Downregulation of Krüppel-like factor 6 expression modulates extravillous trophoblast cell behavior by increasing reactive oxygen species

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Abbreviations: ATB, penicillin-streptomycin; BrdU, bromodeoxyuridine; FBS, fetal bovine serum; H2DCFDA, 2',7'-dichlorodihydrofluorescein diacetate; HMOX1, hemoxygenase 1; JC-1, 5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide; KLF6, Krüppel-like factor 6; NOX4, NADPH oxidase 4; Nrf2, nuclear factor erythroid 2-related factor 2; MTT, 3-(4,5-dimethylthiazol-2yl)-2, 5-diphenyl tetrazolium bromide; PBS, phosphate-buffered saline; PPIA, peptidylprolyl isomerase A; qRT-PCR, quantitative real time-reverse transcription PCR; ROS, reactive oxygen species; SDS, sodium dodecyl sulphate; TBS, Tris buffered saline; siC, scrambled siRNA; siK, KLF6 siRNA; siK3, 3´UTR KLF6 siRNA; Δψm, mitochondrial membrane potential; 2-cys-PRX, 2-Cys peroxiredoxins.

Abstract

Introduction: Placental extravillous trophoblasts play a crucial role in the establishment of a healthy pregnancy. Reactive oxygen species (ROS) may contribute to their differentiation and function as mediators in signaling processes or might cause oxidative stress resulting in trophoblast dysfunction. The krüppel-like transcription factor 6 (KLF6) regulates many genes involved in essential cell processes where ROS are also involved. However, whether KLF6 regulates ROS levels has not been previously investigated. **Materials and Methods**: KLF6 was silenced by siRNAs in HTR8-SV/neo cells, an extravillous trophoblast model. Total and mitochondrial ROS levels, as well as mitochondrial membrane potential and apoptosis were analyzed by flow cytometry. The expression of genes and proteins of interest were analyzed by qRT-PCR and Western blot, respectively. Cell response to oxidative stress, proliferation, viability, morphology, and migration were evaluated. **Results**: KLF6 downregulation led to an increase in ROS and NOX4 mRNA levels, accompanied by reduced cell proliferation and increased p21 protein expression. Catalase activity, 2-Cys peroxiredoxin protein levels, Nrf2 cytoplasmic localization and *hemoxygenase 1* expression, as well as mitochondrial membrane potential and cell apoptosis were not altered suggesting that ROS increase is not associated with cellular damage. Instead, KLF6 silencing induced cytoskeleton modifications and increased cell migration in a ROS-dependent manner. **Discussion**: Present data reveal a novel role of KLF6 on ROS balance and signaling demonstrating that KLF6 downregulation induces an increase in ROS levels that contribute to extravillous trophoblast cell migration.

Introduction

Cells employ several mechanisms to keep reactive oxygen species (ROS) in a physiological range. In this condition, ROS behave as signaling molecules regulating multiple cellular processes such as proliferation, differentiation, programmed cell death, immune responses, adhesion, and migration, among others [1,2]. However, when ROS are overproduced, or their elimination is reduced, these multifaceted molecules contribute to the pathogenesis of many human diseases through oxidative damage [3,4]. On the other hand, if their levels are too low, they can also impair cell fitness due to a reductive stress [5]. The mitochondria electron transport chain and the NADPH-oxidase (NOX) enzymes are two major sources of ROS at the cellular level [1]. The NOX family includes seven transmembrane enzyme complexes, NOXs 1-5 and DUOX 1 and 2, which have primarily evolved to produce ROS [6,7]. NOX4 differs from the other family members since it is mostly regulated at the level of gene expression, and it mainly synthesizes H_2O_2 instead of superoxide [8].

Throughout development, the placenta and its specialized cells: the trophoblasts, face important modifications in ROS levels and require an efficient redox system to maintain a homeostatic range of ROS for normal trophoblast proliferation, invasion, and angiogenesis [9,10]. During placental development, extravillous trophoblasts acquire an invasive and migratory phenotype to anchor the placenta to the uterus. Alterations in these processes are associated to pregnancy pathologies with abnormal redox homeostasis such as preeclampsia and intrauterine growth restriction [11–13].

Krüppel-like factor 6 (KLF6) is a ubiquitous zinc finger transcription factor highly expressed in placenta [14]. It is a developmental essential gene since *Klf6*$^{-/-}$ mice die by embryonic day 12.5 with a phenotype that includes impaired placental development [15]. Although it has been mainly studied as a tumor suppressor gene, it is involved in multiple cellular processes and its activity depends on the cellular context [16]. KLF6 participates in cell cycle progression, apoptosis, angiogenesis, vascular remodeling, and senescence [17–22], as well as in preadipocyte,

macrophage, and villous trophoblast differentiation [23–28]. Interestingly, the contribution of ROS has been recognized in most of the cellular processes mentioned above, as well as in normal and pathological placental development [9,12]. Moreover, low oxygen tension induces an early and transient increase of KLF6 expression in trophoblast cells [29], suggesting that it could be involved in redox balance. Nevertheless, the role of KLF6 on ROS balance has been underexplored. In addition, it has been recently demonstrated that a sub-lethal dose of H_2O_2 increases extravillous trophoblast HTR-8/SVneo cell migration [30] and that downregulation of KLF6 expression facilitates extravillous trophoblast cell differentiation towards a mesenchymal phenotype with increased polarized cell migration [31]. However, whether KLF6 may regulate cell migration through ROS signaling remained unknown.

Herein, we show that downregulation of KLF6 expression leads to an increase in ROS levels in HTR-8/SVneo without compromising cell viability. Instead, cell proliferation was decreased, and cell morphology was modified towards a migratory ROS-dependent profile. In addition, KLF6 silencing led to an increase in NOX4 transcript levels, suggesting that it could be the ROS-generating source. Collectively, present results reveal a novel function for the multifaceted KLF6 transcription factor in ROS-dependent cellular signaling.

Materials and methods

Cell culture

The extravillous trophoblast-derived HTR-8/SVneo cell line was cultured in DMEM-F12 with 10% v/v fetal bovine serum (FBS) and 100 µg/mL penicillin-streptomycin (ATB). HTR-8/SVneo cells were seeded at a density of 1×10^5, 5×10^4, or 8×10^3 cells/well in a 6-, 12-, or 96-multiwell plates, respectively, with complete medium. When required, cells were treated with 10 mM N-acetyl-cysteine (NAC) (Sigma) or 100 µM tempol (Sigma) for 24 h.

Small interfering RNA (siRNA) transfections

For KLF6 silencing purposes, cells were seeded and 24 h later were transfected with 25 nM of siRNAs targeting KLF6 or with the negative control scrambled siRNA (siC) (Silencer Negative™, Applied Biosystems/Ambion), using the JetPrime Transfection Reagent (Polyplus-transfection) or RNAiMAX transfection reagent (Invitrogen), according to the manufacturers' protocols. Two specific KLF6 siRNAs were used: siK (siRNA selected™, Ambion ID no. 9700; targeting the coding mRNA sequence 5'- GGAAGATCTGTGGACCAAA -3´) and siK3 (targeting the 3´UTR mRNA sequence 5'- ACAAAGACATAGAGTGAAA -3´). Mock transfection control was performed treating cells only with the transfection reagent.

Analysis of ROS, mitochondrial membrane potential (Δψm), and apoptosis

Forty-eight hours after siRNA or mock transfection, cells were washed twice with phosphate-buffered saline (PBS) and incubated with the following dyes and concentrations in phenol red-free DMEM without FBS and ATB: 10 μM of the cell-permeant 2',7'-dichlorodihydrofluorescein diacetate (H2DCFDA, Thermo Fisher) for total ROS detection, 5 μM MitoSOX (Invitrogen) for mitochondrial ROS detection, or 2 μM of 5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide (JC-1, Santa Cruz) for mitochondrial membrane potential measurement. Incubations were carried out for 45, 30, or 15 minutes, respectively. Afterwards, cells were washed twice with PBS, trypsinized, inactivated with DMEM without phenol red, washed with PBS, resuspended in 100 μL PBS and kept on ice until flow cytometric analysis. At least 30,000 total events per sample were acquired using the Life Technologies Attune NxT or the BD FACSCanto II cytometer. Samples for autofluorescence control were processed as described above except that the addition of the fluorescent probes was omitted. Apoptotic cells were detected using Annexin V Apoptosis Detection Kit according to the manufacturer's protocol (eBioscience Cat.: 88-8102-72). After subjecting the cells to a similar process than the described for H2DCFDA, MitoSOX, and JC1, Annexin V-PE/ 7-AAD signals were acquired by flow cytometry.

Western blot analysis

Total protein samples were separated by SDS-PAGE on 10 or 14% gels, electrotransferred to nitrocellulose membranes (Hybond-ECL, Amersham Bioscience), and incubated with primary antibodies as previously described [26]. The following antibodies were used: mouse monoclonal anti-KLF6 (1:500; clone 2c11, previously characterized [32]), mouse monoclonal anti-α-tubulin (1:5000; Clone B-5-1-2 Sigma-Aldrich), rabbit polyclonal anti-catalase (1:3000; ab52477 Abcam), mouse monoclonal anti-2-cys-peroxiredoxin (1:1000; ab16765, Abcam), rabbit monoclonal anti-$p21^{Waf1/Cip1}$ (p21) (1:1000; cat. no: 2947, Cell Signaling Technology), rabbit polyclonal anti-p53 (1:1000; sc-6243, Santa Cruz), mouse monoclonal anti-phosphorylated-S15-p53 (1:1000; cat. no: 9286, Cell Signaling Technology). After washing, blots were incubated with IRDye 800CW donkey anti-rabbit or IRDye 680RD donkey anti-mouse IgG antibodies (1:10000; cat no: 926-32213 and 926-68072, Li-Cor Biosciences) in TBS (25 mM Tris, 150 mM NaCl, 2 mM KCl, pH 7.4) for 1 h. After washing with TBS plus 0.1% Tween-20, membranes were visualized and quantified using the Odyssey Infrared Imaging System (Li-Cor Biosciences). Protein expression was normalized to the α-tubulin loading control.

Quantitative real time-reverse transcription PCR (qRT-PCR)

Total RNA was extracted from cultured cells using Trizol Reagent (Invitrogen), according to the manufacturer's instructions. All samples were treated with RQ1 RNase-Free DNase (Promega) to remove trace amounts of genomic DNA. cDNA was synthesized with random primers (Invitrogen) and M-MLV reverse transcriptase (Promega) as previously described [27]. Transcripts for *NADPH oxidase 4* (*NOX4*), *hemoxygenase 1* (*HMOX1*), and *peptidylprolyl isomerase A* (*PPIA*), as the reference gene, were quantified by real time qRT-PCR as previously described [27]. Primer sequences and concentrations are listed in Table 1.

Table 1. Primer sequences and concentrations used in qRT-PCR.

Gene	primer	Sequence 5´-3´	nM
PPIA	sense	GTTTTGCAGACGCCACCG	100
PPIA	antisense	GCAAACAGCTCAAAGGAGACG	100
HMOX1	sense	AGGCCAAGACTGCGTTCC	100
HMOX1	antisense	GCAGAATCTTGCACTTTGTTGCT	100
NOX4	sense	AGTCCTTCCGTTGGTTTG	400
NOX4	antisense	AAAGTTTCCACCGAGGACG	400

Immunofluorescence assays

Immunofluorescence assays were performed on transfected and non-transfected HTR-8/SVneo cells as previously described [27] with the following modifications: samples for p21 immunodetection were blocked with 2.5% v/v normal goat serum in 0.2% v/v Tween-20 in PBS for 15 minutes followed by 15 minutes with 5% v/v bovine serum albumin in PBS plus 0.2% v/v Tween-20 for 45 minutes; samples were incubated overnight at 4 °C with rabbit polyclonal anti-Nrf2 (1:50; sc-722, Santa Cruz) or with rabbit monoclonal anti-p21 (1:800; cat. no: 2947, Cell Signaling Technology); and Alexa Fluor 488-conjugated goat anti-rabbit IgG (1:720; A-11070, ThermoFisher) was used as secondary antibody. Samples were visualized on a Leica DMi8 optical microscope (Leica microsystems). Negative controls without primary antibody incubation were included (data not shown). Non-transfected cells were treated with 100 μM H_2O_2 for 24 h and used as a positive Nrf2 nuclear translocation control.

Cell proliferation and viability assays

Forty-two hours after transfection, cells were exposed to 20 μM bromodeoxyuridine (BrdU, B5002, Sigma-Aldrich) for 6 h to evaluate cell proliferation. BrdU incorporation assay and percentage of BrdU positive nuclei respect to total nuclei were performed as previously described [33].

Cell viability was measured by the 3-(4,5-dimethylthiazol-2yl)-2, 5-diphenyltetrazolium bromide (MTT) assay as previously described [34]. Results were expressed as percentage of cell viability relative to the control. Three independent experiments were conducted in triplicate.

Catalase enzyme activity

Catalase activity was assayed by the UV spectrophotometric method monitoring the continuous decrease in H_2O_2 absorbance at 240 nm (ε= 40 mM^{-1} cm^{-1}) as previously described [29].

Cell morphology characterization

Cell morphology was analyzed by phase-contrast images recorded at 10X with Leica DMi8 optical microscope (Leica microsystems). The shape was quantified using ImageJ software, where the cell aspect ratio was calculated by dividing the length of a cell's major axis by the width of the minor axis as described [35].

Cell migration

HTR-8/SVneo cells (3 x 10^5 cells/well) were plated in 6 multiwell plates and 24 h later were transfected with siRNAs. The next day they were treated with 10 mM NAC, or 100 µM tempol, or remained untreated. Twenty-four hours later, wound assays were performed for which an area of the wells was scraped with a tip. Phase-contrast images were recorded at 10X with Leica DMi8 optical microscope (Leica microsystems) at 0, 4, and 8 h after wounding. The migration area was calculated as the difference between the area remaining at 4 or 8 h and the initial one at 0 h using ImageJ software and expressed as the percentage of closed area.

Statistical analysis

For multiple comparisons of independent samples, one-way analysis of variance (ANOVA) or Kruskal Wallis test with a subsequent Tukey or Dunn's multiple comparison test, respectively, was performed. The one-sample t-test was used to determine whether values were significantly different from the control value set as 1. Comparisons between two groups were performed using the parametric Student's t-test or the nonparametric Mann-Whitney test. Statistical analyses were performed using the GraphPad Prism 5.0 software. A probability value of $p < 0.05$ was considered statistically significant.

Results

KLF6 downregulation increases ROS levels

We have previously reported that KLF6 expression is transiently upregulated by hypoxia in human term placenta and in the extravillous trophoblast-derived HTR-8/SVneo cell line [29], suggesting that it could be involved in redox balance. To explore whether KLF6 modulates redox homeostasis we evaluated ROS levels in HTR-8/SVneo cells transfected with siK, siK3, siC or mock-transfected cells. Successful KLF6 silencing in siK and siK3 cells compared to siC cells was confirmed by Western blotting, and low KLF6 levels were maintained for at least 72 h (Fig. 1A). Since siK showed the highest inhibition efficiency most of the experiments were performed with this siRNA. Flow cytometry analysis using the H2DCFDA probe revealed a significant increase in intracellular ROS production in siK and siK3 cells as compared to siC cells (Fig. 1B). Mean fluorescence intensity was 2.25 ± 0.29 (mean ± SEM) times higher in siK cells than in mock transfected cells; and there was no statistical difference between siC and mock transfected cells, confirming that the difference observed in ROS levels was not due to a reduction of ROS in siC cells (Fig. 1C).

As mentioned, the mitochondrial electron transport chain and the NOX enzymes are the major endogenous source of ROS, and NOX4 activity is mainly regulated at the transcriptional level [1,36]. Thus, in the quest to determine the origin of ROS, we analyzed mitochondrial ROS

through MitoSOX flow cytometry measurement and NOX4 mRNA levels through qRT-PCR. Similar MitoSox mean fluorescence intensity was detected in KLF6-silenced and siC cells (Fig. 1D). Instead, NOX4 mRNA levels were clearly increased in siK compared to siC cells (Fig. 1E), suggesting that this could be the source of ROS generated because of KLF6 silencing.

ROS increase mediated by KLF6 downregulation does not induce an oxidative stress response

When ROS rise over the homeostatic level, cells convert them to other less reactive and harmful species. Catalase and 2-Cys peroxiredoxins (2-cys-PRX) are ones of the antioxidant enzymes that contribute to support redox homeostasis [37]. In addition, PRX-2 is involved in ROS balance in the placenta and its downregulation compromises trophoblast viability [38]. Therefore, we evaluated whether KLF6 depletion alters the expression of these enzymes. As shown in Fig 2, no differences were detected in catalase and 2-cys-PRX levels between siC and siK HTR-8/SVneo cells 48 or even 72 h post-transfection (Figs. 2A & B). In addition, catalase activity also remained unmodified (Fig. 2C).

Nrf2 transcription factor is a master regulator of the cellular response to oxidative stress [39]. In the presence of an excess of ROS, Nrf2 is released from its cytoplasmic degradation complex allowing it to accumulate in the nucleus and to activate the transcription of target genes such as *HMOX1* (alias HO-1) to restore redox homeostasis [39]. Nrf2 nuclear accumulation/translocation and *HMOX1* transcription are clear markers of the activation of this pathway [40]. As shown in figure 2D, the *HMOX1* mRNA level was not increased in siK compared to siC cells, suggesting that the Nrf2 pathway was not activated. Accordingly, the immunofluorescence staining pattern of Nrf2 was indistinguishable between siC and siK cells (Fig. 2E), although a clear nuclear signal was observed in non-transfected HTR-8/SVneo cells treated with 100 µM H_2O_2, demonstrating that these cells can activate the Nrf2 pathway in response to a well-known stressor [41]. Moreover, it has been previously demonstrated that the HTR-8/SVneo

cells increase *HMOX1* expression when treated with 100 μM H_2O_2 [42]. Altogether, present results suggest that the increase in ROS levels after KLF6 downregulation does not elicit an oxidative stress response.

Proliferation is reduced in KLF6-silenced HTR-8/SVneo cells without cell viability alteration

Since an imbalance in ROS levels may trigger cellular damage leading to apoptosis and cell death, we analyzed the impact of ROS induced by KLF6-silencing on cellular homeostasis. To this end, we evaluated cell viability, Δψm, apoptosis, and cell proliferation. One of the early events that characterize some cellular apoptosis systems is a reduction in the Δψm [43]. Analysis of Δψm by flow cytometry assays using the JC-1 dye revealed similar percentage of cells with a high red signal in siK, siC as well as in non-transfected HTR-8/SVneo cells (wild type), suggestive of healthy mitochondria (Figs. 3A & B). In addition, cell viability was not compromised as revealed by MTT assays performed in siK, siC, and mock-transfected cells 72 h post-transfection (Fig. 3C). Flow cytometry analysis with 7-AAD and Annexin V-PE staining further confirmed that apoptosis was not induced in KLF6-silenced cells (Figs. 3D & E).

Remarkably, cell proliferation was reduced in siK compared to siC cells as measured by BrdU incorporation into DNA 48 h post-transfection (Figs. 4A & B). Accordingly, p21 protein level was increased in KLF6-silenced cells without a significant increase in p53 protein level and its Ser-15 phosphorylation status (Figs. 4C & D). Increased expression of p21 in the siK cell population was also proven by immunofluorescence assays (Figs. 4E & F). Thus, downregulation of KLF6 in HTR-8/SVneo cells does not alter cell viability but reduces the proliferation rate and induces p21 expression in a p53-independent manner.

KLF6 downregulation modifies HTR-8/SVneo cell morphology in a ROS-dependent manner

It is well documented that ROS can modulate cytoskeleton dynamics and thus affect cell morphology, fate and migration [44,45]. Therefore, we analyzed cell morphology in siK cells treated or not with NAC, a powerful antioxidant that can scavenge ROS to suppress its activity, and with tempol, a potent radical scavenger and superoxide dismutase mimetic drug. Twenty-four hours after siRNA transfection, cells were cultured in the presence or not of 10 mM NAC or 100 µM tempol for an additional 24 h. KLF6-silenced HTR-8/SVneo cells cultured without treatments became more elongated acquiring a fibroblastic appearance with long extensions that seemed to connect cells between each other. Notably, cellular enlargements were reduced by treatment with either antioxidant (Fig. 5A). To further confirm this, cell aspect ratio was calculated by dividing the length of a cell's major axis by the width of the minor axis, as described [35]. Quantification proved an increase in the cell aspect ratio of siK compared to siC cells, while treatment with NAC or tempol reduced siK cell´s ratio to values similar to those observed in siC cells (Fig. 5B), indicating that ROS are implicated in the phenotypic change observed upon KLF6 silencing.

Antioxidant treatments reduce cell migration induced by KLF6 silencing

Since KLF6 downregulation induced a ROS-dependent phenotypic change and, as previously demonstrated, it promoted HTR-8/SVneo cell migration [31], we evaluated whether the increase in cell migration after KLF6-silencing was also ROS-dependent. To this end, wound healing assays were performed in siC, siK and siK cells treated with NAC or tempol for the last 24 h, and the remaining area was evaluated at 4 and 8 h post-scratching. As shown in figure 6 the increase in cell migration observed in response to KLF6 silencing was abrogated with either NAC- or tempol-antioxidant treatment, returning migration to levels like those observed in siC cells. These results indicate that ROS are involved in the increase in cell migration induced upon KLF6 downregulation.

Discussion

The results of this study demonstrate that KLF6 downregulation in HTR-8/SVneo cells leads to an increase in ROS levels which contribute to a morphological switch and an increase in cell migration. ROS were initially considered just as byproducts of normal cellular oxidative processes with the potential to cause damage. However, a large body of evidence has shown that they are important signal molecules involved in many pathways in diverse cellular contexts [1,46,47]. ROS levels achieved by KLF6 silencing neither affected cell viability nor induced cell apoptosis, suggesting they are not detrimental to the cells. In addition, the Nrf2 pathway was not activated, and the antioxidant enzymes catalase and 2-cys-PRX remained unchanged in KLF6-silenced cells. These results strongly suggest that the elevation in ROS levels were below the threshold that invokes HTR-8/SVneo cell damage, therefore an antioxidant response was not activated.

The mitochondrial electron transport chain is an important supplier of endogenous ROS; in addition, the mitochondria can also be the target of oxidative stress causing mitochondrial dysfunction [48]. However, KLF6 downregulation did not increase mitochondrial ROS levels neither reduced the percentage of cells with high $\Delta\psi m$, suggesting that ROS increase is not generated at the mitochondria, and its function is not impaired in HTR-8/SVneo cells when KLF6 expression is reduced. Instead, KLF6 silencing increased NOX4 mRNA expression implying it as the source of ROS. Interestingly, the human *NOX4* promoter (from -1000 to 100 bp relative to the transcription start site) retrieved from the Eukaryotic Promoter Database [49] and analyzed with the JASPAR database [50], revealed two putative KLF6 binding sites with high scores near the transcription start site (*ccagacgccca*, in the negative strand, and *gaccccgcccg*, in the positive strand with relative scores greater than 0.85). Therefore, present results are compatible with a role of KLF6 as a potential transcriptional repressor of NOX4. Further studies should determine whether KLF6 is a direct or indirect transcriptional regulator of *NOX4.* Although NOX4 mainly synthesizes H_2O_2, we cannot rule out the increase in other ROS since the assay performed herein detect not only H_2O_2, but also other ROS and it may even detect reactive nitrogen species

[51,52]. However, in our model nitrites were undetectable (data not shown) suggesting that they may not be relevant to the observed effects.

A wide number of studies have reported ROS as important signal molecules associated with multiple cellular processes such as epithelial-mesenchymal transition (EMT), cell migration, and neuronal differentiation [53–57]. Particularly, it has been demonstrated that ROS derived from the NOX family enzymes are involved in cell migration, differentiation, and cytoskeletal organization during EMT [58,59]. On the other hand, experimental evidence supports the notion that trophoblast differentiation into the invasive pathway involves the activation of p21 and an EMT-like process [60,61]. Herein, we show that KLF6 downregulation in the extravillous trophoblast cell line HTR-8/SVneo increased the expression of p21 and NOX4, reduced cell proliferation, and enhanced cell fibroblastoid phenotype associated with an increased cellular migratory capacity in a ROS-dependent manner. This is in line with our previous results demonstrating that extravillous trophoblast cell migration and mesenchymal phenotype were induced upon KLF6 downregulation, and a lower KLF6 immunostaining was detected in abnormally invasive placentas compared to normal placentas [31]. By contrast, in the placental bed of preeclamptic pregnancies, where trophoblast invasion is deficient, a higher KLF6 expression was observed compared to the placental bed of uncomplicated pregnancies [29]. Therefore, downregulation of KLF6 may aid in trophoblast differentiation into the invasive pathway through the upregulation of ROS levels.

The increase in p21 in KLF6-silenced HTR-8/SVneo cells was rather unexpected since KLF6 is mainly known as a direct transcriptional activator of p21 expression [62]. In addition, a positive association between KLF6, p21 upregulation, and reduced cell proliferation has been documented in several cell contexts including placental cytotrophoblasts differentiating into the villous pathway [28,63]. However, it has also been reported that KLF6 knockdown impaired cell proliferation in other experimental systems [18,19]. Present results reinforce the fact that KLF6 role and outcome on cell physiopathology depends on the cellular context, as it has been

previously reported [16]. In addition, p21 increase might be an indirect consequence of KLF6 downregulation. In this sense, it has been reported that NOX4 negatively controls the proliferation of liver tumor and untransformed cells and the mechanism proposed includes the upregulation of p21 [64].

KLF6 has been described as an upregulated gene under diverse stress conditions associated with excessive ROS accumulation such as hepatic stellate cell activation, renal ischemia/ reperfusion, and H_2O_2 treatment of neonatal rat cardiac myocytes or bovine granulosa cells [65–69]. Furthermore, podocyte-specific loss of KLF6 in a murine model of diabetic kidney disease and shRNA-mediated KLF6 knockdown in human podocytes increased the susceptibility to mitochondrial injury caused by exposure to adriamycin or streptozotocin [70,71]. In addition, a 1.6-fold increase in KLF6 expression was found in the transcriptome of placentas delivered by labor longer than 15 hours compared with those delivered by cesarean section, in correlation with an increase in oxidative markers in the first group [72]. Thus, it can be proposed that under conditions of oxidative stress KLF6 expression is activated and cellular fitness is threatened if its expression is impaired; conversely, under a eustress condition KLF6 downregulation leads to an increase in ROS levels that contribute to cell differentiation. Altogether our data highlight KLF6 as a new player in cellular redox homeostasis and signaling in extravillous trophoblast cells.

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Author contributions

LTK, GMP-D: designed the research. LTK, ALM, ACR: performed experiments. All authors analyzed the data. CC, GMP-D, SG-R: contributed reagents and resources. LTK, ACR, GMP-D, SG-R: wrote the manuscript. All authors contributed to the discussion and revised the final version.

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Figure captions

Fig. 1. KLF6 silencing in HTR-8/SVneo cells increases ROS levels. (**A**) Representative immunoblots of KLF6 and α-tubulin in protein extracts from siC, siK, and siK3 cells 48 or 72 h post-transfection. KLF6 protein quantification normalized to α-tubulin expressed relative to the corresponding normalized protein level in siC cells. Mean ± SEM of at least 3 independent experiments. (**B**) ROS detection by flow cytometry using the H2DCFDA dye 48 h post-transfection in siC, siK, and siK3 cells. Left panel shows the histograms of one representative experiment; right panel shows ROS levels expressed as mean fluorescence intensity relative to siC of at least 3 independent experiments (mean ± SEM). NC: cells without H2DCFDA. (**C**) ROS levels in mock transfected (MT), siC, and siK cells expressed as mean fluorescence intensity relative to MT of at least 3 independent experiments (mean ± SEM). (**D**) Mitochondrial ROS detection by flow cytometry using the MitoSOX dye. Left panel shows the histograms of one representative experiment; right panel shows mitochondrial ROS levels expressed as mean fluorescence intensity relative to siC of 3 independent experiments (mean ± SEM). NC: cells without MitoSOX. (**E**) *NOX4* mRNA level normalized to *PPIA* mRNA in siK and siC cells determined by qRT-PCR 48 h post-transfection and expressed relative to siC. Statistical significance was evaluated by ANOVA with a Tukey's Multiple Comparison post-test (**A**, **B**, **C**) and one-sample t-test (**D**, **E**). ***p<0.001, **p<0.01, *p<0.05, ns: not significant.

Fig. 2. ROS increase mediated by KLF6 downregulation does not induce an oxidative stress response. (**A**) Western blot analysis for catalase, 2-cys-PRX, and α-tubulin, assayed in protein extracts from siC and siK cells obtained 48 or 72 h post-transfection. (**B**) Catalase and 2-cys-PRX protein level quantification in siK cells normalized to α-tubulin and expressed relative to the corresponding normalized protein levels in siC cells defined as 1. Values are the mean ± SEM

of at least 5 independent experiments. (**C**) Relative catalase activity determined by H_2O_2 decomposition assay; mean ± SEM of 3 independent experiments. wt = wild type non-transfected cells. (**D**) *HMOX1* mRNA level normalized to *PPIA* mRNA in siK and siC cells determined by qRT-PCR 72 h post-transfection and expressed relative to the corresponding normalized mRNA level in siC, mean ± SEM of 3 independent experiments. (**E**) Immunostaining of Nrf2 (green) in HTR-8/SVneo cells treated with 100 µM H_2O_2 for 24 h (positive control of Nrf2 translocation, C+), and in siC and siK cells detected 48 h post-transfection. Nuclei stained with Hoechst dye (blue). Statistical significance *vs.* siC was evaluated by one-sample t-test (**B, D**) and ANOVA with a Tukey's Multiple Comparison post-test (**C**). ns: not significant.

Fig. 3. KLF6 silencing does not alter the percentage of cells with high Δψm, cell viability, and the apoptosis rate. (**A**) Representative flow cytometry plots for Δψm determined by JC-1 dye in siC, siK, and non-transfected (wt) cells, analyzed 48 h post-transfection. NC: negative labelling control. (**B**) Percentage of cells with high Δψm in siC and siK cells. Mean ± SEM of 3 independent experiments. (**C**) Cell viability determined through the MTT assay in MT (mock-transfected), siC, and siK HTR-8/SVneo cells after 72 h of transfection. Bar graph displays the media ± SEM of four independent assays. (**D**) Representative flow cytometry plots of cell apoptosis evaluated with the Annexin V-PE/7-AAD apoptosis detection kit 48 h post-transfection. (**E**) Relative apoptotic rate in siK and siC cells. Mean ± SEM of 3 independent experiments. Statistical significance was evaluated by Student´s t-test (**B**), ANOVA with a Tukey's Multiple Comparison post-test (**C**) and one-sample t-test (**E**). ns: not significant.

Fig. 4. KLF6 downregulation reduces cell proliferation and increases p21 expression. (**A**) Representative images of BrdU (red) incorporation into siC and siK cells immunodetected 48 h post-transfection. Nuclei stained with Hoechst dye (blue). (**B**) Percentage of BrdU-positive cells in each condition (mean ± SEM, n= at least 1600 cells per condition) of one representative of two independent experiments with equivalent results. (**C**) Western-blot analysis of p21, p53, phospho Ser15-p53 (p-p53), and α-tubulin in siC and siK cells assayed 48 and 72 h post-transfection. (**D**) p21, p53, and p-p53/p53 protein quantification in siK cells normalized to α-tubulin and expressed relative to the corresponding normalized protein level in siC cells defined as 1. Mean ± SEM of at least 4 independent experiments. (**E**) Representative images of p21 immunofluorescence (green) in siC and siK cells detected 72 h post-transfection. Nuclei stained with Hoechst dye (blue). (**F**) Percentage of p21 positive cells in each condition (median ± interquartile range, n= at least 4000 cells per condition). Statistical significance was evaluated by Student´s t-test (**B**), one-sample t-test (**D**), and Mann-Whitney test (**F**); ***p<0.001, **p<0.01, *p<0.05 *vs* siC.

Fig. 5. KLF6 silencing modifies HTR-8/SVneo cell morphology in a ROS-dependent manner. (**A**) Representative bright-field images of siC and siK cells, treated or not for 24 h with 10 mM NAC or 100 μM tempol, acquired 48 h post-transfection. Cell prolongations are indicated by arrows. (**B**) Cell aspect ratio calculated as the ratio between the major and the minor axis of the cell. One representative experiment of at least 3 independent experiments is shown (median ± interquartile range). Statistical significance was evaluated by Kruskal Wallis test with Dunn's Multiple Comparison post-test. ***p<0.001 *vs* siC, $^{###}$p<0.001 *vs* siK.

Fig. 6. Increased cell migration in KLF6 silenced cells is restored by antioxidant treatments. Wound healing assay in siC and siK cells after 48 h of transfection treated or not for the last 24 h with 10 mM NAC or 100 µM tempol. (**A**) Representative bright-field images of siC, siK, siK + NAC, and siK + tempol at 0, 4 and 8 h after wounding. (**B**) Percentage of wound closure at 4 and 8 h after scratch calculated from at least ten different positions in each condition. Results of one representative of three independent experiments with NAC and two with tempol are shown. Statistical significance was evaluated by ANOVA with a Tukey's Multiple Comparison post-test. ***$p<0.001$ *vs* siC, $^{###}p<0.001$ *vs* siK