The SUMO E3-ligase PIAS1 couples reactive oxygen species-dependent JNK activation to oxidative cell death

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ABSTRACT Human endometrial stromal cells (HESCs) exposed to reactive oxygen species (ROS) mount a hypersumoylation response in a c-Jun N-terminal kinase (JNK)-dependent manner. The mechanism that couples JNK signaling to the small ubiquitin-related modifier (SUMO) pathway and its functional consequences are not understood. We show that ROS-dependent JNK activation converges on the SUMO pathway via PIAS1 (protein inhibitor of activated STAT1). Unexpectedly, PIAS1 knockdown not only prevented ROS-dependent hypersumoylation but also enhanced JNK signaling in HESCs. Conversely, PIAS overexpression increased sumoylation of various substrates, including c-Jun, yet inhibited basal and ROS-dependent JNK activity independently of its SUMO ligase function. Expression profiling demonstrated that PIAS1 knockdown enhances and profoundly modifies the transcriptional response to oxidative stress signals. Using a cutoff of 2-fold change or more, a total of 250 ROS-sensitive genes were identified, 97 of which were not dependent on PIAS1. PIAS1 knockdown abolished the regulation of 43 genes but also sensitized 110 other genes to ROS. Importantly, PIAS1 silencing was obligatory for the induction of several cellular defense genes in response to oxidative stress. In agreement, PIAS1 knockdown attenuated ROS-dependent caspase-3/7 activation and subsequent apoptosis. Thus, PIAS1 determines the level of JNK activity in HESCs, couples ROS signaling to the SUMO pathway, and promotes oxidative cell death.—Leitao, B. B., Jones, M. C., Brosens, J. J. The SUMO E3-ligase PIAS1 couples reactive oxygen species-dependent JNK activation to oxidative cell death. FASEB J. 25, 3416–3425 (2011). www.fasebj.org

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The human endometrium undergoes rapid cycles of proliferation, differentiation, menstrual shedding, and regeneration in response to the rise and fall of ovarian estradiol and progesterone (2, 3). A key event during these waves of intense tissue remodeling is decidualization of the stromal compartment in preparation for pregnancy. The decidual process, initiated during the midsecretory phase of the cycle, is characterized by vascular remodeling, influx of specialized immune cells and macrophages, and differentiation of the stromal fibroblasts into specialized secretory decidual cells (4). In early pregnancy, decidual cells rapidly encapsulate the implanting embryo, allow invasion of fetal trophoblast, and protect the conceptus against environmental stressors, including reactive oxygen species (ROS; ref. 5).

Several mechanisms work in concert to ensure the integrity of the maternal decidua during the formation of a functional fetomaternal interface. For example, decidualizing human endometrial cells (HESCs) highly express a variety of cellular and extracellular free radical scavengers, including superoxide dismutase 2, glutathione peroxidase 3, monoamine oxidases A and B, thioredoxin, glutaredoxin, and peroxiredoxin, all of which protect against indiscriminate cellular damage caused by environmental ROS (6). In addition, oxidative stress signaling, and especially activation of the stress-responsive c-Jun N-terminal kinase (JNK) pathway, is greatly limited in decidualizing HESCs as a consequence of the induction of MAP kinase phosphatase 1 (MKP1) and the altered expression of several pathway intermediates (7). Moreover, the expression of FOXO3a, a proapoptotic member of the Forkhead family of transcription factors, is firmly silenced on differentiation into decidual cells (8).

Another striking adaptation of decidualizing cells involves the small ubiquitin-related modifier (SUMO) pathway. Sumoylation, which denotes the covalent modification of proteins with SUMO proteins, is implicated in an array of cellular processes, including transcription regulation, DNA repair and stress responses (9, 10). It is mediated by sequential activation of an E1-activating enzyme (the SAE1/SAE2 heterodimer), an E2-conjugating enzyme (UBC9), and several E3 protein ligases that confer substrate specificity. Once a target is sumoylated, the modification can be reversed rapidly.

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through the activities of sentrin-specific proteases (SENP). Decidualization of HESCs is associated with a gradual decline in global cellular sumoylation levels and redistribution of SUMO-1-modified proteins into distinct nuclear foci (11). Notably, this hypusomylation response is not accounted for by altered expression of SUMO, SAE1/SAE2, or Ubc9. However, decidualization profoundly alters the expression of various E3-conjugating enzymes and SENPs, including simultaneous down-regulation of the E3 ligase PIAS1 (protein inhibitor of activated STAT1) and up-regulation of SENP2. An important functional consequence of global hypusomylation and altered expression of key enzymes is a marked decrease in ligand-dependent SUMO-1 modification of key nuclear receptors, such as the progesterone and androgen receptors, which in turn greatly enhances the responsiveness of decidualizing cells to steroid hormones (11, 12). In addition, undifferentiated, but not decidualized HESCs, mount a hypersumoylation response when exposed to ROS levels well below those that cause direct oxidative inactivation of the SUMO pathway (7, 13, 14). This uncoupling of ROS signaling and the SUMO pathway in decidualizing cells is accounted for by silencing of the JNK pathway and ensures that steroid hormone responses remain unimpeded under stress conditions imposed by pregnancy (7). However, the mechanisms responsible for the ROS-dependent hypersumoylation response in undifferentiated cells are not yet defined, nor are the consequences in terms of cell survival or death.

**MATERIALS AND METHODS**

**Primary cell culture and reagents**

Endometrial biopsies were obtained from premenopausal women without uterine pathology, and HESCs were isolated, established in culture, and maintained as described previously (15). The Hammersmith and Queen Charlotte’s and Chelsea Research Ethics Committee approved the study (15). The Hammersmith and Queen Charlotte’s and Chelsea Research Ethics Committee approved the study previously (15). The Hammersmith and Queen Charlotte’s and Chelsea Research Ethics Committee approved the study (15). The Hammersmith and Queen Charlotte’s and Chelsea Research Ethics Committee approved the study (15). The Hammersmith and Queen Charlotte’s and Chelsea Research Ethics Committee approved the study (15).

**Transfections**

Primary HESCs were transfected by the calcium phosphate coprecipitation method using the ProFection Mammalian Transfection kit (Promega, Madison, WI, USA), while COS-1 cells were transfected using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA), according to the manufacturer’s instructions. The expression vectors for pSG5, pSG5-PIAS1, pSG5-PIAS1 W372A/C351S, pCS2-HA-PIAS1 WT, pCS2-HA-PIAS1 W72A/C351S, pCMV5-Flag-PIAS1, pEGFP-SUMO-1, and pBabe-MEKK-1ER® have been described previously (7, 11). pQE30-(His)6-SENP2 was kindly provided by Dr. M. Matunis (Johns Hopkins University, Baltimore, MD, USA) and pSG5-c-Jun by Dr. D. Sooranna (Imperial College, London, UK). The HA-PIAS1 3MT was generated by mutating 3 putative MAP kinase phosphorylation sites (Ser106, Ser185, and Ser488) into alanines using a QuickChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA). Primary cultures in 6-well plates were transfected with 1 μg plasmid/well, unless stated otherwise. The following small interfering RNA (siRNA) duplexes were purchased from Dharmacon (Lafayette, CO, USA): siGenome SmartPool for human PIAS1 (M-008167-01); siGenome SmartPool for human MAPK8 (JNK1; M-003514-04); and siControl nontargeting (NT) siRNA Apoold (D-001260-14-05).

**Western blotting and immunoprecipitation**

Whole-cell protein extracts were obtained by direct lysis in Laemmli buffer heated to 85°C, followed by sonication. Proteins resolved by SDS-PAGE were transferred to a PVDF membrane (Amersham, Little Chalfont, UK) and probed with antibodies raised against SUMO-1 (Zymed, Burlingame, CA, USA); β-actin (Abcam, Cambridge, MA, USA); JNK, phospho-JNK, c-Jun, and phospho-c-Jun (Cell Signaling, Beverly, MA, USA); PIAS1 (Santa Cruz Biotechnology, Santa Cruz, CA, USA); HA tag (Roche, Welwyn Garden City, UK); and Flag tag (Sigma). Following incubation with HRP-conjugated secondary antibodies (Roche), chemiluminescence was visualized using the ECL+ kit (Amersham).

**Densitometry**

Densitometry was performed on scanned immunoblot images using the ImageJ gel analysis tool (http://rsbweb.nih.gov/ij/index.html), according to the users guide. The absolute intensity (AI) was determined for each experimental SUMO-1 smear and corresponding β-actin control band. Relative intensity (RI) for each sample was calculated by normalizing its AI to the corresponding control AI. All the RI values were then normalized to the RI of the untreated sample.

For immunoprecipitation, cells were lysed in HEPES buffer (50 mM HEPES/NaOH, pH 7.0; 150 mM NaCl; 10 mM EDTA; 1% Nonidet P-40; 0.5% Na-deoxycholate; 0.1% SDS; 10 mM DTT; and 1 mM PMSF), with Complete protease inhibitor tablets (Roche). After removal of cell debris by centrifugation, lysates containing 1 mg of protein were precleared with Protein G Plus-Agarose (Invitrogen) and incubated with anti-PIAS1 or anti-HA antibody. Proteins were recovered by precipitation with Protein G Plus-Agarose, washed 3 times with HEPES, and analyzed by Western blot.

**In vitro kinase assay and ELISA**

For the in vitro kinase assays, immunoprecipitated HA-PIAS1, wild-type or W372A/C351S mutant, was incubated with kinase buffer (Cell Signaling), 100 U of purified JNK (Cell Signaling) and 500 μg/μmol γ-32P-ATP for 45 min at 30°C. Reactions were stopped by adding Laemmli buffer and boiling for 5 min. Proteins were resolved by SDS-PAGE; the gels were stained with Coomassie blue, dried, and visualized by autoradiography.

Total and phosphorylated JNK levels in HESC lysates were quantified with PathScan Total and Phospho-SAPK/JNK (Thr180/Tyr185) Chemiluminescent Sandwich ELISA kit (Cell Signaling, respectively), following the manufacturer’s instructions. Activated caspase-3/7 levels were determined using the ApoTox-GloTM Triplex Assay (Promega) and normalized against the percentage of viable cells, also determined by the same kit, according manufacturer’s instructions.

**Flow cytometry**

For cell cycle analysis, primary HESCs were treated with 250 μM H2O2; 24 h later, the cells were trypsinized and centri-
fuged at 1500 rpm. The pellet was resuspended in 300 µl PBS and then fixed with 5 ml of ice-cold 70% ethanol for ≥1 h at −20°C. The fixed cells were then centrifuged and resuspended in 2 ml of cold PBS. After incubation at 4°C for ≥30 min, the cells were centrifuged and resuspended in 1 ml of PBS solution containing RNase (50 g/ml) and propidium iodide (PI; 60 g/ml; Sigma). After incubation at room temperature for 30 min, the stained cells were analyzed in a fluorescence-activated cell sorter (FACSCalibur, BD Biosciences, San Jose, CA, USA). The percentages of cells in various phases of the cell cycle were analyzed using the flow cytometry analysis software FlowJo (Tree Star, Ashland, OR, USA).

**RESULTS**

**PIAS1 is modified in response to ROS-dependent JNK activation**

Modest levels of H₂O₂ (~250 µM) trigger a rapid hypersumoylation response in primary HESC cultures but not in cultures first decidualized for ≥3 d with a cAMP analog, 8-BrcAMP, and MPA, a progestin (7). To explore the mechanism that relays environmental ROS signals to the SUMO pathway, undifferentiated cells and cultures decidualized for various time periods were pulsed with H₂O₂ for 30 min. Western blot analysis confirmed that H₂O₂ increases global sumoylation in undifferentiated cells, and that this response is gradually lost on differentiation of the cultures (Fig. 1A). Densitometry of Western blots from 4 biologically independent experiments indicated that the increase in global cellular sumoylation in undifferentiated HESCs pulsed with H₂O₂ for 30 min averaged ~60%, whereas this response was entirely lacking in cultures first decidualized for 4 d (Supplemental Fig. S1). As reported previously (7, 11), decidualization downregulated PIAS1 expression and conferred gradual resistance to JNK activation in response to H₂O₂ treatment. Unexpectedly, H₂O₂ consistently induced a mobility shift in PIAS1 on SDS-PAGE, suggesting that this E3 SUMO ligase is basally phosphorylated in undifferentiated HESCs and further modified on H₂O₂ exposure.

PIAS1 is a known target of different kinases, including IκB kinase α (IKKα) and casein kinase 2 (CK2) (16, 17). Several lines of evidence indicated that PIAS1 may also be a direct target of JNK. First, siRNA-mediated JNK knockdown in primary cultures attenuated subsequent PIAS1 modification in response to H₂O₂ treatment (Fig. 1C). Second, 4-OHT treatment of COS-1 cells expressing HA-tagged PIAS1 and ΔMEKK1:ER*, a chimeric protein kinase composed of the constitutively active kinase domain of rat MEKK1 (ΔMEKK1) fused to a modified form of the ligand-binding domain of estrogen receptor (ER*), also slowed PIAS1 migration on Western blot analysis (Fig. 1D). Notably, binding of 4-OHT to ΔMEKK1:ER* predominantly activates JNK, with only marginal effects on p38 and ERK1/2 phosphorylation (18). Finally, HA-tagged PIAS1 immunopurified from transfected COS-1 cells became phosphorylated in vitro when incubated with recombinant JNK and γ²53P-ATP (Fig. 1E).

Sequence analysis of PIAS1 revealed the presence of 3 highly conserved putative MAP kinase phosphorylation sites (Ser¹⁰⁶, Ser⁴⁸⁵, and Ser⁴⁸⁸) in human PIAS1 (Supplemental Fig. S2A). However, immunoprecipitation of an HA-tagged PIAS1 mutant (HA-PIAS1 3MT), in which these serines were converted into alanines, had no effect on the mobility shift induced by 4-OHT activation of ΔMEKK1:ER* in COS cells or by H₂O₂ treatment of primary HESCs (Supplemental Fig. S2B, C, respectively). Moreover, liquid chromatography-tandem mass spectrometry (LC-MS/MS) revealed that at least one of these putative MAP kinase phosphorylation sites, Ser⁴⁸⁵, is basally phosphorylated in PIAS1 (data not shown). However, LC-MS/MS failed to identify specific H₂O₂ or ΔMEKK1:ER*-dependent modifications in immunoprecipitated endogenous or overexpressed HA-tagged PIAS1, respectively; probably due to the relatively low sequence coverage that was achieved (<40%). Thus, various lines of evidence suggest that PIAS1...
is a target of activated JNK, although additional experiments are required to identify specific modified residues.

**PIAS1 is a negative regulator of JNK in HESCs**

To test whether PIAS1 plays a role in coupling ROS signaling to the SUMO pathway, we transfected primary HESCs with siRNA targeting PIAS1 before pulsing the cultures with H$_2$O$_2$ for 10 or 30 min. Control cultures were transfected with PIAS1-targeting or NT siRNA. As shown in Fig. 2A, PIAS1 knockdown was sufficient to attenuate the induction of high-molecular-weight sumoylated proteins in H$_2$O$_2$-treated cultures. Unexpectedly, PIAS1 knockdown also enhanced JNK phosphorylation on H$_2$O$_2$ treatment (Fig. 2A), suggesting that this E3 ligase not only plays a role in relaying free radical signaling to the SUMO pathway but also modulates JNK activity. To explore these observations further, we transfected primary cultures with a control vector or an expression vector encoding Flag-tagged PIAS1 and then pulsed the cells with H$_2$O$_2$. Overexpression of PIAS1 increased the levels of protein sumoylation yet prevented a further increase in response to H$_2$O$_2$. Furthermore, ROS-dependent JNK activation was inhibited (Fig. 2B). Total JNK levels were unaffected by PIAS knockdown or overexpression. The ability of PIAS1 to negatively regulate JNK activity was not dependent on primary cellular stress, as coexpression of ΔMEKK1:ER* with HA-tagged PIAS1 strongly attenuated JNK phosphorylation in primary HESCs treated with 4-OHT (Fig. 2C). Next, we quantified by ELISA the total and phosphorylated (Thr$^{183}$/Tyr$^{185}$) JNK levels in primary cultures transfected with wild-type (WT) PIAS1 or a PIAS1 mutant (W372A/C351S) devoid of ligase activity (19, 20). Compared to cultures transfected with a noncoding plasmid, WT and ligase-deficient PIAS1 inhibited basal and H$_2$O$_2$-induced JNK activity with comparable efficacies (Fig. 2D). Western blot analysis confirmed that the E3 ligase function of PIAS was dispensable for the negative regulation of JNK activity (Supplemental Fig. S2D).

**PIAS1 regulates c-Jun sumoylation and activity in response to environmental ROS**

The transcription factor c-Jun, part of the activator protein 1 (AP-1) complex, is a major downstream target in the JNK pathway and is modified by SUMO in a PIAS1-dependent manner (21). We postulated that c-Jun would be an informative protein to dissect further the dual role of PIAS1, as an inhibitor of JNK activity and as a redox-sensitive SUMO ligase. To test whether oxidative stress signals enhance sumoylation of c-Jun in a PIAS1-dependent manner, primary HESCs were first cotransfected with expression vectors encoding EGFP-tagged SUMO-1 and c-Jun. As shown in Fig. 3A, H$_2$O$_2$ rapidly enhanced sumoylation of c-Jun in HESCs, an effect apparent after 10 min and maintained
for ≥1 h. As anticipated, PIAS1 knockdown abolished ROS-dependent c-Jun sumoylation, whereas PIAS1 overexpression enhanced this modification, irrespective of H₂O₂ treatment (Fig. 3B). Overexpression of the ligase-deficient PIAS1 mutant did not enhance c-Jun sumoylation levels but also abolished the ROS-induced hypersumoylation response (Fig. 3C). The observation that overexpression of either WT or mutant (W372A/C351S) PIAS1 negates enhanced sumoylation of c-Jun in response to ROS is in keeping with its ability to antagonize JNK activity in a ligase-independent manner. Thus, SUMO modification of c-Jun is part of the global hypersumoylation response upon ROS-dependent JNK activation.

While JNK-dependent phosphorylation activates the c-Jun/AP-1 complex, sumoylation damps it (22). A major target of this transcriptional complex is JUN, a gene positively autoregulated by its product c-Jun (23). Hence, we examined JUN expression in HESCs exposed to oxidative stress signals to determine the role of PIAS1 in regulating c-Jun transcriptional activity. PIAS1 knockdown enhanced basal as well as H₂O₂-induced JUN transcript levels (Fig. 3D), whereas overexpression of WT PIAS1 or the ligase mutant had the opposite effect (Fig. 3E). Qualitatively, the effect of PIAS1 overexpression, WT or mutant, was comparable to that elicited by JNK knockdown (Fig. 3F). We also monitored the expression of RRAD, another ROS-sensitive gene in HESCs (7). As was the case for JUN, PIAS1 silencing increased the induction of RRAD transcripts upon H₂O₂ treatment (Fig. 3G). JNK knockdown or overexpression of WT or ligase-deficient PIAS1 greatly attenuated this response (Fig. 3H, I). Thus, analysis of JUN and RRAD expression suggests that the cellular levels of PIAS1 in HESCs determine the amplitude of the transcriptional response to ROS, a function that appears to reside foremost in its ability to modulate JNK activity.

Characterization of ROS-sensitive genes regulated by PIAS1

To determine whether PIAS1 also modulates the nature of the transcriptional response to ROS, primary HESC cultures were first transfected with WT or PIAS1-targeting siRNA, were exposed to 250 μM H₂O₂ for 10 or 30 min. Whole-cell protein extracts were subjected to Western blot analysis with antibodies against SUMO-1, PIAS1, phosphorylated JNK (p-JNK), and total JNK. β-Actin served as loading control. (B) Primary HESC cultures transfected with a noncoding vector or Flag-tagged PIAS1 were exposed to 250 μM H₂O₂ for 10 or 30 min, and total cell lysates were immunoprobbed for SUMO-1, Flag tag, p-JNK, and total JNK. (C) Primary HESC cultures were transfected with an expression vector coding for ΔMEKKER*-myc either with or without HA-PIAS1 and treated with 100 nM 4-OHT for 30 min. PIAS1 expression and JNK phosphorylation were assessed by Western blot analysis. (D) Primary cultures, transfected with a noncoding vector or with an expression vector encoding either WT PIAS1 or ligase-deficient (W372A/C351S) mutant, were pulsed with 250 μM H₂O₂ for 30 min. JNK phosphorylation relative to total JNK levels was measured with PathScan phospho- and total SAPK/JNK Chemiluminescent Sandwich ELISA kits, respectively. Activity is measured in relative light units (RLU) and normalized to the levels in untreated cells transfected with a noncoding vector. Data represent means ± sd of triplicate determinations from 3 independent experiments.
siRNA and exposed to H₂O₂ for 8 h, and total RNA was extracted and processed for whole-genome microarray analysis. Using a cutoff of ≥2-fold change, a total of 250 H₂O₂-sensitive genes were identified (P<0.01), 39% (97/250) of which were not modified by the presence or absence of PIAS1 (Fig. 4A, left panel). PIAS1 knockdown abolished the regulation of 17% (43/250) of H₂O₂-responsive genes but also sensitized 110 other genes to ROS. Notably, the proportion of ROS-sensitive transcripts up-regulated in the presence of PIAS1 (11/43; 33%) was significantly lower than the proportion induced on PIAS1 knockdown (84/110; 69%; χ² test; P<0.05).

**Figure 3.** PIAS1 modulates c-Jun sumoylation and activity. A) Primary HESCs, cotransfected with expressing vectors encoding EGFP-SUMO-1 and c-Jun, were exposed to 250 μM H₂O₂ for the indicated time periods, and whole-cell lysates were immunoprobed for c-Jun and β-actin. B) Primary cultures were transfected with expressing vectors coding for EGFP-SUMO-1 and c-Jun in combination with either pSG5-PIAS1 or PIAS1 siRNA. Cells were then treated with 250 μM H₂O₂ for 30 min; total cell lysates were immunoprobe for c-Jun, PIAS1, and β-actin. C) HESCs were transfected with expression vectors encoding EGFP-SUMO-1 and c-Jun in combination with plasmids encoding either HA-tagged WT PIAS1 or ligase mutant (HA-PIAS1 W372A/C351S). Cells were exposed to 250 μM H₂O₂ for 8 h, and subjected to Western blot analysis. D–I) PIAS1 modulates the expression of JUN and RRAD in response to ROS-dependent JNK activation. Primary HESCs were transfected either with PIAS1 siRNA (D, G), expression vector encoding WT or ligase-deficient PIAS1 (E, H), or siRNA targeting JNK (F, I). After 2 d, cultures were treated with 250 μM H₂O₂ for 8 h, and the abundance of JUN (D–F) and RRAD (G–I) transcripts was determined by RTQ-PCR. Data are presented as mean ± so fold induction. Results are representative of 3 independent experiments.

PIAS1 PROMOTES OXIDATIVE CELL DEATH
We speculated that many of the 110 genes that became H₂O₂-responsive on PIAS1 knockdown made the ≥2-fold threshold because of the lack of negative feedback and thus enhanced JNK activity. To test this hypothesis, we repeated the analysis of significantly regulated genes (P<0.05), but this time using a cutoff of ≥1.2-fold change (Fig. 4A, right panel). This analysis yielded many more regulated genes, and cross-referencing of the two gene lists confirmed that 67 of the 110 genes, which included JUN, were indeed responsive to ROS signaling when defined by the lower ≥1.2-fold threshold. However, the responsiveness of 43 genes to ROS remained strictly dependent on low cellular PIAS1 levels. The transcripts in this network, consisting of 30 induced and 13 down-regulated mRNAs, are listed in Supplemental Table S1. The induced genes were analyzed for gene ontology (GO) and molecular pathway enrichment with DAVID. After correction for multiple testing (Benjamini and Hochberg false discovery rate), a single GO category, “antiapoptosis,” was significantly enriched. We also applied this analysis to the genes regulated ≥2-fold in the presence of PIAS1 (Fig. 4A, left panel, and Supplemental Table S2), and this showed enrichment in two GO categories, “cell cycle process” and “phosphoprotein.”

The array data were validated by RTQ-PCR analysis of primary cultures first transfected with NT or PIAS1-targeting siRNA and then treated with H₂O₂ for 4, 8, or 12 h. As shown in Fig. 4B, PIAS1 knockdown not only enhanced the induction of certain ROS-sensitive genes (RRAD and GADD45A) but was conditional for the regulation of others, such as MCL1 and MDM2, which encode the antiapoptotic BCL-2 homologue Mcl-1 and the potent negative regulator of the p53 tumor suppressor Mdm2, respectively.

**PIAS1 promotes oxidative cell death in a SUMO-dependent manner**

The gene expression profiling experiments suggested that PIAS1 is an important modifier of the cellular oxidative stress response in human endometrium, partly through its ability to determine the level of JNK activity and partly by regulating the expression of specific gene networks. To test this conjecture, we monitored the degree of apoptosis induced by H₂O₂ on PIAS1 knockdown. As predicted by the GO analysis, PIAS1 silencing was sufficient to confer a degree of protection against oxidative apoptosis, as measured by the fraction of sub-G₁ cells on flow cytometry analysis of propidium iodide-stained cultures (Fig. 5A), or by the level of caspase-3 and caspase-7 activation (Fig. 5B). We then speculated that this protection must at least partially reflect the attenuated hypersumoylation response to ROS on PIAS1 knockdown. If correct, then enhancing cellular SUMO deconjugase activity should equally confer cytoprotection. As shown in Fig. 5C, overexpression of the SENP2 isopeptidase markedly prevented...
caspase-3 and caspase-7 activation on H₂O₂ treatment of HESCs. Combined, the data suggest that the PIAS1-dependent hypersumoylation response under environmental stress conditions is intricately linked to subsequent cell death.

DISCUSSION

The importance of the decidual process in ensuring survival of the offspring is aptly illustrated by the contrasting responses of undifferentiated and decidualizing HESCs to ROS. Compared to undifferentiated cells, decidualizing cells are remarkably resistant to oxidative cell death (8). Consequently, impaired decidualization predisposes for oxidative damage and cell death at the fetomaternal interface during pregnancy, which in turn underpins a spectrum of obstetrical complications ranging from early pregnancy loss to fetal growth restriction and preeclampsia (24). There are two established mechanisms that confer resistance of decidual cells to environmental ROS. First, decidualization markedly increases the scavenging potential of HESCs; second, it is associated with damping of selective stress-responsive pathways, especially JNK activation (6, 7). A major consequence of JNK silencing in differentiating HESCs cells is that the SUMO pathway becomes refractory to modest fluctuations in environmental ROS levels, thus ensuring that cellular homeostasis and hormone responses are maintained under the stress conditions imposed by pregnancy.

Our observations suggest that PIAS1 integrates ROS-dependent JNK activation, global hypersumoylation, and apoptosis in undifferentiated HESCs. PIAS1 is a multifaceted protein, best characterized for its ability to sense inflammatory signals and restrain the induction of NF-κB- and STAT1-responsive genes (16, 25–27). PIAS1 also binds chromatin directly, represses specific genes by recruiting DNA methyltransferases and other epigenetic modifiers, and is important for DNA-damage response (28, 29). It is increasingly apparent that the distinct functions of PIAS1 are tightly controlled by specific post-translational modifications. For example, IKKα-mediated phosphorylation of PIAS1 at Ser^90 restricts NF-κB activity (16), whereas methylation at Arg^303 is important for recruitment of PIAS1 to STAT1 target gene promoters (30). Further, CK2 phosphorylation of 3 residues (Ser^466–468), located within the SUMO-interacting motif (SIM), mediates SUMO tethering and modulates the transcriptional coregulator function of PIAS1 (17).

Several lines of evidence indicate that PIAS1 senses environmental ROS by being a downstream target of JNK signaling. First, JNK phosphorylation, in response to ROS or ΔMEKK1:ER* activation, correlated with a transient mobility shift of PIAS1 on SDS-PAGE. This mobility shift was attenuated in response to decidualization or in undifferentiated cultures first transfected with JNK siRNA, and lost when protein lysates were first incubated with PPase. Recombinant JNK also phosphorylated immunopurified PIAS1 in vitro. The presence of 3 highly conserved putative MAP kinase phosphorylation sites in PIAS1 further suggested that its function could be controlled by JNK signaling. Two of these sites are in proximity to the PIAS1 SIM module, yet mutation of all three residues did not discernibly alter the SDS migration of PIAS1 species on JNK activation. LC-MS/MS and additional targeted mutations of known phosphorylation sites (e.g., Ser^90 and Ser^466–468, data not shown) were equally uninformative. However, the ligase-deficient PIAS1 mutant (W372A/C351S) was not modified by ROS in vitro or phosphorylated by JNK in vitro (Supplemental Fig. S2E), which may indicate that disruption of the RING-finger motif induces a configurational change that masks putative phosphorylation sites.

PIAS1 has been shown to regulate JNK activity in other cell types. For example, overexpression of PIAS1 in 293T cells or human osteosarcoma U2OS cells is
sufficient to activate JNK (31). One proposed mechanism through which PIAS1 may function upstream of JNK involves sumoylation of Axin, a signal intermediate that interacts with MEKK1 (32). In contrast to these reports, PIAS1 does not serve as positive regulator of JNK activity in HESCs but instead dampens this signaling pathway, independently of its ligase function. PIAS1 also inhibited JNK phosphorylation in response to ΔMEKK1:ER activator, suggesting that the mechanism of negative feedback involves either disabling signal transduction from MEKK1 to JNK or perhaps induction or activation of specific phosphatases.

A striking consequence of JNK activation in HESCs is increased SUMO modification of a variety targets, including c-Jun, in a PIAS1-dependent manner. Activated JNK phosphorylates c-Jun at Ser63 and Ser73, leading to increased transcriptional activity (21). In contrast, sumoylation by PIAS1 negatively regulates c-Jun activity (22). Moreover, these two post-translational modifications in c-Jun are mutually antagonistic, as mutation of Ser63 and Ser73 greatly enhances conjugation by SUMO-1 (33). We used JUN expression as a likely endogenous reporter for c-Jun/AP-1 activity in HESCs. As anticipated, PIAS1 knockdown enhanced JUN expression on H2O2 treatment, which could be accounted for by either increased JNK signaling or decreased c-Jun sumoylation. However, overexpressed WT PIAS1 and the ligase-deficient mutant were equally effective in attenuating JUN induction, suggesting that the JNK-dependent phosphorylation but not sumoylation of c-Jun determines the level of autoregulation of this gene, at least at the time point examined.

Cellular PIAS1 levels also determined the amplitude of RRAD induction in response to ROS, in keeping with the negative feedback on JNK activity. However, genome-wide expression profiling provided compelling evidence that PIAS1 also directs the nature of the transcriptional response in HESCs exposed to oxidative stress. Depending on the threshold used in the analysis (2-fold, 5-fold, and 10-fold), 30–40% of all ROS-sensitive genes were found to be PIAS1 independent. PIAS1 knockdown was a prerequisite for the regulation of 40–44% of genes, a substantial majority of which were induced on H2O2 treatment. Conversely, the presence of PIAS1 was a requirement for ROS-dependent regulation of a smaller fraction (17–24%) of genes, three-quarters of which were repressed. Perhaps the most salient observation was that PIAS1 knockdown enables the induction of various antiapoptotic and antistress genes in response to ROS signaling (e.g., MCL1, MDM2, RIPK2, UBA2, HSPA9). In agreement, PIAS1 knockdown protected HESCs from oxidative cell death.

Sumoylation generally bestows repressive properties onto transcription factors (34–36). This fits well with the observation that PIAS1 knockdown results in 3 times more up-regulated genes on H2O2 stimulation. To provide more insights into the functional consequences of the PIAS1-dependent hypersumoylation response, we considered various strategies for disabling the SUMO pathway prior to exposure of HESCs to ROS. We decided against siRNA-mediated knockdown of key enzymes (e.g., Ubc9) as this approach requires several days to be effective, by which time a multitude of cellular functions may already be perturbed. Pretreatment of HESCs for 2 h with ginkgolic acid, an alkylphenol that blocks the formation of the E1-SUMO thioester complex (37), was sufficient to inhibit ROS-induced sumoylation, but this compound also firmly suppressed JNK activation (data not shown), in agreement with a previous report (38). However, overexpression of the SUMO-specific protease SENP2 markedly protected cells from ROS-dependent caspase-3 and caspase-7 activation. Thus, it appears likely that the ability of PIAS1 to drive cellular sumoylation in response to environmental stress signals is important for the subsequent oxidative cell death response.

In summary, by relaying JNK signaling to the SUMO pathway, PIAS1 determines the fate of endometrial cells under oxidative stress conditions. It exerts this function through two seemingly opposing and independent mechanisms (Fig. 6). On the one hand, cellular PIAS1 levels modulate the amplitude of JNK activation in response to ROS, a function that does not require ligase activity. On the other, PIAS1 is rapidly modified in response to JNK activation, perturbs the cellular sumoylation-desumoylation equilibrium, disables induction of survival genes, and promotes apoptosis. Consequently, down-regulation of PIAS1 on decidualization of HESCs protects against oxidative cell death, while at the same time other mechanisms limit unrestrained JNK signaling, such as the induction of MKP-1 (7).

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