Gene silencing and mitochondrial signalling

D. DE STEFANI [1, 2], A. ROMAGNOLI [1, 2], R. RIZZUTO [1, 2], P. PINTON [1, 2]

[1]*Section of General Pathology*
Department of Experimental and Diagnostic Medicine
Interdisciplinary Center for the Study
of Inflammation (ICS), Ferrara, Italy
[2]*Emilia Romagna Laboratory BioPharmaNet*
University of Ferrara, Ferrara, Italy

Upon physiological stimulation, mitochondria undergo a major rise in mitochondrial [Ca^{2+}], well in the range of the Ca^{2+} sensitivity of the matrix dehydrogenases, in a wide variety of cell types, ranging from non excitable, e.g. HeLa and CHO, and excitable, *e.g.* cell lines to primary cultures of various embryological origin, such as myocytes, adipocytes and neurons. This review briefly summarizes a few basic features of mitochondrial Ca^{2+} handling and its involvement in the modulation of Ca^{2+}-dependent signaling. Afterwards, the role of mitochondrial Ca^{2+} in the control of apoptotic cell death and during oxidative stress is discussed. Finally, data will be presented on RNA interference techniques to study mitochondrial calcium signalling.

Key words: **Gene silencing - Mitochondria - Apoptosis.**

Mitochondria, calcium and apoptosis

With a bacterial evolutionary origin, mitochondria have become perhaps the ultimate symbiont, preserving their own DNA while also deriving many important proteins from the nuclear DNA of the host cell. While they may maintain a modicum of independence from the host cell in some respects, they nevertheless lie at the heart of the life of almost all eukaryotic cells. Mitochondria have long been known to be the major site for aerobic production of intracellular ATP in eukaryotic cells; however, their role in intracellular Ca^{2+} signalling has had a much more checkered past.

The view that mitochondrial Ca^{2+} transport was central to intracellular Ca^{2+} signalling, which was pervasive up until the late 1970s, was summarily dismissed in the 1980s when the ubiquitous Ca^{2+}-releasing messenger inositol 1,4,5-trisphosphate ($InsP_3$) was found to mobilize Ca^{2+} specifically from the endoplasmic reticulum. In addition, use of the newly available Ca^{2+}-sensitive fluorescent dyes revealed that mitochondria released very little Ca^{2+} into the cytosol. Furthermore, intracellular Ca^{2+} concentration could reach, at least globally, the micromolar range upon stimulation, levels considerably smaller than the apparent K_m for mitochondrial Ca^{2+} uptake (10-20 μM). All of this changed in the mid-1990s, and mitochondria have now returned with a vengeance to occupy a key position in intracellular Ca^{2+} dynamics.[1]

*The first two authors equally contributed to this work.

Fundings.—This work was supported by the Italian Association for Cancer Research (AIRC), Telethon, local funds from the University of Ferrara, the Italian Ministry for the University, the EU (fondi strutturali Obiettivo 2), the PRRIITT program of the Emilia Romagna Region, the Italian Space Agency (ASI), NIH (Grant #1P01AG025532-01A1) and the United Mitochondrial Disease Foundation (UMDF).

Received on June 3, 2008.
Accepted for publication on June 3, 2008.

Address reprint requests to: P. Pinton, Department of Experimental and Diagnostic Medicine, Section of General Pathology, via Borsari 46, 44100 Ferrara, Italy. E-mail: pnp@unife.it

Underpinning this mitochondrial renaissance was the localization of genetically targeted Ca^{2+}-selective probes (like the bioluminescent protein aequorin) or fluorescent dyes like rhod-2 to the mitochondrial matrix, which provided a direct and unambiguous readout of mitochondrial Ca^{2+} changes in living cells following receptor stimulation.[1] In addition, following an increase in cytosolic Ca^{2+} to the micromolar range, careful single-cell analysis revealed that the rate of recovery of the Ca^{2+} signal to prestimulation values was sculpted by mitochondrial Ca^{2+} buffering.[2]

At the end of last decade, there was a change from a paradigm in which the nucleus determined the apoptotic process to a paradigm in which mitochondria constitute the centre of death control. It is now well established that mitochondria play a critical role in the regulation of apoptosis by acting as reservoirs for a multitude of apoptogenic proteins, such as cytochrome *c*, Smac/Diablo (second mitochondria-derived activator of caspases/direct IAP binding protein), apoptosis inducing factor (AIF), endonuclease G and procaspases-2, -3, -8, -9. The release of these proteins inevitably leads to a wide array of the morphological hallmarks of apoptosis-ranging from nuclear condensation to the exposure of the phosphatidylserine at the surface of the dying cell. Therefore, it seems that the mitochondria act as the receiving platform, integrating a range of apoptotic stimuli and relaying these to the appropriate downstream signalling cascades. It is possible to assert firmly that the fundamental role played by mitochondria in cellular signalling is now beyond question.

Mitochondria and calcium signalling

The existence of a pathway that allows mitochondria to accumulate Ca^{2+} has been established for about 40 years. Nevertheless, only recently has a consensus begun to emerge concerning the physiological significance of the pathway. Work carried out in isolated mitochondria in the 1960s and 1970s characterized the fundamental properties of Ca^{2+} transport in mitochondria, which, apart from the recent electrophysiological demonstration that the mitochondrial Ca^{2+} uniporter (MCU) is a bona fide channel,[3] still represent the current knowledge of the process. Ca^{2+} is accumulated into the matrix through a Ruthenium red-sensitive electrogenic route (the MCU) and re-extruded, in exchange with monovalent cations (H^+ or Na^+), by two antiporters that prevent the attainment of electrical equilibrium (that would imply, for a mitochondrial membrane potential, $B\Psi\mu$, of 180 mV and a cytosolic Ca^{2+} concentration of 0.1 μM, accumulation of Ca^{2+} into the matrix up to 0.1 M). It was thus logical to assume that mitochondria were loaded with Ca^{2+}, possibly releasing it in a number of physiological and/or pathological conditions.

However, when in the 1980s Ca^{2+} homeostasis emerged as a ubiquitous signalling route, characterized by a remarkable spatiotemporal and molecular complexity, the role of mitochondria took the opposite route, and was greatly downplayed to a that of a low-affinity, high capacity sink coming into action only in the case of major cellular overload with Ca^{2+} (*i.e.* in pathophysiological conditions). Indeed, it became apparent that the endoplasmic reticulum (ER), and not the mitochondria, are the source of rapidly mobilizable Ca^{2+}: it possesses a Ca^{2+} pump for accumulating Ca^{2+}, and the inositol-1,4,5-trisphosphate receptor (IP_3R) for rapidly releasing it upon cell stimulation. At the same time, mitochondria did not appear an important target of the released Ca^{2+}, as the $[Ca^{2+}]$ reached in the cytoplasm (2–3 μM at the peak) appeared well below those required for rapid accumulation by the low-affinity MCU.

This situation was completely reversed when novel gene-encoded targeted probes allowed to unambiguously measure the $[Ca^{2+}]$ of the mitochondrial matrix ($[Ca^{2+}]_m$). This was first achieved by targeting to mitochondria a Ca^{2+}-sensitive photoprotein, aequorin, that allowed to demonstrate that a rapid $[Ca^{2+}]_m$ peak, reaching values well above those of the bulk cytosol, parallels the $[Ca^{2+}]$ rise evoked in the cytoplasm by cell stimulation.[4] Thus, in the 1990s was confirmed that mitochondria promptly respond to cytosolic $[Ca^{2+}]$ rises, but also that the $[Ca^{2+}]c$ oscillations, the typical response to agonists of many cell types, are paralleled by rapid spiking of $[Ca^{2+}]_m$, thus specifically decoding a frequency-mediated signal within the mitochondria, as clearly shown in hepatocytes,[5] cardiomyocytes [6] and HeLa cells.[7]

Ca^{2+} is a key regulator of mitochondrial function and acts at several levels within the organelle to stimulate ATP synthesis. Most of the mitochondrial effects of Ca^{2+} require its entry across the double membrane into the matrix. Although the mitochondrial outer membrane was thought to be permeable to Ca^{2+}, recent studies suggest that the outer membrane voltage-dependent anion channel (VDAC) serves to reg-

ulate Ca^{2+} entry to mitochondrial intermembrane space.[8]

Furthermore, transport across the inner membrane is highly regulated. Mitochondria take up Ca^{2+} primarily through a uniporter, whose molecular nature still eludes us. Some data suggest that this might act like a channel, opening with increased probability once the local $[Ca^{2+}]_c$ rises.[9] The influx of Ca^{2+} into the matrix by this route is dependent on the electrochemical potential gradient for Ca^{2+}. This is developed and maintained firstly by the process of mitochondrial respiration, which establishes a large potential gradient, the mitochondrial membrane potential (??m), generally estimated to be in the order of 150-200 mV or so negative to the cytosol, together with a low resting intramitochondrial Ca^{2+} concentration $[Ca^{2+}]_m$, maintained primarily by the mitochondrial Na^+-Ca^{2+} exchanger. The experimental collapse of ??m using an uncoupler is a simple and much used experimental tool to explore the consequence of preventing mitochondrial Ca^{2+} accumulation. Similarly, the collapse of ??m as a response to pathological states during anoxia or as a response to disordered mitochondrial respiration, for example will also limit mitochondrial Ca^{2+} uptake and may contribute to the emergent cellular pathophysiology. This pathway has often been referred to as the Ruthenium Red (RuR)sensitive uptake pathway, despite the very poor specificity of RuR, which inhibits Ca^{2+} flux through a variety of different channels. In addition to this uniporter, another pathway has been described recently as the rapid uptake pathway,[10] proposed by the authors as a mechanism for the rapid uptake of Ca^{2+} at physiological concentrations. The comparative cell physiology and expression of this mechanism remain to be evaluated. The reequilibration of mitochondrial Ca^{2+} is largely achieved through the activity of the mitochondrial Na^+-Ca^{2+} exchanger, an exchanger distinct from that found in the plasmalemma, and Na^+ is then exchanged for protons through a rapid Na^+-H^+ exchange. It has been suggested that during hypoxia, mitochondria may become Ca^{2+} loaded by the reversal of the Na^+-Ca^{2+} exchanger.[11] This would require that the intracellular Ca^{2+} concentration must be very high, that mitochondria must also be Na^+ loaded and that the rapid Na^+-H^+ exchange be suppressed.

The primary role of mitochondria Ca^{2+} is the stimulation of ox-phos and the control of organelle metabolic activity. Three crucial metabolic enzymes within the matrix, pyruvate, ?-ketoglutarate and isocitrate dehydrogenases, are activated by Ca^{2+}, by two distinct mechanisms. In the case of pyruvate dehydrogenase, a Ca^{2+}-dependent dephosphorylation step is involved; in the other two cases, the activation is through direct binding of Ca^{2+} to the enzyme complex.[12] Given that these enzymes represent the rate limiting step for feeding electrons into the respiratory chain, Ca^{2+} within the matrix is ultimately the positive modulator of mitochondrial ATP synthesis; this aspect was directly addressed by the authors a few years ago using targeted recombinant luciferase to monitor, in living cells, the ATP concentration within the cytoplasm and the mitochondrial matrix.[13]

Many other mitochondrial functions are also regulated by Ca^{2+}. For example, Ca^{2+} activation of N-acetylglutamine synthetase generates N-acetylglutamine, a potent allosteric activator of carbamoyl-phosphate synthetase, the rate-limiting enzyme in the urea cycle. Mitochondria can also exert a more classical buffering role. Petersen *et al.* demonstrated that in pancreatic acinar cells mitochondria strategically located beneath the granular region prevent the spreading of a Ca^{2+} wave from the secretory pole towards the basolateral region by accumulating Ca^{2+}.[14]

While the above mentioned roles are more or less similar in every cell type, there are also other roles of mitochondrial Ca^{2+} that appear to be tissue specific. The two examples best studied are the endocrine pancreas, where mitochondrial Ca^{2+} modulates insulin secretion, and the granulosa cells of the adrenal gland, where it controls a key step in aldosterone synthesis.[15, 16] A fast response of $[Ca^{2+}]_m$ to $[Ca^{2+}]_c$ requires, to be efficient and productive, a rapid Ca^{2+} efflux from the mitochondrial matrix, and several mechanisms exist for this purpose. Primarily, Ca^{2+} efflux is achieved by exchange through the Na^+, which is in turn re-extruded out of the matrix in exchange with protons. Thus both Ca^{2+} uptake and efflux from mitochondria consume ??m and are therefore reliant on H+ pumping by the respiratory chain to maintain this driving force (Na^+/ Ca^{2+} and H^+/ Ca^{2+} uniporters). In addition to these pathways of Ca^{2+} efflux, an additional mechanism exists in the form of the permeability transition pore (PTP). The PTP is assembled from a group of pre-existing proteins in the mitochondrial inner and outer membranes [17] with Ca^{2+} binding sites on the matrix side of the inner membrane believed to regulate pore activity. Normally, "flickering" of the PTP between open and closed states serves to release Ca^{2+} from the matrix. However, prolonged PTP opening due to

$[Ca^{2+}]_m$ overload can result in pathological consequences.

Mitochondria and apoptosis

Organized life requires cell death, and execution of cell death relies on the very machinery of life. Cell death by apoptosis (from Greek: falling off, figurative for the falling of leaves; also termed programmed cell death type I) means an orchestrated collapse of a cell, staging cell shrinkage, chromatin condensation, DNA and protein cleavage, fragmentation of the apoptotic cell accomplished by phagocytosis of corpses by neighbouring cells.[18] Apoptosis is a form of programmed cell death and an outline of the relevant signalling pathways at the molecular level is now well established. Mammalian cells possess two major apoptotic signalling pathways, which are known as the intrinsic pathway and the extrinsic pathway.[19]

Mitochondria are involved in the intrinsic pathway, with an increase of outer mitochondrial membrane permeability that leads to the release of various proteins from the intermembrane space into the cytoplasm, including apoptogenic molecules such as cytochrome c, Smac/Diablo, HtrA2 (Omi), AIF, and DNaseG. In the presence of ATP (dATP), cytochrome c binds to Apaf-1 and triggers its oligomerization, after which pro-caspase-9 is recruited and undergoes autoactivation. The protein complex comprising cytochrome c, Apaf-1, and caspase-9 is called the "apoptosome". In short, an increase of outer mitochondrial membrane permeability is central to apoptosis and mitochondrial membrane permeability is directly regulated by the Bcl-2 family of proteins. However, the detailed mechanisms underlying the increase of outer mitochondrial membrane permeability during apoptosis and how this process is controlled by Bcl-2 family members are still to be determined. The model that was initially developed to explain the apoptotic increase of mitochondrial membrane permeability was based on the "mitochondrial membrane permeability transition", an event which has been appreciated for some time among investigators studying the mitochondria. Mitochondria, the organelles that produce energy through cellular respiration, integrate death signals mediated by proteins belonging to the Bcl-2/Bax family, and kill cells by releasing critical factors such as cytochrome c that activate executioner caspase proteases. Ca^{2+} can be turned into death signals when delivered at the wrong time and place. Mitochondria eventually decide whether Ca^{2+} signals are decoded as life or death signals, but it is not clear whether Ca^{2+} is an additional stress factor that "tips the balance" or is an obligatory signal for death. Because of the toxicity of Ca^{2+} ions, a low Ca^{2+} concentration must be maintained in the cytoplasm, and most of the cellular Ca^{2+} is stored in the ER. Ca^{2+} is pumped into the ER by SERCA ATPases (adenosine triphosphatases) and is released only transiently during bouts of signaling by the opening of inositol 1,4,5-trisphosphate (IP_3) or ryanodine receptor (RyR) Ca^{2+}-release channels. A significant fraction of the released Ca^{2+} is captured by mitochondria, which are strategically located near Ca^{2+}-release channels. This ER-mitochondria connection enables Ca^{2+} signals not only to finetune cellular metabolism but also to modulate the ability of mitochondria to undergo apoptosis. The switch from a life to a death signal involves the coincidental detection of Ca^{2+} and proapoptotic stimuli, and depends on the amplitude of the mitochondrial Ca^{2+} signal. Several studies indicate that the Ca^{2+} content of the ER determines the cell's sensitivity to apoptotic stress. Procedures that decrease the Ca^{2+} concentration in the ER, such as genetic ablation of the ER Ca^{2+}- buffering protein calreticulin or overexpression of plasma membrane Ca^{2+} ATPases, protect cells from apoptosis. Conversely, procedures that increase the ER Ca^{2+} load, such as overexpression of SERCA or calreticulin, sensitize cells to apoptotic stress.[20, 21] Sensitivity to apoptosis correlates with the total ER Ca^{2+} load, rather than with the free ER Ca^{2+} concentration, and depends on the ability of cells to transfer Ca^{2+} from the ER to the mitochondria. Accordingly, procedures that enhance the transfer of Ca^{2+} from the ER to mitochondria augment ceramide induced cell death. It was proposed that mitochondrial Ca^{2+} overload together with oxidative stress is a potent stimulus for the PTP opening which in turn seems to represent the major route for the release of the caspase co-factors.[22] It is not yet clear how increases in $[Ca^{2+}]$, which regulate a diverse range of cellular processes, can be a signal for both physiological events as well as cell death.[23] Interestingly on this topic, Hajnoczky *et al.* recently showed how apoptotic stimuli induce a switch in mitochondrial calcium signalling at the beginning of the apoptotic process. They proposed that in cells exposed to proapoptotic stimuli the Ca^{2+} sensitivity of

PTP increases and that cell stimulation with an $InsP_3$-generating agonist induces $[Ca^{2+}]_m$ transients which in turn cause the PTP opening. In their model prolonged exposure to proapoptotic stimuli or large Ca^{2+} overload are not necessary to induce cell death, but rather PTP opening is the consequence of a coincident detection of short-living signals: it is dependent on a privileged Ca^{2+} signal transmission between $InsP_3$ receptor and mitochondria,[24] in addition to a yet unidentified direct effect of the proapoptotic lipid on mitochondria. Moreover, mitochondrial derangements have been shown to be preliminary signs of ceramide-induced apoptosis that is prevented by all the experimental conditions that reduced the amount of Ca^{2+} releasable from the intracellular stores.[20] Ca^{2+} elevation mediates the induction of apoptosis with a wide range of initiating stimuli. Cytoplasmic Ca^{2+} elevation triggers many pathways to apoptosis, including the activation of proteases and endonucleases. The understanding that cytoplasmic Ca^{2+} elevation induces apoptosis evolved following the seminal findings of Kaiser and Edelman,[25] which indicated that Ca^{2+} elevation mediates the death of immature lymphocytes in response to glucocorticosteroid hormones. A critical role for Ca^{2+} elevation in glucocorticoid-induced apoptosis is now well established [26] and strengthened by evidence that the Ca^{2+} elevation is initiated by the release of Ca^{2+} from the ER and sustained by increased extracellular Ca^{2+} entry. In many cases, proapoptotic Ca^{2+} elevation is generated by Ca^{2+} efflux from the ER *via* IP_3 receptors.[27] This understanding comes from studying the role of Ca^{2+} signaling in lymphocyte cell death. Antisense-mediated repression of IP_3 receptor expression inhibits glucocorticosteroidinduced apoptosis in lymphocytes.[28] Also, antisense-mediated knockdown of IP_3 receptors in Jurkat T cells inhibits apoptosis following TCR activation.[29]

Similarly, consistent with the central importance of mitochondria in apoptosis,[30] considerable attention has been given to apoptosis induction by the redistribution of Ca^{2+} from the ER to mitochondria. The close proximity of the ER to mitochondria facilitates the transfer of Ca^{2+}.[31] Thus, mitochondria play an important role in Ca^{2+} homeostasis and signaling by buffering cytoplasmic Ca^{2+} elevation and modulating Ca^{2+} signals. Also, Ca^{2+} uptake by mitochondria activates oxidative metabolism, which generates high concentrations of ATP and favours cell survival. But IP_3-mediated Ca^{2+} release can also elevate mitochondrial Ca^{2+} concentration sufficiently to induce apoptosis.[20] The opening of IP_3 receptors on the ER can expose the mitochondrial Ca^{2+} uniporter to Ca^{2+} concentrations 20-fold higher than elsewhere in the cytoplasm. Thus, mitochondrial Ca^{2+} signals evoked by IP_3-mediated Ca^{2+} elevation trigger the opening of a mitochondrial permeability transition pore and, in turn, cytochrome c release.[32] Importantly, the permeability transition pore reseals after the Ca^{2+} signal decays, providing an efficient mechanism to establish caspase activation while mitochondrial metabolism is maintained to provide the ATP necessary for the apoptotic process. In many forms of apoptosis, activated Bax or Bak permeabilizes the outer mitochondrial membrane and releases cytochrome c in a Ca^{2+}-independent manner. Ca^{2+} cross talk between the ER and mitochondria mediates apoptosis in response to a variety of initiating factors.

Apoptosis constitutes an essential part of life for any multicellular organism during embryonic development as well as in adulthood; it counterbalances mitosis to maintain cell number homeostasis, it also serves as a protective mechanism to eliminate damaged or (pre)cancerous cells; too little or too much apoptosis is involved in a number of human and animal pathologies incl. infectious diseases, neurodegenerative diseases and cancer; in turn, apoptosis constitutes a major target for prevention or treatment of diseases and for assuring quality of life.

Consequently, apoptosis and mitochondria with it, moved into focus of biomedical research and in the past decade a tremendous progress in elucidation of the underlying molecular mechanisms has been achieved.[33, 34]

Mitochondria and reactive oxygen species

The important role of redox signalling in the regulation of physiological responses is underscored by the apparent dysregulation of physiological responses in various disease-related oxidative stress conditions. Excessive levels of reactive oxygen species (ROS) may be generated by mechanisms that produce ROS "accidentally" in an unregulated fashion. This includes the production of ROS by the mitochondrial electron transfer chain, the quantitatively most important source of ROS in higher organisms. These chemical species are characterized by the presence of an unpaired electron on the oxygen atom that can promptly react with

virtually any biomolecules. Thus mitochondrial structures are particularly susceptible to oxidative damage as evidenced by lipid peroxidation, protein oxidation, and mitochondrial DNA mutations.[35] ROS have been implicated in many pathological conditions, in particular in the ageing process. Indeed, the "free radical theory" of ageing has a long history and it has been originally proposed in the 1950s.[36] This hypothesis was initially hotly debated, at least until the discovery of the first cellular enzyme involved in ROS metabolism, superoxide dismutase (SOD).[37] The existence of a protein whose unique function was to scavenge oxygen free radicals represented the first indirect but strong evidence that cells not only produce ROS but they also need systems to protect against them. ROS are generated by many enzymes, such as cyclooxygenases and NADPH oxidases, and in different subcellular compartments (*i.e.* they are generated by lipid metabolism within peroxisomes). However, the large majority of total ROS are undoubtedly produced by mitochondria, since they are a direct consequence of oxidative phosphorylation.[38] Indeed, at different sites along the mETC (in particular at complex I and III) electrons can "escape" and react directly with molecular oxygen, thus generating superoxide anions. ROS detoxifying enzymes represent the first line of defense against free radicals. SOD is today known to exist in two different isoforms: while SOD1 is a copper containing enzyme present is the cytosol, SOD2 (a manganese containing protein) is located inside mitochondrial matrix where it converts superoxide anion to H_2O_2 which can be further degraded to water and oxygen by catalase. The number of ROS detoxifying enzymes grew very fast in last few decades and includes the large family of glutathione peroxidases (GPx) and peroxiredoxins (Prx), which was recently reported to exist in mitochondrial matrix (Prx III).[39] Mitochondria also have another mechanism to protect against ROS. Indeed, the uncoupling of oxidative phosphorilation through the action of UCP proteins and thus the decrease of mitochondrial membrane potential shortens the half-life of the most reactive steps in the electron transport chain, thereby inhibiting ROS production.[40] Thus, given that prevention is better than the cure, mitochondria can "decide" to slow down their metabolism to prevent oxidative damage. This fact is shiningly demonstrated by observing that the above mentioned PGC1α knockout mice show a much higher sensitivity to oxidative damage, especially in neurons.[41] This means that PGC1α not only promotes mitochondrial biogenesis and oxidative metabolism but it coincidentally takes care of the potentially harmful effect of ROS induction. This is achieved by the two described mechanisms: on one side by increasing ROS scavenging enzymes (SOD1, SOD2, catalase and GPx) and on the other by decreasing ROS production (through the induction of UCPs).

By the way, cells have always been forced to cohabit with free radicals. Thus, it is not unworthy to wonder whether this harmful chemical species could also be exploited to participate in physiological regulation of normal cellular events. Indeed, one of the most fascinating hypotheses is that ROS, besides their obvious toxic effect, could even participate in signal transduction. This notion is supported by recent works on the role of p66shc, the first mammalian protein whose mutation was demonstrated to increase resistance to oxidative stress and to prolong life span.[42] Intriguingly, upon activation, including phosphorylation by protein kinase C (PKC) isoform β (PKCβ) and Pin1 recognition, p66shc translocates to mitochondria [43] (and see below) where it exerts its own oxidoreductase activity.[44] Indeed, p66shc directly oxidizes cytochrome c (thus allowing electron to escape mETC) and generates H_2O_2, leading to mitochondrial permeability transition pore opening (mPTP) and in turn cell death. The existence of a protein that "steals" electrons from the mETC and produces reactive oxygen species represents the first molecular evidence of the role of reactive oxygen species in signal transduction, finally describing the biochemical basis of the free radical theory of ageing.

Interestingly other well studied proteins such as p53, PKC and Apurinic-apyrimidinic endonuclease/Redox effector factor (Ape/Ref-1) play an important role in ROS mediated pathways and translocate to mitochondria during redox stimulation.[45-47]

Tools for gene silencing

In 1998 Fire and Mello demonstrated that the introduction of double-stranded RNA molecules (dsRNA) into *Caenorhabditis elegans* could mediate sequence-specific gene silencing, leading to the Nobel prize awarded discovery of RNA interference (RNAi).[48] In the last 10 years this phenomenon has been extensively exploited to study gene function, becoming a rou-

tinely used technique in many laboratories. Indeed, classical gene targeting by homologous recombination is an expensive and time consuming process, many organism cannot be easily manipulated and in several cases such an approach lead to a lethal phenotype. Conversely, genetic knockdown through RNAi initiated a revolution in cell biology, allowing rapid and inexpensive analysis of gene function in virtually any cell type.

Biochemically, small interfering RNAs (siRNAs) are 21-23 nucleotide long dsRNA with 2-3 nt 3' overhangs, 5' phosphate groups and 3'OH ends. These structures are the products of the Dicer family of RNAse III enzymatic cleavage, and are generated from longer dsRNA molecules. Then, siRNAs enter the RNA-induced silencing complex where the duplex is unwound and the antisense strand target its specific mRNA which is cleaved at a single site in the center of the mRNA-siRNA duplex.[49]

RNAi can be easily obtained in many organisms, such as Drosophila, Mosquitoes, Trypanosoma, Caenorhabditis and plants, by introducing long dsRNA containing various sequences of the target mRNA.[50-52] Unfortunately this approach cannot be applied in mammals, since the introduction of dsRNA longer than 30 nt causes a nonspecific interferon response.[53] However, Tuschl group demonstrated that the direct introduction of 21 nt siRNA into mammalian cells, thus bypassing the Dicer cleavage, can efficient knockdown its target mRNA, leading to the widespread use of RNAi in cell biology.[53] However, chemically synthesized siRNAs show several limitation in everyday use, such as the transient nature of the silencing, the need to use lipid based transfection reagents and the impossibility to be grown in bacteria to produce large amounts of nucleic acids, making this technique quite expensive. In order to overcome these limitations, several groups developed some DNA vectors capable to express substrates which can be efficiently converted into siRNAs in living cells. The most successful approach is to express a short hairpin RNA (shRNA) structure under the control of a RNA polymerase III promoter (like U6 or H1), since Dicer has been shown to be able to process hairpin RNA structures both *in vitro* and *in vivo*. Finally, to overcome the shortcomings of standard DNA-based vectors (*e.g.* the need of transfection and the low efficiency of delivery in some primary cell cultures), retroviral and lentiviral vectors have been developed based respectively on the Moloney murine leukemia virus (MoMuLV) or the murine stem cell virus (MSCV) and on the human immunodeficiency virus-1 (HIV-1). Moreover, recent advances in large scale oligonucleotide synthesis and the development of highly efficient high-throughput technologies led to the ability to construct large arrayed shRNA libraries, covering the most part of mouse and human genomes. The group of Elledge and Hannon created an RNAi Library comprised of multiple short-hairpin RNAs (shRNAs) specifically targeting annotated human genes.[54] The shRNA Library permits rapid, cost-efficient, loss of-function genetic screens and rapid tests for genetic interactions to be performed in mammalian cells. Such a technology thus represents an extraordinary powerful tool to study virtually any cellular event at genome-wide level.

Most of the work conducted in the lab with RNAi is based on a commercial solution, the pSUPER RNAi system. Such a technology provides a mammalian expression vector that directs intracellular synthesis of siRNA-like transcripts.[50] The vector uses the polymerase-III H1-RNA gene promoter, as it produces a small RNA transcript lacking a polyadenosine tail and has a well-defined start of transcription and a termination signal consisting of five thymidines in a row. Most important, the cleavage of the transcript at the termination site is after the second uridine, yielding a transcript resembling the ends of synthetic siRNAs, which also contain two 3' overhanging T or U nucleotides. The pSUPER RNAi System has been used to cause efficient and specific down-regulation of gene expression, resulting in functional inactivation of the targeted genes. Stable expression of siRNAs using this vector mediates persistent suppression of gene expression, allowing the analysis of loss-of-function phenotypes that develop over longer periods of time.

RNA interference techniques to study mitochondrial calcium signalling

Ca^{2+} channeling properties of the mitochondrial porin VDAC1

Many efforts of this research group are focused on deciphering the molecular machinery regulating cellular calcium homeostasis, and in particular on the comprehension of the role of mitochondrial calcium in several cellular events. One of the key component of mitochondrial Ca^{2+} uptake mechanism is the Voltage-Dependent Anion Channel (VDAC), the most

abundant protein located on the outer mitochondrial membrane, which functions also as a Ca^{2+} channel. Indeed it was previously demonstrated that the overexpression of VDAC1 in mammalian cells can effectively stimulate mitochondrial Ca^{2+} accumulation, without affecting Ca^{2+} homeostasis in other cellular compartments (such as cytoplasm or endoplasmic reticulum). In particular, VDAC1 seems crucial for the efficient transfer of high [Ca^{2+}] microdomains formed at the release site on the endoplasmic reticulum (*i.e.* at the mouth of IP_3R).[8] However, it was also observed that the generic overexpression of mitochondrial porins could lead to a nonspecific toxic effect when protein levels are too high, limiting the possibility of studying its function. For this reasons it was decided to use RNAi to knockdown VDAC levels, and observed that genetic silencing is a way better approach when compared to crude overexpression. Indeed, by designing a specific shRNA against VDAC1 it was possible to demonstrate that VDAC1 knockdown cells have a lower mitochondrial Ca^{2+} response after stimulation and are protected against some apoptotic stimuli, thus confirming and enforcing data obtained through VDAC1 overexpression (unpublished data).

Physical coupling of endoplasmic reticulum and mitochondrial Ca^{2+} channels

Mitochondria and ER of eukaryotic cells form two entwined endomembrane networks, and their dynamic interaction controls metabolic flow, protein transport, intracellular Ca^{2+} signalling, and cell death. Vast knowledge on ER-mitochondrial interaction stems from the functional analysis of Ca^{2+} signal transmission between these organelles. However, the structural fundaments and the molecular determinants of this crosstalk, are still largely unknown. Recently, Hajnóczky group showed the existence of a physical linkage between this two organelles, demonstrating that their association is due to the presence of peptidic tethers that link both smooth and rough ER to the mitochondria.[55] At the same time, a biochemical study was carried out to find out the putative VDAC interactors and it was demonstrated that VDAC1 is physically linked to the endoplasmic reticulum Ca^{2+} release channel inositol 1,4,5-trisphosphate receptor (IP_3R), through the molecular chaperone grp75, and this interaction sustains the efficient Ca^{2+} transfer from the endoplasmic reticulum to mitochondria. When studying higher order protein complexes, where the stoichiometry of the elements involved is crucial for the correct function of the macromolecular complex, RNAi approach is often preferable to overexpression. Indeed, the simple upregulation of only one part of the system doesn't lead to any apparent effect. Conversely, the knockdown by RNAi of a single component of the complex can promptly influence its function. Thus the grp75 gene was silenced to demonstrate that the presence of this molecular chaperone is of crucial relevance for the correct coupling of endoplasmic reticulum and mitochondrial calcium channels.[56]

The silencing of PKCβ in the working-out of aging puzzle

As already mentioned, mitochondria are implicated in aging phenomenon especially as source of free radicals. A protein firmly know for its role in determination of organism life-span is the 66-kilodalton isoform of the growth factor adapter Shc (p66Shc).[57] This protein translates oxidative damage into cell death, indeed a fraction of p66Shc localizes to mitochondria where it binds to cytochrome c and acts as oxidoreductase, generating ROS and leading to organelle dysfunction and cell death.[44] In a recent work,[43] this same group studied this protein to find the signalling link between cellular stress and its mitochondrial proapoptotic activity since there were some obscure points regarding p66Shc action's mechanism. An important aspect to consider is that although Serine phosphorylation is critical for p66Shc, the p66Shc mitochondrial form is unphosphorylated, indicating that additional regulatory elements must exist. Different technique were used to identify the molecular route that link an oxidative challenge to the activation of p66Shc, one of which was the siRNA, object of this review. Mitochondria receive, under stimulation by physiological agonists or toxic agents, Ca^{2+}-mediated inputs that are decoded into effects as diverse as metabolic stimulation and apoptosis. Ca^{2+} responsiveness is highly sensitive readout of mitochondrial state: partial defects in mitochondrial energization, as in mitochondrial disease, cause defects in Ca^{2+} handling by the organelle. Moreover, mitochondrial Ca^{2+} uptake is modulated by regulatory proteins such as kinases. In a previous work,[58] authors studied the effect of different member of PKC on Ca^{2+}-signalling. They observed that some isoforms had a mitochondrial

specific effects, for example, the over-expression of PKCβ caused a reduction in $[Ca^{2+}]_m$ and participated into the desensitization of mitochondrial Ca^{2+} uptake.[58]

For the purpose, they initially started to study effect on p66Shc-absence on Ca^{2+} signalling using mouse embryonic fibroblast (MEF) wt and p66Shc-/-. It was demonstrated a close similarity in the global Ca^{2+} signalling pattern between the two cell lines in normal condition. Instead, after treatment with H_2O_2 minor changes in the Ca^{2+} response and mitochondria morphology in MEF p66Shc-/- were visible. This means that without p66Shc the effect of H_2O_2 on mitochondria disappears. Prior data suggested that H_2O_2 actives PKCβ,[59] PKCβ affects mitochondrial Ca^{2+} uptake, and finally that hispidin (a specific blocker of PKCβ) inhibits p66Shc phosphorylation. Thus, the silencing of PKCβ was analysed and no reduction in $[Ca^{2+}]_m$ peak after H_2O_2 treatment (it was confirmed using the blocker hispidin) was observed. Likely a possible link between PKC-dependent phosphorylation of p66Shc and its mitochondrial oxidoreductase activity was the phosphorylation mediated transfer of p66Shc from the cytosol to mitochondria. Moreover, the Prolyl isomerase Pin1 was identified as inducer of p66Shc mitochondrial translocation after Ser36 phosphorylation.

Conclusions

In conclusion, thanks to different tools, among which the gene silencing of cytoplasmic kinase PKCb, it was possible to identify a molecular route that links an oxidative challenge to the activation of p66Shc and the recruitment of mitochondria in apoptosis and may contribute to the aging properties of the protein.[60]

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