endothelial and monocyte-derived exosomes during inflammation. They exhibit particular signatures and may play distinctive diabetogenic and procoagulant roles [65].

2.2. Sensing Redox Signals in DN. In DN, renal cells are exposed and respond both to extra- and intracellular oxidative stress. Key signaling redox-sensitive pathways are either directly activated by ROS or oxidative stress-related mediators signal *via* various receptors. Pathologic consequences arise due to chronic exposure of cells to various cues and/or to transient alteration of signaling, leading to aberrant responses to stress. As multiple stressors persistently challenge renal cells in DN, it is quite difficult to differentiate between the specific pathways. Results are highly dependent on the experimental model used, including the activation system, on the stage of disease, and the time-course of the signaling events.

Cysteine is uniquely suited to sensing redox signals, as the thiol side-chain can be oxidized to several reversible redox states, such as disulphide, sulphenic acid, and S-nitrosothiol. Accordingly, proteins expressing critical cysteine residues are most susceptible to oxidant attack which modifies their structure and interaction ability by formation of disulphide bonds. This will impact on their function, including signaling and transcriptional activities. The disulphide bond status of proteins is tightly controlled by cytoplasmic and mitochondrial thioredoxins (Trx) [66] which reduce disulfides into thiol groups. The activity of Trx1 is regulated by its endogenous inhibitor, Txnip. It acts as a redox rheostat to control Trx1 activity and expression. In DN, Txnip expression is induced by high glucose conditions and Trx-mediated reduction potential is consequently limited [67].

2.2.1. Protein Kinases. Various protein kinases, like PKC, Akt/B, and MAPK (ERK1/2, p38 kinase, JNK), along with phosphatases, are regulated by ROS under physiological conditions. They are also responsible for initiating and propagating disorders related to ROS overproduction [68]. We emphasize that redox and phosphorylation events are interconnected and control signaling. The consequences of these mechanisms are highly dependent on cell type, extracellular milieu, and intracellular signaling machinery, and may trigger either cell death or cell proliferation in apparently similar stressful conditions [12].

2.2.2. FOXO Transcription Factors. The Forkhead box O transcription factors (FOXO1, FOXO3, and FOXO4) are redox sensors and mediators of ROS signaling in various processes and diseases, ranging from glucose metabolism to cell cycle arrest or progression, DNA damage repair, and

apoptosis [69]. The oxidative stress regulates FOXO activity through posttranslational modifications (phosphorylation, acetylation, and ubiquitination). In turn, it dictates subcellular localization and transcriptional activity of FOXO. An example is the stress-activated kinase JNK which phosphorylates FOXO4, leading to its nuclear translocation and increased transcriptional activity [70]. SIRT1-mediated deacetylation of FOXO may repress its transcriptional activity, whilst Akt-dependent phosphorylation dictates ubiquitin-mediated FOXO degradation [70].

FOXO are molecular switches that decide the cell fate in response to oxidative stress, either by promoting prosurvival antioxidant responses or by triggering cell death [71]. FOXO proteins play a critical role in maintaining the redox balance. They upregulate antioxidant genes expression (manganese-superoxide dismutase and catalase) [72] or restrict mitochondrial ROS production along with mitochondria biogenesis *via* enhanced expression of heme oxygenase-1 [73]. Conversely, FOXO1 can regulate genes in both the extrinsic and intrinsic apoptotic pathways [74]. Chronic oxidative stress may induce simultaneously NFκB and FOXO. Both factors have dual roles, as rescue or death-triggering factors [75]. Depending on the context, the effects exerted by each of these transcription factors and their relative balance will decide the target cell fate [76]. In certain cases, FOXO1 may act to amplify NFκB-induced inflammation by binding to a response element nearby the NFκB-binding element, or by physically interacting with NFκB [77].

Hyperglycemia and oxidative stress alter transcription programs in target tissue cells. A "transcriptional memory" spread abnormal gene expression patterns to cell progenitors, even in the absence of the triggering stressor [78]. The existence of metabolic and transcriptional "memories" highlights the importance of early and intensive treatment in DN.

2.2.3. Epigenetic Modulators. Multiple stressors trigger epigenetic changes in diabetes [79], such as DNA methylation and posttranslational modifications (lysine acetylation, methylation and ubiquitination, serine/threonine phosphorylation, and arginine methylation). Transcriptional repression or activation is dependent on the position of the residue within the histone tail and is highly regulated by paired enzymatic systems. For instance, histone lysine acetylation is mediated by histone acetyl transferases acting as transcription coactivators. Conversely, histone deacetylases (HDAC) remove acetylation marks and mostly act as corepressors. Histone-modifying enzymes can be recruited by binding to specific DNA sequences in the promoters or *via* interaction with RNA polymerase II and transcription factors [80].

HDAC can control oxidative stress *via* the transcription of Nox4; HDAC inhibition decreases Nox4 transcription in human endothelial cells by preventing the binding of transcription factors and polymerases to the Nox4 promoter, most likely due to a hyperacetylation-mediated steric inhibition [81]. In mesangial cells exposed to hyperglycemic conditions, nuclear translocation and transcriptional activation of the lysine methyltransferase Set7 are increased upon TGFβ stimulation. This occurs downstream of ROS and is involved in glucose-driven fibrotic gene expression [82].

Sirtuins—nicotinamide adenine dinucleotide (NAD^+)-dependent deacetylases/mono-ADP ribosyltransferases—are regulated by energy metabolism and are involved in various signaling pathways in senescence, apoptosis, DNA damage repair, and autophagy. SIRT1 exerts cytoprotection by an antiapoptotic, antioxidative, and antiinflammatory action, along with regulatory effects on mitochondrial biogenesis and autophagy [83, 84]. In stressed cells, SIRT1 shifts antiapoptotic to proautophagic responses by directly deacetylating essential autophagy proteins [85] and by deacetylation of redox-sensitive transcription factors, such as FOXO [86]. AMPK activates SIRT1 by increasing its substrate, NAD^+ [87], but this interaction is disrupted by hyperglycemia and oxidative stress. As shown above, hyperglycemia decreases AMPK expression, leading to reduced SIRT1 expression.

Another level of epigenetic (de)regulation in DN is mediated by microRNA (miRNA). Decreased miR25 induces upregulation of Nox4 expression in mesangial cells, thus enhancing the local oxidative stress [88]. TGFβ1 can upregulate miR192 in cultured mesangial cells and in glomeruli from diabetic mice, leading to increased collagen production by acting on specific repressors and/or by modulating other miRNA. The susceptibility of podocytes to ROS-mediated apoptosis may be partly induced by decreased miR29c levels [89].

As most molecular studies were focusing on a limited number of signaling pathways and different experimental settings, it is quite difficult to integrate existing data into a complex DN-specific signaling map. Maybe the time has come to apply an "omic" approach to integrate epigenetic changes, gene expression, and signaling in health and disease.

3. Hypertrophy and Death Signals in DN

Main pathological features in DN are hypertrophy of mesangial cells, glomerular extracellular matrix deposition, and podocyte loss. These different types of cellular responses to the same type of stressors reflect subtle differences in the molecular machinery in mesangial cells and podocytes.

3.1. Hypertrophy Responses. In DN, unopposed survival mechanisms are active, dictating mesangial cells expansion and accumulation. Mesangial cells react to high glucose, Ang II, and TGFβ activation, by a biphasic growth response, starting with mitogen-triggered proliferation, followed by cell cycle arrest in the G1/S interphase and consequent hypertrophy. Active cell cycle-dependent hypertrophy, involving PKC activation, is initially mediated by cyclin D and is further developed by the intervention of cyclin E [90].

Mahimainathan et al. (2006) demonstrated that the hypertrophic response of mesangial cells may occur *via* the activation of the PI3K/Akt pathway due to the reduced expression and phosphatase activity of the tumor suppressor PTEN [91].

As reviewed by Brosius et al. (2010), enhanced metabolism and growth of mesangial cells could be also mediated by activation of the mTOR pathway due to reduced AMPK signaling. Activation of downstream substrates of the mTOR complex-1 triggers progrowth and antiapoptotic processes,

whereas the inhibition of mTOR by rapamycin limits early glomerular hypertrophy and mesangial expansion [92].

Decreased expression and activity of the deacetylase SIRT1 favor high glucose-induced mesangial hypertrophy through downregulation of the AMPK signaling pathway and subsequent activation of mTOR [93, 94].

Unlike mesangial cells, mature podocytes do not actively synthesize DNA nor proliferate due to high levels of cyclin dependent kinases [90]. Nevertheless, podocytes might undergo ERK1/2 or Akt-mediated hypertrophic changes in DN in response to high glucose, Ang II, and to increased intraglomerular capillary pressure [95]. mTOR hyperactivation due to decreased AMPK activity in DN might also mediate a sustained hypertrophic stimulus that results in podocyte degeneration, the development of glomerulosclerosis, and proteinuria [96].

Excessive angiogenesis in DN [97] due to increased proliferation and decreased apoptosis of endothelial cells is associated with glomerular hypertrophy. Immature endothelial cells and high levels of VEGFA, which are characteristic for the early stages of DN, trigger abnormal angiogenesis and increased vascular permeability, resulting in extravasation of plasma proteins. Abnormal low levels of endothelial derived NO, along with glomerular hypertension, sustain this pathologic process. Meanwhile, in advanced stages of disease, low levels of VEGFA are registered, probably due to the inability of damaged podocytes and tubular interstitial cells to produce the angiogenic factor.

ECM accumulation contributes to glomerular hypertrophy and is considered as a repair mechanism to glomerular injury, which unfortunately escapes control in DN. ECM accumulation derives from abnormal ECM metabolism in mesangial cells and also from changes in podocytes and endothelial cells metabolism leading to the thickening of the glomerular basement membrane (GBM). Unlike mesangial cells, podocytes normally release matrix-degrading proteinases, but this control mechanism is apparently suppressed or overwhelmed in DN. Mesangium fibrosis in diabetes conditions seems to be mediated by PKCβ stimulation of AP-1 transcriptional activity and ERK pathways, along with TGFβ1 synthesis and signaling [98]. Additionally, JAK2 activation and phosphorylation of its downstream STAT substrates [99] mediate collagen IV and fibronectin production, TGFβ activation, and cell growth in response to Ang II, high glucose exposure, or enhanced ROS generation [100, 101].

3.2. Podocyte Death. Podocytes create and preserve the glomerular perm-selectivity barrier: podocyte interdigitating foot processes are bridged by a sieve-like slit diaphragm; podocytes contribute to the synthesis of GBM; podocytes actively crosstalk with glomerular endothelia *via* VEGF and other paracrine signals. Moreover, podocyte contractile properties can modulate the hydraulic pressures sustained by the glomerulus [92].

DN is characterized by a broadening of the foot processes and podocyte loss due to cell apoptosis or detachment from the GBM. This puts a lot of pressure on remaining cells and leads to glomerulosclerosis. The morphological and functional changes of podocytes in DN are related to abnormal signaling *via* TGFβ, MCP-1/CCR2, Wnt/β-catenin and VEGF [92].

Diabetic conditions increase the expression of TGFβ and its receptor, TGFβRII, in glomerular cells. As reviewed by Lee [102], latent TGFβ complexes released by mesangial cells in DN are stored in the mesangial matrix. Incompletely activated latent TGFβ is released and localizes to the podocyte surface. In an oxidative environment, podocyte-derived plasmin, matrix-metalloproteinases, and thrombospondin-1 may activate the latent TGFβ in podocytes. Activated TGFβ can reduce the binding of podocytes to GBM by specific integrin downregulation, thus promoting podocyte loss [103, 104]. Activated TGFβ delivers PI3K-mediated apoptotic signals to podocytes and subsequent activation of the proapoptotic p38-MAP kinase. This is resulting in synthesis of Bax and its translocation to mitochondria, cytochrome c release, and caspase activation, leading to apoptosis. Activation of Smad7 sustains podocyte apoptosis by inhibiting the survival factor NFκB [104]. Intervention of Notch pathways in the TGFβ-mediated apoptosis of podocytes was also suggested [105]. The activation of the apoptotic machinery by TGFβ may also be dependent on factors controlling the cell cycle, such as the stress-induced p21 [106].

Podocyte apoptosis is aggravated by hyperglycemia and increased production of ROS and AGE, which in turn enhances FOXO4 acetylation and suppresses SIRT1 expression [107].

Autophagy is a stress response involved in the catabolic processes that degrade damaged intracellular proteins and organelles. Autophagy normally has a protective role against renal damage, but in DN it is apparently suppressed due to decreased activity of AMPK and the subsequent activation of mTOR complex-1 [26].

4. The Antioxidant Response in DN

The local and systemic oxidative stress underlining the pathologic features of DN is the net result of the oxidant-antioxidant balance. It may arise not only from increased ROS generation by various mechanisms, as shown above, but also from a downregulated antioxidant response. Apparently, the physiologic mechanism "ROS trigger ROS" is hyperactivated in DN, perpetuating the oxidative stress, whilst cells are unable to react properly to this overwhelming stress.

Overproduction of high glucose-induced ROS decreases *via* the PI3K–Akt–FOXO3a pathway the expression of manganese-superoxide dismutase, as guardian of superoxide generation in mitochondria [108]. Lower erythrocyte and plasma levels of reduced glutathione in type 2 diabetic patients were also registered [109]. Moreover, the sirtuin signal is downregulated and this may contribute to the failure of defense mechanisms to combat chronic oxidative stress in DN [110, 111]. This arises because Sirtuins reduce ROS formation by modulating the acetylation of the respiratory chain and by stimulating mitochondrial superoxide dismutase.

4.1. Dietary Antioxidants in DN. Various attempts to control oxidative stress in type 2 diabetes patients using conventional dietary antioxidants (selenium, vitamins A, C, E) failed to

improve the disease outcome, as shown by the analysis performed by Akbar et al. on 14 studies [112]. The authors concluded that dietary antioxidant supplementation did not affect plasma glucose or insulin levels, but may have some benefit in protecting against the complications of type-2 diabetes. Unfortunately, no definite conclusion could be drawn due to the relatively small number of patients (572) included in these studies.

The meta-analysis performed by Bjelakovic et al. (2007) on 68 randomized trials with over 200.000 adults compared beta carotene, vitamins A, C, E, and selenium *versus* placebo or no intervention [113]. This systematic review showed that beta carotene and vitamins A and E significantly increase all-cause mortality of adults included in prevention trials. No clear evidence was found that vitamin C may increase mortality and only selenium has the tendency to reduce mortality. Biesalski et al. (2010) revised the analysis performed by Bjelakovic and concluded that dietary supplementation for the prevention or treatment of chronic diseases is most effective in those patients with inadequate intakes [114]. Apparently, there is a threshold nutrient status above which additional intake cannot provide further benefit. The threshold for nutrient intake is dependent on age, sex, health status, and gene polymorphisms.

Another approach to control oxidative stress is the adjuvant therapy with phytoantioxidants. For instance, extensive research has been focused on curcumin (diferuloylmethane), a component of the golden spice turmeric (*Curcuma longa*). In various experimental studies curcumin was shown to modulate multiple cell signaling molecules, such as proinflammatory cytokines, apoptotic proteins, transcription factors, TGFβ, and endogenous antioxidants [115]. A small clinical study was performed on 20 patients with type 2 DN [116], showing that short-term curcumin supplementation attenuates proteinuria, TGFβ and IL-8. Curcumin can be administered as adjuvant therapy, but long-term trials with larger numbers of patients are needed for confirmation.

4.2. Targeting the Endogenous Antioxidant System in DN. Advances in ROS biology and the molecular basis of the cellular response to oxidative stress indicated different ROS-associated molecules and signaling pathways as promising therapeutic targets in inflammatory disease.

Limiting ROS production may positively impact on oxidative stress-triggered pathological events, but may also negatively affect normal physiologic processes which rely on ROS. Drawbacks also arise from the multitude of ROS types produced in the respiratory burst, which makes it almost impossible to control. Therefore, a safer strategy would be to enhance the cellular response to the deleterious oxidative stress by activating the antioxidant system at transcriptional level.

The transcription factor Nrf2 (NF-E2-related factor 2), together with its negative regulator, Keap1 (Kelch-like ECH-associated protein 1), play a key role in combating oxidative stress. This is achieved by upregulating important antioxidants, like NADPH quinone oxidoreductase, glutathione S-transferase, hemeoxigenase-1, and γ-glutamylcysteine synthetase [117–119] *via* the antioxidant response element (ARE).

Under unstressed conditions, Keap1 represses Nrf2 transactivation activity. Keap1 appears to act as a sensor molecule of oxidative and electrophilic stresses, and it accelerates Nrf2 degradation. When Keap1 senses oxidative or electrophilic stresses, Nrf2 is liberated from Keap1-mediated repression, accumulates in the nucleus, and induces the expression of cytoprotective genes. The rapid turnover of Nrf2 prevents aberrant expression of Nrf2 target genes [120]. Nrf2 switching on and off, and Nrf2 crosstalk with signaling pathways (p53, Notch1, and NF-κB) protect cells against oxidative damage, prevents apoptosis, and promotes cell survival [121]. Additionally, Nrf2 directly regulates cellular energy metabolism by modulating the availability of substrates for mitochondrial respiration [122].

Despite the chronically enhanced oxidative stress and inflammation in DN, which normally should have induced Nrf2 activation, the diseased kidney has impaired Nrf2 activity and reduced expression of its target genes [123]. Nrf2 activators, like sulphoraphane or cinnamic aldehyde, were shown to attenuate damage and preserve renal function in diabetic mice, indicating that Nrf2 might be a valuable therapeutic target in DN [124].

Nrf2 activators were designed (bardoxolone methyl and its analogues) or were extracted from broccoli (sulphoraphane). They were investigated in preclinical studies and entered clinical trials for the treatment of various diseases, like cancer and Alzheimer's disease. Both promising and disappointing results were obtained, indicating that potential side-effects due to nontargeted effects should be thoroughly considered in the first phases of clinical trials [123].

5. Future Perspectives

(1) We are facing a large amount of experimental information regarding the molecular interferences between oxidative, metabolic, and inflammatory stresses in DN. Most data were obtained in cell cultures and animal models. There is a need to integrate these scattered pieces into a coherent signaling map, to identify missing links, and to solve them. Major drawbacks arise from the differences between the experimental models used in various studies.

(2) Experimental studies in DN focused on small areas of the complex cellular signaling map. Despite progress, what was done until now represents only a preliminary screening, and we should restart studying DN by an "omic" approach. This may provide the "big picture" and unravel new players and connections. In this respect, solving the redox proteome will be a significant progress [125]. Nevertheless, the "omic" approach raises new obstacles, as we generate more and more data, and their integration will be even more difficult. Possibly, the "network medicine" approach may provide the models and the tools for data integration into meaningful molecular maps [126].

(3) There is no animal model of DN closely resembling human disease, especially in the late stages of DN. Therefore, extrapolation of results to humans might be inconsistent. Setup of large studies in humans is mandatory, but this is dependent on the identification of reliable and easy-to-use biomarkers of oxidative stress, which should

allow monitoring of patients all along the disease course. Urinary microsomes/exosomes carry huge promise [127]. They are small vesicular structures (30–100 nm) that may be released by almost all cell types along the kidney structures, and carry a load of transmembrane, soluble, and glycophosphatidyl inositol anchored proteins, along with mRNA and miRNA. This "cargo" faithfully reflects the physiological state of the cells of origin and thus represents a reliable source of biomarkers. Moreover, exosomes participate in the cellular crosstalk which propagates signals related to metabolic, oxidative, and inflammatory stresses [65].

(4) Deciphering the molecular networks in the complex pathophysiology of DN will foster the development of new therapeutic strategies addressing either the source of oxidative stress or the cellular response to this injury. Metabolic, inflammation, and oxidative interference points should be targeted in order to concurrently address the major cues which trigger and sustain pathologic events in DN. Attacking only one of these multiple pathologic pathways is highly unlikely to be effective. Albeit the theoretical promises of such an approach, we should be aware that interfering with signaling pathways might be dangerous, sometimes unpredictable.

Abbreviations

AGE: Advanced glycation end products
Ang II: Angiotensin II
AOPP: Advanced oxidation protein products
DN: Diabetic nephropathy
HDAC: Histone deacetylases
GBM: Glomerular basement membrane
MCP-1: Macrophage chemotactic protein-1
mtDNA: Mitochondrial DNA
PGC1α: Peroxisome proliferator-activated receptor coactivator 1
ROS: Reactive oxygen species
TLR: Toll-like receptors
Trx: Thioredoxins.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors' Contribution

All authors had equal contribution.

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Review Article

Looking for Pyroptosis-Modulating miRNAs as a Therapeutic Target for Improving Myocardium Survival

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Pyroptosis is the most recently identified type of regulated cell death with inflammatory response and has characteristics distinct from those of apoptosis or necrosis. Recently, independent studies have reported that small noncoding RNAs termed microRNAs (miRNAs) are involved in the regulation of pyroptosis. Nevertheless, only a handful of empirical data regarding miRNA-dependent regulation of pyroptosis is currently available. This review is aimed to provide a current update on the role of miRNAs in pyroptosis and to offer suggestions for future studies probing miRNAs as a linker connecting pyroptosis to various cardiovascular diseases (CVDs) and their potential as a therapeutic target for preventing excessive cell death of myocardium during CVDs.

1. Introduction

Heart disease has been the leading cause of death worldwide for many decades [1], and the loss of cells in the myocardium mutually affects the development of various heart diseases with functional demise of myocardium that can ultimately result in heart failure. In a dire situation such as ischemia, where nutrients and oxygen are deprived, individual cells are subjected to a live or die decision, and various cell death mechanisms can be activated as results [2, 3]. Among different cell death mechanisms, pyroptosis is the most recently recognized form of programmed cell death. Pyroptosis, first discovered by Cookson and Brennan in 2001, is characterized by cell lysis and inflammatory cytokine release [4]. Pyroptosis differs from apoptosis in that it does not show the typical membrane blebbing of apoptosis while inducing cell lysis, swelling, and pore formation that are not observed in apoptosis. Furthermore, the type of cysteine-dependent aspartate-specific proteases (caspases) that orchestrates disassembly of the cells in pyroptosis is caspase-1 as opposed to the caspase-3 of apoptosis [2]. In an evolutionary point of view, it is believed that pyroptosis initially developed as a host defense against microbial infections [5]. However, pyroptosis can be induced under noninfectious conditions. For example, typical signs of pyroptosis such as upregulation of caspase-1 and interleukin 1β (IL-1β) in myocardial infarction (MI) have been reported [6], suggesting that modulation of pyroptosis may be a viable therapeutic target for alleviating selective cardiovascular diseases such as MI.

During the last decade, a new class of small, noncoding RNAs termed microRNAs (miRNAs) has emerged as a key regulator of cellular process such as survival, differentiation, and death [7, 8]. Naturally, it is not too far-fetched of an assumption that the development of pyroptosis is also regulated by miRNAs to some extent, and we aim to summarize currently known pyroptosis-modulating miRNAs and others might be involved in pyroptosis based on its expressions in physiologic/pathologic hearts and the analysis of predicted targets. Through this mini review, we aim to provide a glimpse of how miRNA-dependent regulation of pyroptosis fits into a bigger picture of physiologic and pathologic regulation of heart cells by examining any possible connections of those miRNAs to other cardiovascular diseases (CVDs). Furthermore, we hope to offer constructive suggestions for

conducting future studies investigating the role of miRNAs in pyroptosis and their potential as a therapeutic target for preventing undesired loss of the heart cells in CVDs.

2. MicroRNAs at a Glance

MicroRNAs are relatively short (approximately 21~23 nucleotides long), noncoding RNAs that bind to target mRNAs with complementary sequences for degradation and/or translation repression of the target mRNAs, thus acting as a posttranscriptional regulator of genes [9]. Initially, miRNA is transcribed as usually thousands of nucleotide long, primary transcript called pri-miRNA by RNA polymerase II in the nucleus. This pri-miRNA is subsequently processed by the ribonuclease III Drosha to produce approximately hundred nucleotides long premature miRNA (pre-miRNA) with hairpin-like structure. After being transferred to the cytosol by the nuclear export factor Exportin 5, pre-miRNA is further processed by ribonuclease III Dicer to produce a mature miRNA [10]. The double stranded mature miRNA has a short-life so it promptly unwound into two strands and more stable strand associates with argonaute (Ago) protein, which comprises a RNA-induced silencing complex (RISC) [11]. The RISC utilizes miRNA as a template to recognize the complementary sequence in the 3′ untranslated region (UTR) of target mRNA [12]. As a result, gene silencing is achieved via either hindered mRNA translation or mRNA degradation [13]. Since their first discovery in 1993 [14], miRNAs have been implicated in various diseases including CVDs [15–17]. Pertaining to cardiomyocyte death, the importance of miRNA-mediated regulations in cardiac apoptosis [18, 19] and autophagy [20, 21] has been reported.

3. Pyroptosis

Meaning "fire + falling" in Greek, pyroptosis is a proinflammatory cellular suicide program and part of the host defense system responding to pathogens. Initial step of pyroptosis is the formation of caspase-1 binding protein complexes called inflammasome, and this is triggered by the cytosolic receptor-mediated recognition of bacterial and/or viral pathogens or host danger signals such as pathogen-associated molecular patterns (PAMPs) and/or damage-associated molecular patterns (DAMPs) [2, 22].

3.1. Inflammasome. Inflammasome generally consisted of a cytosolic pattern-recognition receptor (PRR), caspase-1, and an adaptor protein that connects caspase-1 to the cytosolic PRR [22].

3.1.1. Cytosolic PRRs. The cytosolic PRRs function as cytosolic molecular sensors and they are either NOD-like receptor (NLR) family proteins or pyrin and HIN domain-containing (PYHIN) proteins [23]. In humans, the NLRs family consists of 22 members and has 3 distinctive subfamilies: the NODs (NOD1-2, NOD3/NLRC3, NOD4/NLRC5, NOD5/NLRX1, and CIITA), the NLRPs (NLRP1-14, also known as NALPs), and IPAF (NLRC4 and NAIP) [24]. Each NLR contains three distinct domains, namely, an N-terminal effector domain, a central nucleotide binding and oligomerization (NACHT) domain, and a C-terminal leucine-rich repeat (LRR) domain. The N-terminal effector domain facilitates signal transduction and four different types have been identified: acidic transactivation domain, caspase activation and recruitment domain (CARD), pyrin domain (PYD), and baculoviral inhibitor of apoptosis protein (IAP) repeat domain. The NACHT domain facilitates signaling complex activation via ATP-dependent oligomerization, and the C-terminal LRR domain is responsible for ligand sensing and autoinhibition [25].

3.1.2. Caspase-1 and Adaptor Protein. Formerly known as interleukin 1β converting enzyme (ICE), caspase-1 was the first caspase identified in mammals [26]. In humans, caspase-1, caspase-4, caspase-5, and caspase-12 comprise inflammatory caspases [27] as opposed to apoptotic caspases such as caspase-3, and, as a member the inflammatory caspases, caspase-1 does not contribute to apoptosis. In relation to CVDs, caspase-1-mediated cardiomyocyte death has been reported [28]. Like caspase-9 which is activated via formation of apoptosome with apoptotic protease-activating factor-1 (Apaf-1) during apoptosis [29], caspase-1 is activated through interaction with inflammasome during the process of pyroptosis. At resting state, NLR monomers stay in inactive conformation. Upon activation, NLR monomers oligomerize via homotypic interaction of NACHT domains and bind to the adapter protein called apoptosis-associated speck-like protein containing a CARD (ASC/PYCARD) through PYD-PYD interaction [30]. Subsequently, these adaptor proteins recruit procaspase-1 via homotypic CARD interaction and cleave to activate the recruited procaspase-1 by induced proximity mechanism [31, 32]. The major mechanism of activated caspase-1-mediated pyroptosis is the formation of ion-permeable pores in the plasma membrane which creates osmotic pressure driving water influx, subsequent cell swelling, and eventual cell lysis with the release of intracellular content [33]. These released cytoplasmic contents become DAMPs and trigger further pyroptotic cascade [34]. Additionally, the activated caspase-1 mediates maturation of IL-1β and -18 inducing inflammatory responses [35]. Although this caspase-1-mediated inflammatory response was proven not to be indispensable for cell death [36], the significant role of both IL-1β and -18 in the development and progression of CVDs has been reported [37–39].

4. Approaches to Finding Candidate Pyroptosis-Modulating miRNAs

Currently, only few miRNAs have been identified as meaningful mediators of pyroptosis. However, this insufficient empirical data on the miRNA-dependent regulation of pyroptosis does not necessarily indicate that the role of miRNAs in the development and progression of pyroptosis is insignificant. Rather, it is probably due to the fact that this field of miRNA-mediated regulation of pyroptosis is in its infancy and the number of studies on this subject is anticipated to increase

in upcoming years. Thus, in this section, we try to offer some feasible research strategies for future studies focusing on how to systematically downsize the number of candidate miRNAs suspected to be involved in the development and progression of pyroptosis. Additionally, currently available information of the miRNA-dependent regulation of pyroptosis will be discussed where appropriate.

4.1. Scenario 1: miRNAs Directly Targeting Key Mediators of Pyroptosis. This is the most intuitive and straightforward case, and the most reasonable approach will be finding miRNAs directly targeting key protein-based mediators of pyroptosis such as pattern recognition receptors (NOD-like receptor (NLR) family of proteins), adaptor proteins, and caspase-1 based on miRNA databases such as, but not limited to, TargetScan [40] and miRWalk [41]. In this case, the first task to be done is to list up possible targets, along with a list of miRNAs (referred to as predicted miRNAs) predicted to target each of the specific proteins of interest (i.e., individual NLR proteins). Next, miRNAs that are dysregulated (referred to as dysregulated miRNAs) in CVDs have to be summarized from individual research articles or review articles providing miRNAs profile in CVDs. Of note, in this particular scenario, "dysregulated" especially means "decreased" because miRNAs are generally a negative regulator of target genes. For example, recent studies have reported that miR-223 directly targets NLRP3 acting as a negative regulator of inflammasome formation [42, 43]. Those were two of the few studies that addressed the evidence of miRNA-dependent regulation of pyroptosis. In this particular case, if miR-223 were to be a link between pyroptosis and CVDs, the expression level of miR-223 would be decreased in CVDs of interests so that the expression of NLRP3 can be increased suppressing inflammasome formation. Nevertheless, we could only identify that one study reported decrease of miR-223 in CVDs [44], and further association between the downregulation of miR-223 and CVDs could not be found. Now, back to the bioinformatic screening process, by crosschecking the dysregulated miRNAs and the predicted miRNAs, one can logically conduct a rough screening of miRNAs for possible involvement in both pyroptosis and CVDs, as exemplified in Table 1.

For example, miR-1 has been reported to be downregulated in various CVDs including myocardial infarction (MI) [45–47], and it is predicted to target multiple NLR proteins (NOD1, NLRP14, and NAIP). Since it has been also documented that acute MI increased caspase-1 activity and formation of inflammasome [48], decreased miR-1 during MI might be an unappreciated propyroptosis mechanism by which the synthesis of inflammasome components is enhanced contributing to the development of pyroptosis. Nevertheless, such deductive reasoning should be empirically tested and validated. In such cases, exogenous delivery of corresponding miRNA mimics or finding ways to increase and/or maintain the endogenous level (i.e., small-molecules increasing specific miRNAs) would be appropriate.

4.2. Scenario 2: miRNAs Indirectly Affect Pyroptosis via Secondary Mediators. In this case, the premise is that miRNAs target regulators of pyroptosis (as opposed to components of inflammasome). This case can be further divided into two possible scenarios: miRNAs target negative regulators or miRNAs target positive regulators. Depending on the type of regulators, the expression patterns of miRNAs in CVDs to look for can be either downregulation or upregulation (Table 2).

Since the same strategy used for the scenario 1 can be applied for the case of miRNAs targeting positive regulators, only the case of miRNAs targeting negative regulators of pyroptosis will be covered in this section. A good example would be the NLRP3 inflammasome. The NLRP3 inflammasome has been recently reported to be involved in ischemia-reperfusion- (I/R-) induced myocardial injury [6], and number of negative regulators have been identified [49]. As laid out above, miRNAs targeting negative regulators should be increased in CVDs to facilitate enhanced pyroptosis. Selected examples of negative regulators and miRNAs targeting those regulators are summarized in Table 3.

4.2.1. Autophagy-Mediated Suppression of Inflammasome Activation. Meaning "self-eating" in Greek, autophagy is a highly conserved process by which long-lived proteins and/or organelles are delivered to the lysosome and degraded within [50]. Negative regulation of pyroptosis by autophagy was demonstrated by the previous study reporting that knockout of Atg16L1 (autophagy-related-gene 16-like 1), one of the key factors of autophagy [51], significantly enhanced inflammasome activation and caspase-1 activation [52]. Additional key components of autophagy, namely, LC3B and beclin1 [53], also have been reported to function as negative regulators of pyroptosis by inhibiting activation of inflammasomes [54]. These data indicate that if miRNAs targeting those proautophagic proteins are upregulated during certain CVDs, those miRNAs can negate the negative regulation of pyroptosis by autophagic process resulting in enhanced pyroptosis.

4.2.2. Transcription Factors Negatively Regulating Inflammasome Activation. Examples of transcription factors acting as a negative regulator of pyroptosis are signal transducers and activators of transcription 1 (STAT1) and forkhead box O3 (FOXO3a). Activated as a downstream signal of type I interferon (IFN) [55], STAT1 repressed the activity of NLRP1 and 3 inflammasomes subsequently hindering pyroptosis [56]. Consequently, miRNAs targeting STAT1 can act as a positive inducer of pyroptosis, and if such miRNAs are upregulated in CVDs, their potential as a linker between CVDs and pyroptosis will be worth examining. Another transcription factor that has been reported to be involved in negative regulation of pyroptosis is FOXO3a [57]. In that particular study, miR-30d increased downregulating one of its targets, FOXO3a, under a high-glucose condition. As a result, the apoptosis repressor with caspase recruitment domain (ARC) that is under the transcriptional regulation of FOXO3a [58] was decreased so that caspase-1 was upregulated and pyroptosis was induced. This was the last study providing the evidence of miRNA-dependent regulation of pyroptosis and is an excellent empirical example of scenario 2 where

Table 1: Examples of miRNA downregulated in CVDs with potential connection on pyroptosis.

Human inflammasome component (NCBI RefSeq)	miRNAs predicted by *TargetScan and reported to be downregulated in CVDs	Related CVDs [reference]
NLR proteins (NODs)		
NOD1 (NM_006092)	miR-1	DCM, AS [45], MI [46], LVH [47]
	miR-133	MI [46], LVH [47]
	miR-208	MI [46]
	miR-499	AS [45], MI [46]
NOD2 (NM_022162)	miR-30e-5p	DCM, AS [45]
	miR-208	MI [46]
	miR-499	AS [45], MI [46]
NOD3/NLRC3 (NM_178844)	miR-10	AS [45]
	miR-24	MI [59, 60]
NOD4/NLRC5 (NM_032206)	miR-21	MI [61]
NOD5/NLRX1 (NM_024618)	miR-24	MI [59, 60]
	miR-133	MI [46], LVH [47]
CIITA (NM_000246)	miR-10	AS [45]
	miR-24	MI [59, 60]
NLR proteins (NOD-like receptor family pyrin domain containing, LNRPs)		
NLRP1 (NM_014922)	miR-10	AS [45]
NLRP2 (NM_017852)	No miRNA meets the screening criteria	
NLRP3 (NM_004895)	miR-17-5p	DCM [45]
	miR-30e-5p	DCM, AS [45]
NLRP4 (NM_134444)	No miRNA meets the screening criteria	
NLRP5 (NM_153447)	No miRNA meets the screening criteria	
NLRP6	Prediction data not available	
NLRP7 (NM_139176)	miR-150	MI [62]
NLRP8 (NM_176811)	miR-155	MI [62], LVH [63]
NLRP9 (NM_176820)	miR-19	DCM, AS [45]
NLRP10	Prediction data not available	
NLRP11 (NM_145007)	No miRNA meets the screening criteria	
NLRP12 (NM_033297)	No miRNA meets the screening criteria	
NLRP13	Prediction data not available	
NLRP14 (NM_176822)	miR-1	DCM, AS [45], MI [46], LVH [47]
NLR proteins (ice protease-activating factor (IPAF))		
NLRC4 (NM_021209)	No miRNA meets the screening criteria	
NAIP (NM_004536)	miR-1	DCM, AS [45], MI [46], LVH [47]
	miR-24	MI [59, 60]
	miR-150	MI [62]
Caspase		
Caspase-1 (NM_001223)	No miRNA meets the screening criteria	
Adaptor protein		
ASC/PYCARD (NM_013258)	No miRNA meets the screening criteria	

*In TargetScan search, only the "miRNA families broadly conserved among vertebrates" for each of the target proteins were used to generate this table. DCM: dilated cardiomyopathy, AS: aortic stenosis, MI: myocardial infarction, and LVH: left ventricular hypertrophy.

Table 2: Screening criteria of miRNAs, dependent on pyroptosis regulator type.

Type of regulators	Corresponding miRNA expressions in CVDs
Positive regulators (promote pyroptosis)	Decreased, abundant positive regulators expected
Negative regulators (inhibit pyroptosis)	Increased, depletion of negative regulators expected

Table 3: Selected examples of miRNAs targeting human negative regulators of pyroptosis and reported to be increased in CVDs.

Negative regulators of pyroptosis (NCBI Refseq, reference)	miRNAs predicted by *TargetScan and reported to be upregulated in CVDs	Related CVDs [reference]
Autophagy		
ATG16L1 (autophagy-related gene 16 like-1) (NM_017974, [52])	Let-7	DCM, ICM, AS [45]
	miR-125	DCM [45, 64], AS [45], LVH [65]
	miR-181	DCM [64], AS [45]
	miR-214	DCM [45, 64], ICM, AS [45], LVH [65]
MAP1LC3B (microtubule-associated proteins 1A/1B light chain 3B) (NM_022818, [54])	miR-30e-5p	DCM, AS [45]
	miR-145	AS [45]
	miR-214	DCM, ICM, AS [45], LVH [65, 66]
	miR-221	LVH [65]
	miR-222	DCM, ICM [45], LVH [65]
BECN1 (Beclin1) (NM_003766, [54])	miR-30e-5p	DCM, AS [45]
	miR-199	DCM, ICM [45], LVH [65, 66]
Transcription factors		
STAT1 (NM_007315, [56])	miR-140	DCM, ICM, AS [45], LVH [65]
	miR-21	LVH [65, 66]
	miR-23	DCM, ICM, AS [45], LVH [65, 66]
	miR-24	ICM, AS [45]
FOXO3	miR-27	LVH [65, 66]
(NM_001455)	**miR-30d**	**Diabetic cardiomyopathy [57]**
*__experimentally proven to be regulated by miR-30d [57]__	miR-99	DCM, AS [45]
	miR-103	DCM, ICM, AS [45], LVH [65]
	miR-125	DCM, AS [45], LVH [65, 66]
	miR-217	LVH [66]
	miR-221/222	LVH [65]
NO synthase		
NOS2 (inducible nitric oxide synthase) (NM_000625, [67])	miR-15	DCM, AS [45]
	miR-214	DCM [64], LVH [65, 66]

*In TargetScan search, only the "miRNA families broadly conserved among vertebrates" for each of the target proteins were used to generate this table. DCM: dilated cardiomyopathy, AS: aortic stenosis, MI: myocardial infarction, and LVH: left ventricular hypertrophy.

miRNAs (i.e., miR-30d) increased in CVDs (i.e., diabetic cardiomyopathy) and such increase caused downregulation of negative regulator of pyroptosis (i.e., ARC transcribed by FOXO3a).

4.2.3. Nitric Oxide-Mediated Suppression of Inflammasome Formation. Nitric oxide (NO) has been reported to negatively regulate NLRP3 inflammasome-mediated caspase-1 activation [67]. Such results indicated that inducible nitric oxide synthase (iNOS), which is responsible for the production of NO [68], also can be a negative regulator of LNRP3 inflammasome. In fact, the significance of iNOS has been demonstrated in a study where IFN-β inhibited NLRP3 inflammasome [69]. Furthermore, since NO itself is not a protein that can be subjected to miRNA-dependent targeting, finding miRNAs that are increased in CVDs, as well as targeting iNOS, would be the next logical strategy for designing studies to elucidate the role of miRNAs in myocardial pyroptosis.

5. Concluding Remarks

Over the last few decades, tremendous interest has been placed on the regulatory nature of miRNAs under various pathologic conditions including CVDs. Considering the complexity of cell signaling in general, it is reasonable to

assume that miRNAs are involved in virtually all biological processes including demise of cells. In this mini review, we tried to provide currently available evidences of miRNA-dependent regulation of pyroptosis. However, due to the limited number of studies, it was not sufficient to draw any significant insights on the role of miRNAs in pyroptosis, and it remains as one of the limitations of this review.

In addition to providing the evidences of miRNA-dependent modulation of pyroptosis, we also tried to offer some suggestions for future studies focusing on what to look for in search of the miRNAs modulating pyroptosis in CVDs. The aim of this review was to encourage scientific interest in examining the need-to-be-further-validated framework of CVD-miRNA-pyroptosis. We hope that, through future studies with well-thought-out strategies, our understanding of the subject of miRNA-dependent regulation of pyroptosis in CVDs is enhanced, and it contributes to the development of an optimized miRNA-mediated therapeutic strategy for targeting cell death of myocardium.

Conflict of Interests

The authors declare no conflict of interests.

Authors' Contribution

Seahyoung Lee and Eunhyun Choi equally contributed to this work.

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Review Article

Necroptotic Cell Death Signaling and Execution Pathway: Lessons from Knockout Mice

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Under stress conditions, cells in living tissue die by apoptosis or necrosis depending on the activation of the key molecules within a dying cell that either transduce cell survival or death signals that actively destroy the sentenced cell. Multiple extracellular (pH, heat, oxidants, and detergents) or intracellular (DNA damage and Ca^{2+} overload) stress conditions trigger various types of the nuclear, endoplasmic reticulum (ER), cytoplasmatic, and mitochondrion-centered signaling events that allow cells to preserve the DNA integrity, protein folding, energetic, ionic and redox homeostasis, thus escaping from injury. Along the transition from reversible to irreversible injury, death signaling is highly heterogeneous and damaged cells may engage autophagy, apoptotic, or necrotic cell death programs. Studies on multiple double- and triple- knockout mice identified *caspase-8*, *flip*, and *fadd* genes as key regulators of embryonic lethality and inflammation. Caspase-8 has a critical role in pro- and antinecrotic signaling pathways leading to the activation of receptor interacting protein kinase 1 (RIPK1), RIPK3, and the mixed kinase domain-like (MLKL) for a convergent execution pathway of necroptosis or regulated necrosis. Here we outline the recent discoveries into how the necrotic cell death execution pathway is engaged in many physiological and pathological outcome based on genetic analysis of knockout mice.

1. Introduction

Cell death is a crucial process in ontogeny, homeostasis, and pathologies [1, 2]. Over 100 billion cells die in our bodies by different cell death pathways every day. The cells die by apoptosis, a physiological and regulated cell death process which is tolerogenic and partially inflammatory, or necroptosis, a pathological and regulated cell death process, which is inherently immunogenic and elicits intense inflammatory reaction [3, 4]. Pyroptosis [5], immunogenic cell death [6, 7], and other distinct cell death processes have been defined at morphological and biochemical levels [3, 4, 8, 9]. Many questions concerning the cross talk among the cell death regulators, their intracellular signaling pathways, and the immunological consequences remain unanswered. Genetic dissection in simple model organisms [10] and mice models [11] has provided us with critical genes of cell-death pathways that control early and late biochemical and morphological events in organ development and cellular homeostasis. A variety of cell death modalities share extrinsic and intrinsic pathways that integrate mitochondrial metabolism, cell proliferation checkpoints, and DNA repair mechanisms [3, 4, 9]. It is now becoming evident that perturbations of intracellular ionic homeostasis induced by certain transmembrane non- and voltage dependent-channels and ion-linked channel receptors play critical roles in the course of cell death processes [1–5, 9]. Here we will summarize common features of necrosis, apoptosis, and necroptosis and the multiple intracellular signal pathways that regulate their cellular triggering in many physiological and pathological situations. In the end, we will outline and discuss important phenotypes of knockout mice models that serve to define the role of *caspase-8*, *flip*, and *fadd* genes and other major components of apoptotic and necroptotic downstream signaling effectors.

2. Cell Death Modalities

2.1. Accidental Necrosis. Necrosis derives from the Greek word "necros" and has long been used by pathologists to describe morphologically the death of cells or tissue as result of pathological infection, cellular injury, and noxious

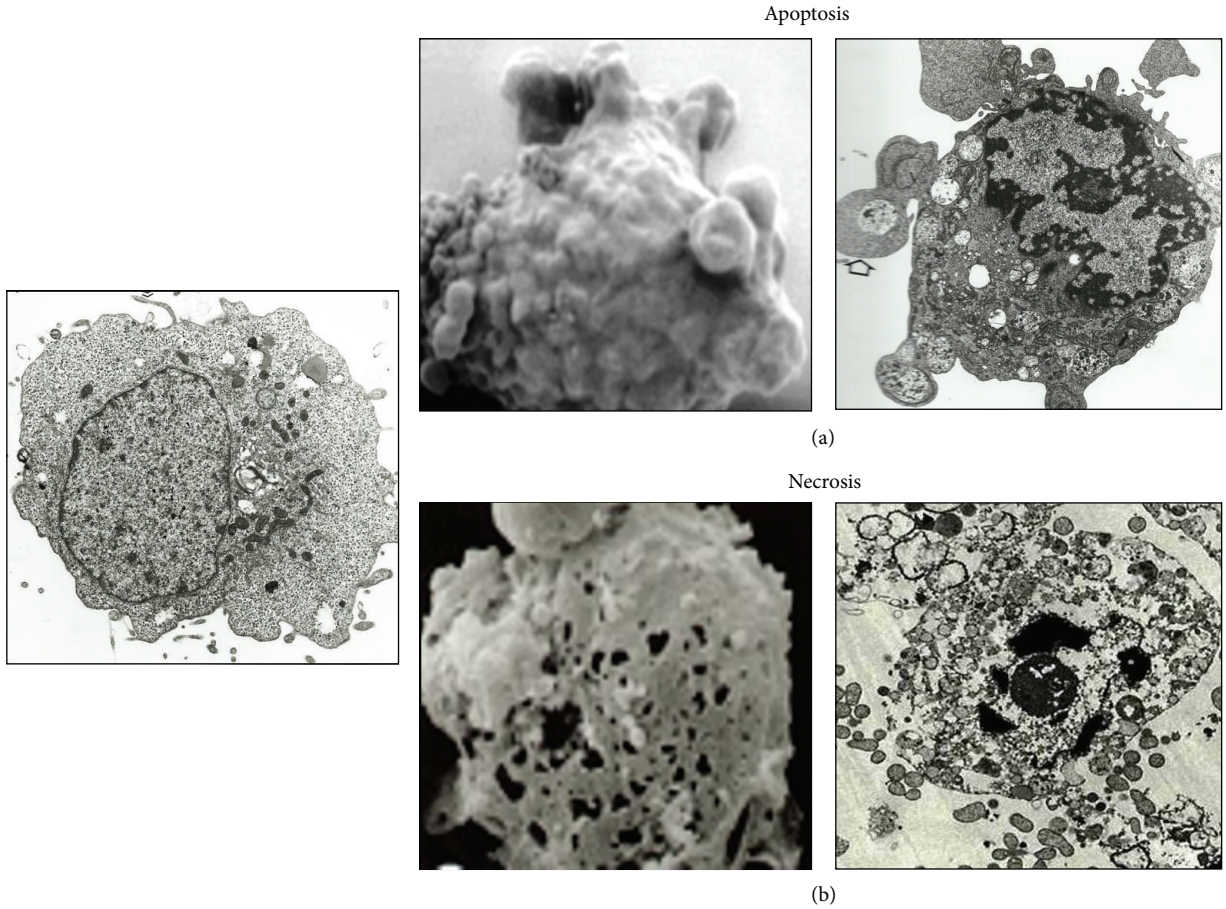

Figure 1: Distinct morphological features of apoptosis and necroptosis. (a) Apoptosis is characterized by cell shrinkage, membrane blebbing condensation, margination of nuclear chromatin, and packaging of apoptotic bodies and its engulfment by neighbor cells. (b) Necroptosis is characterized by the increase in cell volume, swelling of organelles, perforation of plasma membrane, cellular collapse, and release of cellular contents.

stimuli [1, 4]. The term necrosis is now referred to as accidental cell death, which is a form of nonregulated, nonspecific, and uncontrolled cell death by meaning of genetic and biochemical interventions [4]. Necrotic death occurs quickly as a consequence of extreme physicochemical stress, such as heat, acidification, osmotic shock, mechanical stress, and freeze-thawing of cells [2]. Necrotic cells are characterized by loss of plasma membrane integrity, increase in cell volume (also known as oncosis), organelle swelling, lack of internucleosomal DNA fragmentation, and cellular collapse (Figure 1). These events occur at early or late stages of cellular collapse due to cellular energy depletion (ATP), mitochondrial permeability transition, increases in cytosolic calcium concentration, high production of free radicals, reactive (activated) oxygen species (ROS), oxidization of membrane lipids, plasma membrane damage and permeability changes, and critical DNA and protein structural damage [2, 8].

2.2. Apoptosis. The term "apoptosis" was derived from a Greek word that means "falling off" and was first coined by Kerr et al. [1] to describe a morphological manifestation of cellular demise. The complex cellular morphology known as apoptosis can be confidently recognized by a series of morphological changes at the microscopy level (Figure 1). Apoptosis is characterized by cell shrinkage, membrane blebbing, condensation and margination of nuclear chromatin, degradation of DNA into nucleosomal units (200 bp), and formation of apoptotic bodies. However, the hallmark of an apoptotic process is its dependence on caspase activation [1, 8, 12].

Cells undergo apoptosis in response to extrinsic or intrinsic pathways that are regulated by various antiapoptotic and proapoptotic proteins [8, 13]. The extrinsic pathway is mediated by binding of the tumor necrosis factor (TNF) to its receptor (TNFR-1) which is followed the formation of two TNFR complexes [14]. TNFR-1 complex I comprises the adaptor protein TNFR1-associated death domain protein (TRADD), the death domain- (DD-) containing protein kinase receptor-interacting protein 1 (RIPK1), the ubiquitin E3 ligases TNFR-associated factor 2 (TRAF2), and cellular inhibitor of apoptosis protein 1 (cIAP1). TNFR-1 complex II comprises the adaptor FAS-associated death domain protein (FADD), caspase-8, RIPK1 (IIa), and/or RIPK3 (IIb). E3 ubiquitin ligases, including cIAP-1 and -2 and deubiquitinases, including CYLD (cylindromatosis), A20, Cezanne, HOIL-1/HOIP/Sharpin (LUBAC ubiquitin ligase complex),

modify the balance between TNFR-1 complex I and complex II [13–15] leading to either cell survival via activation of nuclear factor-κB (NF-κB) or alternative cell death signaling pathways.

Homodimerization and activation of caspase-8 within either TNFR complex I or II propagate the activation of effector caspases-3, -6, and -7, which then cause cellular destruction by apoptosis without mitochondria participation (known as type I intrinsic pathway). Both caspase-8 and cFLIP (FADD-like IL-1β-converting enzyme-inhibitory protein) possess two DED (death effector domain) domains. Their heterodimerization will block the apoptotic signaling cascade, leading to cellular survival and NF-κB-mediated proinflammatory response. Thus the ratio of different c-FLIP isoforms (long and short isoforms) bound to procaspase-8 is a critical regulator of both procaspase-8 dimerization/activation and cell death by apoptosis and necroptosis [16].

The intrinsic pathway is also called the mitochondrial pathway and is absolutely dependent on BAX and BAK protein activation by BH3-only molecules (BH3s) of The BCL-2 (B-cell lymphoma/leukemia 2) family proteins. Various BH3s proteins of the pro-apoptotic family, including BAD, BIK, BID, BIM, BMF, NOXA, and PUMA participate in this process. These proteins interact and dislocate the BH3s proteins of the anti-apoptotic family, including BCL-2, BCL-XL and MCL-1, allowing conformational changes and association of BAX/BAK proteins at specific sites of the mitochondrial outer membrane. Cytochrome *c* is an abundant protein of the mitochondrial inner membrane and acts as an electron transport intermediate. BAX and BAK promote apoptosis by perturbing the permeability of the mitochondrial outer membrane, referred to as MOMP, which lead to the release of cytochrome *c* [17]. The cytochrome *c* released into the cytoplasm stimulates the formation of the apoptosome, a scaffold for activation of caspase-9, which, in turn, cleaves and activates the effector caspases-3, -6, and -7. Mitochondrial membrane permeabilization leads to the release of other apoptogenic proteins, including apoptosis inducing factor (AIF), Smac/Diablo, HtrA2/Omi (serine protease), and endonuclease G, which execute distinct functions in the downstream apoptosis-signaling pathway. Accordingly, new small-molecule inhibitors of Bcl-2 proteins, including ABT-199 and ABT-263, are viewed as promising new anticancer agents.

In vivo, apoptotic cells maintain their plasma membrane integrity. However, apoptotic cells under certain conditions promote the exposure or release of ecto-calreticulin, phosphatidylserine, HSP70, HSP90, opsonins, thrombospondin, and high mobility group box 1 (HMGB1), which are known as cDAMPs [18]. The uptake of apoptotic cells by macrophages promotes the release proinflammatory cytokines [19, 20] and cell growth and wound healing through the release of vascular endothelial growth factor (VEGF) and transforming growth factor-β (TGF-β), respectively [21]. Several cytokines and lipid mediators produced by immune cells and surrounding tissue are involved in the resolution of acute and chronic inflammation [22].

2.3. Necroptosis. Necroptosis is referred to as cell death initiated by TNF receptors following chemical suppression of caspases [3]. Peter Vandenabeele and Junying Yuan were the first authors to describe the morphological and biochemical features of necroptosis (more details in Section 4). It is morphologically characterized by the increase in cell volume, swelling of organelles, plasma membrane permeabilization, cellular collapse, and release of cellular contents. Necroptotic cells promote highly inflammatory response as consequence of the release of cytokines, cDAMPs, and PAMPs [18, 20]. Necroptosis is now considered a regulated cell death program that ultimately relays in a core execution pathway to promote final cellular demise [4, 9]. A detailed mechanism for necroptotic cell death execution pathway has been described that involves the integration of many downstream signaling pathways with a trio formed by the receptor interacting protein kinase 1 (RIPK1), RIPK3, and MLKL [23, 24]. Importantly, many cell death triggers can induce necroptosis at various pathological scenarios [9].

Recently it has been demonstrated that cells undergo regulated necroptosis after activation canonical and non-canonical extrinsic or intrinsic pathways triggered by TNF-α, TLR (toll-like receptors) and NLR (NOD-like receptors) agonists, interferons, viral and bacterial products, and diverse pathophysiological signals [9]. The emerging connections between necroptosis and apoptosis intrinsic and extrinsic pathways are depicted on Figure 2 described in more details in the next sections.

3. Mitochondrial Channels and Pores That Control Apoptosis and Necroptosis Cell Death

The role of mitochondria is well defined in apoptotic cell death and accidental necrosis. The mitochondrial involvement in necroptosis is still very preliminary and debatable. Given that various transmembrane non- and voltage dependent-channels and ion-linked channel receptors play critical roles in the course of cell death processes, here we will update on some of the putative mechanisms that contribute to apoptotic and necrotic cell death.

3.1. Regulation of Mitochondrial Osmotic Balance. Mitochondria function in a cytosolic milieu containing Na^+, K^+, and Ca^{2+} as well as other anions and cations [25]. The inner mitochondrial membrane, however, is impermeable to these ions and their flux and concentrations in the mitochondrial matrix are controlled by different subtypes of pores, channels, and exchangers. The mitochondrial K^+ balance is controlled by ATP-dependent (KATP) and Ca^{2+}-dependent (KCa) K^+ channels responsible for influx and by K^+/H^+ exchanger responsible for removal of excess of matrix K^+ [26, 27]. Sodium balance is governed by Na^+/Ca^{2+} (influx) and Na^+/H^+ (efflux) exchangers and calcium balance by Ca^{2+} channel (influx) and Na^+/Ca^{2+} exchangers (efflux). The balance of the main cytoplasmic anions, phosphate and chloride is regulated by numerous carriers and channels [25, 27]. Cell size decreases due to water loss from increased K^+

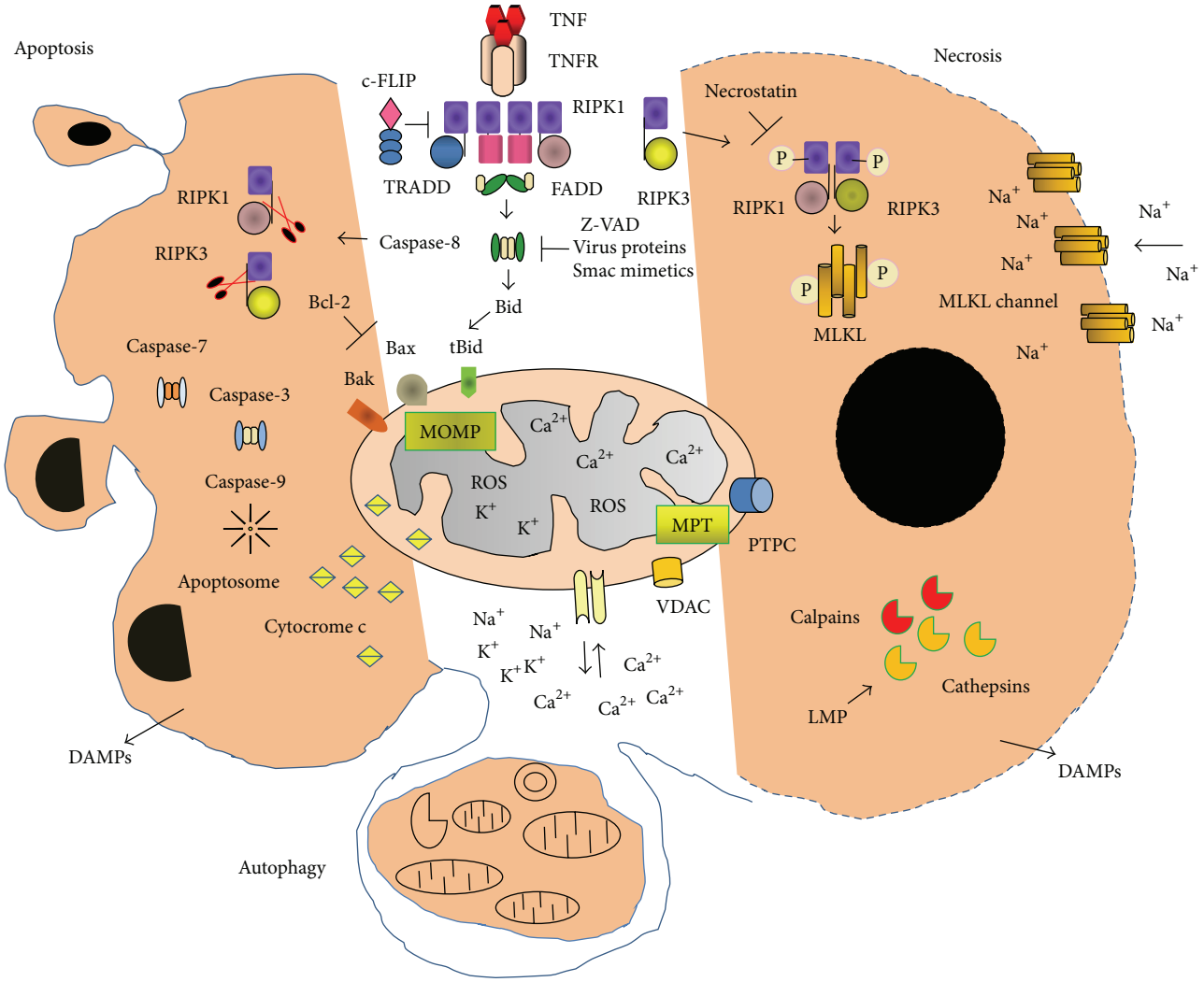

FIGURE 2: Schematic overview of the multiple signaling pathways to apoptosis, necroptosis and autophagy. TNF-α binding to TNFR causes the assembly of a membrane-proximal supramolecular complex including (but not limited to) TRADD, FADD, and RIPK1 (receptor interacting protein kinase 1). Recruitment and activation of caspase-8 play a crucial role in initiation of apoptotic or necrotic cell death. Active caspase-8 cleaves Bid, generating tBid, with together with Bax and Bak promote the mitochondria outer membrane permeabilization (MOMP) allowing the release of cytochrome *c*. Cleavage of both RIP1 and RIP3 by caspase-8 leads to apoptosis, whereas phosphorylation of RIP1 and RIP3 protein kinases causes their activation and in turn the recruitment of MLKL (mixed lineage kinase domain-like). MLKL is phosphorylated by RIP3 and initiated structural changes that led to its insertion in the plasma membrane and formation channels. MLKL channels increase Na^+ influx, osmotic pressure, and membrane rupture, ending with cell death by necroptosis. Membrane rupture promotes the release of cellular contents and, in particular, various endogenous DAMPs. Various chemotherapeutical drugs, chemical and biological stressors, cause mitochondrial dysfunctions and consequently increase the level of ROS (reactive oxygen species, ROS) generation and collapse of electrochemical gradient, which compromise the ADP/ATP exchange transporter. High Ca^{2+} upload in the matrix favors the transient or irreversible opening or closure of the outer/inner mitochondrial permeability transition pore complex (MPTPC) that is well known to participate in the mitochondrial permeability transition (MPT). This is accompanied by mitochondrial depolarization, loss of membrane potential ($\Delta\Psi m$), and massive swelling due to influx of ions and water into the matrix. Depending on the extension of cell injury, the cells undergo apoptosis, necrosis, or autophagy programs. Autophagy of damaged organelles constitutes a survival response that prevents cell death. VDAC: the voltage-dependent anion channel, also known as porin; DAMPs: damaged associated-molecular patterns; TNFα: tumor necrosis factor α; TNFR: tumor necrosis factor receptor; FADD: Fas-associated death domain protein; Z-VAD.fmk: Z-Val-Ala-Asp(OMe)-fluoromethylketone; LMP: lysosomal membrane permeabilization; PTPC: permeability transition pore complex; Smac: second mitochondria-derived activator of caspase.

and Cl^- ionic effluxes. The mitochondrial inner membrane also has unique water channels named aquaporins, which facilitate water or other small-uncharged molecule transport between the cytoplasm and matrix [28]. The rate of water flux in or out of the mitochondrion is determined not only by the osmotic gradient that acts as the driving force for water transport but also by the water permeability of the inner membrane [28].

The mechanisms by which Ca^{2+} ions modulate both oxidative phosphorylation and mitochondrial matrix swelling have been a matter of great debate for decades. Halestrap and colleague [29] demonstrated that an increase in intramitochondrial Ca^{2+} concentration can increase the flux of K^+ into mitochondria. Since then, several mechanisms have been proposed to explain this finding. First, it was proposed that elevated matrix Ca^{2+} could open a putative mitochondrial large conductance Ca^{2+}-activated K^+ channel (KCa channel) [26, 30]. Activation of calcium efflux pathways via Na^+/Ca^+ and Na^+/H^+ exchangers (mitochondrial NHE1) during intramitochondrial Ca^{2+} overload causes dissipation of the mitochondrial membrane potential and loss of proton gradient [27]. Loss of proton gradient suppresses the activity of K^+/H^+ exchange leading to the mitochondrial swelling. Finally, if the Na^+/Ca^{2+} exchanger becomes saturated, a high intramitochondrial Ca^{2+} could rise to the level sufficient to facilitate entry of potassium as well as other ions and solutes and inducing massive swelling of the matrix [26]. An increase of Ca^{2+} concentration up to the micromolar level inhibits pyrophosphatase activity in the matrix, which, in turn, may transiently displace adenine nucleotides from adenine nucleotide translocase (ANT) and convert the latter into a potassium channel. Most likely the total net flux of ions is directed toward the mitochondrial matrix and this ion movement, accompanied by osmotically mediated entrance of water, which leads finally to mitochondrial swelling [31]. The swelling could lead to a rupture of the mitochondrial outer membrane allowing further expansion of the matrix. Thus, rupture of the mitochondrial outer membrane has been proposed as alternative way of permeating the release of cytochrome *c* in the apoptotic process, but this is debatable [32].

3.2. ROS-Induced Mitochondrial Permeability. ROS (reactive oxygen species) overproduction can lead to severe mitochondrial dysfunction commonly observed in the necrotic cell death process [2]. Reactive oxygen species induce mitochondrial structural oxidation and opening of the permeability transition pores, channels, and exchangers [33]. Under normal metabolic conditions, electron-transporting complexes I, II, III, and IV plus a nonredox H^+-translocating complex, ATP synthase (also called complex V, FoF1-ATP synthase), coenzyme Q, and cytochrome *c* carry out oxidative phosphorylation [34]. The a- and b-type cytochromes are inaccessible components of large complexes, but cytochrome *c* is monomeric, freely diffusible in the inner membrane, and in equilibrium between the inner membrane, intermembrane space and cristae. A small percentage of the total O_2 consumed by the mitochondrial electron transport chain in healthy tissues becomes ROS, such as superoxide ($O_2^{\cdot -}$), hydrogen peroxide (H_2O_2), and hydroxyl radical (OH−) [33]. This ROS production occurs primarily in complex I (NADH dehydrogenase) and complex III (ubiquinone-cytochrome *c* reductase). O_2 itself is also a free radical because it has two unpaired electrons in its outer orbit that make it reactive. Increase in the level of products of the one-electron reduction of O_2 is known to induce the mitochondrial permeabilization transition. The hypothesis is that pore formation is involved in the organization of a defense system preventing ROS formation. It is proposed that an ROS-induced nonspecific pore opening lowers ROS production due to (a) maximal stimulation of mitochondrial O_2 consumption and, hence, intracellular lowering of ROS and (b) complete dissipation of mitochondrial membrane potentials and, as a consequence, maximal oxidation of such respiratory chain carriers such as coenzyme Q, which serve as one-electron O_2 reductant [34].

Earlier studies have shown that $OH^{\cdot}$ oxidize thiol (–SH) groups of sensor proteins which directly promote the activation or opening of the mitochondrial permeability transition pore [33, 34]. The mitochondrial permeability transition pore complex (PTPC) is a nonspecific pore in the inner mitochondrial membrane (IMM) whose opening is triggered by high concentration Ca^{2+} in the matrix [35–37]. Ca^{2+} loading increases the production of ROS, including superoxide. PTP is considered to function as a point of no return for both apoptosis and necrosis. This supramolecular complex may contain or be regulated by ANT (adenine nucleotide translocator), the mitochondrial ADP/ATP nucleotide exchanger, VDAC (the voltage-dependent anion channel), also known as porin, protein cyclophilin D (CypD), the peripheral benzodiazepine receptor, the phosphate carrier, and the FoF1 ATP synthase [38, 39]. CyPD binds to the FoF1 ATP synthase into lipid bilayers to form a Ca^{2+}-activated channel that displays features of the mPTP [38]. In this physiological configuration, PTP functions together with other channels and exchangers in the regulation of mitochondrial Ca^{2+} homeostasis [2, 38, 39].

The roles of MOMP and MPT have been mostly considered to participate in the two independent pathways for release of apoptotic and necrotic factors from mitochondria [17]. Bax and Bak interact with the MPT to induce a permeability transition and cytochrome *c* release in isolated mitochondria [40, 41]. Interestingly, intermediate structures common to membrane fusion or fission machineries have also been described to participate in mitochondrial permeabilization phenomenon [17, 42]. New ideas and experiments concerning structures and functions of membrane-permeation channels, exchanger, and pores are under way to confirm whether they act together or separately to promote the mitochondrial events common to apoptotic and necrotic intrinsic pathways [41].

3.3. Mitophagy. The outcome of cells with mitochondrial damage can vary. Apoptosis can eliminate ROS-producing cells (cell selection). Autophagy (self-eating) is a catabolic process that targets organelles and cytoplasmic components for degradation by the lysosome [43]. Autophagy has not

been considered a modality in cell death; nonetheless, many stimuli that activate apoptosis induce autophagy, whereas signals that inhibit apoptosis inhibit autophagy [43, 44]. The pan caspase inhibitor Z-VAD-fmk inhibits caspases but also blocks lysosomal cathepsins and hence cell death by autophagy. Antiapoptotic proteins, such as Bcl-2 family members, bind to and inhibit beclin (Atg 6), and proapoptotic factors, such as BH3-only proteins, to disrupt this inhibitory interaction and thereby activate autophagy [44]. Autophagy is triggered by ROS derived from either the mitochondrial electron transport chain or NAPDH oxidases. Autophagy of damaged mitochondria limits ROS-modulated caspase-1 activation and seems to negatively regulate pyroptosis [44]. Mitophagy is a specialized form of autophagy in which mitochondria are specifically targeted for degradation at the autophagolysosome, with subsequent degradation by cell's own lysosomal system [44]. Depolarization of mitochondrial membranes is a prerequisite for mitophagy. This process occurs for a period of time for clearance of mitochondria in which damage is not too extensive. Therefore, autophagy of damaged organelles constitutes a survival response that prevents cell death.

4. Critical Role of Plasma Membrane Permeabilization by MLKL in the Execution Pathway for Necroptosis

4.1. Necrosome Complex. The interconnected and complex signaling pathways to apoptosis and necroptosis were first recognized by Wallack and Goeddel's groups in 1995-1996. These investigators reported that the interaction of TNF receptors, TRADD and FADD, via DED (death effector domain) was critical for the recruitment of caspase-2, -8, and -10 and the induction of cytotoxicity by TNF family members [45, 46]. The genetic analysis of the caspase-8-deficient and FADD-deficient mice demonstrated the essential role of these genes for embryo development and signaling to tumor necrosis death receptors-induced apoptosis in a physiological setting [45–47]. On the other hand, it was also observed that the cell killing by FADD oligomerization could be caspase-independent suggesting the existence of a particular nonapoptotic cell death program [48–50]. Vercammen and colleagues [51] were the first to report on the induction the morphological features of necroptosis in L929 cells following pharmacological blockage of pan caspase activity by a peptide cell permeable inhibitor named Z-Val-Ala-Asp.fluoromethylketone or ZVAD-fmk.

These studies were confirmed by other authors who identified other cell death genes and small molecule inhibitors that strongly sensitize cells to TNF-α, TRAIL, and FasL in eliciting a necrosis-like cell death [48, 52–55]. Degterev and colleagues identified necrostatin-1, a small molecule inhibitor of RIPK1 enzymatic activity [53, 56]. Mitochondria release the second mitochondria-derived activator of caspase (Smac/DIABLO) which bind and inactivate the inhibitors of apoptosis cIAP-1, cIAP-2, and XIAP. Smac mimetics are peptide antagonists of cIAP-1, cIAP-2, and XIAP. The incubation of cells with these peptides enhances TNF-induced necroptosis by promoting autodegradation of cIAP-1, cIAP-2, and XIAP [57] and the formation of RIPK1-dependent complex named necrosome [9].

RIPK1 bears a DD domain allowing its recruitment to TNF-α, TRAIL, and CD95 large complexes that initiate the necroptotic cell death pathway [50, 58]. Hitomi and colleagues [54] identified RIPK1 among 7 genes out of 432 genes in a large screening for candidates required for TNF plus zVAD-fmk-induced necroptosis in L929 cells. He and colleagues [55] used a combination of genome-wide siRNA screening and immune precipitation assays to identify RIPK3 as key determinant for necrotic cell death downstream RIPK1. Cho and colleagues [59] undertook a large screening using an siRNA library consisting of 691 human kinase genes to identify additional RIPK1 partners in the induction of necroptosis in FADD-deficient Jurkat cells. RIPK3 was identified as crucial upstream activating kinase that regulates RIPK1-dependent necroptosis *in vitro,* because of its physiological importance in the protection against vaccinia virus infection [59].

Caspase-8 forms with its enzymatically inert homolog cellular FLICE-like inhibitory protein long (cFLIPL) protein, an active complex named ripoptosome. This complex contains caspase-8, FADD, RIPK1, and RIPK3 [13]. Caspase-8 inhibition within this complex blocks the cleavage of RIPK1 and RIPK3 allowing RIPK1 to phosphorylate RIPK3. RIPK3 does not have DD domain in its terminus suggesting other physiological roles in cells [58]. The identification of necrosulfonamide, a small molecule that specifically blocks necroptosis in human cells, was the key to further delineate the downstream pathway mediated by RIPK3 [60]. The mixed lineage kinase domain-like protein (MLKL) was identified as the natural target of RIPK3 kinase [60]. Overexpression of MLKL was able to induce the distinguishable hallmarks of necroptosis [61]. These studies led to the assumption that necroptotic cell death occurs upon the assembly of a large, signal-induced multiprotein complex containing RIPK1, RIPK3, and MLKL which was named necrosome [9, 62]. The RHIM (RIP homotypic interaction motif) domains in molecular structures of RIPK1 and RIPK3 proteins allow the homotypic protein–protein interactions which are essential to form filamentous amyloid structures required for necroptotic signaling [15, 63]. A recent study showed that RIPK3 catalytic activity is dispensable for apoptosis but essential for necroptosis and both apoptosis and necroptosis could proceed simultaneously [64].

4.2. MLKL Channel. MLKL is recruited to the necrosome via interaction of its kinase-like domain (KLD) with the kinase domain of RIPK3 [65, 66]. MLKL is a kinase-dead protein. The N-terminal region of MLKL contains a 4HBH (a four-helical bundle domain) with four helices (amino acids 1–125) and, within its N-terminal, two more helices (amino acids 125–181) in its BR domain. MLKL has a pleckstrin homology (PH) domain, similar to the domain of the phospholipase C δ (PLCδ), pleckstrin, spectrin, and dynamin [67]. This PH domain seems to be required for its insertion into the membrane surface, more specifically, through binding to phosphatidylinositol phosphates [66, 68–70]. The PH domain

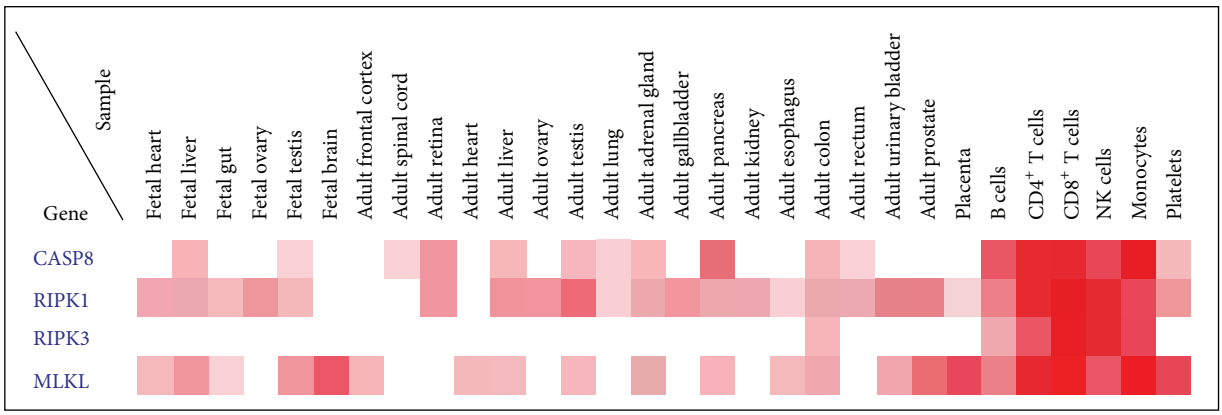

Figure 3: Expression of caspase-8, RIPK1, RIPK3, and MLKL in fetal and adult organs and hematopoietic and myeloid cells. Each row corresponds to each protein concentration displayed as white (no expression) to red color intensity. The heat map was obtained from http://www.humanproteomemap.org/.

can bind to different phosphoinositide polyphosphates and inositol polyphosphates [71]. After its phosphorylation by RIPK3, MLKL translocates to lipid rafts inside of the plasma membrane and interacts with the positively charged patch formed by phosphoinositolphosphate (PIPs) molecules. This leads to formation of a high molecular weight pore-forming structure that allows the diffusion of ions, in particular, sodium [68, 69]. This increases intracellular osmotic pressure and eventually leads to membrane rupture [68, 70]. More importantly, MLKL induced-leakage has been demonstrated after it is bound to PIP, PI(4,5)P2, and PI(3,4,5)P3 containing liposomes [69]. All of the above events are not seen in apoptosis. Nonetheless, MLKL may likely function in a similar mechanism proposed to Bax or Bak proteins for membrane insertion and pore formation. Finally, one study has suggested the possible role of MLKL in regulating extracellular Ca^{2+} influx from the transient receptor potential melastatin-related 7, which deserves further investigation [66].

A recent study showed that the RIPK3-MLKL interaction and translocation of necrosomes to mitochondria associated membranes are essential for necroptosis signaling [68]. It was demonstrated that the assembly of RIPK1-RIPK3 complex initiates the intrinsic necroptosis pathway with the participation of PGAM5L and PGAM5S. These two protein phosphatases cause the activation Drp1 (dynamin-related protein 1) and its translocation to the mitochondria. In mammalian cells, mitochondrial fusion is regulated by mitofusin-1 and -2 (MFN-1/2) and optic atrophy 1 (OPA1), whereas mitochondrial fission is controlled by Drp1. Throughout the necrosis process, Drp1 associated with its mitochondrial anchors Fis1 and Mff to induce mitochondrial fragmentation; however, cytochrome *c* is not released as it occurs in the apoptosis intrinsic pathway [72]. Thus both mitochondrial fission and fusion proteins appear to modulate necroptosis through activities that are distinct from their roles in mitochondrial dynamics, a question that remains to be confirmed [73]. However, recent studies have excluded the requirement of PGAM5 and DRP1 in necroptosis [65, 74, 75].

The production of ROS has been shown to be essential for TNFα-induced programmed necrosis in L929 cells and mouse embryonic fibroblasts [51]. The importance of the mitochondrial axis in the induction of necroptosis was confirmed in one study showing that once activated RIPK3 kinase initiates the phosphorylation of several downstream target proteins including phospholipase A2 and the proteases calpains and cathepsin [76]. Other critical targets include the cytoplasmatic NOXA1/NADPH oxidase complex and mitochondrial complex I, which are responsible for excessive ROS production, ATP depletion, and opening of the mitochondrial permeability transition pores [76]. These events are accompanied by prolonged JNK activation and stimulation of several metabolic enzymes of glycolysis, glycogenolysis, and glutaminolysis as well as the stimulation of the Krebs cycle [76]. In fact, a previous study has shown that the Nox1 NADPH oxidase has a role in the TNF-induction of necrotic cell death [77].

Not surprisingly, RIPK1, RIPK3, and MLKL are strongly expressed in immunologic organs like the thymus and spleen and lymphoid and myeloid cell lines, as compared to fetal and adult organs. The relative expression is shown as dendrogram in Figure 3. It is now well established that necroptosis occurs both in T lymphocytes after HIV-1 infection and neutrophils and macrophages after bacterial infection and during tissues injury [9]. A number of pathogens cause host cell death and caspase-8 and RIPK3 are considered the key regulators of macrophage cell death [78, 79]. Future work is needed to clarify the relationship of these metabolic and bioenergetic mechanisms and their overlapping with either early or later plasma membrane associated biochemical events [65, 74, 75].

5. Knockout Mice Models for *In Vivo* Cell Death Studies

Mice models have been the key biological tools to define apoptotic and necrotic cell death in development, physiology, and homeostasis as revealed in gene-knockout (KO) mice for a broader number of the TNF and Bcl-2 family members and their regulators [11, 80, 81]. Major phenotypes observed in mice deficient in cell death-pathway genes by intercrossing different null alleles are shown in Table 1. The crossing of these transgenic KO mouse models deficient in two and three

Table 1: Summary of phenotypes of double- and triple-knockout mice ablated for genes that regulate apoptotic and necroptotic cell death pathways.

Crossed mice	*ripk1–/–* die postnatal days 1-2	*ripk3–/–* viable	*mlkl–/–* viable	*ripk1–/–* *ripk3–/–* die postnatal day 4	*ripk1–/–* *mlkl–/–* die postnatal day 4
casp8–/– **Embryonic lethal**	**Embryonic lethal**	Viable	**Embryonic lethal**	Viable fertile	Die postnatal day 4
flip–/– **Embryonic lethal**	**Embryonic lethal**	**Embryonic lethal**	**Embryonic lethal**	**?**	**?**
fadd–/– **Embryonic lethal**	**Embryonic lethal**	Viable lympha-denopathy	**Embryonic lethal**	Viable fertile	Viable fertile
casp8–/– *fadd–/–* **Embryonic lethal**	**Embryonic lethal**	Viable	**Embryonic lethal**	Viable fertile	**?**
flip–/– *fadd–/–* **Embryonic lethal**	**Embryonic lethal**	Viable lympha-denopathy splenomegaly	**Embryonic lethal**	Viable fertile	**?**
bax–/– *bak–/–* viable (10%)	**Embryonic lethal**	**?**	**?**	**?**	**?**
cypd–/– viable	**Embryonic lethal**	Viable partial protection of cardiac ischemia	Viable	**?**	**?**

Summary of knockout mice models of apoptosis and necroptosis genes for which the phenotypes reveal a critical role in development, physiology, and homeostasis. Caspase-8, FLIP, and FADD proteins have pivotal roles in the death inducing signaling complex that regulate apoptosis FLIP deficiency causes both massive apoptosis and necrosis. Knockout mice for caspase-8, *Casp-8–/–*, FADD, and *Fadd–/–* or double knockouts for both show an embryonically lethal phenotype due to uncontrolled necrosis. Knockout mice for RIPK1, *Ripk1–/–*, die at birth of systemic inflammation whereas *Ripk3–/–* mice are normal but are resistant to proinflammatory stimuli. *Mlkl–/–* mice are anatomically normal, viable, and fertile. Triple knockouts mice *Fadd–/– Flip–/– Ripk3–/–* have a normal cell-death pathway and develop to normal birth because of absence of necrosis and apoptosis which are modulated by caspase-8. *Casp8–/– Mlkl–/–* double knockout mice are normal and resistant to TNF-induced necroptosis. *Bax–/– Bak–/–* double knockout mice develop perinatal lethality and only 10% survive into adulthood, and these mice develop splenomegaly and lymphadenopathy. The question mark indicates possible or still unknown.

genes has helped elucidate cell death necroptotic pathways and the essential role of downstream regulator genes involved in several inflammatory pathologies. Some examples are shown in Table 2.

5.1. Mice Models for Lethal Genes. Knockout mice for caspase-8, *caspase-8–/–*, FADD, and *fadd–/–* or double-knockouts for both show an embryonically lethal phenotype due to uncontrolled apoptosis or necroptosis [11, 82, 83]. The embryonic lethality in mice lacking FADD or caspase-8 is due to massive necrosis and can be rescued by RIPK1 or RIPK3 deletion, respectively. *Ripk1–/–* mice die at birth of systemic inflammation and the large area of necrosis in the liver and thymus [84, 85]. On the contrary, knockout of RIPK3 in mice did not cause any measurable defect in development, fertility, NF-κB activation, and apoptosis [86]. *Ripk3–/–* mice were resistant to necrotic pancreatitis (cerulein-induced) and vaccinia virus-induced hepatic necrosis [55]. The rescue of a lethal phenotype in RIPK1 knockout is often used as an argument for the implication of necroptosis. Mice deficient in FADD or caspase-8 die during embryogenesis; however, mice with triple deletion of FADD, caspase-8, and RIPK3 are viable [55, 62, 87, 88]. The cardiac, vascular, and hematopoietic defects that occur during the heart development of caspase-8 and FADD knockout mice are caused by RIPK3-mediated necrosis [87, 89]. Therefore, FADD and caspase-8 act as prosurvival factors that suppress the deleterious effects of necrosis by promoting the cleavage and inactivation of RIPK1 and RIPK3. On the other hand, double-knockout mice for *ripk3–/–* and *flip–/–* die during embryonic development due to uncontrolled apoptosis driven by active caspase-8 [89]. These experiments demonstrate that FLIP is an important brake on both apoptotic and necrotic cell death *in vivo.*

Caspase-8 and RIPK3 are all essential for clonal expansion for T and B cell clones [63]. Mice deficient in *Casp8–/–* fail to develop and die *in utero*, which may ultimately be due to failing to maintain the proliferation of T and B cells [90]. After stimulation, T cells lacking caspase-8 or its adaptor protein FADD developed hyperautophagic morphology and die by necroptosis [90]. Proliferation in caspase-8-deficient T cells is fully rescued by crossing with *ripk3–/–* mice, although such rescue ultimately leads to lymphadenopathy [63].

Table 2: Summary of phenotypes in double- and triple-knockout mice ablated for genes that regulate apoptosis, necroptosis, and inflammation.

Crossed mice	*ripk1–/–* die postnatal day 1	*ripk1–/– tnfr–/–* die postnatal day 1	*sharpin–/–* viable die postnatal days 10–14 inflammation	*a20–/–* viable die postnatal days 7–14 inflammation	*cyld–/–* viable reach adulthood inflammation
ripk3–/– casp8–/– viable	Viable	Viable?	**Embryonic lethal**	**Embryonic lethal**	?
ripk3–/– fadd–/– viable	Viable	Viable?	**Embryonic lethal**	**Embryonic lethal**	?
ripk3–/– tnfr–/– viable	Reach adulthood	Viable?	Prevent inflammation skin dermatitis	Prevent inflammation Cachexia	?
ifnar–/– viable	Die postnatal day 1	Die later than *ripk1–/– tnfr1–/–*	?	?	?
trif–/– viable	Die postnatal day 1	Die later than *ripk1–/– tnfr1–/–*	?	?	?
ripk3–/– mlkl–/– die postnatal day 4	Die postnatal day 4	Die later than *ripk1–/– tnfr1–/–*	Prevent systemic inflammation	?	?

Summary of phenotypes for double- and triple-knockout mice models of apoptosis, necroptosis, and inflammation genes. Knockout mice for RIPK1, *Ripk1–/–*, die at birth of systemic inflammation whereas *Ripk3–/–* mice are normal but are resistant to proinflammatory stimuli. *Mlkl–/–* mice are anatomically normal, viable, and fertile. Triple-knockout mice *Fadd–/– Flip–/– Ripk3–/–* have a normal cell-death pathway and develop to normal birth because of absence of necrosis and apoptosis which are modulated by caspase-8. *Casp8–/– Mlkl–/–* double knockout mice are normal and resistant to TNF-induced necroptosis. Deletion of *Tnfr* gene provides protection from *Ripk1–/–* perinatal lethality and double KO mice *Ripk1–/– tnfr–/–* can be partially protected from lethality from systemic inflammation by mating these mice with *ifnar–/–* or *trif–/–* mice. This indicated that both proteins can engage RIPK3-MLKL interaction independent of RIPK1. A20 and CYLD target similar molecular substrates including TRAF2, TRAF6, NF-κB essential modulator (NEMO), and RIPK1. The deubiquitinase CYLD removes the K63-Ub of RIPK1, and A20 promotes the removal of K63-linked ubiquitin chains to terminate signaling induced NF-κB activation. The ablation of A20 and SHARPIN genes is potentially lethal. A20/TNFAIP3, zinc finger and ubiquitin editing protein, CYLD, cylindromatosis, deubiquitylating enzyme; SHARPIN, a protein that together with HOIL-1 and HOIP forms the LUBAC, the heterotrimeric linear ubiquitin chain assembly complex; both are involved in the TNF signaling pathways; IFNAR, the type I IFN receptor. The question mark indicates possible or still unknown phenotype.

Although TNF-α was initially identified by its ability to kill tumor cells, most normal and tumor cells do not undergo cell death in response to this cytokine [91]. The treatment of wild type murine embryonic fibroblasts (MEFs) with TNF does not induce cell death. Overexpression of the *ripk3* gene causes necroptosis in MEFs stimulated with TNF in the absence of RIPK1, caspase-8, Bax, and Bak [92]. RIPK3 activation may be driven by spontaneously high RIPK3 levels or by another RHIM-containing protein such as DAI or TRIF [92]. MLKL is a RHIM-containing protein that is activated in cells with elevated RIPK3 consistent with its important role in necroptosis [92]. RIPK3 can partner also with TLR3 and TLR4 to induce macrophage necrosis and NF-kB activation but this requires TRIF [93].

MLKL is one essential effector of necroptosis since it causes cell death by a caspase- and Bax/Bak-independent mechanism [72]. Mice deficient in *Mlkl* gene are anatomically normal, viable, and fertile [94]. Cells from *Mlkl–/–* mice failed to undergo TNF-mediated necroptosis, as expected. Double deficient mice of *caspase-8* and *Mlkl* genes, *Casp8–/–*; *Mlkl–/–* mice, were normal but showed pronounced splenomegaly, thrombocytopenia, and lymphadenopathy after a few months of age [94]. This phenotype is similar as described by mice lacking functional FAS (*lpr*) or FAS ligand (*gld* mutant mice) [11].

5.2. Mice Models for Inflammatory Genes. Various pathological processes such as ischemic brain injury, myocardial infarction, organ transplantation, and virus replication are accompanied by strong inflammatory response. This inflammatory process is characterized by extensive necrosis of tissues. Studies on mouse models with FADD-TNFR1 and FADD-MyD88 deficiency revealed that both TNF and TLR signaling partially contribute to progression of inflammation [55, 88, 95]. Studies on mouse models have also shown that TNF-induced systemic inflammatory response syndrome (SIRS) and CLP-induced peritoneal sepsis are driven by both RIPK1 and RIPK3-dependent necroptosis [12, 58]. The regulated necrosis is also associated with tissue damage and inflammation driven by activation of sterile inflammatory response, including ischemia-reperfusion injury, Alzheimer's disease, atherosclerosis, and toxic insult to liver and lung [9, 96]. A recent study showed that loss of the RIPK3 or MLKL can provide protection to *Ripk1–/–* mice from systemic inflammation but fails to protect these mice from lethal intestinal inflammation [85]. *Ripk1–/–* and *Ripk3–/–* mice

have significantly lower rates of death and inflammation, whereas the *Ripk1–/–* and *Myd88–/–* mice have reduced inflammation, however, not reduced mortality [85]. Together, these studies demonstrated that only *Ripk1–/–*, *Ripk3–/–*, and *Casp8–/–* mice are protected from inflammation and intestinal disruption, reach adulthood, and are viable and fertile [83, 85, 89]. Future studies using *Ripk1–/– Ripk3–/–* and *Ripk1–/– Mlkl–/–* mice will be important to understand the role of these proteins in inflammation and the release of cytokines and CDAMPs.

Mice with chronic proliferative dermatitis mutation (cpdm mutant) develop TNF-dependent multiorgan inflammation, which is characterized by dermatitis, liver inflammation, splenomegaly, and loss of Peyer's patches [97]. These mice are deficient in SHARPIN, a protein that, together with HOIL-1, is a key regulator of HOIP (LUBAC), an ubiquitin ligase complex that catalyzes the addition of linear ubiquitin to target proteins [15]. SHARPIN is required for the TNFR signaling complex and for prevention of cell death in various cells, including epidermal keratinocytes [15]. A recent study has demonstrated that RIPK3 or MLKL deficiency is able to prevent liver inflammation in *shpn–/–* mice as well as restoring splenic architecture splenic phenotype and leukocytosis [97]. Only combined caspase 8 heterozygosity and RIPK3 deficiencies were able to almost completely prevent chronic proliferative dermatitis in *shpn–/–* mice. The role of SHARPIN in the skin-inflammation phenotype is mainly due to keratinocyte mitochondria-dependent apoptosis [85]. Since RIPK3 and MLKL deletion markedly reduced leukocytosis, it is suggested that the hematopoietic phenotype is mediated by necroptosis [97].

The E3 ligases, cIAP-1 (*Clap 1 gene*), cIAP-2 (*Clap 2 gene*), and Xiap, are responsible for RIPK1 polyubiquitination, but knockout mice of these genes are surprisingly phenotypically normal. Double-knockout *Xiap–/– Clap2–/–* mice are also phenotypically normal. However, *Xiap–/– Clap1–/–* and *Clap1–/– Clap2–/–* double-knockout mice are embryonic lethal and this can be rescued by crossing these animals with *Ripk1–/–* and *Ripk3–/–* mice [98].

Mice deficient in Cylindromatosis (CYLD) gene are viable and fertile during adulthood life. CYLD deficiency leads to hyperubiquitinated RIPK1 in the necrosome and impaired phosphorylation of RIPK1 and RIPK3, thereby blocking caspase-8 activation. CYLD is a caspase-8 substrate and when activated it cleaves CYLD and prevents necroptosis, similar to what occurs with the cleavage of RIPK1 and RIPK3 [99]. CYLD is required for TLR3 or TLR4 receptor-induced necroptosis. The crossing of *cyld–/–* mice with other members of the NLR and TLR families promises to deliver further experimental evidence on its crucial role in innate response. Finally, deficiency of both RIPK3 and caspase-8 or FADD completely abrogated Yersinia-induced cell death and caspase-1 activation. Mice ablated of RIPK3 and caspase-8 genes in their hematopoietic compartment showed high susceptibility to Yersinia infection as well as displayed a very low production of proinflammatory cytokines by monocyte and neutrophils [100]. We are waiting for the participation of additional components in these pathways and more complex interactions among them in the intracellular resolution of infections.

5.3. Mice Models for Mitochondria-Associated Channel and Pore Genes. Ischemia and reperfusion injury (IRI) cause a wide array of functional and structural alterations of mitochondria. Ca^{2+} Overload and MPTP openings are critical steps in this process. MPT is critically dependent on ANT, the ADP/ATP nucleotide exchanger, and the mitochondrial protein cyclophilin D (CypD), the regulator of ANT. Studies have been done to prove the parallel existence of two independent pathways of regulated necrosis in ischemia-reperfusion injury [101, 102]. Knockout of the gene encoding CypD renders mitochondria resistant to Ca^{2+} overload-induced swelling and the heart and brain partially resistant to cell death due to ischemic injury [103]. RIPK3-deficient mice are protected from IRI as well as from hyperacute TNFα-induced shock [104]. The *in vivo* analysis of cisplatin-induced acute kidney injury and hyperacute TNF-shock in Cyp-D and RIPK3 double-deficient mice demonstrated striking differences, but animals died after no longer than 120 h [102]. Protection of RIPK3-knockout mice was significantly stronger than for CypD-deficient mice. Cyclosporin prevents CyP-D binding to the ANT, whereas sanglifehrin (SfA) inhibits the peptidyl-prolyl cis-transisomerase (PPIase) activity of CyP-D but does not prevent its binding to the ANT. The protection from kidney injury upon long ischemia was observed when applying a combination of Nec-1 (necrostatin) and SfA, but the protection was not complete [102]. Necrostatin-1 blocks necroptosis in many settings by interacting and inhibiting RIPK1 kinase activity, which is required for activation of RIPK3. Nonetheless, necrostatin failed to block necroptosis in the absence of RIPK1, suggesting the RIPK1 has a protective role [104]. Moreover, mice with a kinase-dead mutation of RIPK1 show normal development and maturation, although cells from these animals appear to be defective in TNF-induced necroptosis [62].

The *Bax–/–Bak–/–* mice typically die at a perinatal age with multiple developmental defects, and only 10% survive into adulthood [81]. Mouse embryonic fibroblasts (MEFs) from *Bax–/– Bak1–/–* double-knockout (DKO) grown in culture are highly resistant to cell death by the apoptotic inducer staurosporine, confirming their critical role in apoptosis [105]. Recently, Karch and colleagues demonstrated that these cells are also resistant to H_2O_2, ionomycin, and DNA alkylation agents, which are inducers of necrotic phenotypes [106]. Consistent with results in DKO MEFs, cardiac-specific deletion of Bax/Bak1 significantly protected the heart from ischemia-reperfusion (I-R) injury and reduced mortality in mice subjected to permanent myocardial infarction injury [106]. MEFs from *Ppif* (CypD) deficient mice are insensitive to Ca^{2+}-induced MPTP opening, and Bax/Bak does not directly affect the MPTP at the level of the inner membrane. However, the authors concluded that, in the absence of BAX/BAK, the outer membrane resists swelling and prevents organelle rupture, thereby preventing cell death. The anti-apoptotic family members, Bcl-2 and Bcl-xL, act directly or indirectly to preserve the integrity of the outer mitochondrial membrane. To examine this issue, the authors showed that

the Bcl-2/Bcl-xL inhibitor ABT-737 sensitized the MPTP to open with mild Ca^{2+} stimulation or ionomycin treatment in wild type but not in Bax/Bak1 DKO cells. In light on these results, the authors concluded that Bax/Bak oligomers within the outer membrane are required for mitochondrial permeability pore-dependent cell death by serving as a necessary functional component of the MPTP [106]. Similar observations were previously reported by Irrinki and colleagues [107]. These authors investigated necroptosis induced by TNF, cycloheximide (CHX) plus z-VAD-fmk in MEFs derived from mice bearing two or three knockout genes including *fadd*−/−, *rip1*−/−, *NEMO*−/−, *caspase-8*−/−, *bad*−/−*bak*−/− (double-knockout), and *bax*−/−*bak*−/− (double-knockout) genes. Moreover, it was demonstrated that overexpression of Bcl-xL protects cells from necroptosis induced by these agents [107]. Other putative mechanisms through which the outer membrane proteins could affect formation of the MPTP are reviewed in [38].

Future work along these lines using RIPK-3 and MLKL-deficient mice as well as the combination with other mutant mice may uncover the complex regulation and interconnectivity among apoptosis effectors of mitochondrial intrinsic pathway and regulated necrosis execution pathway.

6. Conclusion and Future Directions

In the last decade, a number of cellular and molecular studies have advanced the knowledge and acceptance that in response to a variety of stress conditions, physicochemical insults, viral and bacterial infection, necroptosis rather than apoptosis emerges as predominately cell death in tissues into adult life.

We have highlighted the intricately connected and overlapping extrinsic and intrinsic molecular pathways regulating both apoptosis and necroptosis. We now know that besides TNF family of receptors many other cell-surface and intracellular receptors, including TLRs, NLRs, IFNRs, and T-cell receptors, can induce regulated cell death. Similarly, intracellular deregulation and/or imbalance of synthesis and metabolic pathways inside mitochondria, endoplasmic reticulum, and intracellular organelles can provoke regulated cell death. Exploring and classifying the molecular mechanisms underlying the initiation and execution pathways will help us search for new strategies to enhance or inhibit necroptotic cell death rates in pathological clinical scenarios such as inflammation, cancer resistance to chemotherapy, and excessive tissue death as result of ischemia and injury.

Caspase-8 and cFLIP heterodimers act as key regulators of death checkpoint to alternate the cell death pathway and canonic and noncanonical NF-κB signaling. Intracellular downstream signaling components of the TNFR multicomplexes and NF-κB signaling complexes, such as E3 ligases, cIAP-1, and cIAP-2, deubiquitinases, CYLD, A20, and HOIL-1/HOIP/Sharpin (LUBAC), are equally very important positive and negative regulators of inflammation and cell death decision checkpoints to apoptosis and necroptosis.

Mice models lacking the caspase-8, FADD, or FLIP are embryonic lethal and are rescued with ablation of RIPK1 and RIPK3 genes. With the help of gene-deficient mice models we will advance in the studies aiming to further understand the precise roles of these proteins at cell decision points from apoptosis to necroptosis and inflammation. TNF can activate RIPK3 in the absence of RIPK1 and surprisingly RIPK3 has a proinflammatory role itself. Future studies need to be done to understand how RIPK3-dependent necroptotic cell death can be activated in an RIPK1-independent manner. In this regard, the RHIM-containing protein such as DAI and TRIF as well as IFNAR merits further investigation because of its role in virus-induced necroptosis. Many other landmark genes will have place in the molecular pathways leading to regulated cell death. Knockout mice and their cell lines lacking essential genes will be important to investigate cross talk and kinetics aspects of cell death triggering, propagation, and resolution. These animal models will have critical role to future preclinical tests of new drugs for inhibiting cell death causing human diseases.

The plasma membrane permeabilization induced by MLKL oligomers plays a key role in the execution pathway for necroptosis. The 4HBH phospholipid-binding domain acts by recruiting the MLKL protein to bind to lipid ligands embedded in the membrane surface. Since PIP, $PI(4,5)P_2$, and $PI(3,4,5)P_3$ are abundant phospholipids in ubiquitous cell membranes, it is crucial to establish whether MLKL binding and pore-forming molecular mechanism can be specifically and efficiently modulated by combination of membrane lipids. Elucidating the combinatorial "codes" and critical domain structure for this interaction will help us design and develop new therapeutic strategies to prevent cell death triggered in prolonged degenerative, inflammatory, and infectious diseases.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors' Contribution

All authors equally contributed to this work.

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Research Article

S-Nitrosoglutathione Reductase Plays Opposite Roles in SH-SY5Y Models of Parkinson's Disease and Amyotrophic Lateral Sclerosis

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Oxidative and nitrosative stresses have been reported as detrimental phenomena concurring to the onset of several neurodegenerative diseases. Here we reported that the ectopic modulation of the denitrosylating enzyme *S*-nitrosoglutathione reductase (GSNOR) differently impinges on the phenotype of two SH-SY5Y-based *in vitro* models of neurodegeneration, namely, Parkinson's disease (PD) and familial amyotrophic lateral sclerosis (fALS). In particular, we provide evidence that GSNOR-knocking down protects SH-SY5Y against PD toxins, while, by contrast, its upregulation is required for G93A-SOD1 expressing cells resistance to NO-releasing drugs. Although completely opposite, both conditions are characterized by Nrf2 localization in the nuclear compartment: in the first case induced by GSNOR silencing, while in the second one underlying the antinitrosative response. Overall, our results demonstrate that GSNOR expression has different effect on neuronal viability in dependence on the stimulus applied and suggest that GSNOR could be a responsive gene downstream of Nrf2 activation.

1. Introduction

Oxidative and nitrosative stress has been copiously reported to play a pivotal role in the onset of neurodegenerative diseases [1, 2]. Both endogenous and exogenous sources of nitrooxidative stress, such as mitochondria [3] and immune cells [4] that produce reactive oxygen species (ROS) or reactive nitrogen species (RNS) deriving from nitric oxide (NO), deeply contribute to the persistence of noxious conditions affecting neuron viability. Parkinson's disease (PD) and amyotrophic lateral sclerosis (ALS) are among the neurodegenerative disorders in which nitrooxidative stress has been particularly reported being implied in the loss of specific neuronal populations.

PD is a neurodegenerative disorder that leads to a progressive loss of dopaminergic neurons. The mechanisms underlying this phenomenon are not completely clarified. However, mitochondrial damages (mainly those affecting complex I of the respiratory chain), failure in proteins, and organelles quality control as well as oxidative and nitrosative stress have been suggested to be implicated in the pathogenesis of PD [5]. Several are the mutations associated with the family forms of PD [6]. Many of them affect specific proteins, for example, PINK1, Parkin, and DJ-1. In particular, DJ-1 has been implicated in the protection against oxidative insults as it interferes with the production of mitochondrial ROS, and it has been reported to be undergoing activation by *S*-nitrosylation processes [7–9]. Moreover, the existence

of a signaling axis involving DJ-1 as upstream modulator of Nrf2 (NE-F2-related factor-2) [10, 11], the transcription factor known as one of the master regulators of redox homeostasis, has recently been demonstrated, suggesting that DJ-1 may act in different ways in order to counteract oxidative stress.

ALS is a multifactorial disease caused by motor neuron degeneration that can be divided into a sporadic (90% of the cases) and a familial form (fALS) whose 20% of mutations (2% of total ALS cases) map throughout the *SOD1* gene (coding the Cu, Zn superoxide dismutase) [12, 13]. More than 100 point mutations in SOD1 gene are associated with fALS cases, with some of them affecting its antioxidant activity. One of the most studied SOD1 mutations is the G93A substitution which results in the aggregation of the mutant forms, being, in such a way, detrimental for mitochondrial homeostasis and motor neuron survival. Another common feature of ALS is the chronic inflammatory response activated in the cells surrounding motor neurons (astrocytes and microglia) that leads to the production of cytokines and NO [14–16].

S-Nitrosylation represents one of the main NO-mediated posttranslational modifications [7]. It is based on the attachment of an NO moiety to the thiol group (-SH) of cysteine residues, resulting in the formation of low (amino acids and peptides) and high molecular weight (proteins) *S*-nitrosothiols (SNOs). Although NO could modulate either neuronal viability or death, the role of neuronal *S*-nitrosothiols (SNOs) in the etiopathogenesis of neurodegenerative diseases remains to be fully elucidated yet. Protein aggregates found in brains from PD and ALS patients show a great abundance of nitrated/*S*-nitrosylated proteins, suggesting that SNOs are involved in the pathogenesis and/or in the neuronal defense responses. However, recent results [17] indicate that NO is a potent antioxidant agent that reduces oxidative stress in the central nervous system, such as by increasing the antioxidant capacity of reduced glutathione (GSH) through the formation of *S*-nitrosoglutathione (GSNO), which is a 100-fold more potent antioxidant with respect to GSH.

It has been reported that class III alcohol dehydrogenase [18] also known as *S*-nitrosoglutathione reductase (GSNOR) catalyzes the degradation of GSNO, thus regulating the amount of SNOs and NO bioactivity. Although GSNOR-deficient (KO) mice are viable and fertile, they show some pathological phenotypes, many of which related to aging, such as (i) compromised lymphocytes development [19]; (ii) high morbidity and mortality upon endotoxic shock [20]; (iii) DNA damage accumulation; (iv) high probability to develop hepatocellular carcinoma [21, 22]; (v) early signs of peripheral neuropathy [23]. Altogether, this evidence indicates that (i) excessive *S*-nitrosylation negatively impacts on cell physiology and (ii) GSNOR acts as the principal molecular modulator underneath. GSNOR, in fact, is the best characterized denitrosylating enzyme evolutionarily conserved from bacteria to humans [24], which is widely expressed in all tissues and organs. In particular, brain has been reported to express high levels of GSNOR [24]. However, any alteration linked to GSNOR deficiency in nervous system has never been reported so far.

Based on the evidence that GSNOR deficiency results in ageing-related phenotypes and that brain represents the organ mostly affected during ageing, here we have aimed at characterizing the role of GSNOR in modulating the severity of two neurodegenerative diseases, PD and fALS, taking SH-SY5Y as elective experimental system.

2. Materials and Methods

2.1. Cell Cultures. Human SH-SY5Y cells were purchased from the European Collection of Cell Culture and grown at 37°C in an atmosphere of 5% CO_2. SH-SY5Y cells were cultured in DMEM-F12 containing 25 mM glucose, supplemented with 10% fetal bovine serum, 2 mM L-glutamine, and 1,000 U/mL penicillin-streptomycin. All media were from Lonza. G93A-SOD1 stably expressing SH-SY5Y cells were generated as previously reported [25]. *LowNO* clone was selected from a mixed population of G93A cells subjected to repeated treatments with GSNO.

2.2. Cell Transfection with siRNAs. 24 hours after plating, SH-SY5Y cells were transfected with a commercially available small interference RNA (siRNA) against GSNOR (Sigma). Control cells were transfected with a scramble siRNA duplex (siScr), which does not present homology with any other human mRNAs. In experiments aimed at downregulating DJ-1, double transfection (with both siRNAs against GSNOR and DJ-1) was carried out simultaneously.

2.3. Treatments. All treatments were performed for 24 h in completed culture media. DPTA was used at a concentration of 400 μM, H_2O_2 at 1 mM, MPP^+ at 1.25 or 2.5 mM, and 6OHDA at 50 μM. Trigonelline was preincubated for 30 min before treatments with DPTA and used at a concentration of 0.5 or 1 μM.

2.4. Analysis of Cell Death. Cell death was evaluated by direct cell count at optic microscopy upon Trypan blue staining or, cytofluorometrically, by assessing the percentages of apoptotic cells upon staining with propidium iodide.

2.5. Animal Experimentation. All mouse experiments were conducted in accordance with the European Community guidelines and with the approval of relevant national (Ministry of Health) and local (Institutional Animal Care and Use, Tor Vergata University) ethical committees. All mice employed in the experiments (wild type, G93A, and GSNOR-KO) were on C57BL/6J background. They have been raised and crossed in the indoor animal house in a 12 h light/dark cycle in a virus/antigen-free facility with controlled temperature and humidity and have been provided with water and food *ad libitum*. At the indicated time, mice were anesthetized with ketamine–xylazine, killed, and dissected for the different experiments. All efforts were made to minimize suffering.

2.5.1. Spinal Cords. Spinal cords were collected from wild type (WT) and symptomatic (135-day-old) G93A-SOD1 mice B6.Cg-Tg(SOD1-G93A)1Gur/J (Jackson Laboratory, Bar Harbor, ME, USA) minced and lysed by 40–60 strokes

in Potter-Elvehjem homogenizer and supernatants used for PSNOs detection and GSNOR activity.

2.5.2. Brains. Brains were collected from 2-month-old wild type (WT) and GSNOR-deficient (KO) mice. Mesencephalon (ventromedial zone) was then isolated, since it contains most of the dopaminergic cells (which are affected in PD), minced and lysed by 30–40 strokes in Potter-Elvehjem homogenizer and supernatants used for Western blot analyses.

2.5.3. PCN. Mouse primary cortical neurons (PCN) were obtained from cerebral cortices of E15 C57BL/6 mice (WT and GSNOR-KO) embryos. Minced cortices were digested with trypsin EDTA 0.25% at 37°C for 7 minutes. Cells were stained with 0.08% Trypan blue solution and only viable cells were counted and plated at the density of $1 \times 10^5/\text{cm}^2$ onto poly-d-lysine coated multiwell plates in 25 mM glucose-containing MEM medium supplemented with 10% fetal bovine serum, 2 mM glutamine, and 0.1 mg/mL gentamicin. After 1 hour, the medium was replaced with Neurobasal medium containing antioxidant-free B27 supplement, 2 mM glutamine, and 0.1 mg/mL gentamicin. Cell cultures were kept at 37°C in a humidified atmosphere containing 5% CO_2. Every 3 days, one-third of the medium was replaced up to day 7, the time at which the cells were treated.

2.6. Western Blotting. Cell or tissue were lysed or homogenized in a lysis buffer containing 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1% NP-40, and 12 mM Na-deoxycholate supplemented with protease and phosphatase inhibitors cocktail. Nuclei and cytosol were obtained as previously described [26]. Protein concentration was determined by the Lowry protein assay. Proteins were separated by SDS-PAGE and transferred to nitrocellulose membranes that were probed with the following antibodies: anti-*n*NOS (Santa Cruz), anti-SOD1 (Santa Cruz), anti-LDH (Santa Cruz), anti-poly-ADP-ribose polymerase 1 (PARP1), anti-actin (Santa Cruz), anti-tubulin (Sigma), anti-GSNOR (Thermo Scientific), anti-DJ-1 (Santa Cruz), anti-AKT (Santa Cruz), anti-phospho-AKT (Santa Cruz), anti-Nrf2 (Santa Cruz), and anti-H2B (Santa Cruz). After immunostaining with appropriate secondary horseradish peroxidase-conjugated goat anti-rabbit or anti-mouse antibodies, bands were revealed using the Amersham ECL detection system.

2.7. Detection of Oxidative Stress

2.7.1. ROS Evaluation. 30 minutes before the end of the experimental time, cells were incubated with 50 μM H_2-DCF at 37°C, washed, and resuspended in ice-cold PBS. The fluorescence intensities of DCF, formed by the reaction of H_2-DCF with ROS, were analyzed cytofluorometrically by recording FL-1 fluorescence.

2.7.2. Carbonylation. Carbonylated proteins were detected using the Oxyblot Kit (Intergen, Purchase, NY) after reaction with 2,4-dinitrophenylhydrazine (DNP) for 15 minutes at 25°C. Samples were then resolved by 10% SDS polyacrylamide gel electrophoresis and DNP-derivatized proteins identified by immunoblot using an anti-DNP antibody.

2.8. Evaluation of GSNOR Activity. GSNOR activity was evaluated spectrophotometrically in clarified cell or spinal cord lysates at 340 nm as previously described [20, 24].

2.9. Detection of S-Nitrosylated Proteins (PSNOs) and Pull-Down. *S*-Nitrosylation assays were performed as previously described [27]. In particular, cells or tissues were lysed or homogenized in HEN buffer (25 mM HEPES, 50 mM NaCl, 0.1 mM EDTA1% NP-40, protease inhibitors, pH 7.4). Free cysteine residues were blocked with *S*-methyl methanethiosulfonate (MMTS). More specifically, 0.5–2 mg of protein samples were diluted to 1.8 mL with HEN buffer (100 mM HEPES, 1 mM EDTA, 0.1 mM neocuproine, pH 8.0). Next, 0.2 mL of 25% SDS is added along with 20 μL of 10% MMTS (to reach a final volume of 2 mL and final concentrations of 2.5% SDS and 0.1% MMTS) and incubated at 50°C in the dark for 15–20 min with frequent vortexes. Proteins were then precipitated upon incubation with acetone, resuspended in 0.24 mL HENS buffer (HEN buffer with 1% SDS), and transferred to a fresh tube containing biotin-HPDP (2.5 mg/mL) with or without sodium ascorbate (20 mM). After incubation with the HRP-conjugated streptavidin, biotinylated proteins were revealed using the Amersham ECL detection system. Ascorbate-free samples have been omitted for straightforward visualization and interpretation of data.

Once obtained, the pool of PSNOs was pulled down by incubation with streptavidin and proteins there included were revealed on SDS-PAGE upon incubation with specific primary antibody (e.g., DJ-1).

2.10. Fluorescence Microscopy. Cells were cultured on coverslips, fixed with 4% paraformaldehyde, and permeabilized. Polyclonal anti-Nrf2 and monoclonal Grp75 (Millipore) were used as primary antibodies and probed successively with the appropriate Alexa Fluor 568-conjugated secondary antibody. To visualize nuclei and the actin cytoskeleton, cells were incubated with the cell-permeable DNA-specific dye Hoechst 33342 (Calbiochem-Novabiochem) and fluorescein isothiocyanate- (FITC-) conjugated phalloidin (Invitrogen), respectively. Images were digitized with a Cool Snap video camera connected to a Nikon Eclipse TE200 fluorescence microscope.

2.11. Fluorescence Microscopy Detection of $\Delta\psi_m$. JC-1 (Life Technologies) was added to prewarmed cell medium at the final concentration of 5 μg/mL in order to evaluate $\Delta\psi_m$. After incubation for 10 min at 37°C, cells were washed in PBS and analyzed using a EVOS Floid Cell Imaging Station.

2.12. Data Presentation. All experiments were done at least three different times unless otherwise indicated. The results are presented as means ± SD. Statistical evaluation was conducted by ANOVA, followed by Bonferroni's test. Comparisons were considered significant at $P < 0.05$.

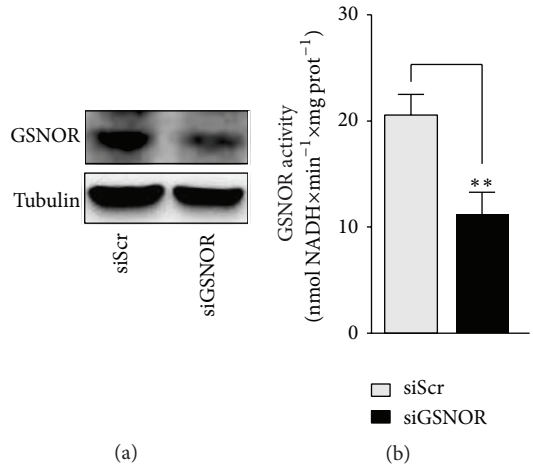

Figure 1: Characterization of siGSNOR cells. (a) Western blotting of GSNOR in total cell lysates of SH-SY5Y cells transiently transfected for 18 h with siRNA against GSNOR (siGSNOR) or control scramble siRNA (siScr). (b) Analyses of GSNOR activity in siGSNOR and siScr cells. Western blots shown are representative of at least $n = 3$ independent experiments that gave similar results. Tubulin was selected as loading control. Graphs shown represent the mean of data ± SD of $n = 3$ independent experiments, $^{**}P < 0.01$.

3. Results

3.1. GSNOR Downregulation Protects SH-SY5Y Cells from MPP$^+$-Induced Toxicity. In order to analyze the effects of *S*-nitrosylation on the viability of dopaminergic cells challenged with PD toxins, we selected the SH-SY5Y cell line in which we transiently knocked down GSNOR (siGSNOR cells) (Figures 1(a) and 1(b)). We then subjected the cells to different doses of the mitochondrial toxin MPP$^+$, used to recapitulate *in vitro* PD-like conditions, and we analyzed cell viability by Trypan blue staining. Dose response experiments indicted that, except for the highest dose employed (namely, 10 mM) where viability was completely compromised, siGSNOR cells were more resistant to MPP$^+$-mediated toxicity (Figure 2(a)). Cytofluorometric analyses upon propridium iodide staining also demonstrated that the extent of Sub-G1 (apoptotic) population was lower in siGSNOR cells (Figure 2(b)) upon treatment with either 1.25 or 2.5 μM MPP$^+$. Coherently, Western blot analyses of poly-ADP ribose polymerase 1 (PARP1) cleavage indicated that siGSNOR cells were almost unaffected by treatment with 2.5 mM MPP$^+$ if compared with the scrambled siRNA transfected controls (siScr) (Figure 2(c)). To confirm these results, we also treated the cells with 50 μM 6-hydroxydopamine (6-OHDA), another toxin usually employed to induce PD-like cell death. As expected, results shown in Figure 2(d) confirmed that siGSNOR cells were more resistant to PD toxin mediated challenge.

3.2. GSNOR Downregulation Correlates with a General Decrease of Oxidative Stress. In order to figure out the molecular events responsible for siGSNOR cell resistance to PD toxins, we cytofluorometrically analyzed H_2O_2 produced in SH-SY5Y under basal conditions by means of incubations with 2′,7′-dihydrodichlorofluorescein (DCF). Figure 3(a) indicates that H_2O_2 production was endogenously lower in siGSNOR than siScr cells. Consistently, primary cortical neurons (PCN) obtained from GSNOR-deficient (GSNOR-KO) mice showed an endogenous production of H_2O_2 significantly reduced with respect to the wild type (WT) counterparts (Figure 3(b)). Dihydroethidine (DHE) staining also showed that GSNOR-KO PCN exhibited low levels of $O_2^{\bullet-}$ (Figure 3(c)), confirming that GSNOR depletion was associated with a general reduction of endogenous levels of ROS. These lines of evidence were in agreement with the observation that the extent of protein carbonylation measured in lysates from mesencephalon of brains obtained by GSNOR-KO mice was lower than the WT counterpart (Figure 3(d)), suggesting that GSNOR deficiency could induce an enhancement of the antioxidant defense.

3.3. GSNOR Downregulation Induces DJ-1 and Nrf2 Activation. In the search for the molecular determinants underlying the decrease of oxidative markers observed upon GSNOR downregulation or complete ablation, we focused on DJ-1, whose increased expression and *S*-nitrosylation have been reported playing a pivotal role in neuroprotection [9, 28, 29]. Results shown in Figure 4(a) indicate that DJ-1 was upregulated in siGSNOR SH-SY5Y cells and that this correlated with an increase in the phosphoactive form of AKT. Interestingly, total brain lysates obtained from WT and GSNOR-KO mouse brains displayed the same trend (Figure 4(b)), which was also associated with an increase of the *S*-nitrosylated form of DJ-1 (Figure 4(c)), suggesting that DJ-1 was upregulated and, reasonably, activated by *S*-nitrosylation upon GSNOR downregulation. Coherently, transfection with siRNA against DJ-1 resulted in a significant increase of MPP$^+$-mediated toxicity in siGSNOR cells, while it seemed to be completely ineffective in siScr SH-SY5Y (Figure 4(d)).

We previously demonstrated that GSNOR-KO skeletal muscle showed an induction of Nrf2 [23]. Moreover data from the literature show that DJ-1 is an upstream modulator

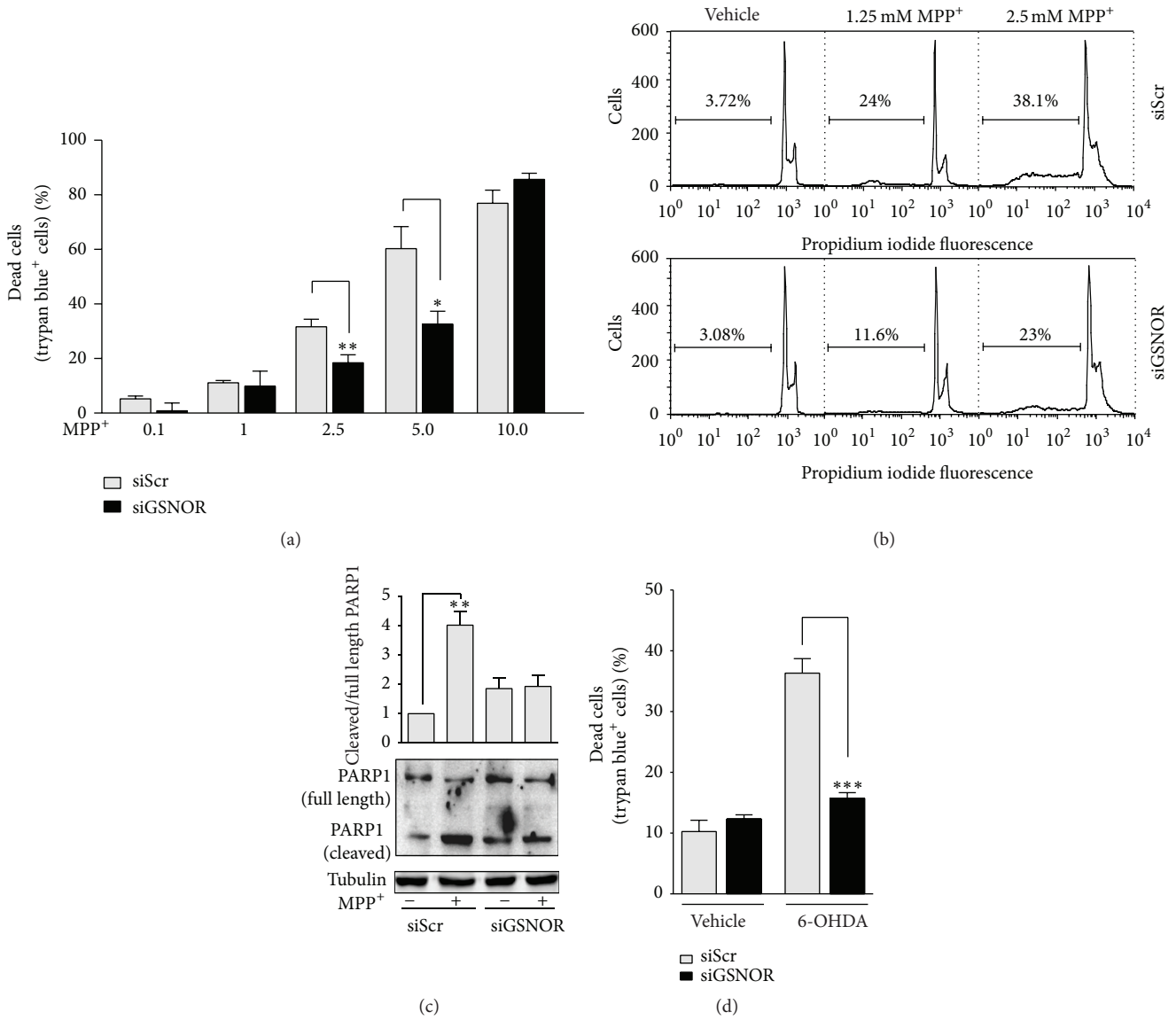

Figure 2: Cell death in siGSNOR cells. (a) Direct cell count upon Trypan blue staining of siScr and siGSNOR SH-SY5Y cells treated for 24 h with 0.1 to 10 mM MPP^+. (b) Cytofluorimetric histogram of siScr and siGSNOR SH-SY5Y cells treated for 24 h with 1.25 or 2.5 mM MPP^+ upon propidium iodide staining percentage of subG1 (apoptotic) population is shown. (c) Western blotting of PARP1 in total cell lysates of siScr and siGSNOR SH-SY5Y cells treated for 24 h with or without 2.5 mM MPP^+. Densitometric analyses reporting the ratio cleaved: full length of PARP1 immunoreactive bands are shown on the top. (d) Direct cell count upon Trypan blue staining of siScr and siGSNOR SH-SY5Y cells treated for 24 h with 50 μM 6OHDA. Western blots shown are representative of at least $n = 3$ independent experiments that gave similar results. Tubulin was selected as loading control. Graphs shown represent the mean of data ± SD of $n = 3$ independent experiments, $^*P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

of the Nrf2-mediated antioxidant response [10, 11]. Therefore, we focused on this transcription factor. Namely, we analyzed its nuclear *versus* cytosolic localization, which is commonly accepted as predictive sign of Nrf2 activation. Immunofluorescence analyses of PCN indicated that Nrf2 localization significantly changed from being exclusively cytosolic and axonal (WT) into predominantly nuclear (GSNOR-KO) (Figure 5(a)). Western blot analyses performed in cytosolic and nuclear fractions of both siGSNOR SH-SY5Y and GSNOR-KO brains indicated that nuclear levels of Nrf2 were higher if compared with siScr and WT controls, respectively (Figures 5(b) and 5(c)), confirming previously obtained results and reinforcing the hypothesis that Nrf2 was activated upon GSNOR downregulation. In agreement with these observations, we inhibited Nrf2 with 1 μM of its pharmacological inhibitor trigonelline and analyzed cell death. Figure 5(d) shows that preincubation with trigonelline significantly exacerbated MPP^+-induced toxicity in siGSNOR cells, arguing for a role of Nrf2 in cell protection against MPP^+ in conditions of nitrosative stress.

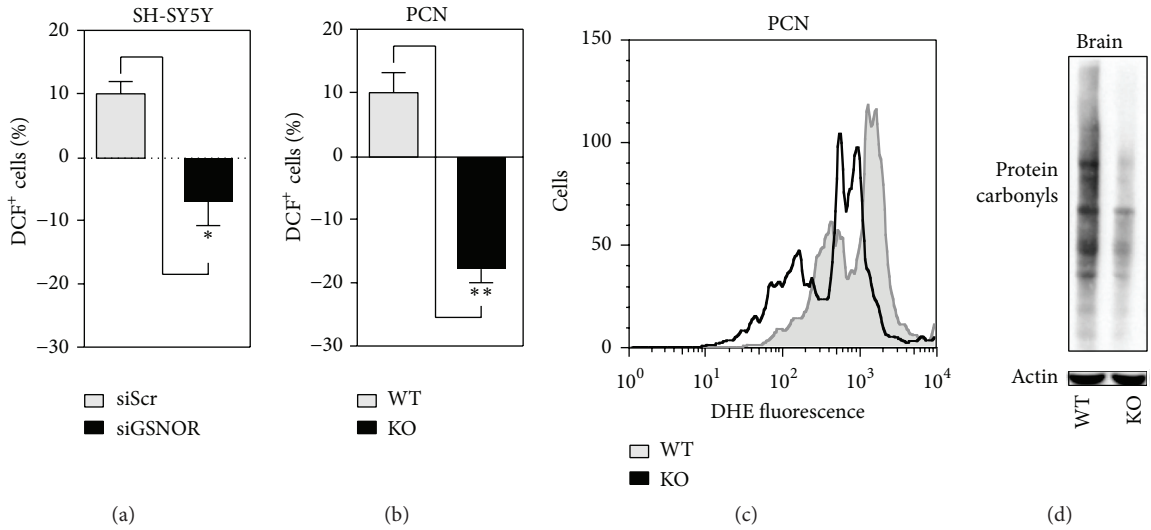

Figure 3: ROS production and protein carbonylation in GSNOR-downregulating systems. (a) Cytofluorimetric analyses of H_2O_2 in siScr and siGSNOR SH-SY5Y cells upon 2′,7′-dihydrodichlorofluorescein (DCF) staining. (b) Cytofluorimetric analyses of H_2O_2 and $O_2^{\bullet-}$ in GSNOR-KO and WT PCN upon 2′,7′-dihydrodichlorofluorescein (DCF) or (c) dihydroethidine (DHE) staining. (d) Western blot analyses of protein carbonyls from mesencephalon obtained from GSNOR-KO and WT brains. Western blots shown are representative of at least $n = 3$ independent experiments that gave similar results. Actin was selected as loading control. Graphs shown represent the mean of data ± SD of $n = 3$ independent experiments, $^{*}P < 0.05$ and $^{**}P < 0.01$.

3.4. GSNOR Decrease Sensitizes G93A-Expressing Models of ALS to Nitrosative Stress. Next, we wondered to extend our analyses and figure out any role of GSNOR in neurodegenerative disturbances where NO-mediated toxicity has been frequently reported being a factor affecting neuronal viability. In particular, we focused on ALS whose progression and severity have been tightly correlated with NO overproduction and nitrosative stress. In order to verify whether excessive *S*-nitrosylation occurred in ALS, we initially performed biotin switch assays of *S*-nitrosylated proteins (PSNOs) in spinal cord of mice overexpressing the fALS associated G93A mutant form of SOD1 that we selected as *in vivo* elective model of ALS; Figure 6(a) shows that PSNOs levels were significantly increased in symptomatic G93A mice (135-day-old), confirming the hypothesis that ALS was accompanied by aberrant *S*-nitrosylation. Interestingly, spectrophotometric analyses indicated that this condition was associated with a marked decrement of GSNOR activity (Figure 6(b)), suggesting that GSNOR decrease could concur to nitrosative stress and motor neuron death observed in ALS.

Coherently, we and others had already reported that SOD1-G93A overexpression rendered SH-SY5Y cells (hereafter named G93A cells) sensitive to nitrosative stress [30], and during our experiments we observed that 24 h treatments with 400 μM of the NO-donor DPTA induced higher extent of apoptotic cells in G93A cells if compared with parental SH-SY5Y cells (Figure 6(c)). Interestingly, this condition was exacerbated by siRNA-induced GSNOR downregulation (Figure 6(c)), adding in such a way a further evidence that GSNOR could play a role in ALS, namely, in protecting ALS motor neurons against nitrosative stress.

3.5. GSNOR Upregulation Induces Resistance to Nitrosative Stress in G93A Cells. We then selected a clone of G93A cells that significantly upregulated GSNOR and downregulated *n*NOS (clone hereafter named as *lowNO*) (Figure 7(a)). This clone still maintained the typical phenotype of fALS mutant SOD1, such as mitochondrial fragmentation (Figure 7(b)) and depolarization (Figure 7(c)), together with an endogenous production of ROS (Figure 7(d)). However, the extent of protein carbonylation decreased in this clone (Figure 7(e)), reinforcing the idea that NO directly contributes to carbonylation.

In agreement with high GSNOR levels, we found out that the *lowNO* clone was highly resistant to 24 h treatment with DPTA (Figure 7(f)), indicatingthat GSNOR induction was protective against nitrosative challenge in *in vitro* fALS models.

3.6. Nrf2 Contributes to Antinitrosative Response. In order to characterize the GSNOR-based preconditioning response underlying *lowNO* clone resistance to nitrosative stress, we performed localization assays of Nrf2 by Western blot. Figure 8(a) shows that Nrf2 preferentially distributed in the nucleus of *lowNO* clone, arguing for Nrf2 being induced in these cells. Consistently, incubation of *lowNO* clone with trigonelline increased susceptibility to DPTA (Figure 8(b)), allowing the hypothesis that Nrf2 activation and GSNOR upregulation, both found in *lowNO* clone, were functionally correlated in mediating resistance of G93A cells to nitrosative stress.

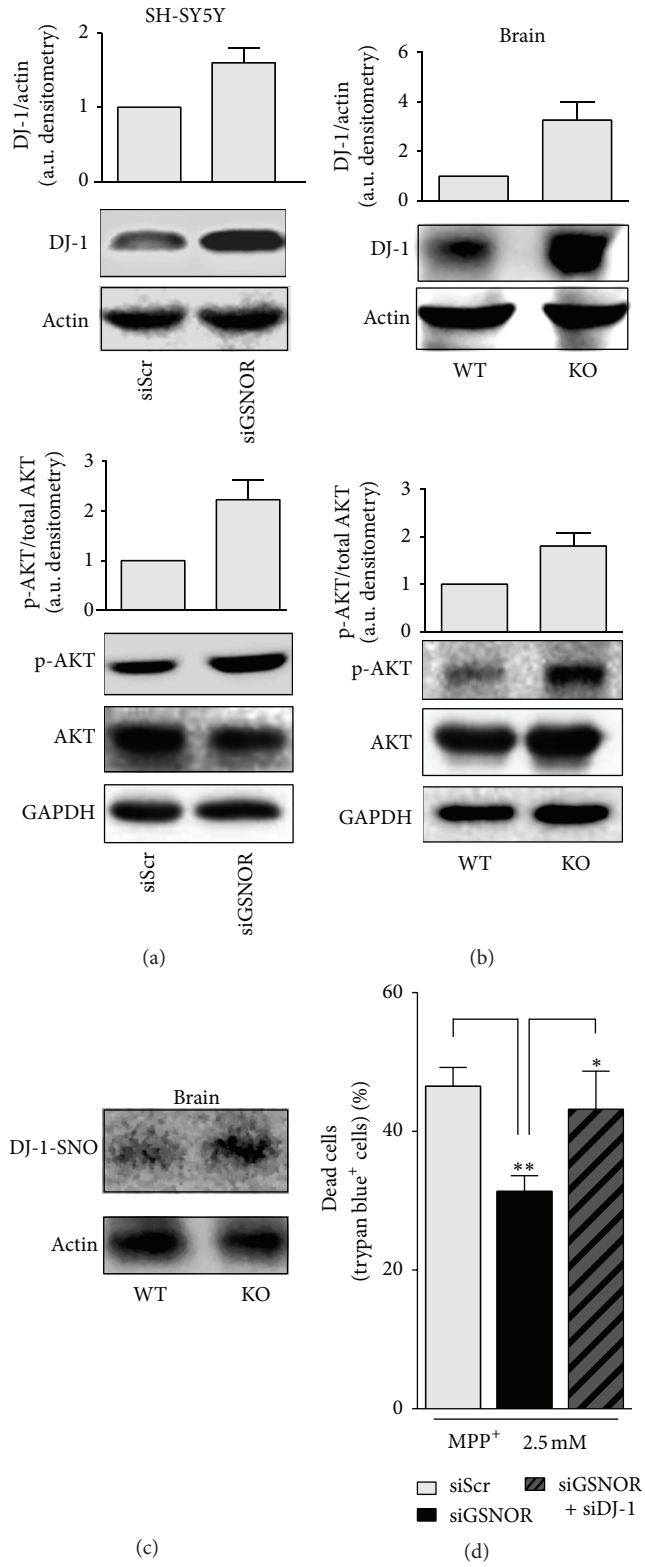

FIGURE 4: DJ-1 and AKT in siGSNOR cells and GSNOR-KO brains. Western blot analyses of DJ-1, as well as basal and phosphoactive AKT in total extracts obtained from (a) siScr and siGSNOR SH-SY5Y cells or (b) GSNOR-KO and WT brains. Densitometric analyses of DJ-1 and phospho-AKT are shown on the top of the corresponding Western blot normalized to actin and basal AKT, respectively. (c) Biotin switch assay followed by pull-down with streptavidin and revealed with anti-DJ-1 antibody, in total extracts obtained from GSNOR-KO and WT brains. Western blot analysis indicates that DJ-1 was *S*-nitrosylated (present in the pull-down). Western blots shown are representative of at least $n = 3$ independent experiments that gave similar results. Actin or GAPDH were selected as loading controls. (d) Direct cell count upon Trypan blue staining of siScr and siGSNOR SH-SY5Y cells transfected or not with siRNA against DJ-1 (siDJ-1) and treated for 24 h with 2.5 mM MPP^+. Graphs shown represent the mean of data ± SD of $n = 3$ independent experiments, $^*P < 0.05$ and $^{**}P < 0.01$.

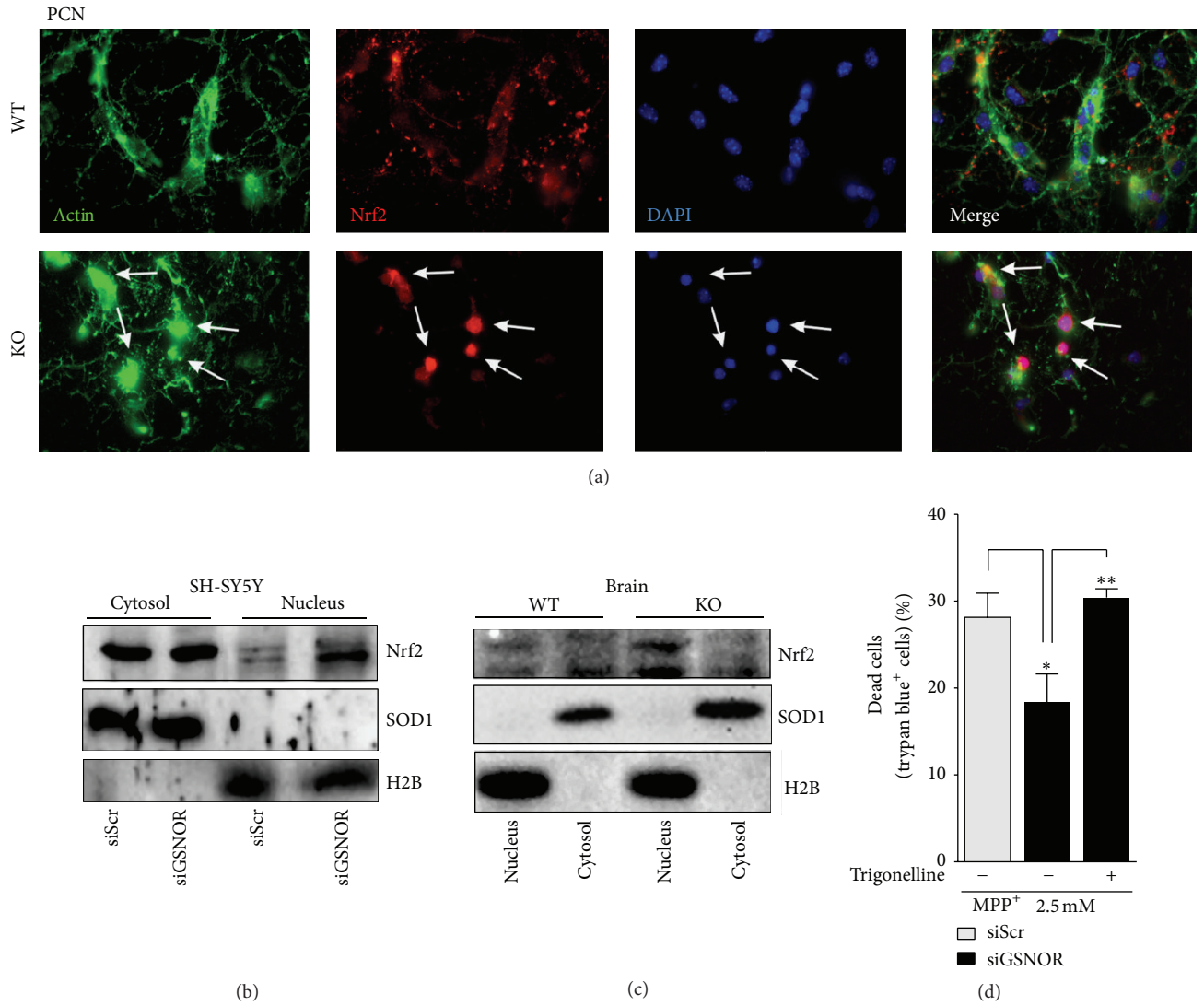

FIGURE 5: Nuclear localization of Nrf2 in GSNOR-downregulating systems. (a) Fluorescence microscopy analyses of WT and GSNOR-KO PCN upon staining with anti-Nrf2 antibody (red), phalloidin (green), and Hoechst 33342 (blue), with the last two probes used to selectively highlight actin (cytoskeleton) and nuclei, respectively. Western blot analyses of Nrf2 in nuclear and cytosolic fractions obtained from (b) siScr and siGSNOR SH-SY5Y cells or (c) GSNOR-KO and WT brains. Western blots shown are representative of at least $n = 3$ independent experiments that gave similar results. SOD1 and histone 2B (H2B) were selected as loading and purity controls of cytosol and nuclei, respectively. (d) Direct cell count upon Trypan blue staining of siScr and siGSNOR SH-SY5Y cells treated for 24 h with 2.5 mM MPP^+ in the presence or absence of 1 mM of the Nrf2 pharmacological inhibitor trigonelline. Graphs shown represent the mean of data ± SD of $n = 3$ independent experiments, $^*P < 0.05$ and $^{**}P < 0.01$.

4. Discussion

In this work we have analyzed and characterized the role of GSNOR and *S*-nitrosylation in modulating phenotype severity of *in vitro* PD and fALS models. Despite the fact that GSNOR is the only alcohol dehydrogenase highly expressed in the central nervous system of mouse, rat, and humans [24], to the best of our knowledge this is the first evidence arguing for GSNOR being a crucial molecular player of neuronal homeostasis. Interestingly, here we have provided evidence that knocking down GSNOR expression results in a significant protection against PD toxins, such as MPP^+ and 6OHDA. We recently demonstrated that muscles from GSNOR-KO mice show a clear fragmentation and depolarization of the mitochondrial network, which are associated with an endogenous activation of Nrf2-mediated antioxidant response [23]. It is therefore plausible that, under these conditions, mitochondria are less affected by MPP^+ and highly protected against any further production of ROS, for example, that induced by 6OHDA. From this perspective, GSNOR downregulation represents a molecular event responsible for neuronal cell adaptation against oxidative stress and a possible tool to be exploited in order to counteract PD onset and progression.

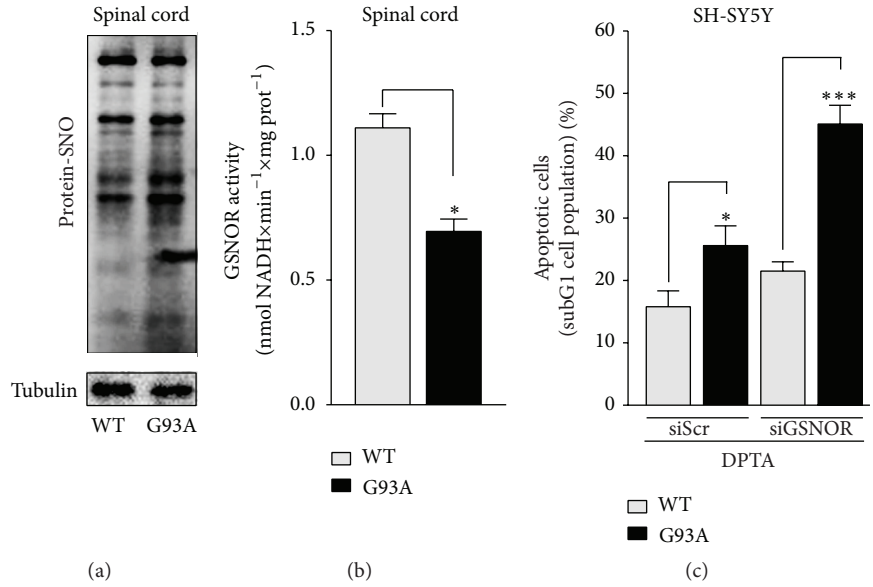

Figure 6: Characterization and sensitivity of G93A models to nitrosative stress. Biotin switch assay of total protein-SNOs (a) and analysis of GSNOR activity (b) performed on spinal cord lysates of WT and G93A-SOD1 expressing mice (G93A). (c) Cytofluorimetric analyses of apoptosis upon propidium iodide staining performed on parental SH-SY5Y cells (WT) and G93A-SOD1 mutants (G93A) transfected or not with siRNA against GSNOR (siGSNOR) and treated for 24 h with 400 μM DPTA. Western blots shown are representative of at least $n = 3$ independent experiments that gave similar results. Tubulin was selected as loading control. Graphs shown represent the mean of data ± SD of $n = 3$ independent experiments, $^{*}P < 0.05$ and $^{***}P < 0.001$; n.s.: not significant.

However, GSNOR downregulation should sensitize cells to nitrosative stress. This is a crucial issue if we take into account neurodegenerative diseases in which NO-mediated toxicity highly contributes to the loss of specific neuronal populations, such as motor neurons in ALS. In particular, a direct role of chronic NO-based inflammatory response activated by astrocytes and microglia has been reported in ALS as detrimental event concurring to motor neuron cell death [4]. In agreement, we observed that *in vitro* models of fALS, namely, the SOD1-G93A SH-SY5Y cells, are highly susceptible to the toxicity of the NO-releasing drug DPTA, which is in line with previously published data obtained with other NO donors [30]. In these conditions, ectopic downregulation of GSNOR expression by siRNA exacerbates NO-mediated cell death, confirming our hypothesis of a protective role of GSNOR in cells susceptible to nitrosative stress. Remarkably, in our studies we found out that G93A-SOD1 expressing mice show an increase of spinal cord PSNOs in association with a decrease of GSNOR activity. This finding suggests for the first time that GSNOR is downregulated in ALS and argues for GSNOR decrease being a new marker of ALS onset and, mainly, progression. This hypothesis is strengthened by the results obtained in the GSNOR-overexpressing *lowNO* G93A clone which shows resistance to DPTA, thereby indicating that *S*-nitrosylation is a posttranslational modification strongly implicated in driving motor neuron death response to nitrosative stress in ALS.

Interestingly, our data support the hypothesis that Nrf2 could play a pivotal role also in these conditions. In particular, we have found out that cell adaptation induced by *lowNO* G93A clone to survive to nitrosative stress conditions (namely, GSNOR induction combined with *n*NOS downregulation) is associated, at least in part, with the induction of Nrf2, as trigonelline significantly increases *lowNO* G93A clone sensitivity to NO.

Altogether, these results argue for Nrf2 being tightly related to GSNOR expression. In particular, while Nrf2 is activated in response to (downstream of) GSNOR depletion to protect against PD toxins-induced cell death, it could also induce (be upstream of) GSNOR transcription to preserve survival in *in vitro* models of fALS in conditions of nitrosative stress. A positive feedback loop between Nrf2 and GSNOR could be therefore hypothesized to occur in order to sustain cell viability in conditions of nitrooxidative stress. However, if GSNOR is a direct target of Nrf2 still waits to be demonstrated.

Conflict of Interests

The authors state that no conflict of interests does exist.

Authors' Contribution

Salvatore Rizza and Claudia Cirotti equally contributed to this work.

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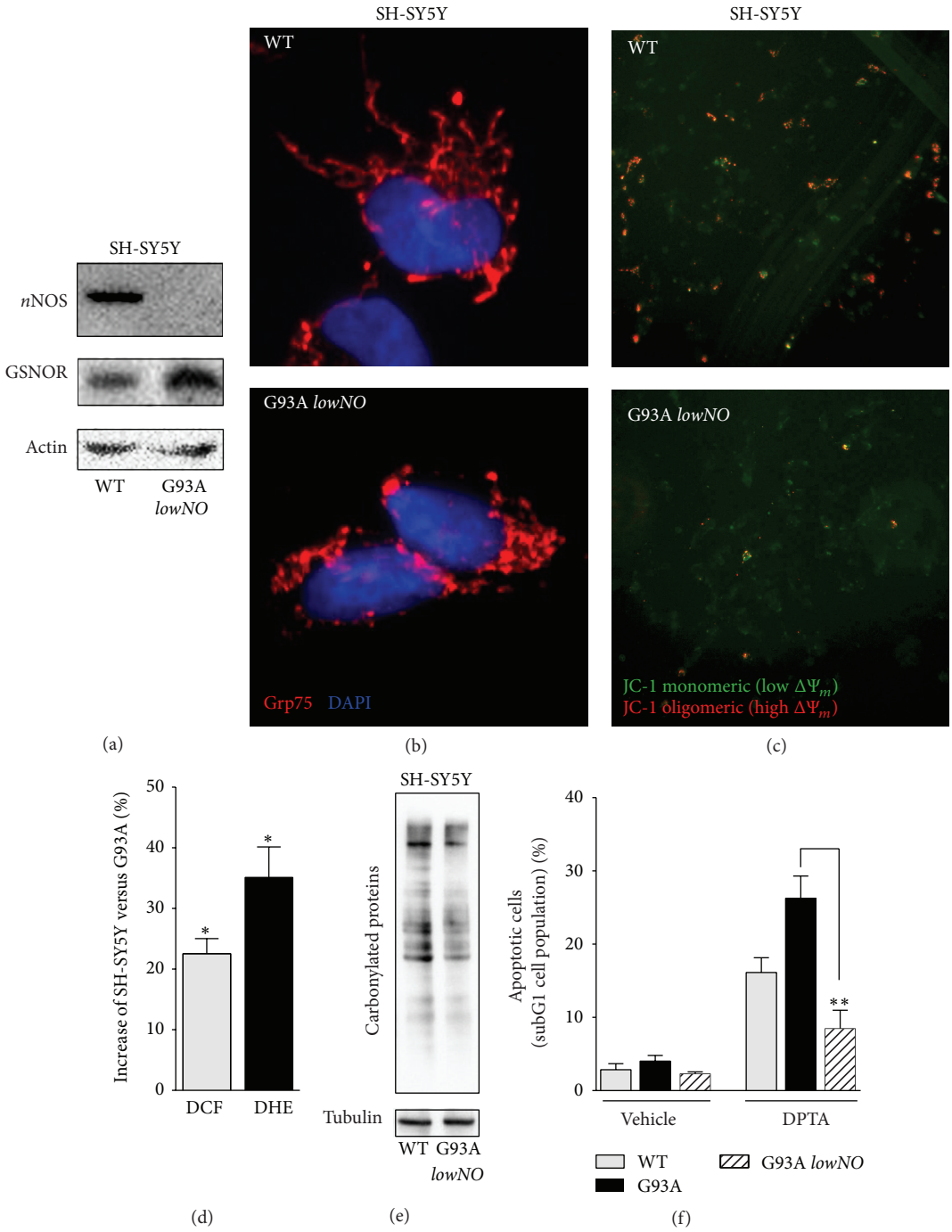

FIGURE 7: Characterization of the *lowNO* G93A resistant clone. (a) Western blotting of *n*NOS and GSNOR in total cell lysates of parental SH-SY5Y (WT) and the resistant *lowNO* G93A clone. (b) Fluorescence microscopy analyses of parental SH-SY5Y (WT) and the *lowNO* G93A clone. Anti-Grp75 (red) and Hoechst 33342 were used to visualize mitochondria and nuclei, respectively. (c) Fluorescence microscopy analyses of mitochondrial transmembrane potential ($\Delta\psi_m$) of WT and the *lowNO* G93A clone by JC-1. Red fluorescence, high $\Delta\psi_m$ and green fluorescence, low $\Delta\psi_m$. (d) Cytofluorimetric analyses of H_2O_2 and $O_2^{\bullet-}$ in SH-SY5Y (WT) and the *lowNO* G93A clone upon 2′,7′-dihydrodichlorofluorescein (DCF) or (c) dihydroethidine (DHE) staining. (e) Western blot analyses of protein carbonyls from total cell lysates obtained from WT and the *lowNO* G93A clone. (f) Cytofluorimetric analyses of apoptosis upon propidium iodide staining performed on parental SH-SY5Y cells (WT), sensitive G93A, and the *lowNO* G93A clone treated for 24 h with 400 μM DPTA. Western blots shown are representative of at least $n = 3$ independent experiments that gave similar results. Actin or tubulin was selected as loading controls. Graphs shown represent the mean of data ± SD of $n = 3$ independent experiments, $^*P < 0.05$ with respect to trigonelline untreated cells.

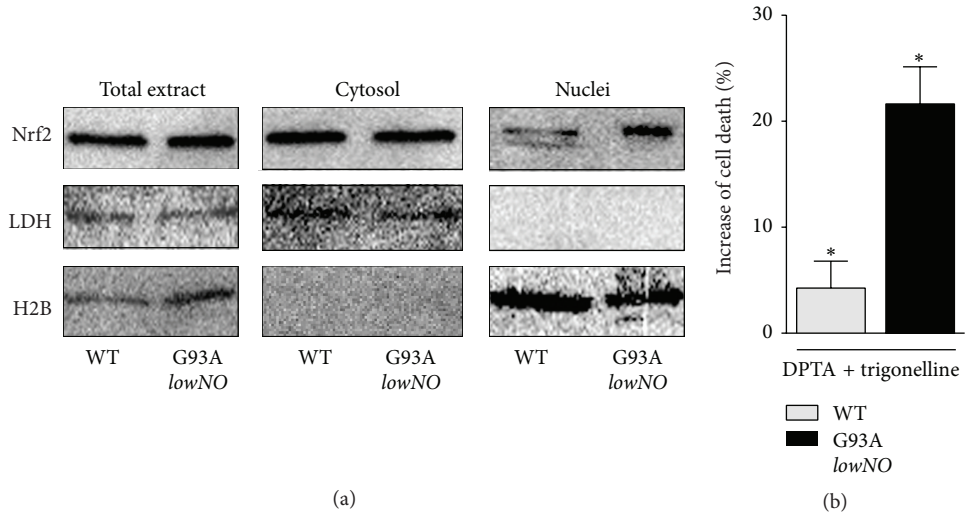

Figure 8: Role of Nrf2 in the *lowNO* G93A resistant clone. (a) Western blot analyses of Nrf2 in nuclear and cytosolic fractions obtained from parental SH-SY5Y (WT) and the *lowNO* G93A clone. (b) Cytofluorimetric analyses of apoptosis upon propidium iodide staining performed on parental SH-SY5Y cells (WT) and the *lowNO* G93A clone treated for 24 with DPTA in the presence or absence of the Nrf2 inhibitor trigonelline (0.5 μM). Western blots shown are representative of at least $n = 3$ independent experiments that gave similar results. Lactate dehydrogenase (LDH) and histone H2B were selected as loading and purity controls. Graphs shown represent the mean of data ± SD of $n = 3$ independent experiments.

mouse strains. This work has been supported by grants from the Italian Ministry of Health, Young Italian Researcher Grant (GR-2008-1138121), the Italian Association for Cancer Research, AIRC-MFAG 2011 (11452), and the Danish Cancer Society (KBVU R72-A4408 and KBVU R72-A4647). The authors are also grateful to the Bjarne Saxhof Foundation, Lundbeck Foundation (R167-2013-16100), and Novo Nordisk (7559) to have sustained this research.

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Review Article

Cancer Microenvironment and Endoplasmic Reticulum Stress Response

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Different stressful conditions such as hypoxia, nutrient deprivation, pH changes, or reduced vascularization, potentially able to act as growth-limiting factors for tumor cells, activate the unfolded protein response (UPR). UPR is therefore involved in tumor growth and adaptation to severe environments and is generally cytoprotective in cancer. The present review describes the molecular mechanisms underlying UPR and able to promote survival and proliferation in cancer. The critical role of UPR activation in tumor growth promotion is discussed in detail for a few paradigmatic tumors such as prostate cancer and melanoma.

1. Introduction

The cellular environment is constantly changing; thus physiological adaptive responses arise in order to maintain the overall cellular equilibrium and tissue homeostasis. Within such framework, numerous ways have evolved to allow optimal adaptation to environmental stress or, under extreme damage conditions, to remove diseased cells and to prevent toxicity [1].

The endoplasmic reticulum (ER) is the intracellular organelle controlling intracellular Ca^{2+} homeostasis, lipid synthesis, and protein folding. Protein folding occurring in the ER is extremely sensitive to environmental changes regarding redox state, nutrient and Ca^{2+} levels, protein synthesis rate, occurrence of pathogens or inflammatory stimuli, altering protein folding, and ultimately causing accumulation of unfolded or misfolded proteins. This condition is generally known as "ER stress" [2] and a sensitive surveillance mechanism ensures degradation of misfolded proteins [3] preventing entry of misfolded proteins in the secretory pathway. When ER stress occurs, ER functions are altered and a number of molecular actions, collectively named "*unfolded protein response*" (UPR), are activated to counteract the ER stress-associated damages. The UPR has a dual function: it mitigates damage associated with ER stress and, if this is not possible, it activates apoptosis [1]. ER stress response/UPR signaling pathways are activated in primary solid tumors as a result of cell-intrinsic defects, such as dysregulation of protein synthesis, folding, and secretion, and also as a consequence of microenvironment changes. Solid tumors microenvironment differs from normal tissues microenvironment, the former being characterized by nutrient (e.g., glucose) deprivation, low pH, hypoxia, and imbalance between production and removal of reactive oxygen species (i.e., oxidative stress) [4, 5].

All such environmental factors contribute to ER stress and cancer cells select effective ways to adapt and prevent ER stress-induced apoptosis [6, 7].

Recent studies have investigated in detail the different ways utilized by cancer cells, under ER stress conditions, to perturb ER-associated cell death signaling and to promote tumor growth [8, 9]. In the present review the known UPR pathways are summarized; then the different ER stressors acting in cancer microenvironment are reported and ultimately

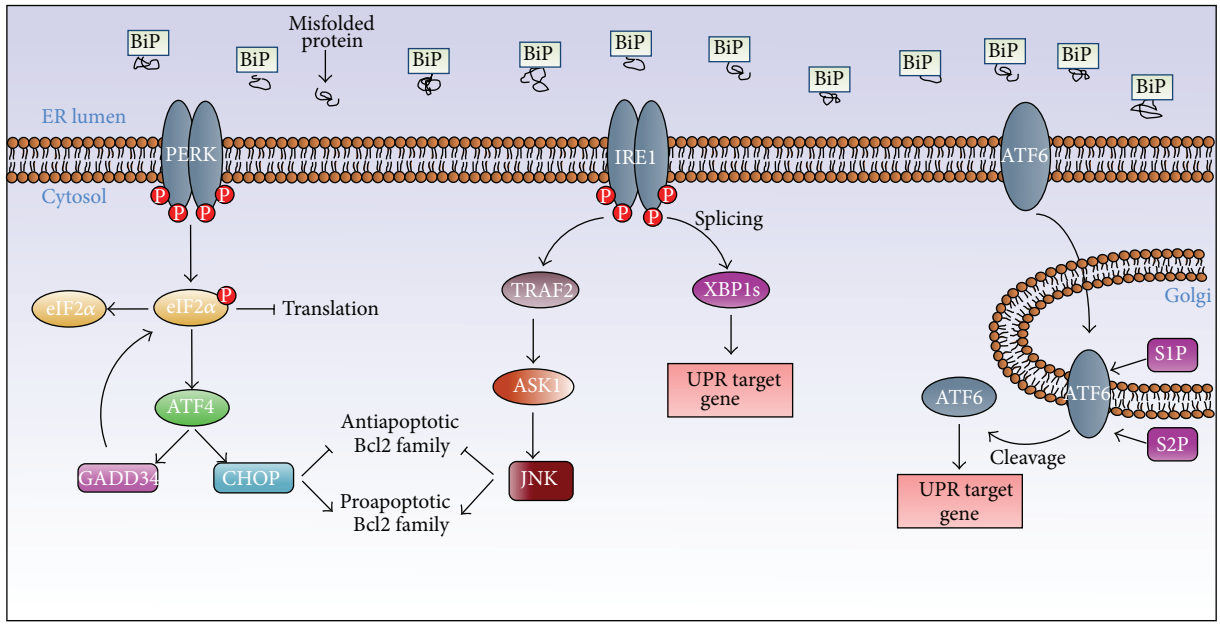

Figure 1: As a consequence of ER stress cells activate signal transduction pathways collectively known as unfolded protein response (UPR). The figure represents the three branches of the UPR and the corresponding UPR sensors (PERK, IRE1, and ATF6).

the altered ER stress responses in cancer are described, emphasizing their possible therapeutic implications.

2. ER Stress Response

Unfolded protein response (UPR) is a cellular response connecting the ER to the nucleus [10]. It represents a key cellular signaling process investigated since the early nineties in yeast [11, 12].

As shown in Figure 1, three ER-associated proteins are key players of UPR, namely, Pancreatic ER Kinase (*PERK*) [13], Inositol-Requiring Enzyme 1 (*IRE1*) [14], and Activating Transcription Factor 6 (*ATF6*) [15]. Under normal conditions, such three transmembrane proteins are bound and inactivated by a chaperone, Glucose Regulated Protein 78 (BiP, also known as GRP78) [16]. As response to ER stress, BiP dissociates from the UPR sensors to allow their proper signaling [17]. The activation of the ER stress sensors and of their downstream targets halts new proteins transcription and increases the synthesis of molecular chaperones. As a first consequence, the UPR promotes cell survival by enhancing ER ability to fold proteins and preventing further protein accumulation that might exacerbate the ER damage. If such response is not sufficient and the stress persists, the UPR leads to apoptosis [18]. Although the exact molecular mechanisms involved are not known, several evidences suggest that cell death induced by ER stress requires continuous signals exchange between ER and mitochondria [19]. This communication depends on the presence of a physical link between the two organelles, represented by specific contact sites between membranes of the ER and mitochondria, known as Mitochondrial Associated Membranes (MAMs) [20]. The integrity of this interaction is modulated by different proteins, as we recently demonstrated [21], and is essential to maintain cellular homeostasis and to modulate important processes such as apoptosis, ER stress, and autophagy [22].

The three ER stress sensors PERK, IRE1, and ATF6 are characterized by an amino-terminal domain important for the stress perception, maintained in an inactive state by interaction with the chaperone BiP under physiological conditions, and a carboxy-terminal domain that interacts with the transcriptional and translational apparatus [16].

PERK activation in response to ER stress leads to phosphorylation of the α-subunit of eukaryotic initiation factor 2 (eIF2α) which, in turn, blocks protein translation. This event promotes cell survival by preventing further ER damage from other nascent proteins [13]. Thus PERK activation initially leads toward a protective cell survival response; however, stress persistence induces the transcription of C/EBP homologous protein (CHOP), a transcription factor positively controlled by the transcription factor 4 (ATF4). Such event is critical to control the shift from survival to apoptosis. Phosphorylated eIF2α activates ATF4, which, in turn, acts on target proapoptotic genes such as growth arrest and DNA damage-inducible 34 (GADD34) and CHOP [23]. CHOP moves to the nucleus, upregulates its proapoptotic target genes, and facilitates the programmed cell death upon ER stress [24].

IRE1, activated in response to unfolded proteins accumulation, determines the splicing of a 26-nucleotide-long intron from the mRNA encoding the transcription factor X box-binding protein 1 (XBP1) [25]. The generated splicing variant, XBP1s, acts as a transcription factor that moves to the nucleus and causes the transcription of genes coding ER chaperones, in order to mitigate the stress [26]. IRE1 overexpression has been also shown to trigger apoptosis [27]. IRE1 has

been demonstrated to recruit the adapter molecule TNF-receptor-associated factor 2 (TRAF2); the complex TRAF2-IRE1 activates a proapoptotic signal by inducing Apoptosis Signal Regulated Kinase (ASK1), which, in turn, transmits the death signal to c-Jun N-terminal kinase (JNK). Once activated, JNK is responsible for the phosphorylation of Bcl2 thus abolishing its antiapoptotic activity [28]; moreover, it is able to determine the phosphorylation of the proapoptotic proteins BAX and BIM [29, 30], enhancing their proapoptotic effect. According to such complex mechanism, it can be concluded that when the stress persists, PERK and IRE1 signaling cascades can converge, mediating the induction of apoptosis. IRE1 RNase is also involved in a process called RIDD (RNA IRE1-Dependent Decay), consisting in cleavage of mRNAs encoding many different proteins and aimed at maintaining ER homeostasis. It has been found that RIDD activity increases as function of ER stress and correlates with apoptosis induction [31].

In response to ER stress, *ATF6* dissociates from the ER membrane and moves to the Golgi apparatus, where its cytoplasmic domain undergoes a proteolytic cleavage by serine proteases S1P and S2P, resulting in the formation of an active transcription factor [15]. Activated ATF6 goes to the nucleus and promotes the transcription of target genes encoding for different proteins such as BiP, GRP94, protein-disulfide isomerase (PDI), and XBP1 that enhance ER ability to fold accumulated proteins, contributing to restoring initial homeostasis.

3. ER Stress Pathways and Cancer

Cancer cells are known to be very resistant to extreme environmental stress and an increasing number of studies indicate that this may be largely due to an altered state of the UPR. The role ER stress and UPR play in cancer is still not completely clarified; however different components are known to be involved and may prove to be promising targets in future anticancer therapy [1].

Cancer cells adaptation to adverse conditions mostly relies on their ability to prevent ER stress-induced apoptosis and perturb the ER stress-associated signaling. A selective advantage occurs in premalignant cells harboring gene mutations able to suppress UPR-induced apoptosis or senescence [2].

Cancer cells have unique modifications enabling them to exploit ER stress responses to promote survival and growth. The ER protein chaperone BiP is commonly found to be highly expressed in breast cancer, lung cancer, prostate cancer, melanoma, and other malignancies [32]. The increased expression of BiP is functionally related to the prosurvival response of cancer cells to major environmental stress. This may occur through a molecular complex formation and inhibition of BIK, a proapoptotic protein [33]. BiP has been also shown to interact with and suppress the activation of caspase-7, preventing apoptosis [34]. Furthermore, BiP is positively regulated by the mitogen-activated protein kinase (MAPK) pathway. In melanoma cells, inhibiting such pathway decreases BiP expression leading to increased caspase-4 mediated ER stress induced apoptosis [35]. BiP is also responsible for cancer resistance to different anticancer therapies. Notably, BiP expression level in breast cancer may have a prognostic value [36]. BiP can therefore represent a molecular target; its inhibition may reduce its cytoprotective effects in combination with photodynamic therapy [37]. In gastric cancer cells treated with multidrug resistance cell-specific binding peptide, decreased BiP expression has been reported and this event prevents multidrug resistance [38].

Human lymphomas demonstrated significantly higher levels of UPR activation compared with normal tissues. In lymphoma models, c-Myc activates the PERK/eIF2α/ATF4 arm of the UPR, leading to increased cell survival via the induction of cytoprotective autophagy by PERK activation [39]. Accordingly PERK deletion inhibits mammary tumor development and reduces lung metastases [40]. PERK/eIF2α pathway largely contributes to the growth and survival of cancer under hypoxic stress [8]. In fact PERK is responsible for activation of many angiogenic genes [41]. Accordingly, PERK inhibition has been found to reduce tumor growth both *in vitro* and *in vivo* [42].

XBP1 increased expression and splicing have been found in hepatocellular carcinoma and breast cancer. It contributes to the adaptive response to ER stress and to survival under hypoxic conditions through positive regulation of BiP. In addition, XBP1 mutations have been described in tumor cells from patients with multiple myeloma [43–45]. XBP1 overexpression in myeloma cells has also been demonstrated and it seems to be critical for multiple myeloma induction. XBP1 therefore represents a regulator of plasma cell differentiation [46]. Interestingly, inhibition of XBP1 splicing has been shown to reduce multiple myeloma cells growth [47]. Furthermore IRE1α may induce XBP1 splicing thus inducing cellular proliferation through increased expression of cyclin A1, a cell cycle regulatory protein [48]. Accordingly IRE1α inhibition has been shown to sensitize multiple myeloma cells to ER stress and reduce their survival [49].

ATF6 is overexpressed in many human solid tumors and is involved in promoting proliferation and survival under nutrient deprivation conditions [50]. The active Ser245-phosphorylated ATF6 is overexpressed in non-small-cell lung cancer cells [51]. Remarkably ATF6 expression contributes to cancer formation by negatively regulating genes involved in cellular senescence [52]. It also mediates survival through upregulation of LC3B, a component of the autophagosomal membrane. In liver cancer ATF6 is also responsible for upregulation of XBP1 expression and the activity of both ATF6 and XBP1 increases BiP expression, leading to hepatocarcinogenesis [45].

Altogether these data explain why the inhibition of ER chaperones level or of one arm of the UPR components has been recently suggested as potential cancer therapies [53]. These approaches may inhibit UPR adaptive and prosurvival pathways leading to increased sensitivity to anticancer therapy.

Remarkably, persistent ER stress and UPR activation by pharmacological approaches can switch the cytoprotective functions of UPR into cell death programs. Therefore both repression of UPR-dependent survival signals [54] and

sustained UPR induction may have beneficial and therapeutic effects against cancer. Some antitumoral agents (e.g., cannabinoids) activate ER stress as the primary mechanism to promote cancer cell death [55, 56]. It is still not known if sustained ER stress and UPR activation can induce tumor cell death activating additional unknown cell death programs. Future work needs to be done to address this issue in the context of cancer therapy.

4. ER-Associated Degradation (ERAD)

ER-Associated Degradation (ERAD) represents an additional cellular adaptive pathway that contributes to restoring ER homeostasis by targeting unfolded/misfolded proteins toward proteasome-mediated degradation. By this pathway such proteins are translocated from ER into the cytosol where they are polyubiquitinated and degraded by the proteasome [57]. The transport into the cytosol involves Sec61 translocation channel as well as other factors identified in ERAD yeast mutants [58, 59]. Indeed the ERAD pathway is conserved from yeast to humans and deletion of ERAD-mediating factors leads to significant UPR induction [60], thus showing significant cross talk between such two pathways. Proteins not correctly folded are firstly selected by molecular chaperones. Then ERAD substrates are modified through ubiquitin binding via specific E3 ubiquitin ligases located near ER membrane. Such modification targets proteins to the proteasome located in the cytoplasm [61].

Among the numerous molecular players in mammalian ERAD pathways, the ER-membrane resident ubiquitin ligase Hrd1 forming a complex with SEL1L [62] plays an important role.

While the role of ERAD pathway in cancer is not fully elucidated, SEL1L has been shown to be involved in cancer pathogenesis. Remarkably, SEL1L overexpression inhibits cell proliferation, growth, motility, and invasion in pancreatic cancer cells. Furthermore, correlation between SEL1L protein levels and poor prognosis has been reported in breast carcinoma patients and other cancers [63]. However further studies are needed to clarify mechanisms underlying SEL1 control of tumorigenesis.

5. ER Stressors in Cancer Microenvironment

UPR activation in transformed cells is attributed to both intrinsic and extrinsic factors. Hyperactivation of oncogenes or loss-of-function mutations in tumor suppressor genes may increase protein synthesis and translocation into the ER in response to high metabolic demand and consequently UPR is activated. In addition, certain types of cancer cells are highly secretory and therefore prone to constitutive UPR activation. Defects in glycoprotein and lipid biosynthesis as a consequence of DNA mutations might also contribute to the induction of ER stress [2]. Mutations in oncogenes and tumor suppressor genes have been shown to inhibit ER stress induced apoptosis [39, 64]. In addition mutations in molecular components of the UPR pathways may also directly contribute to enhanced cancer cell survival upon stress. For example some IRE1α mutants, identified in human cancers, are unable to display proapoptotic RIDD function, thus showing increased cell survival [65]. Furthermore, enhanced activation of IRE1 may have a cytoprotective effect leading to cancer progression via XBP1 mRNA splicing [66]. In response to chronic stress, normal cells usually attenuate the IRE1α–XBP1 and ATF6α pathways, so that the apoptotic signals dominate. On the contrary, some cancer cells have constitutive activation of IRE1α–XBP1 thus inhibiting apoptosis [67, 68]. Although tumors secrete angiogenic factors to promote angiogenesis, this is often not sufficient to satisfy the elevated tumor metabolic requirements. In addition to hypoxia, cells in developing tumors are subject to glucose deprivation, lactic acidosis, oxidative stress, and decreased amino acid supplies [2]. All these changes in the microenvironment contribute to activating the UPR (see Figure 2).

5.1. Hypoxia. Tumor growth with defective microcirculation leads to hypoxia, which activates the UPR [69–72]. Since UPR increases cellular survival and proliferation, these events may produce a positive loop further promoting tumor growth and increasing hypoxia within the tumor. Therefore, hypoxia-mediated UPR activation appears to be essential for tumor cell survival. Although there is a general inhibition of translation under moderate-extreme hypoxia, some proteins are induced under low O_2 conditions including HIF-1 and its downstream targets [73]. Hypoxia induces Ser51 phosphorylation of the translation initiation factor eIF2α via PERK activation, and this is required to downregulate protein synthesis. Hypoxia tolerance is also dependent on the upregulation of ATF4, a downstream effector of eIF2α phosphorylation, both *in vitro* and *in vivo*. The UPR pathway mediated by activation of IRE1 and its downstream target XBP1 is also required to counteract hypoxic conditions leading to tumor growth. Human fibrosarcoma and lung carcinoma cells upregulate BiP level and XBP1 splicing under hypoxia, whereas human colon cancer cells upregulate PERK-dependent phosphorylation of eIF2α and ATF4 translation [74]. It has been demonstrated that XBP1-deficient tumor cell survival is reduced under hypoxic conditions *in vitro*, and these cells are unable to develop tumors *in vivo*. Conversely, spliced XBP1 expression restores tumor growth [8]. Another potential UPR trigger in hypoxic conditions is the ER oxidase 1α (ERO1α) enzyme that catalyses disulfide bond formation in an oxygen-dependent manner. ERO1α activity is reduced by low O_2 conditions, thus compromising correct protein folding and activating UPR [75]. All these hypoxic-modulated molecular responses are differently activated depending on oxygen level and hypoxia duration.

5.2. Oxidative Stress and Inflammatory Stress. ROS play a causal role in tumor development and progression by promoting genetic and epigenetic alterations and inducing protumorigenic signaling [76].

Under protein overload conditions, ROS are generated in the ER as a part of an oxidative folding process. ROS can target ER resident proteins, enzymes, and ER based calcium (Ca^{2+}) channels, leading to calcium release from the ER

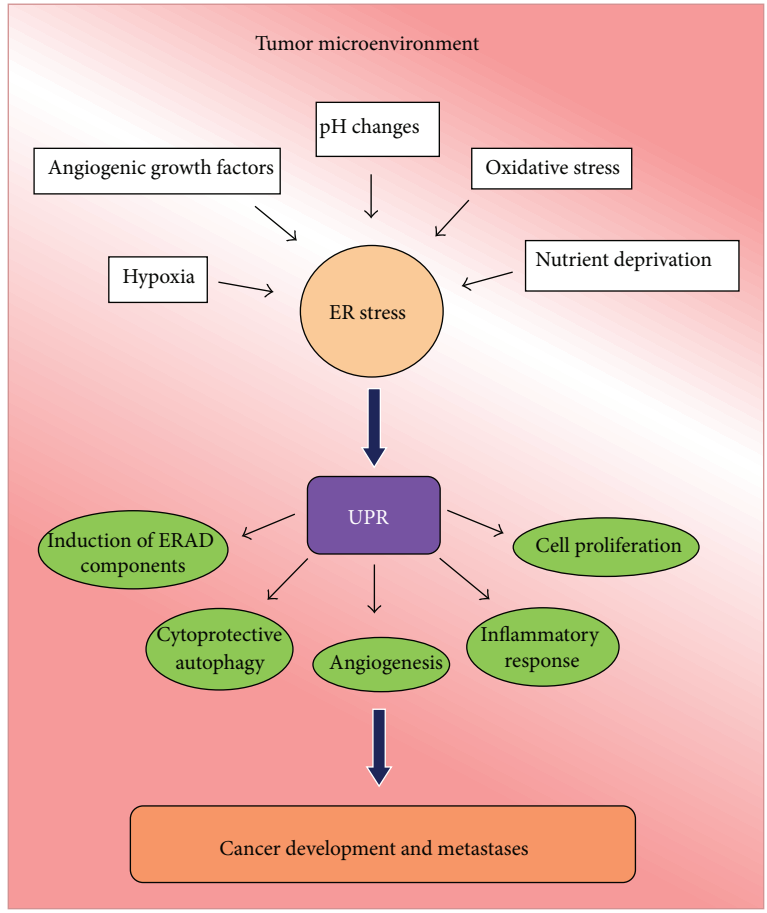

Figure 2: Tumor microenvironment factors activate ER stress and UPR responses leading to cancer development and metastases.

into the cytosol and ER stress signaling. Increased cytosolic calcium and calcium entry in mitochondria from ER via MAM-associated channels can stimulate mitochondria metabolism to further ROS production [77]. Increased ROS accumulation may also occur as a consequence of excessive nutrients thus inducing ER stress and activating the UPR [2]. As a consequence of ROS increase, PERK is activated. PERK activation may limit oxidative DNA damage through Nrf2 transcription factor induction, thus promoting cancer cell proliferation [40].

ROS signaling cooperates with UPR pathway leading to inflammatory responses [78]. Proinflammatory stimuli (e.g., TLR ligands and cytokines) trigger ER stress further amplifying inflammatory responses. The IRE1α–TRAF2 complex can recruit apoptosis signal-regulating kinase 1 (ASK1) and activate JUN N-terminal kinase (JNK), increasing the expression of proinflammatory genes through enhanced AP1 activity [79].

Interestingly, all three UPR branches activate NF-κB which is an important transcriptional regulator of proinflammatory pathways [80]. The PERK–eIF2α and ATF6α branches of the UPR activate NF-κB through different mechanisms. PERK and eIF2α signaling stops protein synthesis and increases the NF-κB/IκB ratio, reducing IκB half-life and leading to NF-κB nuclear translocation [81, 82]. ATF6α activates NF-κB via AKT phosphorylation [83, 84]. NF-κB can be also activated through binding to the IRE1α–TNF receptor-associated factor 2 (TRAF2) complex in response to endoplasmic reticulum (ER) stress, leading to recruitment of the IκB kinase (IKK), IκB phosphorylation and degradation, and nuclear translocation of NF-κB [85].

ER stress also induces transcription of proinflammatory cytokines in macrophages and promotes the type M2 macrophage phenotype that in turn supports tumor growth [86]. In addition ER stress, in combination with TLR agonists, by stimulating IL-23 in dendritic cells, may favour development of T helper 17 (TH17) and tumor growth [87, 88].

5.3. Nutrient Deprivation and Acidosis. Some other environmental factors indirectly induce ER stress and UPR activation. Amino acid deprivation activates eIF2 K4 to phosphorylate eIF2α. Low glucose availability affects protein glycosylation and ATP production leading to misfolded proteins accumulation within the ER [89]. Glucose shortage also leads to disturbed ER–Ca^{2+} homeostasis that is mediated by reduced sarcoplasmic/endoplasmic reticulum calcium ATPase (SERCA) activity. At low glucose concentration SERCA pump inhibition leads to PERK activation [90]. BiP is also upregulated at low glucose concentration. Interestingly BiP identification was originally made

in low glucose experiments [91]. Also XBP1 is involved in response to glucose deprivation. In particular in the XBP1s reporter mouse model, which develops spontaneous mammary tumors, XBP1 splicing was found to increase upon exposure to a nonmetabolizable glucose analog that simulates glucose deprivation [92]. Tumor cells adapt to low glucose levels by switching to a high rate of aerobic glycolysis, which is known as the Warburg effect [93, 94]. The consequent lactic acid production reduces the pH, and low pH is an important feature of the tumor microenvironment, promoting tumor survival and progression also *via* UPR by regulating several BCL-2 family members and CHOP [95].

5.4. Angiogenic Growth Factors. Growth factors synthesized and released within the tumor microenvironment may contribute to UPR activation. In cancer cells a direct link of UPR with growth factors is still to be investigated in detail; however different forms of PDGF (i.e., both PDGF-B and PDGF-A) are known to induce ER stress in nontumor models such as a vascular injury model [96], transgenic mouse crystalline lens models [97] and renal fibrosis [98]. Such data may suggest that the observed role of PDGF family members in melanoma and angiogenesis [99, 100] and in different tumors [101–103] may relate, at least to some extent, to ER stress inducing properties. As far as FGF family members are concerned, one recently published study [104] demonstrates that FGF-2 prevents ER stress induced cancer cell apoptosis in a Nck 1 (Src homology 2/3 domain-containing protein) mediated way. Further, Wang and colleagues recently demonstrated that glucose deprivation induces a PERK/ATF4-mediated UPR which leads to a proangiogenic action by stimulating the expression of a number of proangiogenic factors such as VEGF and FGF-2 and inhibiting the expression of antiangiogenic factors such as THBS1, CXCL14, and CXCL10 [105]. Moreover, VEGF has been shown to induce UPR in an ER stress independent manner, via PLCγ and mTORC1, indicating these players as constitutive parts of the VEGF signaling machine [106]. On the other hand, UPR has been shown to prevent inositol-requiring protein 1 (IRE1) α and ATF6-mediated VEGF degradation [107]. Finally, UPR, via IRE1α/XBP-1, PERK-ATF4, and ATF6α pathways, acts as an upstream regulator of VEGF transcription, directly affecting angiogenesis [108].

6. Recent Evidences on the Direct Role of UPR Regulation in Counteracting Prostate Cancer and Melanoma

Cancer cells generally display increased apoptosis resistance as compared to normal cells, thus bypassing ER stress-induced cell death [109]. Current strategies to counteract cancer growth aim at exacerbating ER stress thus stimulating prodeath UPR. Recent studies underlie the UPR targeting in different cancers such as prostate cancer [110] or melanoma [111]. While UPR role in tumors such as breast cancer [112] and lung cancer [113] has been reviewed in the last three years, recent reviews focused on "melanoma and UPR" or "prostate cancer and UPR" are lacking.

The key UPR relevance in response to cancer may be underlined by the observation that UPR activators may represent valuable novel therapeutic targets in a number of cancer conditions. For instance, SMIP004, a potent inhibitor of prostate and breast cancers growth, has been shown to achieve its proapoptotic effect by altering mitochondrial respiration and activating a MAPK-dependent proapoptotic effect downstream UPR [110]. In addition, death of prostate cancer cells such as PC3 and PNT1a has been observed upon treatment with a standardized green tea extract, acting via UPR activation, leading to cell cycle arrest at G2/M checkpoint in PC3 cells and at G0/G1 checkpoint in PNT1a cells [114]. Furthermore, subtilase cytotoxin catalytic subunit has been shown to sensitize prostate as well as lung cancer cells to photodynamic therapeutic treatments, mostly inducing cell death rather than apoptosis. This effect is related to subtilase targeting of GRP78, a major player in UPR regulation [37]. GRP78 appears to be a potentially relevant molecular target; in fact, also in melanoma, targeting GRP78 via subtilase has been shown to be an effective way to increase the proapoptotic effect of drugs such as fenretinide or bortezomib [115, 116]. Finally, combination of Pim kinase inhibitor and BCL2-antagonist has been shown to induce a strong *in vitro* and *in vivo* apoptosis in prostate cancer cells, mediated by Noxa protein activating UPR [117]. Interestingly, a Noxa-dependent proapoptotic effect has been also observed in melanoma cells, induced by another UPR activator named aurin [118].

7. Concluding Remarks

The UPR appears to adjust cancer microenvironment and represents a mechanism underlying resistance against cancer therapy [119]. Transformed cells may exploit UPR as a survival strategy to survive in a stressful microenvironment. While most studies demonstrate crucial roles for UPR signaling in tumor growth and chemoresistance, only recently UPR activation has been demonstrated to occur during oncogenic transformation and cancer development since UPR signaling molecules have been shown to interact with oncogenes and tumor suppressor genes. Further studies are necessary to understand in more detail the exact interaction of the involved signaling pathways [2]. Identification of such key players has the potential to select additional novel therapeutic approaches to improve the antitumor treatments. Further, selective inhibitors of the ER stress response may be revealed to be useful to counteract drug resistance [89, 120].

Recently several IRE1α inhibitors, namely, STF-083010, 3-ethoxy-5,6-dibromosalicylaldehyde, 2-hydroxy-1-naphthaldehyde, toyocamycin, and irestatin, have been found to induce apoptosis in pancreatic cancer cells [121] and in malignant myeloma cells [122]. Such IRE1α inhibitors have shown promising *in vitro* effects, in combination with other drugs [121]. IRE1α inhibitors clinical potential also comes from the observation that IRE1α inhibition sensitizes cancer cells to apoptosis induced by oncolytic virus therapy [123]. As an example, the novel therapeutic agent eeyarestatin I targets p97, an ATPase involved in the transport of ubiquitinated

proteins, and blocks ERAD pathway inducing cancer cell death [124].

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors' Contribution

Claudia Giampietri, Simonetta Petrungaro, and Silvia Conti equally contributed to this work.

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Review Article

Cellular Response upon Stress: p57 Contribution to the Final Outcome

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Progression through the cell cycle is one of the most important decisions during the life of a cell and several kinds of stress are able to influence this choice. p57 is a cyclin-dependent kinase inhibitor belonging to the CIP/KIP family and is a well-known regulator of the cell cycle during embryogenesis and tissue differentiation. p57 loss has been reported in a variety of cancers and great effort has been spent during the past years studying the mechanisms of p57 regulation and the effects of p57 reexpression on tumor growth. Recently, growing amount of evidence points out that p57 has a specific function in cell cycle regulation upon cellular stress that is only partially shared by the other CIP/KIP inhibitors p21 and p27. Furthermore, it is nowadays emerging that p57 plays a role in the induction of apoptosis and senescence after cellular stress independently of its cell cycle related functions. This review focuses on the contribution that p57 holds in regulating cell cycle arrest, apoptosis, and senescence after cellular stress with particular attention to the response of cancer cells.

1. Introduction

Cells can encounter different kinds of stress during their life and in turn have evolved a wide range of responses. Stress-activated signalling pathways such as ATM/ATR, JNK/SAPK, and p38 pathways are activated in mammalian cells by DNA damage, starvation, heat and osmotic shock, and oxidative stress. Depending on the kind, severity and duration of insult, and on the cell type, these responses can lead to different final outcomes, spanning from cell survival to cell death. Cell cycle delay or arrest is often the first safety step triggered in a stressed cell, followed by injury repair and thus restoration of cellular proliferation, or by the induction of cellular senescence or cell death.

Cellular senescence is defined as the irreversible exit from the cell cycle. In multicellular organisms there are three conditions in which cells stop dividing: quiescence, terminal differentiation, and senescence. Quiescence is reversible and it is usually induced by growth factor's withdrawal or contact inhibition, while in terminal differentiation and cellular senescence cell cycle arrest is permanent. During terminal differentiation cells acquire a distinctive phenotype and specialized functions in response to physiological stimuli. On the other hand, cells become senescent after exposure to peculiar types of stress [1]. Shortening of telomeres has been identified as the main stress inducing senescence in cultured cells *in vitro*, called for this reason replicative senescence. Genotoxic stress and more generally prolonged activation of the DNA damage response pathways results in the so-called premature senescence. Interestingly, cells usually arrest cell cycle in G1 phase during replicative senescence and in G2 phase during premature senescence. Senescent cells often display a flat, enlarged morphology and exhibit an increase in the lysosomal β-galactosidase activity that can be used as senescence biomarker (senescence-associated β-galactosidase activity or SA-β-gal activity). Many senescent

cells also display a characteristic senescence-associated secretory phenotype (SASP) (for a review on cellular senescence see [2]). Senescence is thought to be a major barrier to tumor formation, as it limits the replicative potential of cells and seems to activate the immune system. Indeed, it has been reported that senescence limits the growth of many tumors including epithelial tumors of the colon, head and neck, and thyroid [3–5]. On the other hand, recent studies show that senescence is involved in tumor regrowth and disease recurrence, as senescent tumor cells can serve as a reservoir of secreted factors with mitogenic, antiapoptotic, and angiogenic activities [6].

Regarding cell death, different types of programmed cell death, including autophagy, apoptosis, and necroptosis have been described so far. Starvation is a canonical cellular condition that starts autophagy, but also damaged organelles are recycled by autophagy [7]. DNA damage, instead, represents a common type of cellular stress inducing apoptosis [8]. On the other hand, cells can undergo necroptosis, or necrosis-like caspase-independent programmed cell death, in presence of cellular inhibitor of apoptosis proteins (cIAPs) and caspase inhibitors [9].

Apoptosis is the most common type of programmed cell death by which the body eliminates damaged or exceeding cells without local inflammation. Accordingly, apoptosis plays several physiological and pathological roles, spanning from tissue remodelling during embryogenesis to cancer progression. Two main molecular pathways have been described so far, the so-called extrinsic and intrinsic pathways. The extrinsic pathway is triggered by the activation of death receptors located on the cellular membrane and is usually involved in processes of tissue homeostasis such as the elimination of autoreactive lymphocytes, while the intrinsic pathway is mainly mediated by the release of cytochrome *c* from mitochondria, a well-known cellular response to stress [10]. Both pathways lead to the activation of caspases, aspartate-specific cysteine proteinases, which mediate the apoptotic effects among which the cleavage of proteins responsible for DNA repair and cell shrinkage. Notably, many chemotherapeutic drugs kill cancer cells inducing apoptosis upon DNA damage or sensitize cancer cells to apoptosis to overcome drug resistance. To this regard, much effort has been spent to study and possibly control apoptosis in malignancies and so it is of fundamental importance to understand the molecular pathways and cellular conditions that regulate and trigger apoptosis. It is now clear, indeed, that drug/stress-induced damage can initiate different postdamage responses, including apoptosis and cellular senescence, depending on the balance of pro- and antiapoptotic factors and on the levels of regulators of the cell cycle [11, 12].

p57 (cyclin-dependent kinase inhibitor 1C or KIP2) is considered a master regulator of the cell cycle during embryogenesis and tissue differentiation [13, 14], but recently a broad spectrum of evidence indicates that p57 plays a role, sometimes distinct from cell cycle control, also in the cellular response to different stresses, regulating the induction of apoptosis and senescence. This review summarizes those findings with particular attention to the role that p57 plays in the cellular response to stress of cancer cells.

2. p57 Functions and Regulation

p57 belongs to the CIP/KIP family of cyclin-dependent kinase (CDK) inhibitors (CKIs) along with p21 and p27. The CIP/KIP family counteracts cell cycle progression inhibiting all the cyclin CDK complexes throughout the cell cycle (for a review on CKIs see [15]). In particular, p57 inhibits the complexes formed with CDK2, CDK3, and CDK4 and to a lesser extent CDK1 and CDK6 [16–18]. Induction of p57 causes cell cycle arrest mostly in G1 phase [16], even if cell cycle arrest in G2 phase has also been reported after p57 reinduction in cancer cells [19]. In addition to an N-terminal CDK inhibitory domain, homologs to the ones of p21 and p27, and a C-terminal QT-box, significantly homologous with that of p27, human p57 has a central domain rich in proline-alanine repeats responsible for additional p57 interactions, suggesting that p57 can exert different and/or more complex functions than its siblings [13, 14]. Indeed, the p57 internal domain has been reported to interact with the N-terminal of LIM domain kinase 1 (LIMK-1), a kinase involved in the control of actin dynamics, supporting the idea that the p57 internal domain may be responsible for p57 functions other than CDK inhibition [20, 21]. All the three members of the CIP/KIP family are evolutionary conserved among vertebrates, with p57 more closely related to p27 than p21, as indicated by the structural homology and phylogenetic relationship [22, 23].

Due to its role in cell cycle control, p57 is involved in the regulation of many cellular processes such as embryogenesis and tissue differentiation. In muscle differentiation p57 participates in the regulation of cell cycle exit of differentiating myoblasts [24, 25]. During hematopoiesis decreased levels of p57 are essential for the exit from quiescence and reentry into the cell cycle of hematopoietic stem cells [26–28]. p57 is also involved in the differentiation of several other cell phenotypes, including podocytes [29], placental cells [30], keratinocytes [31], pancreatic cells [32], hepatocytes [33], T-lymphocytes [34], and spermatozoa [35]. Other than differentiation, p57 seems to be involved also in controlling tissue aging. Indeed, Park and Chung analyzed the levels of p57 in muscle and lung during mouse aging showing that these levels go toward a clear decrease [36]. The mechanism of such a decrease was not investigated, but the authors suggested that p57 decrease could be associated to tissue aging as p57 can contribute to muscle differentiation and regeneration.

p57 is considered a tumor suppressor gene due to its ability to inhibit proliferation and the importance of p57 in the suppression of cancer is highlighted by its mutation/inactivation in the Beckwith-Wiedemann Syndrome, a cancer predisposing syndrome [37]. Remarkably, p57 expression is reduced in some human malignancies including lung cancer, hepatocellular carcinoma, and bladder cancer, confirming its involvement during tumorigenesis [38–40].

A cytoplasmic localization for p57 has been described in non-small-cell lung carcinoma and esophageal squamous cell carcinoma [41, 42], suggesting that p57 can exert different functions in different cellular compartments. Indeed, it seems that p57 is important for cytoskeletal dynamics and cell motility. As previously cited, it has been reported that p57 is able to bind LIMK-1, an enzyme that promotes actin

filaments formation, and to sequester it into the nucleus [21]. Accordingly, the absence of p57 causes a delayed migration of neurons in the cortical plate during mouse development [43]. In contrast to these findings, Vlachos and Joseph confirmed in a human cervical adenocarcinoma (HeLa) cell line the interaction between p57 and LIMK-1, but they showed that this interaction does not result in the translocation of the kinase into the nucleus, but instead augments LIMK-1 activity, hence increasing actin-fiber formation [20]. Interestingly, also p21 seems to play different roles depending on cellular localization, as it can localize to the nucleus, where it regulates cell proliferation and differentiation, or in the cytoplasm, where it inhibits apoptosis [22, 44].

Considering that p57 is involved in many cellular processes, it is not surprising that it shows a complex regulation. The expression pattern of p57 has a highly specific spatial and temporal profile, reaching its peak and widespread distribution during embryogenesis and development, while in adults it is restricted to few tissues such as testis and muscle. The importance of p57 during embryogenesis emerges also from the analysis of the knockout mouse phenotype, as p57 null embryos present hyperplasia in several organs and cannot survive [14, 17]. The precise expression pattern of p57 is achieved by complex and multiple levels of transcriptional and posttranscriptional regulation (Figure 1). First of all, in both mouse and human, the p57 gene, *cdkn1c*, is located in the imprinted domain kcnq1/kcnq1ot1. Maternal and paternal allele of an imprinted gene display, despite the fact that they have identical sequences, different epigenetic modifications, such as DNA methylation within CpG islands, histones acetylation, and methylation. The imprinting of a cluster of genes is regulated by specific sequences acting in *cis*, known as imprinted control regions (ICR). In particular, *cdkn1c* is expressed only by the maternal allele, while the paternal allele is silent (Figures 1(a) and 1(c)) [14]. The imprinting regulation is achieved through the ICR KvDMR1, located 150 kbp downstream the p57 promoter. The repressive epigenetic status on the paternal allele is regulated by the long noncoding RNA kcnq1ot1, which is expressed only in the paternal allele from the ICR KvDMR1. Kcnq1ot1 is able to recruit the DNA methyl-transferase 1 and the histone methyl-transferases EZH2 and G9a on the promoters of imprinted genes, leading to the silencing of the paternal allele [45].

In addition to imprinting control, *cdkn1c* promoter harbors the binding sites for many transcription factors that regulate its expression in a cell type dependent manner (Figure 1(c)). For example, Sp1 and p73β are able to bind and induce *cdkn1c* promoter [46, 47], while Hes1, a Notch effector, suppresses the expression of p57 [48].

During muscle differentiation, another mechanism of transcriptional regulation has been described that involves p57 promoter long-range direct and functional association with the ICR KvDMR1 (Figure 1(b)). This association leads to the formation of a repressive intrachromosomal loop mediated by the insulator factor CTCF; this loop is destroyed during muscle differentiation by the binding of MyoD to the ICR KvDMR1 [49, 50].

Moreover, a series of microRNA (miR) have been reported to downregulate the expression of *cdkn1c* (Figure 1(d)). For example, miR-221 and miR-222 have been found overexpressed in many cancer types where they lead to p57 downregulation [51, 52].

Finally, p57 protein stability is regulated by both phosphorylation and ubiquitination (Figure 1(e)). In particular, p57 phosphorylation by different kinases leads to its ubiquitination and 26S proteasome-mediated degradation. CDK2-cyclin E complex phosphorylates p57 at Thr310 of the QT-box domain [53]; Akt, a kinase often deregulated in cancer, phosphorylates p57 at Thr310 or Ser282 [54]; while CHK1 (checkpoint kinase-1) phosphorylates p57 at Ser19 [55].

3. p57 at the Crossroad between Cell Cycle Arrest, Apoptosis, and Cellular Senescence

3.1. p57 Contribution to Cell Cycle Control upon Cellular Stress. All the three members of the CIP/KIP family play an important role in controlling cell cycle exit. In particular, p57 is a master regulator of the cell cycle during embryogenesis and tissue differentiation [13, 14], but nowadays it is emerging that p57 has a specific function in cell cycle regulation upon cellular stress that is only partially shared by the other CIP/KIP inhibitors. Interestingly, this new role of p57 in controlling the cell cycle upon cellular stress has been reported to be both CDK inhibition-dependent and CDK inhibition-independent (Figure 2).

A CDK inhibition-dependent mechanism has been described for the stress-activated protein kinase (SAPK) p38 signalling, which is activated in mammalian cells by several insults, such as osmostress, oxidative stress, ionomycin, and UV [56–58]. It has been shown that SAPK p38 is able to phosphorylate p57 at Thr143 and this modification increases p57 affinity towards CDK2 resulting in cell cycle arrest at G1 in response to stress [59]. The increased activity of p57 is able to confer great resistance to different stimuli as cells lacking p38 or p57 display reduced viability to the previously cited stresses. Interestingly, phosphorylation of p57 by p38 neither affects its stability nor its localization, highlighting a novel mechanism of action of p57 after stress different from that observed during cellular differentiation that instead involves p57 induction/degradation. Notably, while hematopoietic stem cells that lack p57 present elevated levels of p27, suggesting that maintenance of cell quiescence is a common feature of p57 and p27, embryonic fibroblast knockout for p57 subject to stress are not able to increase p27, confirming that response to stress is a peculiar role of p57 [59].

On the other hand, p57 participates in the c-Jun NH_2-terminal kinase/stress-activated protein kinase (JNK/SAPK) pathway with a CDK inhibition-independent mechanism. Indeed, p57 negatively regulates the JNK/SAPK signalling cascade through direct inhibition of JNK/SAPK, independently of its well-known inhibitory function on CDKs [60]. Deletion mutant experiments showed that p57 inhibits JNK and CDK2 by distinct mechanisms. In particular, p57 interacts with JNK1 through its QT-BOX domain and this interaction is able to preclude the interaction between JNK1 and c-Jun [60], while the inhibition of CDK2 is achieved through the CDK inhibitory domain. JNK/SAPK activation

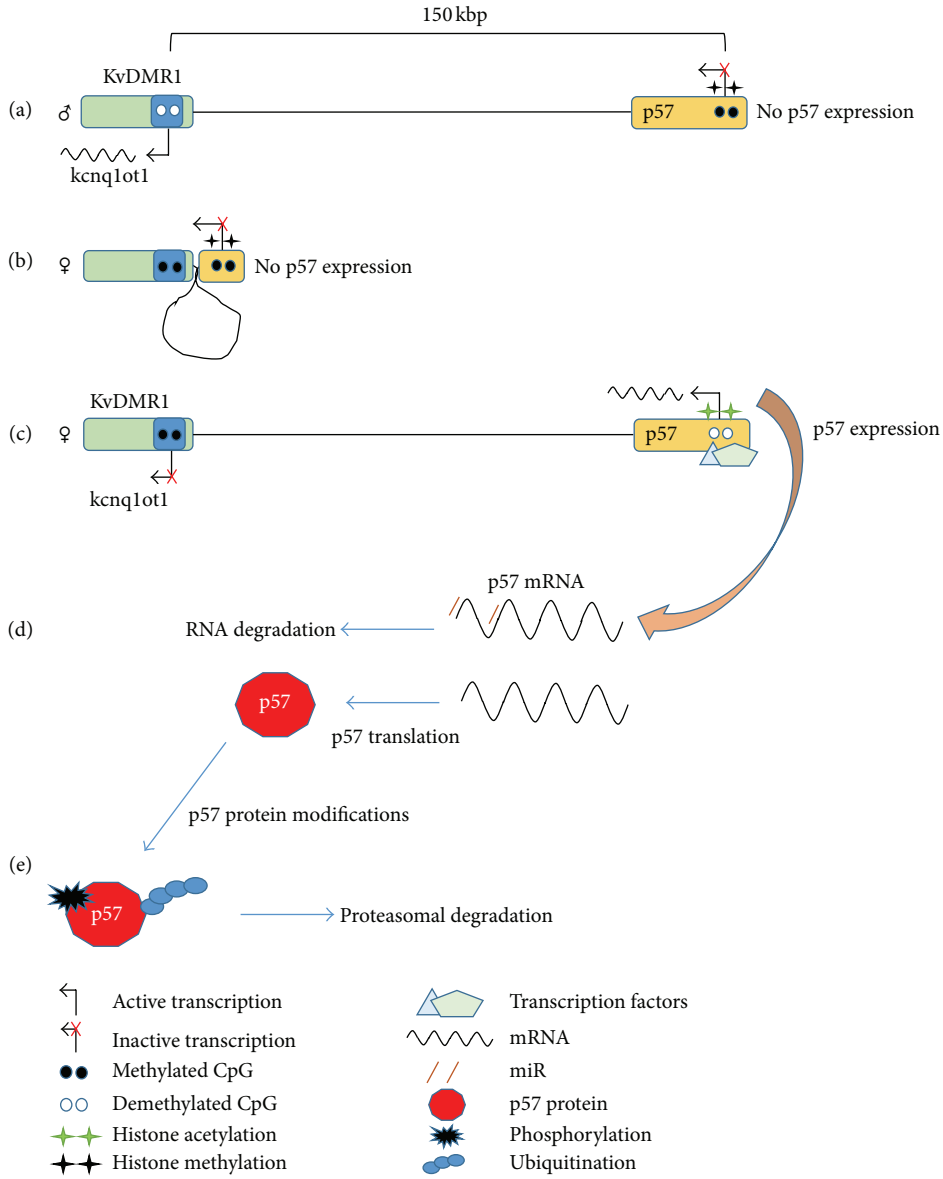

Figure 1: Different mechanisms of transcriptional and posttranscriptional p57 regulation. (a) Imprinting control on the paternal allele; (b) long-range intrachromosomal interactions between p57 promoter and KvDMR1 can repress the transcriptional expression; (c) promoter demethylation, active histone marks, and transcriptional factors binding regulate p57 expression; (d) p57 mRNA stability is regulated by miR; (e) p57 protein stability is regulated by phosphorylation and ubiquitination.

is implicated in the regulation of different cellular activities ranging from cell growth to cell death [61, 62]. Previous studies using p57 knockout mice reported an increase in apoptosis and altered differentiation during mouse development [63, 64]. It is plausible, therefore, to conclude that JNK/SAPK inhibition could be an important mechanism by which p57 exerts its functions not only on proliferation but also on cell death. Notably, this antiapoptotic role of p57 mediated by JNK/SAPK inhibition is in sharp contrast to the proapoptotic effects of p57 overexpression in cancer cells highlighted in the following section of this review.

3.2. p57 and Apoptosis. p21 and p27, the other members of the CIP/KIP family, have been reported to play a role in apoptosis [65, 66] and it is now emerging that p57 is implicated too (Figure 2). Samuelsson and colleagues reported that p57 expression enhances apoptosis in HeLa cells treated with staurosporine, a protein kinase C inhibitor, and showed that p57 itself is a target of caspase activity [67]. In contrast to p27, which has a proapoptotic activity by itself [68], p57 was found to have only a minor proapoptotic effect on its own, but rather to sensitize cells to apoptosis. In agreement, a concomitant expression of the antiapoptotic factors Bcl-x_L or Bcl-2 was found to counteract p57 proapoptotic effect. The molecular mechanism by which p57 promotes cell death was investigated by Vlachos and colleagues [69]. p57 overexpression is able to sensitize cancer cells to apoptotic agents such as cisplatin, etoposide, and staurosporine via a

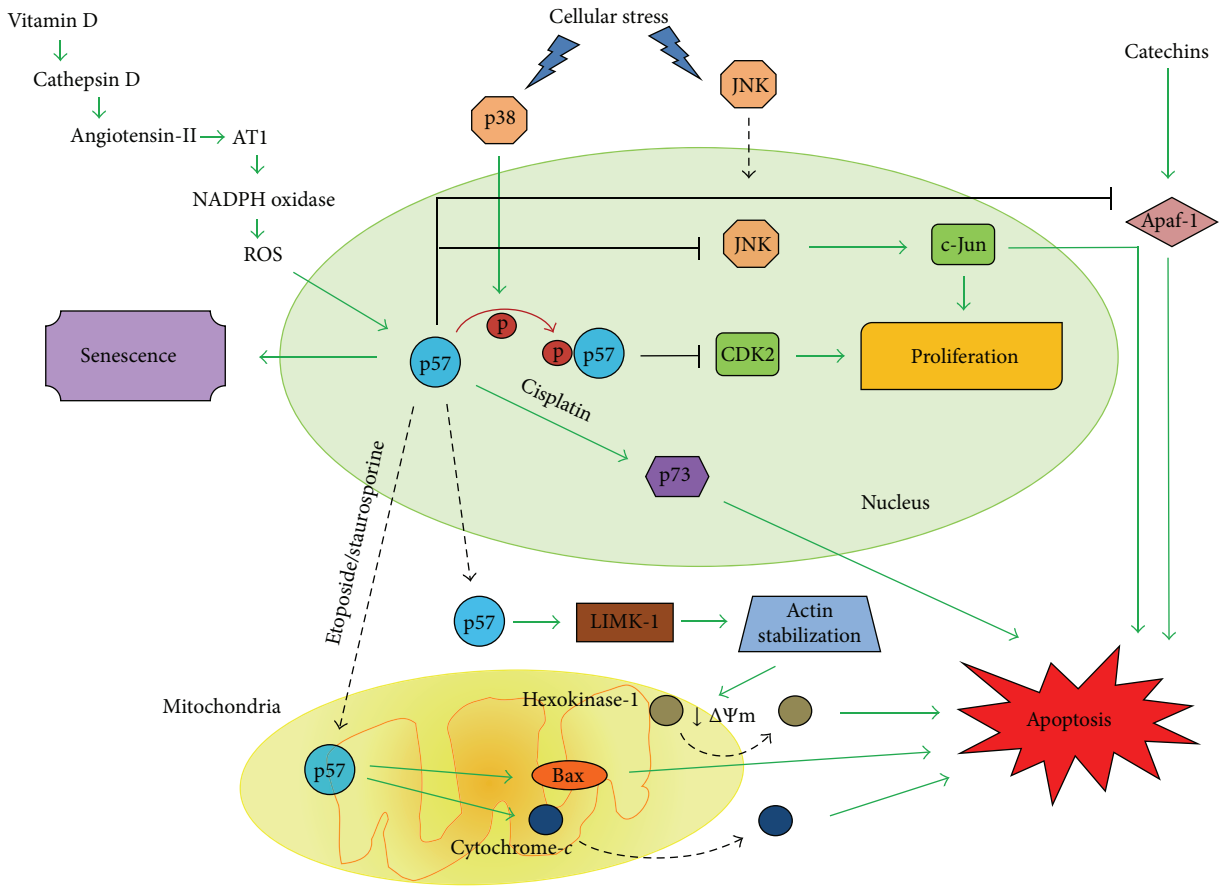

Figure 2: Network of p57 pathways involved in the cellular response to stress. Schematic view of the different mechanisms by which p57 can modulate proliferation, apoptosis, and senescence. Green arrows indicate positive regulation; black T-bar arrows indicate negative regulation; black dotted arrows indicate translocation; red arrow indicates phosphorylation; black facing-down arrow indicates loss of mitochondrial transmembrane potential ($\Delta\Psi m$).

mechanism that is independent of p57 ability to inhibit CDK activity in the nucleus. In particular, p57, within minutes from drug treatment, translocates into mitochondria promoting Bax activation and loss of mitochondrial transmembrane potential, thus triggering the intrinsic apoptotic pathway through the cytochrome *c* release into cytosol and consequent caspase-9 and caspase-3 activation. Mitochondrial pathway specificity was confirmed, as p57 expression was ineffective in promoting death receptor-mediated apoptosis stimulated with agonistic anti-FAS antibodies. The mechanism by which p57 triggers the mitochondrial intrinsic pathway has been linked to the ability of p57 to stabilize the actin cytoskeleton by Kavanagh and colleagues [70]. They demonstrated that p57 directly interacts with LIM domain kinase-1 (LIMK-1) resulting in an increase in LIMK-1 kinase activity, which is required for both p57-mediated actin cytoskeleton stabilization and p57 death promoting effect. Indeed, LIMK-1 is able to inactivate the cytoskeleton remodelling factor cofilin that is involved in the disassembling of actin filaments and it has already been supposed that stabilization of the actin cytoskeleton can promote apoptotic cell death [71]. Furthermore, p57-mediated stabilization of actin leads to the displacement of hexokinase-1, an inhibitor of the mitochondrial voltage-dependent anion channel, from mitochondria, providing a possible mechanism for mitochondrial depolarization and therefore for the promotion of the mitochondrial apoptotic cell death pathway.

p57 proapoptotic effects were observed in different cell lines, including HeLa cervical cancer cells, SH-SY5Y neuroblastoma cells, SKOV3 ovarian carcinoma cells, and mouse embryonic fibroblast cells [69]. In H1299 lung cancer cells and HCT116 colorectal carcinoma cells, silencing of p57 was shown to suppress p73β-mediated apoptosis induced by cisplatin treatment [72]. However, the death promoting effect of p57 was not seen in HEK-293 human embryonic kidney cells, suggesting some degree of cell type specificity, likely due to the balance between survival and death pathways that are active in a particular cell type.

Kuang and colleagues performed an interesting study on different leukemia cell lines that differ for p57 promoter methylation status [19]. They analyzed the expression of p57 after different stimuli such as transforming growth factor-β, lipopolysaccharide, tumor necrosis factor-α, insulin-like growth factor 1, and different forms of cellular stress such as

high-density culture or serum withdrawal. They found p57 reactivation only in cell lines with unmethylated promoter but not in methylated cells, leading to different outcome ranging from no effect on cell growth to G2 arrest and apoptosis, depending on cell type and type of insult. Remarkably, exogenous overexpression of p57 in p57 promoter-methylated leukemic cell lines resulted in marked cell growth arrest and induction of apoptosis, while the overexpression of p57 in partially methylated cells only resulted in a moderate inhibition of cell growth and had no impact on apoptosis, suggesting that the epigenetic status of p57 promoter can influence the cell response to stress stimuli.

It is worthy of notice that the proapoptotic effects of p57 described so far have been mainly reported in cancer cells after p57 overexpression or reinduction, while antiapoptotic activity of p57 has been observed in its physiological regulation of JNK pathway (see previous section) and during embryogenesis, suggesting that cellular context drives a major contribution to the final outcome. For example, antiapoptotic activity of p57 was reported in response to green tea polyphenols administration. Catechins are known to induce cell death in many types of tumor cells, but normal human epithelial cells were found to survive in the presence of polyphenols because of their ability to induce p57 expression. p57 was able to prevent green tea polyphenols-induced Apaf-1 expression thus avoiding apoptosis [73]. Finally, p57 has been suggested to have an antiapoptotic role in the gastrointestinal tract and the lens of the eye during embryonic development [63, 74].

3.3. p57 and Cellular Senescence. In two human hepatocarcinoma cell lines (HepG2 and SNU398) p57 overexpression has been found to affect proliferation and morphology without affecting the apoptotic machinery. In these cells, p57 expression is regulated by neither miR-221 nor the methylation status of the promoter but instead by the Notch target gene *Hes1*. p57 infected cells or Notch1- and Notch3-silenced cells, which upregulates p57, arrest growth with a senescent morphology, SA-β-gal staining, and p16 expression [75].

In a similar manner, Tsugu and colleagues have shown that p57 induction in p57-negative human astrocytoma cell lines (U343, U87, and U373) can block the proliferation and alter the morphology, with cells becoming large and flat with an expanded cytoplasm [76]. These flat cells resemble the senescent phenotype even if the SA-β-gal activity was reported to be partially reversible withdrawing p57 forced expression. Although senescent cells are thought to be resistant to apoptotic cell death, in one of the astrocytoma cell lines induced to express p57 (U373), a small subset of cells (15% of the population) was described to undergo apoptosis. Notably, Bax levels were unchanged. Why this occurs and why this particular cell line responds in a different manner to p57 induction is an interesting yet unanswered question.

A pivotal role of p57 in the premature senescence of vascular smooth muscle cells has been shown by Valcheva and colleagues in vitamin D receptor knockout mice [77]. Interestingly, they show a direct link between oxidative stress, p57 induction, and the onset of the senescent phenotype (Figure 2). Indeed, lack of vitamin D signalling results in increased cathepsin D enzymatic activity, which in turn augments angiotensin-II production. The binding of angiotensin-II to its receptor AT1 increases NADPH oxidase activity and free radical production. The latter induces high levels of p57 that trigger the premature senescence of vascular smooth muscle cells. Senescent vascular smooth muscle cells have been found in atherosclerotic plaques [78] and recent results suggest that vascular smooth muscle cell senescence could even promote atherosclerosis [79], tempting to speculate that p57 could become a therapeutic target. More studies are needed to deepen the interesting correlation between ROS production and p57 increase.

4. Concluding Remarks

Initially identified as a cyclin-dependent kinase inhibitor, p57 has since been shown to have different cellular roles aside from cell cycle inhibition, such as cell migration and regulation of cell differentiation. Nowadays it is emerging that p57 plays a key role also in coordinating the cellular response to stress, being able to drive to both apoptosis and cellular senescence. Different mechanisms of action, both CDK inhibition-dependent and CDK inhibition-independent, have been disclosed and, as highlighted in this review, p57 is now implicated in the crosstalk between several different pathways, among which MAPK signalling, DNA damage response, mitochondrial apoptotic pathway, and cytoskeleton organization. The findings that p57 can induce cell cycle arrest, apoptosis, or cellular senescence depending on cell types and cellular context arise several questions:

(i) Is the final outcome dependent on p57 levels?

(ii) As many data come from *in vitro* studies and overexpression of any gene can lead to experimental artefacts, which is the physiological relevance of p57 induction *in vivo*?

(iii) Which is the grade of overlapping between the three members of the CIP/KIP family?

(iv) Bearing in mind that stopping abnormal proliferation is a key goal of our scientific community, is the reinduction of p57 a promising approach for cancer therapy?

(v) Do cancer cells respond in a different way from normal cells to p57 overexpression?

p57 is now emerging as a new master regulator of cell fate and the mechanisms through which p57 participates in the cellular response to stress have been just started to be dissected.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Review Article

Hypoxia Inducible Factor Pathway and Physiological Adaptation: A Cell Survival Pathway?

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Oxygen homeostasis reflects the constant body requirement to generate energy. Hypoxia (0.1–1% O_2), physioxia or physoxia (~1–13%), and normoxia (~20%) are terms used to define oxygen concentration in the cellular environment. A decrease in oxygen (hypoxia) or excess oxygen (hyperoxia) could be deleterious for cellular adaptation and survival. Hypoxia can occur under both physiological (e.g., exercise, embryonic development, underwater diving, or high altitude) and pathological conditions (e.g., inflammation, solid tumor formation, lung disease, or myocardial infarction). Hypoxia plays a key role in the pathophysiology of heart disease, cancers, stroke, and other causes of mortality. Hypoxia inducible factor(s) (HIFs) are key oxygen sensors that mediate the ability of the cell to cope with decreased oxygen tension. These transcription factors regulate cellular adaptation to hypoxia and protect cells by responding acutely and inducing production of endogenous metabolites and proteins to promptly regulate metabolic pathways. Here, we review the role of the HIF pathway as a metabolic adaptation pathway and how this pathway plays a role in cell survival. We emphasize the roles of the HIF pathway in physiological adaptation, cell death, pH regulation, and adaptation during exercise.

1. Introduction

Hypoxia inducible factor (HIF) senses and coordinates cellular responses to hypoxia. HIF is a heterodimer consisting of one of three alpha (α) subunits and a beta (β) subunit. HIF-β is constitutively expressed, whereas HIF-α is induced by hypoxia. HIF-1α is the most well-established member of the HIF family; the other two members of the basic–helix–loop–helix-PAS (bHLH-PAS) superfamily are HIF-2α (endothelial PAS domain protein 1 or HIF1α-like-factor), which also stabilizes hypoxia and binds to the aryl hydrocarbon receptor nuclear translocator (ARNT) [1, 2] and HIF-3α [3]. HIF-1α is a transcriptional activator and a master regulator for the expression of genes involved in the response to hypoxia in most mammalian cells [4]. HIF-1α is prolyl hydroxylated at P402 and P564 in its oxygen-dependent degradation domain (ODD) under normoxic conditions. This leads to binding of the HIF-α unit to the E3 ubiquitin ligase von Hippel-Lindau protein (pVHL) at L574 for degradation [5, 6]. Homologous proline and leucine residues in HIF-2α also play similar roles [7]. HIF-1/2α transactivation activity is further inhibited by asparaginyl hydroxylation under normoxic conditions [8]. Low oxygen tension stabilizes the α-subunit and leads to nuclear translocation, formation of a dimer with HIF-1β, and recruitment of transcriptional coactivators [9]. This complex binds to an enhancer domain of the hypoxia responsive element (HRE) located either at the 5′ or 3′ region of target genes, including heme oxygenase-1, vascular endothelial growth factor (VEGF), glucose transporter (GLUT)-1, and GLUT-4 [10]. Interestingly, HIF-1α also upregulates glycolytic enzymes and glucose transporters, allowing cells to depend more heavily on glycolysis for energy [11], suggesting that HIF-1α also modulates aerobic metabolism. The vast majority of HIF target genes are regulated by HIF-1α, whereas exclusively HIF-2α-dependent genes are scarce and cell type-dependent [12–15]. Most reports show that HIF-1α and HIF-2α are master regulators of the transcriptional response to hypoxia, but the role of HIF-3α under hypoxic conditions is far less clear. HIF-3α is the newest member of the family and may be more restricted than the other

HIF subunits [3]. HIF-3α mRNA may also be activated by hypoxia [16]. Evidence suggests that HIF-3α may be functionally distinct. HIF-3α sequence has relatively low identity with HIF-1α/2α [3]. HIF-1α and HIF-2α have two terminal transactivation domain (TADs) [1, 10], whereas HIF-3α has only one TAD. HIF-3α has a unique leucine zipper domain and an LXXLL motif [3], and these unique structural features are evolutionarily conserved [17]. HIF-3α has multiple splice variants, and the inhibitory PAS domain protein is the most studied, which is a truncated protein and a dominant-negative inhibitor of HIF-1α [18]. HIF-1α and HIF-2α have 48% amino acid sequence identity and similar protein structures, but are nonredundant and have distinct target genes and mechanisms of regulation. Interestingly, HIF-1α appears the most active isoform during short periods (2–24 h) of intense hypoxia or anoxia (<0.1% O_2) in some cell lines, whereas HIF-2α is active under mild or physiological hypoxia (<5% O_2), and continues to be active even after 48–72 h of hypoxia [19]. Thus, in some contexts, HIF-1α plays key role in initial response to hypoxia whereas HIF-2α drives the hypoxic response during chronic hypoxic exposure [19, 20]. This HIF "switch" results between HIF-1α and HIF-2α suggests physiological and pathological adaptation required to adapt for cell survival. Interestingly, these isoform plays opposite but balancing roles during the hypoxic response under both physiological and pathophysiological conditions in some cells.

2. Prolyl Hydroxylases: Oxygen Sensors

Prolyl hydroxylases (PHDs) [1, 2 and 3] are evolutionary conserved oxygen sensors in metazoans. These dioxygenases were discovered after confirming that oxygen-dependent enzymatic activity covalently modifies an HIF-1α domain known as the ODD domain [21–23]. PHDs require 2-oxoglutarate as a cosubstrate, molecular oxygen, and iron liganded by two histidine and one aspartic acid residues to function as hydroxylases. PHDs play a key role in the HIF-mediated hypoxia signaling pathway, which facilitates cell survival and adaptation in response to capricious environmental oxygen levels [24]. Selective knockdown of PHDs enhances HIF-dependent gene expression *in vitro* [25]. PHDs lose their activity under hypoxic conditions, leading to accumulation and nuclear translocation of HIF-α and activation of HIF target genes by binding to HREs [26]. The first identified function of PHDs was to hydroxylate human HIF-1α h-subunits at Pro402 and Pro564 under normoxic conditions, resulting in their recognition, pVHL ubiquitylation, and degradation by 26S proteasomes. The PHD catalytic domain recognizes a specific LXXLAP motif in the ODD of the HIF-α subunits [27]. PHD1–3 have near ubiquitous tissue expression; PHD-2 is generally the most abundant isoform, with the exception of the testis, where PHD-1 is the most highly expressed isoform, and the heart, where PHD-3 expression predominates [28]. Loss of PHD-1 lowers skeletal muscle and liver oxygen consumption by reprogramming glucose metabolism from primarily oxidative to more anaerobic ATP production, suggesting selective loss of PHD-1-induced hypoxia tolerance [29, 30]. Knockdown of the Phd-2 gene, but not those of Phd-1 and Phd-3, results in embryonic death due to placental and heart defects, suggesting that PHD-2 is essential during mouse embryogenesis [31]. Interestingly, broad spectrum conditional Phd-2 knockout in adult mice leads to hyperactive angiogenesis, angiectasia, and congestive heart failure, suggesting that PHD-2 is a major negative regulator of vascular growth [32, 33]. Furthermore, PHD-3 is required for proper anatomical and physiological integrity of the system, as loss of PHD-3 results in abnormal sympathoadrenal development and systemic hypotension [34]. Targeted deletion of the PHD-3 gene increases angiogenesis and preserves cardiac function by stabilizing HIF-1α after infarction, suggesting a potential target for pharmacological management of ischemic myocardial disease [35].

3. Hypoxia and Reactive Oxygen Species

Oxygen scarcity leads mitochondria to produce reactive oxygen species (ROS) thereby giving alert to cells to the shortage. ROS describe a range of molecules and free radicals (chemical species with one unpaired electron) derived from molecular oxygen. The mitochondrial electron transport chain contains several redox centers that may leak electrons to oxygen, constituting the primary source of superoxide (precursor of most ROS) in most tissues. Superoxide anion is produced both enzymatically and nonenzymatically *in vivo*. Enzymatic sources include NADPH oxidases [36, 37] as well as cytochrome P450-dependent oxygenases [38]. Proteolytic conversion of xanthine dehydrogenase to xanthine oxidase provides another enzymatic source of both superoxide and H_2O_2 (and therefore constitutes a source of $OH^{\bullet}$) and has been proposed to mediate deleterious processes *in vivo* [39]. Nonenzymatic production of superoxide occurs when a single electron is directly transferred to oxygen by reduced coenzymes or prosthetic groups or by xenobiotics previously reduced by enzymes. According to the equation

$$\frac{d\left[\text{superoxide}\right]}{dt} = k\left[O_2\right]\left[R^{\bullet}\right], \tag{1}$$

where $R^{\bullet}$ is an electron donor, the rate of superoxide formation increases with oxygen concentration under normobaric and hyperbaric conditions [40]. As predicted by (1), mitochondrial production of superoxide anion should increase with oxygen concentration, but the proportion of superoxide anion is less than predicted in tissue exposed to atmospheric oxygen [41, 42]. ROS activate the HIF pathway during hypoxia leading to stabilization of the HIF(s) α-isoform. The generation of ROS should decrease with hypoxia according to (1), yet many reports show increased oxidative stress under moderately hypoxic conditions [43–45] but not under severe hypoxic conditions [44]. Moreover, substantial evidence indicates a role for the functional respiratory chain in the generation of ROS under hypoxic conditions. A mutation in the respiratory chain [45, 46] or complex 1 inhibitors [47] prevent stabilization of HIF under hypoxic conditions. More research is needed to clarify the paradoxical ROS formation in the response of tissues to hypoxia.

4. Hypoxia and Mitochondrial Respiration

Mitochondria are the largest consumers of cellular O_2 and are likely candidates for the location of a cellular oxygen sensor. Mitochondria are both targets and important sources of free radicals [48]. Most vital intracellular processes, including blood vessel maintenance, heart contractibility, lung functioning, and neurotransmitter and hormonal support require mitochondria. Mitochondria are the source of ATP and maintain oxygen homeostasis at both the systemic and cellular levels [49]. Initial evidence supporting mitochondria as an oxygen sensor came with the discovery that mitochondrial-depleted Hep3B cells fail to respire and activate mRNAs of erythropoietin, glycolytic enzymes, or VEGF during hypoxia. These cells also fail to increase the generation of ROS during hypoxia, suggesting that mitochondrial (mt) ROS trigger hypoxia-induced transcription [45]. Mitochondria are implicated in multiple HIF-dependent and independent pathways through production of mtROS. Interestingly, decreasing mtROS levels with mitochondrial inhibitors or ROS scavengers prevents stabilization of HIF-1α under hypoxic conditions, suggesting that ROS are important for this effect [45–47]. Interestingly, murine embryonic cells lacking cytochrome *c*, and therefore mitochondrial activity, have impaired cellular oxygen sensing, which prevents stabilization of HIF-1α and HIF-2α under hypoxic conditions, suggesting that mtROS act upstream of PHD to regulate HIF-1α and HIF-2α [50]. Mitochondrial complex III is required for hypoxia-induced ROS production and cellular oxygen sensing [51, 52]. Similarly, embryonic stem cells lacking cytochrome *c* fail to stabilize HIF-1α during hypoxia, as loss of cytochrome *c* leads to complete reduction of cytochrome c1 and the rieske iron-sulfur protein, which inhibit transfer of electrons from ubiquinol at the Q_o site [50]. The complex III Q_o site is necessary to increase cytosolic ROS under hypoxic conditions, which inhibits PHDs from degrading the HIF-1α protein [53]. Additionally, exogenous H_2O_2 or mutations leading to H_2O_2 accumulation stabilize HIF-1α during normoxia [52, 54]. In accordance, antioxidants abolish the hypoxic HIF response, suggesting that generation of mtROS is responsible for propagating the hypoxic signal [45].

5. Physiological Adaptation by HIF(s)

5.1. Physiological Importance of HIF(s). The role of the HIF pathway has been demonstrated under hypoxic conditions but it also plays an important role in normoxia. The physiological roles of HIF-1α and HIF-2α are not known in detail. Hif1$\alpha^{-}/^{-}$ knockout mice have cardiac and vascular malformations and embryos die around mid-gestation [55], whereas HIF-2α knockout mice die presumably of bradycardia due to an inadequate supply of catecholamines during embryonic development [56]. HIF-1α is present in mice under normoxic conditions, increases within distinct cell types in response to systemic hypoxia, and plays an important role in oxygen homeostasis [57]. HIF-2α plays an important role in cardiovascular development and angiogenesis [56, 58]. Murine embryonic stem cells lacking the HIF-2α gene revealed an association with the response to hypoglycemia rather than hypoxia, suggesting that HIF-2α may be more important in the survival response than oxygen level [59]. Hypoxia affects expression of the PER1 and CLOCK circadian genes, and HIF-1α interacts with PER1 under normoxic conditions [60]. The mouse Hif1a gene is expressed from two distinct promoter/first exon combinations, resulting in tissue-specific (mHIF-1alphaI.1) and ubiquitous (mHIF-1alphaI.2) forms. mHIF-1alphaI.1 is a novel HIF-1α mRNA isoform exclusively detected in elongated spermatids. The mHIF-1alphaI.1 protein is located in the mid-piece of the spermatozoal flagellum and expression is oxygen independent [61]. The mHIF-1alphaI.1 isoform is also upregulated in activated T cells under normoxic conditions, suggesting a physiological role for the mHIF-1alphaI.1 isoform in activated lymphocytes [62]. Interestingly, mice exposed to an elevated temperature strongly upregulate HIF-1α in the liver, kidney, and spleen, suggesting a novel mechanism to stabilize HIF-1α under normoxic conditions [63]. Furthermore, a recent study demonstrated that treating normal human cells with low-dose radiation induces a HIF-1-mediated adaptive and protective metabolic response and increased radiation resistance [64]. HIF-1α is induced and activated at physiological oxygen tensions in a mitogen activated protein kinase-dependent manner and determines the increased cell proliferation rate that is common under these conditions [65]. HIF-1α plays an important role in the adaptation of myocardium to mechanical stress via stress-activated channels [66]. As discussed previously, HIF-1α is required for mesenchymal cell survival, and HIF knockout mice have malformed cardiovascular systems and neural tube defects and die during mid-gestation [55, 67, 68]. Hypoxia potentiates interleukin (IL)-1β expression and attenuates selective targeting of IL-1β to autophagic degradation in activated macrophages, suggesting that a novel proinflammatory mechanism may participate in atherogenesis [69]. These observations support the notion that the HIF pathway plays important roles in physiological adaptation and is required for normal growth and development. Interestingly, mice with only one HIF-1α mutant allele develop normally but have impaired physiological responses to chronic hypoxia, such as reduced polycythemia, right ventricular hypertrophy, pulmonary hypertension, pulmonary vascular remodeling, and electrophysiological responses [70, 71]. The carotid body (CB) monitors arterial blood O_2 levels and stimulates breathing in response to hypoxemia to ensure an adequate O_2 supply. Both HIF-1α and HIF-2α are expressed in the CB [56, 72], and CB responses to hypoxia are impaired in *Hif1α*+/− mice [73], whereas they are exaggerated in *Hif2α*+/− mice [74]. Balanced HIF-1α and HIF-2α activity is critical for oxygen sensing by the CB and adrenal medulla and for their control of cardiorespiratory function [75]. Similarly, CBs isolated from HIF-1α heterozygous mice have a dramatic effect on neural activity and ventilatory adaptation after exposure to hypoxia, suggesting a role for HIF-1α at the systemic level [73]. Partial HIF-2α deficiency leads to increased levels of HIF-1α and NADPH oxidase 2, resulting in an oxidized intracellular redox state, exaggerated hypoxic sensitivity, and cardiorespiratory abnormalities, which are reversed by treatment with a HIF-1α inhibitor or a superoxide anion scavenger. In contrast, partial HIF-1α deficiency increases levels of HIF-2α and

superoxide dismutase 2, leading to a reduced intracellular redox state, blunted oxygen sensing, and impaired CB and ventilatory responses to chronic hypoxia, which are corrected by a HIF-2α inhibitor. These observations demonstrate that the redox balance, which is determined by mutual antagonism between HIF-α isoforms, establishes the hypoxic sensing set-point by the CB and adrenal medulla and is required for maintenance of cardiorespiratory homeostasis [75]. HIF-2α plays an important role during the postvasculogenesis stages and is required to remodel the primary vascular network into a mature hierarchical pattern [58]. HIF-2α sense hypoxia during mid-gestational development and translate this signal into an altered gene expression pattern, leading to increased circulating catecholamine levels and proper cardiac function [56]. Despite of normoxic environment several tissues are inherently hypoxic suggesting importance of HIF pathway in normal development. Interestingly, tissue-specific targeting to delete HIF-1α in the cartilaginous growth plate of developing bone showed gross skeletal malformations and die perinatally, probably due to tracheal abnormalities suggesting the role of hypoxia in growth plate development [76]. In addition, hypoxic environment is essential for appropriate embryonic development and placentation. Oxygen tension determines whether cytotrophoblasts, specialized placental cells proliferate or invade, thereby regulating placental growth and cellular architecture [77]. Moreover, ARNT knockout mice placentas shows greatly reduced labyrinthine and spongiotrophoblast layers, and increased numbers of giant cells supporting that HIF-1α is essential for mammalian placentation [78]. Additionally, HIFs play important roles in modulating the developmental plasticity of stem cells by integrating physiological, transcriptional and epigenetic inputs in placenta [79]. Transforming growth factor (TGF) β-3, an inhibitor of extravillous trophoblast differentiation, partly mediates oxygen-regulated early events of trophoblast differentiation through HIF-1α pathway [80]. In addition, HIF-1alpha-deficient mouse embryonic fibroblasts showed impaired migratory capabilities and demonstrated that TGF-β-3 manifests hypoxia and HIF-1α-dependent regulation [81]. Furthermore, hypoxia signaling plays a central role in cartilage, bone, and hematopoiesis [82]. HIF-1α plays a bimodal role in cartilage homeostasis by enhancing anaerobic glycolysis and inhibiting apoptosis suggesting the potential role of this pathway in treatment of osteoarthritis [83, 84]. HIF-2α, was found to be essential for endochondral ossification of cultured chondrocytes and embryonic skeletal growth in mice and its function are independent of oxygen-dependent hydroxylation [85]. Elevated levels of HIF-1α promotes cartilage formation and maintenance whereas elevated levels of HIF-2α favors cartilage destruction and endochondral ossification [85, 86]. Taken together this suggests that both HIF-1α and HIF-2α plays an important role for normal growth and development of skeletal vasculature.

5.2. Regulation of Metabolic Pathways. Increased glycolysis during hypoxia is a crucial step to meet energy demands. Interestingly, HIF-1α stimulates glycolysis and actively represses mitochondrial function and oxygen consumption by inducing pyruvate dehydrogenase kinase-1 (PDK-1) activity [87]. That study also reported that PDK-1 phosphorylates and inhibits pyruvate dehydrogenase from using pyruvate to fuel the mitochondrial TCA cycle, which decreases mitochondrial oxygen consumption resulting in a relative increase in intracellular oxygen tension [87]. Another known mechanism to increase respiration efficiency in hypoxic cells is by regulating of cytochrome *c* oxidase (COX) activity. COX is located in the inner mitochondrial membrane and is a dimer composed of monomers with 13 subunits [88]. Subunits I, II, and III are encoded by the mitochondrial genome, constitute the catalytic core of the enzyme, and are highly conserved in eukaryotes. The crystal structure of bovine COX reveals that subunit IV (COX4) interacts with both COX1 and COX2 [88]. The first step of COX assembly in mammalian cells is the association of COX1 with COX4 [89]. COX4 binds ATP, within the complex, leading to allosteric inhibition of COX activity at high ATP/ADP ratios and demonstrating a regulatory role for COX4 [90]. Under hypoxic conditions, HIF-1α mutually regulates COX4 subunit expression by stimulating transcription of the genes encoding COX4-2 and LON, a mitochondrial protease required for COX4-1 degradation. The effects of manipulating COX-4 subunit expression on COX activity, ATP production, O_2 consumption, and ROS generation indicate that the COX4 subunit switch is a homeostatic response that optimizes respiration efficiency at different O_2 concentrations [11]. Simply, PDK-1 inhibits conversion of pyruvate to acetyl-CoA, thereby preventing pyruvate entry into the TCA cycle and the COX-4 subunits govern mitochondrial respiration efficiency in response to varied oxygen tensions [11, 87]. Autophagy may be the fourth adaptive metabolic response required to prevent increased ROS levels and cell death in hypoxic cells [91]. Mitochondria are replaced every 2–4 weeks in rat brain, heart, liver, and kidney [92]. The destruction of mitochondria is believed to occur via autophagy [93, 94]. Autophagy can be induced by environmental stress such as nutrient deprivation and provides a mechanism to dispose of damaged mitochondria [95, 96]. Autophagy is induced in the heart subject to hypoxic or ischemic conditions and has been proposed to play either a protective or pathogenic role in heart disease [96–98]. BNIP3 is an accepted HIF-1 target gene [99, 100] and is associated with autophagy [98]. BNIP3 may disrupt interactions between Beclin-1, a highly conserved protein required to initiate autophagy, and Bcl2 or Bcl-XL [101]. In contrast to HIF, c-MYC is a proto-oncogene that codes for a transcription factor, regulates the expression of 10–15% of all genes in the genome [102, 103], and promotes mitochondrial respiration by increasing biogenesis [103]. Under physiological conditions HIF-1α inhibits c-MYC activity by directly interacting and stimulating a proteasome-dependent pathway [104, 105]. c-Myc paradoxically collaborates with Hif-1 under stress conditions to induce PDK-1 and hexokinase 2 expression followed by aerobic glycolysis [105, 106] and angiogenesis [106].

5.3. Role of the HIF Pathway in Cell Proliferation and Cell Death. The role of HIF pathway in cell death is controversial; HIF-1 can induce apoptosis [107], prevent cell death, or even stimulate cell proliferation [108]. The oxygen concentration determines whether cell will go apoptosis or not; oxygen level in the range 0–0.5% could induce apoptosis whereas cells with oxygen levels in the range of 1–3% do not undergo apoptosis [109]. ATP is another key determinant of apoptosis; as long as cells have an enough supply of glycolytic ATP during oxygen deprivation, apoptosis can be executed [110]. Moreover, lack of oxygen inhibits the electron transport chain at the inner membrane of the mitochondria thereby causes a reduction in the membrane potential. This reduction of mitochondrial derived ATP causes activation of Bax or Bak, leading to cytochrome C release into the cytosol [111]. Cytochrome c is released and caspase-9 is activated in oxygen-deprived cells undergoing apoptosis [110, 111]. Furthermore, p53 protein, an important regulator of apoptosis can induce the Bax and Bak proteins thereby initiating the cascade leading to apoptosis through cytochrome C [112]. Interestingly, fibroblasts from mice lacking both Bax and Bak genes are resistant to oxygen deprivation induced apoptosis [110]. Similarly, cell over-expressing Bcl-2 or BcL-XL, the anti-apoptotic proteins prevents oxygen deprivation induced apoptosis by inhibiting the release of cytochrome c from the mitochondria [110, 113, 114]. In addition to energy deprivation, ROS generation contributes to hypoxia induced apoptosis. In this case, the initiator caspase 9 is cleaved directly to the active form by caspases 3 and 12, without the involvement of cytochrome C in response to hypoxia [115]. Activation of c-Jun NH2-terminal kinase (JNK) is another mechanism by which hypoxia can induce apoptosis in melanoma cells [116].

HIF pathway is also involved in cell proliferation and promoting metastases [117, 118], VEGF is particularly noteworthy target gene of HIF pathway involved in cell proliferation and upregulated in most cancers [119, 120]. The HIFs can alter cell-cycle progression through putative transcriptional targets such as Cyclin D1 [121] and indirect modulation of p21 and p27 [122]. Furthermore, hypoxic induction of HIF-1α suppresses cell proliferation and acute HIF-1α stabilization at moderate hypoxia (1% O_2) results in cell cycle arrest by inhibiting c-Myc transcriptional activity [122]. In contrast, HIF-2α induction promotes cell cycle progression by enhancing c-Myc function [105].

5.4. Role of the HIF Pathway in pH Regulation and Exercise. The correlation between hypoxia and intracellular pH (pHi) was extensively researched in the 1980–90s. Rapid build-up of intracellular lactate and H^+ as well as an extensive decrease in phosphocreatine concentration is the first sign of hypoxia. ATP is more resistant to hypoxia provided that glucose is present. Furthermore, metabolic damage is considerably greater if glucose is absent during the insult, suggesting that either anaerobic ATP production or low pH may exert some protective effect against acute cell damage [123]. The regulation of Na^+/H^+ exchange (NHE) and pHi is vital for maintaining cell viability. pHi modulates a number of important cell functions, including signal transduction pathways involved in the regulation of cell size and proliferation [124–126]. Alterations in pHi are also associated with hypoxic pulmonary vasoconstriction [127, 128]. Pulmonary arterial smooth muscle cells from chronic hypoxic mice have an elevated basal pHi accompanied by an increase in NHE activity, secondary to increased NHE1 isoform expression [129]. HIF-1α plays an important role governing increased NHE, NHE1 expression, and alkalinization of pulmonary arterial smooth muscle cells in response to hypoxia [130]. Interestingly, NHE inhibitors attenuate hypoxia-induced apoptosis in cardiac myocytes [131]. Tumor cells have a lower extracellular pH (pHe) and a higher pHi than those of normal cells. Low pHe promotes invasiveness, whereas high pHi provides a competitive advantage to growth of tumor cells compared to normal cells for [132, 133]. Low pHe results from lactic acid accumulation solely produced by glycolysis. Hypoxia induces coordinated upregulation of glycolysis, a potential step that may promote tumor cell growth and activate the capacity of tumor-associated carbonic anhydrase (CA) IX to acidify pHe thereby leading to tumor progression [134]. HIF-1α controls CA IX and CA XII expressed by tumor cells. Hypoxia-inducible CAIX and CAXII proteins promote cell survival and growth by maintaining pHi. Moreover, CAIX and CAXII constitute a robust pHi-regulating system that confers a tumor growth and survival advantage to cells exposed to a hypoxic and acidic microenvironment [135].

HIF is a candidate to facilitate training adaptation in skeletal muscle. Muscle training induces negative regulators of HIF (PHD, FIH, and sirtuin-6) but lowers PDK-1 expression in elite athletes, thereby contributing to skeletal muscle adaptation to exercise [136]. Endurance exercise improves muscle oxidative capacity [137], whereas resistance exercise training leads to increased muscle size and strength [138]. One study investigated the effects of 8 weeks of resistance exercise training performed under hypoxic (HRT) or normoxic conditions (NRT) on skeletal muscle. As results, significant increases in muscle endurance, plasma VEGF concentration, and capillary-to-fiber ratio were observed following training in the HRT group compared those in the NRT group, suggesting that HRT may also lead to increased muscular endurance and promote angiogenesis in skeletal muscle [139]. Chuvash polycythemia (CP) is an autosomal recessive disorder in which regulatory degradation of HIF is impaired, resulting in elevated levels of HIF under normal oxygen tensions [140, 141]. Patients with CP show early and marked phosphocreatine depletion, higher blood lactate accumulation, acidosis in skeletal muscles, and reduced exercise capacity [142]. Interestingly, gene therapy with intramuscular administration of Ad2/HIF-1α/VP16 was not effective for patients with intermittent claudication [143]. Pyruvate dehydrogenase (PDH) plays an important role controlling the flux of pyruvate to acetyl-CoA. PDH is inactivated during acute hypoxia thereby promoting conversion of pyruvate to lactate, suggesting an influence of PDH activity on the fate of pyruvate [87]. The transition from acute to chronic hypoxia desensitizes the HIF-1α pathway, leading to a re-establishment of PDH activity and reduced lactate production by exercising muscles [144]. Exercise with intermittent hypoxic training for 3 weeks causes a significant decrease in skeletal muscle HIF-1α mRNA, suggesting that

transcriptional and posttranscriptional regulation of the HIF-1 differ in muscle and other cells [145]. HIF-1α and HIF-2α induce angiogenesis and improve muscle energy recovery [146]. In contrast, the HIF-3α subunit plays a negative role in adaptation to hypoxia because inhibiting HIF-3α expression leads to increased physical endurance [147].

6. Conclusion

In present review we have discussed the physiological adaptations and importance of the HIF pathway. Several studies have demonstrated that manipulating the HIF pathway can help treat diverse disorders. HIF(s) are also upregulated under inflammatory conditions, suggesting their role in maintaining homeostatic conditions and protecting against cellular inflammation. The role of the HIF pathway varies under diverse conditions. For example, HIF inhibitors have been developed to treat cancer and ischemia, whereas HIF activators could be utilized for stroke and spinal cord injuries. Significant developments have been made towards understanding the roles of the HIFs under both physiological and pathophysiological conditions. The roles of HIF(s) are becoming clearer during physiological adaptation. The interaction of HIF-1α with HIF-2α and HIF-3α during physiological adaptation will provide a great deal of understanding of HIF(s). Moreover how these transcription factors interact with other known proteins and pathways will help in designing the future therapeutics with minimal side effect.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Review Article

Targeting the Mitotic Catastrophe Signaling Pathway in Cancer

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Mitotic catastrophe, as defined in 2012 by the International Nomenclature Committee on Cell Death, is a *bona fide* intrinsic oncosuppressive mechanism that senses mitotic failure and responds by driving a cell to an irreversible antiproliferative fate of death or senescence. Thus, failed mitotic catastrophe can promote the unrestrained growth of defective cells, thereby representing a major gateway to tumour development. Furthermore, the activation of mitotic catastrophe offers significant therapeutic advantage which has been exploited in the action of conventional and targeted anticancer agents. Yet, despite its importance in tumour prevention and treatment, the molecular mechanism of mitotic catastrophe is not well understood. A better understanding of the signals that determine cell fate following failed or defective mitosis will reveal new opportunities to selectively target and enhance the programme for therapeutic benefit and reveal biomarkers to predict patient response. This review is focused on the molecular mechanism of mitotic catastrophe induction and signalling and highlights current strategies to exploit the process in cancer therapy.

1. Introduction

Genome instability represents an enabling characteristic underlying the acquisition of the hallmarks of cancer [1]. Mammalian cells have evolved a variety of mechanisms to remove defective and genomically unstable cells. Mitotic catastrophe is a regulated antiproliferative process that occurs during defective or failed mitosis. Although it does not constitute a *bona fide* cell death mechanism in itself, mitotic catastrophe precedes and uses antiproliferative measures including apoptosis, necrosis, and senescence to prevent the proliferation of defective mitotic cells [2, 3]. Mitotic catastrophe is characterised by unique nuclear alterations that lead to multinucleation and/or micronucleation and are used as morphological markers for detection. Giant multinucleated cells arise from clusters of missegregated uncondensed chromosomes, whereas micronucleated cells arise from lagging chromosomes or chromosome fragments during anaphase that are left outside the daughter nuclei formed during telophase, thereby giving rise to a micronucleus in addition to the main nucleus [4]. Figure 1 illustrates the morphological features following normal cell division (a) and a multinucleated cell formed during mitotic catastrophe (b). Failure of the mitotic catastrophe antiproliferative process leads to persistent genome instability and aneuploidy (c–f). Furthermore, as a result of the various antiproliferative pathways adopted by mitotic catastrophe it is often accompanied by morphological and biochemical features of apoptosis and necrosis [2, 3].

The detection and removal of mitotically defective cells are important steps in the prevention of genome instability. Defective or failed mitosis leads to the generation of aneuploid or tetraploid cells, which are a common feature of tumour cells [5, 6]. It was postulated by Theodor Boveri more than 100 year ago that abnormality in chromosome segregation during mitosis could promote tumour formation [7]. It is now known that aneuploidy is present in approximately 90% of solid human tumours and >50% of haematopoietic cancer [8]. Some aneuploid tumours have a minor imbalance in chromosome number whereas others are characterised by a large amount of aneuploidy and contain a near tetraploid chromosome number [5]. During mitosis, the loss or gain of chromosomes can occur through a variety of mechanisms including mitotic checkpoints defects, chromosome cohesion defects that lead to sister chromatid missegregation, and centrosome amplification that promotes multipolar mitosis. The hyperstabilisation of kinetochore-microtubule attachments can also prevent the correction of previous attachment defects [5]. On the other hand, tetraploid cells have twice the normal

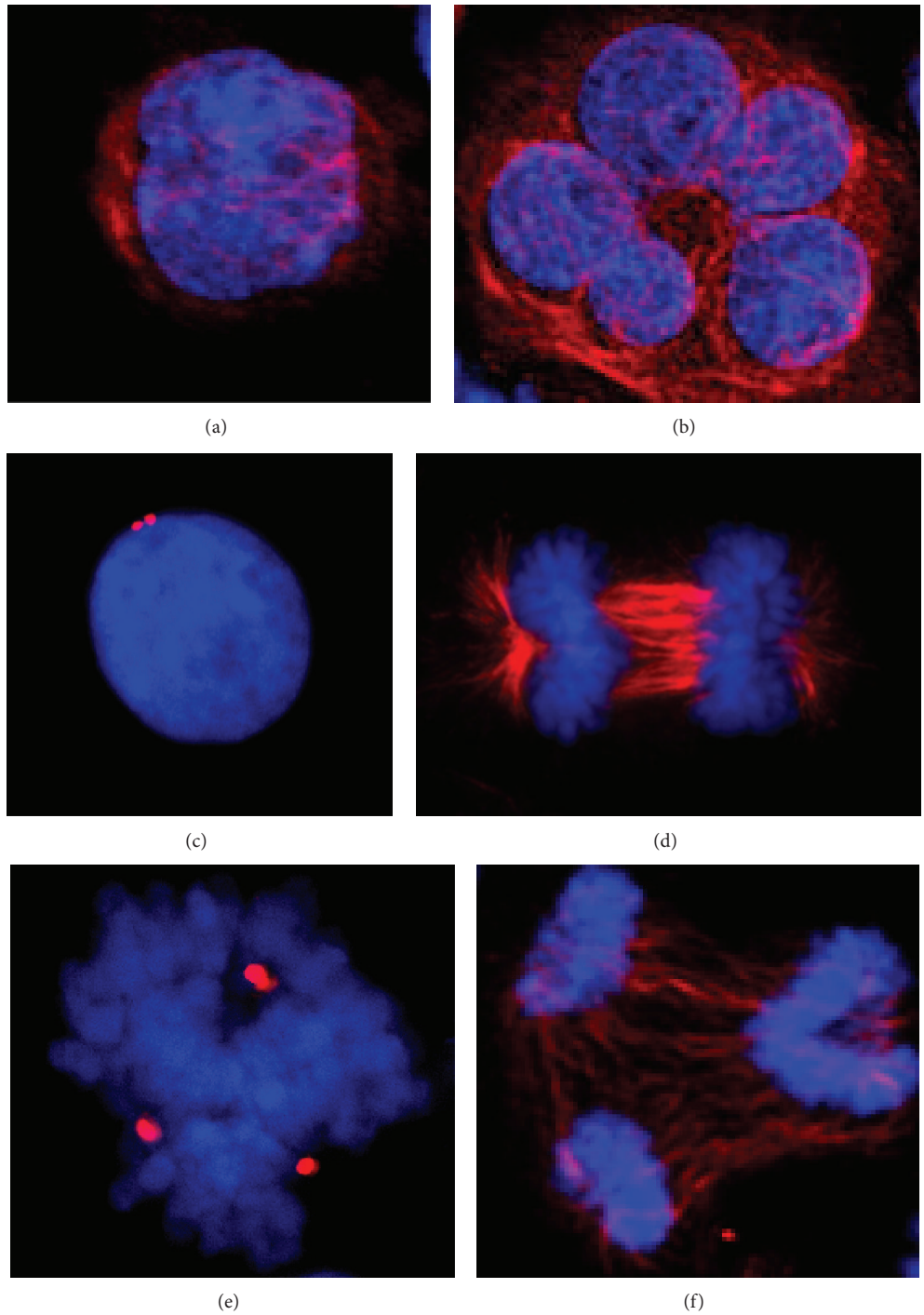

Figure 1: Morphological features of mitotic catastrophe. Human K562 chronic myeloid leukaemia cells during normal interphase (a) and a giant multinucleated cell following mitotic catastrophe induced by microtubule disruption (b). Interphase cell with two centrosomes (c) and normal chromosome segregation during anaphase (d). A cell containing >2 centrosomes (e) forms multipolar mitotic spindles (f) leading to aneuploidy as a result of mitotic catastrophe failure. DNA (blue), α-tubulin (red) (a, b, d, f), and centrosome (red pericentrin staining) (c and e).

diploid chromosome content, which can arise due to mitotic slippage, cytokinesis failure, cell fusion, and endoreplication [5]. Tetraploid cells also contain twice the normal centrosome content, which promotes multipolar mitosis and whole chromosome missegregation, and provides a mechanism for the transition of cells from a tetraploid state to an aneuploid

state. Multipolar mitotic divisions generally lead to catastrophic chromosome missegregation events that are incompatible with survival; however, cancer cells can avoid such catastrophic events and suppress multipolar mitosis by clustering centrosomes into two groups thereby allowing division to occur in a bipolar fashion [9].

While there has been much debate over the role of aneuploidy and tetraploidy in tumour onset, mounting evidence suggests that tetraploid cells can trigger cellular transformation and tumour formation [5, 6]. For example, $p53^{-/-}$ tetraploid mouse cells formed tumours when transplanted into immunocompromised mice, which was not detected with the isogenic diploid cells [10]. Tetraploid cells generated by virus induced cell-cell fusion can proliferate and induce transformation [11, 12]. Mutation of adenomatous polyposis coli (APC) in colorectal cancer resulted in tetraploid genomes *in vivo* due to cytokinesis failure [13]. Furthermore, tetraploidy was identified as an early event during cervical carcinoma [14], and tetraploid cells formed following cytokinesis failure induced transformation *in vivo* [15, 16]. In these cases transformation was coupled with extensive genome instability with abnormalities in the number and structure of chromosomes, providing evidence that tetraploidy represents an intermediate stage to promote aneuploidy and genome instability. Moreover, the loss of two tumour suppressor genes Breast Cancer Susceptibility Gene 2 (BRCA2) or the LATS1 tumour suppressor is accompanied by cytokinesis defects, suggesting a role for these tumour suppressors during cytokinesis [17, 18].

Aneuploidy increases the rate of both spontaneous and carcinogen-induced tumour formation; however, paradoxically, cases where aneuploidy does not promote tumourigenesis or where it suppresses tumourigenesis have also been reported [19]. It is clear that aneuploidy alters the path of tumour development, and a variety of factors influence the final outcome including the combination of chromosomes involved, cell type, genetic context, for example, the presence of additional cooperating mutations in key regulatory genes, as well as the microenvironment within different tissue [19]. This context driven outcome is illustrated in patients with Down syndrome who carry an extra copy of chromosome 21 and have increased incidence of haematological malignancies but reduced incidence of solid tumours [20, 21]. More recently it was suggested that the rate of chromosome missegregation will determine whether aneuploidy will promote or suppress tumour growth, where low rates of chromosome missegregation can promote tumourigenesis, and high rates lead to cell death and thereby prevent tumour growth [22]. In each scenario, the final outcome will be influenced by the functional status of damage sensing mitotic catastrophe signals as well as the cell survival and death machinery. Thus, mitotic catastrophe represents an important part of our genome maintenance machinery and abrogated or compromised signals will contribute to tumour onset. Understanding the molecular mechanism that dictates mitotic catastrophe has important implications for tumour prevention and treatment. Here we provide an update on current knowledge about the mechanism of mitotic catastrophe induction and signalling and highlight approaches to target and exploit the process in cancer treatment.

2. Mitosis

The cell cycle represents a highly coordinated process whereby a cell is divided into two genetically identical daughter cells. Pioneering work over the past four decades has revealed the molecular components that control the cell cycle in eukaryotes [23]. The mammalian cell cycle can be divided into distinct phases, DNA replication (Synthesis (S) phase) and division (Mitosis (M) phase), which are separated by Gap phases (G1 and G2). Mitosis is subdivided into prophase, prometaphase, metaphase, anaphase, telophase and cytokinesis, which together regulate nuclear envelope breakdown, chromosome attachment to spindle microtubules, alignment along the metaphase plate, sister chromatid separation, and finally, the coordinated plasma membrane remodelling and cytoplasmic division to produce two daughter cells [23]. Transition through the cell cycle is controlled by the interplay between cyclin-dependent kinases (cdks) and their respective cyclin binding partners [23, 24]. Activation of cdk1, which occurs upon formation of a cdk1/cyclin B complex, regulates entry and progression through mitosis. Active cdk1/cyclin B phosphorylates substrates involved in nuclear envelope breakdown, assembly of the mitotic spindle, chromosome condensation, and activation of the spindle assembly checkpoint [23, 24]. During metaphase the mitotic chromosomes, which are composed of sister chromatids held together by cohesion, are aligned on the mitotic spindle by stable microtubule attachment through their kinetochores. Properly aligned chromosomes are separated during anaphase and move towards opposite ends of the spindle [25]. A narrow region of overlapping nonkinetochore microtubules forms the central spindle at the midzone between separating chromosomes. This is followed by formation of centralspindlin comprised of MKLP1 and CYK4 and containing a GTPase-activating protein (GAP) domain, and the Chromosomal Passenger Complex (CPC) composed of Aurora B and three additional proteins, INCENP, Survivn, and Borealin that are required for Aurora B regulation [26–29]. Central spindle recruits Ect2, a RhoGTPase leading to RhoA activation and assembly of an actinomyosin contractile ring around the central core of the cell. The contractile ring constricts to form a cleavage furrow that ingresses and packs the midzone microtubules to form the dense region termed the midbody at the centre of a long intercellular bridge holding daughter cells together. During cytokinesis, the midbody acts as a platform for components required during abscission of the plasma membrane and eventual daughter cell separation [26–29].

3. The Spindle Assembly Checkpoint

As well as the coordinated activation and inactivation of cdk1 that controls mitotic progression, the fidelity of the process is maintained by an independent and evolutionary conserved checkpoint known as the spindle assembly checkpoint (SAC) [25]. The SAC is a surveillance process at the transition from

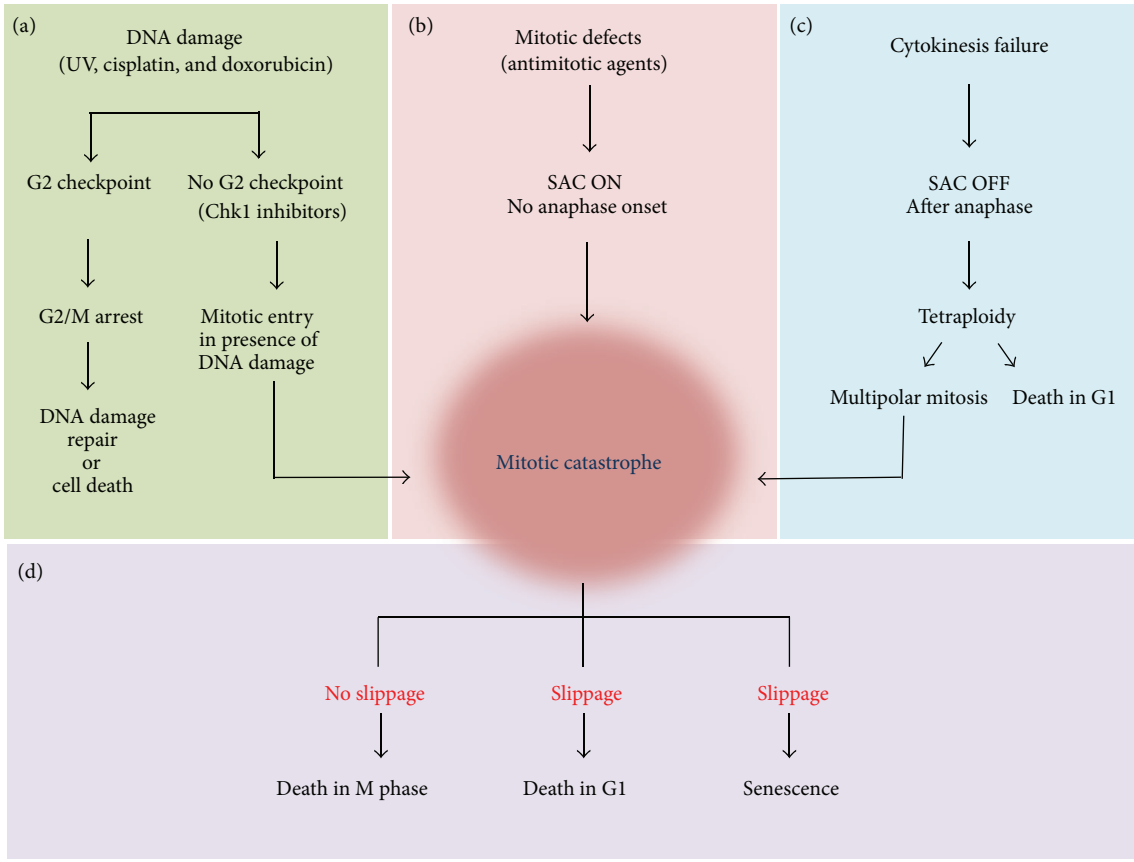

Figure 2: Mechanisms of mitotic catastrophe. (a) Cells with an abrogated G2 checkpoint will enter mitosis prematurely in the presence of damaged DNA and undergo segregation defects leading to mitotic catastrophe. (b) Cells with defects in mitotic apparatus and/or machinery required for faithful chromosome segregation fail to satisfy the spindle assembly checkpoint (SAC) and undergo prolonged mitotic arrest and mitotic catastrophe. (c) Cytokinesis defects that occur after anaphase will lead to a tetraploid progeny that will undergo mitotic catastrophe in the next M-phase. (d). Following activation of mitotic catastrophe, cells arrested in mitosis have three fates; they will undergo death in mitosis in the presence of cyclin B, or cyclin B levels will gradually fall allowing the cells to undergo slippage and exit mitosis where they subsequently undergo death in G1. Alternatively, cells can undergo senescence following slippage.

metaphase to anaphase that monitors the attachment of chromosomes to the kinetochore spindles and halts progression of anaphase until all chromosomes are correctly attached to the bipolar spindle [25]. Upon proper attachment, the SAC is switched off and Cdc20 activates the E3 ubiquitin ligase, Anaphase Promoting Complex (APC), leading to ubiquitination and proteolytic degradation of two substrates, cyclin B, which maintains cdk1 in an active form, and securin, which inhibits separase. Following degradation of securin, the liberated separase targets cohesion causing sister chromatid separation, and, anaphase onset. Furthermore, APC-mediated degradation of cyclin B leads to inactivation of cdk1 and signals mitotic exit [25]. Thus, the SAC is active for a short time during a normal mitosis. A single unattached or incorrectly attached chromosome is sufficient to block progression to anaphase by inhibition of APC activity, thereby leading to mitotic arrest.

4. Mechanism of Mitotic Catastrophe

Mitotic catastrophe senses mitotic damage and directs the defective cell to one of three possible antiproliferative fates (Figure 2). Defective mitotic cells can engage the cell death machinery and undergo death in mitosis, when cyclin B levels remain high. Alternatively, defective cells can exit mitosis, known as slippage, and undergo cell death execution during G1 in the subsequent cell cycle. Finally, defective cells can exit mitosis and undergo senescence [2, 3]. It is clear that mitotic catastrophe is always accompanied by mitotic arrest; however, the mechanisms that dictate cell fate following mitotic catastrophe remain unclear [2, 3]. It was originally proposed that death signals gradually accumulate during mitotic arrest, and therefore the length of mitotic arrest determines cell fate [30]. Since then a model has been proposed whereby cell fate is dictated by two independent, yet competing networks; one involves activation of prodeath signals and the other protects against cyclin B degradation. Both pathways work in opposite directions during prolonged mitosis; that is, cell death signals accumulate and cyclin B levels decline. Both pathways have a threshold and the fate of the cell is determined by which threshold is breached first [31]. It is known that cyclin B levels slowly decline during prolonged mitotic arrest even in presence of an active SAC [32]; thus, if levels fall below

the threshold that dictates mitotic exit, slippage occurs, whereas if the death threshold is met first the cell will undergo death in mitosis. Additional work has focused on determining the molecular events that govern each network and its threshold in order to understand how cells respond to mitotic stress [33].

The activity of the Bcl-2 protein family is a key determinant of fate following mitotic catastrophe [67–71] and phosphorylation mediated by cdk1 is an important signal that controls Bcl-2 family activity [67, 68, 72, 73]. The family is comprised of multidomain prosurvival proteins (Bcl-2, Bcl-$_{XL}$, Bcl-$_{W}$, Mcl1, A1, and Bcl-B) and multidomain proapoptotic effector proteins (Bax, Bak, and Bok) as well as BH3-only proteins (Bim, PUMA, Bad, NOXA, Bik, Hrk, Bmf, and tBid) [74]. The multidomain members of the family (the prosurvival proteins and the effectors Bak, Bax, and Bok) contain four BCL-2 homology regions (BH1–BH4), whereas the BH3-only proteins contain only a BH3 domain, which is important in mediating their interaction with the multidomain members. Various models are proposed to describe how prosurvival and proapoptotic Bcl-2 proteins interact together to control apoptosis. For a recent review see [74].

Bcl-2 proteins are also regulated in a transcriptional and posttranslational manner. Active cyclin B/cdk1 directly phosphorylates Bcl-2, Bcl-$_{XL}$, and Mcl-1 during mitosis and negatively regulates their activity [67, 68]. Cdk1 phosphorylation of Bcl-2 and Bcl-$_{XL}$ blocks heterodimer formation with proapoptotic members, Bax and Bak, promoting their oligomerization at the outer mitochondrial membrane, release of cytochrome C, and thereby apoptosis [75, 76]. In contrast, cdk1-mediated phosphorylation of Mcl-1 during mitosis controls protein stability by ubiquitination and degradation via the proteasome. Harley et al. [68] demonstrated that phosphorylation of Mcl-1 by cdk1/cyclin B initiates its degradation during mitotic arrest in a Cdc20/APC-3 dependent manner. Like Bcl-2 and Bcl-$_{XL}$, loss of antiapoptotic Mcl-1 promotes the oligomerisation of Bax and Bak and thus death during prolonged mitotic arrest. Thus, it is proposed that during a typical mitosis the transient phosphorylation of Mcl-1 by cdk1/cyclin B is not sufficient to drive cell death before cyclin B levels drop sufficiently to inactive cdk1. This transient effect ensures that normal cells are not subject to a fate of apoptosis during normal mitosis. In contrast, however, the sustained cdk1 activity that occurs during mitotic arrest leads to a significant drop in Mcl-1 levels thereby suppressing its antiapoptotic effect and triggering cell death before mitotic exit. Phosphorylation of Mcl-1 also controls interaction with the FBW7 tumour-suppressor and subsequent degradation by the skp-cullin-F-box (SCF) complex during mitotic arrest [77]. Collectively, these reports highlight a role for posttranslational phosphorylation and ubiquitination in the crosstalk between the mitotic and apoptotic machinery to control cell fate during mitotic arrest.

The BH3-only protein Bim undergoes cdk-1-mediated phosphorylation during mitosis. Mac Fhearraigh and Mc Gee [72] demonstrated that two Bim isoforms, Bim$_{EL}$ and Bim$_{L}$, undergo transient phosphorylation during normal mitosis; however, hyperphosphorylation was evident during sustained mitotic arrest. Furthermore, Bim directly binds cyclin B, which acts as a molecular bridge for cdk1 phosphorylation, and serine 44 within Bim$_{L}$ was identified as a novel cdk-1 phosphorylation site [72]. It is suggested that cdk-1-mediated phosphorylation of Bim alters its heterodimer formation with Bcl-2, leading to enhanced activation of Bak and mitochondrial cell death [75], consistent with the view that mitochondrial proapoptotic signalling entails the interplay between pro- and antiapoptotic Bcl-2 proteins [78]. In contrast, cdk1-dependent phosphorylation of Bim$_{EL}$ promotes its polyubiquitylation and degradation via the proteasome during mitotic arrest [73]. Thus, based on the competing model that was proposed by Gascoigne and Taylor, [31] the gradual loss of cyclin B during prolonged mitotic arrest will lead to the loss of cdk-1-mediated Bim phosphorylation, which will alter its cell death activity during prolonged mitosis and following slippage, either through stability or heterodimer formation. This may partially explain the contradictory reports that mitotic catastrophe-induced death occurs via Bim-dependent and Bim-independent mechanisms [79–82].

Cdk1 also phosphorylates members of the proteolytic caspase family, specifically caspase-2, caspase-8, and caspase-9, leading to inhibition of their apoptotic activity, which is believed to be a cytoprotective measure during normal mitosis [83–85]. Furthermore, caspase activity is not required for spindle assembly checkpoint function or mitotic slippage following mitotic catastrophe [86]; however, the downstream cell death can manifest in a caspase-dependent or caspase-independent manner [3]. Cell death can involve mitochondrial perturbations including mitochondrial outer membrane permeabilisation (MOMP) and cytochrome C release induced following Bax/Bak oligomerisation and pore formation on the outer mitochondrial membrane, leading to caspase-dependent apoptosis [87]. Alternatively cell death can occur through the Permeability Transition Pore Complex (PTPC), a large complex that bridges the junction between the inner and outer mitochondrial membranes. A sudden increase in permeability of the inner mitochondrial membrane to small solutes leads to Ca^{2+} overload, oxidative stress, and Mitochondrial Permeability Transition- (MPT-) induced death that is independent of caspase activity [88]. Although details of the mitotic and cell death processes are well characterised, the molecular signals that link these events during mitotic catastrophe remain poorly understood and are the focus of intense investigation. Two interesting candidates are Mad2 and survivin that are reported to have dual functions in regulating spindle checkpoint and cell death [58, 89]. Furthermore, it was recently shown that mitochondrial Protein Tyrosine Phosphatase 1B (PTP1B) undergoes coordinate phosphorylation by cdk1 and plk1 during mitotic arrest. Phosphorylation of mitochondrial PTP1B increases its phosphatase activity and sensitises cells to antimitotic agents [90] representing a new molecular link between the mitotic machinery and the mitochondrion during mitotic catastrophe. The identification of PTP1B substrates at the mitochondria will provide better insight into its precise function and help delineate the downstream execution events. It was also recently demonstrated that cdk1 can directly phosphorylate and regulate numerous mitochondrial proteins, including subunits of the respiratory chain to regulate respiration in

a cell cycle specific manner [91, 92], providing a further link between cdk1 activity and mitochondrial function. It remains to be determined how mitochondrial energetics are altered during mitotic catastrophe.

5. Induction of Mitotic Catastrophe by Mitotic Perturbations

Faithful mitotic progression requires the proper function of various cell components including microtubules, mitotic enzymes, motor proteins, and protein complexes [34, 93]. Microtubules are essential cytoskeletal components composed of subunits of α-tubulin and β-tubulin that dimerise to form linear protofilaments, which together form microtubules. The dynamic nature of microtubule plus ends, which undergo continuous polymerisation and depolymerisation, allow them to form cell structure and enable motility and intracellular transport [93]. During mitosis microtubules form a bipolar spindle array that emanates from the centrosomes located at opposite sides of the cell. The dynamic nature of microtubule ends facilitates proper attachment to chromosomes at their kinetochore [25]. Unattached or incorrectly attached kinetochores, such as merotelic or syntelic attachments, initiate a network of signals to recruit mitotic checkpoint components including Mad1, Mad2, Bub1, Bub3, BubR1, CENP-E, and Mps1 to kinetochores [25, 94–96]. The formation of an inhibitory complex termed the mitotic checkpoint complex (MCC), consisting of three SAC components, Mad2, BubR1, and Bub3 as well as Cdc20, acts as the SAC effector that is enriched at unattached kinetochores. MCC binds to and potently inhibits APC by sequestering Cdc20, thereby preventing mitotic exit [25, 94–96] (Figure 2). Biorientated attachment of all sister chromatid pairs to their kinetochore microtubules promotes displacement of the SAC proteins, allowing release of Cdc20 from the MCC. Released Cdc20 can then activate the APC and promote mitotic exit. Improper kinetochore-microtubule attachment also causes reduced tension across the spindle apparatus which inhibits the APC through a mechanism involving Aurora B kinase [97, 98].

Most cancer cells display a certain level of aneuploidy [5, 6, 19], and it was proposed that mechanisms that induce additional instability constitute a therapeutic strategy. Consistent with that, cancer cells are more susceptible to cell death following mitotic damage in comparison to nontransformed cells [99], and a number of mitotic targets have been identified. These include mitotic kinases such as aurora kinases, monopolar spindle 1 (Mps1), and polo-like kinases (Plks) that play key roles during faithful chromosome segregation [100]. The aurora kinase family of serine/threonine protein kinases includes Aurora A, Aurora B and Aurora C, each with a distinct expression pattern, subcellular localisation pattern, and function [101, 102]. Aurora A localises to centrosomes during interphase and to spindles poles and spindle microtubules during mitosis, where it regulates mitotic entry, centrosome maturation, and spindle formation [35]. Aurora B localises to kinetochores and forms part of the Chromosome Passenger Complex that plays critical roles during chromosome condensation, biorientation, and cytokinesis [97]. Thus, Aurora A and Aurora B act at different stages of mitosis. Aurora C is mainly expressed in testes and required for spermatogenesis and mouse embryogenesis [35]. Dysregulated aurora kinase activity generates mitotic abnormalities and cytokinesis failure [103, 104]. Thus, the critical role of aurora kinases during mitosis makes them indispensible with faithful mitosis.

Mps1 forms a core component of the spindle assembly checkpoint (SAC) and functions in the alignment and orientation of chromosomes during metaphase [105]. Polo-like kinases (Plks) also play critical roles during mitotic progression [102, 106]. Five members of the Plk family have been identified in humans. The most widely studied is Plk1, which is involved in assembly of the mitotic spindle, maturation of centrosomes, activation of the SAC, chromosome segregation, and cytokinesis [102, 106]. Deregulation of the centrosome cycle leading to supernumerary centrosomes generates multipolar mitosis that promotes genome instability, SAC activation, and mitotic catastrophe [107, 108].

Faithful mitosis is also dependent on microtubule motor proteins such as Eg5, a plus-end directed motor from the kinesin superfamily that is responsible for mitotic spindle formation and function. Disruption of Eg5 function during mitosis leads to monopolar spindles and activation of the SAC. Furthermore, the centromere-associated motor protein (CENP-E) is a component of the kinetochore corona fibres of mammalian centromeres and is required for chromosome biorientated attachment and proper mitotic checkpoint signalling [109–111]. In addition to disrupting chromosome segregation, inhibition of cytoplasmic division following anaphase onset will generate genome instability and stimulate mitotic catastrophe in the next cell cycle [109, 112] (Figure 2). The development of pharmacological agents that induce mitotic catastrophe via disruption of bipolar spindle function or faithful chromosome segregation is discussed in more details later.

6. Induction of Mitotic Catastrophe following DNA Damage

DNA damage induced by intrinsic or extrinsic factors threatens genome integrity and stability. Failure to repair the DNA damage leads to mutations and genome instability that ultimately contributes to diseases including cancer [113, 114]. The DNA Damage Response (DDR) is a complex mechanism to sense various types of DNA damage and respond appropriately to maintain genomic integrity. Following DNA damage the phosphatidylinositol 3-kinase-related kinases ATM (ataxia-telangiectasia mutated) and ATR (ATM and Rad-3 related) are activated and coordinate activation of the DNA damage checkpoints through the phosphorylation of numerous downstream substrates. Checkpoint kinase-1 (Chk1) and checkpoint kinase-2 (Chk2) are serine threonine kinases that transduce the DNA damage signal downstream. Chk2, which is expressed throughout the cell cycle, undergoes phosphorylation and activation by ATM, whereas Chk1 is preferentially

expressed in S and G2 and is phosphorylated by ATR. In addition to Chk1 and Chk2, MAPK-activated protein kinase-2 (MK2) regulates cell cycle checkpoint activation [113, 114]. Genomic stress activates the G1 checkpoint, which prevents S phase entry by inhibition of DNA replication. At this point, Chk2, which is activated by ATM, phosphorylates and suppresses the phosphatase Cdc25-A, thereby preventing activation of cyclin E/cdk2 and thus halting the cell cycle. The S phase checkpoint is activated in response to replication errors and DNA damage that occurs during S phase, whereas the G2 checkpoint deals with cells that have either undergone DNA damage in G2, or they have escaped the G1 and S phase checkpoints. Cdk1 activity and mitotic entry are tightly regulated and balanced by inactivating phosphorylation by the protein kinases WEE1 and myelin transcription factor 1 (MYT1), together with the activating Cdc25 phosphatase. Thus, WEE1 and Cdc25 act as a central switch for mitosis and are regulated by posttranslational alterations thereby enabling rapid switching. At G2, Chk1, which is activated by ATR, phosphorylates and suppresses Cdc25-A, -B, and -C thereby preventing cyclin B/cdk1 activation and causing G2 arrest [113–115]. G2 arrest is also initiated by MK2 which inactivates Cdc25-B and -C [113]. Thus, the G2 checkpoint is the last opportunity to halt the cycle and repair DNA damage in cells that have escaped the G1 and S phase checkpoints. Abrogated or compromised G2 checkpoint will allow premature mitotic entry of defective cells that fail to undergo proper chromosome segregation thereby leading to mitotic catastrophe (Figure 2). In support of this, the fusion of interphase and mitotic cells led to mitotic catastrophe which was due to the cyclin B/cdk1 driven-premature entry of cells into mitosis before they had completed S or G2 [116]. Furthermore, knockout of the cytoplasmic binding protein 14-3-3σ in colorectal cancer cells resulted in failure to sequester cyclin B1 and prevented G2 arrest following DNA damage, culminating in mitotic catastrophe [117]. Inhibition of Chk2 also abrogates the G2 checkpoint leading to mitotic catastrophe following DNA damage. [118]. In contrast, cells that harbour DNA damage and undergo death in interphase do not constitute an example of mitotic catastrophe [2, 3, 113]. Furthermore, while an abrogated or defective G2 checkpoint is essential for DNA damage-induced mitotic catastrophe, the eventual mode of cell death induced is determined by whether p53 is present or absent. For example, DNA damage induces two distinct forms of cell death in ovarian carcinoma [119]. Functional p53 triggered apoptosis in ovarian carcinoma cells following mitotic catastrophe whereas loss of p53 in these cells triggered necrosis. The exact mechanism of p53 activation during or after mitotic catastrophe remains to be elucidated; however, it was shown that phosphorylated H2AX-ATM-p53 pathway dictates an apoptotic outcome following mitotic catastrophe. Loss of p53 or depletion of ATM protected against apoptosis and instead led to necrosis [4]. Apoptosis driven by p53 is also associated with caspase activity [120]. Thus it is proposed that the initiation of mitotic catastrophe occurs independently of p53 status and caspase activity; however, the presence of functional p53 is required for a caspase-mediated apoptotic response.

Table 1: Exploiting mitotic catastrophe in cancer therapy.

Mechanism of action	Inducer	References
Microtubule targeting agents		
Microtubule polymerisers	Taxanes	[34]
Microtubule depolymerisers	Vinca alkaloids	
Non-microtubule antimitotic agents		
Mitotic spindle targets	Aurora kinase inhibitors *Alisertib*	[35, 36]
	KSP inhibitors *Eg5* *AZD4877* *Ispinesib* *ARRY-520*	[37–42]
	CENP-E inhibitors *GSK923295*	[43, 44]
	PLK-1 inhibitors *B12536*	[45–47]
Mitotic checkpoint targets	MPS1 inhibitors *NMS-P715* *MPS1-IN-3*	[48–51]
Mitotic exit inhibition	APC inhibitor *TAME*	[52–54]
Centrosome disruption	*Griseofulvin*	[55–57]
G2 checkpoint abrogation	Chk1 inhibitors *UCN-01* *AZD7762*	[58–63]
	HDAC inhibition *Trichostatin A*	[64–66]

7. Exploiting Mitotic Catastrophe in Cancer Therapy

Mitotic catastrophe is induced by a variety of agents classified as those that disrupt mitotic progression or directly damage DNA (Figure 2 and Table 1). The best known antimitotic agents are the microtubule targeting agents (MTAs), also known as spindle poisons [34]. MTAs are grouped into two families: the microtubule polymerisers which include the taxanes (paclitaxel and docetaxel) and the microtubule depolymerisers which include the vinca alkaloids (vinblastine and vincristine). The suppression of microtubule dynamics by both groups precludes normal bipolar spindle formation and prevents chromosome biorientation, leading to mitotic arrest and cell death. Taxol (paclitaxel) originally isolated in 1967 from a Yew tree (*Taxus brevifolia*), was approved for clinical use in 1995 and is widely used across a range of malignancies. For example, taxanes have been used in the treatment of Kaposi's sarcoma, non-small-cell lung cancer, breast cancer, ovarian cancer, and prostate cancer, whereas vinca alkaloids are used to treat haematological malignancies [34, 121]. Although they have been used clinically for decades,

microtubule targeting agents lack specificity towards cancer cells and disrupt other important microtubule-dependent functions leading to severe side effects including neuropathy. Furthermore, the development of drug resistance limits their use, which can be ascribed to drug efflux pumps, overexpression of prosurvival Bcl-2 proteins, and mutations in tubulin that abrogates drug binding [122, 123]. Resistance may also occur as a result of mitotic slippage [32]. Thus, research efforts have focused on the development of non-microtubule antimitotic therapeutics, such as those targeted at mitotic kinases and spindle motor proteins, with the hope that that they would overcome some of the drawbacks associated with microtubule targeting agents.

Primary tumours frequently have overexpressed and/or amplified aurora kinases. Moreover, their depletion or inhibition impairs the proliferation of cancer cells, thus, making them an attractive target for cancer treatment [35, 36]. A number of aurora kinase inhibitors have been developed that target the enzymes ATP binding domain. Early inhibitors did not display specificity towards a family member; however, in recent years work has focused on development of selective inhibitors and a number are in various stages of clinical evaluation including Alisertib (MLN8237) that has displayed promising antitumour properties and is currently in Phases I and II trials [35].

Mps1 is highly expressed in human tumours where it promotes cell proliferation [48]. Mps1 kinase inhibitors have been developed which induce mitotic defects and death in cancer cells, across a variety of preclinical models, either alone or in combination with microtubule inhibitors [49–51].

Plk1 is upregulated in a range of human tumours; thus targeting Plk1 is an attractive therapeutic strategy [45] and a number of inhibitors are under clinical evaluation including BI2536 [46, 47]. Furthermore, agents that disrupt the centrosome cycle in tumour cells, through centrosome amplification and centrosome declustering promote multipolar mitosis, genome instability, and mitotic catastrophe [55–57].

A number of kinesin motor protein inhibitors have been developed [37, 124]. Monastrol is a selective inhibitor of the kinesin-5 motor protein (KSP, also termed Eg5). Eg5 inhibition leads to mitotic arrest and death in tumour cells in culture and in xenograft models. Furthermore, they were found to be free from severe cytotoxic effects and are generally well tolerated [37]. Eg5 inhibitors under clinical development include AZD4877 [38], Ispinesib [39, 40], and ARRY-520 [41]. Eg5 inhibition is also effective in targeting taxol resistant cancers [42]. CENP-E (centrosome-associated protein-E) is a microtubule motor that plays a role in mitosis. The small molecule CENP-E inhibitor, GSK923295, induces defective mitosis and displays antiproliferative effects *in vivo* [43] and has recently entered clinical trial [44].

Cancer cells often harbour a deficient or defective G1 checkpoint due to aberrant p53 signalling, which ultimately leads to increased DNA damage at the G2 checkpoint compared to normal cells [125]. Based on this, the G2 checkpoint has emerged as an attractive anticancer target. Abrogation of the G2 checkpoint allows cells with unrepaired DNA damage to proceed into a premature M phase [59]. Thus, cancer cells that are defective in G1 and G2 checkpoints will undergo mitotic catastrophe following DNA damage induced by radiation, alkylating agents, and doxorubicin [113]. The Chk1 kinase inhibitors, UCN-01 and AZD7762, abrogate the G2 checkpoint and potentiate death in p53-deficient tumours [58, 60, 61] and are currently in clinical development [62, 63]. Evaluation of a panel of therapeutic agents in combination with Chk1 inhibition highlights that the precise drug combinations are important and influence the outcome in a particular genetic background and when treating a certain tumour type [126]. In addition to Chk1, targeting WEE1 kinase activity together with DNA damage can effectively induce mitotic catastrophe [127]. Furthermore, histone deacetylase (HDAC) inhibitors promote mitotic catastrophe and cell death and have shown promise in multiple myeloma and glioma treatments [64–66].

Despite the promising preclinical data displayed by new generation antimitotic agents, their clinical efficacy has been disappointing in comparison to microtubule targeting agents [37, 128]. This may be explained by the shorter doubling time of cells in culture compared to patients [128] and by differences in drug retention times [129]. It has also been suggested that the success of microtubule targeting agents may be due to nonmitotic function of microtubules [130]. Furthermore, the development of resistance to antimitotic agents represents a major challenge that occurs following mitotic slippage when defective cells adapt and survive [131], although in some cases mitotic slippage is required for cell death [132]. Based on this observation, recent research has focused on strategies to block slippage and mitotic exit in order to maximise mitotic arrest-induced death [37]. Such approaches include targeting APC-Cdc20 to prevent cyclin B degradation and mitotic exit that have shown a very promising response [52–54]. An alternative approach is the inhibition of cytokinesis which blocks mitotic exit in postanaphase cells and may be an attractive strategy to overcome resistance in slippage prone cells. Small molecule dynamin GTPase inhibitors have shown antiproliferative effects and induce cytokinesis failure and cell death in cancer cells [133].

It is clear that mitotic catastrophe is an important anticancer strategy that is achieved by a variety of mechanisms that target the cell cycle. Although these approaches target proteins that are upregulated in cancer cells, thereby providing a therapeutic window to preferentially kill the cancer cells, they are not specific to cancer cells and are likely to be accompanied by some side effects. Recent reports highlight that the myc oncogene regulates mitotic events to support its oncogenic program [134, 135] and one way that this may occur is through transcriptional regulation of aurora kinase expression [136, 137]. Moreover, loss of myc activity due to inhibition of sumoylation [134] or transcriptional inactivation by omomyc [135] led to mitotic catastrophe and cell death in *in vivo* models of breast cancer and glioma, respectively. Thus, targeting myc activity, using approaches that inhibit sumoylation and/or mimic omomyc action, represents new approaches to selectively induce mitotic catastrophe in cancer cells. Furthermore, a better understanding of the postmitotic signals that connect to the cell death and senescence pathways will reveal new approaches to push cells down a defined antiproliferative route and is likely to synergise with current

antimitotic drugs to kill cancer cells before adaption and the development of drug resistance. These new approaches may provide more effective strategies to exploit mitotic catastrophe in cancer prevention and treatment.

Conflict of Interests

The author declares that there is no conflict of interests regarding the publication of this paper.

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