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Endometrial epithelial cell organoids as tools for studying the CD39 family of enzymes and for validating enzyme inhibitors

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Summary. Extracellular adenosine triphosphate (ATP) conducts a complex dynamic system of broadly represented cell signaling. Ectonucleotidases are the enzymes with nucleotide hydrolytic ability that regulate ATP levels in physiological and pathological conditions, thus playing a key role in the so-called purinergic signaling. Altered ectonucleotidase expression has been reported in cancer, and the ectonucleoside triphosphate diphosphohydrolase (NTPDase) family of enzymes, with its best-known form NTPDase1 (CD39), is targeted in cancer immunotherapy. The tandem of enzymes CD39-CD73 is responsible for the generation of immunosuppressive adenosine in the tumor microenvironment, and inhibition strategies are of great interest. Organoids have emerged as very convenient models for the study of tumors since they are three-dimensional cultures that retain many of the features of tissue. The present study aims to contribute to improving the methodology and the molecular tools needed for the study of ectonucleotidases in healthy and disease conditions. The study, performed in an endometrial cancer cell model, could be extended to other types of tumors and pathologies in which the purinergic system is involved. We generated organoids from endometrial cancer cells overexpressing NTPDase2 (CD39L1) and NTPDase3 (CD39L3) as fusion proteins with EGFP, and we performed functional assays by adapting *in situ* cytochemistry protocols. This allowed us to simultaneously detect enzyme activity and protein expression and to demonstrate that organoids can be used to test ectonucleotidase inhibitors—a result that can be used to develop new cancer treatment options.

Key words: ENTPD, NTPDase, Endometrial cancer, Cytochemistry, Purinergic signaling, CD39, ATP

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Introduction

Purinergic signaling is a broadly expressed, complex system of substrates, receptors, and enzymes that controls a number of physiological and pathological processes, including cell proliferation, migration, and differentiation, and the regulation of immune responses, among others (Yegutkin, 2014; Haas et al., 2021; Huang et al., 2021; Zimmermann, 2021). Nucleotides, mainly adenosine triphosphate (ATP) and its derivatives, adenosine di- and monophosphate (ADP and AMP, respectively), and nucleosides, such as adenosine, are the main actors in this system, where ectonucleotidases, encompassing four different families of enzymes, finely regulate their extracellular concentrations. The nucleoside triphosphate diphosphohydrolases (NTPDases) constitute one of the families of ectonucleotidases, which includes four membrane-bound members: NTPDase1 (also known as CD39), NTPDase2 (CD39L1), NTPDase3 (CD39L3), and NTPDase8. They hydrolyze ATP and ADP to AMP, which is the substrate of ecto-5'nucleotidase (5'NT, CD73), and which generates adenosine (Robson et al., 2006; Yegutkin,

2008; Zimmermann et al., 2012). One of the checkpoint inhibitory targets in immune oncology is the CD39-CD73 axis (Allard et al., 2017, 2019; Di Virgilio and Adinolfi, 2017), an efficient system of sequentially acting ectoenzymes hydrolyzing extracellular ATP to adenosine, favoring the immunosuppressive tumor microenvironment that supports tumor immune escape, tumor growth, and disease progression (Zhang et al., 2015a,b). In this context, the development and improvement of detection tools for ectonucleotidases and their inhibitors are of great interest (Yegutkin and Boison, 2022).

Three-dimensional (3-D) cell cultures represent a suitable biological model of study for most diseases, especially cancer (Shankaran et al., 2021). In the case of oncogynecological pathology, and compared with current 2-D tools (mainly cell culture), the generation and use of 3-D organoids, organ-on-a-chip, and patient-derived xenografts (PDXs) are emerging as the top experimental techniques in basic, translational, and clinical research (Eritja et al., 2010, 2013; Gu et al., 2020). Endometrial tumor-derived organoids closely resemble the original tissue and maintain both histological and genetic characteristics of tumors, as well as the inter-tumoral heterogeneity of cells (Maru et al., 2019). Currently, there are still some limitations in its use that must be taken into account, such as the lack of intra-tumoral heterogeneity, the species factor with the use of PDX for the study of the tumor microenvironment and the differential response to treatments (Heremans et al., 2021). Conventional histological techniques need to be adapted to these novel sample types. This is the case of ectonucleotidase activities, detected with *in situ* enzyme histochemistry and cytochemistry techniques, known as the lead-phosphate or Wachstein-Meisel staining method. This technique is based on the addition, and incubation, of substrates—ATP and/or ADP, in the case of NTPDases, or AMP for 5'NT/CD73—followed by staining of the lead phosphate precipitates formed at the site of enzyme activity (Wachstein et al., 1960). This approach can be used in frozen tissue sections and fixed cells (Karasaki and Okigaki, 1976; Langer et al., 2008), and can also be combined with the use of antibodies against ectonucleotidases to allow simultaneous visualization of both enzyme expression and hydrolytic activity (Villamonte et al., 2018; Losenkova et al., 2020; Martín-Satué et al., 2020).

Ectonucleotidases are expressed in the non-tumoral endometrium, with changes along the cycle influenced by hormones (Aliagas et al,. 2013; Trapero et al., 2019). As in other types of cancer, ectonucleotidases are dysregulated in endometrial cancer, where the CD39-CD73 axis is increased in the stroma of tumors compared with non-tumoral stroma (Aliagas et al., 2014). Ectonucleotidases are expressed not only in the stroma but also in the epithelial component of endometrial tumors, such as CD73 (Bowser and Broaddus, 2016) and NTPDase2 (Rodríguez-Martínez et al., 2021), whose expression is related to the progression of the disease. Deciphering the regulatory mechanisms of expression and activity of ectonucleotidases would help advance the development of personalized therapies in endometrial cancer.

The main aim of the present work was to improve the methodology for the study of ectonucleotidases and their inhibitors and to corroborate their use in cancer cell-derived organoids. We addressed this by expressing and characterizing green fluorescent protein (GFP)-tagged NTPDase2 and NTPDase3 in human endometrial carcinoma cells, and by obtaining organoids of these cell cultures to validate the use of these 3-D models for the study of ectonucleotidases.

Materials and methods

EGFP-NTPDase2 and EGFP-NTPDase3 cloning

Human *ENTPD2* and *ENTPD3* cDNAs were subcloned into pEGFPC1 expression vector as follows: *ENTPD2* and *ENTPD3* cDNAs were obtained by polymerase-chain reaction (PCR) from pCDNA3.1 vectors, provided by Prof. Jean Sévigny and based on the original sequences reported by Chadwick and Frischauf (1997) and Smith and Kirley (1998), respectively. The sets of primers used were: forward 5'-TCTCTCGAGTGGAATTCGGCTTAATGGCCGGGAAGG-3' and reverse 5'GCCAAGCACCATTAAGCCGAATTCTGGGATCCAGA-3' for *ENTPD2*, and forward 5'-TCTCTCGAGAGATGTTCACTGTGCTGACCCGCCAAC-3' and reverse 5'TCTGTCGACGCGAATCCACTGCATGGTCAA-3' for *ENTPD3*. The two forward primers included a *Xho*I restriction site, and the reverse primers a *Bam*HI restriction site for *ENTPD2*, and a *Sal*I restriction site for *ENTPD3*, for subsequent in-frame subcloning into the pEGFPC1 vector. Amplifications were started by incubating for 2 minutes at 95ºC, followed by 30 cycles of 20 seconds at 95ºC, 10 seconds at 60ºC, and 30 seconds at 72ºC, using a BioRad T100 Thermal cycles apparatus (Bio-Rad Laboratories, Hercules, CA, USA). PCR products of 1487 bp for *ENTPD2* and 1589 bp for *ENTPD3* were excised from the agarose gel and purified using the QIAquick Gel Extraction Kit (QIAGEN, Hilden, Germany). The products were digested and ligated into the *Xho*I and *Bam*HI (or *Xho*I and *Sal*I) digested pEGFPC1 plasmid to generate EGFP-NTPDase2 and EGFP-NTPDase3 fusion proteins, respectively. Plasmids were purified using QIAprep® Spin Miniprep Kit (QIAGEN), and the inserts were verified by restriction mapping and then full-length sequencing using the primer 5'-GACCCCAACGAGAAGCGCGA-3' (Life Technologies, Paisley, UK). The subcloning procedure is summarized in Figure 1.

Cell culture and plasmid transfection

EGFP-ENTPD2- and *EGFP-ENTPD3*-expressing vectors were transfected into the uterine serous papillary carcinoma ARK-2 cell line (USPC-ARK-2; RRID:

CVCL-IV73), a tumor cell line obtained from a 63-year-old stage IV endometrial serous adenocarcinoma patient (English et al., 2013), which was kindly provided for this study by Dr. Alessandro D. Santin of Yale University. Cells were grown at 37ºC in a 5% CO_2 humidified atmosphere in DMEM/F12 medium (Gibco, Paisley, UK) supplemented with L-glutamine, 15mM HEPES, 10% fetal bovine serum (FBS; Gibco), and 1% penicillin/streptomycin (P/S; Sigma-Aldrich, Saint Louis, MO, USA). For transfection, briefly, cells were seeded in 6 well plates, grown at a confluency of 70-90% and transfected with 4 µg of one the following plasmids diluted in 250 µL OPTI-MEM (Gibco): p*EGFPC1-ENTPD2*, p*EGFPC1-ENTPD3*, or p*EGFPC1*, using Lipofectamine (Invitrogen, Waltham, MA, USA) and the protocol provided by manufacturer. Twenty-four hours after transfection, the medium was replaced with DMEM/F12 medium (Gibco) supplemented with 10% FBS (Gibco), 1% P/S (Sigma-Aldrich), and 500 mg/mL of geneticin/G418 (Gibco). Non-transfected cells were also included as controls of the experiments.

Stably-transfected clones of EGFP-tagged NTPDase2 and NTPDase3 were obtained by fluorescent-activated cell sorting using a MoFlo Astrios Cell Sorter (Beckman Coulter, Brea, CA, USA). Briefly, confluent 10 cm^2 plates were washed with 4 mL of PBS, trypsinized, and centrifuged. The resulting pellets were washed twice with PBS and resuspended in 400 µL of a solution containing 0.5% (w/v) bovine serum albumin (BSA; Sigma-Aldrich) and 2 mM EDTA in PBS. Sorted GFP-positive cells were seeded in a 96-well plate at a density of one cell per well to obtain clones. Eight clones per transfection were grown and assayed for NTPDase expression and enzyme activity. One clone from each set was chosen for further experiments. All experiments reported here were performed independently three times. The results shown in this article correspond to one clone, however, sorted GFP-positive pooled cells were analyzed in parallel.

Membrane-enriched protein obtention and Western blotting

One x 10^6 transfected ARK-2 cells were plated on 10 cm^2 plates and grown to 90-100% confluency at 37ºC in 5% CO_2 humidified atmosphere. Plates were washed twice with 10 mL of a cold phosphate-free solution containing 15 mM Tris pH 7.5, 145 mM NaCl, and 0.005% Triton X-100 (v/v) in water, scrapped with 200 µL of the same solution containing Protease Inhibitor Cocktail (Sigma-Aldrich) and kept on ice for 15 minutes. Samples were then sonicated and centrifuged at 8000xg for 15 minutes at 4ºC. Supernatants were centrifuged at 50,000 rpm for 30 minutes at 4ºC and the resulting pellets were diluted in a solution containing 15 mM Tris pH 7.5, 145 mM NaCl supplemented with Protease Inhibitor Cocktail. The protein concentration was determined using the method of Lowry et al. (1951).

Thirty µg of membrane-enriched cell lysates were added per lane and separated in a acrylamide/bis gel under non-reducing conditions, and then transferred to nitrocellulose membranes (Bio-Rad) by electroblotting at 100 V for 1 hour. Membranes were blocked with 2.5%

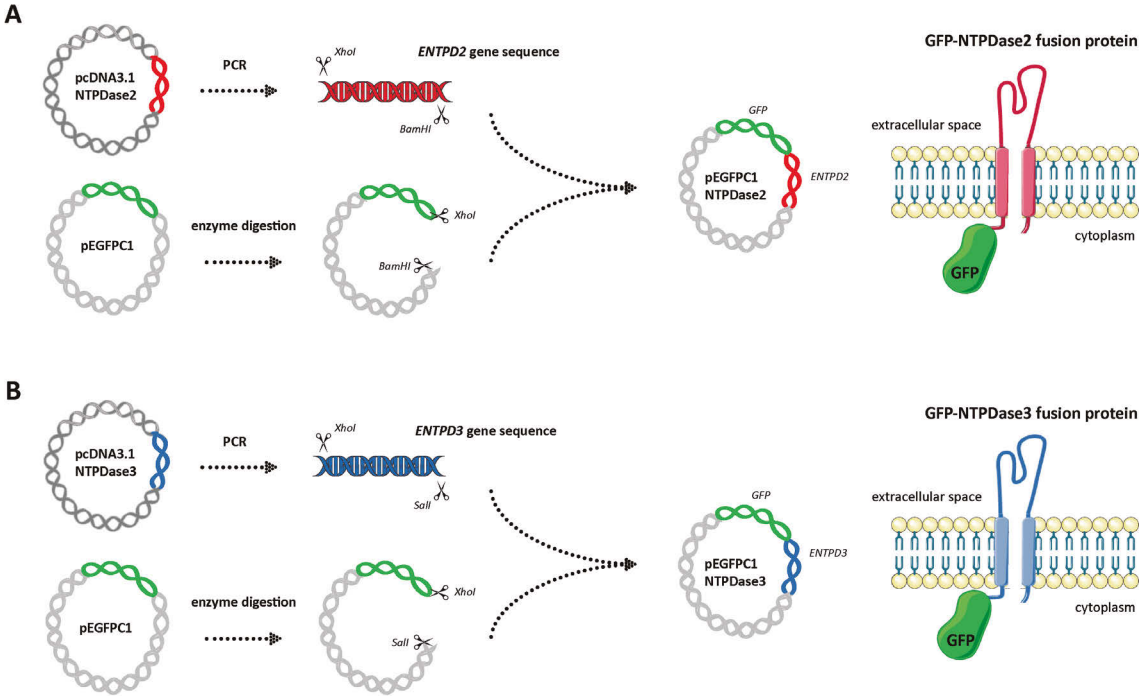

Fig. 1. Cloning strategy to generate the plasmids expressing EGFP-NTPDase2 **(A)** and EGFP-NTPDase3 **(B)** fusion protein used for subsequent transfection into ARK-2 endometrial carcinoma cells.

(w/v) non-fat milk, 1% (w/v) BSA in PBS containing 0.1% (v/v) Tween® 20 for 1 hour at RT. Membranes were then incubated with the primary antibodies: mouse anti-human NTPDase2 (clone H9s; 1:100) (Pelletier et al., 2017) or mouse anti-human NTPDase3 (clone $B3_S10$; 1:500) (Munkonda et al., 2009), both from https://ectonucleotidases-ab.com, in 0.5% (w/v) non-fat milk, 0.1% (w/v) BSA in PBS containing 0.1% Tween® 20 O/N at 4ºC. After this, membranes were incubated with secondary rabbit anti-mouse horseradish peroxidase-conjugated antibody diluted 1:2000 in 0.5% (w/v) non-fat milk, 0.1% (w/v) BSA in PBS containing 0.1% Tween® 20, for 1 hour at RT. Membranes were subsequently revealed using Luminata™ Crescendo Western HRP Substrate (Merck Millipore, Burlington, MA, USA) and the Amersham™ Imager 600 system (GE Healthcare, Chicago, IL, USA). For loading protein control of the experiments, membranes were incubated with mouse anti-flotillin-1 (clone 18/Flotillin-1, BD Biosciences, Franklin Lakes, NJ, USA) diluted 1:1000 in PBS, and revealed as indicated.

Three-dimensional (3-D) organoid generation

Organoids derived from ARK-2 cells overexpressing *EGFP-ENTPD2* or *EGFP-ENTPD3* were generated following the protocol established by Eritja et al. 2010. First, a Matrigel bed (Matrigel® Growth Factor Reduced, Phenol Red Free; Corning, NY, USA) was created by carefully expanding with a yellow tip 10 µL of 100% Matrigel per well in 96-well black plates for microscopy (Cell Culture Microplate, 96 well, PS, F-bottom (chimney well), µClear, black; Greiner Bio-one, Kremsmünster, Austria) and incubated for 30 minutes at 37ºC for Matrigel solidification. Next, cells were suspended at 2000 cells per 100 µL in basal medium containing DMEM/F12 supplemented with 1 mM HEPES, 1mM sodium pyruvate (Sigma-Aldrich), 1% P/S, and 1% amphotericin B (Gibco). Cells were then mixed with 3% Matrigel and 2% Dextran-Charcoal-Coated Fetal Bovine Serum (DCC-FBS; Gibco) and seeded at 100 µL per well. Organoids were left for 5-7 days in the incubator without medium change and fixed with cold 4% paraformaldehyde in PBS for 10 minutes.

Immunofluorescence assays

Three x 10^4 ARK-2 cells were plated on 12 mm poly-L-lysine treated coverslips in 24-well plates and grown to confluence. Coverslips were washed twice with PBS and fixed with 3.5% paraformaldehyde in PBS. Organoids were fixed with cold 4% paraformaldehyde for 10 minutes. Both 2D-grown cells and organoids were permeabilized by incubation with 0.2% (w/v) saponin (Merck Millipore) in PBS for 15 minutes at RT and incubated with PBS containing 20% normal goat serum (NGS; Gibco) and 0.2% gelatin (w/v) (Merck Millipore) for 1h at RT. Coverslips and organoids were incubated O/N at 4ºC with the mouse anti-hNTPDase2 and anti-hNTPDase3 primary antibodies diluted in PBS at 1:100 and 1:500, respectively. After three washes in PBS, samples were incubated with the secondary antibody, goat-anti mouse Alexa Fluor 555 (Invitrogen), for 1 h at RT in the case of coverslips and O/N at 4ºC for organoids. Coverslips were then washed three times in PBS and mounted with an aqueous mounting medium with DAPI (ProLong™ Gold antifade reagent with DAPI, Life Technologies) for nuclei labeling. Organoids were washed three times in PBS and incubated for 30 minutes with DAPI (Invitrogen) diluted 1:10000 in PBS and then three final washes with PBS were made. Samples were observed and photographed under a Zeiss LSM 880 Confocal Laser Scanning Microscope (Zeiss, Oberkochen, Germany) using the objectives 40x and 63x Plan Apochromat with an aperture number (NA) of 1.3 and 1.4 respectively. Fluorescence images were processed with the ZEN 2.3 SP1 software (Zeiss).

In situ ATPase enzyme activity

ATPase *in situ* enzyme activity was detected in ARK-2 cell 2-D cultures and 3-D organoids, using the Wachstein-Meisel staining method with some modifications (Wachstein et al., 1960; Aliagas et al., 2010, 2014). Fixed cells grown on coverslips and organoids obtained as indicated above were washed twice with 50 mM Tris-maleate buffer pH 7.4 and pre-incubated for 30 minutes at RT with 50 mM Tris-maleate buffer pH 7.4 containing 2 mM $MgCl_2$ and 250 mM sucrose (blocking buffer). The enzymatic reaction was performed for 1h at 37ºC in a buffer containing 50 mM Tris-maleate pH 7.4, 250 mM sucrose, 3% Dextran, 2 mM $MgCl_2$, 2 mM $CaCl_2$, 5 mM $MnCl_2$, 2 mM $Pb(NO_3)_2$, and 2.5 mM Levamisole (Sigma-Aldrich), as inhibitor of the alkaline phosphatases, in the presence of 50, 100, 200, 500, or 1000 µM ATP as the substrate (incubation buffer). In the case of ectonucleotidase inhibition experiments with organoids, 1 mM of suramin (Sigma-Aldrich) or 1 mM NF279 (Tocris, Bristol, UK) were included in both the blocking and the incubation buffers. For coverslips, a final incubation volume of 300 µL/well was used; for the organoids, in 96-well plates, a total volume of 100 µL/well was used. Control assays in the absence of nucleotides were routinely included. Released inorganic phosphate was revealed by incubation with 1% $(NH_4)_2S$ (v/v) for exactly 1 minute. Nuclei in coverslips were counterstained with hematoxylin and mounted with Fluoromount™ (Sigma-Aldrich), or, alternatively, directly mounted with an aqueous mounting medium with DAPI for direct fluorescence studies. Coverslips were observed with a light Nikon Eclipse E200 microscope (Nikon, Tokyo, Japan), and photographed with a light Leica DMD 108 microscope (Leica, Wetlzar, Germany). For fluorescence studies, coverslips were observed with a Zeiss LSM 880 Confocal Laser Scanning Microscope (Zeiss), and fluorescence images were processed with the ZEN 2.3 SP1 software (Zeiss). Organoids were maintained at 4ºC

in 100 µL of 15 mM Tris plus 145 mM NaCl in water, and visualized and photographed with a Zeiss LSM 880 Confocal Laser Scanning Microscope (Zeiss) for fluorescence studies, and with an inverted Leica DMIRB microscope (Leica) for *in situ* ATPase activities.

Combined in situ ATPase activity and immunolabeling techniques in organoids

The methodology to simultaneously detect the protein and the enzyme activity described for tissue slices (Villamonte et al., 2018) was adapted to organoids. First, organoids in 96-well plates were washed twice with phosphate-free buffer containing 15 mM Tris and 145 mM NaCl, and blocked for 1h at RT with a solution containing 20% NGS and 0.2% gelatin (w/v) (Merck Millipore). Then the mouse anti-hNTPDase2 or anti-hNTPDase3 primary antibodies were incubated at 1:100 and 1:500, respectively, in 15 mM Tris and 145 mM NaCl, O/N at 4ºC. After three washes in 15 mM Tris and 145 mM NaCl, *in situ* enzyme activity was carried out as indicated above. Once completed, the goat anti-mouse Alexa Fluor 488 (Invitrogen) secondary antibody was added at a 1:500 dilution to 15 mM Tris and 145 mM NaCl, and incubated for 1h at RT. After three washes, organoids were maintained at 4ºC in a phosphate-free buffer solution (100 µL) until visualization. The schematic protocol is shown in Figure 2.

Results and discussion

This manuscript presents new tools for the study of purinergic signaling. We have subcloned two members of the NTPDase/CD39 family of ectonucleotidases, NTPDase2 (CD39L1) and NTPDase3 (CD39L3), and expressed them as fusion proteins with EGFP in the endometrial cancer cell line ARK2. Figure 1 illustrates the subcloning procedure, with the human *ENTPD2* and

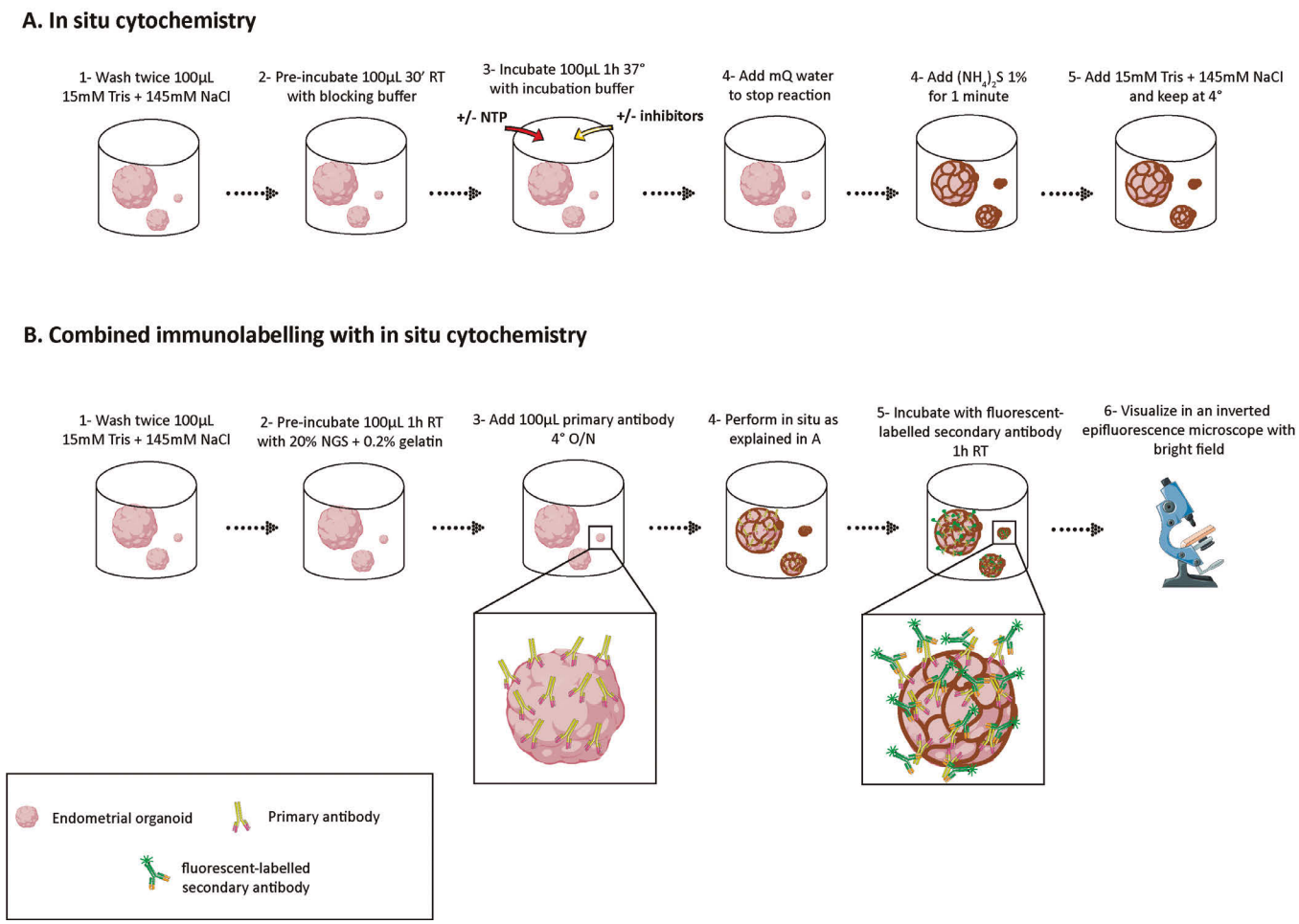

Fig. 2. Illustration detailing the procedure of *in situ* enzyme cytochemistry alone **(A)** and in combination with immunolabeling **(B)** applied to organoids derived from endometrial carcinoma cell cultures for the co-visualization of the protein expression and enzyme activity of NTPDases. The two procedures were carried out in 96-well plates where organoids were formed on a Matrigel bed. Organoids were fixed with cold 4% PFA for 10 minutes. In the combined technique **(B)**, the primary antibody has to be incubated before performing the *in situ* enzyme cytochemistry. Fluorescent secondary antibody was incubated after revealing the ATPase enzyme activity.

ENTPD3 cDNAs inserted in-frame, as assessed by DNA sequencing, at the 3'-terminus of the *EGFP* cDNA. Once transfected into ARK2 cells, protein expression was assessed with Western blot, fluorescence microscopy and direct GFP fluorescence, alone and in combination with specific primary antibodies (Fig. 3). In both cases, NTPDase2 and NTPDase3, the GFP fluorescence was detected at the cell membrane (Fig. 3Aa,g). Transfected proteins were detected by immunoblotting showing specificity for a unique band in the case of NTPDase2 (Fig. 3B), and, two bands for NTPDase3 (Fig. 3C), monomeric and dimeric forms, already described by Munkonda et al. 2009. Moreover, we have demonstrated that the expressed proteins were

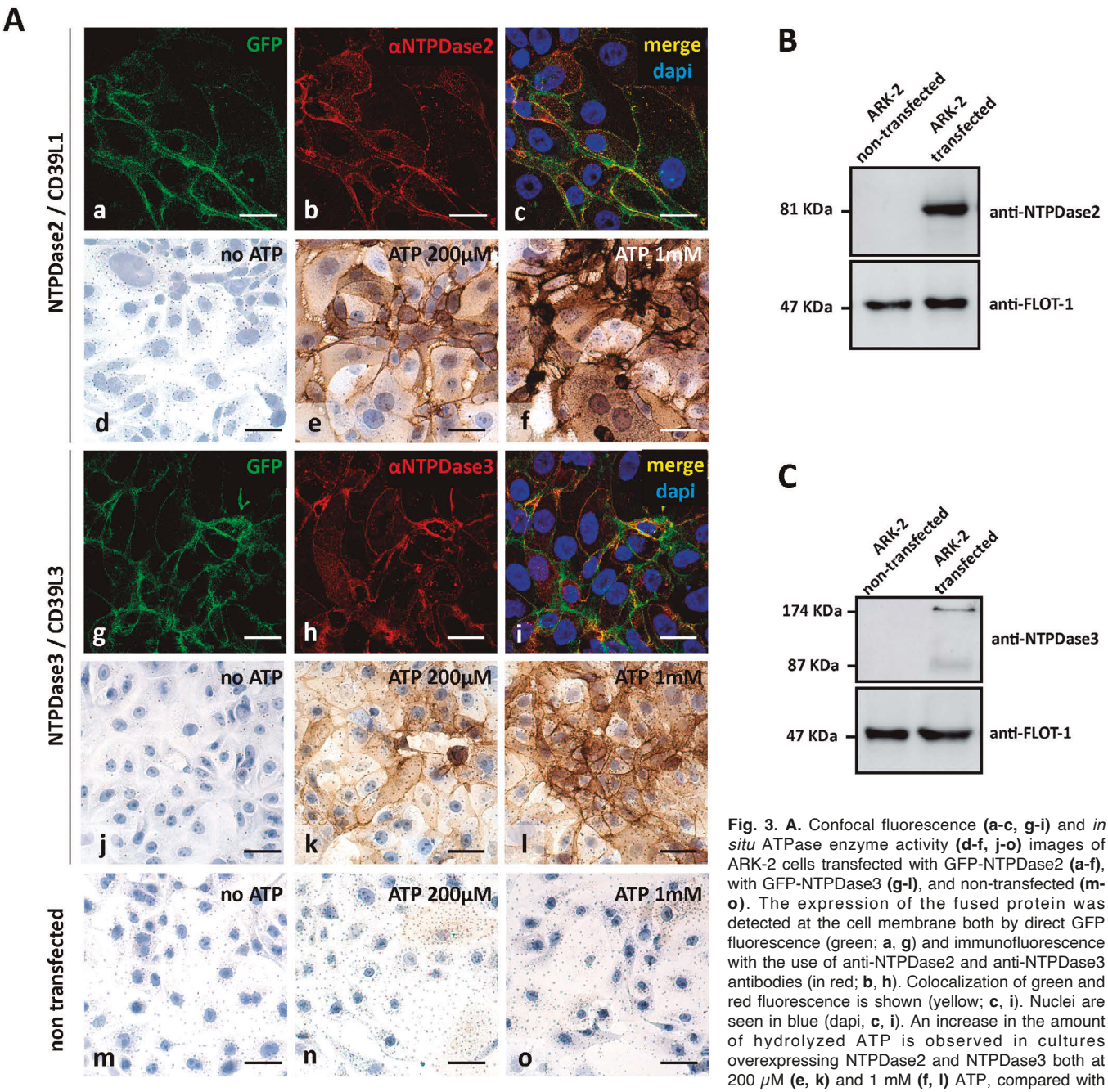

Fig. 3. A. Confocal fluorescence **(a-c, g-i)** and *in situ* ATPase enzyme activity **(d-f, j-o)** images of ARK-2 cells transfected with GFP-NTPDase2 **(a-f)**, with GFP-NTPDase3 **(g-l)**, and non-transfected **(m-o)**. The expression of the fused protein was detected at the cell membrane both by direct GFP fluorescence (green; **a**, **g**) and immunofluorescence with the use of anti-NTPDase2 and anti-NTPDase3 antibodies (in red; **b**, **h**). Colocalization of green and red fluorescence is shown (yellow; **c**, **i**). Nuclei are seen in blue (dapi, **c**, **i**). An increase in the amount of hydrolyzed ATP is observed in cultures overexpressing NTPDase2 and NTPDase3 both at 200 μM **(e, k)** and 1 mM **(f, l)** ATP, compared with non-transfected cultures **(n, o)**. The controls of the experiment were performed in the absence of ATP (*no ATP*, **d**, **j**, **m**). **B and C.** Immunoblotting of membrane-enriched cell lysates showing the presence of the overexpressed proteins: an 81 KDa band in the case of GFP-NTPDase2 and two bands, of 174 and 87 KDa, for those transfected with GFP-NTPDase3. No bands were seen in the lanes of non-transfected cultures. Flotillin (FLOT-1) was used as a loading control of the experiments. Scale bars: d-f, j-o, 50 μm; a-c, g-i, 25 μm.

enzymatically active (Fig. 3Ad-f,j-l). Neither NTPDase2 nor NPTDase3 proteins were immunodetected in non-transfected cells (Fig. 3B,C), which did not display any ATPase activity (Fig. 3Am-o). In conclusion, the two GFP-tagged proteins correctly expressed at the plasma cell membrane, where they can be localized by direct GFP fluorescence, and, importantly, they displayed ATPase activity distinctive of the native enzymes. This GFP-tagged CD39 family of ectoenzymes enables monitoring the activity and visualizing the exact location of these enzymes. The cDNA constructs described here might also be transfected into other cell types to examine the impact of NTPDase expression on other cellular backgrounds, and it has promising applications in *in vivo* models as well.

Ectonucleotidase activities are traditionally assessed in cell and tissue homogenates or with purified enzymes using malachite green approaches, which are highly reliable assays that provide quantitative values of enzyme activity, allowing precise definition of enzyme kinetics and testing of the inhibitors' effectivity (Kirchgesser and Dahlmann, 1990; Repen et al., 2012). *In situ* activity assays, using frozen tissue slides, or fixed cells grown on coverslips, provide valuable complementary information since they identify the enzymes in the context of the cell and tissue environment, also allowing to test inhibitors. Enzyme activity studies on 3D models are challenging.

Organoids have been reported from several organs and tissues, including those from the female reproductive tract such as vulva, vagina, cervix, ovary, and oviduct, and also from both healthy and pathological endometria (Heremans et al., 2021), enabling the testing of molecules and drugs for different purposes such as infertility studies, endometrial cancer research, and the identification of inflammatory pathologies such as

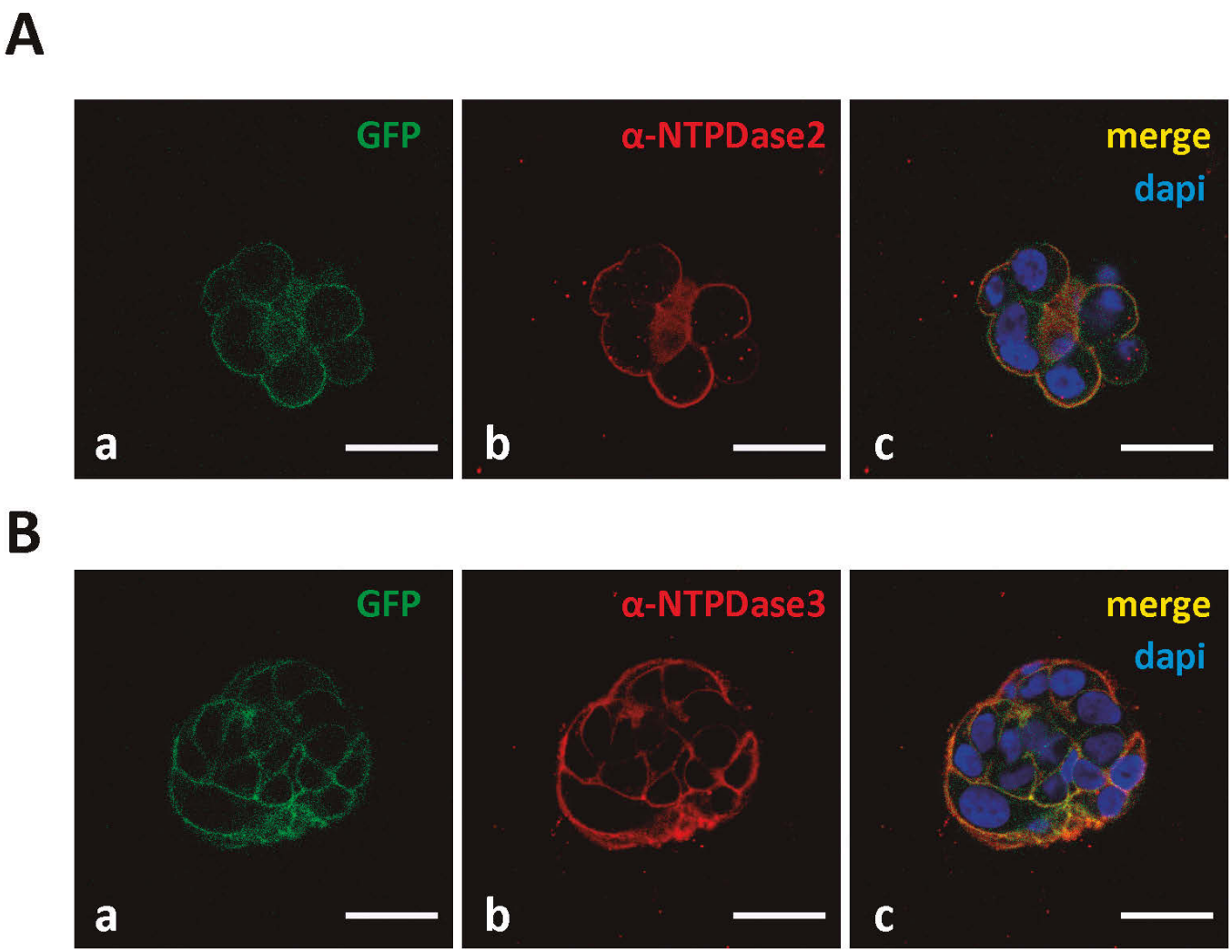

Fig. 4. Confocal fluorescence images of organoids derived from EGFP-NTPDase2- **(A)** and EGFP-NTPDase3- **(B)** transfected ARK-2 cells. The GFP-associated fluorescence of the GFP-NTPDase2- **(Aa)** and GFP-NTPDase3- **(Ba)** expressing organoids lines the cell membrane, coinciding with the expression of NTPDase2 **(Ab)** and NTPDase3 **(Bb)**, respectively, detected by immunofluorescence. Scale bars: 100 μm.

endometriosis (Eritja et al., 2015; Boretto et al., 2019; Stejskalová et al., 2021). Organoids derived from tumoral endometrium maintain the tumor epithelial phenotype and some of the clinical features of the tumors (Boretto et al., 2019; Gu et al., 2020). There have been, however, few studies of ectonucleotidase enzyme activity in organoids. Recently, it was described that cervical cancer cell spheroids decrease CD73 protein expression and enzyme activity compared with a monolayer system, probably affecting cell adhesion, tumor spreading and metastasis (Iser et al., 2021). Here we obtained organoids from serous endometrial carcinoma ARK-2 cells, non-transfected or transfected with the *EGFP-ENTPD2*- or *EGFP-ENTPD2-expressing* constructs. We show that organoids formed correctly and displayed a cell membrane expression of the overexpressed proteins, detectable by direct GFP fluorescence (Fig. 4). We have adapted the *in situ* cytochemistry phosphate-lead method traditionally carried out with 2D samples to these 3D organoids. The complete protocol was carried out in the same 96-well plates where the organoids were generated, taking into account that phosphate-containing buffers should be avoided. We used 15mM Tris with 145mM NaCl, instead of PBS. Brownish phosphate-lead precipitates were already detected at 50 µM ATP for both NTPDase2- and NTPDase3-expressing organoids; the color intensity increased with the nucleotide concentration, reaching saturation at 200 µM (Fig. 5A). Due to the 3D architecture of organoids, which overlaps with the activity of several cell layers, we recommend the use of a lower nucleotide concentration than that for

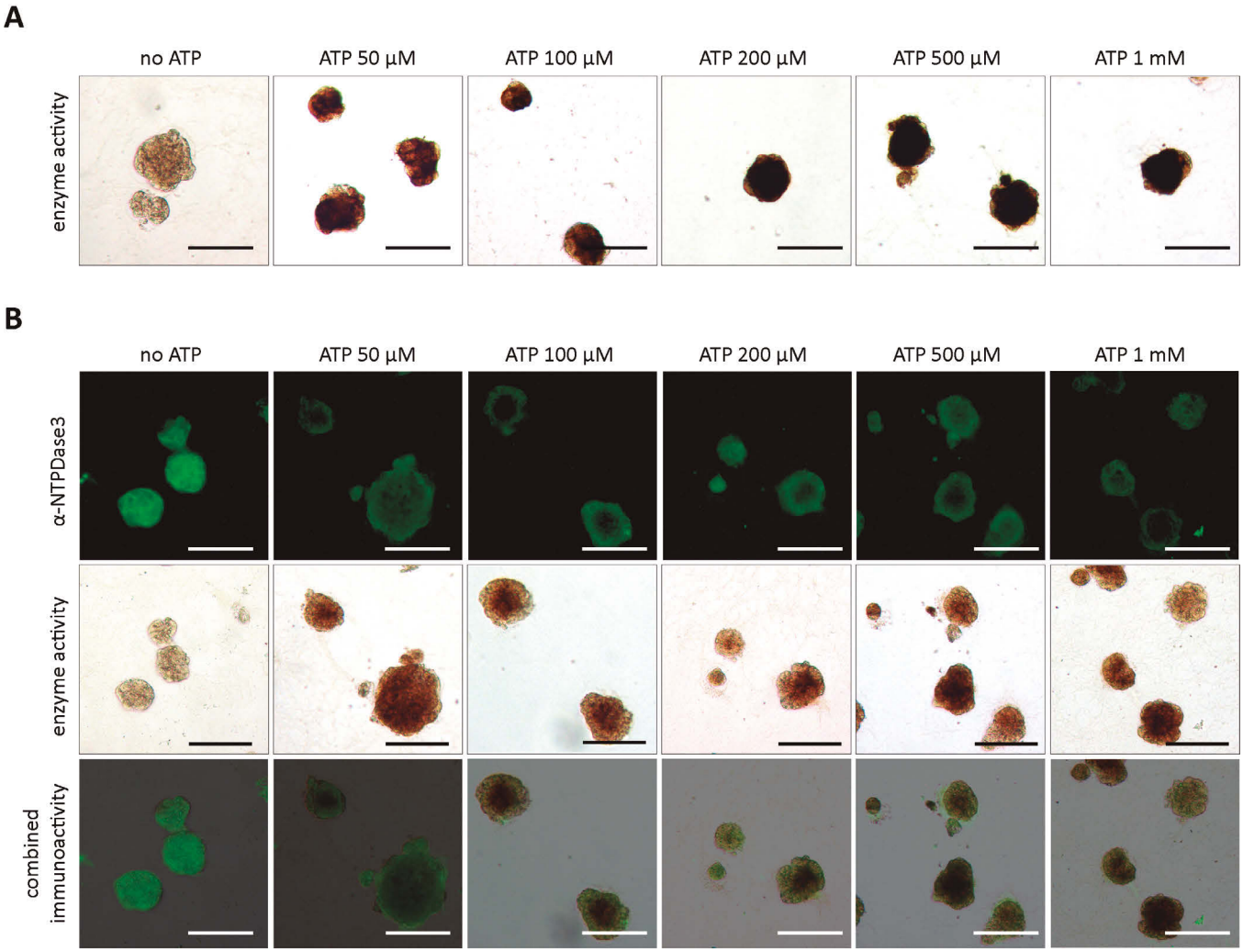

Fig. 5. *In situ* ATPase enzyme cytochemistry alone **(A)** and in combination with immunofluorescence using a specific anti-NTPDase3 antibody **(B)** in organoids derived from EGFP-NTPDase3 overexpressing ARK-2 cells. Brown deposits corresponding to ATP hydrolysis are seen in all the ATP concentrations tested (50, 100, 200, 500 μM, and 1 mM) with the brown color intensity progressively increasing with the ATP concentration. The control of the experiment was performed in the absence of ATP (*no ATP*). The combined technique **(B)** allowed co-visualization of protein expression and enzyme activity in each organoid. Note that in this case, the anti-NTPDase3 antibody used for immunodetection had an enzyme inhibitory effect as seen with the lighter brown deposits compared with **A**. Scale bars: 250 μm.

2D tissue or cell assays. We have also demonstrated that the effect of ectonucleotidase inhibitors can be studied in organoids, where decreased phosphate lead deposits were seen in the presence of inhibitors. This is demonstrated in Figure 6 where organoids were incubated in the presence of two different inhibitors, suramin and NF279.

A step forward with this methodology is the simultaneous detection of the protein, using specific antibodies, and the enzyme activity, a technique that was previously described for tissue slices (Villamonte et al., 2018; Losenkova et al., 2020). We adjusted the technique to organoids (Fig. 2) and demonstrated that enzyme activity and immunodetection are feasible in the same organoid (Fig. 5B). The protocol described here is also compatible with primary cultures, tissues, and patient-derived organoids. For this simultaneous detection of the protein and its activity, we recommend the use of antibodies to immunodetect the protein since, even at low substrate concentrations, brown deposits generated by the activity obstruct the GFP fluorescence associated with the ectoenzyme. This quenching effect is seen in Figure 6Ad,h for organoids and in Figure 7 for cells on coverslips. The method described here also allows the use of inhibitors, as shown for NTPDase3-expressing organoids (Fig. 5), when the antibody $B3_S10$, with an inhibitory effect (Munkonda et al., 2009), was used to detect NTPDase3. In this case, although the protein was well detected, the enzyme activity, identified as brown precipitates, was decreased due to the inhibitory effect of antibody binding to NTPDase3 (Fig. 5B). This is an interesting finding with applications in preclinical cancer models where inhibitory antibodies are used to target the CD39 family of enzymes. In this regard, we believe that the combined *in situ* technique is truly informative in assessing whether an antibody has, and to what extent, any modulatory activity, usually inhibitory, upon the targeted enzyme to which it binds. It can also be useful to characterize antibodies by determining their specificity in cell or tissue contexts where various ectonucleotidases are closely expressed.

In summary, we have updated current methodologies for the study of ectonucleotidases and have proven a suitable molecular approach for directly fluorescent

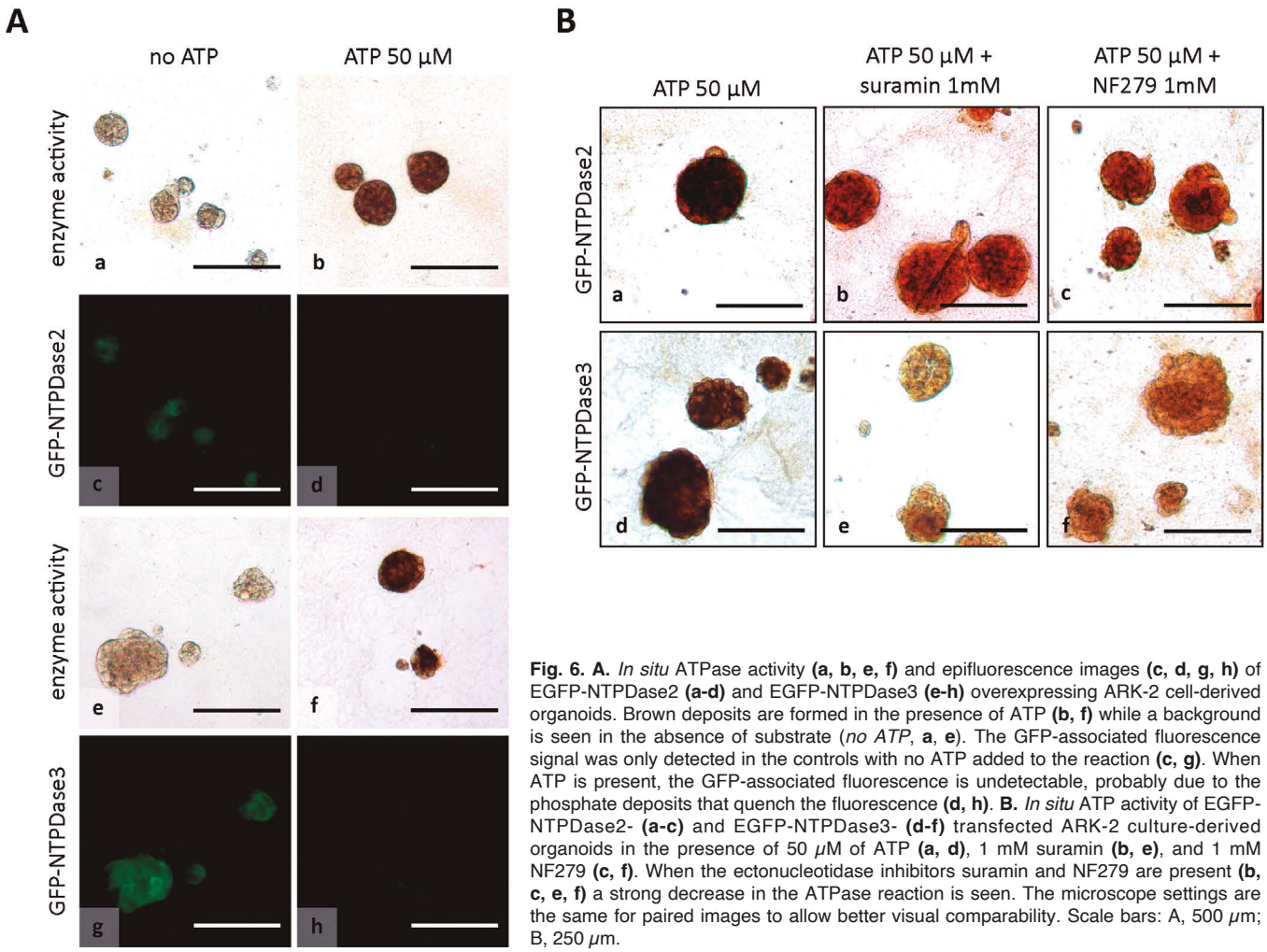

Fig. 6. A. *In situ* ATPase activity **(a, b, e, f)** and epifluorescence images **(c, d, g, h)** of EGFP-NTPDase2 **(a-d)** and EGFP-NTPDase3 **(e-h)** overexpressing ARK-2 cell-derived organoids. Brown deposits are formed in the presence of ATP **(b, f)** while a background is seen in the absence of substrate (*no ATP*, **a**, **e**). The GFP-associated fluorescence signal was only detected in the controls with no ATP added to the reaction **(c, g)**. When ATP is present, the GFP-associated fluorescence is undetectable, probably due to the phosphate deposits that quench the fluorescence **(d, h)**. **B.** *In situ* ATP activity of EGFP-NTPDase2- **(a-c)** and EGFP-NTPDase3- **(d-f)** transfected ARK-2 culture-derived organoids in the presence of 50 μM of ATP **(a, d)**, 1 mM suramin **(b, e)**, and 1 mM NF279 **(c, f)**. When the ectonucleotidase inhibitors suramin and NF279 are present **(b, c, e, f)** a strong decrease in the ATPase reaction is seen. The microscope settings are the same for paired images to allow better visual comparability. Scale bars: A, 500 μm; B, 250 μm.

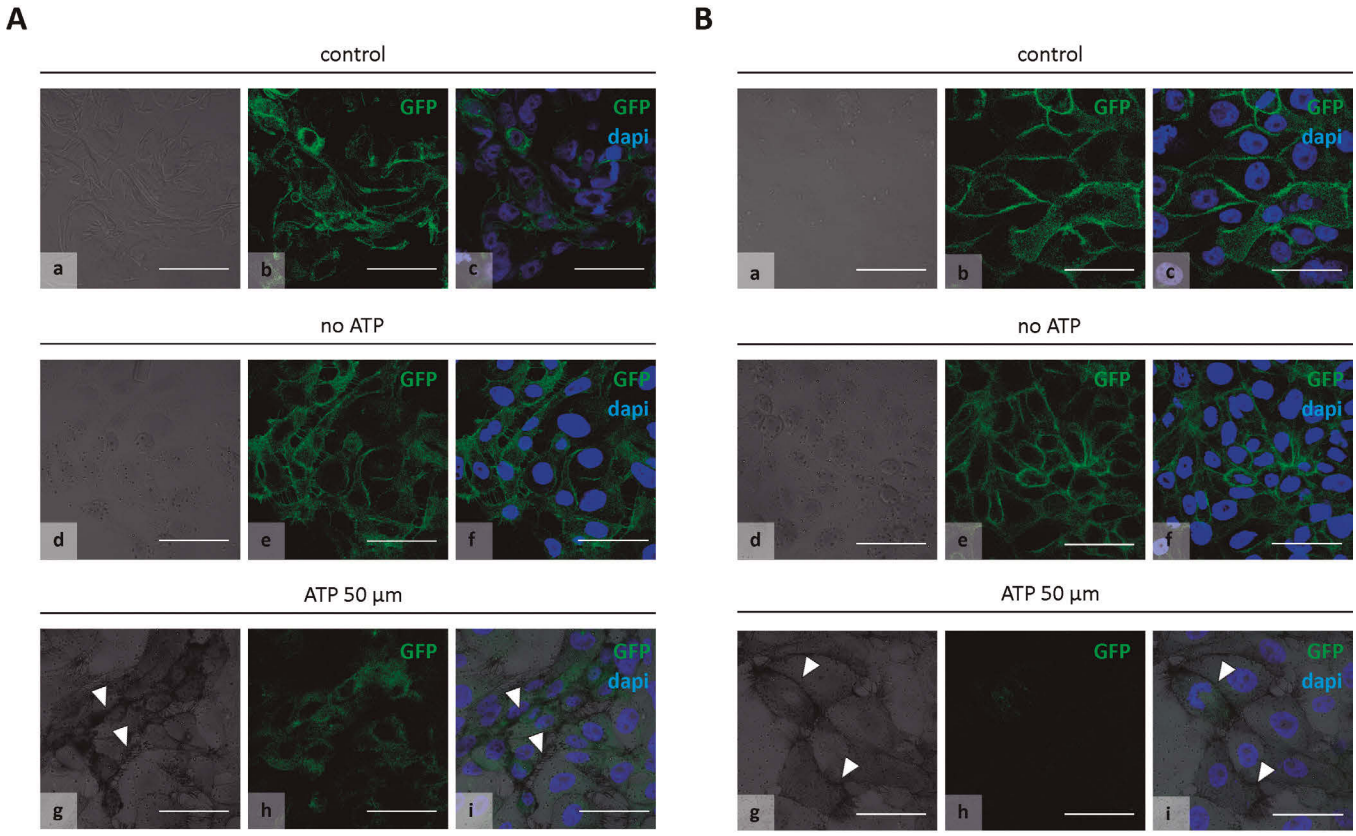

Fig. 7. Confocal microscopy images of *in situ* ATP enzyme cytochemistry performed in EGFP-NTPDase2- **(A)** and GFP-NTPDase3- **(B)** transfected ARK-2 cells. The GFP signal is clearly detected in the control condition **(b, c)**, in which no cytochemistry procedure was applied, and in the absence of ATP **(e, f)**, in which all the reagents of the cytochemistry assay were present except ATP. The GFP-associated NTPDase2 and 3 signals are clearly decreased in the presence of ATP **(h, i)**, where lead phosphate deposits (arrowheads, **g**) mask the fluorescence. Images a, d, and g were taken using bright field parameters. Ai and Bi are merged images of bright field and fluorescent EGFP-NTPDase2 **(A)** and EGFP-NTPDase3 **(B)** signals. Scale bars: 50 μm.

tracking ectonucleotidases of the NTPDase family. This methodology may be useful in the screening of NTPDase inhibitors, including antibodies, for the development of targeted therapies (Imai et al. 2000; Yegutkin and Boison 2022). Relevant recent research in the field has focused on the simultaneous detection of ectonucleotidases and alkaline phosphatase activities (Losenkova et al. 2020), on the precise quantification of CD73 enzyme histochemistry staining in tissue using image processing (Dragić et al. 2019), and on nucleotide-imaging techniques (Conley et al. 2017). In addition, functional studies in organoids are underway, such as the silencing of CD73 in breast cancer cell-derived organoids for epithelial-mesenchymal transition (EMT) status and metastatic potential determination (Petruk et al. 2021). Experiments aimed at improving the tridimensional system (Lou et al. 2022), by co-culturing tumor stromal cells to study the consequences of cell's crosstalk, would benefit from this approach, not only in the case of endometrial cancer but also in other tumor types in which purinergic signaling plays a role. Moreover, this technology can be implemented in nontumoral endometrial pathologies such as endometriosis in which extracellular ATP levels are altered.

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