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N-acetylcysteine protects induced pluripotent stem cells from *in vitro* stress: impact on differentiation outcome

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ABSTRACT Induced pluripotent stem cells (iPSCs) have the ability to differentiate towards various cell types of the adult organism and are a potential source of transplantable material in regenerative medicine. The entire process of conversion of iPSCs into terminally differentiated cells takes place *in vitro* and requires long periods of time. During *in vitro* culture, cells are exposed to environmental factors, which are capable of decreasing cellular performance and viability. Oxidative stress is the major underlying mechanism of such negative impact of *in vitro* environmental factors. We aimed to study the alteration of cellular properties during *in vitro* hematopoietic differentiation of human iPSCs and the ability of N-acetylcysteine (NAC), a potent free radical scavenger, to prevent such alterations. IPSCs were differentiated towards hematopoietic cells in the presence of 1 mM NAC. Intracellular reactive oxygen species (ROS), nitric oxide (NO), senescence, apoptosis and mitochondrial membrane potential (MMP) were evaluated at 1 and 3 weeks of differentiation. In the course of hematopoietic differentiation of iPSCs, cells progressively accumulated intracellular ROS and NO, increased the levels of apoptosis and senescence, and showed a decrease in mitochondrial functionality. NAC supplementation reversed all these phenomena. NAC administration also improved hematopoietic differentiation of iPSCs in terms of production of CD34, CD45 and CD43 positive cells. In conclusion, when supplemented during hematopoietic differentiation of iPSCs, NAC decreased oxidative stress, rescued the decline in cellular properties induced by long-term *in vitro* culture and promoted hematopoietic differentiation of iPSCs.

KEY WORDS: *NAC, ROS, iPSC, hematopoiesis*

Introduction

Transplantation of hematopoietic stem cells (HSC) in disease treatment is not always possible due to insufficient availability of tissue-matched donor material. Therefore, methods for the *in vitro* production of hematopoietic cells are of great interest.

Recently, it was described that terminally differentiated cells can be reprogrammed into cells with key properties of embryonic stem cells. These so called iPSCs are able to self-renew and contribute to the formation of the three embryonic layers both *in vitro* and *in vivo* (Takahashi *et al.,* 2007). Due to these properties and their autologous origin, iPSCs are considered as a potential unlimited source of differentiated cells for regenerative medicine. So far, iPSCs were obtained from different tissues and species (Giorgetti *et al.,* 2009) and successfully differentiated towards various cell types (Szabo *et al.,* 2010). However, the differentiation of human iPSCs towards functional HSC and hematopoietic progenitor cells (HPC) remains a challenge.

For the production of desirable cell types iPSCs must undergo

Abbreviations used in this paper: CBiPSCs, cord blood derived induced pluripotent stem cells; CCCP, carbonyl cyanide m-chlorophenylhydrazone; CD, cluster of differentiation; CFU-A, colony-forming unit assay; CFU-E, colony-forming unit-erythroid; CFU-G, colony-forming unit-granulocyte; CFU-GM, colony-forming unit-granulocyte; macrophage; CFU-M, colony-forming unit-macrophage; DiIC, 1,1',3,3,3',3'-hexamethylindodicarbocyanine iodide; FiPSCs, fibroblasts derived induced pluripotent stem cells; HPC, hematopoietic progenitor cells; HSC, hematopoietic stem cells; iPSCs, induced pluripotent stem cells; KiPSCs, keratinocytes derived induced pluripotent stem cells; MMP, mitochondrial membrane potential; NAC, N-acetylcysteine; NO, nitric oxide; PI, propidium iodide; ROS, reactive oxygen species.

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the whole process of conversion from pluripotent cells into terminally differentiated cells in a dish. Factors which influence cells in their natural *in vivo* niches are not always present *in vitro,* and other factors (enzymatic treatment, mechanical stress, growth on artificial surface, co-culture with other cell types, altered gas composition etc.) are newly introduced. This discrepancy in conditions leads to the acquisition of different properties in cultured cells compared to analogous populations *in vivo.* Proliferation, differentiation potential and homing ability of cells change upon *in vitro* culture (Ramos-Mejia *et al.,* 2012). Cultured cells often show a general decline in their state, loss of viability and increase in apoptosis, a state referred to as "culture shock" (Halliwell, 2003).

To preserve the properties of cultured cells, care should be taken to approximate culture conditions to those *in vivo.* The principal difference between *in vitro* and *in vivo* systems is that *in vitro* conditions impose a state of oxidative stress and trigger oxidative pathways in cells. Some factors of *in vitro* culture are able to stimulate intracellular production of ROS (for review see (Halliwell, 2003). ROS are important for normal cellular functions but they also exert deleterious effects by irreversibly oxidizing biological molecules and altering their properties, and participating in the malignant transformation. Cells trigger mitochondrial apoptosis in response to increased levels of ROS in order to skip the potential hazard (Buttke *et al.,* 1994). Thus, general decline in the state of cultured cells might be explained at least in part through the increase in ROS during *in vitro* cell culture.

NAC is a potent antioxidant. Due to ROS scavenging ability, the primary function of NAC is inhibition of ROS induced cellular damage and apoptosis. The cytoprotective function of NAC is well shown *in vitro* upon stimulation with various oxidative stress inducers and toxic agents (Chaudhari *et al.,* 2007), as well as *in vivo,* where it has beneficial impact on the outcome of surgical procedures and on the course of oxidative stress associated diseases (Knuckey *et al.,* 1995). NAC is an excellent cytoprotective compound for hematopoietic cells, since it preserves parameters of blood formula and hemoglobin level, inhibits apoptosis upon carmustine treatment in rats, (El-Sayed *et al.,* 2010) and decreases oxidative stress upon exposure to pesticides in peripheral blood mononuclear cells in humans (Ahmed *et al.,* 2009). NAC exhibits good radioprotective qualities and improves clonogenic functions and long-term engraftment of HSC and HPC, and decreases genotoxicity and cytotoxicity effects in bone marrow upon irradiation in different mammal species (Demirel *et al.,* 2009). ROS associated defects in FoxO and Atm deficient mice (models with increased ROS level in HSC) (Ito *et al.,* 2004; Tothova *et al.,* 2007) can also be rescued by NAC. In Atm-/- mice, NAC reduces ROS level in HSC and normalizes their size, cell cycling, apoptosis level (Tothova *et al.,* 2007) and improves long-term self-renewal capacity (Ito *et al.,* 2006).

NAC also facilitates the differentiation of various cell types such as mouse calvarial cells (Jun *et al.,* 2008), rat and human dental pulp stromal cells (Paranjpe *et al.,* 2007), human primary keratinocytes and human colony and ovary carcinoma cells (Parasassi *et al.,* 2005), and mouse embryonic stem cells (Qian *et al.,* 2009).

Hematopoietic differentiation of iPSCs is a long lasting process, which in many cases requires several weeks. We hypothesized that this period is sufficient to trigger oxidative stress and associated cellular decline. We anticipated that due to its cytoprotective and antioxidative properties, NAC supplementation could reverse these phenomena and have an impact on hematopoietic differentiation of iPSCs. In the current study we describe the beneficial effects of NAC on differentiating iPSCs.

Results

NAC treatment improves the qualities of iPSCs during differentiation

NAC decreases intracellular ROS production

First, we investigated if the intracellular ROS production was affected during the *in vitro* hematopoietic differentiation of iPSCs, and if NAC treatment had an effect on this phenomenon. In a pilot experiment, different doses of NAC were tested for their physiological effects. As 0.1mM NAC showed no effect and addition of 5mM NAC inhibited iPSCs growth and differentiation, we chose 1mM NAC concentration for our further experiments (data not shown and Fig. 5A). The levels of intracellular ROS were estimated using a derivative of H_2DCFDA, a compound which gains fluorescence upon the intracellular oxidation. We found that the levels of intracellular ROS were increased during the differentiation process (Fig. 1A-B). In the control, intracellular ROS levels augmented about 4 fold from one to three weeks of differentiation. NAC did not change the ROS levels at one week of differentiation, but decreased them at three weeks of differentiation (Fig. 1A-B).

NAC prevents intracellular NO production

The production of reactive nitrogen species can further increase the deleterious effects of oxidative stress on cells. To evaluate if

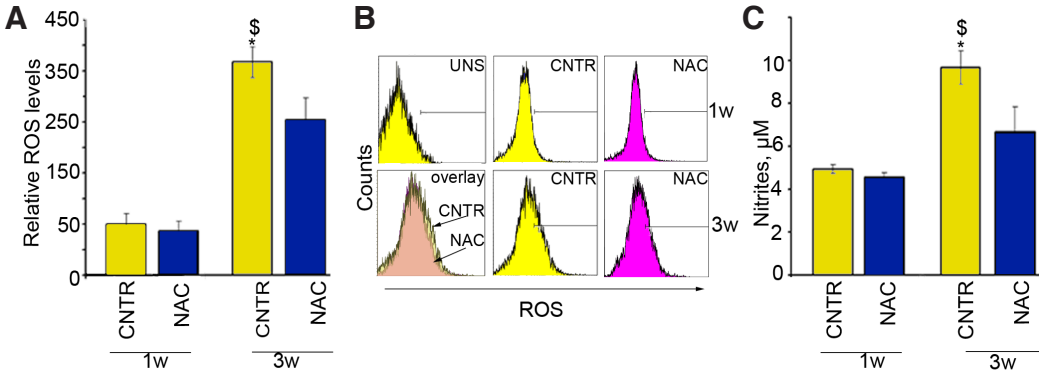

Fig. 1. N-acetylcysteine (NAC) decreases free radical production in differentiating induced pluripotent stem cells (iPSCs). (A) *Relative radical oxygen species (ROS) levels in NAC treated and untreated cultures of iPSCs at 1 and 3 weeks (w) of differentiation.* **(B)** *FACS histograms of control and NAC treated iPSCs upon carboxy-H_2DCFDA staining at 1 and 3 weeks of differentiation.* **(C)** *Nitric oxide (NO) production in NAC treated and untreated cultures of iPSCs at 1 and 3 weeks of differentiation. *p < 0.05 denotes significant difference between control and NAC treated samples, $ - between controls.*

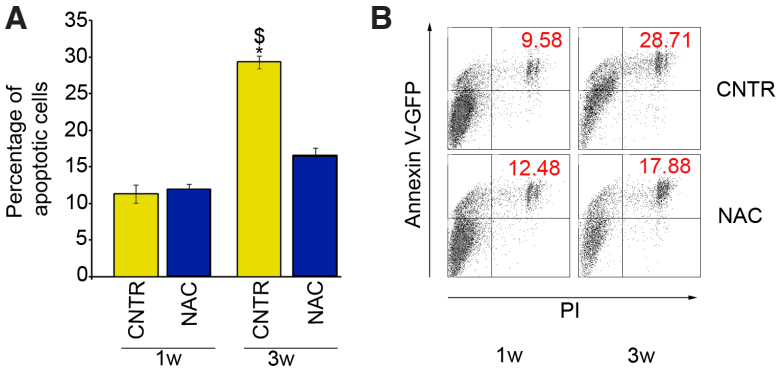

Fig. 2. N-acetylcysteine (NAC) decreases apoptosis in differentiating induced pluripotent stem cells (iPSCs). (A) *The level of apoptotic cells in NAC treated iPSCs and in control upon 1 and 3 weeks of differentiation.* **(B)** *FACS histograms of Annexin V-EGFP-PI stained NAC treated and untreated iPSCs upon 1 and 3 weeks of differentiation. Percentage of apoptotic cells is indicated. *p < 0.05 denotes significant difference between control and NAC treated samples, $ - between controls.*

the level of NO release by iPSCs cultures was increased during differentiation we measured the levels of nitrites, which are stable end products of NO metabolism, in the cell culture media. Fig. 1C shows an increase of NO release over the course of iPSCs differentiation. In control cells, NO release after three weeks of culture was about 2 fold higher than the release of NO after one week. NAC treatment resulted in a significant decrease in the release of NO at three weeks of differentiation (Fig. 1C).

NAC protects against apoptosis

Next we checked if the long-term process of *in vitro* hematopoietic differentiation of iPSCs was associated with an increase in apoptosis. For detection of apoptosis we performed annexin V-EGFP - PI staining of cells at one and three weeks of differentiation. Annexin V is described to bind to phosphatidylserines translocated to outer surface of the cell membrane at early stages of apoptosis (Umeda *et al.,* 1989). We found that the number of apoptotic cells increased with differentiation (Fig. 2 A-B). The percentage of apoptotic cells rose from 11.31% ± 1.22 at one week of differentiation up to 29.30% ± 0.84 at three weeks under basal conditions, but only to 16.6% ± 1.05 after NAC treatment (Fig. 2A).

NAC prevents cellular senescence

Activity of SA-β-galactosidase at pH 6 is a characteristic of senescent cells, and detected by X-gal staining. We found that the number of cells stained positively for SA-β-galactosidase increased with differentiation both in NAC treated and control iPSCs. Already at 6 days of differentiation the amount of SA-β-galactosidase positive cells was higher in the control group than in cells treated with NAC (Fig. 3A-C). Analysis of the area occupied by SA-β-galactosidase positive cells supported these observations (area fraction 5.4% ± 0.34 and 2.23% ± 1.25 in control and NAC treated cultures, respectively) (Fig. 3B).

NAC prevents mitochondrial membrane potential reduction

Mitochondria are key cellular organelles for the regulation of apoptosis. The set of alterations in mitochondrial morphology and functionality is associated with accumulation of proapoptotic signals in the cell and its entry into self-elimination. One of such alterations is the disruption of the mitochondrial membrane potential (MMP), which reflects the energetic potency of mitochondria. We monitored the changes in MMP levels using FACS and staining with DiIC, a cyanine dye which accumulates primarily in mitochondria correspondently to MMP level. The *in vitro* differentiation of iPSCs caused a decrease in MMP (Fig. 4 A-B). Treatment with NAC partially protected against MMP drop at three weeks of differentiation (mean fluorescence intensity 79,38 ± 4.2 and 59.69 ± 6.1 in NAC treated cultures and in control, respectively) (Fig. 4B).

We also investigated how mitochondria from NAC treated and untreated cells were able to maintain MMP upon treatment with carbonyl cyanide m-chlorophenylhydrazone (CCCP). CCCP is a protonophore that renders the mitochondrial inner membrane permeable for protons causing dissipation of the proton gradient. It can induce ROS generation and ultimately apoptosis in some kind of cells or potentiate apoptosis triggered by other stimuli (de Graaf *et al.,* 2004; Chaudhari *et al.,* 2007). As expected, the ability of cells to maintain MMP was decreased upon treatment with CCCP both in control and NAC treated groups at one and three weeks of differentiation. However, NAC treatment protected cells against the MMP loss upon CCCP treatment at three weeks of differentiation (39.36 %± 2.17 and 30.8 % ± 4.6 in NAC treated cultures and in control, respectively) (Fig. 4 A-B).

NAC improves hematopoietic differentiation in vitro

To monitor the hematopoietic differentiation of iPSCs we evaluated the presence of CD34 and CD45 positive cells, since these markers have been reported to be expressed on virtually all progenitor cells from bone marrow and cord blood (Almici *et al.,* 1995; Sutherland *et al.,* 1992). It has also been extensively shown that *in vitro* obtained CD34 or CD45 positive cells are able to

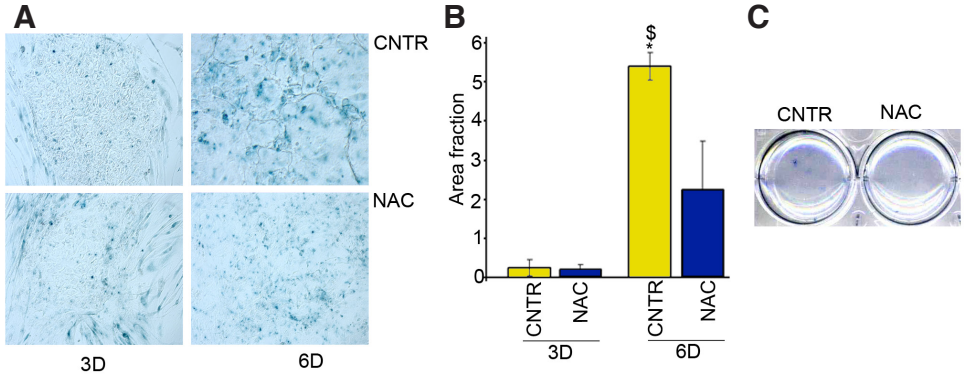

Fig. 3. N-acetylcysteine (NAC) decreases senescence of differentiating induced pluripotent stem cells (iPSCs). (A) *Pictures (200X) of iPSCs stained for SA-β-galactosidase in NAC treated and untreated cultures at 3 (3D) and 6 (6D) days upon hematopoietic differentiation.* **(B)** *Area fraction of SA-β-galactosidase positive cells in control and NAC treated iPSCs.* **(C)** *View of plates with iPSCs treated and untreated with NAC at day 6 of differentiation upon SA-β-galactosidase staining. *p < 0.05 denotes significant difference between control and NAC treated samples, $ - between controls.*

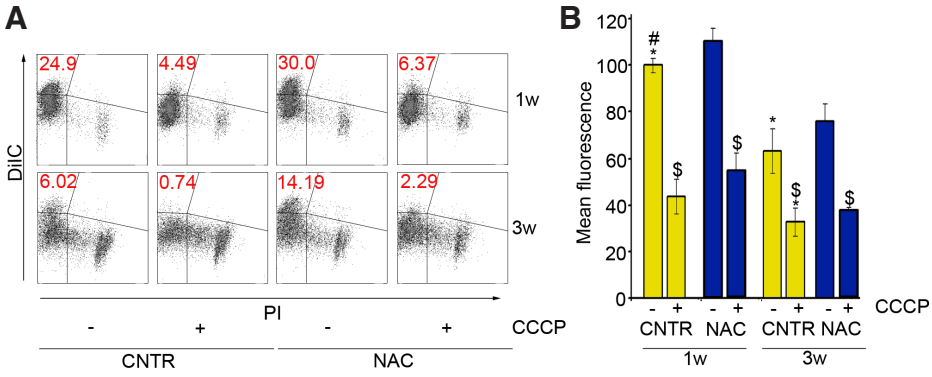

Fig. 4. N-acetylcysteine (NAC) prevents mitochondrial membrane potential (MMP) collapse in differentiating induced pluripotent stem cells (iPSCs). (A) *FACS histograms of differentiating iPSCs upon DiIC staining at 1 and 3 weeks of differentiation in NAC treated cultures and in control. Cells were treated or not with CCCP. DiIC staining of cells treated with CCCP in control was taken as reference to demarcate alive cell population with energized mitochondria (its percentage is indicated).* **(B)** *Mean intensity of fluorescence of alive fraction of DiIC stained cells. *p < 0.05 denotes significant difference between control and corresponding NAC treated samples. $ p < 0.05 denotes significant difference between corresponding samples treated or untreated with CCCP. # p < 0.05 denotes significant difference between control samples at 1 and 3 weeks of differentiation untreated with CCCP.*

form colonies in colony forming unit assays (CFU-A), and in some cases to engraft into the bone marrow of sub-lethally irradiated animals upon intra-bone marrow injection (Szabo *et al.,* 2010). Moreover, it has been recently shown that early blood progenitors in differentiating human embryonic stem cells can be identified by the expression of CD43 (Vodyanik *et al.,* 2006).

We found that upon hematopoietic differentiation the outcome of cells expressing hematopoietic marker CD34 was about 1.8 fold higher in NAC treated cultures of fibroblast - derived iPSCs (FiPSCs) than in untreated cells (Fig. 5A). High doses of NAC (5mM) exerted negative effects on the differentiation of FiPSCs (Fig. 5A). Next, we asked if NAC supplementation could have the same effects in cultures of cord blood – derived iPSCs (CBiPSCs) and keratinocytes – derived iPSCs (KiPSCs). We show that NAC increased the production of CD43 positive cells in KiPSCs, (Fig. 5B) and of both CD45 and CD43 positive cells in CBiPSCs (Fig. 5C) upon hematopoietic differentiation.

CFU-A was performed to evaluate the hematopoietic potential of iPSCs - derived CD34 positive cellular fraction. We found that CD34 positive cells obtained during hematopoietic differentiation of iPSCs both from NAC treated and untreated cultures were capable of forming colonies in CFU-A, stating that they produce real hematopoietic progenitors (Fig. 5D, a-d). Wright-Giemsa staining of cytospin preparations of cells isolated from individual CFU-E colonies showed immature erythroblasts, preparations of CFU-M showed cells with monocytic morphology and preparations of CFU-G showed the morphology of granulocytes (Fig. 5D, e-g). CD34 positive cells obtained upon hematopoietic differentiation of iPSCs in the presence of NAC exhibited equal colony forming ability to the control without changes neither in number nor in qualitative composition of produced hematopoietic colonies (Fig. 5E).

Discussion

Some factors of the *in vitro* culture can dramatically affect the cellular properties (Halliwell, 2003). This phenomenon can be crucial for the production of good quality cells for clinical applications and care should be taken to avoid such negative effects.

Here, we showed on the example of hematopoietic differentiation of iPSCs that standard *in vitro* culture *per se* was quite stressful

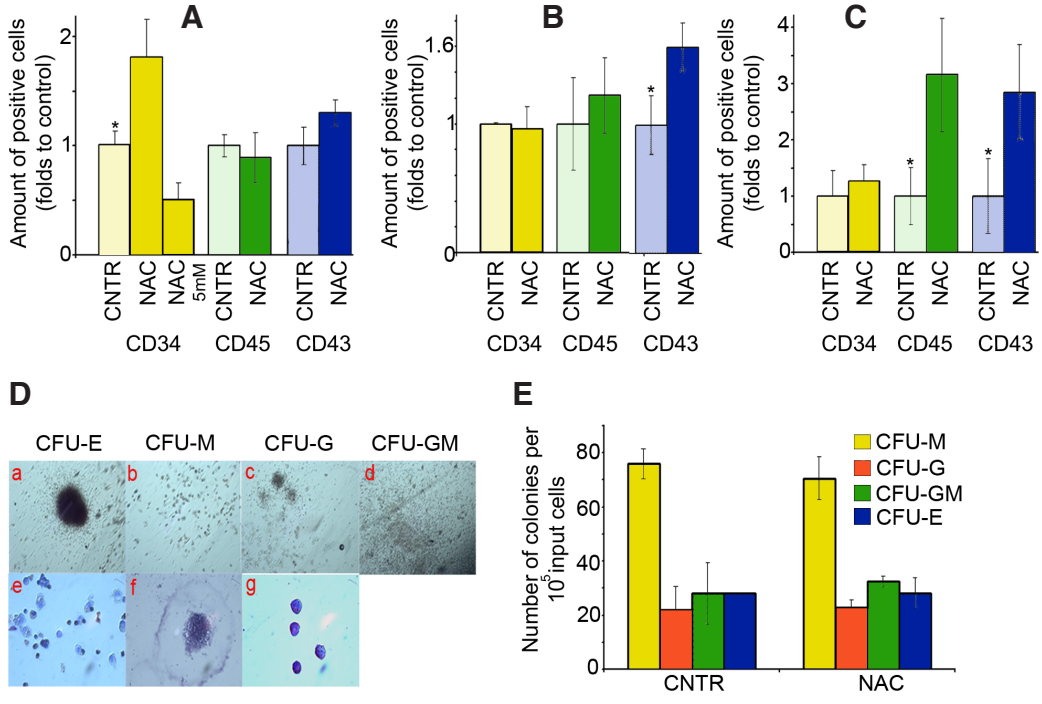

Fig. 5. N-acetylcysteine (NAC) increases efficiency of hematopoietic differentiation of induced pluripotent stem cells (iPSCs). (A) *NAC increases expression of hematopoietic markers in FiPSCs. 5mM NAC exerts inhibitory effect.* **(B)** *NAC increases expression of hematopoietic markers in KiPSCs.* **(C)** *NAC increases expression of hematopoietic markers in CBiPSCs.* **(D)** *Pictures (400x) of hematopoietic colonies obtained during CFU-A from iPSCs-derived CD34 positive cells (a-d) and their cytospins (e-g).* **(E)** *CFU-A of iPSCs-derived CD34 positive cells obtained upon differentiation with NAC. *p < 0.05 denotes significant difference between control and NAC treated samples.*

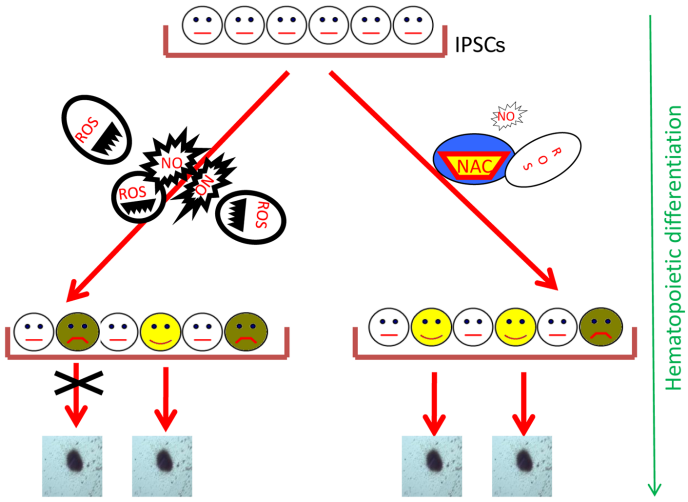

Fig. 6. Model of hematopoietic differentiation of induced pluripotent stem cells (iPSCs) in the abscence or presence of N-acetylcysteine (NAC). *Under normal conditions, differentiating iPSCs are subjected to the deleterious influence of free radicals (ROS and NO). As a result, some cells are damaged and do not participate any more in the process of differentiation and only a few cells undergo the differentiation program completely. When NAC is present in the culture, it decreases the negative effect of free radicals, thus fewer cells are damaged and more cells complete the differentiation program.*

imposing a state of oxidative stress and leading to the progressive deterioration of cultured cells. Supplementation with NAC diminished these effects and was beneficial for the differentiation outcome: iPSCs from various sources produced higher amount of hematopoietic progenitors with preserved ability to give rise to granulopoietic, macrophage and erythropoietic derivatives.

Even though the overall qualitative impact of NAC on differentiation was similar in all tested cell lines, there were specific patterns in each of them. In FiPSCs, NAC preferentially promoted the expression of CD34 positive cells, whereas in CBiPSCs it increased the expression of CD45 and CD43, and only CD45 in KiPSCs. This cell line specific response to NAC may reflect intrinsic properties of iPSCs lines, which show various differentiation potential towards blood under standard conditions in terms of CD34 and CD45 expression and hematopoietic potential in CFU-A (data not shown). It corresponds with the data that human lines of pluripotent stem cells differ in the hematopoietic potential (Ramos-Mejia *et al.,* 2010).

We could not detect any improvement of cellular properties in iPSCs treated with NAC under self-renewal conditions (data not shown). Notably, culture conditions used for propagation of iPSCs and for their differentiation are quite different. Undifferentiated cells are split weekly and undergo daily media change. In this way exhausted feeders, degrading compounds and toxic end products of cellular metabolism are regularly removed from the cellular environment. Instead, in many protocols used for hematopoietic differentiation cells are cultivated *in vitro* during long periods with rare media change that allows the concentration of important factors for hematopoietic differentiation produced by feeders (Ledran *et al.,* 2008; Raya *et al.,* 2009). Therefore we speculate that the detrimental effects caused by oxidative stress *in vitro* become critical when the period of cultivation is long.

NAC treatment has been reported to promote cell-cycle progression of hematopoietic progenitors (Klingler *et al.,* 2002). However, in our hands NAC treatment did not cause significant differences in phase distribution in the total fraction of differentiating iPSCs or in CD34 positive cells only, compared to the control (Fig. S1).

We propose a model explaining the observed effects of NAC. Under hematopoietic differentiation conditions a portion of iPSCs enters the way to hematopoietic differentiation. However, this long-term *in vitro* differentiation process exposes cells to unfavorable environmental factors. That results in the increased intracellular production of ROS and NO, which trigger cell death. Thus, many cells committed to hematopoietic differentiation suffer apoptosis. Instead, when NAC is supplemented to iPSCs cultures, the levels of ROS, NO, senescence and apoptosis are decreased, whereas the resistance of iPSCs to oxidative stress and their mitochondrial functions are improved. This results in a higher number of cells, able to complete hematopoietic differentiation (Fig. 6).

Our data indicate that supplementation with antioxidants such as NAC is beneficial for culture and hematopoietic differentiation of iPSCs.

Materials and Methods

Materials

H_2O_2, NAC, sodium nitrite were from Sigma. SDF1, Flt3, VEGF, TPO, SCF, bFGF, BMP4 were from Peprotech.

Cell lines

IPSCs were previously obtained in our Laboratory, from cord blood (CBiPSCs4F4) (Giorgetti *et al.,* 2009), fibroblasts (FiPSCs3F1) (Rodriguez-Piza *et al.,* 2009) and keratinocytes (KiPSCs4F1) (Aasen *et al.,* 2008). All cultures were performed in standard cell culture incubators at 21% O_2 and 5% CO_2.

Culture and differentiation of iPSCs

For the culture of iPSCs irradiated human foreskin fibroblasts were used as feeders. IPSCs were cultured in growth media (KO-DMEM-HG (Gibco), supplemented with 1% NEAA (Lonza), 1x GlutamMAX (Gibco), 50 U/ml penicillin, 50 μg/ml streptomycin (Gibco), 50 μM β-mercaptoethanol (Gibco), 20% Knockout Serum Replacement (Gibco), 8 ng/ml bFGF). Passing of iPSCs was performed once a week mechanically under the microscope using strippers. For amplification, clusters of iPSCs were transferred to fresh dishes and left for 48 hours to attach. Later on the media was changed daily.

For hematopoietic differentiation colonies of iPSCs were scraped mechanically and transferred to ultra-low attachment dishes for embryoid bodies formation. After 48 - 72 hours embryoid bodies were transferred on OP9 feeders in differentiation media (IMDM supplemented with 10% FBS (Gibco), 100 U/ml penicillin, 100 μg/ml streptomycin, 1x GlutaMAX, 10ng/ml bFGF, 10 ng/ml Flt3l, 10 ng/ml VEGF, 10 ng/ml BMP-4, 20 ng/ml TPO, 25 ng/ml SCF). The media was changed twice a week. NAC was added to the culture medium at 1mM twice a week (if not specified differently in the text). Human cells were separated from mouse cells by FACS upon staining with anti-pan-human marker TRA-1-85 - fluorescine or allophycocyanin conjugated antibodies (R&D) and used for further experiments (and named in the article as differentiating iPSCs).

This study was done in accordance with Spanish laws and regulations regarding the manipulation of human iPSCs and following a protocol approved by the Spanish competent authorities (Comisión de Seguimiento y Control de la Donación de Células y Tejidos Humanos del Instituto de Salud Carlos III).

CD profile analysis

For CD profile analysis cells were stained with monoclonal antibodies against human CD34, CD45, CD43 conjugated with fluorescein, phycoerythrin, allophycocyanin, V450 or tri-color (BD Biosciences) according to the manufacturer's instructions and analyzed by using Moflo High performance

Cell Sorter and Flow Cytometry Analyzer Gallios (Beckman Coulter, Brea, CA, USA). Propidium iodide-stained dead cells were gated out.

CFU-A

For CFU-A CD34 positive cells were sorted by FACS, resuspended in 1 ml of methylcellulose (Stem Cell Technologies, 04434) and plated onto 35 mm dishes in duplicates. Cell plating densities were optimized according to cell subsets tested. Colonies were analyzed and scored under the microscope at day 14 according to the "Colony Atlas" (Stem Cell Technologies).

Wright-Giemsa staining

Few hematopoietic colonies of the same lineage used for the CFU-A were picked and cytospined. Then, cells were fixed in methanol and stained with Wright-Giemsa stain (Sigma) according to manufacturer's instructions.

ROS level determination

For determination of ROS levels suspension of differentiating iPSCs was incubated for 30 minutes with 50 μM carboxy-H_2DCF-DA (Molecular Probes) solution, washed with PBS and incubated for another 90 minutes in carboxy-DCF-DA free media and used for FACS analysis.

NO level determination

NO release by iPSCs was quantified colorimetrically by measuring the accumulation of stable nitrite in 1 ml of culture medium for 15 hours according to published protocols (D'Angelo *et al.,* 2001) with minor modifications. Briefly, 100 μl of culture supernatant was mixed with an equal volume of Griess reagent and incubated at room temperature for 10 min. The absorbance was read at 540 nm. Nitrite concentration was determined by comparison with a standard of $NaNO_2$. The concentration of nitrites produced by pure cultures of OP9 cells grown under equivalent conditions was subtracted from the concentration of nitrites produced by OP9/iPSCs co-culture.

Apoptosis detection

Apoptosis was measured using the Annexin V-EGFP Apoptosis Detection Kit from GeneScript according to manufacturer's instructions.

Senescence determination

Staining of cells for the detection of SA-β-galactosidase was done at days 3 and 6 of differentiation with Senescence β-galactosidase Staining Kit from Cell Signaling following manufacturer's instructions. The area fraction of SA-β-galactosidase positive cells was determined by analyzing areas in the center of embryoid bodies using ImageJ software.

MMP measurements

MMP was measured with MitoProbe DiIC Assay Kit for Flow Cytometry from Molecular Probes according to manufacturer's instructions. In some experiments cells were treated with 150 nM of CCCP.

Statistical analysis

Data are represented as mean ± SD of at least three independent experiments if not mentioned otherwise. Statistical significance was determined using Student's test. The difference between experiments was considered to be significant at $p < 0.05$.

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