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Cellular Functions of Mitogen-activated Protein Kinases and Protein Tyrosine Phosphatases in Ovarian Granulosa Cells

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Abstract. A number of endocrine and paracrine factors regulate the follicular growth and atresia, which are closely associated with granulosa cell survival and apoptosis. However, the molecular mechanisms underlying the intracellular events induced by these factors are poorly understood. Here, we describe the correlation of mitogen-activated protein kinase (MAPK) activities with granulosa cell survival and apoptosis, and the cellular functions of protein tyrosine phosphatases (PTPs) in these cells based on our recent data. MAPKs play key roles in various cellular responses because numerous extracellular stimuli are integrated into MAPKs. The protein phospho-Tyr level regulated by protein tyrosine kinases (PTKs) and PTPs is a major control mechanism for processes as diverse as cell survival, proliferation, differentiation, and metabolism. Although PTKs are critically involved in granulosa cell survival and proliferation, there are no reports indicating the roles of PTPs in the ovary except for ours. Information about MAPKs and PTPs in these cells will provide a basis for the understanding of the molecular mechanisms controlling the fate of follicles.

Key words: Mitogen-activated protein kinase (MAPK), Protein tyrosine phosphatase (PTP), Granulosa cell, Apoptosis, Adenovirus

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Over 99% of ovarian follicles in mammals undergo a degenerative process called atresia, which is characterized by granulosa cell apoptosis [1, 2]. In contrast, only a few follicles mature and reach ovulation under the cyclic gonadotropin stimulation. The selection of granulosa cell apoptosis or survival is a critical process in determining the fate of follicles. To date, a number of endocrine and paracrine factors have been shown as survival or apoptosis-inducing factors for these cells *in vivo* and *in vitro* [1, 2], but the molecular mechanisms underlying the induced intracellular events are poorly understood. In this review, we describe the correlation of mitogen-

activated protein kinase (MAPK) activities with granulosa cell survival and apoptosis, and the cellular functions of protein tyrosine phosphatases (PTPs) in granulosa cells based on our recent data [3–5]. Both MAPKs and PTPs are considered important regulators for granulosa cell survival, proliferation, differentiation and apoptosis as they are in other types of cells.

Correlation of MAPK Activities with Granulosa Cell Survival and Apoptosis

MAPK family

A vast number of extracellular stimuli are integrated into MAPKs that conversely affect many of the signaling pathways induced by these stimuli.

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Therefore, MAPKs play key roles in various cellular responses including survival and apoptosis [6]. Mammals express at least the following distinctly regulated groups of MAPKs: extracellular signal-regulated kinases (ERK)1/2, Jun amino-terminal kinases (JNK1/2/3), p38 proteins (p38 $\alpha/\beta/\gamma/\delta$), and ERK5. ERK acts primarily to positively regulate cell survival and proliferation, whereas p38 and JNK have apparently opposite actions, depending on the types of cells and/or stimulation. For example, the activation of p38 and JNK with concurrent ERK inhibition is critical for the apoptosis of PC12 pheochromocytoma cells induced by the withdrawal of nerve growth factor [7]. In contrast, p38 β mediates the hypertrophic response in rat cardiac myocytes [8]. These findings suggest that ERK, p38, and JNK critically regulate the fate of granulosa cells. However, there are few reports, including ours, indicating the activation of MAPKs in these cells [3, 4, 9–13].

Activation of ERK and p38 by follicle-stimulating hormone

We recently found that follicle-stimulating hormone (FSH), a critical factor for follicular growth, prevented the apoptosis of porcine cultured granulosa cells induced by withdrawal of serum through the activation of both ERK and p38 [4] (Fig. 1). This FSH-induced effect was diminished by PD98059, which inhibits the ERK kinase MEK1, and by SB203580, which inhibits p38. Thus, both ERK and p38 activities are required for the FSH-dependent survival of these cells. Gebauer *et al.* [12], using a model system based on granulosa cells from the equine chorionic gonadotropin-primed immature rats, revealed that activities of ERK and the MEK kinase Raf-1 were reduced with a concomitant decrease in the phosphorylation level of the pro-apoptotic factor Bad prior to the onset of granulosa cell apoptosis. Babu *et al.* [13] demonstrated that FSH activated ERK and that the inhibition of ERK activity with PD98059 distinctly reduced FSH-induced DNA synthesis in immortalized granulosa cells overexpressing a recombinant novel growth factor type 1 receptor for FSH. We [3] also found that the vasoactive peptide angiotensin II suppressed the FSH-dependent activation of ERK and survival in rat cultured granulosa cells via the angiotensin II type 2 receptor, which is expressed at high levels in atretic, but not in healthy follicles. These data

Fig. 1. Correlation of MAPK activities with granulosa cell survival and apoptosis.

indicate that ERK is an important mediator of FSH actions in follicular growth. On the other hand, Seger *et al.* [14] revealed that ERK down-regulated gonadotropin-stimulated progesterone production in granulosa-derived cell lines expressing recombinant gonadotropin receptors. Therefore, ERK appears to be involved in not only survival and proliferation but also in other critical processes closely related to folliculogenesis such as steroidogenesis in granulosa cells.

Although many factors downstream of p38 lead to cell survival, the small heat shock protein Hsp27 might be involved in the process of p38-mediated, FSH-dependent survival of granulosa cells. FSH induces Hsp27 phosphorylation through p38 activation, leading to granulosa cell rounding/aggregation. Bruey *et al.* [15] revealed that Hsp27 binds to cytochrome c released from the mitochondria into the cytosol and blocks the cytochrome-c-mediated interaction of Apaf-1 with procaspase-9, which subsequently prevents the mitochondrial pathway of caspase-dependent cell death in the leukemic cell line U937. Since reactive oxygen species (ROS) such as H₂O₂ generated in the mitochondria have been proposed as early events in the induction of apoptosis, H₂O₂ might also liberate cytochrome c from the mitochondria in granulosa cells. In agreement with this notion, anti-oxidative reagents such as N-acetyl cysteine and catalase partially inhibited the apoptosis of these cells (unpublished data). Therefore, p38 activation might prevent apoptosis caused by H₂O₂ in granulosa cells as it does in U937 cells.

Activation of ERK and p38 by serum

Serum is considered important for folliculogenesis because the influx of serum components is promoted by vascularization for the maintenance and maturation of healthy follicles.

We found that 10% serum with the activation of both ERK and p38 almost totally inhibited the apoptosis of cultured porcine granulosa cells [4]. However, in contrast to FSH-induced survival, the ERK pathway inhibitor PD98059, but not the p38 inhibitor SB203580, suppressed the serum-dependent prevention of apoptosis (Fig. 1). The p38 activity might not be critical for granulosa cell survival elicited by serum containing numerous humoral factors such as growth factors and cytokines.

Activation of ERK and p38 by H₂O₂

We are presently the only group to have investigated the direct effects of reactive oxygen species (ROS) on granulosa cells [4]. We examined the effect of H₂O₂ on granulosa cells because of the following reasons. Firstly, ROS cause apoptosis in some cells but contribute to cell proliferation and hypertrophy in others. For example, Nakamura *et al.* [16] demonstrated that ovarian corpus luteal cells undergo apoptosis in response to H₂O₂. Secondly, the binding of Fas ligand to Fas produces ROS [17] and both Fas and Fas ligand are expressed in granulosa cells [18, 19]. Thirdly, granulosa cell apoptosis in rats is augmented in response to heat stress with a decrease in the number of ovulations [20]. This process is thought to produce ROS through exaggerated respiration such as that associated with exercise. Thus, ROS such as H₂O₂ probably induce granulosa cell apoptosis as an atretogenic factor or as a mediator of such a factor(s) under physiological and pathological conditions.

We found that H₂O₂ causes apoptosis in porcine cultured granulosa cells even in the presence of 10% serum [4]. Like FSH and serum, H₂O₂ also activated ERK and p38 (Fig. 1). SB203580 significantly attenuated the induction of apoptosis, suggesting that p38 is involved in an apoptotic pathway induced by H₂O₂ as well as in a survival pathway evoked by FSH in granulosa cells. Wang *et al.* [8] recently demonstrated that p38 β mediates the hypertrophic response, whereas p38 α contributes to an apoptotic process in rat cardiac muscle cells. Juo *et al.* [21] also implicated p38 α in the Fas-induced apoptotic pathway in T lymphocytes. These reports raise the notion that in granulosa cells, p38 α and β may be respectively involved in H₂O₂-induced apoptosis and FSH-elicited survival. Further analysis is necessary to

determine whether this notion is correct. On the other hand, the ERK pathway inhibitor PD98059 attenuated the SB203580-dependent recovery of apoptosis induced by H₂O₂ in these cells, revealing that ERK functions as an anti-apoptotic mediator under oxidative stress. Consistent with our data, others [22, 23] reported that apoptosis induced by H₂O₂ is accompanied by the activation of ERK, JNK and p38, and that it is augmented or diminished by inhibiting or enhancing ERK activity, respectively.

Similar to p38, JNK was involved in H₂O₂-induced apoptosis in porcine granulosa cells (our unpublished data). Hydrogen peroxide stimulation activated JNK with the induction of apoptosis, which was partially suppressed by adenovirus vector-mediated overexpression of a dominant negative form of JNK. The inhibitor of EGF receptor kinase, AG1478, inhibited the activation of ERK and of JNK evoked by H₂O₂, suggesting that H₂O₂ activates ERK and JNK via transactivation of the EGF receptor.

Cellular Functions of PTPs in Granulosa Cells

PTP family

The regulated phosphorylation of proteins on specific Tyr residues is a major control mechanism for processes as diverse as cell survival, proliferation, differentiation, and metabolism. The opposing activities of protein tyrosine kinases (PTKs) and PTPs accurately regulate protein phospho-Tyr (pTyr) levels. In general, while the functions of many PTKs have been investigated in detail, far less is understood about the physiological roles of PTPs. It is now known that approximately 40 PTP genes exist in the human genome. This large family of PTPs comprises cytoplasmic and receptor-type forms [22, 23], each of which contains at least one catalytic domain of approximately 250 amino acids characterized by the signature motif [I/V]HCxAGxxR[S/T]. A Cys residue and an Asp residue in the catalytic domain are essential for PTP activities.

Several reports have indicated that PTKs play important roles in regulating the intracellular events of granulosa cells after stimulation with various factors [26–29]. For example, growth factors including epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF), the receptors for which possess PTK domains within

their molecular structures, inhibit the spontaneous onset of apoptosis in cultured granulosa cells [26, 27]. Interleukin-1 β (IL-1 β) acts through cytoplasmic PTKs called Janus kinases (JAKs), and this cytokine is an effective survival factor for preovulatory follicles *in vitro* [28]. These findings suggest that the protein tyrosine phosphorylation of intracellular signaling molecules by PTKs is significant for regulating the dynamics of follicular atresia, growth, and maturation. Nevertheless, to date, there are no reports describing the roles of PTPs in the ovary except for ours [5].

Identification of multiple PTPs expressed in granulosa cells

SHP-1, SHP-2, and FAP-1 are expressed in granulosa cells and the SHP-1 mRNA level is influenced by hormones although their cellular functions were not analyzed [30, 31]. We [5] recently identified the expression of at least 25 PTPs including the above three in rat granulosa cells using a PCR-based cloning approach (Table 1). We further examined the expression levels of some of these PTPs during the estrus cycle, using a model

system of immature female rats primed with gonadotropins, as presented in Fig. 2. The results indicated that receptor-type PTP ϵ (PTP ϵ M), cytosolic-type PTP ϵ (PTP ϵ C), PTP-MEG1, and

Table 1. PTPs expressed in rat granulosa cells

Receptor-type form	
LAR	LAR PTP2 (LAR PTP2B)
DEP-1	PTP ϵ M
PTP μ	PTP α
LRP-2	PTPRK
IA-2 β	BPL-1
PTP-BEM2	PTP Like 1
Unknown PTP	
Cytoplasmic form	
PTP1B	PTP-MEG1
PTP-MEG2	PTPS
PTP20	PTP36
SHP-1	SHP-2
PEP	PTP2E
FAP-1	PTP ϵ C

PTP cDNAs were amplified by PCR using degenerate oligonucleotide primers specific for the conserved sequences of PTP catalytic domains.

Fig. 2. PTP mRNA levels in granulosa cells at each estrous cycle. Immature female Wistar-Imamichi rats (21-day-old) were subcutaneously injected with 20 IU of PMSG for 2 (stage 1) or 5 days (stage 2). The ovary contains mostly healthy preovulatory follicles at stage 1 and mostly atretic follicles at stage 2. After an injection with 20 IU of PMSG for 2 days, some rats were further administered with 40 IU of hCG for 7 (stage 3) or 10 days (stage 4). The ovary contains a number of corpora lutea at stage 3, and several degenerating corpora lutea at stage 4.

Fig. 3. Cellular function of PTP20 in granulosa cells.

PTP20 were highly expressed in granulosa cells derived from healthy and/or atretic follicles, but not in corpus luteal cells. Although PCR analysis showed the considerable PTP20 expression in degenerating corpora lutea, in situ hybridization revealed that it was in fact limited to the theca interna and granulosa cells, and absent in luteal cells. This review describes the roles of PTP20 and PTPeM in granulosa cells, and the molecular mechanisms involved.

Role of PTP20 in granulosa cells

PTP20 [32], also called PTP-HSCF [33], FLP1 [34], PTP-K1 [35], or BDP-1 [36], is a cytoplasmic enzyme that constitutes a separate subfamily of PTPs with PTP-PEST [37] and PEP [38]. This subfamily is characterized by one catalytic domain at the N-terminus, a large middle region that is rich in Pro, Glu, Ser, and Thr residues, and a Pro-rich C-terminal region consisting of 24 amino acids.

The biggest problem involved in defining the cellular functions of genes of interest in granulosa cells is the extremely low efficiency of the introduction of foreign genes. Less than 2% of cultured granulosa cells are transfected by standard methods using commercially available transfection reagents. To overcome this hurdle, we used an adenovirus vector and succeeded in transducing more than 95% of cultured granulosa cells. We expressed a wild type and inactive mutant of PTP20 and investigated their roles in rat cultured granulosa cells [5].

Forced expression of the wild type, but not of the inactive mutant of PTP20, induced retraction of the cell body with the extension of long processes, and decreased cell adhesion to the substratum after stimulation with FSH. Inhibiting Rho GTPase activity with C3 botulinum toxin caused similar morphological changes. Wild-type PTP20 selectively reduced the FSH-enhanced pTyr level of p190 RhoGAP, a negative regulator of Rho, whereas inactive PTP20 had no effect. We found that p190 RhoGAP coimmunoprecipitated with the wild type as well as with the mutant, and confocal microscopy revealed that PTP20 intracellularly colocalized with p190 RhoGAP. These results demonstrate that PTP20 induces reorganization of the actin cytoskeleton with subsequent decreased focal adhesion in an FSH-dependent manner by reducing Rho activity via the dephosphorylation of p190 RhoGAP in granulosa cells.

In addition to p190 RhoGAP, Pro, Ser, Thr phosphatase interacting protein 1 (PSTPIP1) and the cytoplasmic PTKs, c-Abl and the Src-related kinases, are known substrates for PTP20 [39–41]. PSTPIP1 interacts with and serves as a substrate for c-Abl, and can bridge interactions between c-Abl and PTP20 with the dephosphorylation of c-Abl by PTP20 [40]. PTP20 associates with the PTK Csk, a potent negative regulator of the Src-family kinases [41]. This association is thought to allow Csk and PTP20 to synergistically inhibit Src-family kinase activity by phosphorylating and dephosphorylating the negative and positive regulatory Tyr residues, respectively. These molecules associated with PTP20 including p190 RhoGAP are implicated in actin cytoskeleton reorganization [39, 42, 43]. Therefore, whereas the physiological roles of PTP20 are still unclear, a principle cellular function of this phosphatase may be to regulate the reorganization of the actin cytoskeleton.

The roles of PTP20 in follicles also remain to be elucidated. Fibronectin is a major secretion product of cultured granulosa cells [44] and enhances their substratum attachment, spreading, survival, and growth [45, 46]. Therefore, interaction between these cells and the extracellular matrix (ECM) is highly significant for controlling follicular growth. One role of PTP20 in the follicles may be to promote granulosa cell proliferation by inducing reorganization of the actin cytoskeleton in response to gonadotropin and/or other humoral factors.

This notion is consistent with the fact that far more PTP20 is expressed in healthy, than in atretic follicles. However, the opposite function of PTP20 is also conceivable. The detachment of granulosa cells overexpressing wild-type PTP20 from the substratum could suggest that cell proliferation is negatively controlled by PTP20 to avoid excess proliferation because the number of these cells noticeably increases within a short period during the estrus cycle.

Role of PTPeM in granulosa cells

Alternative usage of the promoters generates receptor (PTPeM) and cytosolic (PTPeC) isoforms of PTPe [47–50]. We recently found that PTPeM is expressed in atretic as well as in preovulatory follicles [5]. Like PTP20, we expressed a wild type and an inactive, substrate-trapping mutant of PTPeM in porcine cultured granulosa cells using an adenovirus vector. The results suggested that PTPeM controls cell contact with the extracellular matrix (ECM) by inducing cell cytoskeleton rearrangement through decreasing Rho activity (our unpublished data). One of the direct target molecules for PTPeM is focal adhesion kinase (FAK), which is critically involved in the formation and the degradation of focal contacts. The loss of cell-ECM adhesion leads to integrin disengagement, which results in the apoptosis of many types of cells, a phenomenon known as anoikis [51]. The striking change of the follicles during each estrus cycle appears to accompany the remodeling and degradation of ECM, processes that are usually regulated by the plasminogen activator and matrix metalloproteinase (MMP) systems [52]. In fact, these proteinases are expressed in the follicles, and some MMPs are expressed at different levels during the estrus cycle

[53–57]. As described above, fibronectin, a well-known component of ECM, is a major secretion product of cultured granulosa cells [44]. Thus, one of the determinants of follicular destiny may be the extent of granulosa cell adhesion to ECM. It is likely that PTPeM is closely associated with dynamic ovarian functions through coordination with plasminogen activator and/or MMPs to regulate contact between granulosa cells and ECM.

Concluding Remarks

Little is understood about the intracellular signaling pathways in ovarian granulosa cells compared with those in other cells, perhaps because of the extremely low transfection efficiency of foreign genes and the lack of appropriate cell lines derived from granulosa cells. The MAPK family plays central roles in various cellular responses including cell survival and apoptosis. Therefore, elucidating the correlation between MAPK activities and granulosa cell responses will provide a basis for understanding the roles and molecular mechanisms of numerous extracellular stimuli controlling the fate of follicles. Studies of PTPs have proceeded very slowly due to technical and philosophical reasons. However, our data, including the expression of more than 20 PTPs in granulosa cells, suggest that the PTP-dependent regulation of follicular growth and atresia is highly significant.

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