Germ Line Apoptosis in the Mature Human Ovary

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BCL2-gene family, which regulates the intrinsic apoptotic pathway, has been implicated in the process of germ cell loss in the human ovary, especially through the balance of expression between anti-apoptotic *BCL2* gene and pro-apoptotic *BAX*. The developing ovary suffers a massive constitutive germ cell loss driven through the overexpression of *BAX* gene. Almost 85% of the oocytes produced by active proliferation of primordial germ cells colonizing the genital ridges at early fetal life are decimated by the end of gestation leading to a resting primordial follicle reserve at birth of around 400,000 oocytes. The main target of the apoptotic machinery during fetal life is the germ cell proper; after birth, however, apoptosis moves to the somatic granulosa cells which support growth of cyclical activation of dormant primordial follicles. We report here the immunohistochemical analysis of proteins involved in intrinsic apoptosis (BCL2/BAX) as well as in extrinsic apoptosis pathways (FAS/FAS-L), in the mature human ovary. BAX protein was detectable in primordial, primary, secondary, and antral follicles in all samples, whereas BCL2 showed detection restricted to some secondary and antral follicles. FAS/FAS-L proteins were conspicuously expressed both in dormant primordial follicles as well as throughout all follicular stages in the growing pool. TUNEL analysis yield just positive results in some antral follicles and atretic follicles. Apoptosis-related proteins expressed in the mature human ovary in a comparable pattern to that observed in the pre- and pubertal ovary. The level of TUNEL positive follicle detection is not commensurate with the expected magnitude of germ cell loss indicating that concurrent death mechanisms could be acting in the mature ovary. **Key words**: human ovary, *BCL2*/*BAX* genes, *FAS*/*FAS-L*, apoptosis

INTRODUCTION

Adult human ovary function depends on the size of the resting primordial follicle reserve serving as a source of developing follicles. Processes leading to the formation of the resting primordial follicle reserve begin early in fetal life and include differentiation and migration of primordial germ cell (PGC) to the genital ridges, massive mitotic proliferation of PGC to transform into oogonia, entrance to meiosis prophase I, and the formation of the primordial follicle (Macklon and Fauser, 1999). As soon as proliferation begins to increase the few 1,000-2,000 PGCs that reached the developing ovary to produce around 7 x 10^6 potential oocytes at mid fetal life, a counterbalance mechanism of cell death executed through apoptosis takes place. The size of the resting primordial follicle reserve is determined from the fourth month of fetal life, as a consequence of proliferation/cell death balance, when primordial follicle development begins. Germ cell attrition particularly occurs in the transition from meiotic prophase I to primordial follicle formation through massive apoptosis and continuous until the end of gestation, leading the developing ovary to contain just around 400,000 primordial follicles at birth, a wasteful cellular loss of almost 85% of the potential oocyte population reached at mid gestation (Baker, 1963; Forabosco et al., 1991).

After birth, some dormant primordial follicles from the resting reserve activate and are recruited into the growing germinal pool as primary oocytes (McGee and Hsueh, 2000). Intraovarian mechanisms leading to primordial follicle recruitment, or maintenance of the resting stage, remain poorly understood. However, several transcription factors have been very recently identified, that might regulate this first step in folliculogenesis (reviewed by Monget et al., 2011). Approximately 1,000 resting primordial follicles initiate growth per month to develop into primary and secondary stages of folliculogenesis before acquiring an antral cavity (Macklon and Fauser, 1999). In puberty, gonadotropin stimulation acts on a few antral follicles that reach pre-ovulatory stage and eventually are able to respond to pre-ovulatory gonadotropin surge to release a mature oocyte for fertilization. Not activated primordial follicles remain dormant for years or decades before being activated, until less than 1,000 follicles remain in the pool leading to germinal depletion or menopause.

The biological meaning of the exhaustive depletion of germ cell during the entire ontogenetic development of the human female germline is still a matter of discussion. This massive elimination may avoid the persistence in the ovary of germ cells exhibiting nuclear or mitochondrial chromosomal/genetic defects (Tilly, 2001). Alternatively, death may relate to the exhaustion of germ cells acting as nurse cells to the surviving oocyte pool (Pepling and Spradling, 2001). Finally, it has been suggested that massive death may enable the appropriate association between germ cells and pregranulosa cells during ovigerous cords or ovarian cysts breakdown, just before primordial follicles begin to form (Guigon and Magre, 2006). In any case, the balance between germ cell death and survival seems to be critical in determining the size of the resting primordial follicle reserve at birth. Precluding ovarian dysgenesis, preventing premature ovarian failure, and ensuring female fertility largely depends on the size of the departing pool of resting primordial follicles.

Multiple genes are involved in the regulation of both intrinsic and extrinsic pathways of apoptosis. Nevertheless, in the mammalian ovary, it has been shown that *BCL2* gene family plays an essential role (reviewed by Aitken et al., 2011). In particular, apoptosis-inhibiting *BCL2* gene and apoptosis-inducing *BAX* gene act as a rheostat whose bias in favor of one or another determines the survival or death of the germ cell (Tilly, 1996). As a general rule, it has been observed that expression of *BAX* is normally enhanced in the mammalian ovary, whereas BCL2 protein is found at low levels or undetected at all (Kim and Tilly, 2004). This observation gives support to the high rate of apoptosis characterizing the mammalian ovary, and has been experimentally supported by showing that *BCL2*- and *BAX*-deficient knockout mice have decreased or increased primordial follicle reserve respectively (Knudson et al., 1995; Ratts et al., 1995).

In the human ovary, it has also been shown that BCL2 and BAX proteins show expression patterns comparable to those found in mice and rat (Vaskivuo et al., 2001). Much attention has been paid to fetal life since the developing ovary displays the greatest level of oocyte loss reaching 85% of the original germ cell reserve built at early fetal life through the proliferation of PGC and oogonia (De Pol et al., 1997;

Quenby et al., 1999; Abir et al., 2002; Modi et al., 2003; Fulton et al., 2005, Albamonte et al., 2008; Hartshorne et al., 2009). Infant, pubertal and adult ovary deserved less attention but it was shown that germinal loss seems to continue through the same mechanism of BAX over-expression (Vaskivuo et al, 2001; Albamonte et al., 2006). However, after birth, germ cell death takes place from apoptosis mechanisms acting in the somatic stratum, granulosa cells, rather than in the germ cell proper as in fetal life. A switch moving apoptosis machinery from the germinal stratum to the somatic component of resting and growing follicles is believed to take place at birth (Vaskivuo et al., 2001).

Although several data have accumulated on apoptosis in the human developing ovary, and to a lesser extent in the infant and adult ovary, we are still far from having a detailed knowledge on the regulation of germ cell number that is drastically reduced from 7 x 10^6 cells at the fifth month post-conception to around 400,000 oocytes after birth (Baker, 1963; Forabosco et al., 1991). We report here a qualitative immunohistochemical analysis of proteins involved in intrinsic apoptosis, BCL2 and BAX, as well as in extrinsic apoptosis pathways, FAS and its ligand FAS-L, in mature, normal human ovary samples spanning reproductive age in woman from 18 to 45 years old.

MATERIALS AND METHODS

Ovarian Material Collection and Processing

The study reported in this paper was reviewed and approved by the Research Ethics Committee from Universidad Maimónides, Buenos Aires, Argentina. A total of 17 ovaries were obtained from patients, aged 18–45 years old, undergoing total hysterectomy for cervical cancer or ovariectomy as the result of uterine myoma at the Hospital Rivadavia, Buenos Aires city, Argentina, after obtaining the patients' consent. All samples showed normal histology as diagnosed at the Department of Pathology from Hospital Rivadavia. Ovarian tissues were fixed in 10% buffered formalin for 24h and processed for routine paraffin embedding. Embedded samples were treated by standard histology procedures, serially sectioned at 5 µm, mounted onto cleaned, coated slides, and stored at room temperature until used for immunohistochemistry or TUNEL assay. All samples were examined for tissue integrity and general histology by hematoxylin-eosine staining.

Immunohistochemistry

BCL2 and BAX immunodetection. Histological ovarian sections were dewaxed in xylols, re-hydrated through a decreasing series of ethanol, thoroughly rinsed in PBS and incubated 1hr at room temperature with-rabbit polyclonal primary antibodies raised against BAX and BCL2 human proteins (Santa Cruz Biotechnology, CA, USA). In order to reveal tissue protein-antibody reaction, EnVision® Doublestain System (Dako Cytomation, USA) was employed according to the recommendations of the manufacturer. Immunoenzymatic reactions were performed with horseradish peroxidase (HRP) or alkaline phosphatase labeled polymers, and revealed with 3,3'-diaminobenzidine (DAB) and fast-red for BAX and BCL2, respectively. All slides were counterstained with haematoxylin. Negative controls were performed by omitting the primary antibodies.

FAS and FAS-L immunodetection. Ovarian sections were treated as indicated above, and incubated for 1hr at room temperature with polyclonal mouse primary antibodies raised against human FAS and FAS-L proteins (Santa Cruz Biotechnology, USA). Immunoreactions were detected by using LSAB+System-HRP (Dako Cytomation, USA). Negative control was performed by omitting primary antibodies.

TUNEL Assay

Detection of DNA fragmentation was performed in paraffin-embedded sections by terminal deoxynucleotidyl transferase [TdT]-mediated deoxiuridinetriphosphate [dUTP] nick end labeling technique, using the "In Situ Cell Death Detection Kit" (Roche Diagnostics, Germany) with fluorescein tagged nucleotides. Procedure followed the suppliers' recommendations. Treated sections were examined in an Olympus BX40 microscope by conventional epifluorescence with ultraviolet illumination. In order to confirm negative results, TUNEL-processed sections were incubated with 10UI/ml DNase II (Sigma Chemical Co., USA) in 50mM Tris-HCL pH 7.5, 10 mM Mg2Cl and 1 mg/ml BSA for 10 min at room temperature. After incubation, slides were thoroughly rinsed and treated again according to the TUNEL protocol. Images were captured with an Olympus Camedia C-5060 digital camera.

RESULTS

BAX and BCL2 Immunolocalization

Irrespective of women age, BAX protein was immunodetected from primordial to pre-ovulatory antral follicular stages in all samples analyzed (Fig.1). Cytoplasmic signal localized mainly at the somatic

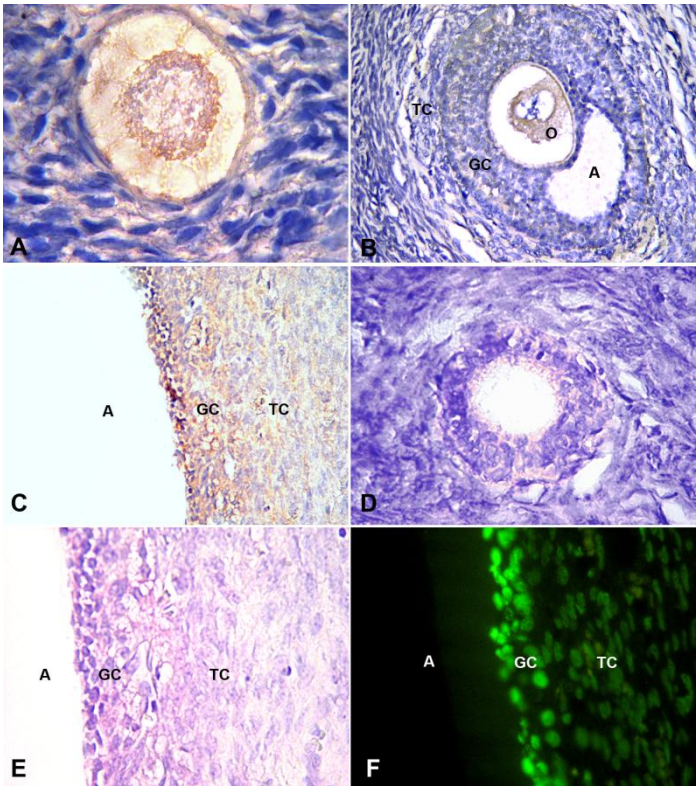


Fig.1. Representative images of BAX and BCL2 immunolocalization and TUNEL in the mature human ovary. A- Resting primordial follicle showing BAX signal (brown staining) both in the germ cell and flattened granulosa (x1000). B- Early antral follicle showing BAX immunostaining in the oocyte and outer granulosa cells (x400). C- Detail of an antral pre-ovulatory follicle with positive staining for BAX both at granulosa and theca cells (x400). D- An occasional secondary follicle showing positive BCL2 signal (red cytoplasmic staining) (x400). E- Partial view of an antral follicle showing BCL2 positivity in granulosa and theca cells (red cytoplasmic staining) (x1000). F- Illustrative picture of TUNEL positive granulosa cells (green nuclei) in an antral follicle (x1000). A: antrum, O: oocyte; GC: granulosa cell; TC: theca cell

stratum both in granulosa and theca cells in antral follicles (Fig. 1C). Preantral follicular stages also showed widespread and strong cytoplasmic staining for BAX (Fig. 1B). Conversely, BCL2 showed a restricted pattern of immunodetection. Some secondary follicles from the growing pool were positive for BCL2 (Fig. 1D) whereas no positive signal was found in primary or primordial stages in any sample (not shown). In some cases too, granulosa cells in antral follicles were positive (Fig. 1E), and co-localized with BAX detection. It is worth to note that signal intensity was always stronger for BAX than BCL2. As two different enzymatic reactions were employed for detecting each protein (see Materials and Methods), we reversed in some experiments the use of DAB or fast-red to detect either BAX or BCL2. Irrespective of this, immunolabeling was always stronger for BAX protein. Negative controls performed by omitting the primary antibodies did not show specific signals in any case.

FAS and FAS-L Immunolocalization

FAS/FAS-L proteins displayed a widespread immunodetection pattern. Primordial, primary and secondary follicles showed positive signal for both proteins in the germ cell proper and the surrounding granulosa cells (Fig. 2). Signal for FAS and its ligand FAS-L protein was mainly detected in the somatic stratum of the antral follicles (Fig. 2B-D). Theca cells showed the strongest signal intensity for both proteins. Degenerating granulosa in atretic follicles also showed strong FAS and FAS-L immunodetection (Fig. 1E, F). No signal was found for either protein when primary antibodies were omitted in control reactions.

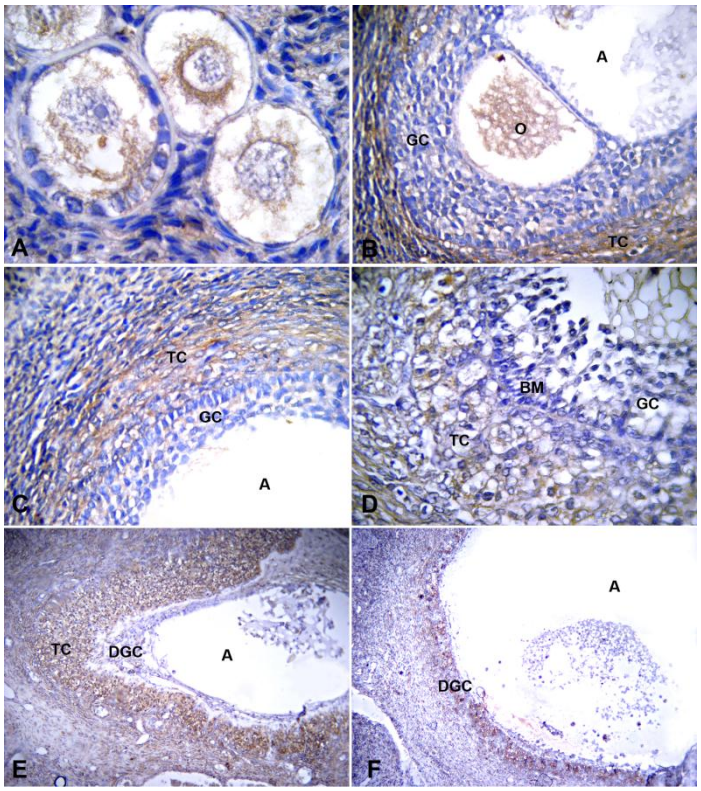


Fig. 2. FAS and FAS-L detection in the mature human ovary. A- Resting primordial follicle and primary follicle in the ovary from a 25 years old women showing FAS positive staining (brown) especially in the oocyte. B- Early antral follicle displaying positive immunostaining for FAX at granulosa and theca cells, note the stronger intensity of the signal in theca cells. C- Positive labeling for FAS-L in an early antral follicle, note that cytoplasmic intensity of the signal is stronger in theca than granulosa cells. D- Detail of FAS-L immunolabeling in an antral follicle with extensive detection in theca cells. E- Partial panoramic view of an atretic antral follicle with strong immunostaining for FAS-L in the theca, and with less intensity in degenerating granulosa cells. F- Atretic antral follicle displaying FAS intense staining in degenerating granulosa cells. A: antrum; O: oocyte; GC: granulosa cell; TC: theca cell; BM: basal membrane; DGC: degenerating granulosa cell. All samples counterstained with haematoxylin, blue nuclei/faint blue cytoplasm.

DNA Fragmentation/Integrity in the Mature Ovary

We did not detect positive cells in resting primordial, primary or secondary follicles at any of the analyzed samples. Some antral follicles from the growing pool were found to be TUNEL positive; positive nuclei were mainly distributed in the granulosa layer of the follicle (Fig. 1F). TUNEL assayed sections were incubated with DNase in order to confirm DNA integrity, especially in negative pre-antral follicles. After enzymatic treatment all nuclei became positive indicating that DNA was originally not fragmented. Negative controls performed by omitting TdT showed no positive reaction.

DISCUSSION

The results presented in this report clearly indicate that both *BAX/BCL2* system from intrinsic apoptosis path as well as *FAS* and its ligand *FAS-L*, known to act in extrinsic apoptosis pathway, are immune-detectable in the mature human ovary.

The pattern of immunolocalization of BAX and BCL2 proteins is comparable and follows what could be anticipated from the expression of both genes during fetal life and the infant human ovary. BAX shows a constant and conspicuous pattern of expression throughout the different stages of folliculogenesis, from dormant primordial follicle reserve to primary and secondary follicles recruited from that reserve, and antral follicles entering cyclical follicular recruitment. This sustained expression of BAX has also been reported to occur throughout fetal life from early proliferative stage of PGC and oogonia, during the entrance to prophase I at mid-gestation and from primordial follicular assembly to the end of gestation (Vaskivuo et al., 2001; Albamonte et al., 2008; Hartshorne et al., 2009). Although the human ovary from birth to puberty has been less investigated, probably due to difficulties in obtaining samples, our previous observations on the infant ovary indicate that BAX is detected in primordial, primary, secondary and pre-antral oocytes (Albamonte et al., 2006).

BCL2 protein displayed a quite different pattern of immunolocalization when compared to BAX. BCL2 protein was not detectable in dormant primordial follicles and primary follicles leaving the resting reserve. BCL2 became detectable at secondary follicles, as well as pre-antral and antral growing follicles, in the somatic stratum, co-existing with BAX protein. In fetal life, BCL2 also shows a time-restricted pattern of expression occurring at the proliferative stage before the entrance to meiosis and follicular assembly stage (Vaskivuo et al., 2001; Hartley et al., 2002; Albamonte et al, 2008). Previous to puberty, the infant ovary displays a comparable pattern to that described here, v.g. no detection in resting primordial follicles and primary follicles, and detection in granulosa cells from secondary follicles (Albamonte et al., 2006).

We have shown that the FAS/FAS-L system involved in the extrinsic pathway of apoptosis, displays an immunohistochemical pattern comparable to that found for the apoptosis-inducing BAX protein. This system has deserved little attention, and a few contributions explore its role in the human ovary (Quirk et al., 1995; Cataldo et al., 2000; Kondo et al., 1996). It is worth to note that both FAS protein and its ligand FAS-L display strong immunolabeling in fully antral and atretic follicles reinforcing the idea that the system acts especially in follicular regression and atresia (Kondo et al., 1996).

It has been proposed that, after birth, apoptotic activity moves from the

germ cell proper to the somatic compartment involving primarily the granulosa cells of resting follicles (Vaskivuo et al. 2001). Our results indicate that the switch occurs when primordial follicles leave the resting primordial follicle reserve to enter the growing follicular pool, a situation that can take place years or decades after birth. After birth, as long as the primordial follicle rests in the dormant follicular reserve, apotosis machinery expresses in the germ cell proper (Albamonte et al., 2006), as it occurs in fetal life (Vaskivuo et al., 2001; Albamonte et al., 2008), and this will be the same in the mature ovary as reported here.

It is worthwhile to note that the constant detection of apoptosis-inducing BAX protein not necessarily implies that a great level of apoptosis cell death is taking place. Members other than *BCL2* and *BAX*, from the *BCL2* gene family may be acting in the mature human ovary. For instance, it has been shown that in the fetal ovary another member of the *BCL2* family, the anti-apoptotic Mcl-1 protein, is strongly expressed in germ cells from gestation week 18, and plays a central role in inhibiting cell death at the time of germ cell–somatic cell association during primordial follicle formation (Hartley et al., 2002). Both BCL2 and Mcl-1 are known to interact with pro-apoptotic BCL2 members, such as BAX, to form heterodimers and suppress apoptotic activity (Hsu et al., 1998). Moreover, it seems that different apoptosis-inhibiting *BCL2* family members act in a concerted temporal way during prenatal oogenesis and folliculogenesis in the developing human ovary, to regulate the default pathway committing germ cells to death (Albamonte et al., 2008). This might well be the case in the mature human ovary, especially in order to preserve the resting primordial follicle reserve which has to be able to deliver healthy oocyte for decades to the growing follicular pool.

We have shown here that TUNEL positive stain is mainly detected in early antral and pre-ovulatory antral follicles from the growing follicular pool, and atretic follicles, in the mature ovary. The resting primordial follicle reserve did not show any sign of apoptosis-related DNA fragmentation detected by the TUNEL assay. Moreover, the frequency at which TUNEL positive antral follicles were observed in the samples analyzed does not seem to be commensurate with the expected extent of intraovarian germ cell dismissal. Puberty is reached with aproximately 400,000 oocyte in the resting primordial follicle reserve (Baker, 1963; Forabosco et al., 1991). In optimal conditions, just around 400 eggs will be released at ovulation throughout reproductive life (0.1% of the reserve); the large majority of them will die in the ovary. Since it is not possible to relate the positive TUNEL stain in a histological section to the actual time it takes to disappear a dying cell from the tissue and DNA degradation is a late event in the sequence of apoptosis (Collins et al., 1997), many follicles already engaged in early steps of apoptosis may have escaped our analysis. However, our preliminary observations on active caspase activity, an early player in the apoptosis pathway, do not seem to account for massive germ cell death (unpubl. results). It is worthwhile to consider that other mechanisms may be at work in the mature human ovary. In the last years, evidence has been gathered that concurrent death mechanisms may be acting in the mammalian ovary such as caspase-independent and autophagic cell death (De Felici et al., 2007; Rodrigues et al., 2009; Tingen et al., 2009). In mice, it has been shown that apoptosis and autophagy contribution to germ cell death may vary at different stages of ovarian development (Rodrigues et al., 2009). In the human ovary, the stage-specific expression of *BCL2* as shown here and in the fetal ovary (Albamonte et al., 2008) might be a clue to illuminating key signaling pathways in survival or death of the germ cell, especially considering that *BCL2* as well as *BCL-X$_l$*, another apoptosis-inhibiting member of the *BCL2* gene family, play essential roles in the crosstalk between autophagy and apoptosis (Zhou et al., 2011). Autophagy is a survival mechanism to escape prolonged nutrient deprivation, but can causes death when taken to extremes. Even if BCL2 and BCL-Xl suppress autophagy by binding to Beclin I protein, crosstalk between both mechanisms is not so simple since autophagy can help to cell survival by suppressing apoptosis or can lead the cell to death in collaboration with apoptosis, depending on cellular environment (Zhou et al., 2011).

CONCLUSIONS

Although some data have accumulated on apoptosis in the human ovary, we are still far from having a detailed knowledge on the regulation of germ cell number that is drastically reduced from 7 x 10^6 cells at the fifth month post-conception to around 400,000 oocytes at puberty. The results presented here show that BCL2/BAX and FAS/FAS-L systems are immunodetectable with differential patterns in the adult human ovary. BCL2/BAX behaves in the resting primordial follicle reserve in a comparable manner as already described for the fetal ovary. Apoptosis-related proteins move from the germ cell proper to the somatic stratum when primordial follicles leave the resting reserve to enter the growing follicular pool. The magnitude of apoptosis-dependent cell death in the growing pool does not seem to explain massive germ cell dismissal. This highlights the need to investigate other mechanisms of programed cell death that may be concurrent to apoptosis. Further studies to understand the multiple mechanisms at work in human germ line development are needed to optimize the rescuing and clinical use of germ cells in infants and adult women.

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