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# Purinergic Signaling: A New Regulator of Ovarian Function

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## Abstract

Purinergic signaling is a sophisticated system of elements in which ATP and related molecules function as intercellular messengers. When ATP is released into the extracellular space, it activates specific receptors that belong to the P2 family. In parallel, ectonucleotidases transform ATP in its dephosphorylated metabolites including adenosine, which stimulates P1 receptors. The activity of both receptors influences various cellular processes. Moreover, metabolic conditions are concatenated with purine signaling to conform a dynamic and continuous informational network. The role of purinergic signaling in ovarian cells has been investigated, for instance, it is known that cells conforming the follicle express functional receptors that modulate basic cellular process such as proliferation, induction of apoptotic cell death, and steroidogenesis. In this chapter, we review contemporary information on purinergic action in ovarian cell physiology and state its relevance in this field.

**Keywords:** purinergic signaling, ovary, granulosa, theca, OSE

## 1. Introduction

The ovary is a complex cell system where folliculogenesis and steroid hormone synthesis take place. These processes involve dynamic changes in the cellular populations of this tissue and highly precise mechanisms of regulation. Folliculogenesis requires a complex coordination of three stages: *recruitment*, which is the initial growth of a group of follicles from the reserve pool; *selection*, during which a subgroup from this pool of recruited follicles survives and grows, while the remainders suffer atresia; and *dominance*, in which the follicles that will be ovulated reach the preovulatory stage and increase their size, while the subordinate follicles arrest their growth. It is well known that the initial stages of folliculogenesis are independent of gonadotropins, whereas the advanced stages depend on these hormones. At the same time, *selection* and *dominance* stages implicate the elimination of subordinated follicles by apoptosis; thus, a coordinated set of events directs growth and surveillance of some follicles and disappearance of others with extreme precision [1].

Folliculogenesis involves constant rearrangement in ovarian cellular architecture. A primordial follicle is formed by an oocyte surrounded by a layer of

squamous epithelial cells and is arrested in the diplotene phase of meiosis I. When it is recruited and enters the growing stage, granulosa cells change their shape from flat to cuboid, starting their proliferation; this entity is known as *primary follicle*. The granulosa layer continues proliferating to adopt a stratified epithelium; then, the theca layer surrounding the follicle emerges. This layer is innervated and vascularized conforming a *secondary follicle*. Inside the follicle, three to four cavities filled with fluid are formed; the cavities fuse and form the antrum. This stage is known as antral follicle. Antrum formation implicates the emergence of two granulosa populations: cumulus cells in contact with the oocyte and mural cells attached to the follicular theca. These follicles grow until reaching the preovulatory stage [1]. Changes in follicle development are orchestrated by diverse cellular messengers (reviewed in [2]).

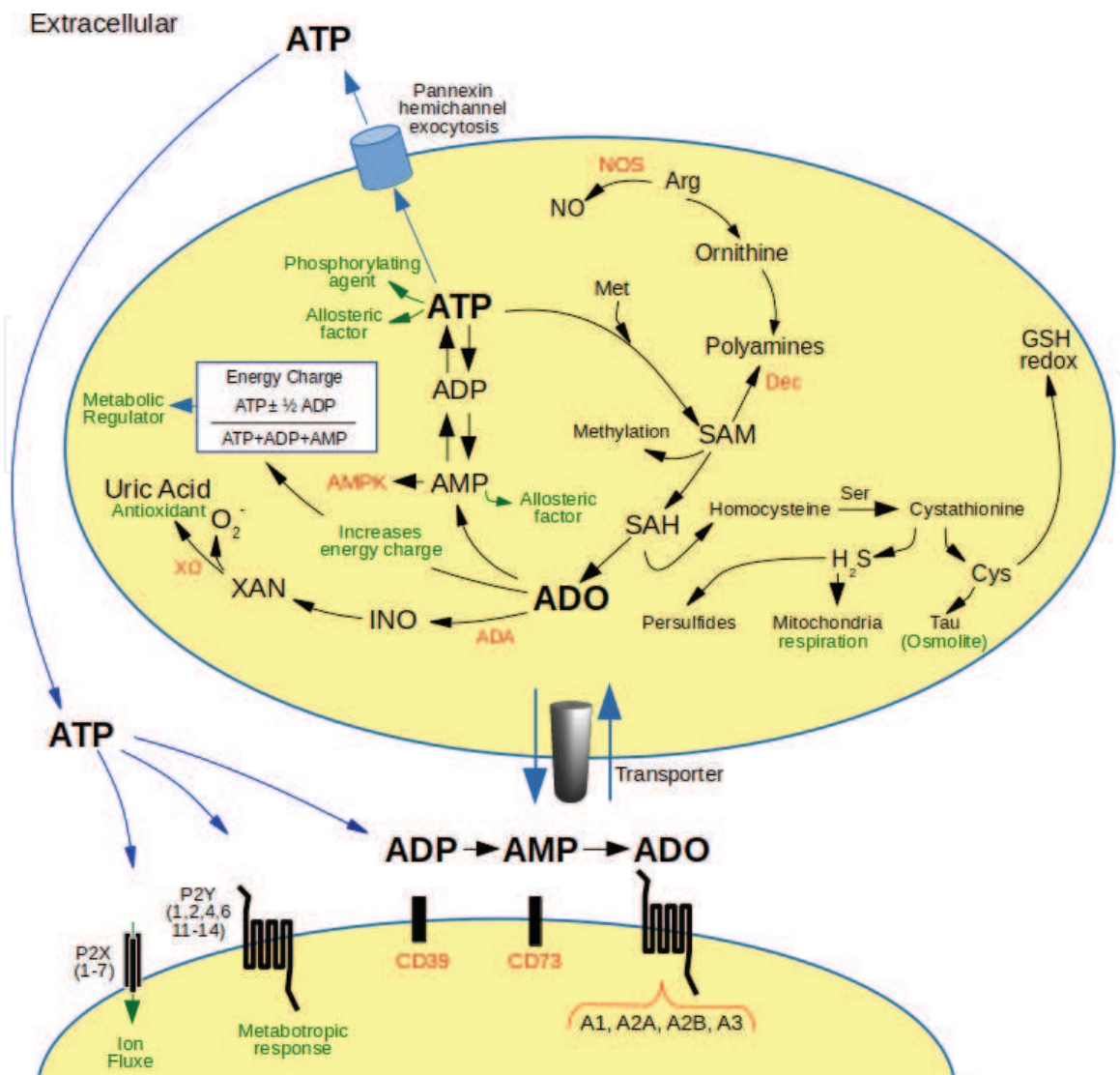
Moreover, synthesis and secretion of ovarian steroid hormones are coordinated by the somatic components of the follicle; thus, cholesterol and acetate uptake from the blood by the theca layer allows the synthesis of androgens that are aromatized into estrogens in the granulosa cells [3]. This process is finely regulated by the coordinated action of intraovarian and endocrine components.

All these dynamic processes are organized at distinct levels by endocrine, nervous, and autocrine-paracrine mechanisms acting with high systematic precision. Recent findings suggest that purinergic signaling participates in the control of cellular process in the follicle cells, making it a new player in the network of signals regulating the cellular biology of ovarian physiology. Given that follicle cell types have defined roles and are typical for each follicular growth stage, receptor expression in each of these cell types suggests a potential physiological role; thus, we organized the information of this chapter to cover the role played by purinergic receptors in ovarian physiology.

## 2. Purinergic system

From a chemical perspective, purines are defined as nitrogen-containing heterocyclic aromatic compounds formed by the fusion of a pyrimidine and an imidazole ring (in total, five carbon and four nitrogen atoms). The purine molecule can be associated with an amino group, such as adenine (6-amino purine), or with amino and keto groups, such as guanine (2-amino-6-oxy purine). The existence of functional groups that are weakly acidic next to a system of conjugated dienes favors the relocation of a proton along the molecule, allowing the formation of constitutional isomers called tautomers. The principal tautomeric equilibriums in purine molecules are amino (predominant)-imino forms and lactam (predominant)-lactim forms [4].

Purines are part of important informational polymers, such as nucleic acids (DNA and RNA), and of various biomolecules with metabolic and functional significance such as bioenergetic factors (ATP and GTP), redox coenzymes (NAD(P)H/NAD(P)<sup>+</sup>, FMNH<sub>2</sub>/FMN<sup>+</sup>, and FADH<sub>2</sub>/FAD<sup>+</sup>), biochemical crossroad metabolites (coenzyme A), and signaling molecules (cyclic AMP, cyclic ADP-ribose, nicotinic acid adenine dinucleotide phosphate [NAADP]) and ligands for purinergic receptors. Caffeine and theobromine are naturally occurring purines in plants [5]. In this section, we will refer exclusively to ATP, adenosine (ADO), and other related molecules as factors in cellular communication and coordinators of metabolic networks (**Figure 1**).



**Figure 1.**

*Purines in cellular signaling and metabolic networks. The figure depicts two cells in communication using purines as signaling molecules. Released ATP is recognized by two types of receptors (P2X, ionotropic, and P2Y, metabotropic). ATP can be hydrolyzed by different ectonucleotidases (such as CD39 and CD73) to be transformed in less phosphorylated nucleotides (ADP and AMP) and eventually to the nucleoside adenosine (ADO). The receptors that mediate ADO's actions are all metabotropic. Thus, purinergic signaling is the result of the types of receptors present in the cellular system, as well as the variable proportion of adenine nucleotides and ADO that result from the enzymatic activities of the expressed ectonucleotidases. There are membrane transporters with the capacity to transfer ADO to the cellular inside. Within the cell, ADO can be transformed in multiple metabolites and act as modulator of strategic metabolic pathways. ADO is catabolized by adenosine deaminase (ADA) resulting in a set of purines that culminates with uric acid (a mild antioxidant) with xanthine as an intermediary with the capability to generate the anion superoxide ( $O_2^-$ ). By the action of adenosine kinase, ADO can turn into a nucleotide (AMP) that is a modulator of the AMPK, which is an important enzyme to regulate energy homeostasis. AMP can be further phosphorylated to ADP and ATP. Eventually, ATP can go to the extracellular space to fulfill its messenger role by exocytosis or by specialized channels (pannexin and connexin hemichannels). The proportion of adenine nucleotides (energy charge) is an important modulator of the equilibrium between catabolic and anabolic reactions. It has been reported that ADO is able to increase the cellular energy charge in the liver. When ADO binds homocysteine to form S-adenosylhomocysteine (SAH), it can also influence the transmethylation and transsulfuration pathways. SAH is usually formed when the methylating agent S-adenosylmethionine (SAM) transfers a methyl moiety to a given substrate. Interestingly, SAM can also participate in the synthesis of polyamines by transferring an aminopropyl group from decarboxylated SAM to putrescine. Homocysteine is joined to serine to form the intermediary cystathionine, which is transformed into cysteine and the intracellular messenger  $H_2S$ . Meanwhile, cysteine can be incorporated into the redox and antioxidant molecule glutathione (GSH) or can be converted into the osmolyte taurine. Hence, a condition in which ADO is increased beyond a threshold may accumulate SAH with the consequent interruption of the methylating reaction and the promotion of polyamine synthesis, as well as the reduction of homocysteine availability and the decrease in cystathionine and all its derivatives.*

Adenine-related molecules are ubiquitously present in all living beings. ATP (a nucleotide) and ADO (a nucleoside) are easily interconverted by a set of three phosphorylation/dephosphorylation steps. Interestingly, this purine conversion involves different mechanisms when it takes place either inside or outside the cellular milieu. Intermediates of these reactions are AMP and ADP nucleotides, whereas some metabolically important ADO derivatives are uric acid, S-adenosyl methionine (SAM), and S-adenosylhomocysteine (SAH). Various physiopathological events modulated by ATP and/or ADO have been reported, including sleep, immunity, tumorigenesis, platelet aggregation, vasodilation, inflammatory and hypoxic responses, and antioxidant status [6]. Overall, adenine nucleotides and ADO are interconnected signaling factors that activate specific membrane receptors and act as metabolic regulators that coordinate the equilibrium between anabolic and catabolic reactions [7].

## **2.1 Purinergic communication**

ATP and ADO are well-known signaling molecules. Both purines have the capacity to promote a set of cellular responses by acting through specific membrane receptors, either by activating ionic conductance or by promoting the formation of second messengers. Two families of receptors for purine ligands have been characterized: (1) P1 or ADORA receptors, which are G-coupled metabotropic adenosine receptors and are classified as A1 and A3 (associated with adenylate cyclase inhibition and formation of IP<sub>3</sub> and diacylglycerol) and as A2A and A2B (both activate adenylate cyclase), (2) P2 receptors, which recognize ATP as a principal ligand but also a variety of related compounds. P2 receptors are divided into ionotropic receptors (P2X1–7) and G-coupled metabotropic receptors (P2Y1, P2Y2, P2Y4, P2Y6, P2Y11–P2Y14). In addition to ATP, P2Y receptors can be recognized by alternative ligands such as ADP, UTP, UDP, and UDP glucose [8].

Unlike other signal transduction systems, purinergic signaling involves the actions of two interconvertible ligands, ATP and ADO, within the same system. Interestingly, these ligands exert complementary or antagonistic actions on each other [9]. For example, epithelial-mesenchymal transition (EMT), a cellular plasticity process important in phenotypic programming of metastatic tumors, can be upregulated by ATP in SKOV-3 cells (from ovarian carcinoma) but downregulated by ADO [10]. This circumstance indicates that enzymes (ectonucleotidases) allowing the sequential conversion from ATP to ADO are a potential regulatory node that controls diverse cellular and physiological responses.

Four families of extracellular enzymes that transform purine nucleotides into ADO and phosphate are known: (1) ENTPD/CD39 (ectonucleoside triphosphate diphosphohydrolase) forms AMP from ATP/ADP; (2) ENPP (ectonucleotide pyrophosphatase) forms AMP from ATP/ADP/ADP-ribose; (3) alkaline phosphatase hydrolyzes different nucleotides to be transformed into ADO; and (4) NT5E (ecto-5'-nucleotidase) forms ADO from AMP [11]. It is expected that the combined and sequential activities of these enzymes in a given cell system result in a highly variable and potentially fine-tuned proportion of adenine nucleotides (ATP, ADP, and AMP) and ADO. Therefore, the physiological equilibrium between ATPergic and adenosinergic transmissions should be considered as a unique and emergent property of each cell system.

## **2.2 Purine-related metabolites**

Adenine purines are key elements in metabolic network control. The proportion of adenine nucleotides dictates the direction of anabolic and catabolic processes by

means of the regulatory parameter known as energy charge ( $ATP + \frac{1}{2}ADP / ATP + ADP + AMP$ ). In this context, ATP and AMP act as allosteric factors for a variety of enzymes that are important in the bioenergetic status of the cell: phosphofructokinase 1 (glycolysis), aspartate carbamoyltransferase (pyrimidine synthesis), and glycogen phosphorylase (glycogenolysis). Interestingly, ADO treatment is one of the few cases that can upregulate the energy charge in vivo [12]. Another example of how the AMP/ATP ratio influences the metabolic networks is AMP kinase (AMPK) activation. AMPK is a strategic kinase that modulates the fasting response by phosphorylating and activating key catabolic enzymes [13].

ADO is a crossroad metabolite; it can be turned into nucleotides (first in AMP by adenosine kinase), or it can originate active catabolites such as xanthine (a source of superoxide) and uric acid (a terminal metabolite and mild antioxidant). The production of ADO in the liver and its transport through the blood are controlled by the circadian timing system; eventually, these mechanisms allow the 24-h rhythmic presence of purine rings in the nervous system, which are necessary for the onset of sleeping [14]. ADO has also been used as a hepatoprotective and antitumoral agent [15].

ADO also plays a role in the transmethylation and transsulfuration pathways, with SAM as a central metabolite for both. Initially, ATP activates methionine, which in turn generates SAM, the main cellular methylating agent. Some principal methylated molecules are phospholipids (phosphatidylethanolamine turns into phosphatidylcholine), catecholamines (adrenaline turns into noradrenaline and serotonin into melatonin), and nucleic acids (during epigenesis and RNA processing). SAM is transformed into SAH, which is hydrolyzed into ADO and homocysteine. In this metabolic step, ADO levels can modulate the methylation process, as high ADO favors SAH synthesis, thus blocking methyl donation. SAM is also a substrate for polyamine synthesis. In the presence of serine, homocysteine is converted into cystathionine. Within the mitochondria, this intermediate is the precursor of both the gasotransmitter  $H_2S$  and the principal antioxidant agent, glutathione [16].

### 2.3 Membrane transporters

Extracellular and intracellular purine dynamics are interconnected, mainly through specialized protein transporters that allow ATP and ADO to transit throughout the plasma membrane.

ATP exists at millimolar levels within the cell. It exits to the extracellular space, where it acts as a cellular messenger through two main paths: (1) exocytic, which involves secretion of vesicles mainly derived from Golgi and secretory ATP-containing granules by means of  $Ca^{2+}$ -dependent membrane depolarization and (2) conductive, in which the ATP efflux is carried out by different ion channels: hexamers of connexin subunits, assemblies of pannexin subunits, volume-regulated anion channels, and maxi-anion channels [17]. As mentioned, ATP can lose its phosphates in the extracellular space and transform into ADO. In turn, ADO can return to the intracellular milieu by the action of two types of carriers; one is driven by a facilitated diffusion event (sensitive to dipyridamole), and the other mobilizes ADO by an active process regulated by the  $Na^+$ -transmembrane gradient [18]. Overall, a net flux of purines can be visualized: first, purine rings exiting as ATP and eventually purines returning to the cell interior as ADO. Indeed, this cycle involves the net efflux of phosphate as well as the net conversion of intracellular ATP into ADO.

### 2.4 Integrative considerations

The transformation among purine molecules inside and outside the cell has the potential of intricately regulating both purinergic signaling and metabolic control.

To propose a model of this interaction, the following considerations are necessary: (1) at least three compartments should be taken in account—extracellular, cytoplasmic, and mitochondrial. However, if there is segregation of receptor populations in different membrane domains (e.g., signalosomes), the extracellular compartment could be more complex. (2) It is important to know the principal metabolite and intermediate levels in the process; indeed, the concentrations of these factors are expected to fluctuate, but knowing average levels is necessary. For example, ATP is at [mM] in the intracellular milieu, but when it is released in the pericellular space, it changes from [nM] to [mM] [19]. (3) It is important to know which receptors are present in a given cell system, as well as their physical constants ( $K_d$  and  $B_{max}$ ). (4) It is convenient to have a clear idea of the activities and regulation of all ectonucleotidases and their corresponding carriers. (5) In the same context, determining the presence of purine metabolizing enzymes and kinetic constants ( $K_m$  and  $V_{max}$ ) is required. (6) It is necessary to know the conformational status of all proteins involved in the purine cycle in order to detect potential allosteric modulation.

### **3. Purinergic signaling in the ovary**

#### **3.1 Granulosa and luteal cells**

Early studies analyzed the effects of ADO over the gonadotropin-induced cAMP production in granulosa and luteal cells. In rat and human granulosa cells, ADO incremented the accumulation of cAMP in response to follicle-stimulating hormone (FSH); similar effects were observed when human luteal cells were stimulated with human chorionic gonadotropin (hCG) or luteinizing hormone (LH), revealing the possibility that ADO is a gonadotropic modulator [20–23]. According to the dual role of ADO in metabolism and cell signaling, it was originally proposed that ADO effects were mediated by two mechanisms acting synergically: ADO translocation to the cytoplasm where the nucleoside can sustain the increment of cytosolic ATP and extracellular activation of specific membrane receptors [20].

In primary cultures of rat granulosa cells, incubation with ADO incremented intracellular ATP; dipyrindamole blocked this effect, indicating ADO uptake through specific transporters [22]. The regulation of adenylate cyclase (AC) activity by ADO analogues was investigated in membranes isolated from the whole ovaries in follicular growth. It was observed that adenosinergic agonists incremented adenylate cyclase (AC) activity. Pharmacological approaches suggested that the effect of ADO analogues was mediated by the A<sub>2A</sub> receptor because it was strongly promoted by 5'-N-ethylcarboxamidoadenosine (NECA) and antagonized by 8-phenyltheophylline (8-PTH) [24]. Similar findings were observed in membranes from luteinized ovaries from superovulated rats induced by injection with pregnant mare serum gonadotropin (PMSG) and in homogenates of isolated cells from luteal bodies; moreover, it was shown that ADO analogues incremented progesterone synthesis. In these preparations, pharmacological evidence also indicated the participation of A<sub>2A</sub> receptor in the adenosinergic induction of cAMP accumulation, suggesting that ADO is a paracrine regulator of the luteal body's endocrine activity [25].

In addition, it has been shown that in ovine luteal cells, ADO increases the effect on luteotropins [hCG] as well as prostaglandins (E<sub>1</sub> and E<sub>2</sub>) over progesterone production and inhibits the antigonadotropic and luteolytic effect of prostaglandin F<sub>2α</sub> (PGF<sub>2α</sub>) [20, 26]. Purinergic responses in ovarian cells were described before cloning P<sub>2</sub> receptors. The effect of adenine nucleotides on cytosolic concentration

of  $\text{Ca}^{2+}$  ( $[\text{Ca}^{2+}]_i$ ) was first investigated in human luteal cells and in porcine granulosa cells; it was observed that nucleotides elicited an increment of  $[\text{Ca}^{2+}]_i$  in both cell types. Moreover, in luteal cells, these compounds also induced an increase in progesterone and estradiol secretion [27]. Consistently, in granulosa from hen preovulatory follicles, ATP, and other adenine nucleotides also induced an increment of  $[\text{Ca}^{2+}]_i$ ; the pharmacological characterization of this response revealed that it was mediated by intracellular  $\text{Ca}^{2+}$  release and dihydropyridine-insensitive  $\text{Ca}^{2+}$  channels according to P2Y receptor activation [28]. In human granulosa cells, it was determined that ATP responses were dependent on  $\text{Ca}^{2+}$  released from intracellular stores [29].

The molecular description of the P2Y2 receptor (then named P2U because of its sensitivity to UTP) in human granulosa-luteal cells (GLC) was made by Leung's group in British Columbia [30]. They detected the transcript of P2U receptor by Northern blot and reported the elevation of cAMP promoted by hCG. The stimulation of these cells with UTP/ATP induced an increment of  $[\text{Ca}^{2+}]_i$  associated with phospholipase C (PLC) activation. Downstream of this pathway, protein kinase C (PKC) was activated, and it negatively modulated the P2Y2-dependent  $[\text{Ca}^{2+}]_i$  response [31]. The molecular machinery and mechanism involved in the purine-induced increment of  $[\text{Ca}^{2+}]_i$  have been studied. ATP-induced  $\text{Ca}^{2+}$  release mediated by activation of PLC and inositol triphosphate ( $\text{IP}_3$ ) production. Indeed,  $\text{IP}_3$  and ryanodine receptor (RyR) expression in GLC has been demonstrated. The increment of ATP-induced  $[\text{Ca}^{2+}]_i$  was modulated by substances interfering with the activity of both RyR and  $\text{IP}_3\text{R}$ , revealing an interplay between both receptors to amplify the purinergic  $[\text{Ca}^{2+}]_i$  signal [32]. Moreover, it was described that GLC expresses three isoforms of  $\text{IP}_3\text{R}$ , RyR, and thapsigargin-sensitive  $\text{Ca}^{2+}$ -ATPase (SERCA) [33].

Electrophysiological and  $\text{Ca}^{2+}$  imaging studies in the GFSHR-17 cell line from granulosa determined that P2Y2 and P2Y4 receptor stimulation induces  $\text{Ca}^{2+}$  mobilization and hyperpolarization. Both responses were sensitive to the PLC inhibitor U73122 and to the  $\text{IP}_3\text{R}$  antagonist 2-aminoethyl diphenyl; hyperpolarization was mediated by  $\text{Cl}^-$  channels, probably dependent on intracellular  $\text{Ca}^{2+}$  [34].

Moreover, it was shown that the P2Y2 (P2U) activation in human GLC induced a decrease in the LH-dependent cAMP levels; this antigonadotropic effect was mediated by PKC $\alpha$  activity [35]. In parallel, it was shown that P2Y2 stimulation also activated extracellular mitogen-regulated kinases (ERK) through a G $\alpha_q$ -dependent pathway; ERK activity was responsible for inhibiting LH-dependent production of progesterone induced by P2Y2 activation [36]. Further experiments revealed that phospho-ERK translocates to the nucleus and regulates cell proliferation by early growth-1 (egr-1) and c-raf-1 responses [37]. These data clearly show that the purinergic response mediated by P2Y2 in GLC can be an important modulator of gonadotropic actions and granulosa cell physiology.

Expression of the P2Y6 receptor was observed in murine GLC. Its stimulation with the selective agonist UDP incremented cell viability and progesterone but did not affect estradiol production. This effect on steroidogenesis was concomitant with a negative regulation of enzymes corresponding to  $\Delta 4$  steroidogenic pathway, CYP11A, 3 $\beta$ -HSD, and StAR. The effects were blocked by the antagonist MRS2578 before UTP stimulus [38]. These results suggested that purines acting through P2Y6 regulate luteal body viability and steroidogenic function.

In human GLC, extracellular ATP promoted apoptosis by activating P2 receptors. These receptors elevated  $[\text{Ca}^{2+}]_i$ , which in turn activated  $\text{Ca}^{2+}$ -dependent  $\text{K}^+$  channels, leading to membrane depolarization [39]; however, the specific receptor involved in this response was not identified. These observations were confirmed by a later study that demonstrated the participation of apoptotic marker caspase-3



[40]. This effect was apparently not consistent with previous observations regarding ATP actions in GLC, but it could be explained by the differential sensitivity of distinct P2 receptors [41]. It is possible that extracellular concentration of ATP and the expression of P2 receptor determine the specific effect of purinergic stimulation.

Recently it was described that the P2X7 receptor is expressed in mouse luteal cells. Its activation with ATP or BzATP induced an antiproliferative effect by regulating the expression of cyclin D2 and cyclin E2, as well as the phosphorylation of mitogen-activated protein kinase p38 [42]. The result suggests a role for the P2X7 receptor in luteal body function.

The purinergic system is well represented in GLC. A set of purinergic receptors can modulate basic cellular processes such as proliferation, apoptosis, and steroidogenesis. Growing evidence indicates that purines are important regulators of GLC, but further studies are necessary to reinforce their role in ovarian physiology.

### **3.2 Theca cells**

The theca cell layer is an enclosure of cells that surrounds the oocyte during folliculogenesis. It is crucial for maintaining the structural integrity of the follicle as well as for regulating nutrient influx to the avascular GCL [43, 44]. Theca is also the site for the synthesis of steroid hormones, specifically androgens (testosterone and dihydrotestosterone), from acetate or cholesterol into estrogens by granulosa cells in an LH-dependent manner [3]. In addition, theca is the only component of the follicle that is innervated by sympathetic and parasympathetic nervous systems, implicating that this layer functions as a complex integrator of endocrine and neural information [45].

When a primary follicle has one or two layers of granulosa cells, an outer granulosa cell layer differentiates into theca cells and, together with recruited theca precursor cells from the stroma, forms the theca cell layer surrounding the oocyte [44, 46]. Some pathophysiological reproduction-related conditions such as infertility or polycystic ovarian syndrome are often the result of dysfunctional activity of theca cells during ovulation and follicle development [47, 48].

The theca cell layer contacts the rich microvasculature system surrounding each follicle and integrates signals from autonomic innervation [49]. It has been demonstrated that ATP can be co-released with noradrenaline from terminals of the peripheral nervous system [50] and as a result of mechanical stress and changes in cell volume in the oocyte [51]; thus, ATP is a relevant modulator of cellular communication between the theca cell layer and surrounding oocyte cells.

Purinergic signaling has been described in female reproductive organs, and evidence has shown that ATP in the extracellular space participates in the physiological regulation of the ovary [52]. The first characterization of purinergic signaling in theca cells showed the functional expression, and activation of P2X7 receptors induced cell death, an important mechanism for the onset and physiological progression of follicle atresia [53]. P2X7 receptors have also been associated with the inhibition of luteal cell survival and proliferation, pointedly in small luteal cells, which have been suggested as theca-derived luteal cells [42].

On the other hand, there is also evidence of the expression of uridine triphosphate (UTP)-sensitive P2Y receptors in theca cells P2Y2 and P2Y6, but not P2Y4 [54]. In this system, stimulation of the expressed P2Y receptors with UTP in cultured theca cells induces the activation of mitogenic-signaling pathways that promote cell proliferation [54]. This finding is a relevant pathophysiological indication, since a slow but maintained proliferation takes place in polycystic ovarian syndrome [47].

Furthermore, an interaction between adenosine receptor A2 and P2Y receptors has been described in theca of *Xenopus* ovarian follicles. The authors suggested that this association took place when both the epithelial and theca cell layers of the oocyte were intact [55].

Collectively, these findings suggest that a tight regulation of purinergic expression and signaling must be in place for the theca cell layer to function properly and communicate with neighboring cells.

### 3.3 Cumulus complex

The organized structure of the cumulus-enclosed oocyte (CEO) complex corresponds to a specialized GLC surrounding the oocyte. Cumulus cells secrete factors to regulate oocyte maturation and maintain meiotic arrest [56]. It was reported that porcine and murine follicular fluid contains purine compounds that presumably participate in CEO functions, suggesting that it could be an important signal to trigger physiological events [57, 56]. Until recently, purinergic receptors were identified and characterized in CEO, indicating that purinergic signaling participates in CEO physiology [58, 59].

When Eppig et al. discovered that the main components of follicular fluid were nucleotide-derived metabolites [60] and established a relationship with follicle maturation [56, 57], they hypothesized that the local purinergic metabolism in the ovarian fluid can be involved in oocyte maturation or may participate in other aspects of follicular functions.

In these studies, the concentration of nucleotide compounds in murine follicular fluid was determined using high-performance liquid chromatography (HPLC). They identified two purine compounds: hypoxanthine and ADO, with concentrations in ranges of 2–4 and 0.3–0.7 mM, respectively. They also showed that these purines affected the CEO by maintaining the meiotic arrest [56].

Eppig et al. also analyzed the same compounds in porcine follicular fluid; they identified that hypoxanthine at 1.4 mM is the major inhibitory component producing a transient inhibition [61]. This observation contrasted with that of other laboratories which had failed to detect inhibitory activity in follicular fluid.

On the other hand, the cellular effects of purinergic ligands were studied by  $\text{Ca}^{2+}$  imaging and electrophysiological approaches. In 2002, two reports elucidated which purinergic receptor was expressed in CEO cells. The first report of P2Y expression in CEO began with an interesting observation that ATP could stimulate an intracellular  $\text{Ca}^{+2}$  transient. Experiments using the CEO complex and applying ATP or UTP to the extracellular solution induced a wave of  $\text{Ca}^{+2}$  mobilization from cumulus cells to the oocyte through gap junctions, suggesting that ATP was involved in oocyte maturation; moreover, they showed that the response involved the P2Y2 receptor. Since gonadotropin hormones, follicle-stimulating hormone (FSH), and luteinizing hormone (LH) had no effect on  $\text{Ca}^{+2}$  changes [59], the authors concluded that ATP was the specific messenger to mediate calcium signals involved in oocyte maturation in the CEO complex.

In another report, the responses generated by a putative purinergic receptor expressed in the CEO were identified and characterized. Employing the voltage clamp technique with two electrodes, the authors observed depolarization responses when extracellular ATP was applied. RT-PCR analysis revealed a product correspondent to the P2Y2 receptor, suggesting that calcium mobilization is dependent on this receptor. A detailed description of distinct currents generated from several ionic channels, such as  $\text{Ca}^{+2}$ -dependent  $\text{Cl}^-$  current, voltage-dependent  $\text{K}^+$  currents, and a cationic current mainly driven by  $\text{Na}^+$ , was provided. The authors concluded that both purinergic receptors and ionic channels were located in CEO

cells that transmitted their electrical signals to the oocyte via gap junctions [58]. These data support the idea that P2Y2 is an important element in paracrine signaling in regulating CEO complex physiology. Future studies are required to determine the mechanisms involved in CEO functions and oocyte maturation by ATP stimulation.

### **3.4 Ovarian surface epithelium**

Ovarian surface epithelium (OSE) is a monolayer surrounding the ovary. It is composed of a single flat layer of squamous-to-cuboidal epithelial cells featuring distinguished epithelial and mesenchymal markers. OSE is essential during ovulation to promote follicular rupture and release the oocyte [62] and for postovulatory repair of the ovary [63].

Initial and important studies were led by Nelly Auersperg to characterize and identify epithelial and mesenchymal markers, hormonal and growth factor receptors, and physiopathological role, with the idea that OSE is determinant in the onset of ovarian carcinoma [64]. During ovulation, the OSE is involved in three main phases: apex formation, rupture, and repair [65].

The initial phase starts with the actions of luteinizing hormone (LH) and triggers apex formation at the rupture site of the ovarian surface [66]. In the second phase, OSE cells initiate a lytic cascade [62, 67, 68], releasing proteolytic enzymes to degrade the basal lamina, the tunica albuginea, and ovarian cells of the mature follicle. The digested matrix, follicular wall disintegration, and peeling of OSE cells create a wound stigma that facilitates oocyte release. Finally, the repair phase consists in wound closure by postovulatory cell proliferation and migration [69].

Nevertheless, the signaling involved in these phases during ovulation in the OSE is unclear. Purinergic signaling was suggested as a part of intraovarian modulation due to a certain purinergic receptor expressed in the OSE committed in physiological processes. Recently, Vazquez-Cuevas et al. demonstrated the expression of ligand-activated ion channel P2X7 in OSE. In primary cultures of mouse OSE, they observed that BzATP induced a non-desensitizing increment of  $[Ca^{+2}]_i$ , and this response was blocked with A438079, a selective antagonist of the P2X7 receptor. The functional role of P2X7 was investigated in situ by TUNEL assay. P2X7 stimulation with BzATP induced apoptosis in OSE cells and was differential throughout the oestrous cycle; DNA fragmentation was greater during proestrous [70]. These findings contribute to the idea that local factors, such as ATP, may participate in a proposed cyclic proliferation-death equilibrium of the OSE cell layer in the ovulatory process.

Understanding purinergic signaling and receptor expression in the OSE will help to decipher the mechanisms underlying ovary physiology and pathology. However, more studies need to contribute evidence related to homeostasis and postovulatory repair. Some studies regarding OSE-derived cancer cells will be discussed in the next section.

## **4. Purinergic signaling in ovarian cancer**

Although the specific roles for purinergic signaling in ovarian physiology are not completely understood, significant advances have been made in deciphering the role of purines in cancer. Plenty of information supports that purinergic system elements have a role in cancer progression, and this implicates that they are potential therapeutic targets. Here we will address the relevance of the purinergic system in ovarian cancer (OC).

Ovarian cancer (OC) is considered the most lethal gynecological malignancy, as it is usually diagnosed by the time the tumor has spread to other regions [71]. OC is the seventh most common type of cancer in women, and patients have a low survival rate [72]. Early detection of the disease proves difficult due to unspecific symptoms such as abdominal pain and bloating, whereas advanced stages are confused with gastrointestinal illnesses [73]. Although OC can arise from any of the cells located in the ovary (germ, stroma, and the epithelium), it is acknowledged that almost 90% of OC is derived from the OSE. According to histological characteristics, epithelial ovarian cancer (EOC) is classified in serous, endometrioid, and clear cells and in mucinous carcinomas [74].

Ovarian tumors frequently show disseminated metastasis through ascites in the peritoneal cavity. OC cells are exfoliated from the primary tumor surface to the peritoneal fluid, where they survive as single cells or multicellular aggregates. These cells acquire resistance to anoikis, have stem cell properties, and are plastic in terms of switching between epithelial and mesenchymal phenotypes. In addition to cancer cells, the malignant ascite microenvironment has normal cell types, such as platelets, associated fibroblast, and immune cells, which support and assist cancer cells [75, 76]. This distinctive tumor microenvironment (TME) has paracrine and autocrine signals that support cancer cell proliferation, death evasion, dissemination, and invasion to peritoneal organs. Therefore, understanding cellular and molecular mechanisms that promote progression of the disease is very relevant.

Purinergic signaling has emerged as an important regulator of tumor growth [77]. The following facts support this assertion: (1) cancer cells increase in metabolic rate [78]; (2) ATP and ADO levels increase in the tumor interstitium [79, 80]; (3) purinergic receptors are expressed in tumor cells [77]; and (4) high CD73 ectonucleotidase expression is a prognosis factor for several types of cancer [81].

Early studies of purinergic signaling in ovarian carcinoma-derived cells (OCDC) evaluated the effects of ATP on  $[Ca^{2+}]_i$  mobilization. It was demonstrated in OVCAR-3 and SKOV-3 cell lines that stimulation with ATP in a  $\mu M$  range induced a biphasic response that consisted in a rapid peak followed by a smaller and sustained plateau phase. In addition, chelation of  $Ca^{2+}$  abrogated the slower response induced by ATP, while the rapid response was maintained. Furthermore, low concentrations of ATP induced SKOV-3 and OVCAR-3 cell proliferation [82, 83]. These pioneer works indicated that OCA cells were responsive to ATP. Furthermore, the biphasic response induced by ATP suggested that these cells could express two types of purinergic receptors: channels operated by ligands and G protein-coupled receptors (GPCRs).

In other OCDC lines (EFO-21 and EFO-27), the presence of P2Y2 receptor was described, and ATP response was monitored through intracellular  $Ca^{2+}$  mobilization and phospholipase D (PLD) activation. In addition, ATP downregulated basal cell proliferation and the proliferation induced by fetal calf serum (FCS) [84]. However, in IOSE-29 (preneoplastic) and IOSE-29EC (neoplastic) cell lines, which also express P2Y2, ATP-stimulated cell growth through the MAPK/ERK kinase activation [85]. These findings showed that ATP effects differ across cell lines, which could be associated with the activated intracellular mechanism.

Regarding the expression of P2X receptors in OCDC lines, some of the first reports evaluated P2X7 expression in OC biopsies as in SKOV-3 and OVCAR-3 cells. P2X7 expression in human ovaries is confined to the OSE, whereas in EOC biopsies, its expression is wider and localized in transformed zones [86, 87]. Additionally, receptor functionality was evaluated in cell lines, and it was demonstrated that its stimulation induces ERK and AKT phosphorylation, whereas its inhibition reduces cell viability [87]. The latter result was surprising due to previous findings that associated P2X7 receptor activation with apoptosis.

An important feature of cancer cells is their ability to migrate and invade secondary organs. One process that allows OC cells to dissociate from primary tumors and survive in peritoneal fluid is the EMT, in which cells switch from an epithelial to a mesenchymal phenotype. Even though this process was first described in embryonic development, today its role in cancer is well accepted. The involvement of purines in EMT has recently been reviewed [9]. It has been proven that P2Y2 stimulation promotes SKOV-3 cell migration, and this effect is associated with epithelial growth factor receptor (EGFR) transactivation. Moreover, expression of EMT inducers such as SNAIL and TWIST was promoted in response to UTP; in addition, the intermediate filament vimentin was augmented by this pharmacological stimulus. Interestingly, addition of apyrase (Apy) to cell medium, with the aim of removing extracellular ATP, decreased cell migration and favored an epithelial-like phenotype due to the relocation of E-cadherin to SKOV-3 cellular junctions [88]. The authors concluded that products obtained by ATP hydrolysis (i.e., ADO) promoted an epithelial phenotype, while P2Y2 activation by ATP analogues promoted a mesenchymal one.

As previously mentioned, ATP and ADO concentrations increase in cancer. Although ATP is within a low nM range in the healthy interstitium, it increases to  $\mu\text{M}$  concentrations ( $\sim 800 \mu\text{M}$ ) near the tumor. This new evidence is relevant because extracellular ATP was monitored in vivo in tumors induced with OVCAR-3 cells in nude mice [80]. Given that extracellular ATP can be hydrolyzed by membrane ectonucleotidases (CD39 breaks ATP into ADP and AMP, and CD73 hydrolyzes AMP to ADO), it could be assumed that the increase in extracellular ATP is directly correlated with the increase in ADO. Even though extracellular ADO has not been measured in tumors in vivo, there is evidence that ADO levels increase in tumor microdialysates and are more abundant at the core of the tumor [79]. In vitro studies have also indicated that OCDC-derived cell lines release ATP to the cell medium [10, 89].

Correspondingly, ADO function has been evaluated in several types of cancer, and an immunosuppressive role has been proposed for this molecule through ADORA2A receptor activation. Specifically, in OCDC cell lines, it has been demonstrated that ADO inhibits antitumor activity of T and natural killer (NK) cells. Moreover, OC biopsies, OC-derived primary cultures, and cell lines express functional CD39 and CD73 [89]. Therefore, strategies such as CD39 and CD73 inhibition with the aim of reducing extracellular ADO concentrations have been performed, and an improved immune response was described using antibodies against these enzymes [89]. CD73 expression has also been associated with poor prognosis in high-grade serous ovarian carcinoma (HGSOC) [90]. Recent evidence demonstrated that CD73 expression in primary-derived OC promotes stemness and tumor growth and proved that this enzyme acts as an EMT promoter [91], allowing us to recognize CD73 as a promising target for OC.

Eventually, ADO in the extracellular milieu activates ADORA receptors (also known as P1), whose expression has also been characterized in different OCDC lines (e.g., in SKOV-3, CAOV-4, and OVCAR-3). Transcript and protein presence of the four ADORA receptors has been described in the previously mentioned cell lines, with ADORA2B and ADORA3 expression being more abundant; their functionality was demonstrated through cAMP measurement in response to specific agonists [92, 93].

SKOV-3 cell incubation with Apy, ADO, or NECA, an ADORA2 receptor agonist, decreases cell migration. A transcriptional study using a microarray demonstrated that treatment with ADO reduced expression of WNT2, WNT6, WNT10B, and FGF-18, all of which activate signaling pathways involved in EMT in OC [10]. On the other hand, expression of ARPC4 and RAPGEG1 transcripts associated with

cytoskeleton rearrangement increased [10]. Regarding ADORA2 expression, it has been proven that the addition of NECA reduces cell viability and promotes apoptosis in CAOV-4 and OVCAR-3 cell lines [92]. Activation of ADORA3 in OCA-derived cell lines is associated with apoptosis induction and G1 phase cell cycle arrest [94]. Altogether, the evidence highlights purinergic signaling as an important regulator in EOC progression.

## 5. Conclusion

Since Geoffrey Burnstock proposed his purinergic hypothesis in the early 1970s, enormous advances have been achieved in describing the molecular elements that conform the purinergic system and in our understanding of a complex system that is constituted as a continuous metabolic network together with the dynamic events in extracellular signaling. Indeed, the specific actions of purinergic signaling in each system are still being discovered, and its study is a growing field of knowledge. In this chapter, we summarize current knowledge of purinergic signaling in the ovary, where an extensive and specialized expression of purinergic receptors and purine-handling enzymes are observed. The accumulated evidence depicts an emergent and complex system, and at the same time, it raises important questions with deep physiological and pathological implications.

## Acknowledgements

This work was funded by PAPIIT-UNAM, number IN201017 to F.G.V.-C. and IN201618 to M.D.-M. and CONACyT number 284-557 to M.D.-M. We thank Jessica González Norris for editing the English version of this manuscript.

## Conflict of interest

The authors declare that there is no conflict of interest regarding the publication of this article.

## Abbreviations

AC	adenylate cyclase
ADO	adenosine
ADP	adenosine diphosphate
AMP	adenosine monophosphate
AMPK	AMP kinase
Apy	apyrase
ATP	adenosine triphosphate
$[Ca^{2+}]_i$	intracellular concentration of $Ca^{2+}$
CEO	cumulus-enclosed oocyte
EGFR	epithelial growth factor receptor
egr-1	early growth-1
EMT	epithelial to mesenchymal transition
ENPP	ectonucleotide pyrophosphatase
ENTPD/CD39	ectonucleoside triphosphate diphosphohydrolase
EOC	epithelial ovarian cancer


ERK	extracellular mitogen-regulated kinases
FCS	fetal calf serum
FADH <sub>2</sub> /FAD <sup>+</sup>	reduced/oxidized flavin adenine dinucleotide
FMNH <sub>2</sub> /FMN <sup>+</sup>	reduced/oxidized riboflavin-5'-phosphate
FSH	follicle-stimulating hormone
GLC	granulosa-luteal cells
GPCRs	G protein-coupled receptors
GTP	guanosine triphosphate
hCG	human chorionic gonadotropin
HGSOC	high-grade serous ovarian carcinoma
HPLC	high-performance liquid chromatography
LH	luteinizing hormone
NAADP	nicotinic acid adenine dinucleotide phosphate
NAD(P)H/NAD(P) <sup>+</sup>	reduced/oxidized nicotinamide adenine dinucleotide phosphate
NECA	5'-N-ethylcarboxamidoadenosine
NK	natural killer
NT5E	ecto-5'-nucleotidase
OC	ovarian cancer
OCDC	ovarian carcinoma-derived cells
OSE	ovarian surface epithelium
PGF2 $\alpha$	prostaglandin F2 $\alpha$
PLD	phospholipase D
PLC	phospholipase C
PKC	protein kinase C
PMSG	pregnant mare serum gonadotropin
8-PTH	8-phenyltheophylline
RyR	ryanodine receptor
SAM	S-adenosylmethionine
SAH	S-adenosylhomocysteine
SERCA	Ca <sup>2+</sup> -ATPase
TME	tumor microenvironment
UTP	uridine triphosphate

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