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Programmed cell death in mouse primordial germ cells

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ABSTRACT In a number of mammalian species, the main events of development of the primordial germ cells (PGCs), the embryonic precursors of the oocytes and spermatozoa, were described during the early twentieth century. Actually, the concept of the origin of germ cells in extragonadal sites before the formation of the gonadal anlagen, was put forward for the human embryo around the first decade of the 1900s (for a review, see De Felici, 2013). PGC development is characterized by two major cellular processes, a movement from the wall of the yolk sac, where the germline is determined, to the gonadal anlagen and an increase in number due to active proliferation. As far as we know, the notion that programmed cell death (PCD) might physiologically occur in mammalian PGCs was for the first time put forward by us in 1993 in the case of the mouse. How we arrived to such a concept and the progress made up to now in the characterization of this process in our and other laboratories mainly in the mouse are the topics of the present review.

KEY WORDS: *MAPK, ROS, apoptosis*

Isolated primordial germ cells in culture as a model of apoptotic cell death for neglect

In the mouse, germ cell line is induced in the epiblast at around 6.0 days post coitum (dpc) by Bone morphogenetic protein (BMP) signalling from extraembryonic tissues. PGCs are then determined at about 7.5 dpc as a small cluster of alkaline phosphatase (AP)-positive cells in the extraembryonic mesoderm (for a review, see De Felici, 2010). During the subsequent 3-4 days of development (8.0-11.5 dpc), PGCs move to the gonadal anlagen and proliferate rapidly to establish a population estimated approximately of 20,000 cells in the MF-1 and CD-1 mouse strains (Tam and Snow, 1981; La Sala *et al*., 2012). About two days after their arrival in the gonadal ridges, at around 13.5 dpc, PGCs stop mitotic divisions and enter cell cycle arrest or meiosis in the developing testis or ovary, respectively. At this stage, the life history of PGCs can be considered over since they are now differentiated into prospermatogonia or primary oocytes (for a review, see, De Felici and Farini, 2012).

Our first attempts to *in vitro* culture isolated mouse PGCs obtained from 11.5-12.5 dpc gonads performed at the beginning of the eighty years were a failure (De Felici and McLaren, 1983). In fact, we observed that under a variety of culture conditions, PGCs underwent a rapid loss of viability (De Felici and McLaren, 1983). We obviously suspected that at this stages PGCs required a support from the neighbouring somatic cells. For this reason, we begun to devise *in vitro* coculture systems of PGCs onto various cell monolayers. These experiments lead we and others to identify two cytokines namely the Stem cell factor (SCF, then also called Macrophage growth factor, MGF and then known as Kit ligand, Kl or Steel factor, Sl) and the Leukemia inhibitory factors (LIF), essential for maintaining PGC survival in culture (Pesce *et al*., 1993). On the wave of the emerging notion of the cell death for neglect suggesting that most if not all cells require growth factor signalling to avoid activation of default intrinsic PCD pathways in the form of apoptosis, we thought to investigate whether dying PGCs in culture showed morphologies and biochemical features characteristic of apoptosis (Fig.1). Besides obtaining morphological and biochemical evidence of PGC apoptosis in culture such as nuclear chromatin margination and cell fragmentation, elevated tissue transglutaminase (tTGase) activity and laddering DNA degradation (Pesce et a., 1993; Pesce and De Felici, 1984), we also found mainly in extragonadal sites of 11.5-12.5 dpc embryos, rare PGCs showing apoptotic markers such as tTGase and TUNEL positivity, (Pesce *et al*., 1993; 1996).

These observations together with the important finding that the addition of either SCF or LIF significantly reduced the number of

Abbreviations used in this paper: SCF, stem cell factor; Kl, kit ligand; BMP, bone morphogenetic protein; tTGase, tissue transglutaminase; AP, alkaline phosphatase; LIF, leukemia inhibitory factor; PCD, programmed cell death; PARP, poly ADP ribose polymerase; AGM, aorta-gonad-mesonephros; ECM, extracellular matrix; FAK, focal adhesion kinase, Src, Rous sarcoma oncogene cellular homolog); ILK, integrin-linked kinase; GSK3, glycogen synthase kinase 3.

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PGCs showing apoptotic features in culture (Pesce *et al.*, 1993), paved the way to subsequent studies by us and others aimed to characterize this process and investigate at which extent PGC apoptosis was involved in normal and altered conditions of the germ cell development.

Morphological and biochemical features of PGC apoptosis

*The i*dentification of ongoing cell death in a cell type relies on a number of morphological and biochemical markers that are also useful to distinguish among the various forms of PCD, basically including apoptosis, autophagy, necroptosis and necrosis.

As reported above, our first studies indicated that *in vitro* cultured dying PGCs showed morphological and biochemical features of classical apoptosis. Apoptotic morphologies and DNA degradation were also recognized in a small percentage of fluorescence-activated cell sorting (FACS) analysed mouse PGCs freshly isolated from 12.5 and 13.5 dpc mouse gonads (about 1% and 4%, respectively) (Coucouvanis *et al.*, 1993). In our subsequent studies, we also found that flow cytometry was a suitable method to identify apoptotic PGCs on the basis of their reduced size (forward scatter) and DNA content (<2C DNA) (Fig. 2).

Detection of DNA fragmentation by TUNEL, caspase activity (mainly caspase-3) and cleaved Poly ADP ribose polymerase (PARP) were then used in a number of works to identify apoptotic PGCs both in culture and in the embryo (Fig. 3 and see for example Pesce *et al.*, 1997; Kasai *et al.*, 2003; Francis and Lo, 2006; Runyan *et al.*, 2006; Watanabe *et al.*, 2013).

A spectacular demonstration of PGC undergoing apoptosis *in vivo* was given by time-lapse video microscopy of aorta-gonad-mesonephros (AGM) slices cultures from 10.5 dpc OCT4-PE:GFP embryos (Stallock *et al.*, 2003). In these videos, live migrating PGC-labelled GFP are seen to rapidly fragmentize and disappear. Apoptosis was confirmed by cleaved caspase-3 staining of these cells and by the observation that fragmentation did not occurred in embryo slices obtained from null mutants of the pro-apoptotic gene Bax (see also below).

Defining the causes and the molecular pathways of PGC apoptosis

The identification of apoptotic markers in PGCs, *in vitro* culture experiments and the characterization of natural or induced gene mutations resulting in altered germ cell number, have all contributed to our present knowledge of the possible causes of PGC apoptosis and the involved pathways. It is generally considered that as in other cell types, PGC apoptosis may basically occur as a consequence of three processes: absence of specific growth factors (death for neglect), loss of contacts with neighbouring somatic cells or certain extracellular matrix (ECM) molecules (anoikis) and DNA damage.

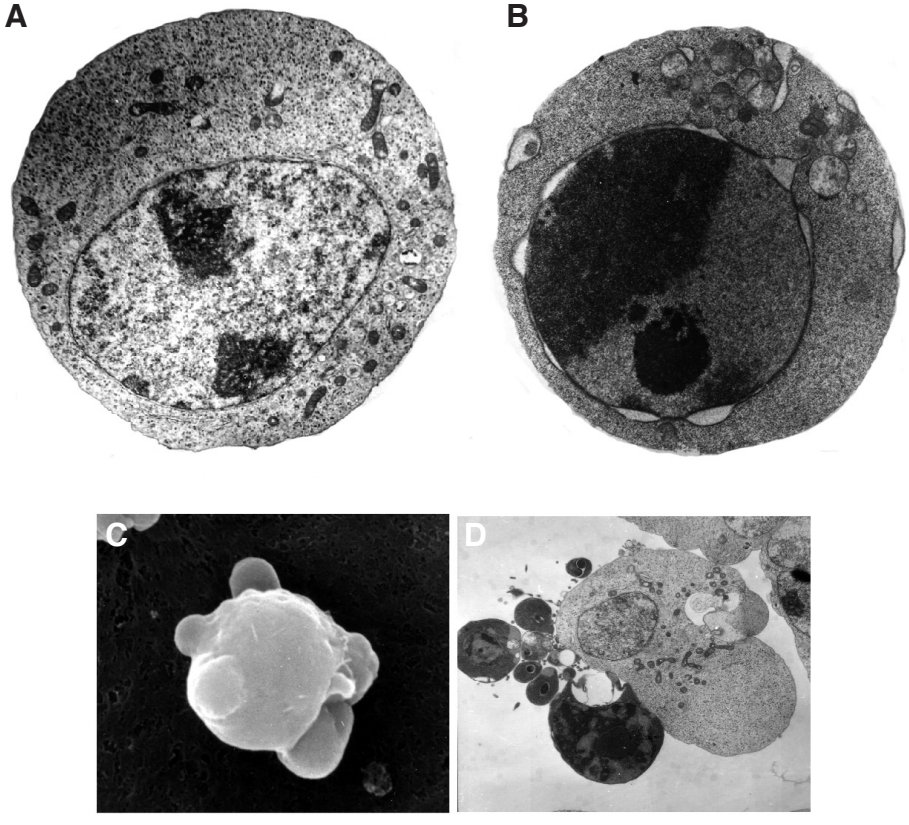

Fig. 1. Morphologies of mouse primordial germ cells (PGCs) in culture by TEM and SEM. **(A)** *A PGC showing normal morphology;* **(B)** *a PGC with typical morphologies of apoptosis. TEM x 10,000.* **(C)** *A PGC showing blebs, likely foreseeing cell fragmentation, SEM x 7000.* **(D)** *A fragmenting PGC, TEM x4000.*

PGC death for neglect: anti-and pro-apoptotic pathways

As reported above, it is believed that in normal tissues many cells should undergo a form of default intrinsic apoptosis known as death for neglect, if not prevented to do this by survival factors, generally a combination of growth factors and cytokines. Growth factors and cytokines can be present in soluble form in the cell microenvironment, exposed to cell plasma membrane or attached to ECM. Cell attachment to ECM can represent a further level of control for their survival. In fact, when some cell type lose their normal ECM interactions mainly mediated by integrins, cell cycle is arrested and a specific form of caspase-mediated apoptosis, known as anoikis, is initiated.

Current information indicate that all these conditions might contribute to PGC apoptosis. Actually, early observations reported above indicated that mouse PGCs underwent apoptosis when isolated from their environment and that this process was at least partly prevented by the addition of certain soluble cytokines or co-culture onto cell feeder layers, especially those expressing the membrane bound form of SCF.

The relevance of adhesion to ECM in PGC apoptosis has been never investigated in details. We and others, however, have identified several integrins expressed by mouse

PGCs and characterized their preferential developmental-stage dependent adhesion to certain ECM substrates (for a review, see De Felici *et al*., 1998). Moreover, PGC adhesion to serum-derived compounds or fibronectin has been reported to be crucial for the pro-survival action of growth factors *in vitro* (Farini *et al*., 2005; Leitch *et al*., 2013)

Three primary integrin signalling molecules that have been linked to cell survival, FAK (focal adhesion kinase), Src (Rous sarcoma oncogene cellular homolog) and ILK (integrin-linked kinase), may impinge upon PI3K/Akt on which the signalling from several growth factors and cytokine also converge. As we will discuss below, the PI3K/Akt pathway plays a pivotal role in PGC survival and the lack of signals from this system can activate a default intrinsic apoptotic pathway in such cells.

The first indication of possible death by neglect in mouse PGCs caused by cytokine deprivation came from studies showing that the mouse *White spotting* (W) and *Steel* (Sl) loci, known for mutations leading to marked reduction or absence of germ cells within the gonads, encoded for the receptor tyrosine kinase Kit and its cognate cytokine ligand Kl or Steel factor (also known as Stem cell factor or SCF), respectively (for a review, see Besmer *et al*., 1991). These observations were timely followed by *in vitro* culture studies (Dolci *et al*., 1991; Matsui *et al*., 1991; Godin *et al*., 1991), including ours (see previous section), demonstrating that the SCF/Kit system was crucially involved in sustaining the survival/proliferation of migrating and gonadal mouse PGCs by preventing their apoptosis. Several years later, Runyan *et al.,* (2006) provided the first molecular evidence that the loss of Steel factor caused actually PGC apoptosis in vivo. These authors showed that in *Steel-/-*mice, PGC apoptosis revealed by cleaved PARP activity, begun at 9.0 dpc, but not before, and continued until most germ cells were lost by 10.5 dpc. Besides proving the important role of the SCF/Kit system for PGC survival in the mouse embryo, these results confirmed previous observations (Buehr *et al*., 1993), that this system is not important for the survival of PGCs in early stages of formation.

Another cytokine as the Leukemia inhibitor factor (LIF), well known for its pivotal role in the formation and maintenance of mouse embryonic stem (ES) cells, was found to be required for the *in vitro* survival and/or proliferation of migrating and gonadal mouse PGCs (De Felici and Dolci, 1991; Dolci *et al*., 1993; Pesce *et al*., 1993; Koshimizu *et al.,* 1996, Matsui *et al.,* 1991, Matsui *et al.,* 1992 and Resnick *et al.,* 1992). The biological activity of LIF on PGC survival, at least partly due to its anti-apoptotic action (Pesce *et al*., 1993), was substituted partially or fully by its close relative Oncostatin M (OSM) and IL-11 (Cheng *et al*., 1994; Hara *et al*., 1998; Koshimizu *et al*., 1996). Moreover, the effect of LIF on the survival of PGCs was fully inhibited by antibodies blocking the LIF gp130 receptor. Although *in vitro* cell culture studies have convincingly shown that these IL-6 family of cytokines plays important roles in PGC survival, inactivation of either LIF or its specific receptor does not cause obvious defects in PGCs in vivo, either suggesting redundancy or compensation by other classes of growth factors *in vivo* (Molyneaux *et al*., 2003; Ware *et al*., 1995).

Some evidence exist that the fibroblast growth factor 2 and 7 (FGF2 and FGF7) through FGFR2-IIb receptor (Takeuchi *et al*., 2005), Stromal cell-derived factor 1 (SDF1) through CXCR4 receptor (Molyneaux *et al*., 2003), and Interleukin 4 (IL-4) through IL-2R/4R receptors (Cooke *et al*., 1996), may also promote the survival of migrating mouse PGCs by reducing their apoptosis.

Finally, TIAR, an RNA recognition motif/ribonucleoprotein-type RNA-binding protein, highly expressed in mouse PGCs, may affect the stability of mRNA encoding for PGC anti-apoptotic growth factors or their respective receptors. In fact, the numbers of PGCs populating the gonadal ridge in TIAR-deficient embryos were severely reduced compared to wild-type embryos by E11.5 and germ cells completely absent at 13.5 dpc (Beck *et al*., 1998).

In general, cytokines and growth factors exert their anti-apoptotic action through signals converging in maintaining an adequate level of cell metabolism and/or a favourable balance between pro- and anti-apoptotic factors. Both activities can require regulation of gene expression at various level or modulate directly various signal transduction pathways.

As far as we know, the only evidence that cytokines and growth factors may exert an anti-apoptotic action on PGCs by regulating their metabolism, is the observation that in 8.5 dpc mouse PGCs, the mTOR/FRAP signalling, centrally involved in cell metabolism, can be activated downstream of the Kit receptor (De Miguel *et al*., 2002). In addition, it is to mention that the targeted deletion of the gap junction protein connexin 43, results in apoptosis of migrating mouse PGCs associated with abnormal p53 activation (Francis and Lo, 2006). Thus suggesting that metabolic communication through gap junctions not only are present between PGCs and the surrounding somatic cells, as also showed by our early studies (De Felici *et al*., 1989), but are able to modulate PGC apoptosis.

Typically metabolic stresses also including growth factor deprivation, may activate cell autophagy to mitigate damage and provide nutrients for short-term survival. If such process occurs in PGCs *in vivo* or *in vivo* in the absence of growth factors is not known but it certainly deserves to be investigated. Likewise scant information is available about the involvement of extrinsic pathways in PGC apoptosis. In this regard, it was reported that mRNAs encoding the extrinsic pathway receptor Fas, and Casp8, are not expressed by 10.5 dpc mouse PGCs (Runyan *et al.,* 2006) and that ablation

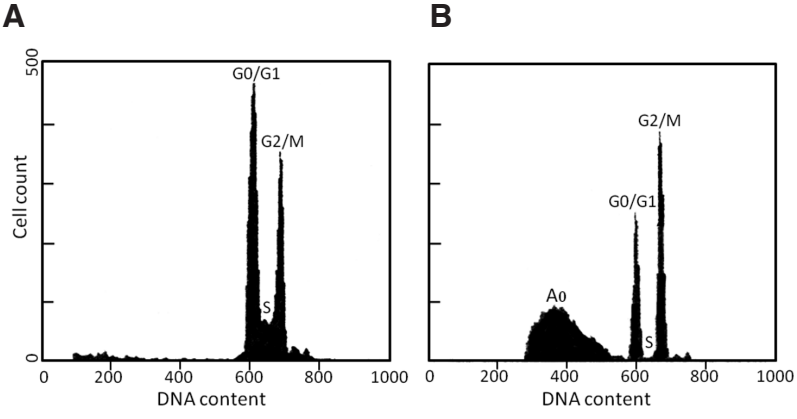

Fig. 2. Identification of apoptotic primordial germ cells (PGCs) by flow cytometry using propidium iodide dye uptake. (A) *12.5 dpc PGCs at the beginning of culture show the normal distribution of cells between the G0/G1, S, and G2/M phases.* **(B)** *The same cells after 16-18 hours of culture, a proportion of cells has undergone apoptosis and appears below the G0/G1 peak in the distribution (Ao; <2C DNA).*

of Fas in W^v/W^v mutant mice while rescued testicular germ cells and oocytes in the adult, did not affect the number of germ cells at birth (Sakata *et al*., 2003). It is to be mentioned, however, that in humans, BMP4 appears to negatively regulate post-migratory PGC numbers by promoting their apoptosis (Childs *et al*., 2010).

Akt kinase-dependent anti-apoptotic pathways

The signal transduction pathways activated by the SCF/Kit system that prevent apoptosis in PGCs has not be clearly identified and only fragmentary information exists about the involved players.

It is well known that in many cell types, including mouse PGCs (de Miguel *et al*., 2002; Farini *et al*., 2007), the Akt kinase is downstream the auto-phosphorylation of Kit occurring after SCF binding. Three main pathways can lead then to Akt activation: PI3K, Src and Ras/Raf/MEK/MAPK pathways. The relationship among these pathways is complicated by various crosstalks and the fact that they respond to several extracellular and intracellular cues. In addition, some evidence suggest that these three pathways might be differently active in modulating PGC survival and apoptosis depending on the developmental stages. In migratory PGCs, Kit appears to preferentially activate Akt through Src and at the same time to stimulate JNK (de Miguel *et al*., 2002; Chen *et al*., 2013). At the same time, FGF2 or FGF7 stimulate Ras/Raf/MEK/MAPK pathways (Takeuchi *et al*., 2005). In gonadal PGCs, Kit-dependent Akt activation is regulated by PI3K, and at lesser extent by Src and Ras/Raf/MEK/MAPK (Farini *et al*., 2007). In addition, a Kit-dependent Akt activation mediated by compound different from SCF such as estrogens and retinoic acid is possible (La Sala *et al*., 2010; our unpublished observations). Whatever the pathways leading to Akt activation, how this kinase may regulate PGC apoptosis?

In a number of cell types, Akt is pivotal in the regulation of Bad (Bcl-2-antagonist of cell death), a BH3 only pro-apoptotic member of Bcl-2 family. Bad heterodimerizes strongly with Bcl-xL, and at lesser extent with Bcl-2, inhibiting the death repressor activity of Bcl-xL, but not that of Bcl-2. This allows Bax and Bak to aggregate, form channels on the mitochondrial membranes and induce apoptosis. Actually, Akt phosphorilates Bad that in this form is sequestered in the cytosol by binding to 14-3-3 proteins and makes Bcl-xL and Bcl-2 free to exert their anti-apoptotic action. Unfortunately, no information exists if Akt activation in PGCs leads to Bad phosphorylation. Several data indicate, however, that PGC survival, as that of many other cell types, depends on the balance between members of the pro-and anti-apoptotic Bcl-2 family controlled via SCF/Akt mainly Bcl-xL and Bax. Early experiments showed that, the transfection and expression of Bcl-xL in 11.5 dpc mouse PGCs significantly promoted their survival (Watanabe *et al*., 1997). Runyan and colleagues (2006) reported that the mRNAs for pro-apoptotic Bcl-2 proteins Bax, Bak, Bad and Bim, and for Casp3 (caspase 3), were all upregulated in 10.5 dpc mouse PGCs undergoing apoptosis in the midline of the dorsal body wall and down-regulated in gonadal PGCs. These authors also showed that at this stage, midline expression of SCF has become down-regulated and PGCs still in the midline do not migrate directionally, but move randomly, then fragment and disappear (Runyan *et al*., 2006; Stallock *et al*., 2003). The transcripts for other genes such *Bcl-2*, *Bcl-w*, *Bid* and *Casp7* were present both in migratory and post-migratory PGCs, but did not have significant differential regulation.

As for Bcl-2 and Bax proteins, we found very low or absent expression in the ovary of 12.5-13.5 dpc mouse embryos, but a marked increased expression for both proteins in PGCs from such ovaries after 16-18 hr of culture associated to ongoing apoptosis (De Felici *et al*., 1999). Interestingly, we found that the rescue of PGC survival by SCF was linked to a decrease in the amount of Bax but did not affect Bcl-2 expression. In line with our results, a crucial role of Bax in inducing PGC apoptosis was demonstrated by the findings that in *Bax–/–* embryos, midline germ cells fail to die in the 10.5 dpc to 11.5 dpc period. Instead, they colonize extragonadal regions (Stallock *et al*., 2003). Moreover, the absence of Bax *in vivo* did rescue gonadal PGC from apoptosis caused by the loss of SCF (Runyan *et al*., 2006), Nanos3 (Suzuki *et al*., 2008) and aploinsufficiency of Bcl-xL (Rucker *et al*., 2000).

It is to be noted that the severe PGC loss resulting from mutation of in *Dnd1*$^{Ter/Ter}$ gene encoding a not characterized RNA-or DNA-binding protein, which makes PGCs prone to give rise teratomas, can be partly rescued by the absence of Bax (Cook *et al*., 2009). Thus suggesting that Bax may normally eliminate mutant germ cells before their transition into teratomas but that Bax-dependent apoptosis is not the only mechanisms for clearing defective germ cells.

Interestingly, Kasai *et al.,* (2003) found that there is a distinct elevation in the frequency of TUNEL detected apoptosis in male, but not in female PGCs as soon as they start sex differentiation (between 12.5 and 13.5 dpc). At the same time, Bcl-xL expression became higher in female than in male PGCs, while no significant difference was detected in the expression levels of Bax. Finally, these authors described an apoptotic–inducing activity by male gonadal somatic cells on the PGCs after 12.5 dpc, perhaps linked to their inhibitory action on the meiotic entry (see, also Dolci and De Felici, 1990).

A number of studies using transgenic mice also supported a pivotal role of Bcl-2 family proteins in regulating PGC apoptosis. Histological analyses revealed that ovaries collected from Bcl-$2^{-/-}$ mice possessed numerous aberrantly formed primordial follicle-like structures containing a single layer of granulosa cells without an oocyte. Additionally, the total number of primordial follicles present which contained a healthy oocyte was markedly reduced in Bcl-$2^{-/-}$ mice as compared to heterozygote (Bcl-$2^{-/+}$) or wild-type (Bcl-$2^{+/+}$) mice (Ratts *et al*., 1995). However the impact of Bcl-2 ablation in postnatal oocytes number was not dramatic. Targeted over-expression of the human Bcl-2 driven by the Kit promoter in the mouse ovary, increased the size of the primordial follicle pool at birth likely by decreasing the number of germ cells that undergo apoptosis in embryonic life (Flaws *et al*., 2001). Finally, young adult female Bax$^{-/-}$ mice possess threefold more primordial follicles in their ovarian reserve than their wild-type sisters (Perez *et al*., 1999).

In some cell types, Akt promotes cell survival, at least in part, by stimulating gene expression via the phosporylation of CREB (the transcriptional cAMP response element binding protein). Interestingly, a coactivator of CREB, CBP (coactivator/histone acetyltransferase CREB-binding protein) is highly expressed in mouse PGCs and its ablation leads to increased apoptosis and subsequent reduction in PGC numbers. (Elliot *et al*., 2007).

Kimura *et al.,* (2008) noted that hyper-activation of Akt signalling in proliferating PGCs at the proliferative phase dramatically augmented the efficiency of their trans-differentiation in embryonic

stem (ES) cell-like cells termed embryonic germ (EG) cells. These authors reported that, Akt activation induced phosphorylation of Glycogen synthase kinase 3 (GSK3), which inhibits its kinase activity; enhanced the stability and nuclear localization of Mouse double minute 2 homolog (Mdm2) and suppressed p53 phosphorylation, which is required for its activation. The p53 deficiency, but not GSK3 inhibition, recapitulated the effects of Akt hyper-activation on EG cell derivation, suggesting that inactivation of p53 is also a crucial downstream targets of the PI3K/Akt signal. In this contest, p53 might be involved in the block of mitosis and the clearance of refractory PGCs by apoptosis (see also below).

As far as we know, no information is available about the involvement in PGC apoptosis of two other possible Akt targets such as FoxOs (Forkhead box O transcription factors) and IKKα, a component of the upstream kinase complex that regulates NF-κB (nuclear factor κB) activation.

Likewise, although, Mcl-1 seems to play an important anti-apoptotic SCF/Kit-dependent role in neonatal mouse oocytes (Jones and Pepling, 2013), no information about the expression of this gene in PGCs is available.

MAP kinase-dependent apoptotic pathways

Mitogen-activated protein kinase (MAPK) family members are crucial for the maintenance of many cell types. Three subfamilies of MAPKs have been identified: extracellular signal-regulated kinases (ERKs), c-Jun N-terminal kinases (JNKs), and p38-MAPKs. It has been originally shown that ERKs are usually important for cell survival, whereas JNKs and p38-MAPKs were deemed stress responsive and thus involved in promoting apoptosis. However, the regulation of apoptosis by MAPKs is more complex than initially thought and often controversial.

As anticipated above, De Miguel *et al.,* (2002), first suggesting a role of MAP kinase in preventing PGC apoptosis via Kit showing that two potent MEK/MAPK inhibitors reduced the number of migratory PGC culture onto cell feeder layers producing membrane bound SCF. Subsequently, Takeuchi *et al.,* (2005) reported that FGF2 and FGF7 through FGFR2-IIb receptor were able to promote the survival of migrating mouse PGCs by reducing their apoptotic rate via MEK/ERK signalling. Interestingly, contrary to the expecting, mouse PGCs carrying a constitutively activating Kit mutation undergo an extensive Bax-dependent apoptosis both *in vivo* and in vitro. In these PGCs, the basal and SCF-dependent Akt activity remained unperturbed but the activity (phosphorylation) of ERK1/2 and JNK was significantly decreased. Moreover, the addition to the culture medium of a JNK inhibitor, but not of an ERK inhibitor, caused increased apoptosis both in mutated or wild type PGCs (Chen *et al*., 2103). These results clearly implicate that also JNKs are involved in promoting PGC survival via Kit. Another indication that MAPKs control positively PGC survival by decreasing apoptosis comes from the observation that REST (repressor element 1-silencing transcription factor) via regulation of the LEK5 kinase is necessary for the survival of migratory mouse PGCs. In fact, target ablation of the REST locus resulted in a significant increase of apoptosis in such cells and a significant depletion of their number. Interestingly, there were a normal number of PGCs in the REST mutants at birth, and both the male and female REST-mutant adults were fertile. These final observations revealed that the PGC population was very robust and could recover from a genetically induced reduction in cell number.

ROS-dependent PGC apoptosis

Reactive oxygen species (ROS) represent a major player in cell apoptosis. Different stimuli will lead to the production of different ROS species and in turn these will elicit different responses. ROS levels are increased in response to stress likely due to increase in mitochondrial damage. It appears to stimulate the apoptotic cascade by causing damage to many cellular components including proteins, lipids and the mitochondria which in turn facilitates the release of pro-apoptotic factors.

Moreover, Bax activity and ROS are intimately linked such that increases in either one of the three leads to either increased levels of the other or an increase in their ability to function. Commonly proposed mechanisms of cross-talk between Bax and ROS mediated apoptosis involves the convergent activation of common pro-apoptotic proteins such as JNK kinase.

Antioxidants considerably increase the survival of pig PGCs cultured in the absence of cell feeder layers (Lee *et al*., 2000). Moreover, we found that the addition of a potent antioxidant N-acetyl-L-cysteine (NAC) to the culture medium significantly increased mouse PGC survival after 24 hr and implemented the anti-apoptotic action of a cocktail of growth factors and cytokine including SCF and LIF. However, decreased oxygen tension from 21% to 5% did not affect significantly the survival of cultured PGCs (Farini *et al*., 2005). Although these results imply a role of ROS in PGC apoptosis no other information is available on this topic.

DNA damage and PGC apoptosis

DNA damage is able to activate enzymatic complexes able to detect the DNA damage and trigger the repair and/or intrinsic apoptotic pathways. Key components of DNA damage signalling networks include Atm (Ataxia telangiectasia mutated protein), Chk2 (Checkpoints factor-2), p53 family members and Bax. Mutations in the damage signalling systems are linked to tumorigenesis and developmental abnormalities. The maintaining of the DNA integrity is of vital importance in the germline where DNA repair is essential to ensure the faithful transmission of the genome from one generation to the next; failure to do so has catastrophic consequences for the offspring. Furthermore, tumours arising from cells of the germline are the most common solid cancers in newborns. In this contest, apoptosis could be crucial to eliminate defective PGCs. p53 family members are major sensors of DNA damage, and critical activators of the intrinsic apoptotic pathway. For example, the DNA checkpoints proteins, Atm and Chk2 directly phosphorylate and stabilize p53 and inhibit Mdm2 (mouse double minute-2 homolog)-mediated ubiquitination of p53. Mdm2 binds p53 and mediates its nuclear export. When bound to Mdm2, p53 can no longer function as an activator of transcription. In vertebrates, p53 family of genes includes three genes for the p53, p63, and p73 proteins.

In *Drosophila* PGC death is mediated by p53 and mutations in p53 result in excess PGCs that are ectopic to the gonads (Yamada *et al*., 2008). In mammals, although direct and indirect evidence reported below indicate that p53 and p63 are expressed both in migrating and gonadal mouse PGCs, their ablation did not seem to significantly affect their number (Matsui *et al*., 2000; Guerquin

et al., 2009; Donehower *et al.*, 1992). Nevertheless, p53 might have the function to activate apoptosis in PGCs in various conditions related both to DNA damage and cell cycle control.

Heyer *et al.,* (2000) observed that during specification (between 6.25 and 7.25 dpc) mouse PGC precursors are extremely sensitive to very low irradiation (<0.5 Gy) that caused apoptosis of about half of the forming PGCs. Activation of ATM and p53 controlled this process, since it did not occurred in embryo lacking these genes. Since in cell lines, Blimp1 (or Prdm1), a transcription factor pivotal for mouse germline specification, suppresses p53 transcription and promotes normal cell growth (Yan *et al.*, 2006), it should be interesting to investigate whether Blimp1 exerts such action also in mouse PGC precursors. At later stage, ionizing radiation sensitivity of PGCs seems regulated both by p53 and p63, but in a sex-dependent manner. In fact, gonadal male mouse PGCs resulted more sensitive to irradiation and underwent massive apoptosis driven by p53 and p63 while female PGCs suffered less p53 and p63 independent apoptosis.

Germ cells tumour development *in vivo* and EG cells formation *in vitro* from PGCs are favoured by ablation or reduced activity of p53 likely associated to refractoriness to anti-proliferative signals (Donehower *et al.*, 1992; Kimura *et al.*, 2008). In this contest, p53 might be involved in the block of mitosis and clearance by apoptosis of PGCs refractory to cell cycle block. Observations in mice with *Dnd1*$^{Ter/Ter}$ mutation appear to be consistent with such a model (Youngren *et al.*, 2005; Cook *et al.*, 2011). Examination of the developing gonads revealed that *Ter* embryos possessed reduced number of PGCs even though the *Ter* mutation causes PGCs to undergo an extended proliferative period. This observation is consistent with the *Ter* mutation contributing to a lack of responsiveness to anti-proliferative signals among the PGC population, with most of the cells being subject to clearance but a few escaping to form tumours. In this contest, PGC loss in *Dnd1*$^{Ter/Ter}$ mice was only partly rescued by the absence of Bax thus suggesting that Bax-dependent apoptosis is not the only mechanisms for clearing defective germ cells and that this could riley also on p53 family members (Cook *et al.*, 2009). A role for these guardians in regulation the number of PGCs is also supported by the finding that targeted disruption of the *Puma* gene (p53 up regulated modulator of apoptosis), also known as Bcl-2-binding component 3 (Bbc3), a pro-apoptotic BH3-only protein belonging to the Bcl-2 family downstream p53 activation, resulted in a significant increase in the number of mouse PGCs as early as embryonic day 13.5 dpc. The number of germ cells remained elevated in *Puma-/-* female mice compared with WT female mice throughout the rest of embryonic and early postnatal life, resulting in a about two fold increase in the number of primordial follicles in the ovary on postnatal day 10 (Myers *et al.*, 2014). Finally, as reported above, connexin 43 ablation, results in apoptosis of migratory PGCs associated with p53 activation (Francis and Lo, 2006).

Contrary to the canonical pro-apoptotic role of p53, in female PGCs entering into meiosis and in male PGCs coming close to mitotic block, p53 phosphorylation seems necessary to avoid apoptosis. Although phosphorylation of p53 does not appear to be essential for p53 function, several studies have suggested that phosphorylation of p53 plays an important role in regulating p53 activities such as its stability and DNA binding capability. Actually, haploinsufficiency of the transcription factor Zfp148, highly expressed in mouse PGCs and able to phosphorylate p53 in Ser15, caused a marked reduction of the PGC number by apoptosis (Takeuchi *et al.*, 2003).

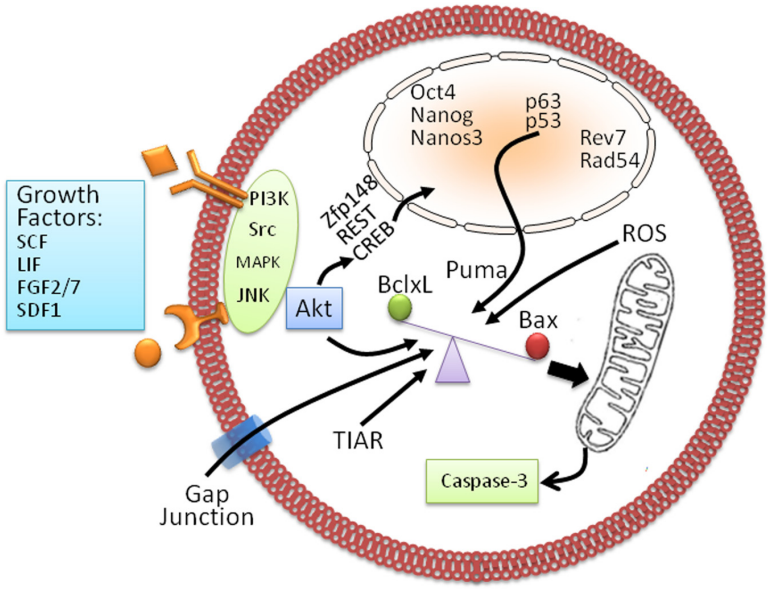

Fig. 3. Schematic drawing of the current knowledge about the main pathways of mouse primordial germ cell (PGC) apoptosis. *The balance between Bcl-xL and Bax represents a pivotal regulator of the intrinsic apoptotic pathways of PGCs. External signals by growth factors converge on Akt and JNK to prevent apoptosis; the RNA-binding protein TIAR and molecules from gap junctions contribute to this process. Internal signals from sensors of DNA damage act as pro-apoptotic factors. Among these, p53 phosphorylation modulated by Zfp148 is necessary to prevent apoptosis, whereas p53 or p63-dependent Puma activation is involved in inducing apoptosis. At the same time, the activity of enzymes involved in DNA repair such as Rev7 and Rad54 and of a variety of transcription factors such as CREB and REST and of the pluripotency core circuit (Oct4, Nanog, Nanos3) is necessary to avoid apoptosis. Further details are given in the text.*

Although the predominant p63 isoform expressed during development are amino-terminally truncated ΔN form, strong expression of ΔNp63 and TAp63 is observed in the nucleus of both female and male PGCs as early as 8.5 dpc and throughout the embryo/foetal life (Nakamuta and Kobayashi 2004; Kurita *et al.,* 2005; Suh *et al.,* 2006; Petre-Lazar *et al.*, 2007). The meaning of such expression remained to be clarified.

Mutations in the DNA damage signalling systems have been recently identified leading to PGC apoptosis. In mouse embryos lacking Rev7/Mad2l2, a chromatin binding protein encoding a subunit of DNA polymerase ζ (Polζ), involved in both cell cycle control and DNA repair during S phase, PGCs were specified normally, but became eliminated by apoptosis during the subsequent phase of development (Pirouz *et al.*, 2013; Watanabe *et al.*, 2013; Khalai et a., 2014). PGC apoptosis induced by defects in DNA replication is also suggested by the marked reduction in germ cell number at birth in mice lacking Mcm9, a DNA helicase, This defect was not compensate by the absence of p53 (Hartford *et al.*, 2011)

Finally, Rad54 an important factor in the homologous recombination pathway of DNA double-strand break repair, is required for the

normal development of PGCs and contributes to the maintenance of their genome integrity after genotoxic stress. In fact, KO mice for Rad54 showed reduced number of PGCs as early as 11.5 dpc and increased irradiation sensitivity (Messiaen *et al*., 2013).

Deregulation of genome reprogramming as cause of PGC apoptosis

In addition to the causes of apoptosis discussed above, recent evidence indicate that PGCs are subjected to another source of cell death, abnormal genome reprogramming. In the last few years, some studies reported that PGCs undergo genome wide reprogramming during their development involving histone changes and DNA demethylation and the activity of a mutual regulatory circuit of pluripotency transcription factors (for a review, see De Felici, 2011). Pirouz and coll. (2013), showed that in the majority of Rev7/Mad2l2 knockout PGCs which failed to arrest in the G2 phase the normal switch from a H3K9me2 to a H3K27me3 histone configuration did not occur, while Watanabe and coll.(2013), reported increased level of histone methylation. Conditional ablation of two master pluripotency transcription factors such as *Nanog* and *Oct4* resulted in a significant reduction of the number of PGCs in 12.5 dpc and 10.5 dpc embryos, respectively, revealed by TUNEL staining (Kehler *et al*., 2004). Knockout of *Nanos3*, an exclusive germline transcription factor, results in the complete loss of PGCs in 12.5 dpc embryos both via Bax-dependent and independent way (Suzuki *et al*., 2008). Importantly, a common consequence of the loss of gene expression is apoptotic cell death, not cell differentiation. Single-cell microarray analysis demonstrated that abnormal transcription of various types of core regulators, including the RNA-binding protein *Tial1*(or *Tiar*), differentiation inhibitor *Id1*, and PRC2 subunit *Suz12*, occurred within 24 hours of Nanog downregulation in 10.5 dpc PGCs. Surprisingly, under such conditions, the apoptotic death of PGCs is triggered by deregulation in the gene regulation network. The molecular mechanism involved in this control remains to be characterized. It is also unknown whether the apoptosis of Nanog KO PGCs depends on the Bax pathway. Apoptotic cell death triggered by a deficiency in any core gene, including Nanog, might play an important role in preventing the transmission of abnormal genetic information to the next generation.

Reprotoxicants and PGC apoptosis

A number of studies have reported that exposure of mammalian embryos to a number of chemicals cause reduced fertility or cause sterility in the adults. In some cases, such defect was traced to the activation of PGC apoptosis. For example, we found that *N*-ethyl-*N*-nitrosourea (ENU) and adriamycin (ADR) caused a marked reduction of isolated mouse PGC growth in culture by activating apoptosis (Iona *et al*., 2002). Similarly, we reported that exposure by gavage of pregnant mice to 15 or 30 mg/kg of lindane during the period of PGC migration and gonad colonization (from 8.5 to 11.5 dpc, dpc) resulted in a significant reduction of the number of germ cells within 12.5 dpc testis and ovaries and a dose-dependent decrease of cultured PGCs associated to a decrease of Akt activity and increased level of active caspase-3, poly-ADP-ribose polymerase cleavage, and terminal dUTP nick-end labelling (TUNEL) (La Sala *et al*., 2009). Interestingly, using a model of human embryonic stem cells (hESCs) differentiation to germ cells, Kee and coll. (2010) showed that polycyclic aromatic hydrocarbons (PAHs) caused apoptosis in PGCs mediated through the aromatic hydrocarbon receptor (*AHR*) and *Bax* pathway.

Conclusions

From our early studies it was clear that PGCs require multiple factors to avoid activation of intrinsic apoptotic pathways. The advantages of having PGC survival depending on signals produced by the surrounding somatic cells may be to provide a simple mechanism for eliminating cells that end up in abnormal locations during migration. In fact, we first observed that *in vivo* apoptotic PGCs were mainly found in extragonadal sites. Moreover, an active process of cell death may provide a further means of precisely regulating germ cell number by signals that can stimulate or inhibit apoptosis. The aberrant survival of proliferating germ cells in ectopic regions or a prolonged proliferation of gonadal PGCs refractory to mitotic block associated to apoptotic failure might lead to development of germ cell tumours. Finally, precise control of DNA damage in PGCs is essential to maintain the continuity and integrity of the germline. While some players of the PGC apoptosis were identified such as the balance of Bcl-xL/Bax likely controlled by growth factors and cytokines via Akt and the p53/Puma pathway activated by DNA damage, others wait to be recognized and characterized (i.e. extrinsic apoptotic pathways, MAPKs, ROS, metabolism and autophagy crosstalk).The identification of the apoptotic players activated by unbalance in the regulatory gene circuit of pluripotency remain particularly challenging. Fig. 3 summarizes schematically our current knowledge of causes and pathways of PGC apoptosis in mammals.

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