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ORIGINAL CONTRIBUTION

Doxorubicin induces senescence and impairs function of human cardiac progenitor cells

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Abstract The increasing population of cancer survivors faces considerable morbidity and mortality due to late effects of the antineoplastic therapy. Cardiotoxicity is a major limiting factor of therapy with doxorubicin (DOXO), the most effective anthracycline, and is characterized by a dilated cardiomyopathy that can develop even years after treatment. Studies in animals have proposed the cardiac progenitor cells (CPCs) as the cellular target responsible for DOXO-induced cardiomyopathy but the relevance of these observations to clinical settings is unknown. In this study, the analysis of the DOXO-induced cardiomyopathic human hearts showed that the majority of human CPCs (hCPCs) was senescent. In isolated hCPCs, DOXO triggered DNA damage response leading to apoptosis early after exposure, and telomere shortening and senescence at later time interval. Functional properties of hCPCs, such as migration and differentiation, were also negatively affected. Importantly, the differentiated progeny of DOXO-treated hCPCs prematurely expressed the senescence marker $p16^{INK4a}$. In conclusion, DOXO exposure severely affects the population of hCPCs and permanently impairs their function. Premature senescence of hCPCs and their progeny can be responsible for the decline in the regenerative capacity of the heart and may represent the cellular basis of DOXO-induced cardiomyopathy in humans.

Keywords Cardiotoxicity · Doxorubicin · Cardiac progenitor cells · Senescence

Introduction

Doxorubicin (DOXO) is a very effective anticancer drug that belongs to a class of anthracyclines. The most serious side effect of DOXO treatment is a cardiomyopathy followed by congestive heart failure (CHF) [45]. The time of clinical manifestation of cardiotoxicity is broadly variable in patients receiving chemotherapy. Toxic effects can be present acutely after treatment, can occur within months or manifest even years or decades after drug administration [49]. Therefore, it is possible that myocardial damage progresses over time, independently from the acute, direct cytotoxic action of DOXO. Several mechanisms have been proposed to explain DOXO-induced cardiomyopathy including DNA damage, attenuation in protein synthesis, changes in the adrenergic system and defects in Ca^{2+} homeostasis, together with the increase in oxidative stress and lipid peroxidation [19, 34, 35, 43, 46, 51], but the causal mechanism of this complication remains unclear.

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For a long time, death of cardiomyocytes has been considered as the basis of progressive alterations of the cardiac muscle [3, 25, 53, 58, 60]. Recent studies have focused their attention on the evaluation of the effects of DOXO on other cell types suggesting an alternative explanation of the pathogenesis of DOXO-induced cardiomyopathy [18, 24, 48, 57].

The adult heart contains a population of primitive cells with stem cell characteristics that are responsible for tissue homeostasis in normal conditions and mediate myocardial regeneration in pathological states [4, 6, 37, 47]. Adult CPCs express the stem cell antigen c-kit, are self-renewing, clonogenic and multipotent, giving rise to cardiomyocytes, smooth muscle cells and endothelial cells. The involvement of CPCs in chronologic aging and in several pathological conditions has been documented in animals and humans [12, 15, 41, 42, 55], indicating this cell class as a pathophysiological target. Furthermore, increasing evidence indicates that senescence of stem cell population is a fundamental process that contributes to the onset and progression of heart failure [12, 15, 20, 26, 41, 55]. It has been shown in different animal models that DOXO cardiotoxicity is not restricted to cardiomyocytes, but severely affects also resident CPCs. DOXO exposure impaired vascular development and reduced the number of CPCs in juvenile mice, resulting in a higher susceptibility of the heart to stress in the adult life [24]. In anthracycline-exposed rats, DOXO decreased the number of functionally competent CPCs by inhibiting their proliferation together with accumulation of oxidative DNA damage, apoptosis and progressive cellular senescence. The depletion and premature senescence of the CPC population interfered with the physiological turnover of the myocardium [18]. Thus, the determining event responsible for the initiation and evolution of the myopathy arises at the level of the CPC compartment [18, 24]. However, the studies that identify new, stem cell-related mechanisms of DOXO cardiotoxicity are currently limited to animals. The cardiotoxicity induced by the cancer therapy is a growing epidemiologic problem, related to the late morbidity and mortality, that affects the quality of life of otherwise successfully treated cancer patients. For this reason, it is fundamental to establish the relevance of the animal findings to humans, providing information that may have important clinical implications. For this purpose, the aim of the present study was to determine the effects of DOXO on hCPCs. The hearts from cancer patients who died of CHF following chemotherapy with the anthracycline were analyzed to determine whether cellular senescence occurred in hCPCs. Moreover, to shed light on cellular processes that may be affected by DOXO, isolated hCPCs were exposed to the drug. Cell growth, death, senescence and functional properties such as migration and differentiation, together with the related molecular pathways were studied. In addition, since the time is an important variable in the pathogenesis of DOXO-induced cardiomyopathy, the early and late effects on these cellular events in DOXO-treated hCPCs were investigated.

Materials and methods

Human heart samples

Histological and immunohistochemical analyses were performed on hearts obtained at autopsy from oncologic patients who died of CHF developed after treatment with chemotherapeutic regimens including anthracycline ($n = 6$) and on two samples from patients with normal cardiac function that died from other complications early during chemotherapy with anthracycline. Control tissue was obtained from age-matched subjects that died from non-cardiovascular causes ($n = 6$). Patients' characteristics are shown in Table 1. Sections were stained with Masson's trichrome for the morphologic and morphometric analysis of myocardial damage or processed for immunofluorescence and confocal microscopy.

Isolation of hCPCs

The present study conforms with the national ethical guidelines (Italian Ministry of Health; D.L.vo 116, January 27, 1992) and has been performed upon approval of local ethics committee in accordance with the ethical standards of the 1964 Declaration of Helsinki and its later amendments. Informed consent was obtained from each patient to allow the collection of myocardial samples. c-kit-positive hCPCs were isolated from discarded fragments of myocardium obtained from atrial appendages of patients who underwent elective coronary bypass surgery or mitral valve replacement ($n = 6$). Cells were sorted by immuno-magnetic beads and cultured in alpha-MEM supplemented with FBS and bFGF.

DOXO treatment of hCPCs

c-kit positive hCPCs, from passage 3 to 7, were treated for 24 and 48 h with 0.1, 0.5 and 1.0 μM DOXO. These concentrations of DOXO were selected because they are comparable to peak or steady state plasma concentrations observed in patients after standard bolus infusion [34]. To evaluate long-lasting effects of anthracycline, hCPCs were cultured for 7 days in a fresh medium after 24 and 48 h of DOXO treatment. Differentiation of control and DOXO-treated hCPCs was induced with 10^{-8} M dexamethasone.

Table 1 Patient population

Case	Age (years)	Sex	B Wt (kg)	H Wt (g)	Disease	Anthracycline cumulative dose		Interval from therapy to CHF (years)	Cause of death
						(mg)	(mg/m^2)		
Anthracycline									
CHF									
1	50	F	60	271	NHL	180	122	1	CHF
2	48	F	59	343	AML	675	450	2	CHF
3	53	F	67	392	AML	680	453	1	CHF
4	61	M	81	480	NHL	600	316	1	CHF
5	60	M	75	470	AML	680	378	1	CHF
6	47	F	78	515	NHL	800	421	5	CHF
Mean ± SD	53 ± 6		70 ± 9	412 ± 93		603 ± 217	357 ± 126	1.8 ± 1.6	
No. CHF									
1	63	F	65	400	NHL	100	58	–	JCV encephalopathy
2	61	M	74	470	AML	75	40	–	GI hemorrhage
Controls									
1	58	M	68	339					Acute trauma
2	45	F	65	325					Acute trauma
3	49	F	61	343					Cerebral hemorrhage
4	52	F	64	342					Cerebral hemorrhage
5	59	F	67	307					Acute trauma
6	35	M	78	567					Acute trauma
Mean ± SD	50 ± 9		67 ± 6	337 ± 20					

NHL non-Hodgkin lymphoma, *AML* acute myeloid leukemia, *B Wt* body weight, *H Wt* heart weight, *CHF* congestive heart failure, *JCV* John Cunningham virus, *GI* gastrointestinal

hCPC viability, proliferation and death

Cytotoxicity of DOXO was detected by MTT assay. Cell proliferation was determined by the number of BrdU-positive hCPCs identified by immunostaining. In addition, Ki67 labelling was performed. TUNEL assay was used for the detection of apoptosis.

Senescence-associated β-galactosidase and telomere length

Senescent hCPCs were detected by senescence-associated β-galactosidase (SA-β-gal) activity using the artificial substrate X-gal. Telomere length was evaluated by quantitative fluorescence in situ hybridization (Q-FISH). The intensity of fluorescence was measured using Image Pro-Plus software.

Cell migration assay

Untreated and DOXO-treated hCPCs were resuspended in serum-free fresh medium and placed in 8 μm pores inserts in order to migrate towards complete medium. The number of migrated cells was counted in the whole area of the membranes at 20 × magnification.

Western blotting

Protein expression in control and DOXO-treated hCPCs lysates was determined for: cyclin D1, cdk4, $p16^{INK4a}$, $p21^{Cip1}$, phospho-$p21^{Cip1–Thr145}$, Bax, Bcl-2, Rb, phospho-Rb^{Ser798}, p53, phospho-$p53^{Ser15}$, ATM kinase, phospho-$ATM^{Ser1981}$, IGF-1R and c-Met.

Immunofluorescence

hCPCs were fixed in 4 % paraformaldehyde and labelled with c-kit antibody. Senescence was recognized by the expression of $p16^{INK4a}$ and phospho-$p53^{Ser15}$. DNA strand breaks were visualized by γH_2AX staining. Cell differentiation was determined by the expression of α-sarcomeric actin, myosin heavy chain, connexin43, α-smooth muscle actin, calponin, von Willebrand factor and CD31. Formalin-fixed tissue sections of human hearts were incubated overnight with primary antibodies.

Data analysis and statistics

Results are presented as mean ± SD. Significance was determined by Student's t test and by ANOVA with Bonferroni post-test. All P values are two-sided and $P < 0.05$ was considered to be significant.

An expanded Methods section is available in the Online Supplement.

Results

hCPCs in patients with DOXO cardiomyopathy

Increasing evidence demonstrates that senescence of CPC population significantly contributes to the development of heart failure [12, 15, 20, 26, 41, 42, 55]. To determine whether hCPCs were affected by anti-cancer therapy with anthracyclines, the hearts from oncologic patients who developed CHF at variable time intervals after treatment were analysed (Table 1). Confocal microscopy of cardiac tissue revealed the presence of c-kit-positive hCPCs labelled with $p16^{INK4a}$, a well established and widely used marker of cellular senescence (Fig. 1a) [5, 11, 41]. Importantly, the percentage of hCPCs positive for $p16^{INK4a}$ in pathological hearts was nearly fivefold higher than in controls (Fig. 1b). Moreover, compared to control myocardium, in the hearts from patients who died of DOXO-induced CHF, a 2.9-fold increase in the fraction of hCPCs with the nuclear expression of the phosphorylated form of histone H2AX on serine 139 (γH2AX, control: 4.2 ± 0.2 %; DOXO: 12.3 ± 4.2 %) was detected, indicating the occurrence of DNA damage (Fig. 1c). Widening of the interstitial space and focal collagen accumulation were constantly present in cardiomyopathic hearts, and myocardial fibrosis was 2.2-fold higher than in age-matched control tissues (Fig. 1d, e).

To address the question whether the senescence of hCPCs in the hearts of patients who received anthracycline can be a process independent from the presence of overt heart failure, the myocardial samples obtained from the age-matched cases who died during the course of the primary disease but did not develop symptoms of heart failure were examined (Fig. 1f). The analysis of two available hearts revealed that after DOXO treatment, already 38 and 49 % of hCPCs were senescent. These values were higher than in control hearts. The results document that the senescence of hCPCs occurs at high frequency in the hearts of patients treated with DOXO, even in the absence of evident cardiac dysfunction. It is conceivable that $p16^{INK4a}$-positive hCPCs are not able to control myocardial cell turnover and therefore cannot compensate the loss of myocytes. The large fraction of senescent hCPCs in patients treated with DOXO can affect cardiac homeostasis and accelerate the development of heart failure [20, 41].

Isolated hCPCs

c-kit positive hCPCs were isolated from non-injured myocardium and expanded. Amplified hCPCs continued to express c-kit (Fig. 2a) were clonogenic and negative for hematopoietic markers such as CD45, CD34, CD14 (< 1 %), 1.4 % expressed kinase insert domain receptor (KDR) and 18 % were positive for CD105 (Supplementary Fig. 1 in Online Resource 1). These cells were capable to differentiate into myocyte (documented by the presence of α-sarcomeric actin, myosin heavy chain, connexin43), smooth muscle (shown by the expression of α-smooth muscle actin and calponin) and endothelial lineages (detected by the expression of von Willebrand factor and CD31) (Supplementary Fig. 2 in Online Resource 1, see also Fig. 7 later in the text).

Doxorubicin and hCPC death and growth

The documentation that the population of hCPCs is negatively affected in patients treated with DOXO, and raised the question which cellular events are triggered by the anthracycline in these cells. For this purpose, expanded c-kit positive hCPCs were exposed to clinically relevant concentrations of DOXO that reflect the peak or steady state plasma concentrations in patients after drug infusion [34]. Colorimetric MTT assay was performed to evaluate the effects of DOXO on the viability of hCPCs exposed for 24 and 48 h at the concentrations of 0.1, 0.5 and 1 μM. 0.1 μM DOXO did not affect hCPC survival. However, with higher doses of the anthracycline, hCPC viability was dramatically reduced. After 24 h, DOXO at 0.5 and 1 μM decreased hCPC viability by 45 and 62 %, respectively. The reduction of cell viability was even more apparent at 48 h, reaching 79 and 89 % for 0.5 and 1 μM DOXO, respectively (Fig. 2b). In addition, apoptotic death measured by TUNEL assay was markedly increased. The rate of apoptosis rose in a dose-dependent manner and peaked after 48 h of treatment with 1 μM DOXO (Fig. 2c). At the same time, the ratio between pro-apoptotic protein Bax and anti-apoptotic protein Bcl-2 was increased in DOXO-treated hCPCs (Fig. 2d). To determine whether DOXO affected hCPC proliferation, cells were incubated with BrdU. The analysis of BrdU incorporation showed a dramatic decrease in hCPC growth. With respect to untreated cells, DOXO induced almost complete inhibition of cell proliferation with longer exposure (Fig. 2e). Therefore, hCPCs are highly sensitive to DOXO with increased cell death and decreased growth. In order to understand the possible alterations in the cell cycle checkpoints, the molecular

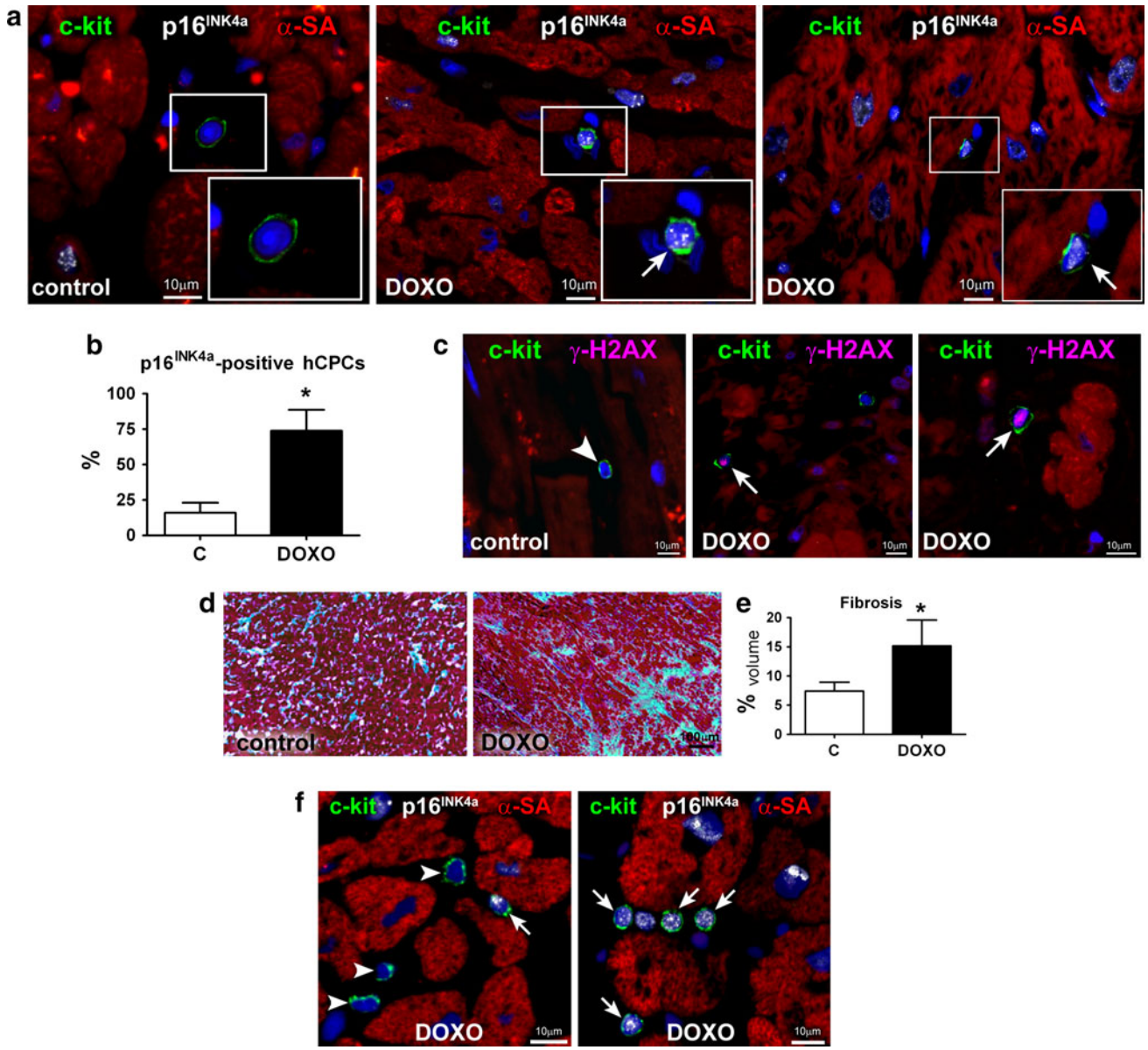

Fig. 1 hCPCs in doxorubicin cardiomyopathy patients undergo premature senescence. **a** c-kit-positive hCPCs (*green*) in control (*left panel*) and diseased hearts (*central* and *right panels*). The *areas* in the *rectangles* are shown at higher magnification in the *insets*. The expression of senescence-associated protein $p16^{INK4a}$ (*white*, *arrows*) is shown in the nuclei of c-kit-positive hCPCs present in the cardiac tissue of cardiomyopathic patients 2 years (*central panel*) and 5 years (*right panel*) after chemotherapy. Cardiomyocytes are labelled by the α-sarcomeric actin antibody (α-SA, *red*). Nuclei are stained with DAPI (*blue*). **b** The fraction of senescent, $p16^{INK4a}$ labelled hCPCs in controls (C) and patients with DOXO cardiomyopathy (DOXO). $*P < 0.005$ vs control. **c** *Left panel* shows c-kit-positive, γH2AX-negative hCPC (*arrowhead*) in control heart. γH2AX (*magenta*, *arrows*) is present in the nuclei of hCPCs (c-kit, *green*) in the myocardium of DOXO-treated patients (*central* and *right panels*). **d** Masson's trichrome staining of the section of a control heart (*left panel*) showing interstitial fibrosis (*blue*) surrounding cardiac muscle (*red*). Widening of the interstitial spaces by focal collagen accumulation is apparent in the myocardium of a patient who died of CHF 5 years after chemotherapy (*right panel*). **e** The quantification of fibrosis in controls (C) and patients with DOXO cardiomyopathy (DOXO). $*P < 0.001$ vs control. **f** hCPCs positive for $p16^{INK4a}$ (*white*, *arrows*) are present in the myocardium of DOXO-treated patients in the absence of heart failure. $p16^{INK4a}$-negative hCPCs are also visible (*arrowheads*)

regulators of G1 and G1/S transition were measured. Cyclin D1, which is required for the cell cycle progression from G1 to S, is activated by the cyclin-dependent kinase 4 (cdk4). Cyclin D1/cdk4 complex by phosphorylation of retinoblastoma protein (Rb) on serine 798 (phospho-Rb^{Ser798}) releases its inhibition on cell cycle progression. Cyclin D1, cdk4 and phospho-Rb^{Ser798}/Rb ratio were significantly decreased after exposure to DOXO, further documenting the ability of this drug to negatively interfere with cell cycle in hCPCs (Fig. 2f).

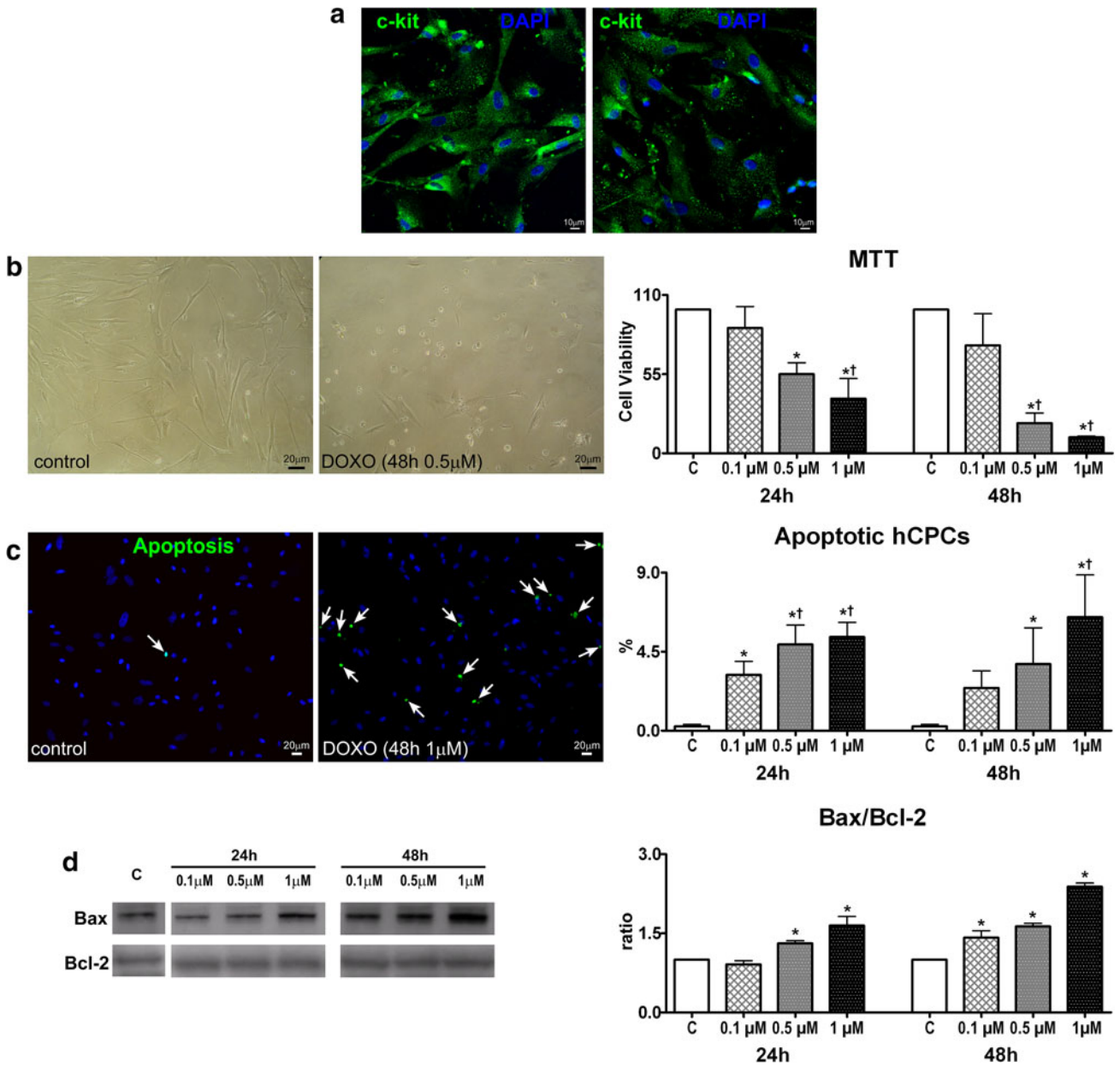

Fig. 2 Doxorubicin induces death and inhibits growth of hCPCs. **a** Isolated hCPCs express the stem cell antigen c-kit (*green*). **b** Control and DOXO-treated hCPCs are shown by phase contrast microscopy. The viability of hCPCs determined by MTT assay was negatively affected by DOXO. The results are shown for different concentrations (0.1, 0.5, 1 μM) and time of exposure (24 h and 48 h). **c** Apoptosis of hCPCs is shown by TdT labelling (*green*, *arrows*). DOXO treatment increased the fraction of apoptotic hCPCs. **d** Western blotting for Bax and Bcl-2 and the ratio between the expression levels of these proteins are shown. $^{*}P < 0.05$ vs control. **e** hCPC proliferation detected by BrdU labelling (*green*, *arrows*). The cytoskeletal protein phalloidin is shown in red. The fraction of BrdU positive cells decreased after DOXO treatment. **f** DOXO affected the expression of cell cycle related proteins. Western blotting analysis showed the reduction of cyclin D1, cdk4 and phosphorylated Rb^{Ser798}/Rb ratio. Viability, BrdU incorporation and protein expression are shown as fold changes with respect to control. $^{*}P < 0.05$ vs control; $^{\dagger}P < 0.05$ vs 0.1 μM; $^{\ddagger}P < 0.05$ vs 0.5 μM. *C* control

Doxorubicin and DNA damage in hCPCs

Doxorubicin can induce DNA damage by the generation of reactive oxygen species and by interfering with the function of enzymes topoisomerase II and gyrase [34]. Activated form of the ataxia-telangiectasia mutated (ATM) protein kinase, phosphorylated on serine 1981 (phospho-$ATM^{Ser1981}$), and the γ-H2AX are sensitive reporters of DNA damage, particularly of the presence of DNA double-strand breaks. Moreover, phospho-$ATM^{Ser1981}$ is required for phosphorylation of p53 at serine 15 (phospho-$p53^{Ser15}$), which is a central element of cell response to the DNA

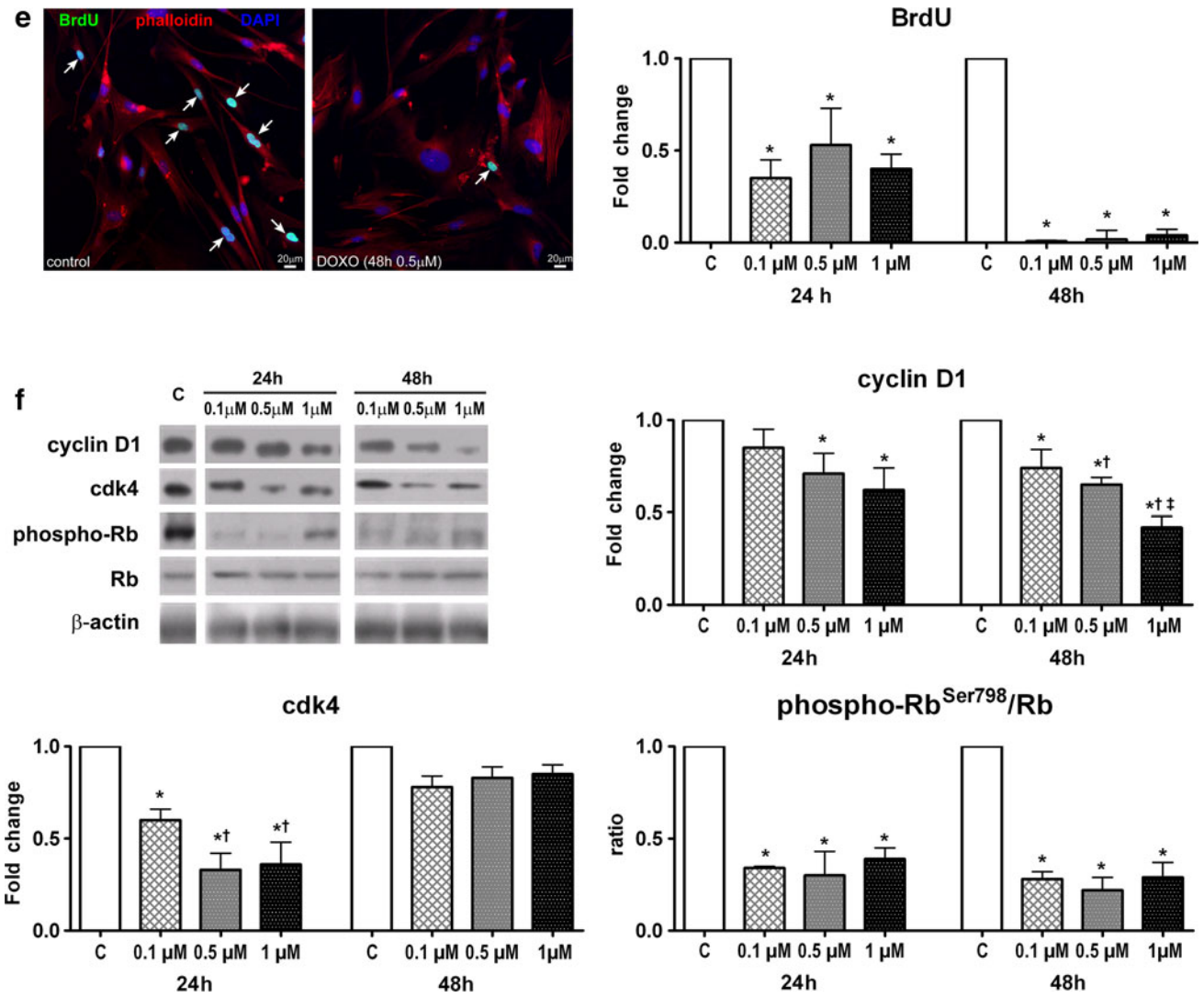

Fig. 2 continued

damage [10, 16]. Phospho-ATM^{Ser1981} kinase, γH2AX and phospho-p53^{Ser15} were upregulated in DOXO-treated hCPCs (Fig. 3a–c). In comparison with control cells, phospho-ATM^{Ser1981} expression was similarly increased at 24 and 48 h (Fig. 3a), whereas phospho-p53^{Ser15} peaked at 24 h (eightfold increase) and remained elevated to a lesser extent at 48 h (threefold increase) (Fig. 3c). Histone H2AX phosphorylation, that is mediated by ATM [16], was markedly increased in a dose and time-dependent manner, reaching values up to 30-fold higher than in control (Fig. 3b).

Doxorubicin and hCPC senescence

The loss of proliferative capacity in DOXO-treated hCPCs was associated with a change in the phenotype from typically spindle-shaped small cells to enlarged, flat and vacuolated cells, a characteristic morphology of senescent cells. Cellular senescence is a stress response that results in permanent withdrawal from the cell cycle, independently from the activated pathways [11, 61]. The two major pathways implicated in the induction of senescence are p53-p21^{Cip1} and phospho-Rb-p16^{INK4a}. The altered expression of cell cycle regulators, such as phospho-Rb^{Ser798} and p53, observed in DOXO-treated hCPCs suggests growth arrest as a result of cellular senescence. Therefore, the protein levels of the cyclin-dependent kinase inhibitors p21^{Cip1} and p16^{INK4a}, often expressed by senescent cells, were determined. Interestingly, DOXO did not affect expression of p21^{Cip1} (Fig. 4a), suggesting that DNA damage-induced activation of p53, in these specific conditions, was not followed by the activation of p21^{Cip1}. In addition, expression of phospho-p21$^{Cip1–Thr145}$ and the ratio of phospho-p21$^{Cip1–Thr145}$ to total p21^{Cip1} remained unchanged after DOXO treatment. This observation indicates that phosphorylation of p21^{Cip1} with a possible

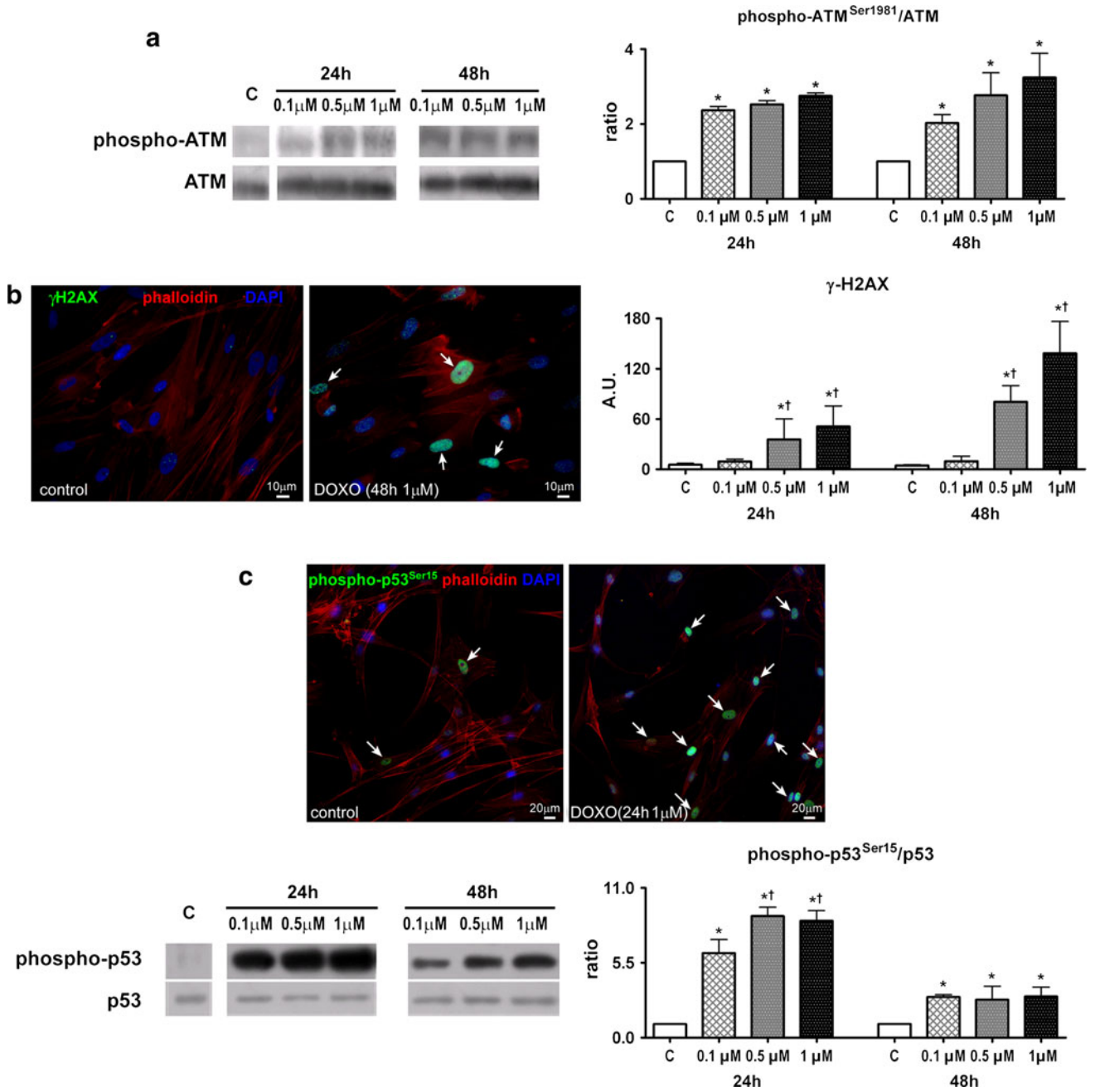

Fig. 3 Doxorubicin induces DNA damage response in hCPCs**. a** Phosphorylation of ATM^{Ser1981}, detected by western blotting, increased in response to DOXO. **b** γH2AX detected by immunofluorescence (*green*, *arrows*), increased in DOXO-treated hCPCs. **c** Phosphorylated p53^{Ser15} shown by immunofluorescence (*green*, *arrows*) in the nuclei of untreated cells and hCPCs exposed to DOXO. Phospho-p53^{Ser15}, detected by western blotting, increased predominantly at 24 h after treatment. *$P < 0.05$ vs control; $^{\dagger}P < 0.05$ vs 0.1 μM. *C* control; *AU* arbitrary units

subsequent activation of a proliferative response was not induced in hCPCs exposed to DOXO [40]. In contrast, the level of p16^{INK4a} was markedly increased (Fig. 4b). The presence of high quantity of p16^{INK4a} and a significantly higher fraction of p16^{INK4a}-positive cells (Fig. 4c) together with the low level of phospho-Rb^{Ser798} (see Fig. 2f) indicates that DOXO induces irreversible growth arrest and cellular senescence in hCPCs by the activation of the p16^{INK4a}-pRb pathway. To confirm that loss of growth capacity was associated with premature senescence, an additional marker, the senescence-associated β-galactosidase (SA-β-gal) was used. The high activity of SA-β-gal supported the occurrence of cellular senescence in DOXO-treated hCPCs (Fig. 4d). Finally, increasing drug

concentration and duration of treatment was associated with the larger fraction of senescent hCPCs.

Doxorubicin and growth factors in hCPCs

hCPCs express insulin-like growth factor-1 receptor (IGF-1R) and hepatocyte growth factor (HGF) receptor, c-Met, and synthesize and secrete the corresponding ligands. IGF-1-IGF-1R system is an important regulator of cell growth and survival. Stimulation of IGF-1R mediates mitogenic and antiapoptotic effects of IGF-1 that are essential for CPC-mediated myocardial regeneration. HGF-c-Met mediate migration of CPCs to the site of injury that is required for normal cell turnover and efficient tissue regeneration in case of damage [17, 20, 52, 54]. Western blotting and immunocytochemistry showed that the treatment with DOXO reduced the expression level of IGF-1R in hCPCs (Fig. 5a) documenting that this pro-survival and cell-protective system was compromised. DOXO exposure decreased the expression of c-Met and markedly impaired the migratory capacity in a dose- and time-dependent manner. The ability of hCPCs to migrate was almost abolished after 48 h of 1 μM DOXO (Fig. 5b, c).

Long-lasting effects of DOXO on hCPCs

It is possible that after the treatment is finished, the damage induced by DOXO can be reversed and the cells can return to their initial status. Alternatively, with time, the effects of the treatment could persist or become even more dramatic. To determine whether the early molecular and functional changes that occurred in hCPCs in response to DOXO were permanently present, the drug was removed and cells were allowed to grow in a fresh medium. The results obtained in hCPCs at the early time points (DOXO-early) were compared to those collected in cells after 7 days washout period (DOXO-late).

The analysis of cell proliferation measured by incorporation of BrdU and Ki67 labelling showed that cell growth continued to be significantly reduced in DOXO-late hCPCs (Fig. 6a). Conversely, the high rate of apoptosis present at early time point was markedly lower at late time point. Specifically, DOXO-late hCPCs had 11-fold reduced apoptosis with respect to DOXO-early hCPCs (Fig. 6b). This was consistent with the initial increase in phospho-$p53^{Ser15}$ detected early after treatment with DOXO (Fig. 6c). In addition, the strikingly elevated apoptotic rate in DOXO-early may be the effect of the direct and instant cytotoxic action of the drug. DOXO and its highly reactive metabolites can rapidly increase production of superoxide and directly release cytochrome *c* from mitochondria, promote the release of the iron from intracellular stores, directly damage cell membrane or trigger a sphingomyelin cycle with formation of ceramide. These mechanisms can induce cell apoptosis independently from DNA damage or activated signaling pathways [21, 27, 34]. Consistently, the expression levels of proteins involved in DNA damage response, phospho-ATM and phospho-p53, that were initially increased, returned to normal values in DOXO-late hCPCs (Fig. 6c).

In contrast, the markers of cellular senescence $p16^{INK4a}$ and SA-β-gal increased significantly with time. Nearly all DOXO-late hCPCs were positive for $p16^{INK4a}$ and 80 % of cells showed SA-β-gal activity (Fig. 6d, e). These data are consistent with the notion that, while p53 acts at the initiating stage of the senescence response, $p16^{INK4a}$ operates to maintain this state [7]. In addition, when cells become senescence, they can be less susceptible to pro-apoptotic signals [14, 22]. The senescence marker $p16^{INK4a}$ once induced, decreases phosphorylation of Rb which, in turn, leads to growth arrest [10, 44]. The persistent Rb hypophosphorylation detected in DOXO-late hCPCs confirmed the continuous activity of this $p16^{INK4a}$-dependent growth arrest pathway (Fig. 6f). Moreover, cellular senescence and growth arrest can be initiated and maintained by dysfunctional telomeres [16, 39, 50]. Indeed, 1 week after exposure to DOXO, hCPCs had the telomeres on average 30 % shorter than untreated cells. Importantly, the length distribution curve was shifted to the left with majority of cells with telomeres shorter than 3 kilobase pairs (kbp) (Fig. 6g).

These results indicate that hCPCs, that survived the initial exposure to DOXO and were not anymore massively dying, were still affected by the treatment and reached irreversible growth arrest and senescence.

In addition, at later time point after DOXO exposure, the expression of IGF1-R and c-Met receptors (Fig. 6h) and the migratory capacity (not shown) continued to be reduced. These results represent another aspect of long-term impairment of progenitor cell function that further compromises their regenerative capacity in DOXO-induced cardiomyopathy.

The differentiation of CPCs into cardiomyocytes, smooth muscle cells and endothelial cells is required for progenitor cells to be fully functional and is essential for tissue repair process. Differentiation of hCPCs into cells of different cardiac lineages was not significantly different at early time point. In contrast, in DOXO-late cells, differentiation of hCPCs into cardiomyocytes and smooth muscle cells was significantly reduced, while the formation of endothelial cells was moderately lowered but did not reach statistical significance (Fig. 7a and Supplementary Fig. 2 in Online Resource 1). In particular, the fraction of cells expressing α-sarcomeric actin and α-smooth muscle actin was reduced by 54 and 56 % respectively. However, it is critical to point out that the generated progeny of hCPCs

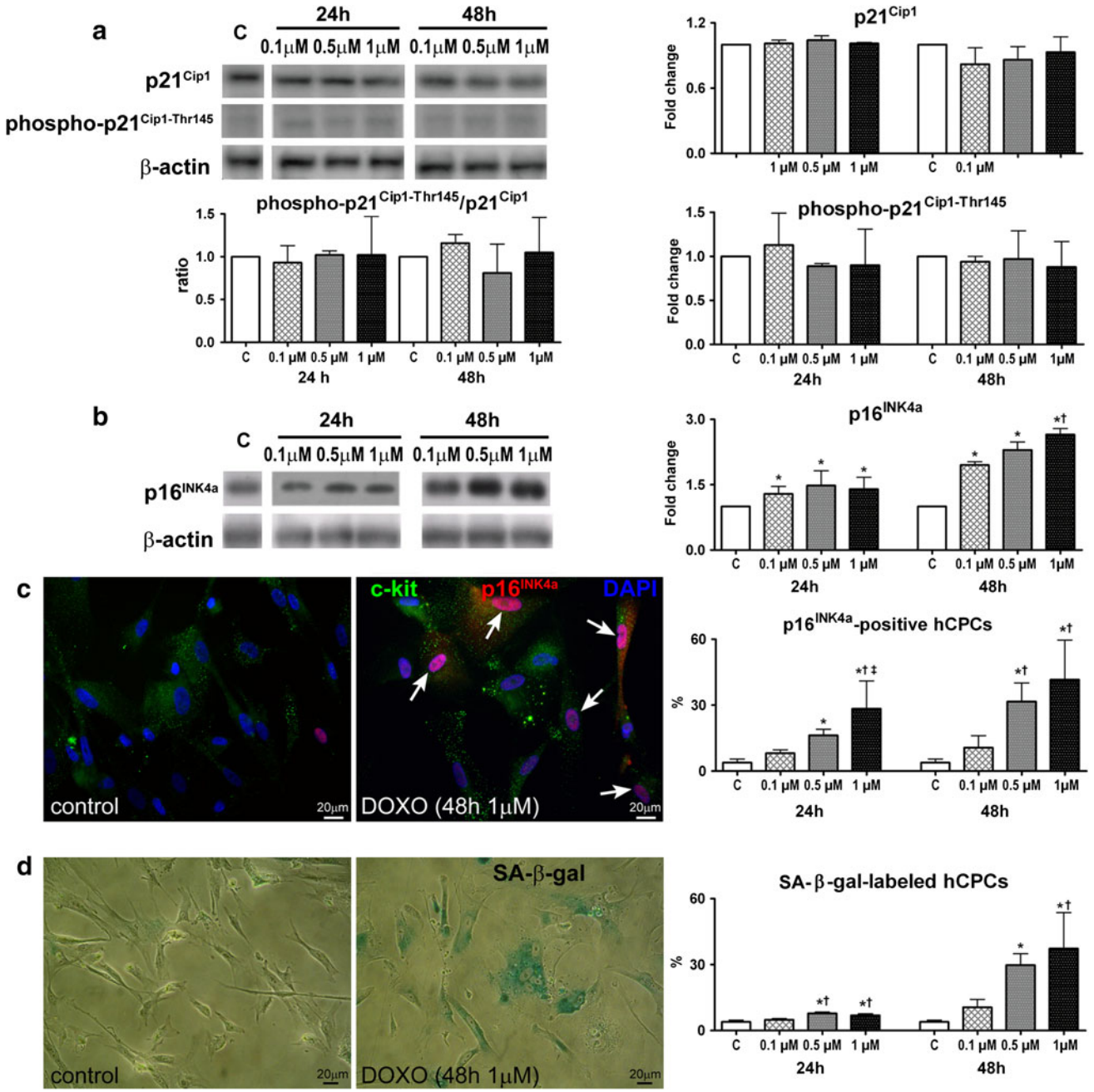

Fig. 4 Doxorubicin promotes senescence of hCPC. **a** DOXO treatment did not affect the expression of the cell-cycle inhibitor $p21^{Cip1}$. The levels of phospho-$p21^{Cip1-Thr145}$ and the ratio of phospho-$p21^{Cip1-Thr145}$ to total $p21^{Cip1}$ were not changed. **b** DOXO administration induced dose-dependent increase in $p16^{INK4a}$ expression, detected by western blotting. **c** Senescent hCPCs (c-kit, *green*; $p16^{INK4a}$, *red*) are shown by immunolabelling. The percentage of hCPCs expressing $p16^{INK4a}$ increased with time and drug concentration. **d** DOXO-induced an increase in the SA-β-gal activity (*blue*). The fraction of hCPCs positive for enzyme activity is shown. Protein expression is shown as fold changes with respect to control. $^{*}P < 0.05$ vs control; $^{\dagger}P < 0.05$ vs 0.1 μM. *C* control

was already senescent (Fig. 7b, c), and therefore, their regenerative capacity was permanently reduced. The presence of $p16^{INK4a}$-positive differentiated cells may be due to the commitment of hCPCs with short telomeres, which form a progeny that rapidly reaches cellular senescence [20].

Discussion

The advances in the treatment of neoplastic diseases result in the growing population of oncologic patients with long-term survival. However, the patients in whom the chemotherapy protocols included anthracyclines are at higher risk

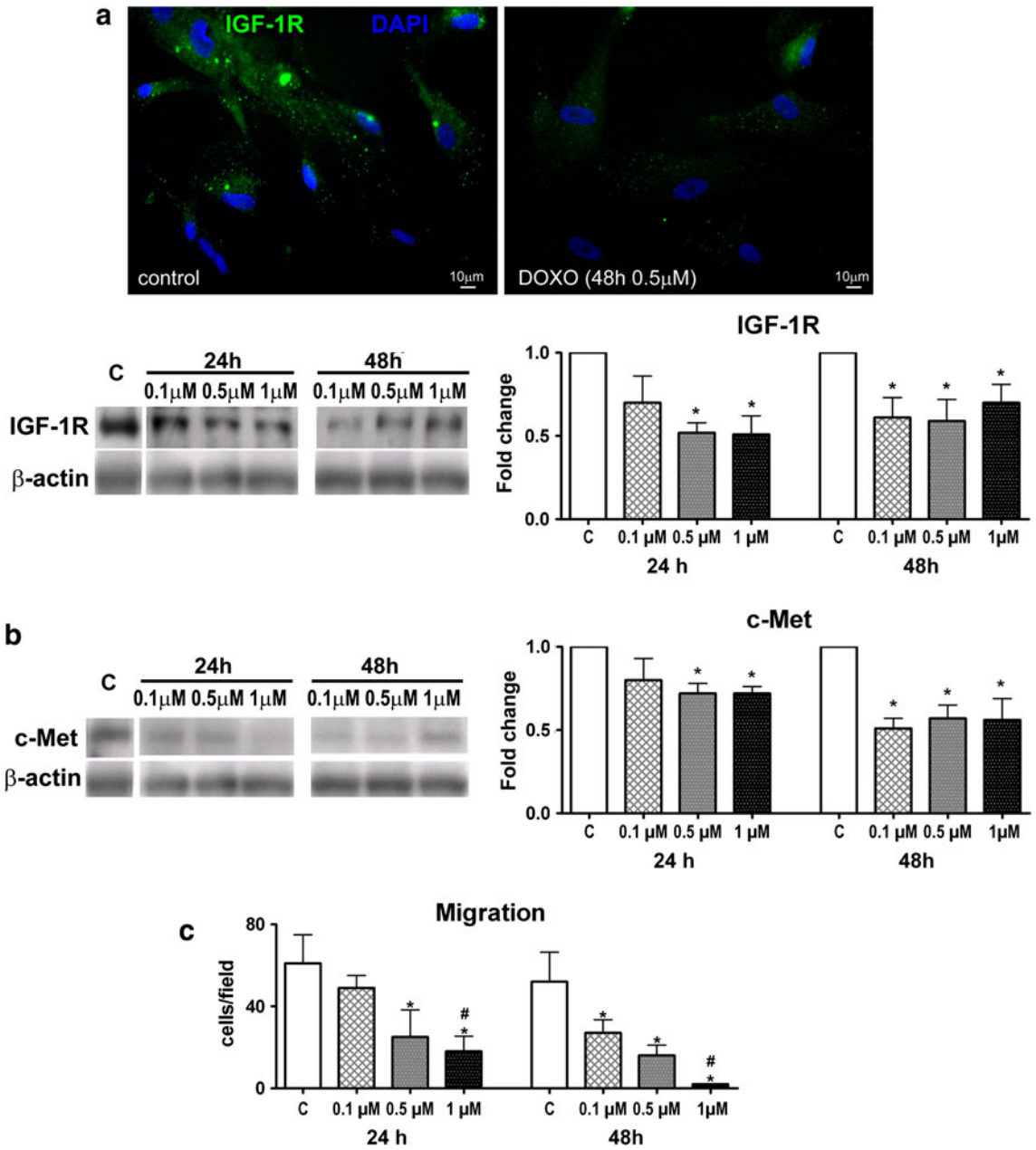

Fig. 5 Doxorubicin reduces the expression of growth factors receptors and impairs migration of hCPCs. **a** IGF1-R expression, shown by immunolabelling (*green*) and western blotting, decreased after DOXO treatment. **b** The reduced expression of c-Met receptor was detected by western blotting. **c** hCPC migration was severely impaired by DOXO. Protein expression is shown as fold changes with respect to control. $^{*}P < 0.05$ vs control; $^{\dagger}P < 0.05$ vs 0.1 μM. *C* control

of developing heart failure due to the treatment [19, 45, 51]. Up to date, the mechanisms responsible for anthracycline-induced cardiomyopathy are not fully elucidated. Recent animal studies provided a new explanation of DOXO cardiotoxicity documenting that the determining event responsible for the initiation and evolution of the myopathy arises at the level of the CPC compartment [18, 24]. DOXO treatment provoked the depletion and premature senescence of the CPC population that interfered with their functional capacity and physiological turnover of the myocardium. Increasing evidence indicates that senescence of stem cell population is a fundamental process that contributes to the onset and progression of heart failure [12, 15, 20, 26, 41, 55]. Therefore, the cardiotoxicity in patients receiving DOXO may originate from the premature senescence and impaired function of CPCs.

The current study documents, for the first time, the presence of senescent hCPCs in the hearts of patients with

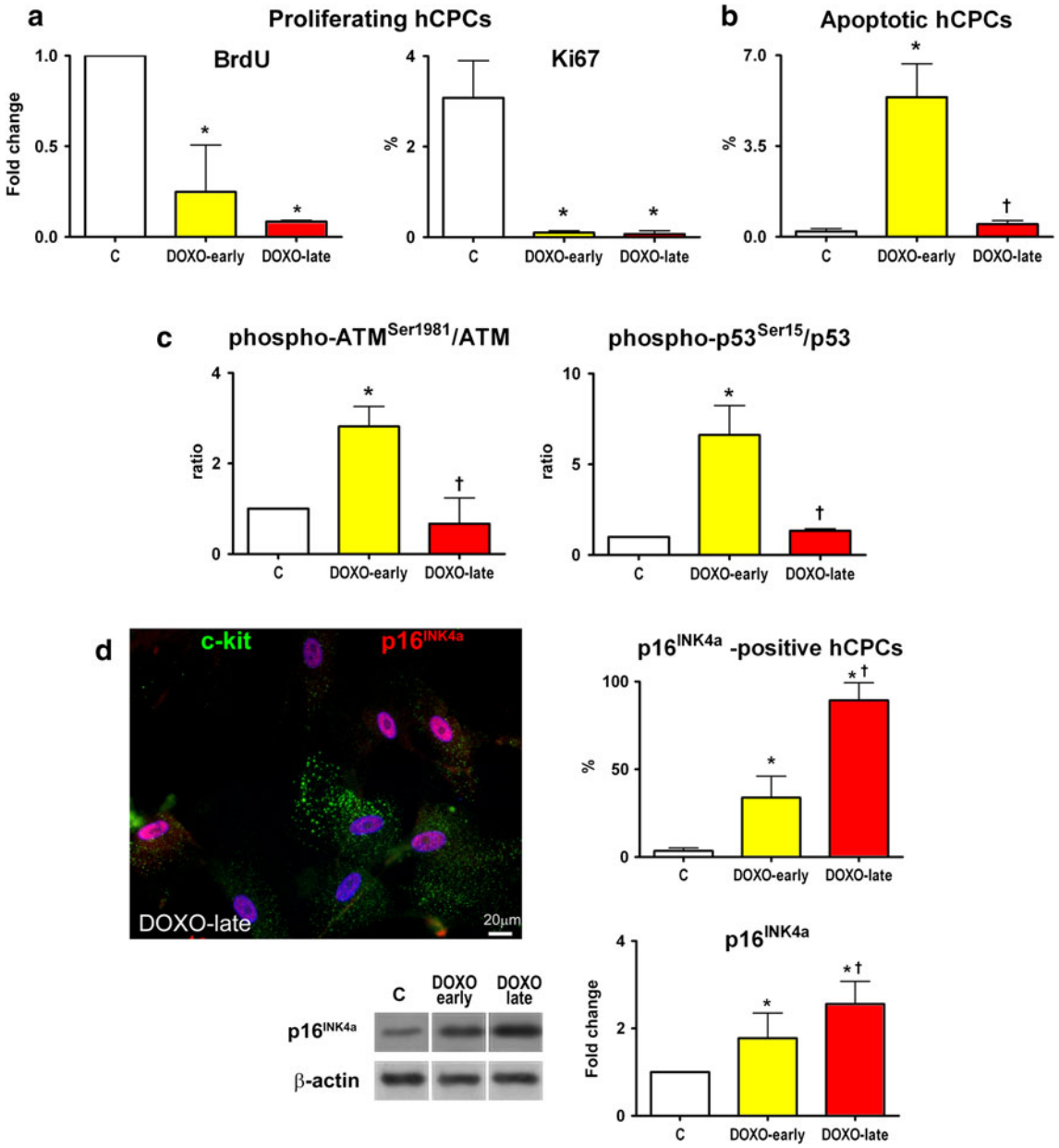

Fig. 6 Long-lasting effects of doxorubicin on hCPCs. **a** hCPC proliferation, detected by BrdU labelling and Ki-67 was significantly decreased both early and late after treatment. **b** Apoptosis in hCPCs was dramatically increased early after treatment, with subsequent decrease at later time point. **c** DOXO triggered DNA damage response in hCPCs. The phosphorylation of ATM^{Ser1981} and p53^{Ser15}, detected by western blotting, strongly increased early after treatment and returned to normal values at later time point. **d** Senescence of hCPCs (c-kit, *green*) was detected by p16^{INK4a} expression (*red*). DOXO administration increased the percentage of p16^{INK4a} positive cells. p16^{INK4a} protein level detected by western blotting showed a similar trend. The expression of p16^{INK4a} was further increased at later time point. **e** Senescence of hCPCs, detected by the SA-β-gal activity (*blue*) increased after DOXO treatment and was strikingly higher at later time point. **f** In DOXO-late hCPCs, the drug induced persistent Rb hypophosphorylation, confirming the continuous activity of growth arrest pathway. **g** Telomere shortening in DOXO-treated hCPCs. Nuclei (*blue*) were stained with a PNA telomere probe (*green*). Lymphoma cells with long telomeres are shown for comparison. In DOXO-treated hCPCs (*red bars*) the telomere length distribution is shifted towards shorter values as compared with untreated cells (*green bars*). **h.** The expression of IGF1-R and c-Met receptors continued to be reduced late after DOXO exposure. Protein expression is shown as fold changes with respect to control. $^{*}P < 0.05$ vs control; $^{\dagger}P < 0.05$ vs DOXO-early. *C* control

anthracycline cardiomyopathy. Importantly, senescence marker p16^{INK4a} was present in the vast majority of c-kit-positive hCPCs exceeding the values reported for normal aging and other cardiomyopathies [12, 15, 26]. Damage of DNA, shown by the expression of γH2AX in hCPCs and the accumulation of senescent cells may account for the

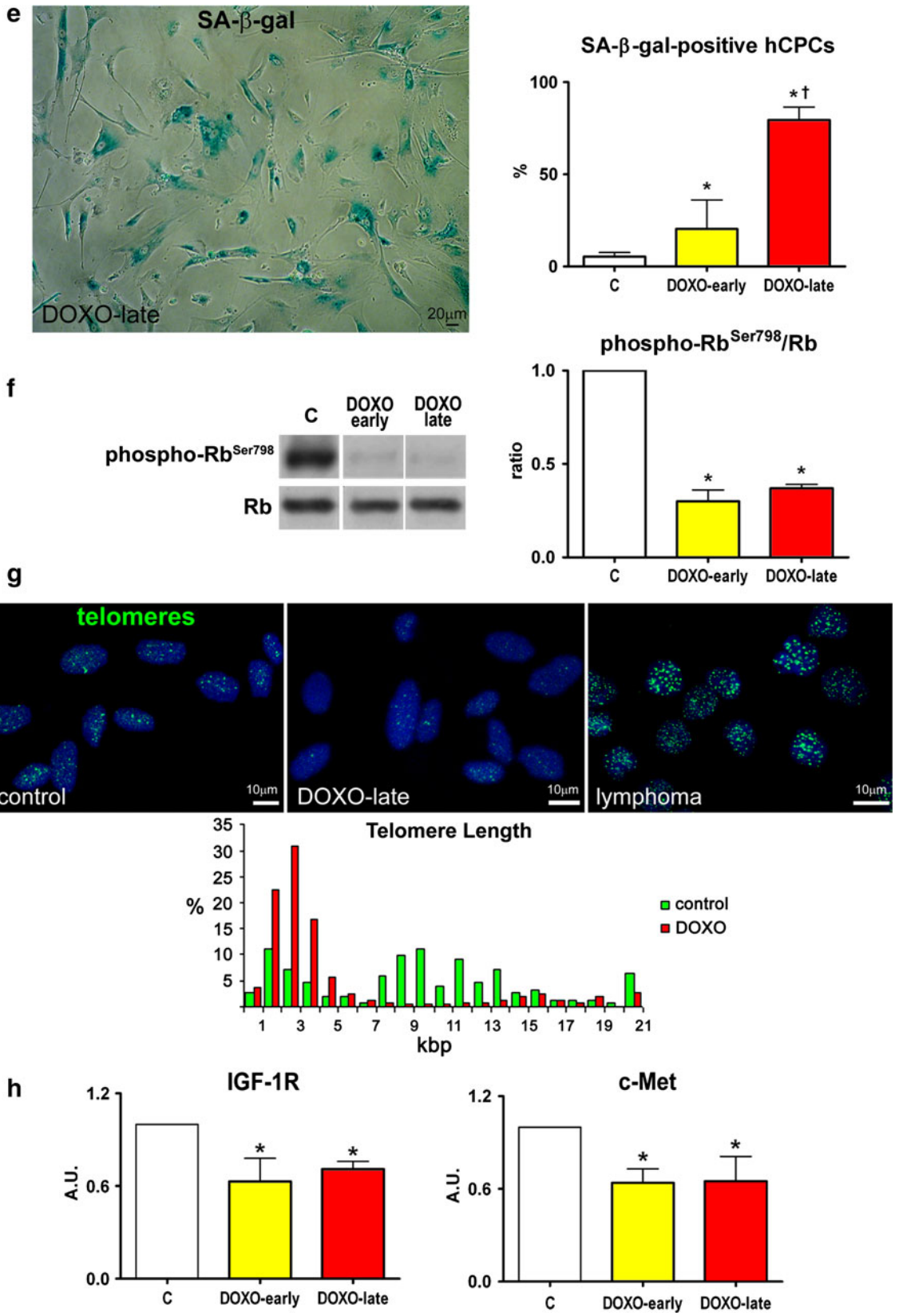

Fig. 6 continued

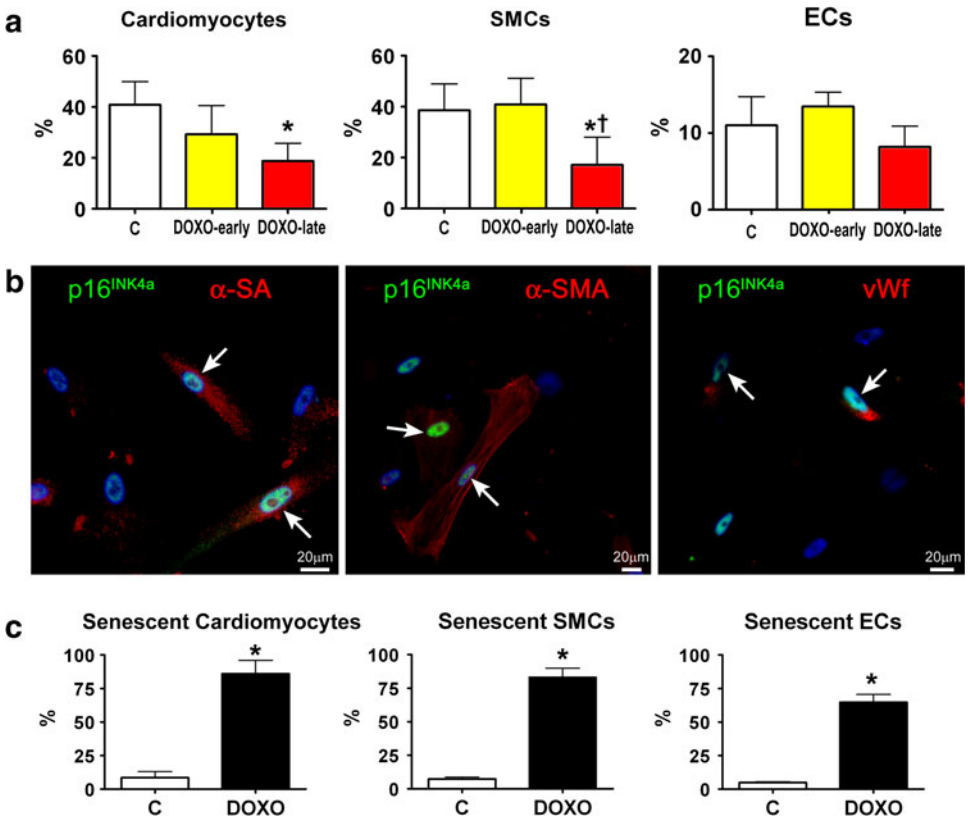

Fig. 7 Doxorubicin impairs differentiation potential of hCPC. **a** The differentiation capacity into cardiomyogenic, smooth muscle and endothelial cell lineages of DOXO-early hCPCs was not significantly different, while in DOXO-late hCPCs the formation of cardiomyocytes and smooth muscle cells was significantly reduced. **b**, **c** The generated progeny of these cells was prematurely senescent. Differentiated senescent cells ($p16^{INK4a}$, *green*, *arrows*) expressing α-sarcomeric actin (α-SA, *red*), α-smooth muscle actin (α-SMA, *red*) and von Willebrand factor (vWf, *red*) are shown by immunolabelling. The percentage of $p16^{INK4a}$ labelled cardiomyocytes, smooth muscle and endothelial cells is dramatically higher in the progeny derived from DOXO-treated hCPCs. $^{*}P < 0.05$ vs control; $^{\dagger}P < 0.05$ vs DOXO-early. *C* control

premature, progressive myocardial aging in DOXO-treated patients. In such case, the resulting irreversible cell cycle arrest in hCPCs could affect cardiac homeostasis and reduce regenerative capacity leading directly to the heart failure or increasing susceptibility to the myocardial damage associated with other conditions. These important observations prompted us to perform in vitro study in order to better understand the cellular processes that occur in hCPCs exposed to DOXO. Isolated hCPCs were sensitive to DOXO, and their survival, growth and function were negatively affected.

The time interval between therapy with anthracyclines and onset of cardiac complications varies from months to years or even decades. In patients treated with anthracyclines, the cardiomyopathy and heart failure can be triggered later in life even by minor pathological events that in the healthy person would not have such severe consequences. It is possible that therapy with anthracyclines can leave a specific cellular "signature" to the heart that persists with time and reveals itself later with the devastating outcome. The long-lasting damage of the progenitor cell that controls turnover of myocardial cells in a healthy individual and drives cardiac repair in case of injury can be responsible for late-onset complications. Therefore, the early effects of DOXO on hCPCs and those that occur after drug removal were studied. Early after exposure, DOXO reduced hCPC viability and induced significant level of apoptosis. Moreover, the expression of proteins involved in the DNA damage response was highly elevated. In particular, phospho-$ATM^{Ser1981}$ kinase, γH2AX and phospho-$p53^{Ser15}$ were upregulated. The activation of ATM was coupled with the increase of γH2AX indicating DNA damage. Serine 15 phosphorylation induces posttranslational modifications of p53 which, in turn, leads to transcription of p53 target genes and subsequent activation of apoptosis or cellular senescence [10]. In hCPCs, the highest amount of phospho-$p53^{Ser15}$ was detected already at 24 h after DOXO treatment. This initial increase triggered the apoptotic pathway with the peak of cell death at 48 h. Interestingly, at this time, the expression of cyclin-dependent kinase inhibitor $p16^{INK4a}$ began to rise in parallel with the increased activity of SA-β-gal. These data indicate that in hCPCs early after DOXO treatment, p53 could trigger both apoptosis and cellular senescence pathway depending upon the intensity and duration of the stimulus. Similar cellular events can take place in the hearts of patients during or immediately after administration of anthracyclines. However, the early and late cellular adaptations that occur after treatment is finished may profoundly differ. In fact, 1 week after removal of DOXO, the rate of apoptotic death of hCPCs and the expression levels of proteins involved in DNA damage response (phospho-$p53^{Ser15}$, phospho-$ATM^{Ser1981}$) returned to almost normal values. In such situation, in the absence of other pro-senescence

stimuli, cells could potentially resume their proliferative capacity [5]. However, as documented by the very high fraction of $p16^{INK4a}$ and SA-β-gal-positive cells, nearly all hCPCs were senescent. This indicates that hCPCs entered the irreversible phase of growth arrest after permanent activation of $p16^{INK4a}$-Rb pathway. These data show that activation of p53 was a transient event, fading off with the rise of $p16^{INK4a}$ expression. Therefore, while p53 initiates the senescence response, $p16^{INK4a}$ operates to maintain this state. In fact, it has been shown that $p16^{INK4a}$ represents a delayed cellular response, which can follow the induction of p53 and $p21^{Cip1}$ [10, 50]. Unexpectedly, the marked activation of p53 was not paralleled by the increased levels of $p21^{Cip1}$, which suggests that DOXO-induced senescence of hCPCs was a p53-mediated, but $p21^{Cip1}$-independent process. Further studies will be required to understand the details of the cross-talk between p53-$p21^{Cip1}$ and pRb-$p16^{INK4a}$ senescence pathways. Finally, DOXO-induced telomere shortening further demonstrated the presence of persistent genotoxic stress in hCPCs.

IGF-1-IGF-1R and HGF/c-Met systems are critical determinants of CPCs functional capacity to preserve tissue homeostasis and repair [17, 30]. Stimulation of IGF-1R activates mitogenic and antiapoptotic effects favouring CPC-mediated myocardial regeneration [17, 52, 54]. c-Met is the receptor for HGF, a cytokine that stimulates cell migration to the sites of injury [38, 54]. It is possible that in addition to an induction of CPC death and senescence, DOXO negatively interferes with growth factor systems that regulate cardiac repair, further aggravating the inadequate response of the human heart to pathological insults. Our findings indicate that DOXO-treated hCPCs had a reduced expression of IGF-1R and c-Met and that this negative effect persisted with time. This represents an additional mechanism involved in pathogenesis of anthracycline cardiomyopathy and may be of high clinical relevance.

To fulfil their role, the viable progenitor cells need to reach the area of injury and give rise to differentiated progeny capable to repair the damage. Studies in rodents showed that DOXO can induce multiple and complex changes in the transcription profile of CPCs and interfere with their lineage commitment [18]. hCPCs exposed to DOXO had reduced, although not abolished differentiation capacity. Importantly, the majority of cardiomyocytes, smooth muscle and endothelial cells deriving from DOXO-treated hCPCs inherited a senescent phenotype, prematurely expressing $p16^{INK4a}$. Even though lineage commitment of hCPCs can occur in DOXO-treated patients, the defective, senescent pool of differentiated progeny could not expand properly in response to organ needs. Thus, the inability of defective hCPCs with short telomeres to give rise to competent progenitors, precursors and amplifying cells may play a central role in the onset of DOXO cardiomyopathy. Although the reported phenomena per se may not directly lead to the heart failure, they can make the myocardium of an apparently healthy person more vulnerable. The late onset of cardiomyopathy in patients, who already have sustained subclinical cardiac damage as a result of DOXO chemotherapy, could be attributed to an additional pathological or physiological stress like ischemia, acute viral infection, exercise, pregnancy, or the increase of body mass in children during normal growth. These factors can transform the silent myopathy into overt heart failure [2, 13, 31, 36].

Furthermore, although it was not specifically addressed here, it is also possible that the effects of DOXO on hCPCs may be more profound by affecting stem cell niches [56]. Cardiac fibroblasts activation triggered by the necrotic damage and its pro-inflammatory bulk, changes in extracellular matrix and collagen deposition are the characteristic findings in the cardiomyopathic hearts, but the cytotoxic action of DOXO can also directly affect this cell class [1, 57, 58]. Because fibroblasts play a role as supporting cells for CPCs within the niches, the potential damage produced by DOXO can result in the derangement of the cardiac niche [56]. This reasonable possibility requires specific future investigations.

The present study clarifies the mechanisms underlying cardiotoxicity of anthracyclines and is particularly important because of the increasing population of cancer survivors. Only in the United States, there are 2,000,000 women after breast cancer treatment and more than 380,000 people cured from the childhood cancers who live or can live long enough to develop anthracycline cardiomyopathy [19, 33]. This cohort is at higher risk of heart failure for the rest of their lives. At present, there are no specific clinical practice guidelines for treating cardiac dysfunction in cancer survivors that received anthracyclines. As for all patients with heart failure, the treatment includes a combination of β-blockers, ACE inhibitors, angiotensin receptor blockers, diuretics, nitrates, and hydralazine [19] and, for end-stage failure, heart transplantation.

In the view of the human heart as a self-renewing organ in which tissue repair is controlled by the resident progenitor cells, the observation that hCPCs represent new target for DOXO with premature senescence and impaired function as the long-lasting effects, can be the foundation of new, more specific therapeutic strategies. In fact, novel approach to reverse DOXO-induced heart failure was successful in a rat model of DOXO cardiomyopathy, where CPC administration improved anatomical and functional parameters and decreased mortality [18]. Cell-based strategies for myocardial repair are extensively investigated in multiple experimental and clinical settings and hopefully the near future will clarify the most appropriate form of

cell therapy for the damaged myocardium [9, 28, 29, 32, 59]. Importantly, human cardiac stem cells isolated and expanded from small samples of human myocardium harvested during cardiac surgery are currently administered to patients with heart failure of ischemic origin with extremely positive results [8, 23]. Therefore, the application of this form of cell therapy may be forthcoming in DOXO cardiomyopathy in humans. Because, even in the end-stage ischemic cardiomyopathy, a pool of functionally competent hCPCs can be found in the human heart [55]; the possibility is raised that although with extreme caution, the preserved small fraction of hCPCs in cardiomyopathic hearts of oncologic patients may be obtained with the purpose to be administrated back to the same patient. Alternatively, in patients at high risk of developing CHF, myocardial biopsy samples could be obtained before chemotherapy, and autologous hCPCs could be isolated and expanded for future cell therapy.

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Conflict of interest The authors declare that they have no conflict of interest.

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