the FEBS Journal

Vanadium-induced apoptosis of HaCaT cells is mediated by *c-fos* and involves nuclear accumulation of clusterin

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Keywords
apoptosis; Bcl-2; c-fos; clusterin (CLU); vanadyl(IV) sulfate

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(Received 22 January 2009, revised 28 April 2009, accepted 13 May 2009)

doi:10.1111/j.1742-4658.2009.07093.x

Vanadium exerts a variety of biological effects, including antiproliferative responses through activation of the respective signaling pathways and the generation of reactive oxygen species. As epidermal cells are exposed to environmental insults, human keratinocytes (HaCaT) were used to investigate the mechanism of the antiproliferative effects of vanadyl(IV) sulfate ($VOSO_4$). Treatment of HaCaT cells with $VOSO_4$ inhibited proliferation and induced apoptosis in a dose-dependent manner. Inhibition of proliferation was associated with downregulation of cyclins D1 and E, E2F1, and the cyclin-dependent kinase inhibitors $p21^{Cip1/Waf1}$ and $p27^{Kip1}$. Induction of apoptosis correlated with upregulation of the c-fos oncoprotein, changes in the expression of clusterin (CLU), an altered ratio of antiapoptotic to proapoptotic Bcl-2 protein family members, and poly(ADP-ribose) polymerase-1 cleavage. Forced overexpression of *c-fos* induced apoptosis in HaCaT cells that correlated with secretory CLU downregulation and upregulation of nuclear CLU (nCLU), a pro-death protein. Overexpression of Bcl-2 protected HaCaT cells from vanadium-induced apoptosis, whereas secretory CLU overexpression offered no cytoprotection. In contrast, nCLU sensitized HaCaT cells to apoptosis. Our data suggest that vanadium-mediated apoptosis was promoted by *c-fos*, leading to alterations in CLU isoform processing and induction of the pro-death nCLU protein.

Introduction

Vanadium is a transition metal that is widely distributed in the environment and in biological systems. Vanadium is a member of group VB of the periodic table and can form compounds mainly in valencies III, IV, and V. V(III) species are unstable at physiological pH and in the presence of oxygen. Under physiological conditions, V(IV) is easily oxidized to V(V) species, which are found as vanadate anions. Vanadium-containing compounds regulate growth factor-mediated signal transduction pathways and exert potent toxic and

Abbreviations
AP-1, activator protein 1; CLU, clusterin; DAPI, 4′,6-diamidino-2-phenylindole; ER, endoplasmic reticulum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IL-6, interleukin-6; nCLU, nuclear clusterin; NF-κB, nuclear factor kappaB; PARP1, poly(ADP-ribose) polymerase-1; pnCLU, pre-nuclear clusterin; psCLU, pre-secretory clusterin; ROS, reactive oxygen species; sCLU, secretory clusterin; TGF-β1, transforming growth factor-β1.

anticarcinogenic effects on a wide variety of biological systems. Several experimental studies in animal models showed that vanadium compounds exerted chemopreventive and antitumor effects against chemically induced carcinogenesis and in tumor-bearing animals [1,2].

Although the biochemical mechanisms of the action of vanadium are still not fully understood, recent studies on various cell lines revealed that vanadium exerts its antitumor effects through modulation of the activities of protein tyrosine phosphatases and tyrosine kinases, leading to antiproliferative cell responses. Furthermore, vanadium compounds exert cytotoxic effects by generating reactive oxygen species (ROS), generated by Fenton-like reactions and/or during intracellular reduction of V(V) to V(IV), mainly by NADPH, that contribute to the induction of apoptosis [1,2]. The existing evidence indicated that the cellular mechanisms of the anticancer effects of vanadium compounds were due to both inhibition of cellular proliferation and induction of apoptosis [1,2]. Vanadium compounds inhibited the growth of several tumor cell lines [3–6] by suppressing the expression of cyclin D1 [3], Cdc25 [4], and cyclin B1, reducing the phosphorylation of Cdc2, and upregulating $p21^{Cip1/Waf1}$, through ROS generation [4,5]. In mouse epidermal $JP6^+$ (C141) cells, an S-phase arrest was induced through the p53–p21 pathway [6]. In addition to effects on cell cycle progression, vanadium compounds can cause DNA damage and apoptosis in several human cancer cell lines [1,2] and in mouse epidermal $JP6^+$ cells via H_2O_2-mediated reactions leading to p53 transactivation [7,8]. The negative effects of vanadium compounds on cell cycle progression and survival also appear to be mediated through the regulation of growth factor-stimulated signal transduction pathways [9], leading to the induction of oxidative stress and activation of the transcription factors nuclear factor kappaB (NF-κB) [10] and activator protein 1 (AP-1) [10–12] in several cell types.

AP-1 is a transcription factor composed of homodimers and/or heterodimers of basic leucine zipper proteins that belong to the Jun (c-Jun, JunB, and JunD), Fos (c-Fos, FosB, Fra-1, and Fra2), Maf and ATF subfamilies that recognize either 12-*O*-tetradecanoylphorbol-13-acetate response elements or cAMP response elements. Fos proteins, which cannot homodimerize, form stable heterodimers with Jun proteins, thereby enhancing their DNA-binding activity. The regulation of AP-1 activity is complex and is induced by various physiological stimuli and environmental insults, including growth factors, cytokines, tumor promoters, and chemical carcinogens [13,14]. In addition, the activity of AP-1 is modulated by the redox state of the cells [15]. In turn, AP-1 regulates a wide range of cellular processes, including cell proliferation, death, survival, differentiation, and neoplastic transformation [13–16].

Clusterin (CLU) has been functionally implicated in cell cycle regulation and apoptotic cell death, and a prominent feature is its differential expression in many pathological states, including tumor formation and metastasis [17–20]. Two alternatively spliced forms of the CLU gene that encode secretory CLU (sCLU) or nuclear CLU (nCLU) have been reported [21,22]. sCLU is a heterodimeric glycoprotein that was identified as apolipoprotein J, and it primarily functions as an extracellular chaperone. sCLU is initially targeted in the endoplasmic reticulum (ER), where proteolytic removal of the ER-targeting signal peptide and glycosylation results in the ER-associated high-mannose form of $\sim$ 60 kDa [pre-secretory CLU (psCLU)]. Following further processing in the Golgi apparatus, psCLU matures to the secreted heterodimeric sCLU protein form of $\sim$ 75–80 kDa (sCLU) [19]. In general, sCLU exerts a prosurvival effect during cell death and confers resistance to various cytotoxic agents both *in vitro* and *in vivo* [18–20]. In contrast, the precursor form of nCLU [pre-nuclear CLU (pnCLU), $\sim$ 49 kDa] is translated from an alternatively spliced truncated CLU transcript that bypasses the ER signal peptide and remains dormant in the cytosol. Upon cytotoxic stress, pnCLU migrates to the nucleus and is post-translationally modified by an as yet unknown mechanism, and the $\sim$ 55 kDa mature nCLU triggers cell death by interacting with and interfering with Ku70–Ku80 [21,22].

Considering that epidermal cells are mostly exposed to environmental insults, and that both AP-1 [10–12] and CLU [20] are induced and activated in these cells following oxidative stress, a spontaneously immortalized human keratinocyte line, HaCaT (bearing mutant, transcriptionally inactive p53), was used to investigate the effects of vanadyl(IV) sulfate ($VOSO_4$) on cell proliferation and apoptosis. We also explored the possible involvement of the *c-fos* oncogene or CLU in vanadium-modulated cell responses. We report that vanadium-induced apoptosis of HaCaT cells was mediated by *c-fos* and involved induction of total Bax and upregulation and accumulation of nCLU. Furthermore, forced expression of nCLU sensitized HaCaT cells to apoptosis.

Results

$VOSO_4$ inhibited cell proliferation of HaCaT cells by affecting the expression of cell cycle regulatory proteins

To investigate the effects of $VOSO_4$ on cell growth, we performed cell proliferation and colony formation

assays. Actively proliferating HaCaT cells were treated with 0–1000 μM $VOSO_4$ for 24 h, and cell numbers were determined. Cell proliferation was inhibited in a dose-dependent manner, with an EC_{50} of ~ 75 μM $VOSO_4$ (Fig. 1A). Concentrations of $VOSO_4$ > 200 μM did not result in a linear reduction of cell numbers.

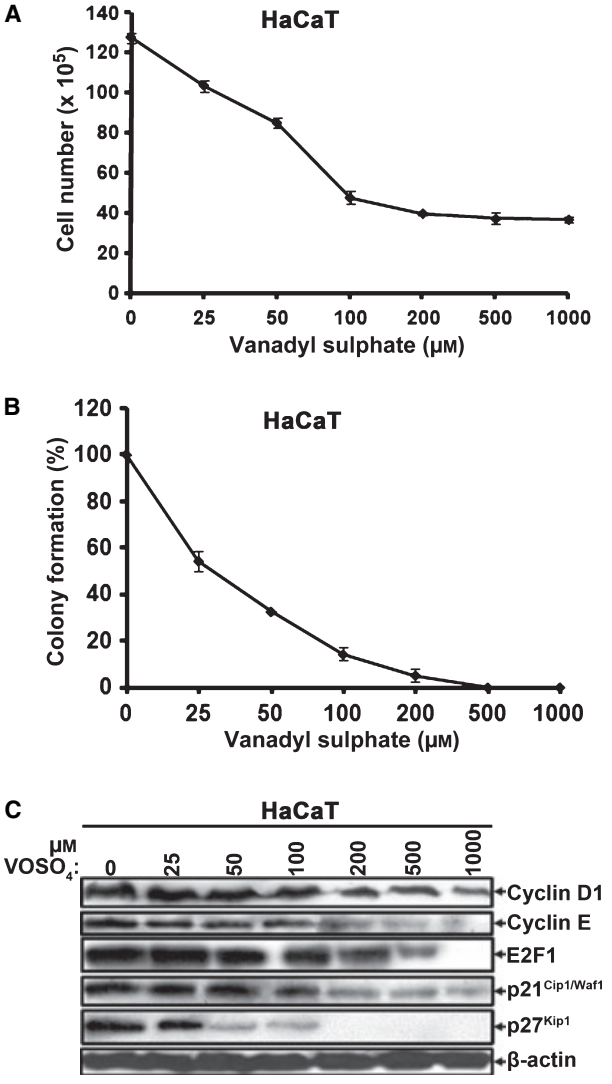

Fig. 1. $VOSO_4$ inhibited HaCaT cell proliferation. (A) HaCaT cells (1×10^5) were treated with increasing concentrations of $VOSO_4$, ranging from 0 to 1000 μM, for 24 h, and cell numbers were determined. (B) For colony formation assays, 200 cells were plated per 60 mm dish in triplicate and treated with $VOSO_4$ for 24 h. Colonies were fixed and stained with crystal violet after 14 days. Colony formation was expressed as percentage of the number of cells plated. (C) HaCaT cells (1.5×10^6) were treated with $VOSO_4$ for 24 h, and total proteins extracted were analyzed for the expression of selected cell cycle regulatory proteins such as cyclin D1, cyclin E, E2F1, $p21^{Cip1/Waf1}$ and $p27^{Kip1}$ or β-actin, using appropriate antibodies. Graphs represent the means of experiments in quadruplicate, and error bars denote ± standard deviation.

To further investigate the long-term antiproliferative effects of $VOSO_4$, actively proliferating HaCaT cells were treated with 0–1000 μM $VOSO_4$ for 24 h, and colonies were allowed to develop for 14 days. $VOSO_4$ inhibited the growth of HaCaT cells in a dose-dependent manner (Fig. 1B). Inhibition of colony formation was evident at 25 μM, marked at 50 μM, and more profound at 100 μM. Although higher concentrations of $VOSO_4$ further inhibited colony formation of HaCaT cells, the reduction was not linear.

The inhibition of cell proliferation by $VOSO_4$ prompted us to determine whether $VOSO_4$ affected the expression of cell regulatory proteins, such as cyclins D1 and E, the proliferation-associated transcription factor E2F1, and the cyclin-dependent kinase inhibitors $p21^{Cip1/Waf1}$ and $p27^{Kip1}$. Treatment of HaCaT cells with increasing concentrations of $VOSO_4$ for 24 h reduced the protein expression levels of cyclins D1 and E, E2F1 and $p21^{Cip1/Waf1}$ (at concentrations ≥ 100 μM $VOSO_4$) and the protein level of $p27^{Kip1}$ (at concentrations ≥ 50 μM $VOSO_4$) (Fig. 1C).

$VOSO_4$ induced apoptosis of HaCaT cells

To determine whether $VOSO_4$ affected cell viability, HaCaT cells were treated with 0–1000 μM $VOSO_4$ for 24 h. $VOSO_4$ reduced the survival of HaCaT cells in a dose-dependent manner (Fig. 2A). Treatment of HaCaT cells with $VOSO_4$ caused marked morphological changes, cytotoxic effects, and dose-dependent cell detachment characteristic of apoptosis (data not shown). Untreated and $VOSO_4$-treated HaCaT cells were stained with 4′,6-diamidino-2-phenylindole (DAPI) to visualize cell nuclei (Fig. 2B). $VOSO_4$ reduced the number of cell nuclei in a dose-dependent manner, and nuclei of apoptotic cells were brightly stained, owing to chromatin condensation (Fig. 2B).

To further investigate the effects of increasing concentrations of $VOSO_4$ on HaCaT cell viability, low molecular weight DNA was extracted from control and $VOSO_4$-treated HaCaT cells and analyzed by agarose gel electrophoresis (Fig. 2C, upper panel). Whereas mock-treated control cells did not undergo apoptosis, treatment of HaCaT cells with $VOSO_4$ resulted in the induction of internucleosomal DNA fragmentation, producing a DNA ladder characteristic of cells undergoing apoptosis, at all concentrations studied. Induction of apoptosis by $VOSO_4$ in HaCaT cells was evident at 25 μM and became more pronounced with increasing concentrations of $VOSO_4$ (Fig. 2C, upper panel).

Whereas antiapoptotic Bcl-2 family members such as Bcl-2 induce resistance to apoptosis, proapoptotic

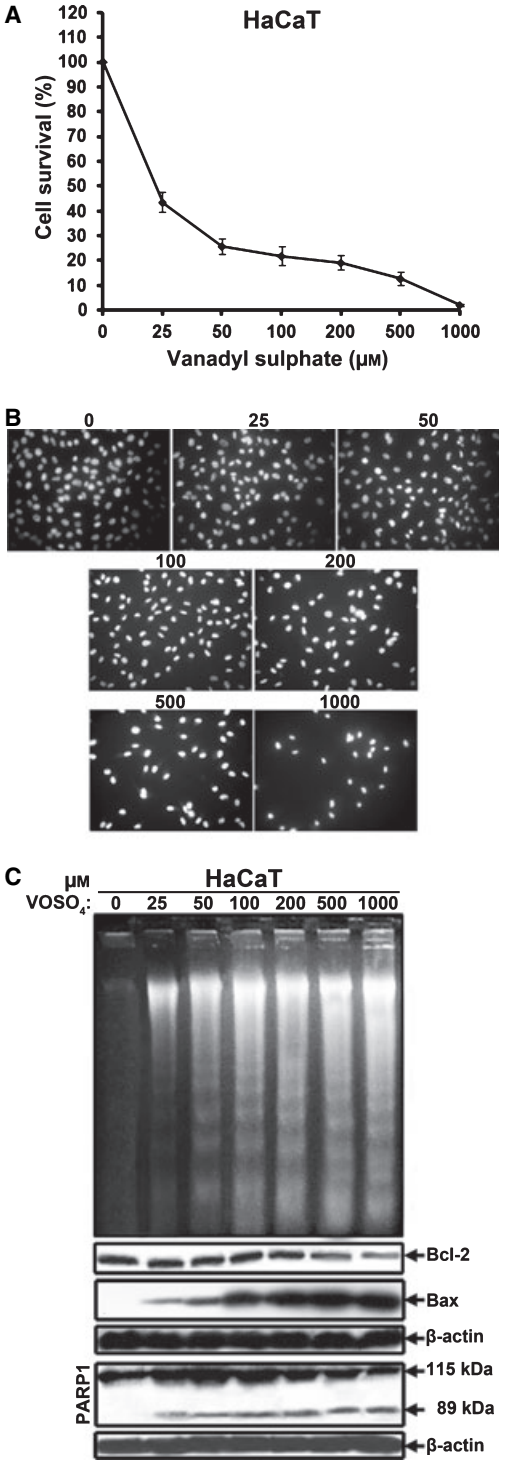

Fig. 2. $VOSO_4$ induced apoptosis of HaCaT cells. (A) HaCaT cells (1×10^5) were treated with increasing concentrations of $VOSO_4$, ranging from 0 to 1000 μM, for 24 h, and cell viability was determined by the Trypan blue exclusion assay. (B) HaCaT cells were treated with $VOSO_4$ for 24 h and then stained with DAPI and visualized under a fluorescence microscope and photographed. (C) HaCaT cells (1.5×10^6) were treated with $VOSO_4$ for 24 h, and DNA isolated from floating and attached cells was analyzed by agarose gel electrophoresis. Total proteins isolated from HaCaT cells treated with $VOSO_4$ for 24 h were analyzed by immunoblotting for the expression of Bcl-2, Bax and PARP1 or β-actin, using appropriate antibodies. The intact and cleaved forms of PARP1 are indicated. The graph shown represents the means of experiments performed in quadruplicate, and error bars denote ± standard deviation.

members such as Bax sensitize cells to apoptosis [23]. To this end, the expression of Bcl-2 and Bax was investigated by immunoblot analysis following treatment of HaCaT cells with increasing concentrations of $VOSO_4$ for 24 h (Fig. 2C, lower panels). Expression of Bcl-2 was reduced mainly at high (500 and 1000 μM) $VOSO_4$ concentrations. In contrast, the expression of total, but not conformationally active, Bax exhibited dose-dependent induction, as it was evident at 25 μM and further upregulated with increasing concentrations of $VOSO_4$ (Fig. 2C, lower panels). Whereas untreated HaCaT cells expressed an intact form of poly(ADP-ribose) polymerase-1 (PARP1), treatment of HaCaT cells with increasing concentrations of $VOSO_4$ for 24 h induced PARP1 cleavage, producing the 89 kDa cleaved form which correlated with apoptosis. Cleavage of PARP1 was detected at 25 μM $VOSO_4$ and became more pronounced with increasing concentrations of $VOSO_4$ (Fig. 2C, lower panel).

Thus, $VOSO_4$ altered the proapoptotic/anti-apoptotic Bcl-2 family member ratio, shifting it to the former, and hence sensitizing HaCaT cells to Bax-mediated apoptosis and promoting cleavage of the caspase-3 substrate, PARP1. Collectively, these data showed that $VOSO_4$ exhibited both cytostatic and cytotoxic effects on HaCaT cells.

Induction of apoptosis correlated with upregulation of the *c-fos* proto-oncogene and changes in the expression of CLU

Next, we investigated the involvement of the *c-fos* proto-oncogene in $VOSO_4$-induced antiproliferative responses of HaCaT cells, as *c-fos* has been implicated in keratinocyte homeostasis [13,16]. HaCaT cells were treated with 0–1000 μM $VOSO_4$ for 24 h, and total cell lysates extracted from untreated and vanadium-treated HaCaT cells were analyzed by immunoblotting for the expression of c-fos oncoprotein (Fig. 3A). $VOSO_4$ markedly upregulated the expression of c-fos oncoprotein in a dose-dependent manner, suggesting that induction of c-fos oncoprotein may be related to the $VOSO_4$-mediated cytostatic and cytotoxic effects in

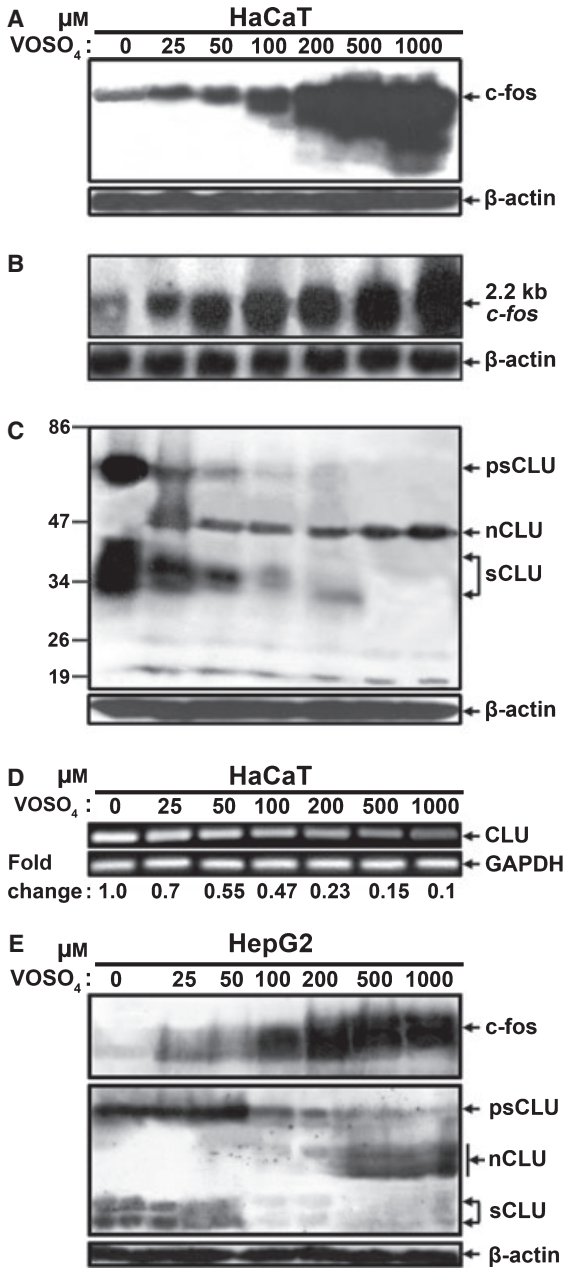

Fig. 3. Dose-dependent induction of c-fos oncoprotein and changes in CLU expression induced by $VOSO_4$. (A) HaCaT cells (1.5×10^6) were treated with $VOSO_4$ for 24 h, and total proteins extracted were analyzed for the expression of c-fos (A), CLU (B) or β-actin, using appropriate antibodies. (C) HaCaT cells (1.5×10^6) were treated with $VOSO_4$ for 24 h, and total RNA extracted was analyzed for the expression of *c-fos* or β-actin, using specific cDNA probes. (D) Total RNA was extracted from untreated HaCaT cells and from cells treated with $VOSO_4$ for 24 h, and subjected to RT-PCR analysis, using specific primers for sCLU or GAPDH as a reference/control. (E) HepG2 cells (1.5×10^6) were treated with $VOSO_4$ for 24 h, and total proteins extracted were analyzed for the expression of c-fos (upper panel) and CLU (lower panel) or β-actin, using appropriate antibodies.

HaCaT cells. To determine whether changes at the protein level correlated with changes at the mRNA level, total RNA was isolated from untreated and $VOSO_4$-treated HaCaT cells and subjected to northern blot hybridization analysis using a *c-fos*-specific cDNA probe or β-actin (Fig. 3B). $VOSO_4$ induced the expression of the 2.2 kb *c-fos* transcript in a dose-dependent manner (Fig. 3B).

Considering that CLU has been implicated in increased resistance of cells to various apoptotic stimuli, including oxidative stress [18–22], CLU protein kinetics were studied in $VOSO_4$-treated HaCaT cells. Exposure of HaCaT cells to $VOSO_4$ resulted in a dose-dependent reduction of the psCLU and sCLU isoform expression levels (Fig. 3C). Interestingly, these changes were accompanied by upregulation of an ~ 49 kDa polypeptide, most likely corresponding to nCLU (Fig. 3C), a nuclear CLU isoform implicated in the induction of cell death [21,22]. Thus, changes in the upregulation of c-fos oncoprotein in response to $VOSO_4$ correlated with changes in the expression and processing of CLU.

To determine whether changes at the protein levels correlated with changes at the mRNA level, we performed RT-PCR analysis, as described in Experimental procedures, for the expression of at least sCLU (Fig. 3D). Treatment of HaCaT cells with $VOSO_4$ resulted in dose-dependent downregulation, but not total loss, of sCLU mRNA (Fig. 3D), suggesting that $VOSO_4$ also affected the expression of CLU at the protein level, perhaps by affecting protein stability.

To investigate whether other cell lines behave similarly in response to $VOSO_4$, we next used HepG2 cells treated with $VOSO_4$ in exactly the same way as HaCaT cells, and total proteins isolated were probed for the expression of c-fos and CLU by immunoblotting (Fig. 3E). Indeed, $VOSO_4$ induced the expression of c-fos oncoprotein (Fig. 3E, upper panel) and altered the expression and processing of CLU (Fig. 3E, lower panel). Thus, the effects of $VOSO_4$ on c-fos protein expression and CLU expression and processing were not unique to HaCaT epidermal cells.

Ectopic overexpression of c-fos oncoprotein promoted the induction of nCLU and apoptosis in HaCaT cells

To further investigate the effects of c-fos oncoprotein upregulation on HaCaT cell homeostasis, HaCaT cell lines stably overexpressing human *c-fos* proto-oncogene or a Neo vector control were generated. As shown in Fig. 4A, HaCaT *c-fos* cells expressed higher levels of the c-fos oncoprotein than their Neo-express-

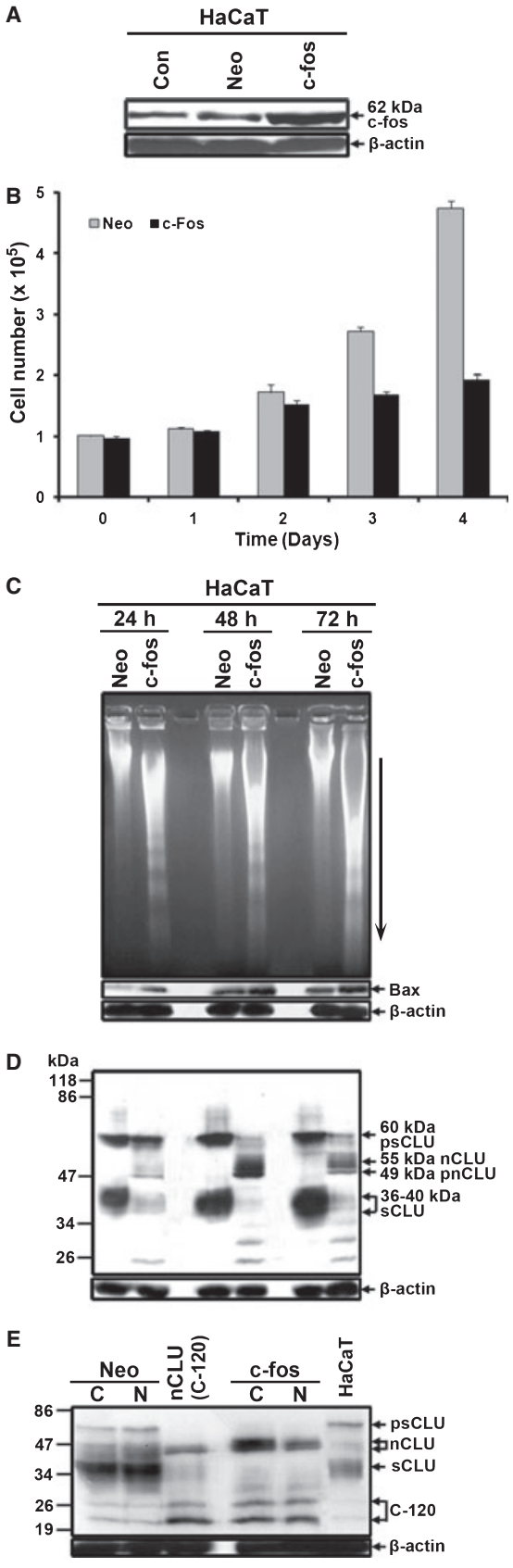

Fig. 4. *c-fos* inhibited proliferation and induced apoptosis of HaCaT cells through changes in CLU expression. (A) HaCaT cells were infected with high-titer recombinant retroviruses carrying either Neo or human *c-fos* cDNA, selected in G418 and analyzed for the expression of c-fos oncoprotein or β-actin. (B) HaCaT cells (1.5×10^5 per well) were plated in 24-well plates in triplicate, and cell numbers were determined over a period of 4 days. (C) Confluent monolayers of HaCaT Neo and HaCaT *c-fos* cells were cultured for 24, 48 and 72 h in the presence of serum, and DNA isolated from floating and attached cells was analyzed by agarose gel electrophoresis. Total proteins extracted from the same cell types and under the same culture conditions were analyzed by immunoblotting for the expression of Bax or β-actin. (D) Total proteins extracted from the different cell types cultured under the conditions described in (C) were analyzed for the expression of CLU or β-actin. (E) Cytoplasmic (C) and nuclear (N) extracts isolated from confluent monolayers of HaCaT Neo and HaCaT *c-fos* cells and total proteins isolated from HaCaT cells and HaCaT cells expressing nCLU (C120) were analyzed for the expression of CLU or β-actin. In (E), the two faster-migrating bands corresponded to the minimal Ku70-binding domain of nCLU. The graph shown represents the means of experiments performed in triplicate, and error bars denote ± standard deviation.

ing control counterparts, indicating that the infected cells expressed the corresponding exogenously introduced c-fos oncoprotein.

Next, we analyzed cell proliferation and apoptosis of the transgenic cell lines to determine whether c-fos oncoprotein expression affected the homeostasis of HaCaT cells. Cell proliferation assays showed that *c-fos* inhibited HaCaT cell growth, over a period of 4 days, as compared with HaCaT Neo control cells (Fig. 4B).

Semiconfluent (70–80%) monolayers of HaCaT Neo and HaCaT *c-fos* cells were exposed to fresh serum-containing medium, and DNA was extracted after 24, 48 and 72 h and analyzed by agarose gel electrophoresis. In contrast to Neo-expressing control cells, overexpression of the *c-fos* oncogene induced apoptosis of HaCaT cells within 24 h (Fig. 4C, upper panel). As is evident by the pattern of ethidium bromide staining, apoptosis was more severe with time, as more higher molecular weight DNA was converted to smaller fragments in the HaCaT *c-fos* cells than in HaCaT Neo cells (Fig. 4C, upper panel, arrow). To further confirm that *c-fos* overexpression was directly related to the observed apoptotic outcome of HaCaT cells (which bear mutant p53), we analyzed the expression of Bax, a proapoptotic protein regulated by AP-1 [24] and p53 [25], by immunoblotting (Fig. 4C, lower panel). Overexpression of the *c-fos* proto-oncogene induced expression of total, but not conformationally active, Bax in p53-defective HaCaT cells as compared with HaCaT Neo cells in a time-dependent manner (Fig. 4C, lower panel).

To determine whether induction of apoptosis by *c-fos* correlated with changes in the expression of CLU, immunoblot analysis was performed in HaCaT Neo and HaCaT *c-fos* cells, under the conditions described above. Both cell types expressed psCLU and sCLU (Fig. 4D). However, the levels of both forms of sCLU were reduced in HaCaT *c-fos* cells as compared with control cells, and a doublet at $\sim$ 49–55 kDa, most likely corresponding to pnCLU and nCLU, was detected in *c-fos*-expressing HaCaT cells. Both pnCLU and, to a lesser extent, nCLU were detected at 24 h, and were strongly upregulated after 48 h, with nCLU accumulating at higher levels. Whereas both pnCLU and nCLU protein levels were reduced in HaCaT *c-fos* after 72 h of incubation, perhaps because of extensive cell death, nCLU levels were sustained at higher levels than at 24 h but at lower levels than at 48 h (Fig. 4D). It should also be noted that growth curves over a period of 12 days showed that HaCaT *c-fos* cell proliferation began to recover after 4 days, and this correlated with the loss of nCLU expression and the re-expression of psCLU and sCLU (Doc. S1 and Fig. S1). Thus, ectopic overexpression of the c-fos oncoprotein suppressed the expression of the prosurvival psCLU and sCLU isoforms and induced nCLU, a cell death signaling protein [21,22,26–28].

To further verify the effects of c-fos oncoprotein on CLU processing, cytoplasmic and nuclear fractions were extracted and analyzed for the expression of CLU following serum stimulation of semiconfluent monolayers for 24 h (Fig. 4E). Whereas the expression of psCLU and sCLU was detected in both cytoplasmic and nuclear extracts of HaCaT Neo cells, it was absent in HaCaT cells overexpressing c-fos oncoprotein. Instead, the expression of nCLU, appearing as a doublet, was detected in *c-fos*-expressing HaCaT cells, with a higher expression level in the cytoplasmic than in the nuclear fraction, suggesting that nCLU may be produced in the cytoplasm and translocate to the nucleus (Fig. 4E). In fact, it has been shown that nCLU is produced as a cytoplasmic precursor, which, upon apoptosis, is converted to nCLU [28].

Collectively, these data suggested that c-fos-induced apoptosis of HaCaT cells was Bax-mediated and involved downregulation of sCLU and upregulation of nCLU.

Differential effects of constitutive expression of sCLU and Bcl-2 on vanadium-induced apoptosis

As $VOSO_4$ dramatically affected the expression of sCLU (Fig. 3) and, to a lesser extent, of Bcl-2 (Fig. 2), the effects of sCLU or Bcl-2 forced overexpression on vanadium-induced apoptosis were investigated. HaCaT cells were transfected with pcDNA3.1B (Neo) or with expression vectors carrying the entire human sCLU or Bcl-2 cDNA to generate stable cell clones [29,30]. HaCaT Neo^T, HaCaT sCLU and HaCaT Bcl-2 cells were treated with increasing concentrations of $VOSO_4$ for 24 h. HaCaT Neo^T and HaCaT sCLU cells displayed marked morphological changes with increasing $VOSO_4$ concentrations, including cell shrinkage, rounding up, and detachment from the substratum, characteristic of an apoptotic phenotype (data not shown), and similar to those observed in untransfected HaCaT cells (Fig. 2). In contrast, Bcl-2-overexpressing HaCaT cells displayed normal morphology at all concentrations used (data not shown). To investigate the effects of sCLU and Bcl-2 on cell survival following treatment with increasing concentrations of $VOSO_4$ for 24 h, HaCaT Neo^T, HaCaT sCLU and HaCaT Bcl-2 cells were subjected to Trypan blue exclusion assay (Fig. 5A). Although $VOSO_4$ reduced the viability of HaCaT Neo^T and HaCaT sCLU cells (Fig. 5A) in a manner similar to that observed in HaCaT cells (Fig. 2), it caused no cytotoxic effect in Bcl-2-overexpressing HaCaT cells (Fig. 5A).

To further investigate the effect of sCLU and Bcl-2 overexpression on $VOSO_4$-induced apoptosis of HaCaT cells, DNA and proteins were isolated from floating and attached HaCaT Neo^T, HaCaT sCLU and HaCaT Bcl-2 cells treated with 0–1000 μM $VOSO_4$ for 24 h and analyzed by agarose gel electrophoresis to detect DNA fragmentation or by immunoblotting to detect PARP1 cleavage (Fig. 5B). Whereas treatment of HaCaT Neo^T and HaCaT sCLU cells with increasing concentrations of $VOSO_4$ resulted in the induction of DNA fragmentation at concentrations of $VOSO_4$ as low as 25 μM, enforced expression of Bcl-2 completely blocked $VOSO_4$-induced apoptosis of HaCaT cells (Fig. 5B, upper panel). Analysis of PARP1 expression showed that $VOSO_4$ induced PARP1 cleavage in HaCaT Neo^T and HaCaT sCLU cells, but not in HaCaT Bcl-2 cells (Fig. 5B, lower panel). Thus, Bcl-2 but not sCLU blocked $VOSO_4$-induced apoptosis of HaCaT keratinocytes.

Next, we investigated by immunoblotting whether $VOSO_4$ affected the expression of c-fos oncoprotein and the expression and/or processing of CLU in HaCaT Neo^T and HaCaT Bcl-2 cells (Fig. 5C). Whereas treatment of HaCaT Neo^T cells with $VOSO_4$ induced the expression of c-fos oncoprotein, which was evident at 50 μM $VOSO_4$ and increased dose-dependently, enforced expression of Bcl-2 delayed $VOSO_4$-

induced c-fos oncoprotein expression, which was evident at 100 μM and at lower levels than that detected in HaCaT Neo^T cells (Fig. 5C, upper panel). Immunoblot analysis of CLU expression showed that $VOSO_4$ induced nCLU expression with the concomitant downregulation of psCLU and sCLU in HaCaT Neo^T cells (Fig. 5C, lower panel) in a similar way as in control HaCaT cells (Fig. 3B). In contrast, induction of nCLU was much lower in HaCaT Bcl-2, was evident at higher $VOSO_4$ concentrations, and correlated with c-fos oncoprotein expression (Fig. 5C, upper panel). Collectively, the data suggested that induction of HaCaT cell apoptosis by $VOSO_4$ was promoted through the induction of both c-fos and nCLU, the expression of which was affected by Bcl-2.

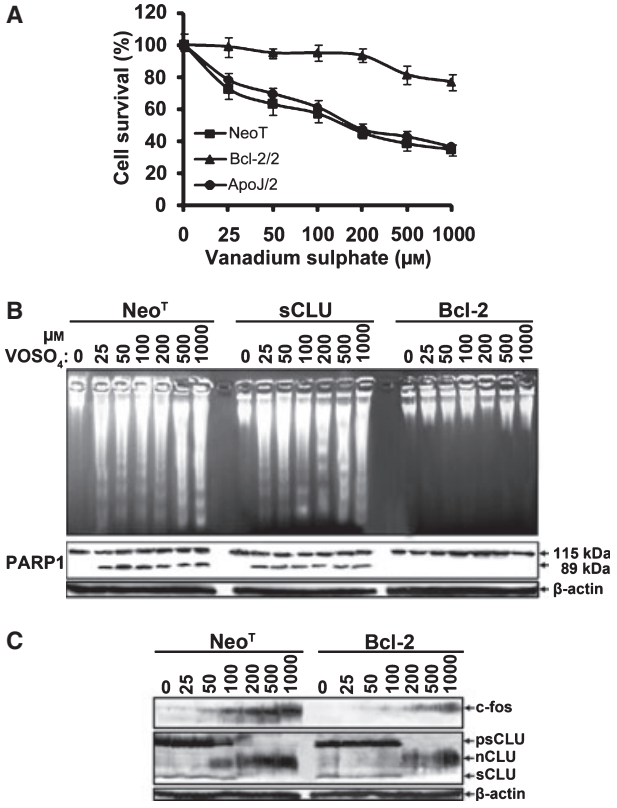

Fig. 5. Bcl-2 but not sCLU protected HaCaT cells from $VOSO_4$-induced apoptosis. (A) HaCaT Neo^T, HaCaT sCLU or HaCaT Bcl-2 cells (1×10^5) were treated with increasing concentrations of $VOSO_4$, ranging from 0 to 1000 μM, for 24 h, and cell viability was determined by the Trypan blue exclusion assay. (B) DNA isolated from $VOSO_4$-treated HaCaT floating and attached cells was analyzed by agarose gel electrophoresis. (C). Total proteins isolated from $VOSO_4$-treated HaCaT cells for 24 h were analyzed by immunoblotting for the expression of PARP1, c-fos and CLU or β-actin, using appropriate antibodies. The intact and cleaved forms of PARP1 are indicated.

Overexpression of nCLU (C120) sensitized HaCaT cells to apoptosis

Induction of nCLU expression following treatment with $VOSO_4$ or after *c-fos* transduction of HaCaT cells prompted us to generate nCLU (C120)-expressing HaCaT cells to investigate whether nCLU overexpression sensitized them to apoptosis.

Immunoblot analysis of total lysates showed that HaCaT nCLU (C120) cells strongly expressed a doublet of 49 kDa and two smaller fragments of ~ 26 and 20 kDa, corresponding to nCLU (C120) (Fig. 6A, lane 2), as compared with HaCaT Neo^T cells (Fig. 6A, lane 1). Interestingly, overexpression of nCLU (C120) resulted in the loss of both psCLU and sCLU (Fig. 6A, compare lanes 1 and 2).

To further verify this differential expression of CLU, cytoplasmic and nuclear extracts and total

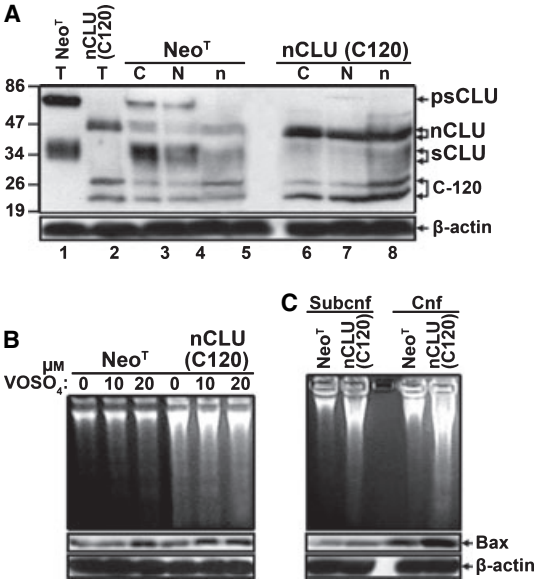

Fig. 6. nCLU (C120) sensitized HaCaT cells to apoptosis. (A) HaCaT cells were transfected with either pcDNA or a vector carrying nCLU (C120), selected in G418 to generate HaCaT Neo^T and HaCaT nCLU (C120), respectively, and total proteins (T), cytoplasmic extracts (C), nuclear extracts (N) and isolated nuclei (n) were analyzed by immunoblotting for the expression of CLU or β-actin. (B,C) Neo-expressing or nCLU (C120)-expressing HaCaT cells (1.5×10^6) were treated with $VOSO_4$ at the indicated concentrations for 24 h (B), or cultured as subconfluent and confluent monolayers for 24 h in the presence of serum (C), and DNA isolated from floating and attached cells was analyzed by agarose gel electrophoresis. Total proteins isolated from cells in (B) or from cells in (C) were analyzed by immunoblotting for the expression of Bax or β-actin, using appropriate antibodies. The graph shown represents the means of experiments performed in quadruplicate, and error bars denote ± standard deviation.

proteins from 'purified' nuclei isolated through sucrose gradients were immunoblotted and probed for the expression of CLU (Fig. 6A). Whereas HaCaT Neo^T cells expressed the intracellular, the secreted and, to a lesser extent, the nuclear forms of CLU, with higher expression of all forms being seen in the cytoplasmic extracts (Fig. 6A, lanes 3–5), HaCaT nCLU (C120) cells expressed only the 49 kDa nuclear form and two faster-migrating bands of $\sim$ 26 and 20 kDa, at higher levels than in HaCaT Neo^T cells (Fig. 6A, lanes 6–8). Both of these faster-migrating bands were expressed at higher levels in the nuclei of HaCaT nCLU (C120) cells than in the nuclei of HaCaT Neo^T cells, and corresponded to the minimal Ku70-binding domain (120 amino acids of the CLU/XIP8 C-terminus) of CLU [26,27]. Thus, ectopic overexpression of nCLU (C120) resulted in the loss of psCLU and sCLU, suggesting that these forms of CLU/apolipoprotein J were reciprocally regulated.

To determine whether nCLU (C120) sensitized HaCaT cells to apoptosis, HaCaT Neo^T and HaCaT nCLU (C120) cells were treated with low concentrations of $VOSO_4$ (Fig. 6B) or cultured at low and high density in the presence of serum (Fig. 6C), and low molecular weight DNA was isolated and analyzed on agarose gels. Whereas no DNA fragmentation was detected in untreated HaCaT Neo^T cells, cells treated with 10 and 20 μM $VOSO_4$ exhibited low levels of apoptosis. In contrast, untreated or $VOSO_4$-treated HaCaT nCLU (C120) cells exhibited higher levels of apoptosis than their Neo-expressing control counterparts (Fig. 6B). $VOSO_4$-induced apoptosis of HaCaT Neo^T and HaCaT nCLU (C120) cells correlated with the dose-dependent induction of Bax (Fig. 6B, lower panel), which was higher in the latter cell type (Fig. 6B, lower panel).

To further verify that overexpression of nCLU (C120) sensitizes cells to apoptosis, DNA was isolated from subconfluent and confluent HaCaT Neo^T and HaCaT nCLU (C120) cell monolayers and analyzed by agarose gel electrophoresis (Fig. 6C, upper panel). Ectopic overexpression of nCLU (C120) induced apoptosis of HaCaT cells under both culture conditions, as compared with HaCaT Neo^T cells (Fig. 6C, upper panel), resulting in the induction of Bax expression in both subconfluent and confluent HaCaT nCLU (C120) cells as compared with their HaCaT Neo^T control counterparts (Fig. 6C, lower panel). Thus, nCLU (C120) induced spontaneous apoptosis and sensitized HaCaT cells to $VOSO_4$-induced apoptosis through upregulation of Bax protein expression.

Discussion

Vanadium inhibited HaCaT cell proliferation in a dose-dependent manner by affecting the expression of genes that regulate cell cycle progression. Specifically, it downregulated the expression of cyclins D1 and E, E2F1, and the cyclin-dependent kinase inhibitors $p21^{Cip1/Waf1}$ and $p27^{Kip1}$ (Fig. 1). Both $p21^{Cip1/Waf1}$ and $p27^{Kip1}$ act as positive and negative regulators of the cell cycle [31], and, in particular, as assembly factors contributing to cyclin D1–CDK4/6 or cyclin E–CDK2 complex formation. In addition, both $p21^{Cip1/Waf1}$ and $p27^{Kip1}$ act as antiapoptotic factors [32], suggesting that their downregulation by $VOSO_4$ most likely contributed to sensitization of HaCaT cells to apoptosis.

In addition to the inhibition of cell proliferation, $VOSO_4$ induced dose-dependent morphological changes (not shown), a reduction in cell nuclei and chromatin condensation and DNA fragmentation characteristic of apoptosis, by shifting the proapoptotic/antiapoptotic Bcl-2 family member ratio towards the former and by inducing PARP1 cleavage (Fig. 2). Thus, $VOSO_4$ inhibited cell proliferation and induced apoptosis of HaCaT cells in a dose-dependent and p53-independent manner, as HaCaT cells bear mutant, transcriptionally inactive p53. Our results conflict with other findings showing that p53 transactivation was required for vanadium-induced apoptosis of mouse epidermal cells [8].

A major factor that appeared to contribute to $VOSO_4$-induced apoptosis was the profound dose-dependent induction of c-fos oncoprotein expression, which correlated with *c-fos* mRNA levels (Fig. 3). In addition, induction of c-fos oncoprotein expression was not specific to HaCaT epidermal cells, as c-fos was also induced in HepG2 liver tumor cells by $VOSO_4$ (Fig. 3E). Thus, in addition to confirming the role of ROS in vanadate-induced inhibition of cell proliferation and apoptosis [4–8], the present study extended these investigations and examined the mechanism of c-fos-mediated apoptosis of HaCaT cells in response to $VOSO_4$. Prior studies showed that vanadocene complexes triggered activation of the *c-fos* promoter in epithelial HepG2 liver cells [12], and exposure of murine transformed 3T3 fibroblasts [33] or C127 mammary cells [34] to vanadate induced expression of *c-jun* and *junB*, both encoding for components of AP-1, through ROS [34]. Similarly, vanadate induced the activity of AP-1 in murine $JB6^+$ epidermal cells through generation of ROS [10,11]. In contrast, in short-term experiments, sodium orthovanadate was shown to inhibit the serum-mediated induction of c-fos [9]. *c-fos* has been implicated in skin homeostasis

[13,16] and in life-and-death decisions [14]. Ectopic overexpression of the *c-fos* oncogene in HaCaT cells inhibited cell proliferation and induced apoptosis (Fig. 4). Previous studies showed that c-fos oncoprotein was expressed at high levels in normal adult skin [35] and its expression was increased in epidermal cells in late stages of differentiation, but not in proliferative cell populations [36,37], suggesting a role for *c-fos* in developmental apoptosis. Moreover, it was shown that *c-fos* was involved in mediating epidermal keratinocyte growth arrest in response to differentiation-inducing agents such as serum, 12-*O*-tetradecanoylphorbol-13-acetate, and high calcium levels [38]. Furthermore, *c-fos* was activated during apoptosis of epithelial cells [39], and *c-fos* was shown to increase the sensitivity of keratinocytes [40] and other epithelial cells [41,42] to apoptosis, but with no indication of the mechanism involved.

It was shown here that vanadium-induced c-fos-mediated apoptosis of HaCaT cells involved upregulation of total Bax and changes in the expression profile of CLU (Fig. 3). Indeed, enforced expression of the *c-fos* proto-oncogene, in addition to inhibiting cell proliferation, also induced apoptosis of HaCaT cells through the induction of total, but not conformationally active, Bax, downregulation of sCLU, and upregulation of nCLU (Fig. 4), in a p53-independent manner [43]. However, HaCaT *c-fos* cell proliferation recovered over a growth period of 12 days, and this correlated with the loss of nCLU expression and the re-expression of psCLU and sCLU (Fig. S1). It was previously shown that transforming growth factor-β1 (TGF-β1) induced the expression [44,45] and nuclear localization of CLU in epithelial cells [46]. C-fos oncoprotein repressed CLU gene expression, maintaining low basal levels in the absence of TGF-β1, and TGF-β1, presumably through its effects on c-fos oncoprotein synthesis and/or stability, abrogated repression of c-fos oncoprotein, thereby resulting in gene expression [47]. As TGF-β1 is an inducer of epithelial cell apoptosis [48], it is tempting to speculate that this effect could be mediated through the induction of nCLU. Indeed, overexpression of nCLU (C120) sensitized HaCaT cells to $VOSO_4$-induced apoptosis through loss of psCLU and sCLU, suggesting reciprocal regulation of the different forms of CLU (Figs 3 and 6). Previous studies demonstrated that, although in certain cellular contexts sCLU may suppress cellular growth [49–51] or promote cell death [50], it mostly exerts a prosurvival effect, conferring resistance to cytotoxic agents both *in vitro* and *in vivo* [18–20]. Indeed, overexpression of sCLU did not alter the proliferative capacity of normal and SV40-transformed human fibroblasts [52], and it was shown to protect cells from apoptosis induced by oxidative stress [53–57], tumor necrosis factor-α [58,59], and genotoxic stimuli [60], but not from C_2-ceramide [29]. CLU ablation sensitized osteosarcoma [61] and prostate cancer cells [62] to both genotoxic and oxidative stress induced by chemotherapeutics and H_2O_2 [61] and to TRAIL-induced apoptosis [62], further supporting a cytoprotective role for sCLU.

In contrast, nCLU induced apoptosis of human tumor epithelial cells [21]. Accumulation of nCLU correlated with inhibition of cell proliferation and induction of apoptosis of human tumor epithelial cells caused by cell detachment and anoikis [63], chemotherapy [50,64–66] and tumor necrosis factor-α treatment [65], calcium depletion [67], or heat shock treatment [68]. Furthermore, transient but not stable ectopic overexpression of an intracellular form of CLU (psCLU) in PC-3 androgen-independent prostate cancer cells resulted in signal-independent massive nuclear localization of the protein, leading to G_2–M-phase blockade followed by caspase-dependent apoptosis [69]. In contrast, in stable psCLU-overexpressing surviving cells, CLU was confined to the cytoplasm, suggesting a negative correlation between nCLU accumulation and cell survival [69]. Enforced expression of sCLU in prostate epithelial cells inhibited cell cycle progression and induced apoptosis that correlated with the relocation of sCLU from the cytoplasm and nuclear accumulation of the protein [50]. Indeed, overexpression of nCLU was shown to induce apoptotic cell death [26,27]. Thus, whereas the secreted form of CLU possesses antiapoptotic properties, its nuclear form signals cell death. Because interleukin-6 (IL-6) induces CLU antiapoptotic isoform production (sCLU), Bax activity inhibition, and Bcl-2 overexpression [70], we also investigated the expression of IL-6 in untreated and $VOSO_4$-treated HaCaT cells by RT-PCR (Doc. S1 and Fig. S2).

Although one of the findings in the present study was the reciprocal expression of sCLU and nCLU, we can only speculate at this stage. First, apoptotic signals in human and rodent cells can induce the production of various CLU protein isoforms, including nCLU [20]. Second, the induction of nCLU and the reduction in sCLU expression may be linked to calcium homeostasis. Previous studies showed that calcium depletion induces nCLU, a novel effector of apoptosis in human tumor cells [66,67,71]. It was shown that calcium deprivation caused translocation of a 45 kDa CLU isoform to the nucleus in human prostate epithelial cells, leading to inhibition of cell proliferation and caspase cascade-dependent anoikis [67]. Addition of

the intracellular calcium ion chelator BAPTA-AM [67] or the use of EDTA, which reduces the intracellular and extracellular calcium levels, stimulated nuclear expression of CLU protein [66], and in both cases induction of nCLU was accompanied by extensive cell death. Thus, an alternative explanation is that $VOSO_4$ may interfere with calcium homeostasis, which in turn affects CLU expression and processing.

It was mentioned above that loss of nCLU expression and the reappearance of psCLU and sCLU correlated with the recovery of HaCaT *c-fos* cell proliferation (Fig. S1). However, whereas forced expression of Bcl-2 protected HaCaT cells from $VOSO_4$-induced apoptosis, sCLU failed to do so (Fig. 5). Similar results were obtained with HeLa cells (data not shown). Although Bcl-2 overexpression delayed c-fos and nCLU induction following treatment with $VOSO_4$ (Fig. 5), the possibility that CLU and Bcl-2 affect different signaling pathways cannot be excluded. One possible explanation may be related to the different subcellular localization of Bcl-2 and CLU: Bcl-2 is found in mitochondria (in addition to the ER and nucleus), organelles that are affected in response to apoptotic stimuli, but there is no evidence so far that CLU is also localized in mitochondria. A second factor contributing to the differential effects of Bcl-2 and CLU on cell physiology in response to apoptotic stimuli may be related to the effects that the different subcellular pools of Bcl-2 [72] and CLU [73–76] may have on the activity of transcription factors such as NF-κB, which mainly acts as an inhibitor of apoptosis but can also act as a proapoptotic factor [77].

Collectively, the present studies showed that vanadium upregulated c-fos oncoprotein expression, leading to the induction of Bax and nCLU, a death signal protein, and to the downregulation of sCLU, a survival protein, both of which are AP-1 target genes. Thus, our studies revealed a novel mechanism through which c-fos reciprocally regulated the expression of the different forms of CLU, leading to vanadium-induced antiproliferative responses of human keratinocytes. Most importantly, our results strongly suggest that overall sCLU and nCLU expression in the cell is tightly regulated and that cells try to maintain a homeostatic ratio of sCLU/nCLU. In cells undergoing vanadium-induced apoptotic cell death, this ratio decreases dramatically, owing to the simultaneous decrease in sCLU levels and increase in nCLU levels. Thus, the sCLU/nCLU ratio is an important factor in homeostasis as well as in carcinogenesis, with the ratio increasing as cells move towards promotion and progression [78].

Experimental procedures

Cell culture

HaCaT, a spontaneously immortalized human keratinocyte cell line, its derivatives HaCaT Neo (referred to as HaCaT Neo^T, thereafter), HaCaT Bcl-2 and HaCaT CLU [29,30], HepG2, a human hepatoma cell line and the Phoenix amphotropic retroviral packaging line were cultured in DMEM supplemented with 10% fetal bovine serum, 1.4 mM L-glutamine, 100 units·mL^{-1} penicillin and 100 μg·mL^{-1} streptomycin (Seromed-Biochrom KG, Germany) at 37 °C in 5% CO_2.

Retroviral vectors and retroviral HaCaT cell infections

The pZipNeoSV(X) retroviral vector carrying neomycin phosphotransferase resistance gene (Neo^R) and pZipNeoSV(X)-*c-Fos* (PM43.1) carrying human *c-fos* proto-oncogene cDNA have been described previously [79]. Phoenix cells were transfected with retroviral plasmid DNA using calcium phosphate precipitation, and viral supernatants were used to infect HaCaT cells, which were then selected in 500 μg·mL^{-1} G418 for 3 weeks to generate stable HaCaT Neo and HaCaT *c-fos* keratinocyte pooled cell lines.

Generation of the nCLU (C120) plasmid and nCLU (C120)-expressing HaCaT cells

Using specific primers: (C120, *Hin*dIII, forward, 5′-CGAA TTCGCGGAAGCTTCATGTCTGTGGACT-3′; and *Bam*HI, reverse, stop, 3′-ATCAGATGGATCCTTATCACTCCTCC CGGTGCTTTTTGC-5′), the C120 cDNA encoding for the minimal Ku70-binding domain of nCLU (120 amino acids of the C-terminus) was amplified from the original pACT2–C120 vector [27]. The cDNA of interest was excised and subcloned directly into pcDNA3.1/Myc-His^+ (Invitrogen, Athens, Greece). Within the C120 peptide, a Met residue was inserted just before the first CLU-relevant amino acid (Ser310), yielding a polypeptide with a theoretical molecular mass of ~ 16.3 kDa. Correct cloning was verified by dsDNA sequencing. HaCaT cells were transfected with pcDNA or pcDNA carrying nCLU (C120) by the calcium phosphate precipitation method. Transfected cells were selected in 500 μg·mL^{-1} G418 for 3 weeks to generate cells stably expressing the nCLU (C120) fragment [referred to as HaCaT nCLU (C120) hereafter].

Treatment of HaCaT cells with $VOSO_4$

For analyses of cell proliferation, 1×10^5 cells per well were plated in 24-well plates in quadruplicate and allowed to grow for 24 h in complete DMEM. The medium was aspirated, and the cells were treated with 0–1000 μM $VOSO_4$

for 24 h. Cell numbers were estimated by counting on a hemocytometer.

For colony formation assays, 200 cells were plated per 60 mm dish in triplicate. Following attachment for 24 h, the cells were treated with 0–1000 μM $VOSO_4$ for 24 h. Colonies were allowed to develop over a period of 14 days, and then fixed and stained with crystal violet. The numbers of cells in colonies containing 100 or more normal-appearing cells were expressed as percentages of the number of cells plated and normalized to colonies in mock-treated controls. The experiment was performed twice.

For analyses of cell survival, cells were plated in 24-well plates in quadruplicate at a density of 1×10^5 cells per well and allowed to grow for 24 h in complete DMEM. The medium was aspirated, and the cells were treated with 0–1000 μM $VOSO_4$ for 24 h. Cell viability was determined by a Trypan blue exclusion assay. The experiment was performed twice.

Cell nuclei in untreated HaCaT cells and in cells treated with 0–1000 μM $VOSO_4$ for 24 h were visualized using the fluorochrome stain DAPI. Cells were stained with 0.4 $\mu g \cdot mL^{-1}$ DAPI for 3 min, and examined and photographed under a fluorescence microscope (Nikon eclipse TS100; Nikon Corp., Tokyo, Japan) fitted with a camera (Nikon cool pix 990; Nikon Corp., Tokyo, Japan).

DNA fragmentation assay

DNA fragmentation analysis of $VOSO_4$-treated and untreated cells was performed as described previously [29,30]. HaCaT cells were seeded in duplicate at a density of 1.5×10^6 cells per 10 cm dish, allowed to grow for 24 h in complete DMEM, and then treated with 0–1000 μM $VOSO_4$ for 24 h. DNA was extracted and analyzed by agarose gel electrophoresis.

Untreated confluent or subconfluent monolayers of HaCaT cells were exposed to fresh complete DMEM growth medium, and DNA was isolated at 24, 48 and 72 h following serum stimulation and analyzed by agarose gel electrophoresis.

Preparation of cytoplasmic and nuclear extracts

Cytoplasmic and nuclear extracts were prepared as previously described [80]. The protein concentration was determined using a Roti-Quant reagent (Carl Roth GmbH & Co. KG, Karlsruhe, Germany).

Extraction of total proteins from isolated nuclei

HaCaT or HepG2 cells were collected by centrifugation at 3100 ***g*** for 2 min and washed in ice-cold NaCl/P_i at 4 °C. The cell pellets were lysed in TITE buffer (50 mM Tris/HCl, pH 8.0, 100 mM NaCl, 0.2% Triton X-100) by incubation for 5 min on ice, and disrupted by vortexing. The resulting cytoplasmic and nuclear suspension was layered over a cushion (10% sucrose in TITE buffer) and centrifuged at 800 ***g*** for 10 min at 4 °C. The supernatant was removed without disrupting the nuclear pellet, and the nuclei were resuspended in lysis buffer [46]. The protein concentration was determined using a Roti-Quant reagent (Roth).

Western blot analysis

Proteins were extracted from subconfluent, confluent or 1.5×10^6 untreated and $VOSO_4$-treated HaCaT or HepG2 cells plated 24 h prior to treatment, as previously described [29], and the protein concentration was determined using a Roti-Quant reagent (Roth). Protein samples were analyzed by SDS/PAGE followed by immunoblotting. Antibodies against cyclin D1 (sc-20044), cyclin E (sc-481), E2F1 (sc-251), $p21^{Cip1/Waf1}$ (sc-817, sc-6246), Bcl-2 (sc-509), $p27^{Kip1}$ (sc-1641, sc-528), c-fos (sc-7202) and CLU (sc-6419) were from Santa Cruz Biotech (CA, USA). Antibodies against $p21^{Cip1/Waf1}$ (OP64), PARP1 (CII-10), β-actin (A5441) and Bax (A3533) were from Calbiochem/MERCK (Athens, Greece), BD Biosciences (Transduction Laboratories; Athens, Greece), Sigma-Aldrich Ltd (Athens, Greece) and Dako/Kordopatis Ltd (Athens, Greece), respectively. Horseradish peroxidase-conjugated secondary antibodies were purchased from Santa Cruz Biotechnology (Heidelberg, Germany). Antibody binding was detected by using the ECL detection kit (GE HealthCare, Athens, Greece).

Isolation of total RNA and RT-PCR and northern blot hybridization analysis

Isolation of total RNA and RT-PCR were performed using cultured cells as described previously [81]. One hundred nanograms of the cDNA was amplified in a 50 μL reaction volume using the following primers (Invitrogen); human CLU forward and reverse primers have been described previously [57], and produce an amplicon of 118 bp; and human glyceraldehyde-3-phosphate (GAPDH) (reference/normalization control), forward (5′-TGGTATCGTGGAAGGACTCA-3′) and reverse (5′-GCAGGGATGATGTTCTGGA-3′), producing an amplicon of 126 bp. The PCR reaction conditions were as follows: one cycle of 95 °C (2 min) and 72 °C (2 min); 35 cycles of 95 °C (1 min), 53 °C (1 min) and 72 °C (1.5 min); and one cycle of 72 °C for 5 min. The reaction products were analyzed on a 1.5% agarose gel stained with ethidium bromide.

Northern blotting was performed essentially as previously described [81,82], using a ^{32}P-labeled rat *c-fos* cDNA fragment of β-actin [82].

Acknowledgements

We thank M. Piechaczyk [Institut de Génétique Moléculaire de Montpellier (IGMM), CNRS, Univer-

site de Montpellier II, France] for kindly providing the human *c-fos* retroviral vector (PM43.1). We also thank P. Pappas (Department of Pharmacology, University of Ioannina Medical School) for critical comments. This research work was funded by grants from the Research Committee of the University of Ioannina and the Empeirikeion Foundation, Athens, Greece to E. Kolettas, and was also partially supported by DOE grant DE-FG-022179-16-18 to D. A. Boothman.

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Supporting information

The following supplementary material is available:
Fig. S1. Effects of c-fos on HaCaT cell proliferation and CLU expression.
Fig. S2. Effects of $VOSO_4$ on IL-6 expression in HaCaT cells.
Doc. S1. Construction of growth curves and isolation of total RNA and RT-PCR for the expression of IL-6.

This supplementary material can be found in the online version of this article.

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