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ORIGINAL PAPER

Mammalian Ste20-like protein kinase 3 mediates trophoblast apoptosis in spontaneous delivery

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Abstract The placenta is essential in transferring gases and nutrients from the mother to the developing fetus. Trophoblast apoptosis may cause labor or other pregnancy-related disorders. This study demonstrated the essential role of Mst3, a human Ste20-like protein kinase, in the oxidative stress-induced apoptosis of trophoblasts of term placenta in normal spontaneous delivery. Oxidative stress, but not hormones released during labor such as prostaglandin E_1, oxytocin or angiotensin II, induces the expression of Mst3 and apoptosis of human term placenta after elective Cesarean section without labor pain. The role of Mst3 in oxidative stress-induced apoptosis was further demonstrated in the 3A-sub-E, a human trophoblast cell line. The H_2O_2-induced apoptosis of 3A-sub-E cells was largely suppressed by overexpressed $Mst3^{KR}$, the kinase-dead mutant or by selective knockdown of endogenous Mst3. Further studies showed that Jun N-terminal kinase (JNK) may participate in the signaling pathway of H_2O_2-induced apoptosis by mediating the level of Mst3. Subsequently, caspase 3 and other downstream apoptotic components may be activated by Mst3 and trigger the apoptotic process in human trophoblasts.

Keywords Mst3 · Oxidative stress · Placenta · Trophoblasts · Apoptosis · Parturition

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Introduction

The placenta, a unique organ developed from embryonic tissue, is juxtaposed between fetal and maternal tissue. With its extensive villous or tree-like projections, it forms a direct link between mother and fetus [1–3]. On the surface of placental villi, a layer of multinucleated syncytiotrophoblasts in direct contact with maternal blood is essential for continuously supplying essential gases and nutrients to the developing fetus and removing metabolic waste. The multinucleated syncytiotrophoblast layer is maintained by a continuous fusion of proliferated cytotrophoblasts during pregnancy. The aged syncytiotrophoblast cells are replaced by younger populations of trophoblasts without affecting neighboring cells [2, 3]. The older material in the syncytiotrophoblast layer is then packed into a syncytial knot/sprout, undergoes apoptosis, and sheds into maternal circulation [2–4]. Apoptosis, characterized by cell shrinkage and nuclear condensation and fragmentation, is an intrinsic cellular mechanism essential for the development, remodeling and aging of placenta [4–8]. Apoptosis is also required in embryo development and cellular stress to maintain normal functions of tissues by removing injured or dysfunctional cells [9].

Trophoblast apoptosis is a highly regulated process triggered internally or externally by stimuli such as Fas/FasL [10, 11], TNF-α [12] and oxidative stress [13–16].

Upon stimulation, certain key pro-apoptotic proteins, such as Bax and Bak (members of Bcl-2 family protein) [17, 18] as well as caspases (Cys-dependent, Asp-specific proteases) [4, 19], may be activated or expressed. Caspase activation causes cleavage of structural proteins, anti-apoptotic factors and functional proteins essential for cell survival. Typically, the balance between cytotrophoblast proliferation and syncytiotrophoblast apoptosis is maintained throughout pregnancy. However, the rate of trophoblast apoptosis changes under normal physiological and pathological conditions. For example, increased trophoblast apoptosis is observed in normal spontaneous deliveries (NSDs) [6–8, 13, 20]. Trophoblast apoptosis may disrupt the physical connection between the placenta and the endometrial layer of the uterus during the final stage of gestation, enabling the uterus to naturally discharge the placenta after delivery [13]. Additionally, increased trophoblast apoptosis at term may signal the initiation of parturition. Dysregulation of trophoblast apoptosis, however, may result in pregnancy complications, such as preeclampsia and intrauterine growth retardation [21–23].

Recently, Mst3 (mammalian Ste20-like protein kinase 3) was identified in human placental tissue by Northern blotting [24] and further verified in this study by immunohistochemical study of human placental tissue from normal spontaneous delivery. The Mst3 is a member of the GCK-III subfamily of Ste20-like serine/threonine protein kinases with a molecular mass of 47.5 kDa [24–28]. This protein kinase is believed to mediate apoptosis in response to environmental cues [25–27]. The Mst3 protein kinase was recently found to phosphorylate NDR, a serine/threonine protein kinase, at Thr442 and may regulate cell cycle progression and cell morphology [25]. The Mst3 may also regulate the phosphorylation of paxillin and hence cell migration [26]. Moreover, overexpression of Mst3 causes the nuclear condensation and DNA fragmentation, characteristics of apoptosis, in several cell lines [27, 28]. Although an early study has indicated that Mst3 is largely present in human placenta and many other tissues [24], the biological and physiological function of Mst3 in human placenta remains unknown.

This study revealed for the first time the presence of Mst3 in human placental tissue from NSD but not in that of first trimester and term-with-no-labor Cesarean section. The presence of Mst3 in human placental tissues is associated with DNA fragmentation and nitrotyrosine formation, sign of oxidative stress. This suggests Mst3 has a role in regulating oxidative stress-induced trophoblast apoptosis in human term placenta. The importance of Mst3 in the oxidative stress-induced apoptosis of human trophoblasts was also demonstrated in explant culture and in the human trophoblast cell line 3A-sub-E.

Materials and methods

Materials

Restriction enzymes were from New England Bio-Labs. Fetal bovine serum (FBS), DMEM, penicillin G, streptomycin, and Lipofectamine were obtained from Life Technologies. Staurosporine was bought from Sigma. Oligonucleotides were synthesized by BioBasic Inc., UK. Other reagents used were reagent grade.

Histology and immunohistochemistry

Human placental specimens from first trimester placenta were obtained via uterine evacuations ($n = 3$), while samples of third trimester placenta were obtained immediately following NSD ($n = 3$) and term-of-no-labor Cesarean sections ($n = 3$). All samples were obtained with informed consent. The gestational stage of the first trimester samples ranged from 5 to 14 weeks, while that of the third trimester specimens ranged from 28 to 40 weeks. The 4 μm sections were prepared following the general protocol used in immunohistochemistry. Sections of placental specimens were first washed twice with 1× PBS, pH 7.4 and fixed with 3.7% formaldehyde under the room temperature for 25 min. After fixation, placental specimens were rinsed three times with 1× PBS and permeabilized with 20 μg/ml proteinase K in 1× PBS at 50°C for 20 min. After incubation, specimens were rinsed twice with 1× PBS, followed by equilibrating with 100 μl of equilibration buffer at room temperature for 10 min. The placental specimen was stained by hematoxylin and eosin and examined by a pathologist to confirm the histological preservation of the microanatomical structure and the absence of any disease in placental pathology.

The immunohistochemical study of human placental specimens was performed by incubating fixed sections with anti-Mst3 (1:100 dilution, Santa Cruz), anti-active caspase 3 (1:100 dilution, Santa Cruz) or anti-nitrotyrosine (1:100 dilution, Santa Cruz) antibodies for 2 h. Placental specimens were then washed once with PBST (0.13 M NaCl, 0.1 M NaH_2PO_4, 0.05% Tween 20) and further incubated with peroxidase-conjugated anti-rabbit or -mouse IgG (1:200 dilution, Santa Cruz). The activity of antibody-conjugated peroxidase was determined using diaminobenzidine (DAB) or 3-amino-9-ethylcarbazole (AEC) at room temperature for 10 min. Placental specimens were then washed with PBST, followed by washing with distilled, deionized H_2O (ddH_2O). Slides were then dried and sealed for microscopic analysis on a confocal laser scanning microscope (FV500, Olympus). Photoimages were

obtained using a digital camera (Olympus, F70) and processed by using the free PLUS software supplied with the camera.

The explant culture experiments were performed by using fresh placental tissues of term-of-no-labor Cesarean sections ($n = 2$). The placental specimens were first sectioned to a size of $2 \times 2 \times 0.3$ cm^3 and incubated in the absence or presence of 200 μM H_2O_2, 1 mIU/ml oxytocin, 1 ng/ml PGE_1, 10 μM angiotensin II or 100 μM dexamethosone for 6 h prior to histological and immunohistochemistry analysis. The placental specimens were immediately frozen at −80°C and fixed with 10% neutral formaldehyde for 12–16 h prior to paraffin embedding. The 4 μm sections were then prepared from paraffin embedded tissues. The human placental section was attached to the glass slide, incubated at 60°C for 20 min and then placed in xylin to deparaffin. After a series of soakings in 70%, 80%, 90% and 100% ethanol, the slides were put into 1× PBS, pH 7.4 for histological and immunohistochemistry analysis.

Cell lines and cell culture

Human normal placental trophoblast, SV40 transformed cell line (3A-sub-E) from American Type Culture Collection (ATCC # CRL-1584) was maintained in the α-MEM (GIBCO Life Technology) containing 10% fetal bovine serum (FBS) and 100 units/ml penicillin and 100 μg/ml streptomycin (GIBCO Life Technology). Cells were then incubated in a humidified 37°C incubation chamber containing 5% CO_2. The 3A-sub-E stable clones that contain pcDNA 3.0, pcDNA/$Mst3^{KR}$ and siRNA vector were maintained under 0.3 mg/ml G418-sulfate (Invitrogen).

Flow cytometry

The 3A-sub-E cells (2×10^6) in 100 mm dishes were incubated with or without 150 μM H_2O_2 in α-MEM containing 10% FBS at 37°C for the indicated time. After incubation, cells were rinsed, harvested and suspended in 1× PBS buffer. The suspended cells were then fixed with 95% ethanol for 16 h, followed by permeabilization with PBS containing 0.1% Triton X-100. After permeabilization, cells were rinsed twice with 1× PBS, followed by incubating with 50 μg/ml propidium iodide in 1× PBS for 30 min. Cells were then rinsed three times with PBS and gently resuspended in 1× PBS. The stained cells were analyzed on a fluorescence-activated cell sorter scan flow cytometer (CyFlow® SL, Partec).

TUNEL assay

The TUNEL (TdT-mediated, dUTP-incorporated Nick-End Labeling) staining of human placenta specimens were performed by using APO-$BrdU^{TM}$ TUNEL assay kit (Oncogen). After incubation, specimens were covered with the TUNEL mix (TdT, HRP-12-dUTP, and cobalt chloride) at 37°C for 1.5 h. A negative control was performed by omitting TdT; while DNase I-treated slides were used as the positive control. After incubating with HRP-12-dUTP, specimens were rinsed three times with 1× PBS and then incubated with 5 mg/ml diaminobenzidine (DAB) or 3-amino-9-ethylcarbazole (AEC) at room temperature for 10 min. The specimens were then washed twice with 1× PBS and once with ddH_2O before drying and mounting for the microscopic analysis.

The TUNEL assay of 3A-sub-E cells was carried out by using DeadEnd™ Fluorometric TUNEL System kit (Promega). 3A-sub-E cells were harvested by scraping from 100 mm dishes and washed once with 1× PBS. Cells were then re-suspended in 1× PBS buffer and fixed with 0.1% formaldehyde for 10 min. After fixation, cells were rinsed three times with 1× PBS and then suspended in 5 ml 70% ice-cold ethanol. After incubating at −20°C for at least 4 h, cells were then rinsed three times with PBS before equilibrating with 100 μl equilibration buffer (10 mM EDTA, 10 mM Tris–HCl pH 7.5, 10 mM DTT) at room temperature for 10 min. TUNEL staining was done by incubating cells with the TUNEL mix (TdT, FITC-12-dUTP, and cobalt chloride) at 37°C for 1 h. A negative control was performed by omitting TdT. DNase I-treated slides were used as the positive control. The reaction was terminated by adding 1 ml of 20 mM EDTA. Cells were then rinsed twice with 1× PBS and soaked in 1× PBS for flow cytometry analysis.

Western blotting

Cell lysates were prepared by incubating 3A-sub-E cells in a 100 mm culture plate with RIPA buffer (1% Triton X-100, 0.1% SDS, 50 mM Tris, pH 8.0, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Na-deoxycholate, 5 μg/ml leupeptin, and 5 μg/ml aprotinin) at room temperature for 15 min. Cell lysate was collected by scraping. The particulates were removed from cell lysates by centrifugation at 4°C and 14,000× rpm for 5 min. Cell lysate (40 μg each lane) was loaded onto 10% SDS-polyacrylamide electrophoresis gel (SDS-PAGE). Following electrophoresis, the protein bands were transferred to a polyvinylidine difluoride (Millipore) membrane by electroblotting. The Western blot analysis was performed using antibodies specifically against Mst3 (1:2000 dilution; Santa

Cruz), active caspase 3 (1:2000 dilution, Santa Cruz), p-JNK (phosphorylated form of c-Jun kinase) (1:2000 dilution, Biovision) and actin (1:2000 dilution; Santa Cruz). The protein bands were visualized by using ECL Chemiluminescence kit.

Selective knockdown of endogenous Mst3 by siRNA

The pNEO-siMst3 vector for the generation of siRNA of Mst3 (siMst3) was constructed using the *Sal*I–*Hin*dIII-digested pNEO vector [29, 30] and the complementary oligonucleotide pair 5′-TCGACGGCATTGACAATCGGA CTCTTCAAGAGAGAGTCCGATTGTCAATGCCTTTT TA-3′ and 5′-GCCGTAACTGTTAGCCTGAGAAGTTCT CTCTCAGGCTAACAGTTACGGAAAAATTCGA-3′ for siMst3 [25]. The target sequence of Mst3 was 5′-GGCATTGACAATCGGACTC-3′ (nucleotides 121–139 of Mst3 from the start codon). Cells were transfected with the pNEO vectors containing siRNA of targeting sequence by using Lipofectamine transfection reagent (Invitrogen). At least two stable clones were selected with G418. The efficacy of siRNA for Mst3 was determined by Western blotting with anti-Mst3 antibody to monitor the expression of Mst3 in 3A-sub-E stable clones.

Results

Immunohistochemical study of human placental tissue

The Mst3 protein kinase has been identified in human placenta by Northern blot analysis [24]. However, the distribution of Mst3 protein in human placental tissue at different stages of pregnancy is still unknown. Therefore, the expression of Mst3 in human placental tissue samples from first trimester, term-with-no-labor Cesarean section and term labor was determined by immunohistochemical analysis. As noted in previous studies [24], Mst3 was specifically expressed in both villous cytotrophoblasts and syncytiotrophoblasts of human term placental tissues obtained from NSD (Fig. 1a). However, Mst3 was not observed in the placental tissue of term-with-no-labor Cesarean section (Fig. 1e) or in the fetal membrane of the first trimester (Fig. 1i). Human placenta trophoblasts are known to undergo apoptosis during parturition [7, 8, 13, 20]. Therefore, this study postulated that Mst3 may trigger trophoblast apoptosis during labor. Therefore, DNA fragmentation was investigated in human placental tissue by TUNEL assay. A clear indication of nicked ends, indicative of DNA fragmentation, was observed in the cytotrophoblasts of human term placenta from NSD (Fig. 1b). Conversely, mild or no staining was observed in trophoblasts of human placental tissues from term-with-no-labor Cesarean section (Fig. 1f) and fetal membrane of the first trimester (Fig. 1j). In the negative control, the primary antibody or TdT enzyme was replaced by PBS buffer displayed no positive staining in any of the placental specimens (data not shown). These results indicate that trophoblast apoptosis in human placenta from NSD may be correlated with the increased Mst3 level in these cells.

Recent studies have postulated that inflammation and oxidative stress play a central role in inducing trophoblast apoptosis [13–15]. Further, increased oxidative stress was also observed during labor [31–35]. Therefore, nitrotryosine, an indicator of oxidative stress, was measured in placental tissue. Notably, AEC staining was clearly visible in cytotrophoblasts of human term placenta from NSD (Fig. 1c) but was absent in trophoblasts of human placental tissue from term-with-no-labor Cesarean section (Fig. 1g) and fetal membrane of the first trimester (Fig. 1k). This finding suggests that oxidative stress occurs mainly in the trophoblasts of human term placenta from NSD, in which apoptosis may also be triggered, presumably through elevation of endogenous Mst3.

The caspases, particularly caspase-3, are key players in the process of apoptosis by cleaving and destroying various downstream proteins essential for cell survival [36–38]. Hence, activated caspase-3, an important mediator of cell apoptosis, was studied by using antibody specifically against active caspase-3. Similar to the results observed in studies of Mst3 expression and DNA fragmentation, active caspase-3 was also observed in the cytotrophoblasts of human term placenta from NSD (Fig. 1d). However, human term placenta obtained from term-with-no-labor Cesarean section (Fig. 1h) and the fetal membrane of first trimester (Fig. 1l) exhibited little or no active caspase-3. Similar results, such as Mst3 expression, DNA fragmentation, nitrotyrosin formation and caspase 3 activation in the villous syncytiotrophoblasts of human placenta from NSD were also observed (data not shown). These experimental results suggest that apoptosis of placental trophoblasts occurs at full term, whereas placental cells in pre-term and first-trimester placenta remain intact. This finding is consistent with the observation that trophoblasts in the chorion laeve tissue of human fetal membrane undergo apoptosis at the final phase of pregnancy. Thus, trophoblast apoptosis may play an important role in spontaneous disruption of the fetal membrane tissue during the final stage of gestation.

Expression of Mst3 in human placental tissue is induced by oxidative stress

Previous study demonstrated the elevation of Mst3 level, trophoblast apoptosis and oxidative stress occurred only in

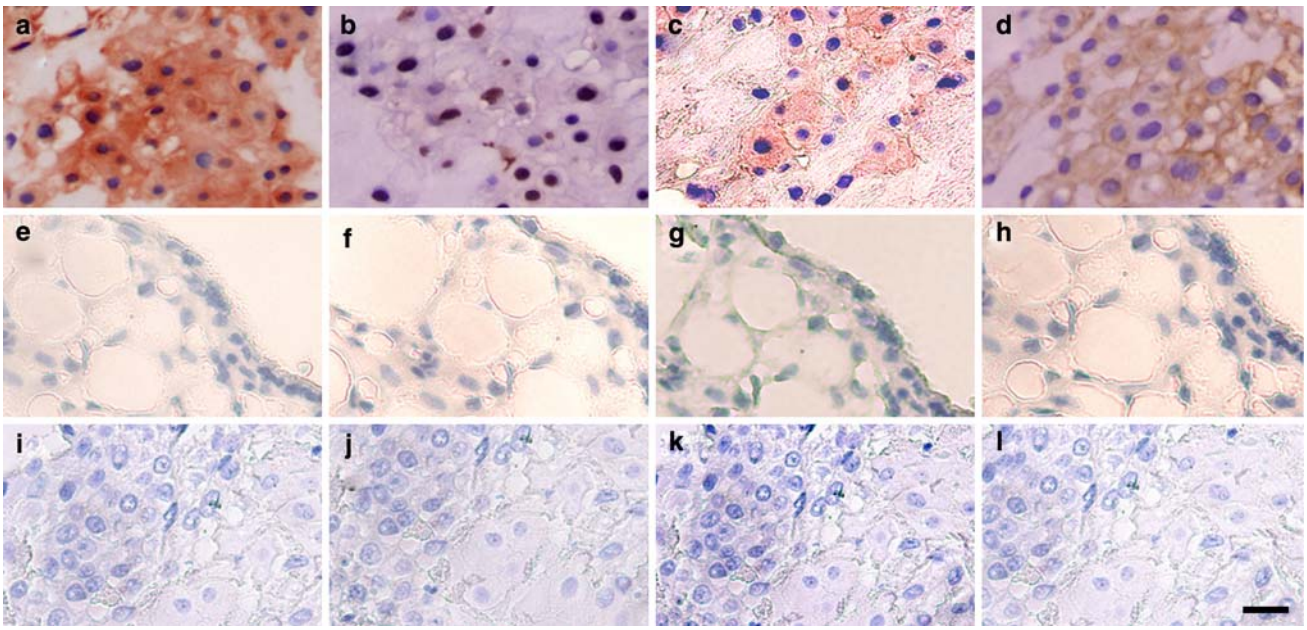

Fig. 1 Immunohistochemical studies of Mst3 expression, caspase-3 activation, DNA fragment and nitrotyrosine in the syncytiotrophoblasts and cytotrophoblasts of human placenta. Human tissue specimens were placenta from spontaneous delivery (**a**–**d**), selective Cesarean section (**e**–**h**) and fetal membrane of first trimester (**i**–**l**). The stains were developed by AEC, 3,3-diaminobenzidine (DAB) and TUNEL assay. The AEC stain was confined to Mst3 expression in the cytotrophoblasts (**a**, **e**, **i**). The DAB stain was confined to TUNEL assay in the cytotrophoblasts (**b**, **f**, **j**). The AEC stain was confined to nitrotyrosine in the cytotrophoblasts (**c**, **g**, **k**). The DAB stain was confined to activated-caspase3 in the cytotrophoblasts (**d**, **h**, **l**). In negative controls, the first antibody or TdT enzyme was replaced by PBS buffer (data not shown). [A representative bar in (**l**) is 50 μm and applies to all the panels]

human term placenta from NSD, which suggests some correlation between these events. Therefore, the biological response of trophoblasts in the human placenta were studied in an explant culture of fresh human placental tissue [39, 40]. Accordingly, fresh human placental tissues from term-with-no-labor Cesarean section were incubated in a cultivation medium containing H_2O_2 or hormones, including angiotensin II, oxytocin and prostaglandin E_1 (PGE_1), an analog of PGE_2, before immunohistochemical study. Oxytocin and PGE_2 are released during labor when the fetus stimulates the cervix and vagina and then facilitate delivery by enhancing contraction of uterine smooth muscle [41]. Angiotensin II is also released during term pregnancy. Abortion and premature delivery can be induced by a high level of angiotensin II in the serum or its receptor in the placental tissue [42]. This study postulated that these hormones play a dual role in facilitating fetus delivery as well as signaling trophoblast apoptosis and placental delivery. As expected, Mst3 was specifically expressed in fresh human placental tissue from term-with-no-labor Cesarean section treated with 200 μM H_2O_2 (Fig. 2d). Conversely, the AEC stain of Mst3 was not observed in human placental specimens treated with 10 μM angiotensin II (Fig. 2m), 1 mIU/ml oxytocin (Fig. 2j) or 1 ng/ml PGE_1 (Fig. 2p). These results indicate that oxidative stress induces Mst3 expression in placental trophoblasts during labor.

Similarly, DNA fragmentation (TUNEL assay) in trophoblasts of fresh human placental tissue was inducible only by H_2O_2 (Fig. 2e) but not by oxytocin (Fig. 2k), angiotensin II (Fig. 2n) or PGE_1 (Fig. 2q). Furthermore, nitrotyrosine (AEC stain) occurred only in H_2O_2-treated human placental tissues from term-with-no-labor Cesarean section (Fig. 2f) and not in placental specimens treated with oxytocin (Fig. 2l), angiotensin II (Fig. 2o) or PGE_1 (Fig. 2r). Untreated human placental tissue from term-with-no-labor Cesarean section was used as a control and exhibited little or no staining (Fig. 2a–c). The experimental results of this study reveal that trophoblast apoptosis in the human placenta is markedly induced by H_2O_2, presumably by inducing Mst3 expression in trophoblast. In contrast, hormones released during labor, such as oxytocin and PGE_1, do not induce the Mst3 protein level or induce apoptosis in trophoblasts.

Glucocorticoid, an anti-inflammatory steroid hormone, is known to effectively suppress oxidative stress-induced trophoblast apoptosis in human placenta [13, 16]. Therefore, the suppressive effect of dexamethasone, an analog of glucocorticoid, on H_2O_2-induced Mst3 and trophoblast apoptosis in fresh human placental tissue

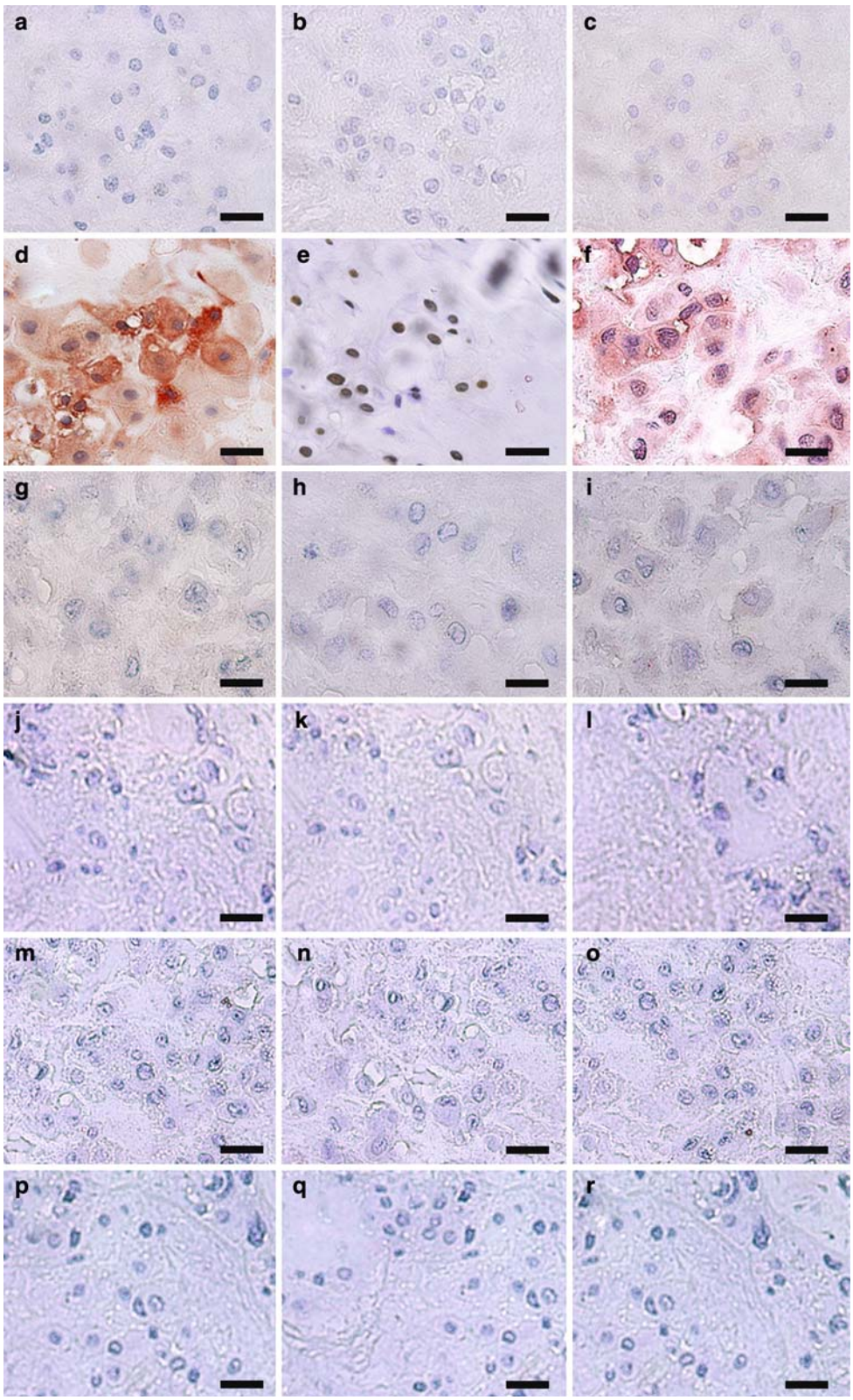

Fig. 2 Immunohistochemical studies of fresh human placental specimens from ECS. Fresh human 38-week placental specimens obtained from ECS without labor pain were sectioned into 2 × 2 × 0.3 cm^3 specimens and incubated in cultivation medium containing 200 μM H_2O_2 (**d**–**f**), 200 μM H_2O_2 and 100 μM dexamethosone (**g**–**i**), 1 mIU/ml oxytocin (**j**–**l**), 10 μM angiotensin II (**m**–**o**) or 1 ng/ml PGE_1 (**p**–**r**) prior to histochemical study. The human placental specimen incubated only in cultivation medium served as a negative control (**a**–**c**). The Mst3 expression, the apoptosis and the presence of nitrotyrosine, a marker of oxidative stress, in cytotrophoblasts were determined by specific antibody against Mst3 (AEC stain) (**a**, **d**, **g**, **j**, **m**, **p**), TUNEL assay (DAB stain) (**b**, **e**, **h**, **k**, **n**, **q**) and by specific antibody against nitrotyrosine (AEC stain) (**c**, **f**, **i**, **l**, **o**, **r**), respectively. In the negative control, the first antibody or TdT enzyme was replaced by PBS buffer (**a**–**c**). [Bar = 50 μm]

from term-with-no-labor Cesarean section was also studied. Notably, the H_2O_2-induced Mst3 expression (Fig. 2g), DNA fragmentation (Fig. 2h) and nitrotyrosine formation (Fig. 2i) in trophoblasts of human placental tissue were considerably suppressed by treatment with 100 μM dexamethosone. This observation implies that the inhibiting

effect of dexamethasone on apoptosis in trophoblasts may result from suppression of oxidative stress as well as Mst3 expression.

Role of Mst3 in H_2O_2-induced trophoblast apoptosis

To further clarify the role of Mst3 in H_2O_2-induced trophoblast apoptosis, the human trophoblast cell line 3A-sub-E was examined. The effect of oxidative stress on Mst3 expression was first demonstrated in the 3A-sub-E cells. As Fig. 3a shows, the protein level of endogenous Mst3 was markedly increased by 200 μM H_2O_2 in 3A-sub-E (top panel). The protein level of Mst3 in 3A-sub-E cells increased approximately 2.0- and 3.3-fold after 6 h and 8 h of treatment (Fig. 3a, bottom panel), respectively. Conversely, PGE_1, oxytocin and angiotensin II could not induce endogenous Mst3 expression in 3A-sub-E cells (data not shown). This experimental result further confirms that oxidative stress induces endogenous Mst3 expression in human trophoblasts. The H_2O_2-induced cell death of 3A-sub-E cells occurs in a time-dependent (2–8 h) manner (Fig. 3b). The viability of 3A-sub-E cells decreased by about 50% following incubation in 200 μM H_2O_2 for 4 h. Decreased viability of 3A-sub-E cells was also induced by H_2O_2 in a dose-dependent (50–600 μM) manner with an EC_{50} of approximately 150 μM (data not shown). Closely examining the sub-G_0/G_1 level, a characteristic of apoptosis, by flow cytometer demonstrated that H_2O_2 induces apoptosis in 3A-sub-E cells (Fig. 3c). The sub-G_0/G_1 level of 3A-sub-E cells increased from $3.70 \pm 0.01\%$ to $14.20 \pm 0.02\%$ ($P < 0.0001$) following treatment with 150 μM H_2O_2 for 6 h (Fig. 3c). These experimental results suggest that H_2O_2 induces apoptosis of 3A-sub-E cells by increasing Mst3 level. However, oxidative stress may have caused trophoblast apoptosis by inducing endogenous Mst3 activity.

Suppression of H_2O_2-induced apoptosis by $Mst3^{KR}$ and Mst3 siRNA

The role of Mst3 in the H_2O_2-induced apoptosis of 3A-sub-E cells was further investigated in cells over-expressing $Mst3^{KR}$, a kinase-dead mutant of Mst3 [27], or in selective knockdown of Mst3. For this purpose, stable clones of

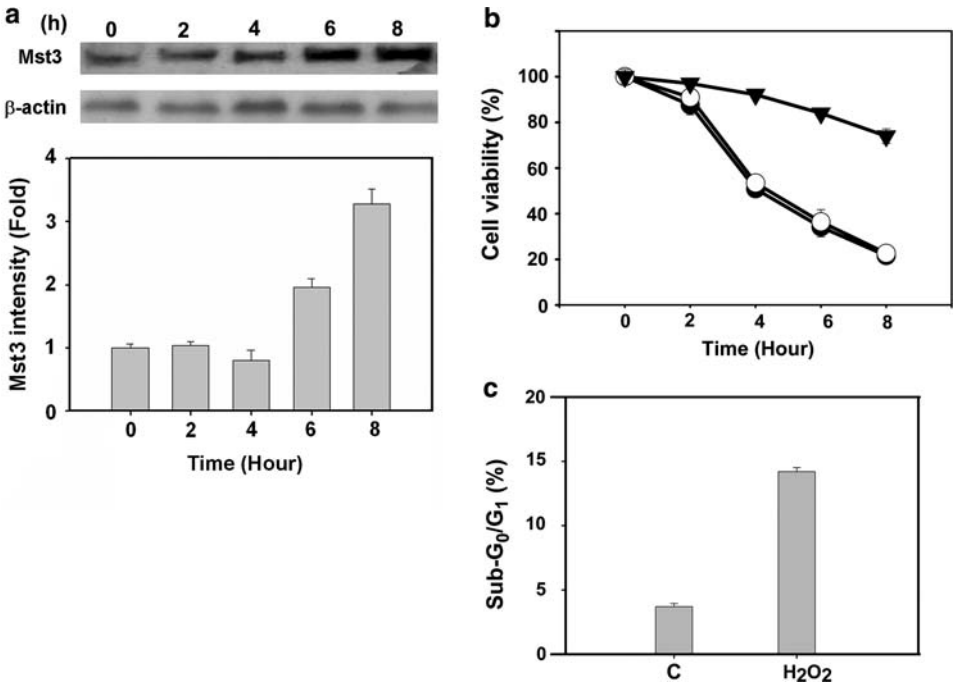

Fig. 3 The Mst3 is essential in the oxidative stress-induced apoptosis of 3A-sub-E cells. (**a**) The Mst3 expression in 3A-sub-E cells could be induced by H_2O_2. The 3A-sub-E cells were treated with 200 μM H_2O_2 at 37°C for 6 h before preparing cell extract. The level of Mst3 in the cell lysates was determined by Western blotting and further quantified by densitametric scanning. Data presented in the bottom panel are means ± SD from three independent experiments. (C, Control) (**b**) The H_2O_2-induced cell death was attenuated in stable clone 3A-sub-E($Mst3^{KR}$). The 3A-sub-E, 3A-sub-E(pcDNA) and 3A-sub-E($Mst3^{KR}$) cells were treated with 200 μM H_2O_2 at 37°C for 0, 2, 4, 6 and 8 h prior to MTT assay. (▼, 3A-sub-E($Mst3^{KR}$); ○, 3A-sub-E(pcDNA); ●, 3A-sub-E). Data are presented as means ± SD from three independent experiments (**c**) The apoptosis of 3A-sub-E cells was induced by H_2O_2. The sub-G_0/G_1 level in cells, a characteristic of apoptosis, was determined by flow cytometry. Trophoblast 3A-sub-E cells were treated with 150 μM H_2O_2 at 37°C for 6 h before PI staining. Data are presented as means ± SD of three independent experiments. (C, Control)

3A-sub-E, 3A(pcDNA) (containing pcDNA only), 3A(pNEO) (containing pNEO vector only), 3A(Mst3^{KR}) (containing expression vector pcDNA-Mst3^{KR}) and 3A(siMst3)-1 and -2 (containing siRNA of Mst3) were generated. The 3A-sub-E and 3A(pcDNA) and 3A(pNOE) cells were used as controls. Interestingly, the cytotoxic effect of H_2O_2 was markedly attenuated in 3A(Mst3^{KR}) cells. After 8 h incubation with 200 μM H_2O_2, the viability of both 3A(pcDNA) and parental 3A-sub-E cells was reduced approximately 80%. However, the viability of 3A(Mst3^{KR}) cells was reduced only ~25% after treatment (Fig. 3b). Apparently, Mst3^{KR} antagonized the H_2O_2-induced cell death of 3A-sub-E. The role of Mst3 in the H_2O_2-induced apoptosis was further studied by DNA fragmentation via TUNEL assay. The percentage of parental 3A-sub-E cells exhibiting DNA fragmentation was increased by 150 μM H_2O_2 in a time-dependent manner from 1.89 ± 0.85% at 0 h to 20.21 ± 2.20%; 45.12 ± 2.38% and 66.10 ± 1.93% after 4, 8 and 12 h of treatment, respectively (Fig. 4a). A similar experimental result was observed in 3A(pcDNA) control stable clone before and after treatment. Interestingly, the DNA fragmentation in 3A(Mst3^{KR}) stable clone after H_2O_2 treatment was significantly lower than that of controls (Fig. 4a). The percentages of 3A(Mst3^{KR}) stable clones with positive TUNEL signals after 150 μM H_2O_2 treatment for 0, 4, 8 and 12 h were 2.06 ± 1.03%, 4.30 ± 1.13%, 13.96 ± 2.14% and 35.03 ± 3.05%, respectively (Fig. 4a). These experimental results indicate the essential role of Mst3 in the pathway of H_2O_2-induced apoptosis of the human trophoblast cell line 3A-sub-E.

To further demonstrate the essential role of Mst3 in trophoblast apoptosis, two independent Mst3 stable knockdown clones of 3A-sub-E, 3A(siMst3)-1 and -2, were examined for oxidative stress-induced apoptosis. Western blot indicated endogenous Mst3 protein was suppressed approximately 80% in stable clones 3A(siMst3)-1 and -2 (Fig. 4b). The H_2O_2-induced apoptosis of stable clones with either vector only or Mst3 siRNA was determined by TUNEL assay. Without H_2O_2-treatment, the percentages of apoptotic cells in 3A(pNEO) and Mst3 knockdown stable clones 3A(siMst3)-1 and -2 were 2.31 ± 0.82%, 2.09 ± 0.81% and 1.96 ± 0.79%, respectively. Interestingly, compared with controls, H_2O_2-induced apoptosis was largely attenuated in Mst3 knockdown stable clones (Fig. 4a). After treatment with 150 μM H_2O_2, the percentage of apoptotic cells in stable clones, 3A(siMst3)-1 and -2 at 4, 8 and 12 h were 5.31 ± 1.13% and 6.04 ± 0.86%; 15.40 ± 2.16% and 17.10 ± 1.14%; and 29.18 ± 1.92% and 31.13 ± 1.73%, respectively (Fig. 4a). The percentage of cells exhibiting DNA fragmentation in the control stable clone 3A(pNEO) was similar to that observed in parental 3A-sub-E and 3A(pcDNA) stable clones (Fig. 4a). These

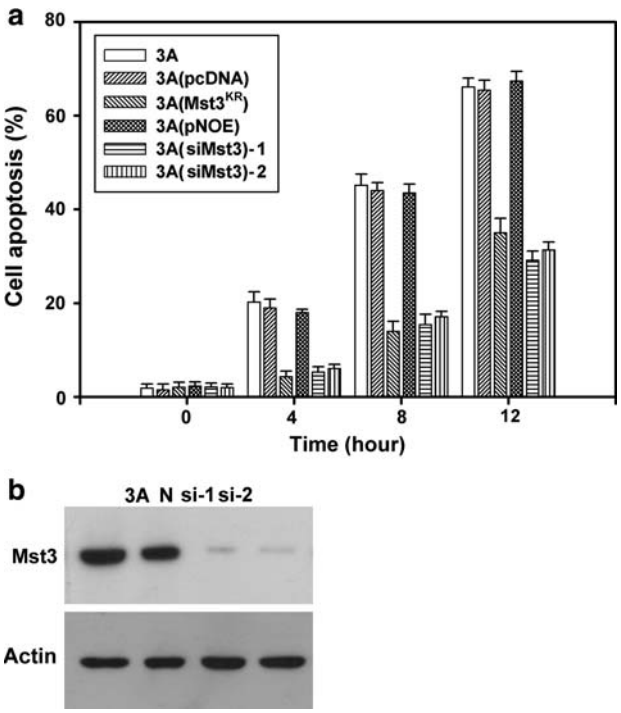

Fig. 4 The H_2O_2-induced apoptosis of human trophoblasts was attenuated in stable clones with overexpressed Mst3^{KR} and selective knockdown of Mst3. (**a**) The H_2O_2-induced apoptosis of 3A-sub-E was attenuated in stable clones 3A(Mst3^{KR}) and 3A(siRNA)-1 and -2. The 3A-sub-E, 3A(pcDNA), 3A(Mst3^{KR}), 3A(pNEO), 3A(siMst3)-1 and 3A(siMst3)-2 were treated with 150 μM H_2O_2 at 37°C for 0, 4, 8 and 12 h prior to TUNEL assay. Following TUNEL assay, the total number of cells and the number of cells with positive fluorescent staining were detected and counted by flow cytometry. These data were used to calculate the percentage of cell apoptosis in each sample. 3A, 3A-sub-E cells. Data are presented as means ± SD from three independent experiments. (**b**) The protein level of Mst3 in stable clones 3A(siMst3)-1 and -2. Cell lysates (50 μg) were separated on a 10% SDS-PAGE, followed by Western blotting by using specific antibody against Mst3. Actin was used as an internal control

results confirm that H_2O_2-induced apoptosis is largely suppressed in stable clones of 3A-sub-E expressing Mst3^{KR}, indicating the importance of Mst3 in H_2O_2-induced apoptosis in human trophoblasts.

Mitogen-activated protein kinase (MAPK) cascade participates in H_2O_2-induced apoptosis of human trophoblast

H_2O_2 is believed to induce apoptosis by activating JNK (c-Jun N-terminal protein kinase) cascade [43]. This study thus examined the involvement of JNK in the H_2O_2-induced apoptosis of human trophoblasts. As Fig. 5a shows, the H_2O_2-induced DNA fragmentation of 3A-sub-E cells was significantly suppressed by SP600125, a JNK inhibitor. Interestingly, in the presence of 1 nM SP600125,

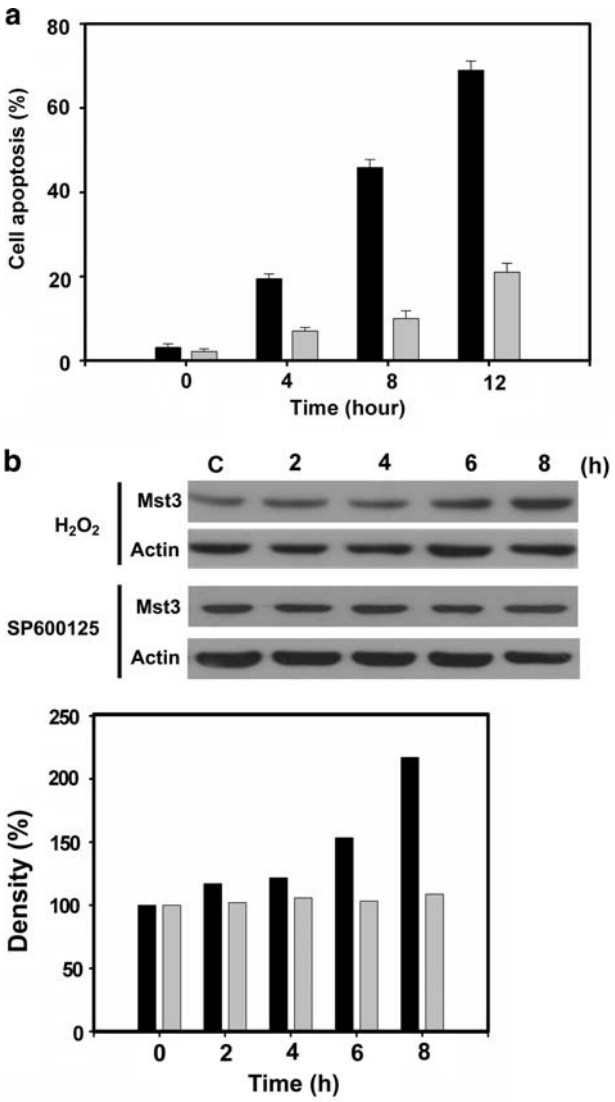

Fig. 5 The H_2O_2-induced Mst3 expression and apoptosis in trophoblasts was suppressed by JNK inhibitor. (**a**) The JNK inhibitor suppressed the apoptotic effect of H_2O_2 in human trophoblast 3A-sub-E cells. The 3A-sub-E cells were co-treated with buffer only (black bar) or 1 nM SP600125 (gray bar) and 150 μM H_2O_2 at 37°C for 0, 4, 8 and 12 h. Apoptosis of 3A-sub-E cells was determined by flow cytometry using FITC-conjugated TUNEL assay kit (Oncogen). Data are presented as means ± SD from three independent experiments. (**b**) The Mst3 expression in 3A-sub-E cells could be suppressed by JNK inhibitor SP600125. The 3A-sub-E cells were pretreated with 1 nM SP600125 at 37°C for 3 h before treatment with 150 μM H_2O_2 at 37°C for 2, 4, 6 and 8 h. The Mst3 level in the cell lysates was detected via Western blotting. The expression level of Mst3 in 3A-sub-E cells with or without treatment was quantitated by the densitometry (bottom panel). The western blot is the representative of two independent experiments

the H_2O_2-induced DNA fragmentation of 3A-sub-E cells was reduced from 19.50 ± 1.13% to 7.03 ± 0.91% (after 4 h treatment), from 45.90 ± 1.89% to 10.03 ± 1.85% (after 8 h treatment) and from 69.01 ± 2.17% to 21.01 ± 2.14% (after 12 h treatment) (Fig. 5a). A similar experimental result was observed in the presence of 10 nM PD98059, an inhibitor of MEK1 (MAP kinase kinase and ERK activator kinase 1) (data not shown). These results indicate that the MAPK/JNK cascade may be involved in the H_2O_2-induced apoptosis of human trophoblasts. The suppressive effect of JNK inhibitor on H_2O_2-induced Mst3 expression in 3A-sub-E cells was further examined. As Fig. 5b shows, the H_2O_2-induced expression of Mst3 in 3A-sub-E cells could be inhibited by SP600125. Thus, Mst3 may be downstream of JNK cascade in the oxidative stress-induced apoptosis of human trophoblast. In response to oxidative stress, JNK may regulate both the expression and activity of Mst3 in human trophoblasts.

Role of caspase in H_2O_2-induced apoptosis

The results of Fig. 1 (d, h, l) demonstrate the appearance of active caspase 3 in human placental tissue from NSD but not in placental tissue from first trimester and term-with-no-labor Cesarean section. Caspases are believed to be involved in the apoptosis of human trophoblasts [19]. It is possible that caspases may also participate in the oxidative stress-induced apoptosis of human trophoblasts. In the presence of caspase inhibitor 50 μM Z-DEVD-fmk, H_2O_2-induced apoptosis of 3A-sub-E cells could be reduced 37–50% (Fig. 6a). The percentages of apoptotic 3A-sub-E cells after H_2O_2 treatment were reduced from 19.12 ± 1.89% to 8.42 ± 2.07% (after 4 h treatment), from 46.25 ± 2.52% to 23.1 ± 1.69% (after 8 h treatment) and from 68.36 ± 2.31% to 43.02 ± 2.91% (after 12 h treatment) (Fig. 6a). Increased active caspase 3 after 4 and 8 h treatment of H_2O_2 was also demonstrated by using antibody specifically against active caspase 3 (Fig. 6b). After 12 h treatment, however, the active caspase 3 was markedly reduced. Interestingly, the level of active caspase 3 increased only slightly in 3A(siMst3)-1 and -2, the stable clones with selective knockdown of endogenous Mst3, in response to H_2O_2 treatment (Fig. 6b). The presence of universal caspase inhibitor further reduced the level of caspase 3 in 3A(siMst3)-1 and -2 stable clones even after H_2O_2 treatment (Fig. 6c). Conversely, Z-DEVD-fmk suppresses neither the H_2O_2-induced expression of Mst3 (data not shown) nor the phosphorylation of JNK in 3A-sub-E cells (Fig. 6c). These findings indicate that the activity of caspase 3 may be regulated by Mst3 in human trophoblasts in response to oxidative stress.

Discussion

Oxidative stress caused by elevated reactive oxygen species (ROS) is known to occur throughout pregnancy due to

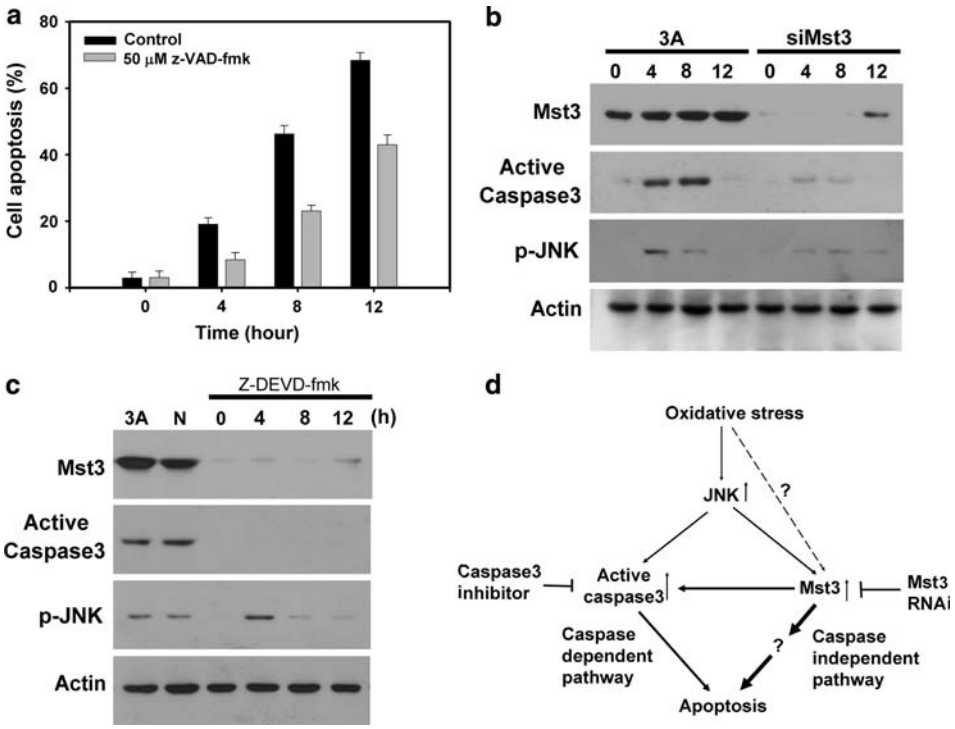

Fig. 6 The activity of caspase 3 and JNK in H_2O_2-treated 3A-sub-E and 3A(siMst3) cells. (**a**) The caspase inhibitor (Z-DEVD-fmk) moderately suppressed the apoptotic activity of H_2O_2 in human trophoblast 3A-sub-E cells. The 3A-sub-E cells were co-treated with buffer only (black bar) or 50 mM Z-DEVD-fmk (gray bar) and 150 μM H_2O_2 at 37°C for 0, 4, 8 and 12 h. Apoptosis of 3A-sub-E cells was determined by flow cytometry using FITC-conjugated TUNEL assay kit (Oncogen). (**b**) Activated caspase 3 and JNK in parental 3A-sub-E and stable clone 3A(siMst3) in response to oxidative stress. Cells were treated with 150 μM H_2O_2 at 37°C for 0, 4, 8 and 12 h prior to Western blotting. Cell lysates (50 μg each) were separated on a 10% SDS-PAGE, followed by Western blotting using specific antibody against Mst3, active caspase 3 and phospho-JNK (p-JNK). Actin was used as an internal control. (**c**) The effect of Z-DEVD-fmk on the activation of caspase 3 and JNK in parental 3A-sub-E cells (left panel) and 3A(siMst3) stable clone (right panel). Cells were co-treated with 50 mM Z-DEVD-fmk and 150 μM H_2O_2 at 37°C for 0, 4, 8 and 12 h prior to the Western blotting. Cell lysates (50 μg each) were separated on a 10% SDS-PAGE, followed by Western blotting by specific antibody against Mst3, active caspase 3 and phospho-JNK (p-JNK). Actin was used as an internal control. 3A, parental 3A-sub-E cell; N, stable clone 3A(pNEO). (**d**) Schematic illustration of possible role of Mst3 in an H_2O_2-induced apoptotic pathway in a human trophoblast

increased metabolic activity in placental mitochondria [44]. Oxidative stress is a pathophysiological process leading to oxidative damage in cells, tissues, or organs by damaging lipids, inhibiting protein synthesis and depleting ATP [15]. Mechanisms to prevent oxidative stress, such as antioxidants and avoidance of excess iron, can be adopted throughout pregnancy until labor to reduce maternal and early fetal damage [45]. When labor begins, the disrupted balance between ROS generation and anti-oxidation may cause oxidative stress [31–35]. The oxidative stress-induced trophoblast apoptosis in term or pre-term placenta may cause detachment of the placenta from the endometrial tissue of the maternal uterus, resulting in spontaneous labor [13, 16] or the pathogenesis of various pregnancy disorders [46–49]. However, the molecular mechanism underlying oxidative stress-induced apoptosis of the placenta has not been fully elucidated.

The Mst3, a human counterpart of the yeast Ste20-like serine/threonine protein kinase, was recently shown to be involved in apoptosis of several cell lines in response to treatment with staurosporine and Fas ligand [27]. A previous investigation revealed Mst3 in human placenta by Northern blotting [24]. In the current study we observed Mst3 expression in the human placenta as measured by immunocytochemistry and Western blotting. However, it can only be seen in the human placental tissue from NSD, but not in tissues from term-with-no-labor Cesarean section and first trimester (Fig. 1). Interestingly, Mst3 expression was co-present with nitrotyrosine formation as well as DNA fragmentation in human placental tissue from NSD. These observations suggest that Mst3 may play an important role in parturition, presumably by mediating oxidative stress-induced trophoblast apoptosis and placental degeneration. The involvement of Mst3 in the oxidative stress-induced apoptosis of human trophoblasts was further demonstrated in the human trophoblast cell line 3A-sub-E by overexpressed $Mst3^{KR}$, a kinase-dead form of Mst3, and by selective knockdown of endogenous Mst3. Compared

with controls, H_2O_2-induced apoptosis was markedly reduced in 3A(Mst3^{KR}), a stable clone of 3A-sub-E over expressing Mst3^{KR} (Fig. 4a) as well as in 3A(siMst3)-1 and -2, stable clones with the selective knockdown of Mst3 (Fig. 4b). These experimental results further indicate that Mst3 plays an important role in the oxidative stress-induced apoptosis of human trophoblasts.

Previous study [43] suggests that JNK participates in oxidative stress-induced apoptosis. The current study further demonstrates that the level of phosphorylated and active JNK (p-JNK) rapidly increased in human trophoblast cell line 3A-sub-E (Fig. 5). The importance of Mst3 in the H_2O_2-induced apoptosis has also been demonstrated in this study. Hence, both Mst3 and JNK may participate in the signaling pathway of H_2O_2-induced human trophoblast apoptosis. However, the relationship between Mst3 and JNK is unknown. Previous study demonstrates that overexpression of Mst3 does not affect the activity of ERK-1, ERK-6, JNK or p38 [24]. On the other hand, Mst3 overexpression-induced HeLa apoptosis cannot be suppressed by MAPK cascade inhibitors [27]. These results indicate that Mst3 is not the upstream of MAPK cascades. Interestingly, in human trophoblasts, both H_2O_2-induced apoptosis as well as the elevation of Mst3 can be markedly inhibited by SP600125, a JNK specific inhibitor (Fig. 5b). Furthermore, the knockdown of Mst3 in 3A-sub-E cells markedly attenuates the H_2O_2-induced apoptosis but not the phosphorylation of JNK in human trophoblasts (Fig. 6b). These results suggest that Mst3 is downstream of JNK cascade in the pathway of oxidative stress-induced human trophoblast apoptosis.

Caspase-3 is believed to be involved in trophoblast apoptosis [19]. In this study, activation of caspase 3 was also demonstrated in human placental tissue from NSD (Fig. 1), which suggests that caspase 3 is involved in placental apoptosis during labor. Caspase 3 activated by oxidative stress was also observed in the human trophoblast cell line 3A-sub-E (Fig. 3). However, the presence of the universal caspase inhibitor Z-DEVD-fmk could only moderately suppress the H_2O_2-induced apoptosis of 3A-sub-E cells (Fig. 6a), suggesting the presence of another signaling pathway. Further studies showed that the oxidative stress-induced activation of caspase 3 was largely suppressed in stable clones 3A(siMst3)-1 and -2, which exhibit a selective knockdown of Mst3 (Fig. 6b). These experimental results indicate that the oxidative stress-induced activation of caspase 3 in human trophoblasts is mediated primarily by Mst3, which is regulated by JNK. However, the possibility that caspase 3 is activated by JNK cannot be ruled out, because the activation of caspase 3 by oxidative stress cannot be completely suppressed by selective knockdown of Mst3 (Fig. 6b). These experimental results indicate that JNK may be the upstream of both Mst3 and caspase 3 in the signaling pathway of H_2O_2-induced apoptosis. Although caspase 3 can be controlled by both JNK and Mst3, the latter is apparently important in up-regulating caspase 3 in the signaling pathway of H_2O_2-induced apoptosis (Fig. 6d). Activated Mst3 may facilitate apoptosis by inducing the activity of unknown downstream components since H_2O_2-induced apoptosis of 3A-sub-E cells cannot be completely suppressed by the caspase inhibitor Z-DEVE-fmk. Thus, oxidative stress may mediate both the availability and possibly activity of Mst3 in human trophoblasts by regulating the activity of JNK. Activated Mst3 then triggers human trophoblast apoptosis by activating caspase 3 and other downstream pro-apoptotic components (Fig. 6d). Further investigation of the role of Mst3 in regulating the apoptotic process in the human placenta is required to further elucidate the molecular origin of this process.

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