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Pharmacologic Inhibition of Cardiac Stem Cell Senescence

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1. Introduction

Mammalian aging may be viewed as a reduction in the capacity to adequately maintain tissue homeostasis or to repair tissues after injury(Sharpless and DePinho, 2007). When homeostatic control diminishes to the point at which tissue/organ integrity and function are no longer sufficiently maintained, physiological decline develops, and aging becomes apparent. Cells that express senescence markers accumulate at sites of chronic age-related pathology, such as osteoarthritis, atherosclerosis and chronic heart failure(Blasco, 2007; Campisi and d'Adda di Fagagna, 2007; Chimenti, et al., 2003; Deng, et al., 2008; Jeyapalan and Sedivy, 2008; Minamino and Komuro, 2008; Sharpless and DePinho, 2007; Shawi and Autexier, 2008; Torella, et al., 2004; Urbanek, et al., 2005). Thus, senescent cells are associated with aging and age-related diseases in vivo(Campisi, 2011).

The discovery of tissue-resident stem and progenitor cells has suggested that these cells are responsible for tissue homeostasis and regeneration(Hosoda, et al., 2009; Hsieh, et al., 2007; Li and Clevers, 2010). For this reason, pathological and patho-physiological conditions characterized by altered tissue homeostasis and impaired regenerative capacity can be viewed as a consequence of the reduction in stem cell number and/or function. Following the evolutionary theory of antagonistic pleiotropy, stem cell senescence can be considered a double edged-sword that exerts both a tumor-suppressor effect, by preventing the expansion of injured self-renewing cells, and detrimental effects, contributing to tumor invasiveness in a paracrine fashion or to aging by causing stem cell arrest or attrition (cancer-ageing hypothesis)(Campisi, 2005; Sharpless and DePinho, 2007). In line with this, stem cell aging has been demonstrated in hematopoietic stem cells, as well as in other self-renewing compartments(Beltrami, et al., 2011a).

The recognition that the heart possesses a pool of primitive, clonogenic, self-renewing, and multipotent cells responsible for tissue homeostasis has opened a new era of research aimed at harvesting, expanding and utilizing these cells for cardiac repair(Beltrami, et al., 2003).

However, experimental studies have demonstrated that, although the cardiac stem cell (CSC) pool is expanded acutely after myocardial infarction, this response is attenuated in chronic heart failure(Urbanek, et al., 2005). In addition, a significant accumulation of senescent CSC in cardiac tissue both in pathological settings and with aging has been described(Cesselli, et al., 2011; Chimenti, et al., 2003; Rota, et al., 2006). More recently, our group has demonstrated that both age and pathology exert detrimental effects on human CSC (hCSC). Specifically, they attenuate CSC telomerase activity, reduce telomeric length, determine telomere erosion, are associated with the presence of telomere induced dysfunction foci and impair CSC function(Cesselli, et al., 2011). Importantly, comparing the gene expression profile of CSC obtained from normal and pathological tissues we identified several possible molecular targets for pharmacological interventions aimed at reverting or attenuating the senescence processes.

Aims of this chapter will be to review the knowledge on the impact that CSC senescence exerts on cardiac function, to discuss interventions aimed at reverting it and to focus on original results investigating the effects of Rapamycin, Resveratrol and DETA/NO on CSC senescence.

2. Cellular senescence

In 1961 Hayflick applied the term *cellular senescence* to cells that ceased to divide in culture despite favorable growth conditions, based on the speculation that their behavior recapitulated organism aging(Hayflick and Moorhead, 1961). Since then, this phenomenon was proposed to be either a detrimental cause of aging or a beneficial tumor suppression mechanism. In fact, cell senescence plays both these roles, supporting the evolutionary theory of antagonistic pleiotropy that postulates that cellular processes, selected to benefit young organisms, may have unselected deleterious effects in older organisms(Campisi and d'Adda di Fagagna, 2007).

Cellular senescence is currently defined as a specialized form of growth arrest, confined to mitotic cells, induced by various stressful stimuli and characterized by several, although not specific, markers(Sharpless and DePinho, 2007). Specifically, senescent cells are characterized by a permanent growth arrest, resistance to apoptosis, an altered pattern of gene expression, and the expression of proteins that are characteristic of, although not exclusive to, the senescent state(Beltrami, et al., 2011a), such as the senescence-associated β-galactosidase (SA-βgal) (Dimri, et al., 1995).

Recently identified markers of cellular senescence are p16, DEC1, p15, and DCR2(Collado, et al., 2005), and the cytological markers: senescence-associated heterochromatin foci (SAHFs), and senescence associated DNA-damage foci (SDFs)(Di Micco, et al., 2008; Narita, et al., 2003). SDFs are present in senescent cells from mice and humans and contain proteins that are associated with DNA damage. When these foci result from dysfunctional telomeres they are defined as telomere-induced dysfunctional foci (TIFs) (Campisi and d'Adda di Fagagna, 2007; Jeyapalan and Sedivy, 2008; Sharpless and DePinho, 2007).

Regarding the molecular mechanisms responsible for cellular senescence, intrinsic and extrinsic pathways have been described. While the first one is initiated by intracellular damages/stimuli, the second one is related to extracellular molecules. Importantly, these two mechanisms are strictly interconnected since senescent cells are characterized by the

production of molecules able to alter the microenvironment thus inducing senescence on the neighborhood cells through a paracrine mechanism(Campisi, 2005).

Intrinsic inductors of cellular senescence are either the progressive telomere erosion that is associated with cell proliferation (i.e. replicative senescence) (Deng, et al., 2008; Shawi and Autexier, 2008) or the formation of irreparable DNA lesions that induce a persistent DNA damage response (DDR) which keeps the cells alive, but arrests their proliferation (i.e. telomere independent, stress-induced premature senescence)(Beltrami, et al., 2011a). In this latter case, DDR is induced by activated oncogenes, and DNA double strand break-inducing agents, such as reactive oxygen species (ROS). Many proteins participate in the DDR, including protein kinases (e.g. ataxia telangiectasia mutated -ATM- and checkpoint-2 -CHK2-), adaptor proteins (e.g. 53BP1 and MDC1 -mediator of DNA damage checkpoint protein‑1-) and chromatin modifiers (for example, γ-H2AX)(von Zglinicki, et al., 2005). Therefore, intrinsic inductors of cellular senescence initiate a DDR, consisting of the activation of ATM and ataxia telangiectasia- and Rad3-related (ATR), and downstream kinases CHK1 and CHK2, and phosphorylation of p53. Phosphorylated p53 transcriptionally up-regulates genes, such as p21, that mediate cellular senescence and/or apoptosis to inhibit tumorigenesis. Although less well-understood, telomere dysfunction could also activate the $p16^{INK4A}$–RB pathway and inhibit cellular proliferation(Campisi and d'Adda di Fagagna, 2007; Deng, et al., 2008; Sharpless and DePinho, 2007; Shawi and Autexier, 2008).

Recent reports have demonstrated that autophagy plays a crucial role in the induction of cellular senescence(Adams, 2009), either replicative (Young and Narita, 2010) or stress-induced premature senescence (Patschan and Goligorsky, 2008; Young and Narita, 2010). However, several interventions that extended lifespan in various species (e.g. caloric restriction, and negative regulation of insulin and mTOR pathways) are associated with the activation of autophagy(Vellai, 2009). To reconcile this observation with the observed accumulation of senescent cells in aged tissues and organs, Authors hypothesize that autophagy may play a beneficial role in mild but long-term stress conditions, counteracting the accumulated damage, while it contributes to senescence establishment in more severely damaged cells(Young and Narita, 2010). Until recently, the general notion was that once senescence was established, cells were locked into a senescent phenotype through a global induction of heterochromatin, which results in the formation of Senescence Associated Heterochromatin Foci (SAHF). In this process, the cyclin dependent kinase inhibitor p16INK4A seemed to play a primary role. However, recently it was shown that SAHF are induced mainly in response to activated oncogenes in a cell type- and insult- dependent manner(Kosar, et al., 2011).

Several *extrinsic inductors of cellular senescence* have also been described so far(Beltrami, et al., 2011a). Specifically, it has been demonstrated that: Advanced Glycation End-products (AGE) (Patschan and Goligorsky, 2008), Angiotensin II (Fukuda and Sata, 2008; Imanishi, et al., 2005; Kunieda, et al., 2006), IGFBP7, IL-6, IL-8(Kuilman, et al., 2008; Orjalo, et al., 2009), GROα, urokinase- or tissue-type plasminogen activators (uPA or tPA), the uPA receptor (uPAR), and inhibitors of these serine proteases (PAI-1 and -2) (Blasi and Carmeliet, 2002; Kortlever, et al., 2006), can induce cellular senescence in different cell types. In this regard, a special role is played by the altered secretome of senescent cells (e.g. Senescence Associated Secretory Phenotype - SASP-). In fact, it has been demonstrated that senescent cells may

alter profoundly their microenvironment, by inducing cellular senescence in neighboring cells in a paracrine fashion, by remodeling the extracellular matrix and by stimulating inflammation(Acosta, et al., 2008; Coppe, et al., 2008; Wajapeyee, et al., 2008).

3. Cardiac stem cell senescence

The recognition that the human adult heart possesses a pool of resident cardiac progenitor cells (hCSC), which are self-renewing, clonogenic, and multipotent(Bearzi, et al., 2009; Bearzi, et al., 2007; Beltrami, et al., 2007; Castaldo, et al., 2008; Messina, et al., 2004; Smith, et al., 2007), changed the dogma of the heart as a terminally differentiated organ, offered new hints in the understanding of the pathophysiology of heart diseases and opened a new area of research focused on the use of stem cells for cardiac repair(Beltrami, et al., 2011b; Dimmeler and Leri, 2008). Several different hCSC populations have been identified and characterized on the basis of the expression of specific markers, i.e. c-Kit(Bearzi, et al., 2009; Bearzi, et al., 2007; Castaldo, et al., 2008), ABCG2(Meissner, et al., 2006) and Islet-1(Bu, et al., 2009), or utilizing selective culture conditions, i.e. cardio-spheres(Messina, et al., 2004; Smith, et al., 2007) and multipotent adult stem cells(Beltrami, et al., 2007). Whether these cells are distinct populations or whether they represent different stages of maturation of the same cell type is still a debated question (Beltrami, et al., 2011b; Laflamme and Murry, 2011). Nonetheless, moving from the robust evidence of the efficacy of cardiac stem cell therapy in animal models(Bearzi, et al., 2007; Smith, et al., 2007), the feasibility, safety and some hints on the efficacy of autologous CSC therapy in patients suffering from cardiac pathology is currently under investigation in several clinical trials (ClinicalTrials.gov identifier NCT00474461, NCT00893360, and NCT00981006). Autologous CSC represent a population of cells intrinsically committed to cardiac lineages and would offer the advantage to avoid immunological issues(Dimmeler and Leri, 2008). Nonetheless, it would be important to identify whether and at which extent cardiac diseases can affect this resident stem cell reservoir.

3.1 CSC senescence in cardiac pathologies

The first evidence that hCSC could undergo cellular senescence was given by Anversa's group showing that aged diseased hearts were characterized, at tissue level, by an accumulation of $p16^{INK4a}$-positive/c-Kit-positive CSC(Chimenti, et al., 2003). Later it was shown that chronic heart failure was associated, in human heart tissues, with an increase in the number of $p16^{INK4a}$-p53-positive senescent hCSC, further characterized by short telomeres(Urbanek, et al., 2005). More recently, our group provided a direct demonstration of the impact that both aging and pathology exert on hCSC function(Cesselli, et al., 2011). Specifically, we observed that age and pathological state are both associated with: a reduction in telomerase activity, telomeric shortening, and an increased frequency of CSC with telomere induced dysfunction foci, and eventually expressing $p16^{INK4a}$ and $p21^{CIP}$. These pathologic alterations were coupled with a reduced hCSC function; in fact, hCSC obtained from failing hearts showed, with respect to those obtained from healthy hearts, a significant reduction in clonogenic, proliferative, and migratory potential. Moreover, senescent hCSC displayed an altered gene expression profile, enriched in transcripts of proteins involved in the senescence associates secretory phenotype (SASP), such as IL6 and IGFBP7(Cesselli, et al., 2011). Of note, the underlying diseases of the patients enrolled in this study were different, ranging from ischemic cardiomyopathy to hypertrophic and dilated

cardiomyopathy(Cesselli, et al., 2011), suggesting that, independently from the etiology, end stage heart failure is characterized by a progressive loss of the compartment of hCSC with high regenerative potential, paralleled by an increase in the pool of stem cells with minimal or no ability to divide and acquire cardiac cell lineages. Moreover, animal models showed an involvement of CSC senescence in other pathologies such as the diabetic cardiomyopathy (Rota, et al., 2006) and the antracyclin-induced cardiomyopathy(De Angelis, et al., 2010).

3.2 Pathways involved in hCSC senescence

Whith regard to the mechanisms responsible for the replication, differentiation, senescence, and death of hCSC, different growth-factor receptor systems have been shown to play a key role: IGF-1-IGF1R, IGF-2-IGF2R, HGF-c-Met and the renin angiotensin system (RAS)(Dimmeler and Leri, 2008). While IGF-1-IGF1R and HGF-c-Met seemed to exert a protective effect, IGF-2-IGF2R and the RAS up-regulation is associated with CSC senescence.

Specifically, the expression of IGF-1R and the production of IGF-1 are attenuated in aging CSC, and this negatively interferes with oxidative damage and telomere shortening(D'Amario, et al., 2011a; Torella, et al., 2004). In fact, IGF-1 – IGF-1R induces CSC division, upregulates telomerase activity, maintains telomere length, hinders replicative senescence, and preserves the population of functionally competent cardiac stem cells in animals(Torella, et al., 2004) and in humans(D'Amario, et al., 2011a).

Ageing is also associated with a reduction in HGF production, thus impairing the migratory ability of CSC in response to tissue damage(Gonzalez, et al., 2008; Khan, et al., 2011); importantly, CSC dysfunction was shown to be partially restored by HGF injection(Gonzalez, et al., 2008). Regarding IGF-2-IGF2R, it has been recently demonstrated that hCSC expressing IGF-2R are characterized, with respect to IGF-1R positive hCSC, by a more senescent phenotype and by a reduced in vivo regenerative capacity(Gonzalez, et al., 2008).

Similarly, it has been documented that a local RAS is present on hCSC and that the formation of Angiotensin II (Ang II), together with the expression of AT1R, increases with age in hCSC(D'Amario, et al., 2011a). Ang II generates ROS possibly contributing to the age-dependent accumulation of oxidative damage in the heart(Fiordaliso, et al., 2001; Smith, et al., 2007). In fact, the use of ACE-inhibitors positively interferes with heart failure and prolongs life in failing patients(McMurray and Pfeffer, 2005). Moreover, sustained oxidative stress can trigger telomere shortening and uncapping initiating a permanent DNA-damage response(von Zglinicki, et al., 2005). The importance of oxidative stress has been confirmed in a murine model of diabetes, where it has been shown the association of cardiomyopathy with the premature senescence and apoptosis of CSC; importantly, in this model the deletion of p66shc could prevent CSC senescence and was associated with the preservation of myocyte number and cardiac function(Rota, et al., 2006).

Despite these important data, it remains to be determined whether other pathways, that are involved in the senescence of other cell compartments, could contribute to hCSC senescence as well. For example, data on the role played by autophagy, mitochondrial dysfunction, nucleolar dysfunction and epigenetic changes are still missing(Beltrami, et al., 2011a). However, comparing the gene expression profile of hCSC isolated from end-stage failing hearts with that

of hCSC isolated from normal hearts, it was possible to demonstrate changes in the expression of genes strictly related to these senescence associated pathways(Cesselli, et al., 2011). Importantly, the analysis identified several possible molecular targets for pharmacological interventions aimed at reverting or attenuating the senescence processes. Interestingly, some of them were very well known target of drugs commonly used in clinical practice, such as beta-blockers and ACE-inhibitors(Cesselli, et al., 2011).

4. How to interfere with cardiac stem cell senescence

Cell therapy is a promising option for treating ischemic disease and heart failure(Dimmeler and Leri, 2008). In fact, various experimental studies documented that tissue-resident primitive cells improve recovery after ischemia(Beltrami, et al., 2011b). Moreover, different groups have demonstrated the feasibility of isolating and expanding hCSC even from end-stage failing hearts(Bearzi, et al., 2007; Beltrami, et al., 2007; Itzhaki-Alfia, et al., 2009; Smith, et al., 2007). However, accumulated evidences indicate that both ageing and pathology are associated with hCSC senescence and functional impairment (Cesselli, et al., 2011; D'Amario, et al., 2011a; D'Amario, et al., 2011b; Itzhaki-Alfia, et al., 2009). In fact, hCSC obtained from failing hearts present reduced migration, proliferation and differentiation(Cesselli, et al., 2011), features considered to be crucial for the regenerative potential of this autologous cell source. Moreover, these cells are characterized by a gene expression profile enriched in elements that are part of the senescence associated secretory phenotype (SASP). Therefore, senescent hCSC can contribute to create a microenvironment favoring, through a paracrine mechanism, senescence on neighbor cells, inflammation and extracellular matrix remodeling, thus creating a vicious circle hampering regenerative purposes.

For this reason, it would be extremely intriguing any attempt aimed at “improving” the quality of the expanded cells, selecting the fraction of cells with the highest regenerative potential or devoid of senescent cells. Conversely, we can hypothesize an intervention aimed at attenuating/reverting the molecular pathways characterizing senescent cells. In this regard, three main strategies can be envisioned: a sorting-based strategy, a function-based strategy and a drug-based strategy.

4.1 Sorting-based strategy to enrich in non-senescent cells

The sorting-based strategy would consist in the physical selection of the cells of interest. Sorting can be achieved, for example, utilizing a fluorescence-activated cell sorting (FACS) or a magnetic activated cell sorting (MACS). The selection strategy can be either positive (we choose and sort “young” cells on the basis of specific surface antigens) or negative (we enrich in non-senescent cells removing from the un-fractioned population those cells we believe to be senescent). Both approaches require the knowledge of specific surface antigens able to recognize the right population to sort. D’Amario et al have recently given examples of positive selection, utilizing antibodies recognizing insulin-like growth factor (IGF)-1 receptors to select, within hCSC isolated from end-stage failing patients, a population of young cells characterized by high telomerase activity, intact telomere length and endowed with a high regenerative ability, being able to restore a large quantity of infarcted myocardium, thus representing a potent cell population for cardiac repair(D'Amario, et al.,

2011a). Although not yet utilized for a negative selection, AT-2(D'Amario, et al., 2011a), IGF-2R and CD49a(Cesselli, et al., 2011) are surface markers that, being more expressed in senescent cells, could be utilized to deplete hCSC culture of the most senescent cells.

The major drawback of the sorting-based approach is the fact that it adds a further grade of complexity to the procedure aimed at producing clinical grade hCSC, since it requires Good Manufacturing Practice-compliant cell sorting and large-scale expansion starting from a reduced number of cells.

4.2 Function-based strategy to enrich in non-senescent cells

It is possible to take advantage of the fact that non-senescent cells are functionally impaired to select cells whose stem cell properties are still preserved. For example, we showed that single-cell derived clones, obtained from hCSC isolated from end-stage failing patients, are less senescent than the overall population(Cesselli, et al., 2011). Again, this approach would be hard to transfer to clinical practice, since it requires Good Manufacturing Practice-compliant cell sorting and large-scale expansion starting from very few cells. In fact, we have recently shown that only 0.7% of the hCSC obtained from end stage failing hearts gives rise to highly proliferating clones(Cesselli, et al., 2011) and, since hCSC are finite cell lines(Beltrami, et al., 2007; Cesselli, et al., 2011), hCSC-derived clones could undergo replicative senescence as a consequence of the high number of population doublings that are required to obtain a number of cells suitable for clinical purposes.

Whether selecting cells on the basis of the ability to actively extrude Hoechst 33342 (side population) could enrich in less senescent cells is still unknown(Hierlihy, et al., 2002; Martin, et al., 2004). However, also in this case a Good Manufacturing Practice-compliant cell sorting would be required.

4.3 Drug-based strategy to enrich in non-senescent cells

Several molecular pathways have been either associated with the development of cell senescence or, on the contrary, with organism longevity (Beltrami, et al., 2011a). Interestingly, the key elements of these two are common and the possibility to act on them can be explored to interfere with stem cell senescence and dysfunction, ameliorating stem cell regenerative approaches and organ pathology.

Briefly, as shown in Figure 1, the pathways main involved are: Insulin/Insulin-like Growth Factor Signaling (IIS), mTOR, AMPK/Autophagy, Nitric Oxide/Estrogen/Telomerase, Sirtuins, and p38MAPK(Beltrami, et al., 2011a).

Although the *Insulin/Insulin-like Growth Factor Signaling (IIS)* is critical for nutrient homeostasis, growth and survival, experimental evidences show that reduced IIS signaling in animals is associated with life extension(Beltrami, et al., 2011a). Insulin like Growth Factors and insulin inhibit the FoxO family of transcription factors through a pathway involving Insulin Receptor Substrate (IRS), PI3K and Akt. FoxO transcription factors promote a variety of cellular responses that include apoptosis, cell cycle arrest, differentiation, resistance to oxidative stress, and autophagy(Ronnebaum and Patterson, 2010; Salih and Brunet, 2008). The transcriptional activities and biological effects of FoxO depend on post-translational modifications and, in this regard, Sirt1 is believed to increase

the ability of FoxO to respond to stress through cell cycle arrest and other adaptations but inhibits FoxO transcription of apoptotic genes. Last, FoxO is required for preventing Akt-mediated cardiac hypertrophy(Ronnebaum and Patterson, 2010). Regarding the possibility to interfere with this pathway, we have previously reported that hCSC expressing IGF1-R represent a subset of young and fully functional cells(D'Amario, et al., 2011a), and that IGF1 was able to support proliferation and differentiation of IGF-1R-positive hCSC.

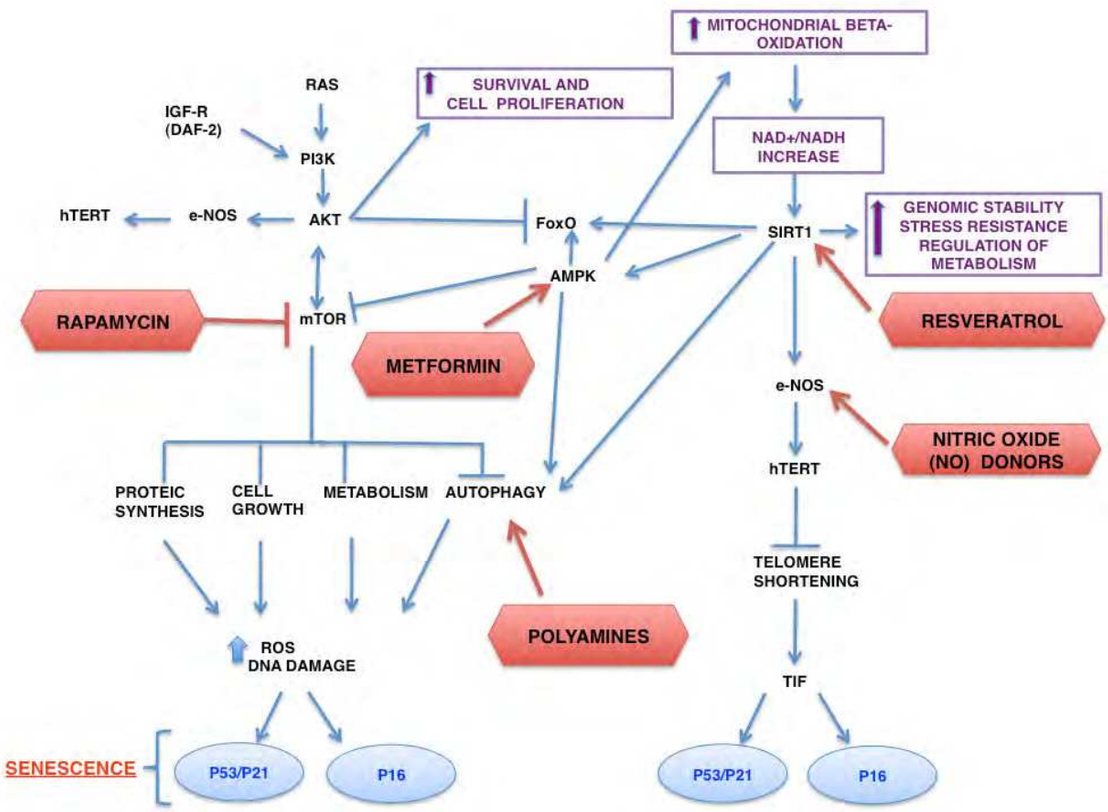


Fig. 1. Senescence pathways and possible pharmacological targets.

In aging, the *mammalian Target Of Rapamycin (mTOR)* plays a prominent role, which is, at least in part, mediated through IIS (Bhaskar and Hay, 2007). In fact, mTOR is activated by insulin, growth factors, nutrients and, indirectly, by Akt. mTOR forms two protein complexes; the Rapamycin-sensitive mTORC1, when bound to Raptor, and the Rapamycin insensitive mTORC2, when bound to Rictor. mTORC1 phosphorylates S6 kinase (S6K), eukariotic translation initiation factor 4E (eIF4E), and other factors involved in protein synthesis and hypertrophy. S6K, in turn, inhibits IRS by phosphorylation, while mTORC2 has a positive feedback on Akt. Nutrients and energy balance can regulate mTORC1, where aminoacids can activate it through the Rag family of GTPases, while AMPK, which is activated by ATP depletion, inhibits it (Bhaskar and Hay, 2007). Because of the central role of mTOR in ageing, Rapamycin has emerged as a very promising drug able to interfere with aging and, possibly, cell senescence(Blagosklonny, 2010). Importantly, Rapamycin is already

used in clinical practice for its immunosuppressant and antiproliferative effects. Moreover, accumulated evidences display a possible role of Rapamycin in ageing and cell senescence. In fact, Rapamycin can extend the maximum lifespan of mice, when given late in life, restore self-renewal of hematopoietic stem cells of aged mice, and prevent epidermal stem cell exhaustion induced by Wnt-1 in mouse skin(Blagosklonny, 2010). Last, it has recently been shown that Rapamycin, the mTOR inhibitor PP242 or the IGF1R inhibitor PQ401, are able to increase the efficiency of iPS generation(Chen, et al., 2011). Despite the fact that rapamyicin is utilized in heart transplanted patients to avoid immunrejection, the effects of Rapamycin on hCSC senescence are still unexplored.

Nitric Oxide (NO) and estrogen signaling have been shown to counteract endothelial progenitor cell senescence through the catalytic subunit of human telomerase (hTERT)(Farsetti, et al., 2009). Estrogens' action is mediated either via genomic or nongenomic signaling pathways. The first ones follow the binding of estrogens to nuclear hormone receptors, which are capable of regulating transcription of a number of genes involved in development, metabolism, and differentiation following interaction with a hormone molecule. Therefore, estrogen receptors are ligand-dependent transcription factors. In addition, estrogens can trigger nongenomic signaling pathways through membrane associated estrogen receptors (mER) that activate both the PI3K and the Mitogen Activated Protein Kinase (MAPK) pathways. Estrogens can also activate Adenylate Cyclase and c-Src through the G-protein coupled estrogen receptor (GPER)(Meyer, et al., 2009). NO, on the other hand, is a free radical and an ancestral regulator of biological functions that include endothelial function, vasodilation, inflammation, and heart and muscle organogenesis(Farsetti, et al., 2009). NO is produced by a family of NO synthases (NOS) starting from L-arginine: neuronal NOS (n-NOS), endothelial NOS (e-NOS), and inducible NOS (i-NOS). Despite their names, the distribution of these enzymes is ubiquitous, but, while e-NOS and n-NOS are activated following an increase of intracellular calcium levels, i-NOS is calcium insensitive and is activated by inflammatory cytokines(Farsetti, et al., 2009). Importantly, it has been shown that VEGF-induced angiogenesis is mediated by NO and relies on hTERT activity. Estrogens, on the other hand, exert a beneficial role on the cardiovascular system which is, at least in part, mediated through the induction of e-NOS and hTERT(Farsetti, et al., 2009). Last, it has been recently shown that e-NOS and estrogen receptor (ERα) physically interact and cooperate in regulating hTERT and possibly other genes, thus delaying vascular senescence. Although NO production and endothelial nitric oxide synthase have been shown to be greater in longer living rodents, NO donors do not seem to influence animal maximum lifespan(Csiszar, et al., 2007). However, it has been shown both that NO can regulate telomerase activity(Farsetti, et al., 2009) and that it has a profound impact on mouse embryonic stem cell differentiation towards a cardiovascular fate(Spallotta, et al., 2010). Data on the effects of NO on hCSC are still missing.

Mammalian Sirtuins (Sirt) are yeast Sir2 orthologs possessing both NAD+ dependent- protein deacetylase and ADP-ribosyltransferase activity(Beltrami, et al., 2011a). Although Sir proteins are key regulators of *S. Cerevisiae*, *Drosophila*, and *C. Elegans* lifespan, the effect of Sirtuins on mammalian lifespan is less dramatic. Nonetheless, in mice lacking Sirt1, caloric restriction is unable to extend lifespan. Mammalians, in fact, possess at least 7 sirtuins, that act as metabolic sensors directly linking environmental signals to metabolic homeostasis and

stress response. Sirt1, the most studied mammalian Sirtuin, controls gene expression, metabolism and aging, through a continuously growing list of substrates, that include: p53, members of the FoxO family, HES1 (hairy and enhancer of split 1), HEY2 (hairy/enhancer-of-split related with YRPW motif 2), PPARγ (peroxisome proliferator-activated receptor gamma), p300, PGC-1α (PPARγ coactivator), and NF- κB (nuclear factor kappaB)(Rahman and Islam, 2011). Although aging has been associated with stem cell senescence and dysfunction, the molecular mechanisms through which Sirt1 could protect primitive cells have not been completely delineated yet. However, the most prominent ones are: the positive regulation of telomeric length(Palacios, et al., 2010), the reduction of ROS production, the inhibition of p53(Rahman and Islam, 2011) and the induction of autophagy(Lee, et al., 2008). In this regard, Resveratrol is emerging as a potent drug able to delay age-related deteriorations and in mediating cardio-protection, conceivably by activating Sirt1(Petrovski, et al., 2011). In fact this polyphenolic compound has the ability to mimic the effects of caloric restriction by activating sirtuins and therefore acting modulating cell cycle, inhibiting apoptosis, increasing resistance to stress, and, finally, interfering with mTOR (Petrovski, et al., 2011). Accordingly, Resveratrol has shown beneficial effects against most degenerative and cardiovascular diseases from atherosclerosis, hypertension, ischemia/reperfusion, and heart failure to diabetes, obesity, and aging (Petrovski, et al., 2011). Importantly, pretreatment of either the infarcted heart or of cardiac stem cells with Resveratrol prior to cell injection results in an improvement of the regenerative capacities of the injected cells that eventually leads to improved heart function(Gorbunov, et al., 2011). However, in this specific case it was only evaluated the ability of Resveratrol to increase the engraftment of "normal" donor cells. In fact, up to now, the effects of Resveratrol on hCSC senescence remain to be elucidated. In addition, Resveratrol has been shown to be the most potent drug able to enhance iPS generation(Petrovski, et al., 2011).

p38MAPK is rapidly and transiently activated, by phosphorylation, following acute cellular stress. It is involved in senescence growth arrest by activating both p53 and pRb/p16INK4A pathways. Additionally, p38MAPK activity is required for the oncogene-induced premature senescence caused by oncogenic RAS, while its inhibition is able to delay replicative senescence, and to reverse the accelerated aging phenotype of fibroblasts obtained from Werner syndrome patients(Freund, et al., 2011). Further, p38MAPK is necessary and sufficient for the development of SASP in cells undergoing cellular senescence as a result of direct DNA damage or by oncogenic RAS(Freund, et al., 2011). Last, it was recently shown that p38MAPK inhibits Sirt1 by inducing its proteasomal degradation(Hong, et al., 2010). Although P38MAPK inhibitors have been successfully used to counteract in vitro the accelerated senescence phenotype seen in Werner syndrome progeria, it is still unclear whether this effect could be generalized to more physiological aging conditions. Importantly, it has been shown that p38MAPK inhibition can maintain hematopoietic stem cell quiescence, inhibiting the exhaustion of the hematopoietic stem cell pool(Ito, et al., 2006). In addition, p38MAPK inhibition can reduce cellular senescence in EPC exposed to doxorubicin (Spallarossa, et al., 2010). No data are available regarding hCSC. However, a role played by p38MAPK inhibition in inducing myocyte differentiation of embryonic stem cells has been reported(Gaur, et al., 2010).

Altogether, we can conclude that, although extremely interesting, the possibility to pharmacologically interfere with hCSC senescence has not yet been exploited.

5. Experimental data

In order to establish whether drugs known to interfere with the ageing processes could positively interfere with hCSC senescence and rescue their functional competence, hCSC obtained from failing hearts were cultured in the presence of increasing concentration of Rapamycin (1nM, 10nM, 100nM), Resveratrol (0.2μM, 0.5μM, 1μM) and DETA/NO (5μM, 10μM, 50μM). To reduce cell line variability, we selected hCSC obtained from ≈60 year old, male patients affected by end stage ischemic cardiomyopathy. After a 3-day treatment, cell lines (n=8) were analyzed both in terms of stem cell marker expression and cell proliferation, death and senescence. The ability of hCSC to differentiate and migrate was further assessed.

5.1 Methods

5.1.1 hCSC isolation and culture

Human atrial specimens, weighing 3-6 g, were collected over a period of five years from explanted hearts of patients in NHYA class 4 undergoing cardiac transplantation at the Cardiac Surgery Unit of the University Hospital of Udine, Italy. Informed consent was obtained in accordance with the Declaration of Helsinki and with approval by the Independent Ethics Committee of the University of Udine. Samples were employed for the isolation and expansion of c-kit-positive human cardiac stem cells (hCSC), as previously described (Bearzi, et al., 2009; Bearzi, et al., 2007; Beltrami, et al., 2007; Cesselli, et al., 2011). Specifically, two protocols were employed for the isolation of hCSC: enzymatic dissociation of the samples with collagenase and primary explant technique(Cesselli, et al., 2011). These two methodologies yielded comparable results up to 20-25 population doublings; efficiency and viability of hCSC were superimposable. Collagenase treatment was not found to affect these variables.

5.1.2 Pharmacological treatment of hCSC

After about 20 population doublings, growing cultures of hCSC were exposed to Rapamycin (1-100 nM, Sigma-Aldrich), 1-[N-(2-Aminoethyl)-N-(2-ammonioethyl)amino]diazen-1-ium-1,2-diolate or DETA/NO (5-50 μM, Sigma-Aldrich) and Resveratrol (0.2-1 μM, Sigma-Aldrich) for three days. At the end of the treatment, part of the cells was analyzed in terms of immunophenotype and to quantify cellular senescence, cell proliferation, and cell death. Part of the vehicle-treated and drug-treated cells was switched for n=2 days to a drug-free medium and subsequently assayed in terms of growth kinetic, differentiation and migration ability (see below).

5.1.3 Cell growth kinetic

Cells were seeded at a density of 2,000 cells/cm^2 in expansion medium. Cells were detached and counted at 1-2-5-9-12 and 14 days.

5.1.4 Cell differentiation assay

Muscle cell differentiation was achieved plating 0.5 to 1x10^4/cm^2 cells in expansion medium containing 5% FCS (Sigma-Aldrich, st. Louis, MO, USA), 10 ng/mL bFGF, 10 ng/mL VEGF,

and 10 ng/mL IGF-1 (all from Peprotech EC, London, UK), but not EGF. Cells were allowed to become confluent and cultured for up to 4 weeks with medium exchanges every 4 days(Beltrami, et al., 2007; Cesselli, et al., 2011). Endothelial cell differentiation was obtained plating 0.5 to $1x10^4/cm^2$ hCSC in *EGM®-2* Endothelial Cell Growth Medium-*2* (Lonza, Switzerland) for 2 weeks.

5.1.5 Migration assay

In order to evaluate in vitro cell migration of drug treated or untreated hCSC, a scratch assay was performed(Liang, et al., 2007). In 33mm-plates at high confluence, scratches were created utilizing 200µl tips. Phase contrast images of the scratches were acquired at 3-hour intervals, until their complete closure, utilizing Leica DMI6000B. Images were then compared and quantified by ImageJ in order to calculate the rate of cell migration. The mean scratch width did not differ significantly in the different culture conditions ($p>0.05$).

5.1.6 Flow cytometry

Proliferating cells were detached with 0.25% trypsin-EDTA (Sigma-Aldrich) and, after a 20 minutes recovery phase, were incubated with either properly conjugated primary antibodies: CD13, CD29, CD49a, CD49b, CD49d, CD90, CD73, CD44, CD59, CD45, HLA-DR, CD117, CD271, CD34, (BD Biosciences), CD105, CD66e (Serotech), CD133 (Miltenyi Biotec), E-cadherin (Santa Cruz Biotechnology), ABCG-2 (Chemicon International), or with an unconjugated primary antibody: N-cadherin (Sigma-Aldrich). Unconjugated antibody was revealed using PE or FITC conjugated secondary antibodies (DakoCytomation). Properly conjugated isotype matched antibodies were used as a negative control.

Apoptosis and necrosis were evaluated utilizing the Annexin V-FITC Apoptosis Detection Kit (Bender MedSystem), following manufacturer's instructions.

The analysis was performed either by FACS-Calibur (BD Biosciences) or by CyAn (Dako Cytomation).

5.1.7 Immunofluorescence and fluorescence microscopy

Cells cultured either in expansion or in differentiation medium were fixed in 4% buffered paraformaldehyde for 20 minutes at room temperature (R.T.). For intracellular stainings, fixed cells were permeabilized for 8 minutes at R.T. with 0.1% Triton X-100 (Sigma-Aldrich) before exposing them to primary antibodies. Primary antibody incubation was performed over-night at 4°C using following dilutions: Oct-4 (Abcam, 1:150); Sox-2 (Chemicon, 1:150); Nanog (Abcam, 1:150), cKit (R&D; 1:100), p21 (Santa Cruz; 1:40), $p16^{INK4A}$ (CIN-TEK, pre-diluted), γH2A.X (Upstate, 1:500), Ki67 (Novocastra, 1:1000); α-Sarcomeric Actin (Sigma, 1:100) and CD31 (Dako, 1:50). To detect primary antibodies, A488 and A555 dyes labeled secondary antibodies, diluted 1:800, were employed (Molecular Probe, Invitrogen). Finally, 0.1 µg/ml DAPI (Sigma) was used to identify nuclei. Vectashield (Vector) was used as mounting medium. Confocal image acquisition was carried out by a Confocal Laser Microscope (Leica TCS-SP2, Leica Microsystems) utilizing either a 63x oil immersion objective (numerical aperture: 1.40) or a 40x oil immersion objective (numerical aperture: 1.25). Epifluorescence and phase contrast images were obtained utilizing a live cell imaging dedicated system consisting of a Leica DMI 6000B microscope connected to a Leica

DFC350FX camera (Leica Microsystems, Wetzlar, Germany). 10X (numerical aperture: 0.25), 40X oil immersion (numerical aperture: 1.25), and 63X oil immersion (numerical aperture: 1.40) objectives were employed for this purpose. Bright field images were captured utilizing a Leica DMD108 microscope (Leica Microsystems). 10X (numerical aperture: 0.40), 20X (numerical aperture: 0.70), and 40x (numerical aperture: 0.95) objectives were employed. Adobe Photoshop software was utilized to compose, overlay the images and adjust the contrast (Adobe, USA).

5.1.8 Statistics

Two-tailed unpaired- Student t- test and one-way Anova followed by Bonferroni post-test were utilized to compare means between two or more groups, respectively (Prism, version 4.0c). Results are expressed as mean±standard deviation. *P* values less than 0.05 were considered significant.

5.2 Results

We evaluated the effects of Rapamycin, Resveratrol and DETA/NO on hCSC stem cell marker expression, proliferation, senescence, death and function.

5.2.1 Effects of drugs on hCSC stem cell marker expression

As previously mentioned, hCSC obtained from failing hearts presented a mesenchymal immunophenotype and largely expressed the pluripotent state specific transcription factors Oct-4, Nanog and Sox-2. Drug treatment did not alter the mesenchymal immunophenotype and left unchanged the faction of cells expressing the pluripotent state specific transcription factors (data not shown).

5.2.2 Effects of drugs on hCSC proliferation, senescence and death

The effects exerted by Rapamyicin, Resveratrol and DETA/NO on hCSC proliferation, senescence and death resulted to be drug- and concentration- dependent; therefore, the effects exerted by each drug will be presented separately.

Rapamycin

As shown in Figure 1, Rapamycin mainly acts inhibiting mTOR-related pathway, thus inhibiting cell growth, autophagy and reducing oxidative stress. However, the anti-proliferative effect of the drugs is partially counteracted by a positive effect on Akt.

Accordingly, after a 3-day treatment hCSC, with respect to vehicle-treated cells, did not display changes in proliferation, as testified both by Ki67 expression and nuclear density (Figure 2).

Rapamycin was instead effective in reducing the fraction of senescent cells acting primarily on the fraction of cells expressing p16 that, at a 10nM concentration, resulted to be halved. No changes in the fraction of cells with DNA-damage foci were observed (Figure 2). DNA-damage foci positive cells were identified by the presence of the histonic protein γH2AX in the absence of Ki67 expression (Lawless, et al., 2010).

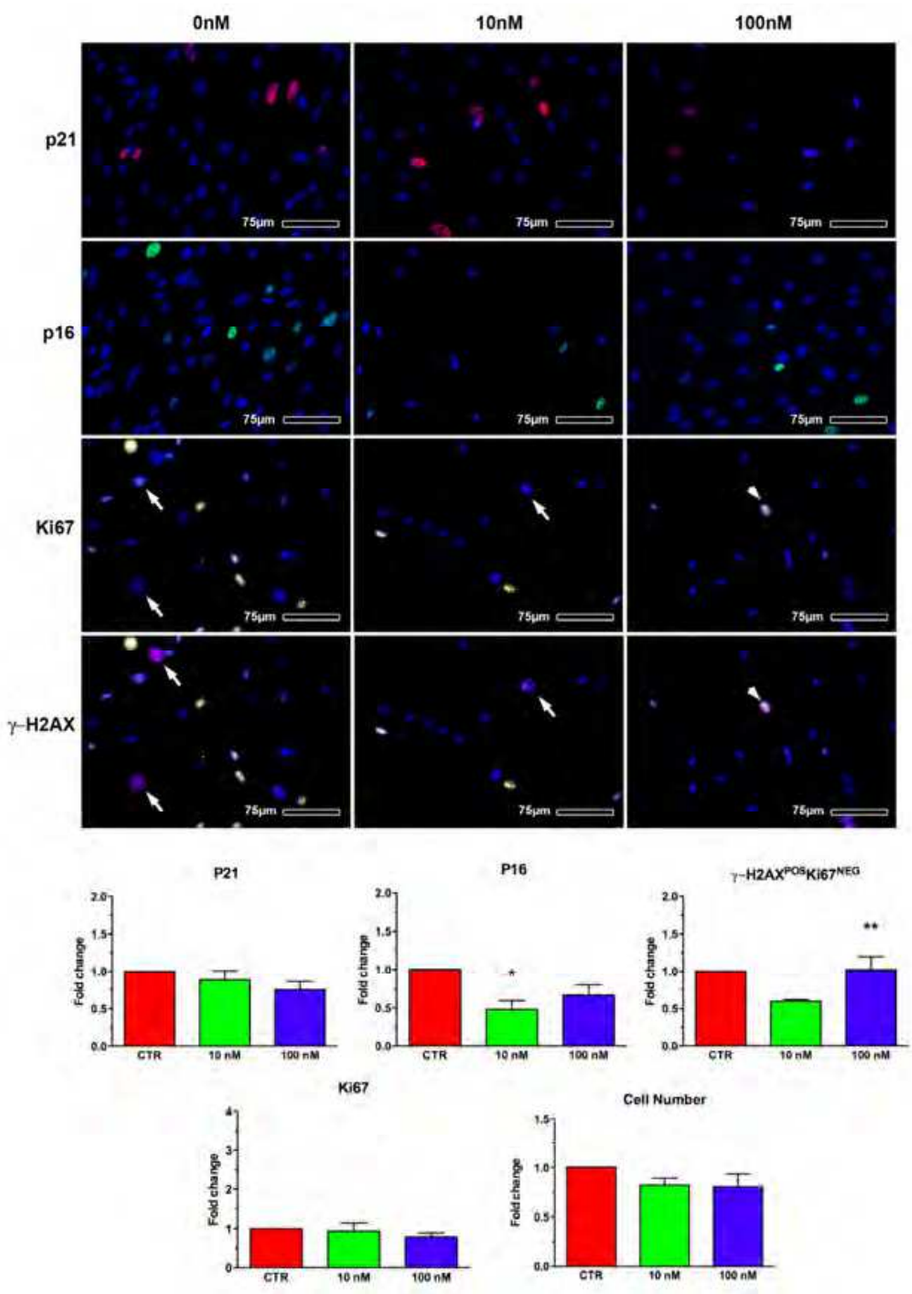


Fig. 2. **Effects of Rapamycin on hCSC.** hCSC were exposed for 3 days to 0nM (left panels), 1nM (central panels) and 10nM (right panels) Rapamyicin. Cells were then stained for p21 (red fluorescence), p16 (green fluorescence), Ki67 (yellow fluorescence) and γH2AX (magenta fluorescence). DNA-damage foci positive cells (arrows) were recognized as cells positive for γH2AX (magenta fluorescence) but negative for Ki67 (yellow fluorescence). Cells positive for both Ki67 and γH2AX (arrowheads) were excluded from the count. Histograms represent the fold changes in the fraction of cells expressing senescence (p21, p16, $\gamma H2AX^{+}Ki67^{-}$) and proliferation markers (Ki67) and in hCSC number of treated cells with respect to vehicle-treated cells (CTR). *, **, $p<0.05$ with respect to CTR and 10nM treated cells, respectively.

Interestingly, Rapamyicin at both concentrations tested increased the fraction of cells undergoing cell death through apoptosis (Figure 3).

In conclusion, Rapamyicin seemed to act reducing the fraction of p16-positive senescent cells, without affecting cell proliferation.

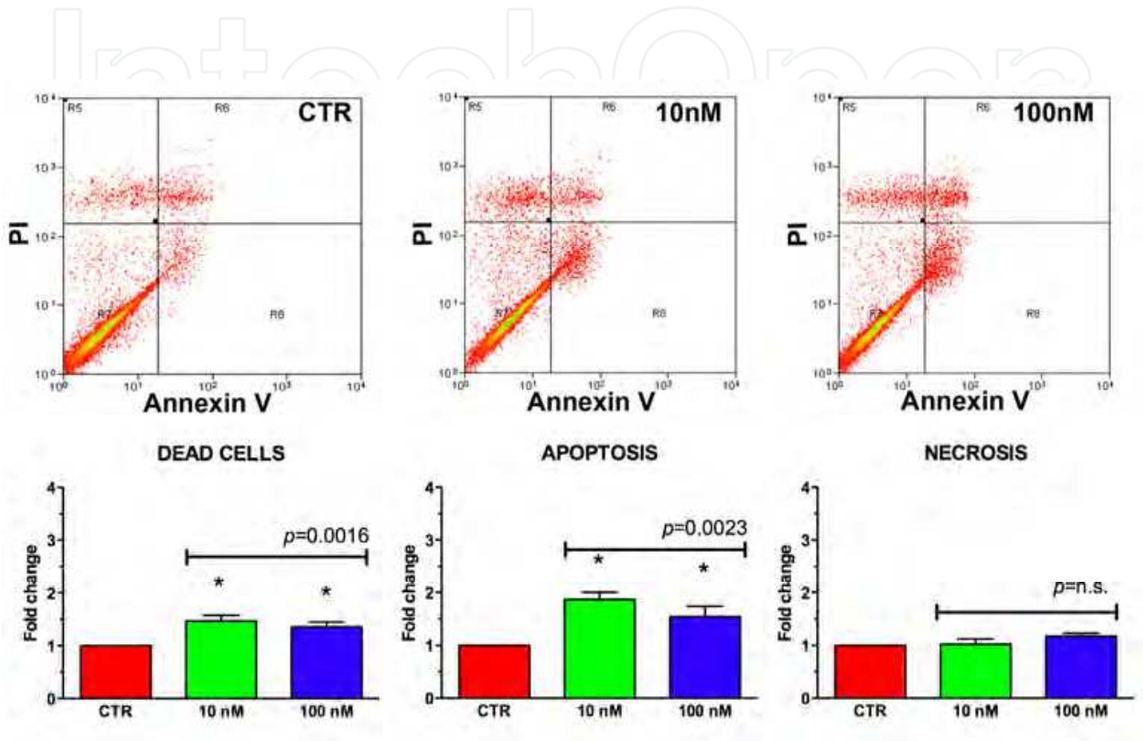


Fig. 3. **Effects of Rapamycin on hCSC death.** In the upper panel dot-plots graphically represent hCSC cultured in the presence of different concentrations of Rapamycin, stained for PI and AnnexinV and analyzed by FACS. Apoptotic cells were defined as AnnexinV^{+}PI$^{+/-}$ cells, necrotic cells as AnnexinV^{-}PI^{+}cells. In the lower panels, histograms represent the quantitative analysis of the fold change in the fraction of dead cells. *, $p<0.05$ with respect to vehicle-treated cells (CTR).

Resveratrol

As displayed in figure 1, Resveratrol has the ability to mimic the effects of caloric restriction by activating sirtuins and therefore acting modulating cell cycle, inhibiting apoptosis, increasing resistance to stress, and, finally, interfering with mTOR (Petrovski, et al., 2011).

Accordingly, Resveratrol-treated cells presented a larger fraction of Ki67-positive cells and an increased nuclear density (Figure 4). Importantly, the fraction of senescent cells resulted to be significantly reduced at both drug concentration used. Differently from Rapamyicin, acting on p16-positive cells, Resveratrol was effective in reducing the fraction of cells presenting DNA-damage foci and expressing p21 (Figure 4).

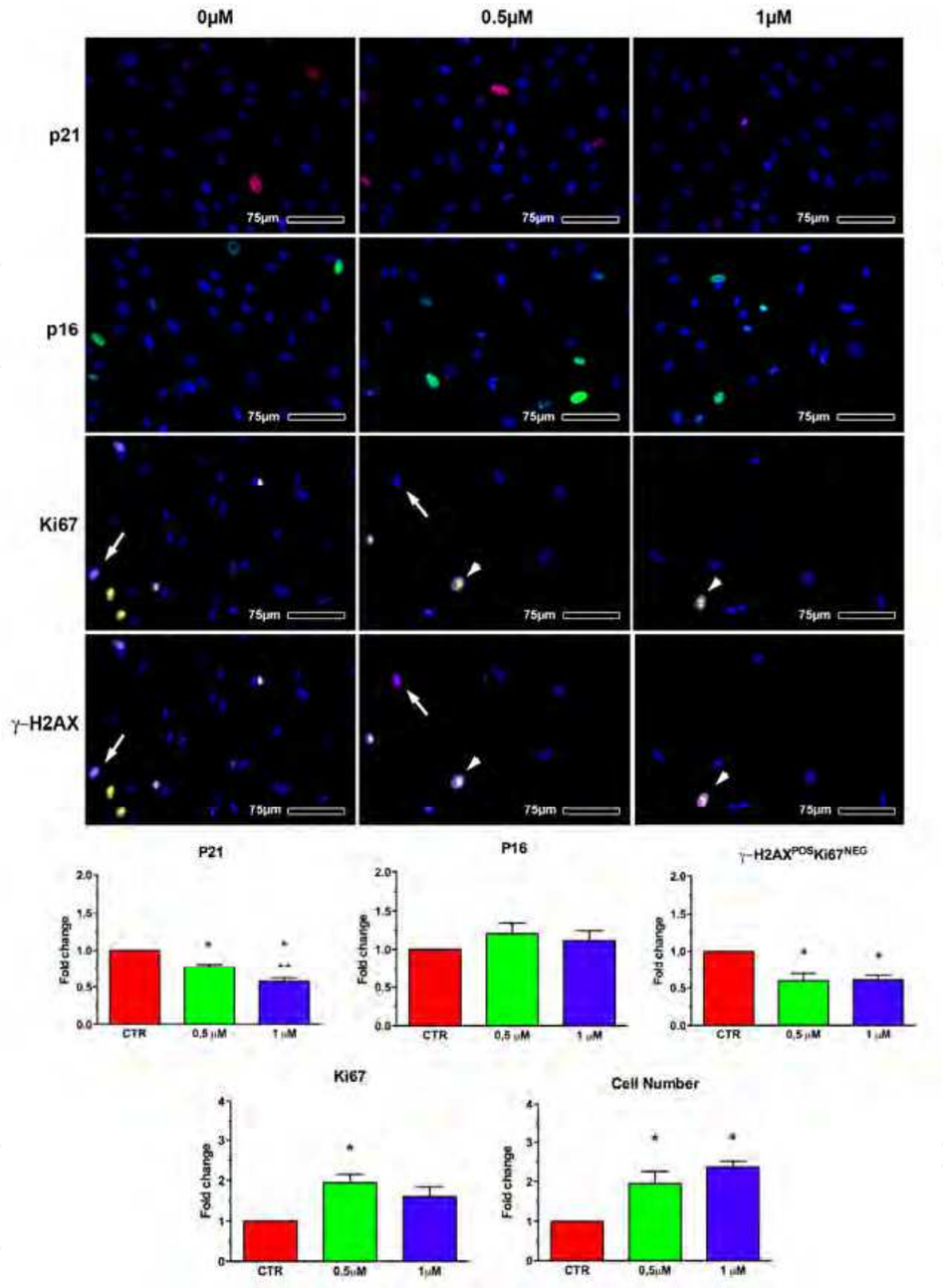


Fig. 4. **Effects of Resveratrol on hCSC.** hCSC were exposed for 3 days to 0μM (left panels), 0,5μM (central panels) and 1μM (right panels) Resveratrol. Cells were then stained for p21 (red fluorescence), p16 (green fluorescence), Ki67 (yellow fluorescence) and γH2AX (magenta fluorescence). DNA-damage foci positive cells (arrows) were recognized as cells positive for γH2AX (magenta fluorescence) but negative for Ki67 (yellow fluorescence). Cells positive for both Ki67 and γH2AX (arrowheads) were excluded from the count. Histograms represent the fold changes in the fraction of cells expressing senescence (p21, p16, $\gamma H2AX^{+}Ki67^{-}$) and proliferation markers (Ki67) and in hCSC number of treated cells with respect to vehicle-treated cells (CTR). *, $p<0.05$ with respect to CTR.

Moreover, Resveratrol significantly reduced the fraction of cells dying by necrosis (Figure 5).

Altogether these results indicate that Resveratrol presented beneficial effects on hCSC stimulating cell proliferation, reducing DNA-damage induced senescence and cell death.

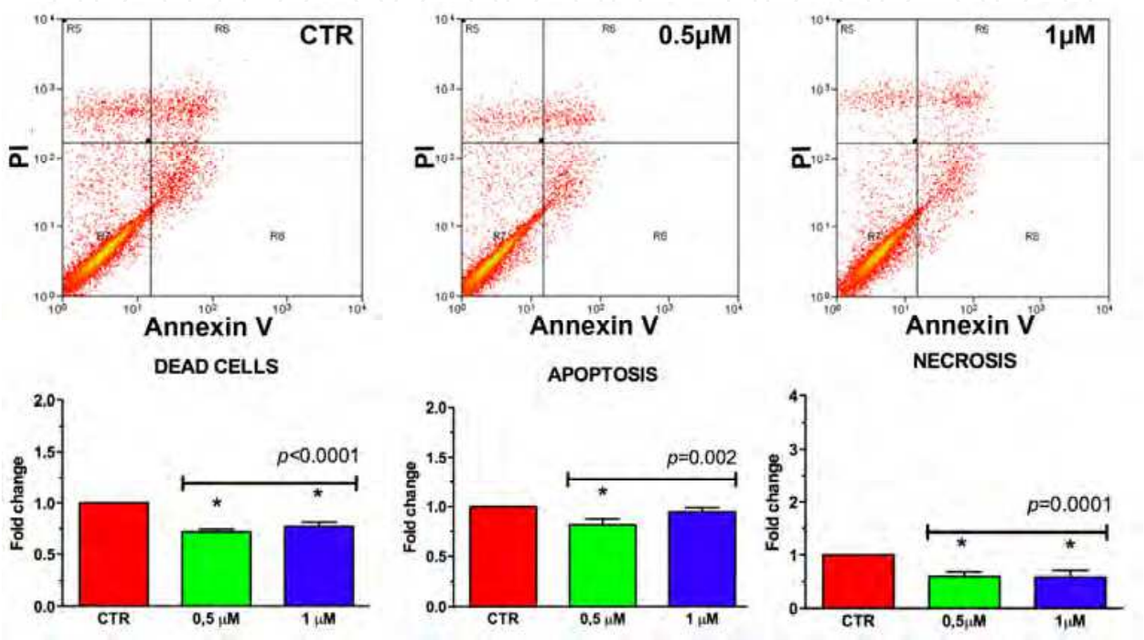


Fig. 5. **Effects of Resveratrol on hCSC death.** In the upper panel dot-plots graphically represent hCSC cultured in the presence of different concentrations of drug, stained for PI and AnnexinV and analyzed by FACS. Apoptotic cells were defined as AnnexinV^{+}PI$^{+/-}$ cells, necrotic cells as AnnexinV^{-}PI^{+}cells. In the lower panels, histograms represent the quantitative analysis of the fold change in the fraction of dead cells. *, $p<0.05$ with respect to vehicle-treated cells (CTR).

DETA/NO

As displayed in Figure 1, NO donors regulate telomerase activity. Moreover, it has been shown that it has a profound impact on stem cell differentiation towards a cardiovascular fate (Farsetti, et al., 2009).

After a 3-day treatment, DETA/NO-treated cells did not differ, with respect to vehicle-treated cells, in terms of nuclear density, while Ki67 resulted to be increased only in 10μM-treated cells (Figure 6). Importantly, all DETA/NO used concentrations significantly decreased the fraction of γH2AX-positive cells, with a trend to reduce the fraction of p21 positive cells only at 10 μM (Figure 6).

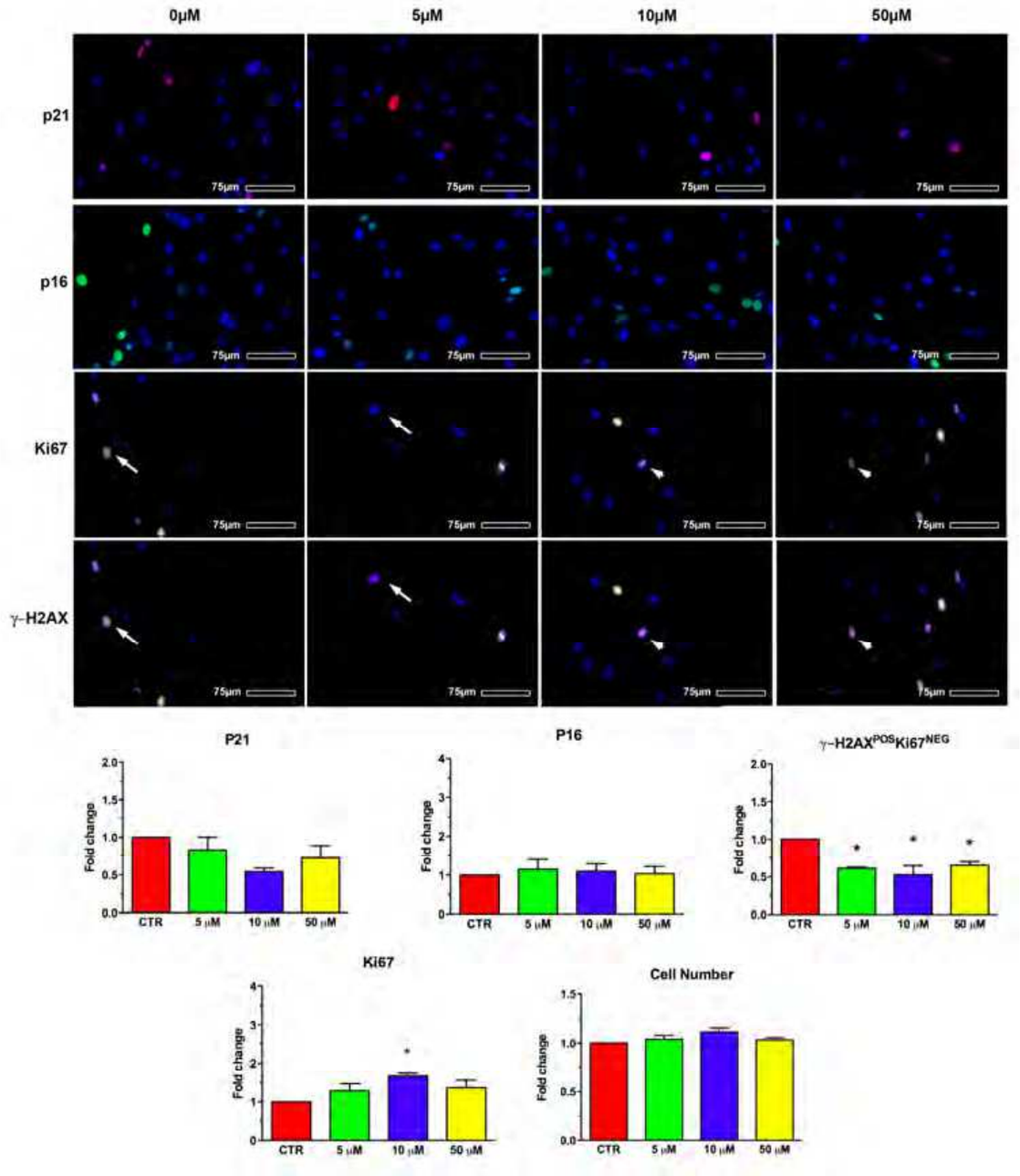


Fig. 6. **Effects of DETA/NO on hCSC.** hCSC were exposed for 3 days to DETA/NO 0μM, 5μM, 10μM and 50μM. Cells were then stained for p21 (red fluorescence), p16 (green fluorescence), Ki67 (yellow fluorescence) and γH2AX (magenta fluorescence). DNA-damage foci positive cells (arrows) were recognized as cells positive for γH2AX (magenta fluorescence) but negative for Ki67 (yellow fluorescence). Cells positive for both Ki67 and γH2AX (arrowheads) were excluded from the count. Histograms represent the fold changes in the fraction of cells expressing senescence (p21, p16, $\gamma H2AX^{+}Ki67^{-}$) and proliferation markers (Ki67) and in hCSC number of treated cells with respect to vehicle-treated cells (CTR). *, $p<0.05$ with respect to CTR.

No significantly changes in the fraction of dying cells was assessed (Figure 7).

In conclusion, DETA/NO seemed to act specifically by reducing the fraction of cells with DNA-damage foci eliciting a DNA-damage response. This is in line with its ability to activate telomerase activity.

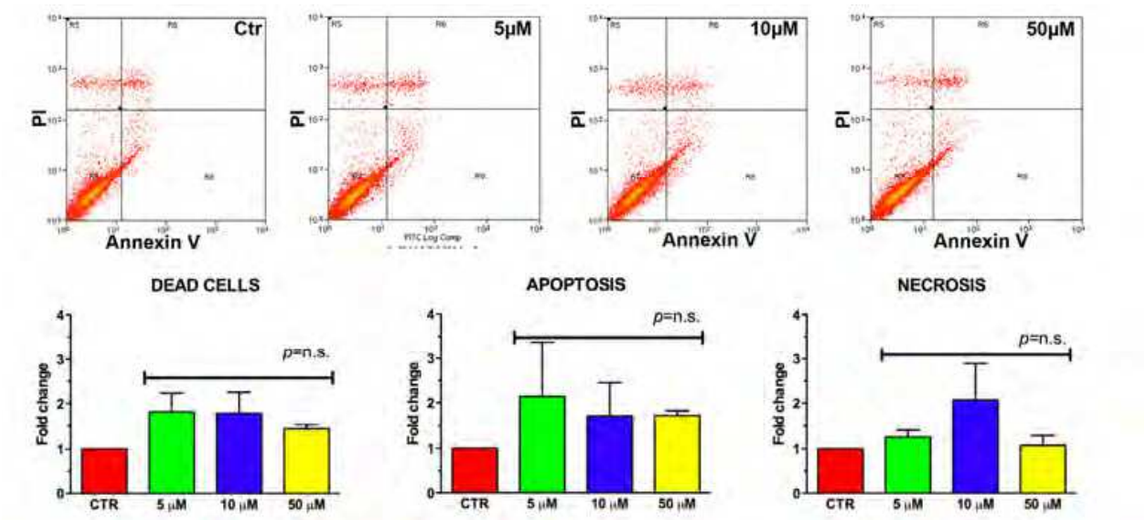


Fig. 7. **Effects of DETA/NO on hCSC death.** In the upper panel dot-plots graphically represent hCSC cultured in the presence of different concentrations of drug, stained for PI and AnnexinV and analyzed by FACS. Apoptotic cells were defined as AnnexinV^{+}PI$^{+/-}$ cells, necrotic cells as AnnexinV^{-}PI^{+}cells. In the lower panels, histograms represent the quantitative analysis of the fold change in the fraction of dead cells.

Altogether, the analysis of the effects of a three-day drug treatment of senescent hCSC, showed that, although all the utilized drugs exerted a beneficial effect in reducing the fraction of senescent cells, they differed not only in the pathway of cell senescence specifically targeted (p16 vs γH2AX/p21), but also in their ability to interfere with other key-processes such as cell proliferation and cell death. Table 1 summarizes the effects of the drugs on the principal cell processes and indicates the identified optimal drug concentration.

	Variable	Rapamycin	Resveratrol	DETA/NO
Senescence	**P16**	↓		
	P21		↓	↓
	γH2AX		↓	↓
Proliferation	**Ki67**		↑	↑ (10 μM)
	Nuclear density		↑	
Cell Death	**Apoptosis**	↑		
	Necrosis		↓	
Optimal drug concentration		10 nM	0.5 μM	10 μM

Table 1. Summary of drug effects on hCSC senescence, proliferation and death.

5.2.3 Effects of drugs on hCSC function

In order to verify whether the beneficial effects exerted by drugs on hCSC senescence were paralleled by an improvement in hCSC function, hCSC treated for three days with the optimal drug concentration were assayed, after two days of recovery, for: growth kinetic, differentiation capacity and migration abilities.

Growth kinetic

Despite the fact that during the three-day treatment only Resveratrol-treated cells resulted to increase their number (Figure 3, 5 and 7), all the drugs resulted to be effective in significantly reduce the population doubling time (Figure 8, p=0.002), suggesting that the reduction in the fraction of senescent cells was afterward associated with an increased proliferation rate.

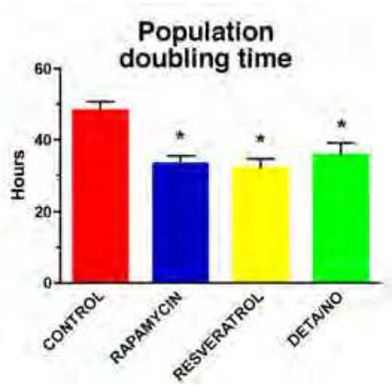


Fig. 8. Effects of drugs on hCSC population doubling time. *, $p<0.05$ with respect to vehicle-treated cells (CONTROL).

Differentiation ability

We investigated the ability of drug-treated hCSC to differentiate along the endothelial and myogenic fate. CD31 was utilized as endothelial marker, while alpha-sarcomeric actin as myogenic markers.

Interestingly, we have seen that cells treated for three days with drugs and then exposed to endothelial-differentiation inducing conditions displayed different behaviour. Specifically, while Rapamycin-treated cells significantly improved their ability to differentiate into endothelial cells expressing CD31, Resveratrol and DETA/NO did not (Figure 9).

Regarding, myocyte differentiation capacity, we noticed that cell cultures differed not only in the percentage of alpha-sarcomeric actin (ASA) positive cells, but also in the level of organization of the filaments. Therefore, we decided to use a score able to taking into account these two factors and defined as the product of the fraction of ASA-positive cells and an index expressing ASA organization, which ranged from 1 (not-organized) to 3 (well defined filaments)(Cesselli, et al., 2011). Applying these criteria, we established that Rapamycin did not interfere with the differentiation ability of hCSC, while the other two, especially DETA/NO, improved the myogenic potential of hCSC (Figure 10).

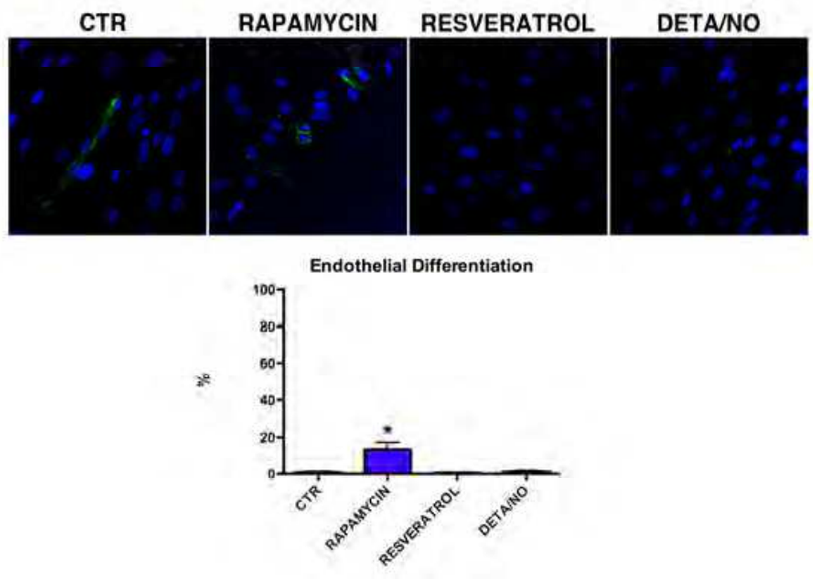


Fig. 9. **Effects of drugs on hCSC endothelial differentiation ability.** Green fluorescence represent CD31 expression on hCSC exposed to endothelial differentiation medium. Nuclei are depicted by the blue fluorescence of DAPI staining. Histograms represent the quantitative analysis of the fraction of CD31-positive cells in the cells treated with different drugs. *, $p<0.05$ vs vehicle-treated cells (CTR).

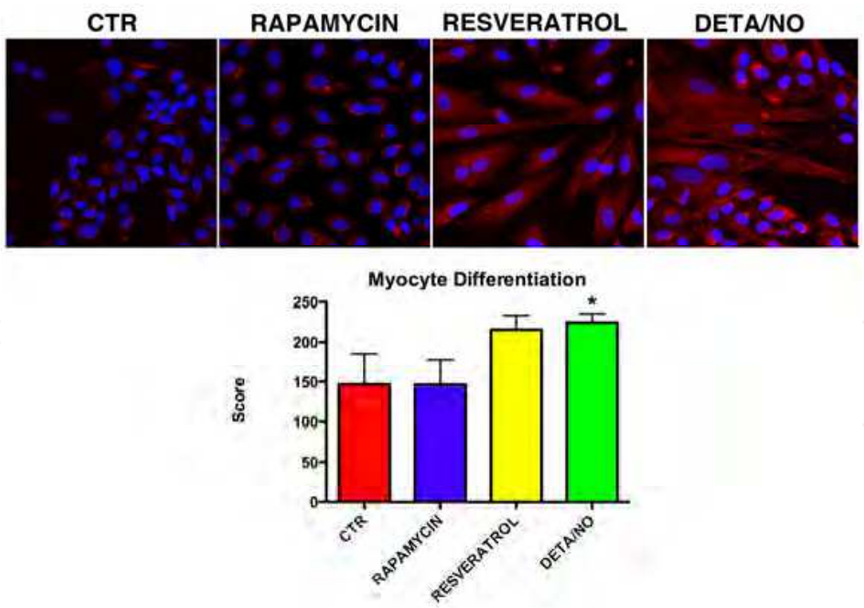


Fig. 10. **Effects of drugs on hCSC myocyte differentiation ability.** Red fluorescence represent alpha-sarcomeric actin expression on hCSC exposed to myocyte differentiation medium. Nuclei are depicted by the blue fluorescence of DAPI staining. Histograms represent the quantitative analysis of the level of myocyte differentiation of the cells treated with different drugs. See text for score meaning. *, $p<0.05$ vs vehicle-treated cells (CTR).

It remains to be demonstrated whether the improved differentiation ability of drug-treated cells is a consequence of the benefical effects of the drug on senescence or if it is due to a direct effect of the drug on differentiation pathways. In fact, the ability of DETA/NO to favor stem cell differentiation towards a cardiovascular fate has already been demonstrated in mouse embryonic stem cells (Farsetti, et al., 2009). Moreover, oxytocin, a hormone present also in the heart, induces embryonic and cardiac somatic stem cells to differentiate into cardiomyocytes, possibly through nitric oxide(Danalache, et al., 2007).

Migration capacity

In order to establish the migration speed of hCSC, a scratch assay was performed. With respect to vehicle treated cells, only DETA/NO-treated cells showed a trend to increase their migration ability (Figure 11), while Rapamycin and Resveratrol treated cells did not. Even in this case, it is difficult to establish whether DETA/NO would act directly on the migration ability of the cells, since it has already demonstrated a role of NO on SDF-1/CXCR4-mediated bone-marrow cell migration(Cui, et al., 2007).

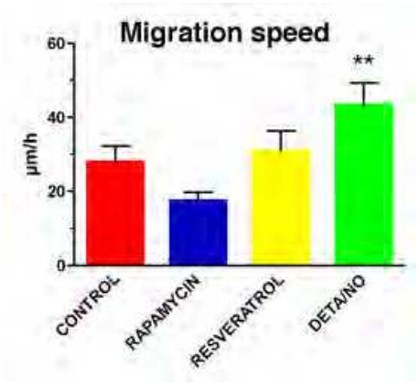


Fig. 11. **Effects of drugs on hCSC migration ability.** Histograms represent the quantitative analysis of migration speed of the cells treated with different drugs. **, $p<0.05$ vs Rapamycin-treated cells.

As a whole, drug treatment did not modify hCSC phenotype and stem cell marker expression. However, different effects were observed with respect to cell death, where Rapamycin increased of about 1.5 fold hCSC apoptosis ($p=0.002$), whereas Resveratrol showed a protective effect on cell necrosis, reducing it by 50% ($p=0.0001$). Although all drugs were associated with a significant decrease in the fraction of senescent cells, different pathways of cellular senescence were involved. Specifically, while Resveratrol and DETA/NO treatment were associated with a significant reduction by half of cells with DDR and p21 expression, Rapamycin treatment was mainly associated with a ≈60% reduction in p16 expression ($p<0.05$). Importantly, although all drug-treated cells showed, with respect to vehicle, an increase in cell proliferation, the effects on hCSC differentiation and migration ability were different. Specifically, Rapamycin treated cells displayed an improved endothelial differentiation capacity, while Resveratrol seemed to positively affect only the myogenic potential of hCSC. Finally, DETA/NO improved both the myocyte differentiation capacity and the migration ability of hCSC, without effects on endothelial differentiation capacity.

6. Conclusions

Severe heart failure is characterized by the loss of the growth reserve of the adult heart, dictated by a progressive decrease in the number of functionally-competent hCSC(Cesselli, et al., 2011). Despite these limitations, autologous CSC therapy is feasible and can be considered a therapeutic option for the large population of patients affected by severe heart failure(Beltrami, et al., 2011b; Segers and Lee, 2008). In fact, even in patients with advanced cardiomyopathies hCSC can be isolated from small myocardial biopsies and expanded in vitro(Cesselli, et al., 2011; D'Amario, et al., 2011b; Smith, et al., 2007). For this reason, it would be extremely intriguing any attempt aimed at "improving" the quality of the expanded cells, selecting the fraction of cells with the highest regenerative potential.

In this regard, Anversa's group showed that different membrane receptors influence the regenerative ability of hCSC and that IGF-1 receptor-positive hCSC are endowed with a high regenerative ability, representing a potent cell population for cardiac repair(D'Amario, et al., 2011a). However, this approach would require the sorting of cells expressing specific surface antigens, thus adding a further grade of complexity to the procedure aimed at producing clinical grade hCSC.

The strategy we wanted to undertake in this project was slightly different, since we decided to treat hCSC with drugs in culture. The results we obtained indicate that, although hCSC isolated from failing hearts are senescent and functionally impaired, it is possible to interfere pharmacologically, at least in vitro, with the senescence processes, rescuing the properties of the primitive cells. Specifically, we have shown that a three-day treatment with Rapamycin, Resveratrol or DETA/NO was able to reduce the fraction of senescent cells, improving their proliferative capacity. Importantly, the tested drugs seemed to exert their effects on different subpopulations of senescent cells; in fact, while Rapamycin mainly reduced the p16-positive fraction, DETA/NO and Resveratrol principally acted on the pool of cells characterized by DNA-damage foci and expressing p21. Similarly, different drugs showed different effects on hCSC function. In fact, while Rapamycin increased endothelial differentiation ability, DETA/NO improved hCSC myogenic and migration capacity.

These results represent the first demonstration that hCSC senescence can be attenuated in vitro, and that this is associated with an improved proliferative capacity.

Future research will be aimed: 1) at understanding more in depth the mechanism through which drugs exert their effects on cellular senescence, e.g. removal of senescent cells, modulation of SASP-mediated pathways; 2) establishing whether drug-treated cells possess an increased in vivo regenerative potential; 3) establishing criteria to define which is the best drug to use.

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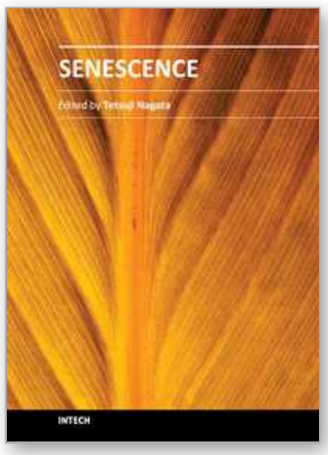
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The book "Senescence" is aimed to describe all the phenomena related to aging and senescence of all forms of life on Earth, i.e. plants, animals and the human beings. The book contains 36 carefully reviewed chapters written by different authors, aiming to describe the aging and senescent changes of living creatures, i.e. plants and animals.

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