Histochemistry and Cell Biology (2018) 149:269–276
https://doi.org/10.1007/s00418-017-1627-8

SHORT COMMUNICATION

Characterization of ecto-nucleotidases in human oviducts with an improved approach simultaneously identifying protein expression and in situ enzyme activity

María Lina Villamonte[1] · Benjamín Torrejón-Escribano[1,2] · Aitor Rodríguez-Martínez[1,3] · Carla Trapero[1,3] · August Vidal[1,3,4] · Inmaculada Gómez de Aranda[1] · Jean Sévigny[5,6] · Xavier Matías-Guiu[3,4] · Mireia Martín-Satué[1,3]

Accepted: 15 December 2017 / Published online: 22 December 2017

Abstract

Extracellular ATP and its hydrolysis product adenosine modulate various reproductive functions such as those taking place in oviducts, including contraction, beating of cilia, and maintenance of fluid composition that, in turn, influences sperm capacitation and hyperactivation, as well as oocyte and embryo nourishing. Ecto-nucleotidases are the enzymes that regulate extracellular ATP and adenosine levels, thus playing a role in reproduction. We have optimized a convenient method for characterizing ecto-nucleotidases that simultaneously localizes the protein and its associated enzyme activity in the same tissue slice and characterizes ecto-nucleotidases in human oviducts. The technique combines immunofluorescence and in situ histochemistry, allowing precise identification of ecto-nucleotidases at a subcellular level. In oviducts, remarkably, ectonucleoside triphosphate diphosphohydrolase 2 (NTPDase2) and NTPDase3, with the ability to hydrolyze ATP to AMP, are expressed in ciliated epithelial cells but with different subcellular localization. Ecto-5′nucleotidase/CD73 is also expressed apically in ciliated cells. CD73, together with alkaline phosphatase, also expressed apically in oviductal epithelium, complete the hydrolysis sequence by dephosphorylating AMP to adenosine. The concerted action of these enzymes would contribute to the local increase of adenosine concentration necessary for sperm capacitation. The use of this method would be an asset for testing new potential therapeutic drugs with inhibitory potential, which is of great interest presently in the field of oncology and in other clinical disciplines.

Keywords Ecto-nucleotidases · CD39 · CD73 · Alkaline phosphatase · ATP · Adenosine · Oviduct

Abbreviations

ADA	Adenosine deaminase
AP	Alkaline phosphatase
α,β-meADP	Alpha, beta-methylene adenosine 5′-diphosphate
E-NTPDase	Ectonucleoside triphosphate diphosphohydrolase
PLAP	Placental alkaline phosphatase

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s00418-017-1627-8) contains supplementary material, which is available to authorized users.

✉ Mireia Martín-Satué
martinsatue@ub.edu

[1] Departament de Patologia i Terapèutica Experimental, Facultat de Medicina i Ciències de la Salut, Universitat de Barcelona, Campus Bellvitge, Barcelona, Spain

[2] Serveis Científics i Tecnològics, Universitat de Barcelona, Campus Bellvitge, Barcelona, Spain

[3] Institut d'Investigació Biomèdica de Bellvitge (IDIBELL), Oncobell Program, CIBERONC, Barcelona, Spain

[4] Servei d'Anatomia Patològica, Hospital de Bellvitge, Barcelona, Spain

[5] Centre de Recherche du CHU de Québec, CHUL, Université Laval, Québec, Canada

[6] Département de microbiologie-infectiologie et d'immunologie, Faculté de Médecine, Université Laval, Québec, Canada

Introduction

Oviducts are the anatomical structures that host fertilization and early stages of embryo development. They collect and transport the oocyte from the ovary, afford protection to the sperm after ejaculation, and create the appropriate milieu for sperm capacitation and hyperactivation to occur (Coy et al. 2012). The oviductal fluid is generated by secretory cells that define the mucosa layer together with ciliated cells, necessary for oocyte and embryo transport (Li and Winuthayanon 2017).

Purinergic signaling, the group of cell responses to extracellular nucleotides, such as ATP, and nucleosides, such as adenosine, is involved in oviduct function (Burnstock 2014). Extracellular ATP mediates oviduct smooth muscle contraction, increases ciliary beat frequency (Barrera et al. 2004), and determines luminal fluid composition (Keating and Quinlan 2008) by inducing ion secretion by oviduct epithelial cells. Adenosine is a key molecule in sperm capacitation, the series of biochemical changes that sperm undergo in the female reproductive tract to acquire fertilizing ability (Fraser 2008; Torres-Fuentes et al. 2015; Bellezza and Minelli 2017).

Extracellular ATP and adenosine levels are mainly regulated by the action of ecto-nucleotidases, the group of enzymes acting sequentially hydrolyzing ATP to ADP, AMP and adenosine (Yegutkin 2014). Amongst cell surface ecto-nucleotidases, the ectonucleoside triphosphate diphosphohydrolase (E-NTPDase) family is predominant. This family of enzymes hydrolyzes ATP (and ADP) to AMP which in turn is hydrolyzed to adenosine by the action of ecto-5′-nucleotidase (CD73). Adenosine inactivation is produced by the action of adenosine deaminase (ADA) which catalyzes its deamination to inosine. ADA in humans is often associated with CD26/dipeptidyl peptidase IV expressed at the cell membrane (Yegutkin 2008). Ecto-nucleotidases and also CD26 were previously characterized in human endometrium showing differences along the cycle (Aliagas et al. 2013). In the case of oviducts, expression of NTPDase3 and CD73 has been described in mice in epithelial oviductal cells (Martín-Satué et al. 2009; Aliagas et al. 2010), suggesting a possible sequential activity of the two enzymes in the hydrolysis of ATP to adenosine in the luminal environment. Although the relevance of ecto-nucleotidases in the control of ATP and adenosine concentrations in oviductal fluid is considerable, no studies have yet been conducted in human oviducts.

Ecto-nucleotidase activities can be identified at a subcellular level in frozen tissue sections, taking advantage of the ability of these enzymes to generate inorganic phosphorus (Pi) when incubated in the presence of appropriate substrates, e.g., AMP for CD73 (Yegutkin 2014). Considering that the same substrate might usually be hydrolyzed by more than one enzyme, specific inhibitors, or the use of antibodies, are needed for complete characterization of these ecto-enzymes (Langer et al. 2008). We implemented a new methodological approach combining immunofluoresce and in situ histochemistry that allows direct identification of an ecto-enzyme and its associated enzyme activity.

In the present study we analyzed, with this optimized method, the expression and activity of NTPDase1 (CD39), NTPDase2, NTPDasae3, CD73, and alkaline phosphatases (AP) in human oviducts.

Materials and methods

Samples

The study was performed in accordance with the ethical principles of the Declaration of Helsinki and the protocol was approved by the ethics committee for clinical investigation of Bellvitge Hospital. Human oviduct samples ($n = 13$) were obtained from salpingectomy specimens without tubal malignancy at the Pathology Service of Bellvitge Hospital (mean age 52, standard deviation 9.87, range 35–64 years).

Tissue samples were fixed with 4% paraformaldehyde, then embedded in 30% sucrose at 4 °C for 24 h and snap frozen in O.C.T freezing media (Tissue-Tek®; Sakura Finetk, Zoeterwoude, Netherlands). 15 µm sections were obtained using a CM1950 Leica Cryostat (Leica, Wetzlar, Germany). Sections were put onto poly-L-lysine-coated glass slides and stored at − 20 °C until use. Routine hematoxylin and eosin staining was performed.

Antibodies

Primary antibodies used in this study are listed in Table 1. Secondary antibodies used were horseradish peroxidase (HRP)-conjugated goat anti-mouse (EnVision™ + System; DAKO, Carpinteria, CA, USA), Alexa Fluor 488-goat anti-mouse IgG2b and anti-rabbit, Alexa Fluor 555-goat anti-mouse IgG1 and anti-rabbit (Thermo Fisher Scientific, Franklin, MA, USA). Secondary antibodies were used at 1:500 and dilutions were made in PBS.

Inmunolabeling experiments

Sections were washed three times with PBS to remove the O.C.T freezing media. The slices were pre-incubated for 1 h at room temperature (RT) with PBS containing 20% normal goat serum (NGS; Gibco, Paisley, UK), 0.2% Triton, and 0.2% gelatin (Merck, Darmstadt, Germany). For immunohistochemistry experiments, a previous blocking of

Table 1 List of primary antibodies used for inmunolabeling experiments

Antibody specificity	Name/clone	Source (isotype)	Supplier	Dilution
CD26	202-36	Mouse (IgG2b)	Abcam (ab3154)	1:100
NTPDase1 (CD39)	BU-61	Mouse	Ancell (188–820)	1:500
Ecto-5′-nucleotidase (CD73)	4G4	Mouse (IgG1)	Abcam (ab81720)	1:50
Human placental alkaline phosphatase (PLAP)	8B6	Mouse	Sigma (A2951)	1:1000
NTPDase2 (CD39L1)	–	Rabbit	Enzo (ALX-215-045)	1:100
NTPDase3	B_3S_{10}	Mouse	http://ectonucleotidades-ab.com	1:500

endogenous peroxidase activity was performed with 10% methanol (v/v) and 2% H_2O_2 (v/v) in PBS for 30 min. Slices were incubated overnight at 4 °C with the primary antibodies (listed in Table 1) diluted in PBS. After three washes in PBS, tissue sections were incubated with the appropriate secondary antibodies for 1 h at RT. Secondary antibodies alone were routinely included as control for the experiments (Supplementary Fig. 1).

For immunohistochemistry, the peroxidase reaction was performed in a solution containing 0.6 mg/mL 3,3′-diaminobenzidine substrate (DAB; D-5637, Sigma–Aldrich, Saint Louis, MO, USA) and 0.5 µL/mL H_2O_2 in PBS for 10 min, and stopped with PBS. Nuclei were counterstained with hematoxylin, and slides were then dehydrated and mounted with DPX mounting medium. Samples were observed under a light Nikon Eclipse E200 and photographed under a light Leica DMD 108 microscope. In fluorescence assays, for nuclei visualization, samples were incubated with To-Pro®-3 (Life Technologies, Paisley, UK) for 7 min. After three washes, slides were mounted with aqueous mounting medium (Fluoromount™, Sigma–Aldrich). Fluorescence samples were observed and photographed under a Leica TCS-SL spectral confocal microscope.

In situ nucleotidase activity experiments

A protocol based on the Wachstein/Meisel lead phosphate method was used (Wachstein et al. 1960; Aliagas et al. 2010, 2014). The sections were washed twice with 50 mM Tris-maleate buffer pH 7.4 and pre-incubated for 30 min at RT with 50 mM Tris-maleate buffer pH 7.4 containing 2 mM $MgCl_2$ and 250 mM sucrose. The enzymatic reaction was carried out by incubating tissue sections for 1 h at 37 °C with 50 mM Tris-maleate buffer pH 7.4 supplemented with 250 mM sucrose, 2 mM $MgCl_2$, 5 mM $MnCl_2$, 3% Dextran, 2 mM Pb $(NO_3)_2$ and 2 mM $CaCl_2$. All experiments were performed in the presence of 2.5 mM levamisole, as an inhibitor of alkaline phosphatase (AP) activity, and in the presence of 1 mM AMP, ADP, or ATP as a substrate. Control assays were performed in the absence of a nucleotide. For CD73 inhibition experiments, 1 mM α,β-methylene-ADP (α,β-meADP) was added to both pre-incubation and enzymatic reaction buffers. The reaction was revealed by incubation with 1% $(NH_4)_2S$ (v/v) for exactly 1 min. Nuclei were counterstained with hematoxylin. Samples were mounted with aqueous mounting medium (Fluoromount™; Sigma–Aldrich), observed under light Nikon Eclipse E200 microscope and photographed under a light Leica DMD 108 microscope.

Combined NTPDase1 (CD39) immunolabeling and in situ ADPase activity experiments

Sample sections were washed twice with PBS and blocked in PBS containing 20% NGS (Gibco), 0.2% Triton and 0.2% gelatin (Merck) at RT for 1 h. The samples were incubated overnight at 4 °C with the anti-human CD39 antibody. The sections were then washed three times with PBS and twice with 50 mM Tris-maleate buffer. In situ ADPase activity experiment was performed in the same sections as indicated above, using 1 mM ADP as substrate. Subsequently, the tissues were washed three times in PBS before Alexa Fluor-488 goat anti-mouse secondary antibody was added. After three final washes with PBS, samples were mounted on a glass slide with Prolong Gold antifade reagent with DAPI mounting medium (Thermo Fisher Scientific). The sections were observed and photographed under a Nikon Eclipse E800 microscope.

Combined NTPDase2 (CD39L1) immunolabeling and in situ ATPase activity experiments

Immunolocalization of NTPDase2 (CD39L1) and ATPase activity assay were performed in the same slides using the protocol indicated above. The samples were incubated with rabbit anti-human CD39L1 primary antibody and Alexa Fluor-488 goat anti-rabbit secondary antibody.

Combined immunolabeling of human placental alkaline phosphatase (PLAP) and in situ AP activity experiments

The sections were rinsed twice with PBS and pre-incubated for 1 h at RT with PBS containing 20% NGS (Gibco, Paisley,

UK), 0.2% Triton and 0.2% gelatin (Merck). The samples were incubated overnight at 4 °C with the anti-human PLAP primary antibody. Subsequently, the sections were incubated with Alexa Fluor 488-goat anti-mouse secondary antibody for 1 h at RT. The histochemical localization of AP was addressed in the same slides using the Gossrau method (Schelstraete et al. 1985; Aliagas et al. 2013) with some modifications. Briefly, slices were rinsed with 0.1 M Tris–HCl buffer pH 7.4 containing 5 mM $MgCl_2$ and then incubated with the same buffer at pH 9.4 for 15 min at RT. The enzymatic reaction was detected using the revealing reagent BCIP (Sigma-Aldrich) for 20 min at RT. The reaction was stopped by rinsing with 0.1 M Tris–HCl buffer, pH 7.4. For AP inhibition experiments, 5 mM levamisole was added to both pre-incubation and enzymatic reaction buffers. In control experiments, the revealing reagent BCIP was omitted. The samples were mounted in ProLong Gold antifade reagent with DAPI mounting medium (Thermo Fisher Scientific) and observed and photographed under a Nikon Eclipse E800 microscope.

Results and discussion

In the present study, we characterized the ecto-nucleotidases in human oviducts with a method that we set up to simultaneously detect, in the same tissue section, both protein expression and enzyme activity with immunofluorescence and in situ histochemistry, respectively. Our results show that ecto-nucleotidases are abundantly present in human oviducts, where they might act in concert to regulate extracellular nucleotide and nucleoside concentrations.

Methodologically, although other protocols are feasible, in our hands, the most suitable combination for optimal visualization and further photographing is the sequence of techniques shown here, with histochemistry being performed between primary and secondary antibody incubations. Equivalent results were obtained when immunofluorescence and histochemistry were analyzed separately in different tissue slices (data not shown). We recommend testing different nucleotide concentrations and incubation times to optimize the results for a particular tissue, since dark brown deposits can hamper fluorescence capture. Notably, phosphate buffer used for incubation of antibodies and for immunolabeling washing steps did not increase the background of the tissue after histochemistry, performed in Tris-maleate (phosphate-free) buffer (Supplementary Fig. 1). Since the use of primary antibodies with possible enzyme inhibitory action, such as the one to NTPDase3 (Munkonda et al. 2009), can impede the subsequent in situ enzyme activity reaction, in these cases, the in situ histochemistry should be performed at the beginning of the procedure. This consideration, which is apparently a limitation, indeed represents an asset, since the technique might well be used to validate antibodies with potential inhibitory action, such as those against CD39 and CD73, which are already being used in cancer clinical trials (Allard et al. 2017).

The hydrolysis of ATP to AMP is catalyzed mainly by the E-NTPDase family. As expected, NTPDase1 (CD39) label was prominent in the vascular endothelial cells and in smooth muscle cells (Kaczmarek et al. 1996; Sévigny et al. 2002). The strong ADPase activity detected coincided with the CD39 immunolocalization (Fig. 1). Moreover, NTPDase2 and 3 were not detected in these structures.

NTPDase2 was largely expressed in ciliated epithelial cells (Fig. 2A) and in fibroblasts of the lamina propria (not shown). NTPDase3 was also detected in ciliated epithelial cells (Fig. 2B). Strong signal for ATPase activity was detected in cilia, coinciding with NTPDase2 and NTPDase3 expression (Fig. 2A). Double immunolabeling for NTPDase2 and NTPDase3 revealed that both proteins were expressed at the apical pole of ciliated cells, but with different subcellular distribution. While NTPDase2 is expressed throughout the entire length of the cilia, NTPDase3 expression is limited to the apical side of the cell membrane, basally in the cilia (Fig. 2B).

The next step in the metabolism of purine nucleotides is the dephosphorylation of extracellular AMP to adenosine, mainly accomplished by ecto-5′nucleotidase (CD73). This enzyme was abundantly detected on the apical side of ciliated epithelial cells. As expected, the enzyme was also expressed in smooth muscle, connective tissue and endothelial cells (Fig. 3). In situ AMPase activity experiments demonstrated that the enzyme was active where the protein was immunodetected. In situ AMPase activity was inhibited by α,β-meADP, a specific CD73 inhibitor, further demonstrating the identity of the immunodetected protein in the abovementioned structures (Fig. 3A).

Placental alkaline phosphatase, an ecto-nucleotidase that catalyzes the hydrolysis of phosphate from a broad range of substrates, was expressed and active in luminal epithelium and also, as expected, in the endothelial cells of blood vessels. In the present study, we demonstrate with this new method the correlation between PLAP protein expression and AP activity in the fallopian tubes (Fig. 4). The activity was inhibited with the AP inhibitor levamisole, confirming the specificity of the activity (data not shown).

The generated adenosine can be further inactivated by other enzymes such as adenosine deaminase (ADA), which may be expressed as a soluble ecto-enzyme or as membrane-associated enzyme often forming larger complexes with CD26/dipeptidyl peptidase IV, converting adenosine to inosine. CD26 receptor expression was detected in the secretory, non-ciliated, epithelial cells (Supplementary Fig. 2). Double immunofluorescence showed that CD26 staining and CD73 staining were mutually exclusive.

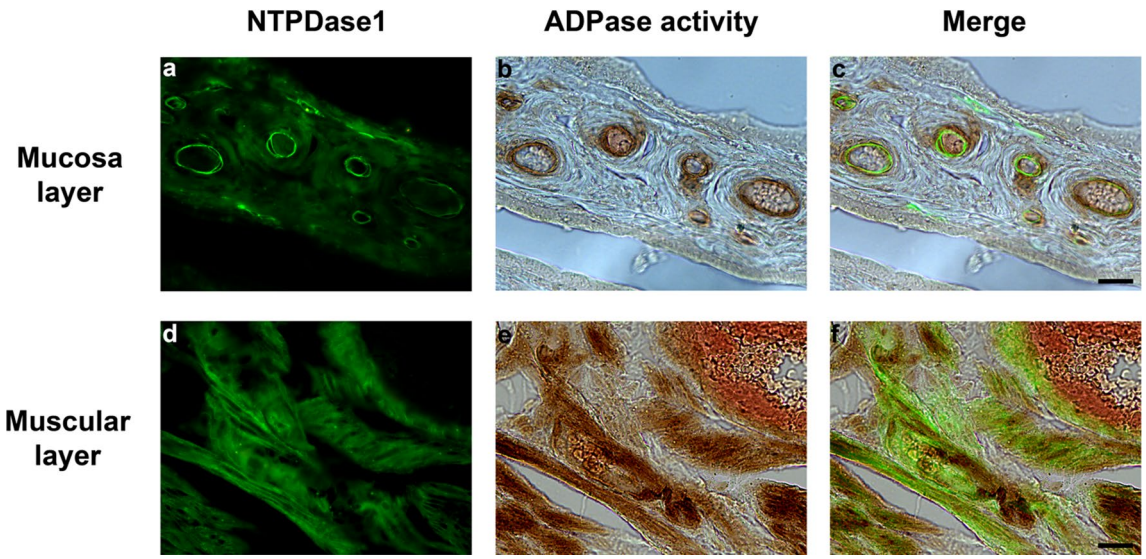

Fig. 1 Immunolocalization of NTPDase1 (**a, d**) and in situ ADPase histochemistry (**b, e**) in cryosections of human oviducts. NTPDase1 was detected with immunofluorescence in endothelial cells of lamina propria (**a**) and in smooth muscle cells (**d**). Microphotographs **b** and **e** show dark brown deposits corresponding to in situ ADPase activity. Merged images (**c, f**) confirms that NTPDase1 is active in the same structures where it immunolocalizes. Reddish structure at the top right of the image is the blood inside the vessel. Scale bars 25 µm

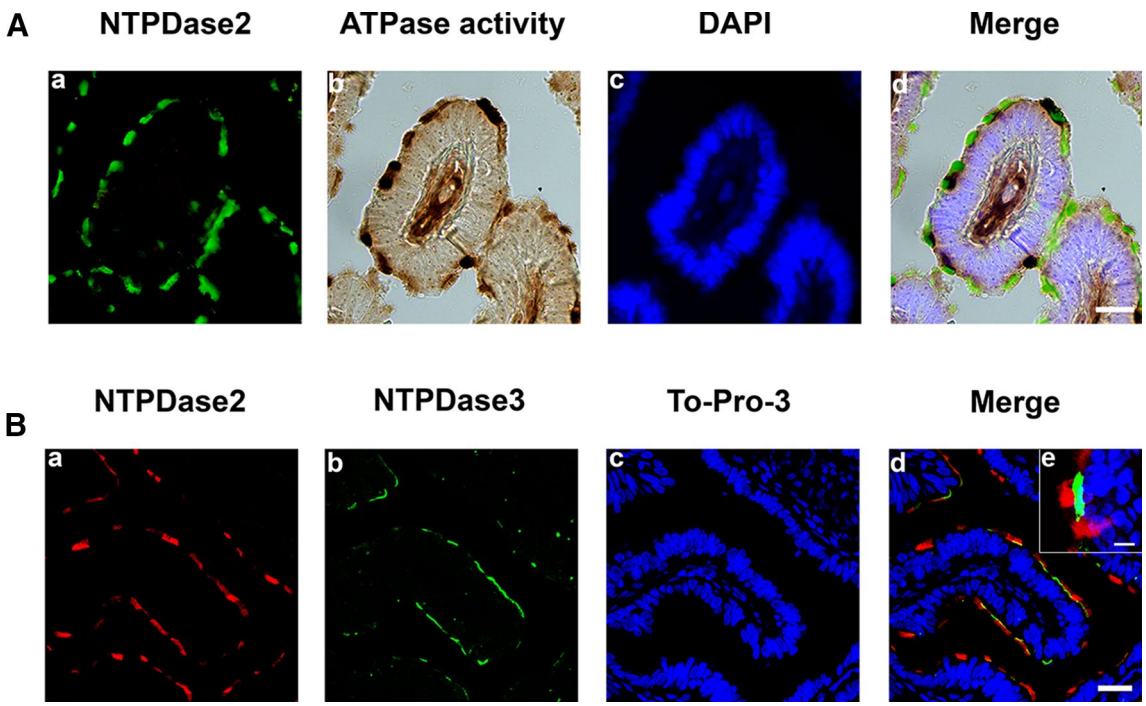

Fig. 2 **A** Immunolocalization of NTPDase2 (**a**) and in situ ATPase histochemistry (**b**) in cryosections of human oviducts. NTPDase2 was largely detected with immunofluorescence in ciliated epithelial cells. Microphotograph **b** shows dark brown deposits corresponding to in situ ATPase activity. Nuclei were labeled with DAPI (**c**). Merged image (**d**) confirms that NTPDase2 is active in the same structures where it immunolocalizes. Scale bar 25 µm. **B** Confocal fluorescence images of the mucosa of oviducts with antibodies against NTPDase2 (**a**) and NTPDase3 (**b**). Nuclei were labeled with To-Pro-3 (**c**). Merged image shows that NTPDase2 and NTPDase3 are expressed in ciliated epithelial cells; NTPDase2 is detected throughout the entire length of the cilia and NTPDase3 expression is confined to the apical side of the cell membrane, basally in the cilia (**d, e**). Scale bars 40 µm (**d**) and 5 µm (**e**)

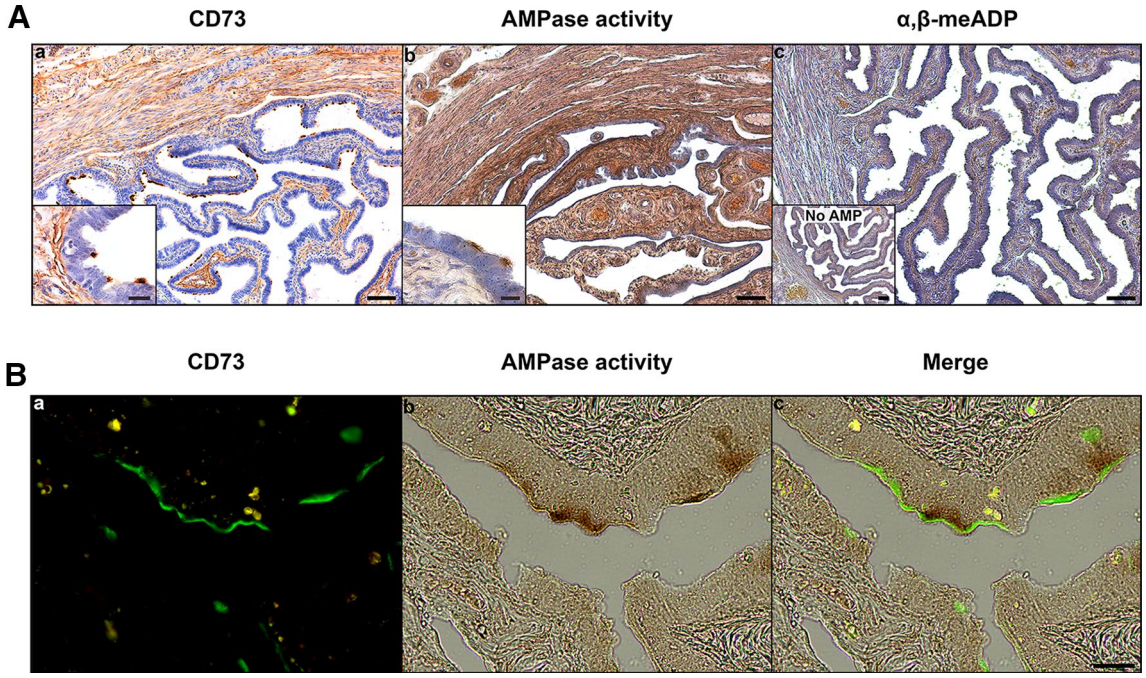

Fig. 3 **A** Immunolocalization of CD73 (**a**) and in situ AMPase histochemistry (**b, c**) in cryosections of human oviducts. **a** CD73 was abundantly immunodetected on the apical side of ciliated epithelial cells (inset). CD73 was also detected in smooth muscle cells, connective tissue and endothelial cells. Microphotograph **b** shows dark brown deposits corresponding to in situ AMPase activity, coinciding with CD73 localization. **c** Activity experiment performed in the presence of the inhibitor *α,β*-meADP shows complete inhibition of AMPase activity. Inset in **c** corresponds to the activity experiments in the absence of substrate. Nuclei were counterstained with aqueous hematoxylin. Scale bars 100 µm and 25 µm in insets. **B** Immunolocalization of CD73 (**a**) and in situ AMPase histochemistry (**b**) in cryosections of human oviducts. **a** CD73 was detected with immunofluorescence on the apical side of ciliated epithelial cells. Microphotograph **b** show dark brown deposits corresponding to in situ AMPase activity. **c** Merged image confirms that CD73 is active in the same structures where it immunolocalizes. Scale bar 25 µm

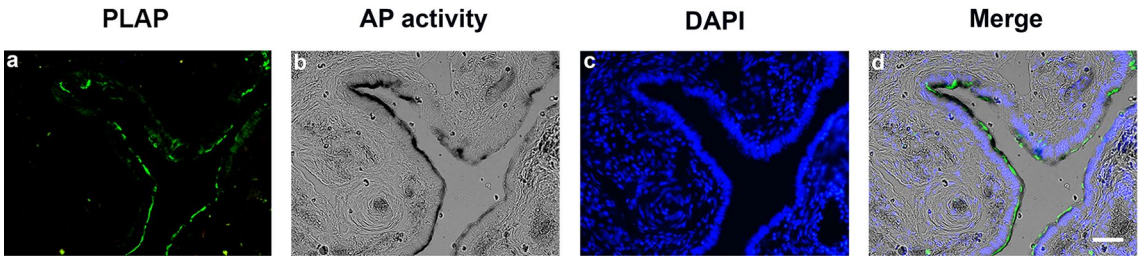

Fig. 4 Immunolocalization of PLAP (**a**) and in situ AP histochemistry (**b**) in cryosections of human oviducts. PLAP was detected with immunofluorescence in luminal epithelium. Microphotograph **b** shows dark deposits corresponding to in situ AP activity. Merged image (**d**) confirms that PLAP is active in the same structures where it immunolocalizes. Scale bar 50 µm

A schematic representation of the ecto-enzymes studied in human oviducts can be seen in Fig. 5. In summary, ciliated cells, remarkably, have the complete enzyme mechanism needed to efficiently hydrolyze extracellular ATP, ADP and AMP to adenosine, and thus they might well be the key cells in the control of purinergic signaling in the lumen of oviducts. A local increase of adenosine would favor sperm capacitation. This would add a new function to the already versatile ciliated cells that, besides triggering fluid movement by cilia beating, contribute, amongst other functions, to the anchorage of the sperm favoring successful fertilization (Spassky and Meunier 2017).

Considering that ecto-nucleotidases are involved in the physiology of tissue homeostasis as well as the physiopathology of many diseases, the improvement of methods for their study in the context of tissue architecture is of relevance. As mentioned above, an example is the study of specific inhibitors for developing new therapeutic strategies in oncology (Allard et al. 2017). This protocol optimizes resources and, remarkably, saves tissue samples, which is of

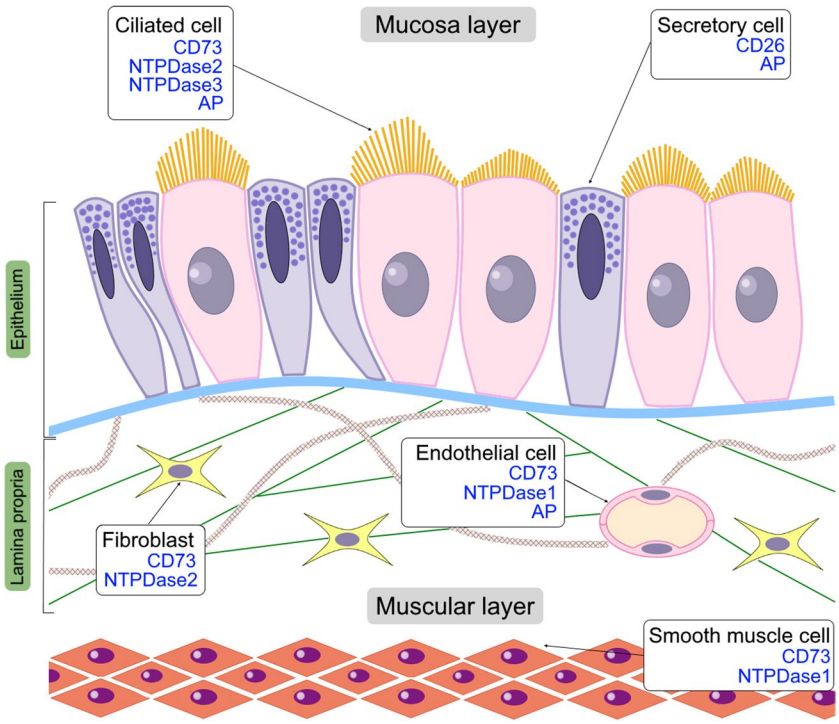

Fig. 5 Schematic representation showing the distribution of ecto-nucleotidases in human oviducts

particular interest in those cases in which only a small piece of tissue sample is available.

Acknowledgements This work was supported by a grant from the *Instituto de Salud Carlos III* (*FIS PI15/00036*), co-funded by FEDER funds/European Regional Development Fund (ERDF) "a Way to Build Europe"-// *FONDOS FEDER* "una manera de hacer Europa", and a grant from the *Fundación Merck Salud* (*Ayuda Merck de Investigación 2016-Fertilidad*). ARM was awarded a fellowship from the *Asociación Española Contra el Cáncer* (*AECC*). JS received support from the Canadian Institutes of Health Research (CIHR) and was the recipient of a *Chercheur National* Scholarship from the *Fonds de Recherche du Québec–Santé* (*FRQS*). We are grateful for the technical support of Serveis Científics i Tecnològics, Campus Bellvitge, Universitat de Barcelona. The authors thank Tom Yohannan for language editing.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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