PARP1 during embryo implantation and its upregulation by oestradiol in mice

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Abstract

Pregnancy requires successful implantation of an embryo, which occurs during a restricted period defined as ‘receptivity of the endometrium’ and is influenced by the ovarian steroids progesterone and oestradiol. The role of poly(ADP-ribose)polymerase-1 (PARP1) in apoptosis is well established. However, it is also involved in cell differentiation, proliferation and tissue remodelling. Previous studies have described the presence of PARP in the uterus, but its exact role in embryo implantation is not yet elucidated. Hence, in this study, we studied the expression of PARP1 in the uterus during embryo implantation and decidualisation, and its regulation by ovarian steroids. Our results show upregulation of the native form of PARP1 (~116 kDa) in the cytosolic and nuclear compartments of implantation and non-implantation sites at day 5 (0500 h), followed by downregulation at day 5 (1000 h), during the embryo implantation period. The transcript level of Parp1 was also augmented during day 5 (0500 h). Inhibition of PARP1 activity by the drug EB-47 decreased the number of embryo implantation sites and blastocysts at day 5 (1000 h). Further, cleavage of native PARP1 was due to the activity of caspase-3 during the peri-implantation stage (day 5 (0500 h)), and is also required for embryo implantation, as inhibition of its activity compromised blastocyst implantation. The native (~116 kDa) and cleaved (~89 kDa) forms of PARP1 were both elevated during decidualisation of the uterus. Furthermore, the expression level of PARP1 in the uterus was found to be under the control of the hormone oestrogen. Our results clearly demonstrate that PARP1 participates in the process of embryo implantation.

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Introduction

In mammals, embryonic development takes place in the uterus, requiring the implantation of an embryo in a specific time frame known as the window of uterine receptivity. In this period, the uterus acquires maximal receptivity for the implantation of the blastocysts (Duc-Goiran et al. 1999). During implantation, uterus and embryo synchronise for their intimate interaction (Burghardt et al. 2002). The endometrium undergoes many morphological changes, including cell proliferation, differentiation and apoptosis, to provide the best environment for implantation of the embryo (Demir et al. 2002, Joswig et al. 2003, Correia-da-Silva et al. 2004). However, when embryo implantation occurs, many changes begin in the endometrium, where stromal cells proliferate in response to oestrogen and progesterone to form the decidual cells, a process called decidualisation (Dey et al. 2004, Lei et al. 2012). Poly(ADP-ribose) polymerase (PARP) is reported to be linked with the tissue remodelling process (Pagano et al. 2007), which has already been studied in the uterus (Ghabreau et al. 2004, Brustmann 2007, Postawski et al. 2011). Furthermore, the double mutation of both ataxia–telangiectasia (Atm) and Parp1 genes in the mouse leads to an early post-implantation lethality of the embryo at embryonic day 8.0 (E8.0), with extensive cell death in the embryo at E11.5 (Menisser-de et al. 2001). These studies suggest a possible role of PARP in the reproduction process.

Although PARP1 is a hallmark of apoptosis, it is also reported to be involved in other cellular signalling, e.g. expression of adhesion molecules (von Lukowicz et al. 2008), transcriptional regulation of cyclooxygenase-2 (Lin et al. 2011), transforming growth factor-β1 (TGF-β1) receptor (Sterling et al. 2006), syndecan-4 (Lacal et al. 2009), NF-κB (Kauppinen et al. 2013), SMAD3 (Huang et al. 2011), sex determining region Y-Box-2 (Musard et al. 2001) and E-cadherin (McPhee et al. 2008), which are essential during embryo implantation (Nakamura et al. 2004, San et al. 2004, Jha et al. 2006, Lin et al. 2006, St-Louis et al. 2010). Furthermore, cell differentiation, cell
proliferation (Lei et al. 2009, Kaloglu & Onarlioglu 2010, Macdonald et al. 2011, Afshar et al. 2012) and the tissue remodelling process also take place in the uterus (Fazleabas & Strakova 2002, Rosario et al. 2003, Kaloglu & Onarlioglu 2010), and can be influenced by PARP1 (Pagano et al. 2007, Kobayashi 2011). In endometrial carcinoma, PARP1 can regulate the progesterone receptor (PR; Ghabreau et al. 2004), suggesting the involvement of PARP1 in ovarian steroid signalling. Conversely, the activity of PARP can be under oestradiol regulation (Suzuki et al. 1990), suggesting cross-talk between PARP1 and ovarian steroid signalling. These findings raise the possibility of association of PARP1 with uterine receptivity for successful embryo implantation. Therefore, in this study, we explored the role of PARP1 in the uterus during embryo implantation and also examined its regulation by ovarian steroids.

Materials and methods

Reagents

Anti-PARP1 (sc25780), anti-STAT3 (sc483), anti-caspase-3 (sc7148), caspase-3 inhibitor (sc300325) and inhibitor of PARP1 (EB-47, sc222125) were obtained from Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA and have already been characterised (Jagtap et al. 2004, Watzlakw et al. 2010). Cleaved PARP1 (Asp214, 7C9), mouse mAb (Mouse Specific, 9548) and p-STAT3 (Serine 727) (91345) were from Cell Signaling Technology Inc., Danvers, MA, USA. The secondary antibody conjugated with HRP (goat anti-rabbit IgG, 621140380011730) was from Merck-Millipore, Molsheim, France. The steroids hormones (progesterone and oestrogen), phosphatase inhibitor cocktail (P5726), protease inhibitor (S8830), anti-β-actin (A3854), Trizol reagent (T9424) and anti-histone H-3 (H9289) were from Sigma–Aldrich, Inc. The Immobilon-P PVDF membrane (0.2 μm) and ECL reagent (WBKLS0500) kits were from Merck-Millipore, MA, USA and GE Healthcare Life Sciences, Pittsburgh, PA, USA respectively. Superscript III first-strand cDNA synthesis supermix (18080-400) and Platinum blue PCR supermix (12580-015) were from Life Technology, Carlsbad, CA, US. Light cycler 480 SYBR green 1 master (04707516001, lot 14555300) was purchased from Roche Applied Science, Indianapolis, IN, USA. PCR primers of PARP1 and β-actin were synthesised by Integrated DNA Technology (IDT), Leuven, Belgium. The caspase-3 activity assay kit (K106-100) was supplied by BioVision Inc., Milpitas, CA, USA. Protein standard (SM0671) was purchased from Fermentas, St. Leon-Rot, Germany.

Experimental animals

The entire study was conducted on Mus musculus (Swiss strain, albino) as used earlier (Jha et al. 2006, Maurya et al. 2013). Animals (out-bred) were 3 months old and they were housed in polycarbonate cages under a 12 h light:12 h darkness regimen with a controlled temperature of 27 ± 1 °C. All the mice had free access to food and water. The animal experimental protocol was in accordance with the guidelines of the CSIR-CDRI Animal Ethical Committee, Lucknow, UP, India.

Embryo implantation mouse model

Pregnancy was established in mice by caging sexually mature (3–4 months old) and virgin females with males (3–4 months old) of proven fertility. Pregnancy was confirmed by the presence of a whitish vaginal plug the following day, designated day 1 of pregnancy (Pakrasi & Jain 2008). Female mice were killed at different days of embryo implantation i.e. pre-implantation (day 4 (1000 h)), late pre-implantation (day 4 (1600 h)), peri-implantation (day 5 (0500 h)) and post-implantation (day 5 (1000 h)) according to a previous study (Maurya et al. 2013). During peri- and post-implantation stages, implantation sites were rendered visible via tail-vein injection of 100 μl Evans blue dye (1%) suspension in saline. Extra-vascular Evans blue dye accumulation in the areas of blastocyst-induced increased capillary permeability reveals sites of implantation as discrete blue bands (Psychoyos 1986). The uterine tissue was obtained by the cervical dislocation method. The implantation sites were demarcated by discrete blue bands (Evans blue stain) and the spaces between the blue bands (without/faint colour) were termed inter-implantation or non-implantation sites (Kondoh et al. 2009). The Evans blue coloured implantation sites and the non-/less coloured non/inter-implantation regions were separated and processed for protein extraction.

Experimental decidualisation induction in pseudopregnant mice

Experimental decidualisation was induced in pseudopregnant female mice. The pseudopregnancy was obtained by natural mating with vasectomised male mice. The male mice were vasectomised as described previously (Itttner & Gotz 2007) and allowed to rest for a week to recover from the injury. Thereafter, sexually mature female mice were housed with these vasectomised males. Females displaying a whitish vaginal plug on the following day were considered pseudopregnant. The presence of a vaginal plug confirmed successful mating and the day was designated day 1 of pregnancy. Experimental decidualisation was stimulated in the pseudopregnant female mice at day 3 (1600 h) by the introduction of an intra-uterine lumen infusion of 10 μl corn oil into one uterine horn lumen. The contralateral non-infused horn served as a control non-decidualised horn. The decidualised and non-decidualised uterine horns were collected 72 h after corn oil infusion (Nakamura et al. 2006).

Delayed implantation mouse model

Delayed implantation was induced in the pregnant female mice after confirmation of their successful mating by the observation of a vaginal plug. The ovaries of the pregnant female mice were excised by bilateral ovariectomy on day 3 at 1600 h. This process was carried out under ketamine (30 mg/kg body weight) and xylazine (4 mg/kg body weight) anaesthesia, under aseptic conditions. The ovariectomised female mice were divided randomly into two groups comprising a minimum of five animals each. Group 1 animals received progesterone (1 mg/20 g body weight) at 1700 h from day 3 to day 7 of the
pregnancy, while group 2 animals received progesterone from day 3 to day 7 along with oestradiol on day 7 (25 ng/20 g body weight). These steroids were administered subcutaneously. Progesterone (4-pregn-3,20-dione) was dissolved in corn oil at a concentration of 20 mg/ml and oestrogen (1,3,5(10)-oestratriene-3,17-β-diol) (β-oestradiol) was dissolved in corn oil at a concentration of 500 ng/ml. The hormone treated mice were killed at 1700 h on day 8 of pregnancy by cervical dislocation. The uterine horn was flushed gently to obtain embryos and the collected embryos from various stages of delayed pregnancy were verified as reported earlier (Maurya et al. 2013). Non-implanted or hatched dormant blastocysts were seen in the progesterone-treated animal group, whereas in the progesterone +17β-oestradiol-treated group implanted blastocysts were visible by their trophoblast cell protrusions sites (Paria et al. 1993, Lee et al. 2011, Maurya et al. 2013). The uterine adherent fat was cleared by washing thoroughly in PBS and processed for protein extraction.

**Protein extract (cytosolic and nuclear) preparation from the uterus**

To isolate the uterine cytosolic protein fraction, excised uterus (~100 mg) was minced thoroughly in 200 μl buffer containing 100 mM KCl, 3 mM NaCl, 3.5 mM MgCl₂, 10 mM Pipes, 1.5 mM EGTA, phosphatase and protease inhibitor (pH 7.4) in accordance with the previously described method (Maurya et al. 2013). This was followed by homogenisation of uterine tissue and centrifugation at 4 °C, 200 g to separate unbroken cells. Then the 200 g supernatant was centrifuged at 1475 g to remove nuclei. The post-nuclear supernatant was further centrifuged at 12,000 g (10 min at 4 °C); the resultant supernatant, designated the whole uterine tissue crude cytosolic protein extract, was stored at −80 °C until use.

The nuclear pellet obtained by centrifugation at 1475 g was reconstituted in hypotonic buffer (20 mM HEPES, pH 7.9; 1 mM EDTA, 10 mM KCl, 1 mM EGTA, 1 mM MgCl₂, 1 mM dithiothreitol (DTT) and 400 mM NaCl, pH 7.8) containing protease and phosphatase inhibitor cocktail and incubated in a rocking mode at 4 °C for 30 min. Thereafter, the mixture was centrifuged at 20,800 g at 4 °C for 30 min (Davoodi-Semiromi et al. 2004). The supernatant (nuclear extract) was collected and stored at −80 °C. Protein concentration was determined using the Bio-Rad DC protein assay kit method and the protein concentration was adjusted to 20 μg.

**SDS-PAGE and western blotting**

Uterine cytosolic and nuclear protein fractions were denatured in Laemmli buffer (Laemmli 1970, Laemmli et al. 1970) containing β-mercaptoethanol followed by heating at 95 °C for 5 min. A total of 20 μg protein was loaded into each well and resolved on 10% SDS-PAGE at 100 V using the Tetra-Protein Cell vertical electrophoretic system (Bio-Rad). Then the proteins were electro-blotted onto PVDF membrane using transfer buffer containing 20% v/v methanol, 25 mM Tris, pH 8.2, 190 mM glycine at 50 mA per gel for 12 h (Towbin et al. 1992).

**Immunoblotting**

The blotted membranes were first incubated with 5% non-fat milk blocker in PBS containing 0.1% Tween-20 (PBS-T) for 2 h. Then they were incubated with the respective primary antibody, i.e., for PARP1 (1:1000 dilution), caspase-3 (1:500 dilution), STAT3 (1:200 dilution), p-STAT3 (1:500 dilution), histone H-3 (1:3000 dilution) and β-actin (1:3000 dilution) for 2 h at room temperature in the presence of 2% milk blocker. Finally, membranes were probed with secondary antibody (anti-rabbit IgG) conjugated with HRP in PBS-T containing 2% milk blocker. PBS-T was used throughout the procedure and each step was followed by three washes with PBS-T. The dilution of the secondary antibody (anti-rabbit IgG HRP) was 1:3000 for PARP1 and β-actin antibodies. The blots of histone H-3 were incubated with anti-rabbit IgG HRP at 1:4000 dilution. The presence of antibody–protein complexes on the blot was detected with the ECL/ECL plus kit. Immunoblots were imaged through the chemiluminescence documentation system (ImageQuant LAS 4000, GE Healthcare Life Science). Antibody specific for β-actin was used as an internal loading control for proteins. The protein bands were scanned densitometrically using the TotalLab Quant software (Nonlinear Dynamics, Newcastle upon Tyne, UK). Results are expressed as a ratio (protein of interest/β-actin) to correct for the loading of each sample.

**PARP1 in vivo assay**

Each male mouse (Swiss Strain, 3 months old) was co-habited with two females. Day 1 of pregnancy was the day when a whitish vaginal plug was seen in the morning after mating. Animals were anaesthetised with ketamine (30 mg/kg) and xylazine (4 mg/kg) at day 4 (1000 h) of pregnancy. The anaesthetised animal was placed in a dorso-ventral position, a cut of ~1 cm was made in the dorsal mid-lumbar area to visualise the uterus, and an inhibitor of PARP1 (EB-47, 2 μM concentration and 3 μl volume; Jagtap et al. 2004) was infused via the intra-uterine route into the left horn of the uterus and the corresponding vehicle (sterile distilled water, 3 μl volume; Jagtap et al. 2004) was infused into the right horn. On day 5 (1000 h) of pregnancy, Evans blue dye was injected into the mice via the tail vein 1 h before they were killed. The animals were killed by cervical dislocation and the numbers of implanted and non-implanted sites in PARP1-inhibited as well as sham-treated horns were observed. Thereafter, the number and morphology of blastocysts were recorded.

**Caspase-3 in vivo assay**

Intra-luminal delivery of caspase-3 inhibitor was performed on day 4 (1000 h) and its effect on embryo implantation (sites of embryo implantation) was evaluated by administering Evans blue dye (0.01% in PBS). Pregnant female mice were anaesthetised with ketamine (30 mg/kg) and xylazine (4 mg/kg) and placed in a dorso-ventral position. Using a surgical blade, an incision (1–2 cm) was made near the dorsal mid-lumbar area just below the kidney region. The uterine horn was held gently with forceps and caspase-3 inhibitor (5 μg/3 μl) was delivered into the left uterine horn while the corresponding
vehicle (3 μl volume) was infused into the right horn. Both the dermis and skin were stitched back with sutures. On day 5 (1000 h) of pregnancy, Evans blue dye (1%) in PBS was given via the tail vein, the mice were killed by cervical dislocation, and implantation sites and implanted embryos were counted.

Caspase-3 activity assay

Caspase-3 activity assay was performed in the uterine samples by means of a caspase-3/CPP32 colorimetric assay kit (K106-100) as per the manufacturer’s instructions. Briefly, a total of 50 μg protein from the uterus (cytosolic+nuclear) fraction was added to 50 μl of 2 × reaction buffer (containing 10 mM DTT), followed by 5 μl of 4 mM DEVD-pNA substrate. The suspension was incubated at 37 °C for 1–2 h and absorbance was recorded at 405 nm with a micro plate reader (ELISA plate reader, BioTek, Winooski, VT, USA).

PCR

All the experiments were performed under RNase-free conditions. Total RNA was extracted from uterine tissue using Trizol reagent according to the manufacturer’s protocol. The concentration of RNA was measured by Nanodrop (Thermo Fisher Scientific, Wilmington, DE, USA). Uterine tissue cDNA was synthesised using 1 μg RNA and the SuperScript III cDNA synthesis kit as per the manufacturer’s instructions. Each semi-quantitative PCR had the following components: 1 μl of RT product, 23 μl of Platinum blue PCR Super Mix and 20 pmol of forward and reverse primers. PCR was performed on a C1000 thermal cycler (Bio-Rad Laboratories) for 38 cycles (95 °C for 1 min; 53 °C for 1 min, 72 °C for 1 min and a final extension of 10 min at 72 °C) after an initial 5 min incubation at 95 °C. The primer sequences used to detect the Parp1 gene were 5'-CACCCCTCAAGAAGACGA-3' (sense) and 5'-CTCT-TCTTGTCCAGACTCAG-3' (antisense). The expression of β-actin (primers 5'-TACTCCTGCTTGGTTA TCCAC-3' (sense) and 5'-ATCTGGACACCCACCTCTAC-3' (anti-sense)) was used as endogenous control (Spina-Purrello et al. 2010). Amplified products were resolved in 1.5% agarose gel and imaged using the Gel documentation system (Bio-Rad). Band intensity was analysed using Total Lab Quant 1D software version 5.01 (Nonlinear Dynamics) and mean value, along with S.D., error and t-test, were calculated.

Real-time PCR

Quantitative RT-PCR was carried out on a LightCycler 480 instrument II (Roche Diagnostics). Uterine cDNA was diluted with nuclease-free water. One microlitre of cDNA was amplified with nuclease-free water. The primers used for Parp1 were 5’-GGACGAGGCGATCAAGAC-3’ (sense) and 5’-TGAGTGATAGAGC-3’ (antisense) (NC_000067.6). The primers used for the reference gene (β-actin) were 5’-AGCCATGTACGTAGCCATCC-3’ (sense) and 5’-GCTGGTGGTGAAGCCTGA-3’ (antisense) (NC_000071.6). These primers were intron-spanning and were designed by means of Primer3 (http://bioinfo.ut.ee/primer3-0.4.0/) to amplify a <200 bp product. Thermal cycling was carried out as follows. The first segment of the amplification cycle consisted of denaturation at 95 °C for 10 min; the second segment consisted of a four-step denaturation (15 s at 95 °C), primer annealing (30 s at 58 °C) and extension (30 s at 72 °C) for 40 cycles. The third segment consisted of a melting curve programme (95 °C for 5 s, 58 °C for 15 s). The final segment consisted of cooling to 40 °C. The threshold cycle (Ct) was used to represent the relative mRNA amounts. All samples were run and analysed in triplicate using the 2^-ΔΔCt method (Livak & Schmittgen 2001).

Statistical analysis

All the experiments were repeated a minimum of three times using separate animals for each replicate (n=5). The protein band intensity was analysed by TotalLab Quant gel analysis software version 5.01 (Nonlinear Dynamics). Protein band intensities were averaged and the S.E.M. was calculated. All values were expressed as the mean ± S.E.M. of at least three independent experiments. Histograms were plotted of protein band intensity for various stages/groups using Microsoft Excel 2007. The band intensities obtained from the immunoblots were compared within the phases of embryo implantation using Student’s paired t-test. The data were subjected to one-way ANOVA using Microsoft Excel 2007. The statistical significance level was considered to be 0.05 for all tests analysed. β-Actin antibody was used to normalise the PARP1, caspase-3 and STAT3 values. Histone H-3 was used as a nuclear compartment marker.

Results

Expression of PARP1 is upregulated in the uterus during embryo implantation

Expression of native PARP1 protein in uterine protein extracts was studied by immunoblotting to examine its relation with endometrial receptivity/implantation. The invasive period in rodents is day 4–10 of pregnancy, when the endometrial stromal cells undergo extensive remodelling in response to blastocyst implantation; the loss of the luminal epithelium is evident at this stage (Enders 2000, Kennedy et al. 2007). Therefore, we analysed PARP1 expression levels at day 4 (1000 h), day 4 (1600 h), day 5 (0500 h) and day 5 (1000 h) of embryo implantation. We separated implantation and non-implantation sites at the peri-implantation to post-implantation stages and analysed the expression levels of the native and cleaved forms of PARP1 in the cytosol as well as in the nuclear compartment (Figs 1 and 2).

The native form of PARP1 (~116 kDa) was present at a basal level in the cytosol at the pre-implantation (day 4 (1000 h)) and late pre-implantation stages (day 4 (1600 h)) (Fig. 1A and B). The expression level of native PARP1 was higher at implantation sites at the peri-implantation stage (day 5 (0500 h)) (P<0.05) than at the
late-pre-implantation stage (Fig. 1A and B). However, it showed a no change at non-implantation sites during the peri-implantation period (day 5 (0500 h)) \((P<0.04)\). At the following stage (day 5 (1000 h)), there was no change in the expression level of native PARP1 at implantation sites (Fig. 1A and B). We observed a fall in the expression level of native PARP1 at non-implantation sites at post-implantation stage (day 5 (1000 h)) \((P<0.04)\) compared with implantation sites (Fig. 1A and B). Similar results were observed for the cytosolic cleaved form of PARP1 \((\sim 89 \text{kDa})\), which was noted to be higher during late pre- to peri-implantation stages, and declined at post-implantation stage \((P<0.02)\) (Fig. 1C and D).

We also studied the native and cleaved forms of PARP1 in the nuclear fraction. We observed an augmentation in the expression level of the native form during day 4 (1600 h) and day 5 (0500 h) in comparison with day 4 (1000 h) \((P<0.003\) and \(P<0.0001\) respectively) (Fig. 2A and B), and this level remained high at the non-implantation sites at day 5 (0500 h). At the advanced stage (day 5 (1000 h)), the expression level of native PARP1 was downregulated \((P<0.005)\) (Fig. 2A and B), and no difference was observed at the non-implantation sites at day 5 (1000 h). Expression of the cleaved form of PARP1 \((\sim 89 \text{kDa})\) followed a similar pattern (Fig. 2C and D).

**Figure 1** Determination of expression level of PARP1 in the uterine cytosol. Using immunoblotting, the expression level of PARP1 (native and cleaved form) was evaluated in the uterine cytosolic compartment of mice during different stages of embryo implantation (A, B, C and D). The expression of cytosolic PARP1 was analysed by densitometry (B and D). \(\beta\)-actin was used as a loading control for the immunoblot of PARP1 in the cytosolic protein fraction.
Expression level of nuclear native PARP1 during embryo implantation

Expression level of cleaved PARP1 in the nuclear fraction

Relative expression level of native PARP1 in the nuclear fraction

Relative expression level of cleaved PARP1 in the nuclear fraction

Figure 2 Expression of the native and cleaved forms of PARP1 in the nuclear compartment of the uterus. The native and cleaved forms of PARP1 in the nuclear protein fraction isolated from the uterus during embryo implantation were analysed simultaneously (A, B, C and D). β-actin was used as a loading control for the immunoblot of PARP1 in the nuclear protein fraction. Histone H3 was used as a nuclear compartment marker.
To investigate the transcript level of Parp1, we carried out PCR using cDNA (synthesised from isolated mRNA) from various stages of implantation. Our study revealed a low/basal level of Parp1 transcript at day 4 (1000 h), and an elevated level at late pre-implantation stage (day 4 (1600 h)) ($P<0.01$) (Fig. 3A and B). The expression level of Parp1 transcript was further increased at day 5 (0500 h) (implantation and non-implantation sites) ($P<0.009$), but at the advanced stage, post-implantation (day 5 (1000 h)), this expression was downregulated ($P<0.01$) (Fig. 3A and B). Similar results were exhibited by quantitative real-time PCR analysis. Using qRT-PCR, Parp1 transcripts were detected at all of the stages of implantation that were studied. We observed that the expression level of Parp1 was maximum (twofold) at implantation sites at day 5 (0500 h), whereas it was least at day 4 (1000 h Fig. 3C). The non-implantation sites showed reduced expression of Parp1 compared with implantation sites at day 5 (0500 and 1000 h Fig. 3C). The mRNA expression levels of Parp1 correlated well with the protein expression data.

**Elevated expression of PARP1 and caspase-3 in the decidualised uterus**

Decidualisation involves stromal cell proliferation and differentiation into morphologically distinct decidual cells as a remodelling process, followed by embryo implantation (Fazleabas & Strakova 2002). In fact, programmed cell death also occurs in the uterus (Pampfer & Donnay 1999), and we speculated that PARP1 might be involved in this process. Therefore, we next investigated the relation of PARP1 with decidualisation in the uterine cytosol using western blotting. The native form of PARP1 ($\sim 116$ kDa) was detected in protein samples of uterine tissue of decidualised and non-decidualised groups (Fig. 4A). Decidualised uterus showed increased expression of native PARP1 in comparison with the non-decidualised group ($P<0.03$) (Fig. 4A and B). Later, we examined the cleaved form of PARP1 in the decidualised uterus and its expression level was noted to be similar to that of native PARP1 (Fig. 4C and D).

PARP1 is a known substrate for caspase-3 (Brustmann 2007); therefore, to confirm the cleavage of PARP1 in the decidualised uterus, we investigated the action of caspase-3. We analysed the expression levels of pro-caspase-3 and caspase-3 (p17/active caspase-3) in the decidualised uterus. Our results showed a non-significant difference ($P>0.05$) in pro-caspase-3 expression between the decidualised and non-decidualised uterus groups, whereas active caspase-3 was found to be significantly elevated in the decidualised group in comparison with the non-decidualised group ($P<0.0001$) (Fig. 4E and F).

**Effect of PARP1 inhibition on embryo implantation**

To confirm the functional role of PARP1 during embryo implantation, we administered an inhibitor of PARP1 during the pre-implantation period and observed its effect on the post-implantation stage. Intra-luminal delivery of PARP1 inhibitor at the pre-implantation stage showed a reduction in embryo implantation sites ($P<0.01$) and blastocyst numbers ($P<0.01$) (Fig. 5A, B, C and D). To examine the signalling of PARP1 for embryo implantation in the receptive uterus, we determined the expression level of STAT3 in the PARP1-inhibited group. STAT3 is known to be an essential signalling molecule for embryo implantation/uterine receptivity (Nakamura et al. 2006). We observed a compromised expression level of STAT3 at the implantation sites of the PARP1-inhibited group compared with the sham/vehicle-treated group ($P<0.04$) at day 5 (1000 h) (Fig. 5E and F). A similar pattern was noticed at the non-implantation sites ($P<0.008$) (Fig. 5E and F). Further, active (phosphorylated) STAT3 demonstrated downregulation at the non-implantation sites of the PARP1-inhibited uterus group compared with the implantation sites of the sham and PARP1 inhibitor-treated groups ($P<0.05$, $P<0.009$) (Fig. 5G and H). No significant difference was observed between the implantation sites of the PARP1-inhibited and sham-treated groups (Fig. 5G and H). The PARP1 inhibition effect was also seen in the non-implantation sites containing STAT3, where the expression level of STAT3 was decreased (Fig. 5E and F). The activity of STAT3 was maintained in the implantation sites and decreased in the non-implantation sites at the day 5 (1000 h) stage (Fig. 5G and H).

**Consequence of inhibition of caspase-3 activity on day 4 (1000 h) embryo implantation**

In mice and hamsters, uterine cells undergo apoptosis during days 5–8 of pregnancy (Zhang & Paria 2006). Therefore, we determined the activity of caspase-3 during the stages of embryo implantation. The activity of caspase-3 was moderate during the pre-implantation stage, and augmented at late-pre-implantation stage ($P<0.005$) (Fig. 6A). The activity was further increased at implantation sites at day 5 (0500 h), but at the following stage, day 5 (1000 h), demonstrated reduced activity. Non-implantation sites at day 5 (0500 h) showed decreased activity of caspase-3 compared with implantation sites (Fig. 6A).

To confirm the action of caspase-3 at day 5 (1000 h), its activity was analysed in the post-caspase-3-inhibited uterus. As expected, the activity of caspase-3 was significantly reduced in the treated samples compared with sham-treated/control samples at both implantation ($P<0.005$) and non-implantation ($P<0.05$) sites at day 5 (1000 h) (Fig. 6B).
As shown in Fig. 6C and D, administration of caspase-3 inhibitor at pre-implantation stages reduced the number of sites of embryo implantation compared with the sham-treated group ($P < 0.03$; Fig. 6C and D). The number of embryo implantation sites (blue bands) was recorded visually; the inhibitory effect of caspase-3 activity blocker was nearly 70% (Fig. 6D).

PARP1 is a substrate of caspase-3; therefore, to confirm the action of caspase-3 on PARP1, we measured the expression levels of the cleaved and native forms of PARP1 protein in the caspase-3-inhibited uterine protein samples using western blotting. The expression level of the cleaved form of PARP1 was seen to be decreased in caspase-3-inhibited samples (Fig. 6E and F) from Parp1 β-actin

[Graph showing relative expression level of Parp1 gene transcript with statistical significance marks around the bars, indicating $P > 0.05$, $P < 0.009$, $P < 0.01$, $P > 0.05$, and $P < 0.01$ as relevant p-values.]

**Figure 3** Analysis of the gene transcript level of PARP1 during embryo implantation. PCR and real-time PCR were used to analyse the expression levels of the Parp1 gene transcript in the uterus at different stages of embryo implantation (A, B, C). β-actin was used as a reference gene.
Figure 4 Association of the native and cleaved forms of PARP1 with decidualisation. The expression level of the native form of PARP1 in the crude cytosolic fraction from the uterus was studied using immunoblotting (A and B). The cleaved form of PARP1 was also investigated in the decidualised and non-decidualised uterus (C and D). The expression levels of pro- and active caspase-3 were also analysed in the decidualised and non-decidualised uterus (E and F). The values of PARP1 and caspase-3 expression in the cytosolic compartment were normalised with the β-actin of the corresponding replicate.
Figure 5 Effect of inhibition of PARP1 on embryo implantation sites. The role of PARP1 at embryo implantation sites at day 5 (1000 h) was studied by administering an inhibitor at day 4 (1000 h) (A, B, C, D). Further, the response of PARP1 inhibition to STAT3 signalling was determined by measuring the expression levels of both total STAT3 and its phosphorylated form (E, F, G, H).
Role of PARP1 in embryo implantation

Figure 6 Analysis of PARP1 cleavage and embryo implantation sites in response to caspase-3 inhibition. Activity of caspase-3 was determined in the uterus during different stages of embryo implantation (A). Further, the activity of caspase-3 was evaluated in the uterus after inhibition on day 4 (1000 h) (B). Inhibition of caspase-3 reduced the number of embryo implantation sites (C and D). Additionally, immunoblot analysis of cleaved PARP1 was done following caspase-3 inhibition at day 5 (1000 h) (E and F). The expression level of native PARP1 (116 kDa) was analysed at day 5 (1000 h), after caspase-3 treatment (G and H).
implantation sites ($P<0.02$) compared with the sham-treated group. The intensity of the band due to the cleaved form of PARP1 from the non-implantation sites was reduced for the sham-treated as well as caspase-3-treated uterus groups ($P<0.005$; Fig. 6E and F). Expression of the native form of PARP1 was unaffected at the implantation sites of sham-treated and PARP1 inhibitor-treated uterus, but higher than at the non-implantation sites of the respective groups (Fig. 6G and H).

**Hormonal regulation of PARP1 and caspase-3 expression during delayed embryo implantation**

Establishment of the receptive uterus to support embryo development and implantation is primarily coordinated by ovarian hormones, which modulate uterine events in a spatiotemporal manner. Oestrogen and progesterone prime the uterus for implantation (Dey et al. 2004); thus, these ovarian steroid hormones are the key factors influencing embryo implantation. Expression of the native form of PARP1 was found to be low in the progesterone-treated uterine group (Fig. 7A and B). It showed a sudden increase in expression in the progesterone primed oestriadiol group ($P<0.03$) (Fig. 7A and B). The cleaved form of PARP1 was assayed in the uterus treated with progesterone alone and in combination with oestriadiol. Progesterone treatment gave a lower level of cleaved PARP1 (Fig. 7C and D), but this level was elevated on supplementation with oestriadiol ($P<0.001$; Fig. 7C and D). Additionally, the expression levels of pro-caspase-3 and active caspase-3 (p17) were found to follow the same pattern as PARP1, showing significant increase in the progesterone + 17β-oestriadiol groups ($P<0.01$ and $P<0.006$ respectively) (Fig. 7E and F).

**Discussion**

Remodelling of the uterus for embryo implantation during the female reproductive cycle involves epithelial cell and endometrial gland proliferation (Sato et al. 1997, Fazleabas & Strakova 2002, Maruyama & Yoshimura 2008, Tanaka et al. 2009) along with the apoptosis (Joswig et al. 2003). Various other factors also come into the picture, including caspase-3 (Joswig et al. 2003), which is a known upstream molecule in the signalling of PARP1 (Tewari et al. 1995). In our study, the expression level of PARP1 was moderate in pre- and late pre-implantation stages (day 4 (1000 and 1600 h)), when the uterus is non-receptive and undergoes various changes at the molecular and cellular level in order to become receptive. At the peri-implantation stage, the expression level of native PARP1 was elevated at implantation sites, which suggests a requirement for native PARP1 in the implantation compartment during blastocyst/embryo implantation. This was followed by downregulation of PARP1 at the post-implantation stage (at implantation as well as non-implantation sites). In addition, the cleaved form of PARP1 was also comparatively elevated during late pre- and peri-implantation stages, which suggests a significant role of PARP1 in the process of embryo implantation, in particular at day 5 (0500 h).

As PARP1 is required for various nucleus related functions; we examined its native form in the uterine nuclear compartment at the different stages of embryo implantation. An elevated expression level of nuclear native PARP1 was found at implantation and non-implantation sites at day 5 (0500 h). Interestingly, the cleaved form of PARP1 showed the same pattern as the native form, its expression being elevated at during day 4 (1600 h) and day 5 (0500 h) (implantation sites). This suggests a requirement for native PARP1 in the nuclear compartment in order for embryo implantation to occur; moreover, cleavage of PARP1 inevitably also takes place in the uterus during embryo implantation. Increased expression of Parp1 transcript during day 5 (0500 h), as evidenced by PCR and real-time PCR, might possibly maintain the equilibrium between the native and cleaved form of PARP1 in the nucleus due to supply of new transcript. Having looked at cleaved PARP1, we investigated the enzyme that cleaves PARP1, caspase-3, in the endometrium during embryo implantation. Elevated activity of caspase-3 was seen at the site of embryo implantation at day 5 (0500 h), along with the cleaved form of PARP1. Interestingly, inhibition of caspase-3 activity reduced the number of embryo implantation sites along with the decrease of PARP1 cleavage during the post-implantation period. These results confirm the role of PARP1 during embryo implantation and the requirement for its cleavage by caspase-3 during this phase.

To facilitate embryo implantation, the uterus undergoes decidualisation during the process of embryo invasion from day 5 (1000 h) onwards (Carson et al. 2000). It is well-known that the uterine epithelium undergoes remodelling at the site of the implanting embryo/blastocyst (Schlaeke et al. 1985, Welsh & Enders 1991, Ramathal et al. 2011, Estella et al. 2012). The proliferating stromal cells differentiate to form the uterine interface with the placenta, which is referred to as the decidua (Bell 1983), and decidualisation is essential for successful reproduction (Lydon et al. 1995, Robb et al. 1998). PARP1 is reported to be linked with the tissue remodelling process (Pagano et al. 2007). Thus, we examined its possible involvement in the decidualisation of the uterus. The expression level of the native form of PARP1 ($\sim 116$ kDa) was on the higher side in the decidualised uterus, along with its cleaved form. This cleavage of PARP1 is possibly mediated by caspase-3 as an elevated expression level of the active form coincided with the level of cleaved PARP1, which suggests that the cleavage of PARP1 is due to the action of caspase-3 in the decidualised uterus. This is a totally
Figure 7 Role of ovarian hormones in regulation of PARP1 expression using delayed embryo implantation. The expression of native/full length/parental forms of PARP1 in response to progesterone and progesterone + oestradiol treatment was measured (A and B). The expression levels of cleaved PARP1, pro- and active caspase-3 (p17) in the pregnant uterus after progesterone and progesterone + oestradiol treatment were also determined (C, D, E and F). β-actin was used to normalise PARP1 and caspase-3 densitometric values.
new observation of PARP1 signalling in uterine tissue associated with embryo implantation. This observation does not necessarily mean that PARP1 is required for decidualisation, but clearly indicates a role in this process. Further studies are required to elucidate the exact signalling mechanism of PARP1 in decidualisation.

We next determined the functional role of PARP1 in embryo implantation. Administration of PARP1 inhibitor at the pre-implantation stage blocked embryo implantation at day 5 (1000 h) and reduced the number of blastocysts recovered. Furthermore, it also affected the signalling of the embryo implantation associated with the gene Stat3. Inhibition of PARP1 downregulated the expression of STAT3 and its activated form (p-STAT3) at day 5 (1000 h). This clearly suggests an important role of PARP1 in the embryo implantation process. It is important to note that many previous studies have hinted at a role of PARP1 in endometrial carcinomas (Brustmann et al., 2007, Postawski et al., 2011) and pre-eclampsia (Crocker et al., 2005), but our study for the first time clearly demonstrates an upregulation of PARP1 in the uterus during the window of uterine receptivity for embryo implantation, functional cleavage of which can result in failure to develop embryo implantation sites, suggesting an essential role of PARP1 in embryo implantation or maintenance of uterine receptivity. Further, cleavage of PARP1 is equally important; this is controlled by the action of caspase-3 in the endometrium, as the cleavage of caspase-3 action reduced embryo implantation. The action of caspase-3 during embryo implantation has already been demonstrated (Zhang & Paria, 2006); however, we found that its activity was elevated at implantation sites during the peri-implantation stage. Thus, the cleavage of PARP1 by caspase-3 takes place during embryo implantation, and is essential in this process.

Ovarian steroids play a vital role in uterine tissue during the female reproductive cycle and make the uterus receptive for embryo implantation (Paulson, 2011). In mice, implantation of embryos depends on an ovarian oestrogen surge that occurs in the morning of day 4 (1000–1200 h; Yoshinaga & Adams, 1966; McCormack & Greenwald, 1974). PARP1 has been shown to regulate the progesterone receptor in endometrial carcinomas (Ghabreau et al., 2004). To evaluate whether PARP1 expression is modulated by the ovarian steroids progesterone and oestrogen, we selected a delayed implantation mouse model. We ovarioctomised the pregnant female mice on day 3 (1600 h), before the oestrogen surge, and supplied either progesterone alone or a mix of progesterone and oestrogen to the animal (delayed implantation mice model). Administration of progesterone alone gave a moderate level of PARP1, but this level was elevated in the progesterone-primed oestradiol-treated uterus. Our delayed implantation model clearly suggests that the upregulation of PARP1 expression is an oestradiol-dependent mechanism. Surprisingly, the cleaved form of PARP1 replicated the pattern of the native form in the progesterone and progesterone + 17β-oestradiol-treated groups. Furthermore, the increased expression level of active caspase-3 in response to progesterone in combination with oestradiol indicates a role in the cleavage of PARP1.

The picture emerging from this study underlines an important role of PARP1 in the preparation of the uterus for embryo implantation and decidualisation, and PARP1 is cleaved by the action of caspase-3 at day 5 (0500 h). Our findings unveil for the first time a possible role of PARP1 in the uterus during the window of embryo implantation and decidualisation; it is too soon to define the exact signalling role of PARP1 in the female reproductive cycle, but our study indicates that it is an important one.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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