Expression of Fas and Fas ligand protein and mRNA in mouse oocytes and embryos

R. L. Kelkar, S. J. Dharma and T. D. Nandedkar*

National Institute for Research in Reproductive Health, JM Street, Parel, Mumbai-400012, India

During mammalian embryonic development, abnormal eggs and embryos are eliminated by apoptosis; however, the precise apoptotic pathways remain as yet unidentified. In the present study, expression of Fas and Fas ligand – the proximal members of the death receptor pathway, was evaluated in mouse preimplantation embryos by immunofluorescence and *in situ* hybridization techniques. Ovulated oocytes were collected from oviducts of cyclic mice on the day of oestrus (day 0), and one-cell, two-cell embryos and eight-cell morulae were collected from oviducts of mated animals on days 1, 2 and 3 of pregnancy, respectively. Blastocysts were flushed from the uterine horns on day 4. Expression of Fas and Fas L mRNAs and

proteins was absent from embryos at days 0, 1 and 2. A marked increase in Fas and Fas L mRNA and protein expression was detected in all morphologically normal embryos on day 3 and day 4. In addition, embryos on days 3 and 4 were positive for terminal deoxynucleotidyl transferase-mediated dUTP nick-end labelling (TUNEL) staining; however, absence of caspase 8 and 3 and intense localization of proliferating cell nuclear antigen confirmed the proliferative status of these embryos. Furthermore, TUNEL staining was absent in postimplantation embryonic sections obtained on day 6. The results of the present studies thus indicate an equilibrium between proliferation and apoptosis in the preimplantation embryo.

Introduction

Apoptosis is a process of selective and programmed death of cells occurring in almost all tissues (Kerr *et al.*, 1972) and is finely regulated by a number of genes and gene products. Morphological and biochemical studies have shown that the demise of both somatic and germ cells in ovarian follicular atresia is mediated by apoptosis (Hughes and Gorospe, 1991; Morita and Tilly, 1999).

In contrast to atretic follicles, apoptotic processes seem to be dormant in follicles destined to ovulate. However, after ovulation and fertilization, these cell death processes are activated during preimplantation development (Warner *et al.*, 1998). Studies in mice, rats and humans have demonstrated that isolated cells in the inner cell mass as well as the trophectoderm are eliminated by apoptosis (Hardy, 1997, 1999; Pampfer and Donnay, 1999), which is under the regulatory control of growth factors (Brison and Schultz, 1997; Byrne *et al.*, 2002). The function of this cell death may be to control the number of developing embryos, to eliminate abnormal embryos or trim excess or dysfunctional stem cells. The expression of caspases and Bcl2 family members has been reported in mouse preimplantation embryos (Jurisicova *et al.*, 1998; Exley *et al.*, 1999), indicating an involvement of this mitochondrial pathway

of cell death in embryo demise. Similarly, expression of mRNA encoding Fas and Fas L has been demonstrated in rat and human preimplantation embryos by RT-PCR (Kawamura *et al.*, 2001). However, the precise pathways governing this phenomenon of embryonic apoptosis remain unknown.

Fas (48 kDa) is a cell membrane integral glycoprotein similar to tumour necrosis factor α receptor (TNF α -R), and its ligand (Fas L) is a type II membrane protein homologous to TNF- α . Apoptosis is induced in Fas-bearing cells when Fas L binds to Fas. This interaction results in the activation of the Fas-associated death domain (FADD), which in turn forms a docking site to facilitate the autocatalytic activation of procaspase 8 (Chinnaiyan *et al.*, 1995; Nagata and Goldstein, 1995; Ashkenazi and Dixit, 1999). Once activated, caspase 8 can stimulate other downstream proteases such as caspase 3. These proteolytic effectors of cell death cleave key cellular substrates, thereby disorganizing the cellular assembly and executing apoptosis (Thornberry *et al.*, 1997; Thornberry and Lazebnik, 1998).

In the present study, the expression of Fas and Fas L and the mRNA encoding them has been studied in mouse oocytes after ovulation and after fertilization in embryos up to the blastocyst stage. Apoptosis was detected by terminal deoxynucleotidyl transferase-mediated dUTP nick-end labelling (TUNEL) (Gavrielli *et al.*, 1992) and immunohistochemical localization of caspases 8 and 3. Proliferating cell nuclear antigen (PCNA) is a protein

*Correspondence
Email: cellbioirr@hotmail.com

expressed during the S-phase of the cell cycle and is involved in DNA replication and repair (Kelman, 1997). Immunolocalization of PCNA has been used in a number of studies as an index of proliferation and in the present study was used to assess the proliferative status of the embryos (Hall *et al.*, 1990; Leibovici *et al.*, 1992). The results indicate a role for the Fas–Fas L signalling system in preimplantation embryo development.

Materials and Methods

Animals

Swiss mice bred from the animal colony at the National Institute for Research in Reproductive Health were maintained at constant light (12 h light:12 h dark), temperature (24°C) and humidity (60%), and were supplied with food and water *ad libitum*. The animal requirement for the present study was approved by the Institutional Animal Ethics Committee and experiments were performed in accordance with the guidelines set by the Committee for the Purpose of Control and Supervision of Experiments on Animals, India.

Collection of oocytes, eggs and embryos

Vaginal smears of adult female Swiss mice aged 2.5–3.0 months were monitored daily. After assessing normal cyclicity of 4–5 days for three consecutive cycles, animals were killed by decapitation on the day of oestrus (day 0) and oviducts were teased to obtain ovulated oocytes with cumulus mass.

For collection of embryos, female mice were cohabitated with males and the day of appearance of vaginal plug was considered as day 1 of pregnancy. Fertilized eggs, two-cell embryos and morulae were retrieved from the oviduct on days 1, 2 and 3 of pregnancy, respectively, and blastocysts were flushed from the uterine horns on day 4 using 0.01 mol PBS l⁻¹. The oocytes and embryos at the various stages (days 0–4) were fixed on slides coated with silane for *in situ* hybridization or slides coated with poly-L-lysine (Sigma, St Louis, MO) for indirect immunofluorescence and immunohistochemistry. Each group consisted of 10 animals and 5–10 embryos were recovered per animal.

Post-implantation embryonic sections

Five animals were administered 1% (w/v) Evan's blue in saline through the tail vein on day 6 of pregnancy and were autopsied after 5 min. The implantation sites were cut and 10 µm sections taken on Leitz cryostat were mounted on silanated slides for TUNEL staining.

In situ hybridization

Unfertilized oocytes at day 0 and fertilized embryos at days 1, 2, 3 and 4 were fixed on slides in 4% (w/v) paraformaldehyde in PBS for 20 min at room temperature

(25°C) and then were treated with proteinase K (10 µg ml⁻¹) in Tris–EDTA buffer (pH 7.6) at 37°C for 30 min. *In situ* hybridization was performed for Fas and Fas ligand as described by Nandedkar and Dharma (2001). The oligoprobe sequences selected for Fas–Fas ligand mRNA localization in the present study were taken from Roughton *et al.* (1999). These oligoprobes were tail-labelled with Digoxigenin Oligonucleotide Tailing Kit (Roche Molecular Biochemicals, Mannheim). Briefly, after washing in PBS (0.1 mol l⁻¹), the slides were incubated in sodium saline citrate (2 × SSC) for 10 min. Prehybridization was carried out at room temperature for 1 h in prehybridization cocktail. Overnight hybridization at 42°C was performed with digoxigenin-labelled cDNA antisense and sense oligoprobes dissolved in the prehybridization mixture at a concentration of 0.5 pmol µl⁻¹. After stringent post-hybridization washings with various concentrations of SSC and incubation in Tris buffer (pH 7.4) for 10 min, the slides were blocked with 2% (v/v) normal sheep serum in Tris buffer containing 0.3% (v/v) Triton X-100 (Sigma) for 1 h at room temperature. Sections were incubated overnight in alkaline phosphatase-conjugated anti-digoxigenin antibody (1:500 dilution; Roche). The next day, slides were washed twice in Tris buffer for 5 min each. Colour reaction was carried out by incubating all embryos and sections in a mixture of nitro-blue tetrazolium (NBT; Roche) and 5-bromo 4-chloro 3-indolyl phosphate (BCIP; Roche) for 20 min at room temperature in the dark. Levamisole (0.2% (w/v); Sigma) was added in the colour mixture to block endogenous phosphatases. After a wash in distilled water, slides were mounted in aquamount and viewed. The representative areas were photographed under bright field. The slides were subjected to an identical procedure using digoxigenin-labelled sense probes to provide negative controls.

Immunofluorescence

Ovulated oocytes and embryos were fixed in acetone at –20°C for 10 min, mounted on poly-L-lysine-coated slides and air dried. After washing in PBS, the specimens were permeabilized by incubating in 0.01% (v/v) Triton-X 100 (Sigma) in PBS for 5 min and then incubating 3% (w/v) non-fat dried milk in PBS for 30 min to block non-specific binding. Slides were then incubated with primary antibody (5 µg ml⁻¹) of either hamster anti-mouse Fas or Fas L monoclonal antibody (Pharmingen, San Diego, CA) overnight at 4°C. Controls were incubated in 1% (v/v) SP₂O culture supernatants in PBS. After PBS washes, secondary antibody (R-phycoerythrin conjugated mouse anti-hamster IgG; Pharmingen) was applied at a dilution of 1:1000 in the dark at room temperature for 1 h. Slides were washed in distilled water, air dried, mounted in glycerol and observed under blue light (Carl Zeiss, Gottingen). Photographs were taken at a magnification × 400 using a 400ASA Kodak colour film.

Table 1. Staining intensities for Fas mRNA and protein in mouse embryos

	Day 0	Day 1	Day 2	Day 3	Day4
Fas mRNA (<i>in situ</i> hybridization)	0.1166	0.1836	0.2074	0.4291	0.6154 (0.1931)
Fas protein (indirect immunofluorescence)	0.0691	0.0769	0.0719	0.6560	0.6955 (0.0393)

Results are expressed in terms of mean absorbance.
Figures in parentheses indicate values for negative controls.

DNA fragmentation detected by TUNEL

DNA fragmentation in embryos at days 0, 1 and 4 and in postimplantation embryonic sections taken on day 6 was detected using the non-radioactive detection method described by Gavrielli *et al.* (1992). The specimens were fixed in 1% (w/v) paraformaldehyde in PBS for 20 min at room temperature and washed twice in PBS. The slides were then incubated in Proteinase K ($20 \mu\text{g ml}^{-1}$) for 15 min at 37°C and washed in PBS. The labelling of 3' ends of fragmented DNA with digoxigenin was facilitated using terminal transferase (Tdt; Roche) after incubation of slides in terminal transferase buffer for 10 min at room temperature. Reaction mixture (Roche) containing 200 mmol potassium cacodylate l^{-1} , 5 mmol $\text{CoCl}_2 \text{l}^{-1}$, 1 U terminal transferase μl^{-1} , 1 μmol dig dUTP l^{-1} and 49 μmol dideoxyATP l^{-1} was applied to the specimens for 2 h at 37°C in a humidified chamber. After three washes in Tris buffer, the slides were incubated in blocking buffer (100 mmol Tris l^{-1} , 150 mmol NaCl l^{-1} , pH 7.5 and 2% (w/v) blocking reagent) for 1 h at room temperature before addition of antidigoxigenin antibody conjugated to alkaline phosphatase. After incubation with antidigoxigenin antibody (1:10000 in 2% blocking solution; Roche) at 4°C overnight in a humidified chamber the slides were washed twice in Tris buffer (pH 7.5) and finally equilibrated in alkaline phosphatase buffer (100 mmol Tris l^{-1} , 150 mmol NaCl l^{-1} , 50 mmol $\text{MgCl}_2 \text{l}^{-1}$, pH 9.5). Enzyme substrate (33 μg NBT ml^{-1} and 16.5 μg BCIP ml^{-1}) for alkaline phosphatase was applied for colour development for 15 min in the dark. Colour reaction was terminated with 10 mmol Tris l^{-1} , 1 mmol EDTA l^{-1} , pH 8.0. Blue-brown staining represented a positive reaction. Slides were observed and photographed under bright field using a Carl Zeiss microscope. Controls were processed in a similar manner with the omission of terminal transferase in the reaction mixture.

Immunohistochemistry

The primary antibodies used were mouse monoclonal antibodies to recombinant caspase 8, caspase 3 (Novocastra Laboratories, Newcastle-upon-Tyne) and PCNA (Santacruz Biotechnology, Santa Cruz, CA) at dilutions of 1:50 for caspases and 1:500 for PCNA. Specimens were fixed in acetone at -20°C for 10 min and then incubated in 0.3% (v/v) H_2O_2 in methanol at -20°C for 30 min to block endogenous peroxidase activity. After

Table 2. Staining intensities for terminal deoxynucleotidyl transferase-mediated dUTP nick-end labelling (TUNEL) in mouse embryos

Stage of the embryo	Mean absorbance
Day 3	0.4584
Day 4	0.5530 (0.2441)
Day 6 (embryo)	0.1314 (0.1715)
Day 6 (decidual cells)	0.7292 (0.2882)

Figures in parentheses indicate values for negative controls.

two PBS washes, non-specific binding was inhibited by incubation in 3% (w/v) non-fat dried milk powder in PBS for 30 min at room temperature and primary antibody was applied overnight at 4°C ; controls were incubated in 1% (v/v) SP₂O culture supernatants. Slides were then washed in PBS and incubated with secondary antibody (rabbit anti-mouse IgG; Dako, Glostrup) at a dilution of 1:1000 for 1 h at room temperature. Colour was developed using 1% (w/v) diaminobenzidine (Sigma) and 0.3% (v/v) H_2O_2 in PBS as substrate mix. Slides were then washed in distilled water, mounted in Canada balsam and photographed at a magnification of $\times 400$.

Quantitation by image analysis

Photographs were scanned and comparisons of staining intensities for Fas and mRNA encoding Fas and of TUNEL in oocytes and embryos at various stages were made using Biovis Image Analysis Software (Expert Vision Laboratories, Mumbai). Results are expressed in terms of mean absorbance (Tables 1 and 2).

Results

Expression of Fas and Fas L in oocytes and embryos by *in situ* hybridization and indirect immunofluorescence

Expression of Fas and mRNA encoding Fas in unfertilized oocytes, fertilized eggs and embryos is shown (Fig. 1). On the day of estrus, day 0, eggs collected from the oviduct showed weak staining for Fas mRNA by *in situ* hybridization (Fig. 1a) and for Fas protein by immunofluorescence (Fig. 1g). However, the cumulus

Fig. 1. Expression of Fas mRNA and protein in mouse oocytes and embryos by *in situ* hybridization (a–f) and immunofluorescence (g–l), respectively. Staining was weak in (a,g) ovulated oocytes on day 0, (b,h) fertilized eggs on day 1 and (c,i) two-cell embryos on day 2. There was a marked increase in both Fas and mRNA encoding Fas in (d,j) 8–16-cell embryos on day 3 and (e,k) blastocysts on day 4 as compared with (f,l) negative controls on day 4. Scale bars represent (a–f) 10 μ m and (g–l) 16 μ m.

granulosa cells were positive for the death receptor. Similarly, on the day of appearance of vaginal plug, day 1, fertilized eggs obtained from the oviduct expressed Fas mRNA (Fig. 1b) and Fas (Fig. 1h); staining was weak

in the zygote but moderate-to-intense in the dispersed cumulus cells. Staining for both Fas mRNA and protein was negligible in two-cell (day 2) embryos (Fig. 1c,i). On day 3, a marked increase in expression of Fas mRNA

(Fig. 1d) and Fas (Fig. 1j) was observed in the morulae retrieved from the oviduct. Blastocysts recovered from the uterus on day 4 had 16–32 cells with intact zona pellucidae. Intense expression similar to that observed at day 3 was observed in all morphologically normal embryos (Fig. 1e,k). The controls did not stain for Fas mRNA (Fig. 1f) and Fas (Fig. 1l).

Quantitation by image analysis is summarized (Table 1). The results confirmed a gradual increase in intensity for both Fas mRNA and Fas from day 0 to day 4 reaching a peak intensity on day 4. The intensity for Fas mRNA increased 5–6-fold (0.1166 at day 0 versus 0.6154 at day 4) whereas the intensity for Fas was 10 times higher (0.0691 at day 0 versus 0.6955 at day 4) during the embryonic development.

Similar results were obtained with Fas L, although the expression of mRNA and protein was lower than that of Fas (data not shown).

Detection of DNA fragmentation by in situ 3' end labelling (TUNEL)

One of the hallmarks of apoptotic cell death is internucleosomal DNA fragmentation. The TUNEL assay was used to detect 3'-hydroxy DNA fragments in mouse eggs and embryos. On day 0, ovulated oocytes were negative but cumulus cells were positive for TUNEL (Fig. 2a), indicating apoptosis in the cumulus cells but not in the oocyte. Similarly on day 1 of pregnancy, the zygote was negatively stained for TUNEL but most of the cumulus cells exhibited a positive reaction (Fig. 2b). However, day 3 morulae (Fig. 2c) and day 4 blastocysts (Fig. 2d) showed positive TUNEL reactions consistent with an increase in Fas and Fas L expression observed at these stages. Negative controls were processed in the absence of terminal transferase and did not show any staining (Fig. 2e,f).

The implanted embryo was negative for TUNEL but the decidual cells and the uterine epithelium showed a strong positive reaction, as observed in sections of implantation sites obtained on day 6 of pregnancy (Fig. 2g). Negative controls did not show any staining (Fig. 2h).

The quantitation of intensity for TUNEL is shown (Table 2). A slight increase in intensity for TUNEL was observed from day 3 to day 4 (0.4584 versus 0.5530, respectively). However, on day 6, the implanted embryo showed a low (0.1314) intensity but the decidual cells showed significantly high intensity (0.7292) for TUNEL.

Immunohistochemical localization of PCNA and caspases 8 and 3

Localization of PCNA and caspase 3 in ovulated oocytes (day 0) and preimplantation embryos recovered on day 4 is shown (Fig. 3). A weak positive immunoreaction for PCNA was detected in the oocyte but not in cumulus cells on day 0 (Fig. 3a) and an increase in the

intensity of staining was observed in the day 4 blastocyst (Fig. 3c). Both trophectoderm and inner cell mass were positive for the proliferative marker. Control day 0 and day 4 embryos incubated in SP₂O culture supernatants instead of primary antibody did not show any staining (Fig. 3b,d).

Caspase 3 was localized weakly in the oocyte as well as cumulus cells on day 0 (Fig. 3e), indicating initiation of apoptosis in these cells. However, no staining for caspase 3 could be detected in the blastocysts retrieved on day 4 (Fig. 3g) as compared with the negative controls (Fig. 3f,h). Similar results were obtained using a mouse monoclonal to caspase 8, the proximal caspase of the Fas–Fas L pathway (data not shown).

Discussion

In the present study, expression of Fas and Fas L, the pro-apoptotic members of the death receptor pathway was observed in mouse oocytes and embryos. On day 0, after ovulation, oocytes do not express Fas–Fas L. After fertilization, the egg continues to be negative for both Fas and Fas L antigens on day 1. Absence of Fas and Fas L expression as observed by *in situ* hybridization confirmed these results. The cumulus cells demonstrated expression of Fas and Fas L on day 1. After fertilization, the fate of these cumulus cells is cell death. TUNEL-positive staining in the cumulus cells but not in the fertilized egg confirms the presence of apoptotic processes in these cells.

Oocytes on day 0 and embryos on day 1 and day 2 did not express Fas and Fas L; however, embryos on day 3 and day 4 expressed both Fas and Fas L, mRNA and protein, and DNA fragmentation as indicated by TUNEL was also observed in embryos on day 1 and day 4. These results indicate that apoptotic processes might be present in these developing embryos. However, (i) both TUNEL and Fas staining was observed consistently in all morphologically normal embryos retrieved, and (ii) day 4 embryos failed to express caspases 8 and 3 while intensely expressing the proliferation marker, PCNA. Therefore, the present observations could be explained by one or more of the following:

First, the membrane-anchored receptor Fas is activated by trimerization after ligand binding and facilitates caspase activation through adaptor proteins such as FADD or TNF- α receptor-associated death domain (TRADD) (Chinnaiyan *et al.*, 1995; Nagata and Goldstein, 1995; Ashkenazi and Dixit, 1999). Earlier studies demonstrated that Fas expression may not always lead to apoptosis (Payne *et al.*, 1999). In the present study, activation of caspase 3 antigen was not observed as revealed by immunohistochemical localization. Absence of caspase activation may be the result of alternative splice forms of Fas that lack either the death domain or the membrane-anchoring sequences (Cascino *et al.*, 1995, 1996) or lack of trimerization of the receptor.

Fig. 2. Detection of apoptosis by terminal deoxynucleotidyl transferase-mediated dUTP nick-end labelling (TUNEL) assay in (a) ovulated oocyte, (b) fertilized egg and preimplantation embryos at (c) day 3 and (d) day 4 of pregnancy in mice. An increase in staining was observed at days 3 and 4. (e) Day 0 and (f) day 4 negative controls were processed in the absence of terminal transferase. (g) Staining was not observed in postimplantation embryos (Em) sectioned on day 6 of pregnancy, whereas only decidual cells (Dc) were TUNEL-positive as compared with (h) negative controls. Scale bar represents 10 μ m.

Fig. 3. Immunolocalization of (a–d) proliferating cell nuclear antigen (PCNA) and (e–h) caspase 3 in ovulated oocytes on day 0 and blastocysts on day 4 of pregnancy. (a) Positive staining for PCNA observed in oocytes but not cumulus cells at day 0 and (c) intense staining is observed in embryos at day 4. (b,d) No staining was observed in negative controls. Weak caspase 3 expression was detected in (e) oocyte and cumulus cells at day 0 but not in (g) blastocysts at day 4 as compared with negative controls at (f) day 0 and (h) day 4. Scale bar represents 16 μm .

Second, an increasing number of studies have reported false positive results with TUNEL in cardiac myocytes (Kano *et al.*, 1999), in mouse kidney and liver cells (Pullkkanen *et al.*, 2000) and histological sectioning (Sloop *et al.*, 1999). A similar possibility cannot be denied in case of this study yielding intense TUNEL staining in all normal embryos.

In contrast to the above two conditions, in which it is assumed that no apoptosis occurs in spite of Fas expression or positive TUNEL staining, numerous studies can be cited that demonstrate apoptosis in developing embryos (Hardy 1997, 1999; Warner *et al.*, 1998; Pampfer and Donnay, 1999). Therefore, it is probable that, in the present study, apoptosis is indeed triggered in the developing embryos. However, there is a balance between proliferation and cell death as indicated by a strong immunoreaction to the PCNA antibody seen in day 4 blastocysts. The results of the present study are in agreement with those of Makarevich and Markkula (2002) who demonstrated colocalization of PCNA and TUNEL-positive cells in bovine blastocysts, indicating an involvement of PCNA in DNA repair processes in TUNEL-positive cells. Thus, the PCNA may be involved in DNA repair (Warbrick, 2000) and therefore the rescue of the preimplantation embryo from undergoing apoptosis so as to facilitate implantation. The rescue of the implanting embryo from apoptosis is confirmed through the present observations in day 6 embryonic sections that TUNEL staining is seen in the decidual cells of the endometrium but not in the implanted embryo.

Another important factor in rescuing the embryo from apoptosis is the action of growth factors. Studies *in vitro* in mouse (Brison and Schultz, 1997; Byrne *et al.*, 2002) and bovine (Makarevich and Markkula, 2002) embryos indicate that factors such as insulin-like growth factor I (IGF-I), IGF-II and transforming growth factor α (TGF- α) act as survival factors for the developing embryo. Thus, growth factors secreted by the endometrium around implantation may play a role in inhibiting embryonic apoptosis.

Furthermore, the increased concentrations of oestradiol that occur before implantation are essential for the preparation of receptive endometrium for the attachment of the blastocyst (Shelesnyak and Kraicer, 1963). Oestrogen is an anti-apoptotic agent (Hseuh *et al.*, 1994) and is able to rescue the blastocyst from apoptosis and propel it towards implantation. Therefore, it seems probable that the TUNEL-positive day 4 blastocyst is rescued after the peak oestrogen concentrations and can implant on day 5 and thus, on day 6, the implanted embryo is TUNEL-negative. Therefore, a failure of oestradiol secretion from the ovary or blockage by anti-oestrogens can cause degeneration of embryos and failure of implantation. The preovulatory LH surge has been reported to reactivate-retrieve the TUNEL-positive cells (Richards, 2001). Therefore,

a role for gonadotrophins and steroids in embryonic development, apoptosis and implantation is proposed.

In conclusion, the blastocyst on day 4 is positive for TUNEL and the death receptor Fas. However, the blastocyst also expresses PCNA and is negative for caspase 3, which is the terminal protease in the apoptotic cascade. Thus, the present studies indicate a balance between apoptosis and proliferation in the developing preimplantation embryo.

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