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In situ monitoring of PTHLH secretion in neuroblastoma cells cultured onto nanoporous membranes

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ABSTRACT

In this work, we propose for the first time the use of anodic aluminum oxide (AAO) nanoporous membranes for *in situ* monitoring of parathyroid hormone-like hormone (PTHLH) secretion in cultured human cells. The biosensing system is based on the nanochannels blockage upon immunocomplex formation, which is electrically monitored through the voltammetric oxidation of Prussian blue nanoparticles (PBNPs). Models evaluated include a neuroblastoma cell line (SK-N-AS) and immortalized keratinocytes (HaCaT) as a control of high PTHLH production. The effect of total number of seeded cells and incubation time on the secreted PTHLH levels is assessed, finding that secreted PTHLH levels range from approximately 60 to 400 ng/mL. Moreover, our methodology is also applied to analyse PTHLH production following PTHLH gene knockdown upon transient cell transfection with a specific silencing RNA (siRNA). Given that inhibition of PTHLH secretion reduces cell proliferation, survival and invasiveness in a number of tumours, our system provides a powerful tool for the preclinical evaluation of therapies that regulate PTHLH production. This nanoporous membrane – based sensing technology might be useful to monitor the active secretion of other proteins as well, thus contributing to characterize their regulation and function.

KEYWORDS: Nanopores/nanochannels, PTHLH, neuroblastoma, cancer cells, electrochemical detection.

1. INTRODUCTION

Neuroblastic tumors encompass a heterogeneous group of developmental malignancies derived from peripheral nervous system precursor cells. Disseminated cases in infants and local-regional, well-differentiated tumors are usually benign. However, metastatic neuroblastomas in patients older than 18 months and some local-regional cases display a notable capacity to recur and/or become refractory and, unfortunately, there is no therapy known to be curative for these patients (Maris, 2010). Moreover, long term survivors suffer from secondary effects inflicted by current multimodal therapies (chemotherapy, surgery, radiotherapy, autologous bone marrow transplant, differentiating agents and immunotherapy) on their normal, developing organs. In this context, there is unmet clinical need for novel drugs that specifically target neuroblastoma cells.

Data gathered over the years by our group are consistent with the hypothesis that the calcium-sensing receptor (CaSR) exerts tumor-suppressor functions in neuroblastoma (De Torres et al., 2009; Casalà et al., 2013; Masvidal et al., 2013; Rodríguez-Hernández et al., 2016; Mateo-Lozano et al., 2016; Masvidal et al., 2017). In a number of cancers, CaSR activation promotes increased secretion of parathyroid hormone-like hormone (PTHLH) (Moseley et al., 1987) a protein that exerts relevant roles in the initiation, growth and metastatic processes of several tumors (Suva et al., 1987). PTHLH is a secreted factor present in almost all organs and tissues of the body. It was first identified in patients with hypercalcemia, being later known its involvement in the initiation, survival and progression of primary tumors (Li et al., 2011; Park et al., 2013) as well as in the generation and development of metastases (Iguchi et al., 1996; Urosevic et al., 2014; Wang et al., 2014; Bilezikian, 1992).

We first described that PTHLH is also expressed in neuroblastic tumors and upregulated in benign, differentiated cases, thus potentially indicating novel functions of this cytokine (De Torres et al., 2009). However, the factors that regulate the production and secretion of this protein have not been fully characterized, thus making the monitoring of such production of

great relevance for i) a better understanding of PTHLH regulation and function and ii) the evaluation of novel cancer therapies based on drugs that regulate PTHLH production.

Radioimmunoassays (RIAs) and immunoradiometric assays (IRMAs) (Fraser et al., 1993; Pandian et al., 1992) are the only methods available so far for the detection of PTHLH. Implementation of these technologies in laboratories focusing on cell biology is hampered by the hazardous and time consuming experimental procedures required, which involve the use of sophisticated and expensive equipment and facilities. With the aim of overcoming such limitations, we previously reported biosensing systems based on both lateral-flow assays (Chamorro-García et al., 2016) and nanochannel technology (Espinoza-Castañeda et al., 2015) for *in vitro* quantitation of PTHLH. Furthermore, anodic aluminum oxide (AAO) nanoporous membranes have been proposed as outstanding materials for the detection of protein biomarkers in complex matrixes, thanks to their ability to act as both filtering and sensing platforms. Such sensing is based on nanochannels blockage upon protein recognition, which is monitored through the inhibition of an electrical signal (De la Escosura-Muñiz and Merkoçi, 2010a; De la Escosura-Muñiz and Merkoçi, 2010b; De la Escosura-Muñiz and Merkoçi, 2011; De la Escosura-Muñiz and Merkoçi, 2012; De la Escosura-Muñiz et al., 2013). In this context, we propose for the first time the use of AAO nanoporous membranes for *in situ* monitoring of parathyroid hormone-like hormone (PTHLH) secretion in cultured human cells, as schematized at **Figure 1**. Also, this secretion is monitored following PTHLH gene knockdown upon transient cell transfection with a specific silencing RNA (siRNA).

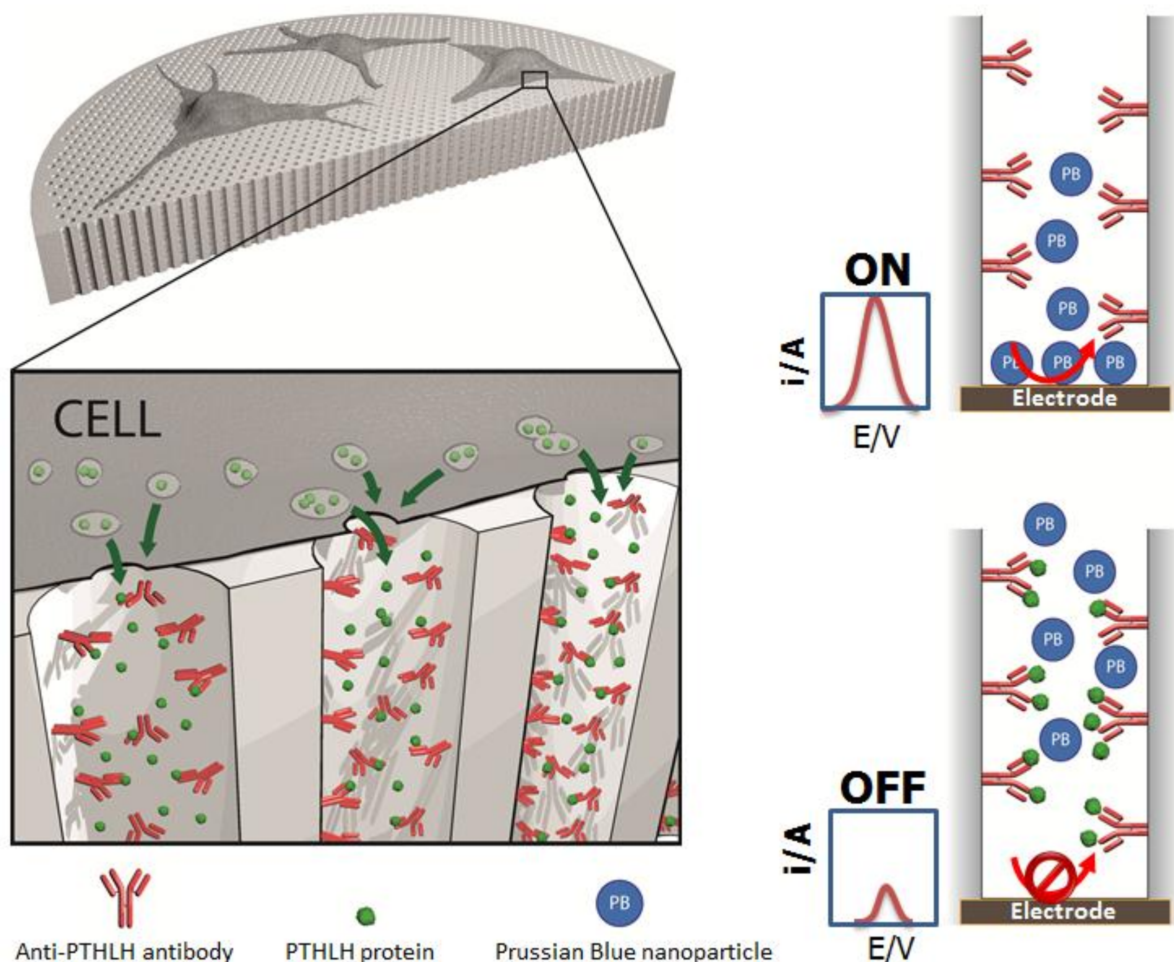

Figure 1. Scheme (not in scale) of the biosensing system. HaCaT and SK-N-AS cells are cultured onto AAO nanoporous membranes previously modified with anti-PTHLH antibodies. Secreted PTHLH is selectively captured and detected through the decrease in the electrochemical signal of the Prussian blue nanoparticles used as red-ox indicators (oxidation of Prussian blue to Berlin green at approx. +500 mV, analytical signal), due to the nanochannels blockage by the immunocomplex formation.

2. EXPERIMENTAL SECTION

2.1. Cells, proteins and antibodies

Neuroblastoma cell line SK-N-AS was purchased from the European Collection of Cell Cultures and immortalized keratinocytes HaCaT were obtained from the repository at Institut de Recerca Sant Joan de Déu, (Barcelona, Spain). Cells were grown in Roswell Park Memorial Institute (RPMI)-1640, supplemented with 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, penicillin (100 U/ml) and streptomycin (100 μg/ml), at 37ºC and 5% CO_2. Mycoplasma

polymerase chain reaction (PCR) tests were routinely performed. Characterization of SK-N-AS cells included analysis of *MYCN* and *TP53* status, and authentication by STR profiles (Mateo-Lozano et al., 2016).

Parathyroid hormone-like hormone (PTHLH) (SRP4651, human recombinant, expressed in E.coli) was purchased from Sigma-Aldrich (Spain). Anti-PTHLH (PC09, 34-53, polyclonal antibody developed in rabbit) was purchased from Calbiochem (Spain). Aliquots of this antibody were prepared in 0.01 M PBS buffer pH 7.4, containing 5 mM EDC/sulfo-NHS.

ON-TARGETplus^{TM} Human PTHLH Smart Pool, ON-TARGETplus^{TM} Non-Targeting Pool and DharmaFECT^{TM} were purchased from Dharmacon (GELifeSciences). Both siRNAs consist on a pool of four siRNA designed to either specifically target PTHLH mRNA (siRNA-PTHLH) or for a minimal targeting of human genes (siRNA-NT).

2.2. Chemicals and equipment

$FeCl_2 \cdot 4H_2O$ and $K_3[Fe(CN)]_6$ were purchased from Panreac Química SA (Spain). Polyvinylpyrrolidone (PVP) (average MW 40 KDa), 3-aminopropyltrimethoxysilane (APS), [N -(3-dimethylamino) propyl)]-N-ethylcarbodimide (EDC), sulfo N-hydroxysuccinimide (sulfo-NHS), phosphate buffer solutions tablets (PBS) were purchased from Sigma-Aldrich (Spain). RPMI-1640 w/o L-glutamine (CE), L-glutamine 200mM (100X), penicillin streptomycin solution (100X) and trypsin 0.25%–EDTA solution were purchased from Fisher Scientific (Spain). Fetal Bovine Serum EU approved (South Amer.) was purchased from Hyclone (Cultek). All chemicals were of analytical grade and used as received. All aqueous solutions were prepared in Milli-Q water. Anodized aluminum oxide filter membranes (Whatman anodisc AAO, 13 mm diameter, 60 μm thickness, containing 20 nm pores) were purchased from Scharlab (Spain).

Prussian blue nanoparticles (PBNPs) 4 nm-sized were synthetized following the experimental procedure described at the S*upplementary Material*.

Thermostatic bath ISOTEMP was purchased from Fisher Scientific (Spain). Incubator NU-5100E/GCO2 was purchased from Nuaire (USA). A BIOII A/P biosecurity cabinet (from Telstar, Spain) was used for cells manipulation. TC Flask 75 cm², Multiple Well Cluster Plate, TC-Treated, Sterile, Stripettes individual 25, 10, 5, 2 mL were purchased from Cultek (Spain). The homemade screen-printed carbon (SPCEs) used as transducers were prepared following the experimental procedure detailed at the S*upplementary Material*. The experimental set-up for the in situ cell culture/electrochemical detection (AAO membranes assembled to SPCEs) is detailed at the S*upplementary Material*. Electrochemical measurements were performed with an Autolab 20 (Eco-chemie, The Netherlands) connected to a PC. All the measurements were carried out at room temperature with a working volume of 200 µL, which was enough to cover the three electrodes contained in the SPCEs connected to the potentiostat by a homemade edge connector module. Olympus IX71 (Spain) inverse microscope and Scanning Electrochemical Microscopy FEI Quanta 650 FEG ESEM (The Netherlands) were used for the optical characterizations.

2.3. Methods

AAO nanoporous membranes functionalization and in situ cell culturing

AAO membranes (20 nm-sized pores) functionalization with specific antibodies was performed following a previously optimized methodology (De la Escosura-Muñiz and Merkoçi, 2010a). Briefly, membranes were first boiled in milli-Q water for 1 h. After drying in argon they were immersed in a 5% acetone solution of APS for 1 h. Then they were washed in acetone and baked at 120° C for 30 min. After that a 1 mg/mL solution of anti-PTHLH antibodies in PBS buffer containing 5 mM EDC/sulfo-NHS (30 µL) was added and left there for 2h. The same experimental procedure was followed for the immobilization of the non-specific antibodies (anti-human IgG) used as control. As alumina is well known to exhibit reduced protein adsorption, no blocking reagents are necessary to avoid unspecific

absorptions in complex matrixes such as cell culture medium (Espinoza-Castañeda et al., 2015) or even whole blood (de la Escosura-Muñiz and Merkoçi, 2011). A thorough washing of the membranes before the electrochemical measurement allows to remove unspecific proteins present in the nanochannels.

The design of the experimental set-up for the *in situ* cell culture/electrochemical detection (AAO membranes assembled to SPCEs) is detailed at the S*upplementary Material*. AAO nanoporous membranes were fixed onto the SPCEs by physical attachment, which consisted of placing the SPCEs onto a polyethylene terephthalate glycol-modified (PTEG) block (circular shape) and putting the membranes over the three-electrode surface. Then, a second circular PTEG block containing holes of the same size as the working area of the SPCEs was placed onto the AAO nanoporous membranes, with a red silicone (RS) insulating ring between them to avoid liquid leakage. Finally, the carousel-like system was fixed with a screw. Different materials were evaluated for building such set-up so as to find the optimum one which allows the cells to normally grow, without exerting any toxic effect (see materials optimization at the S*upplementary Material*).

Finally, 1.5 mL of the desired amount of cells were placed on the AAO nanoporous membranes there and incubated at 37 °C in humidified atmosphere with 5% CO_2 during a determined time.

Electrochemical detection of PTHLH

After washing with PBS buffer, the electrochemical cell containing the AAO nanoporous membrane was filled with 200 µL of a 10 µg/mL PBNPs suspension. A pre-treatment at -550 mV was applied during 30s and immediately after, a differential pulse voltammetric (DPV) scan from -550 mV to +700 mV (step potential 10 mV, modulation amplitude 50 mV) was applied resulting in an analytical signal due to oxidation of Prussian blue (PB) to Berlin green

(BG) at approximately +500 mV, whose peak current is chosen as analytical signal. The measurements were carried out at room temperature under non- stirring conditions.

Preliminary studies for i) the evaluation of the ability of the developed sensing system for the determination of PTHLH in a complex matrix such as the cell culture medium and ii) building the calibration plot for PTHLH determination were also performed. The medium was extracted from the cell culture and placed in Eppendorf tubes where different quantities of standard solutions of PTHLH were spiked. After that, 50 μL of this solution were placed onto the membranes (with the anti-PTHLH antibody previously immobilized) assembled with the SPCEs (set-up described at the S*upplementary Material*) and incubated during 2 h at room temperature.

Cell transfection procedure

Cell transfections were performed in two steps. First, 24000 HaCaT cells were cultured for 20h onto the AAO nanoporous membranes assembled with the SPCE electrode (set-up described at the S*upplementary Material*). Then cells were exposed to a mixture of 0.25 μL of 20 μM siRNA solution and different amounts of DharmaFECT^{TM} solution (0.6 μL and 0.3 μL for the "high" and "low" amounts, respectively, according to the manufacturer's indications) for 28h before collecting media for PTHLH analyses. "Blank" assays were performed following the same experimental procedure using cell culture medium instead of siRNA. "Control" assays were carried out using non-targeting siRNA (siRNA-NT) instead of siRNA.

3. RESULTS AND DISCUSSION

3.1. Cell growth onto AAO nanoporous membranes

Initially, the ability of the cells to adhere and grow onto AAO nanoporous membranes was evaluated. As shown at **Figure 2**, both neuroblastoma cells (SK-N-AS) and immortalized human keratinocytes (HaCaT cells) adhered and grew well onto these membranes (a and c respectively) showing a similar morphology to those grown onto standard culture flasks (b and d respectively).

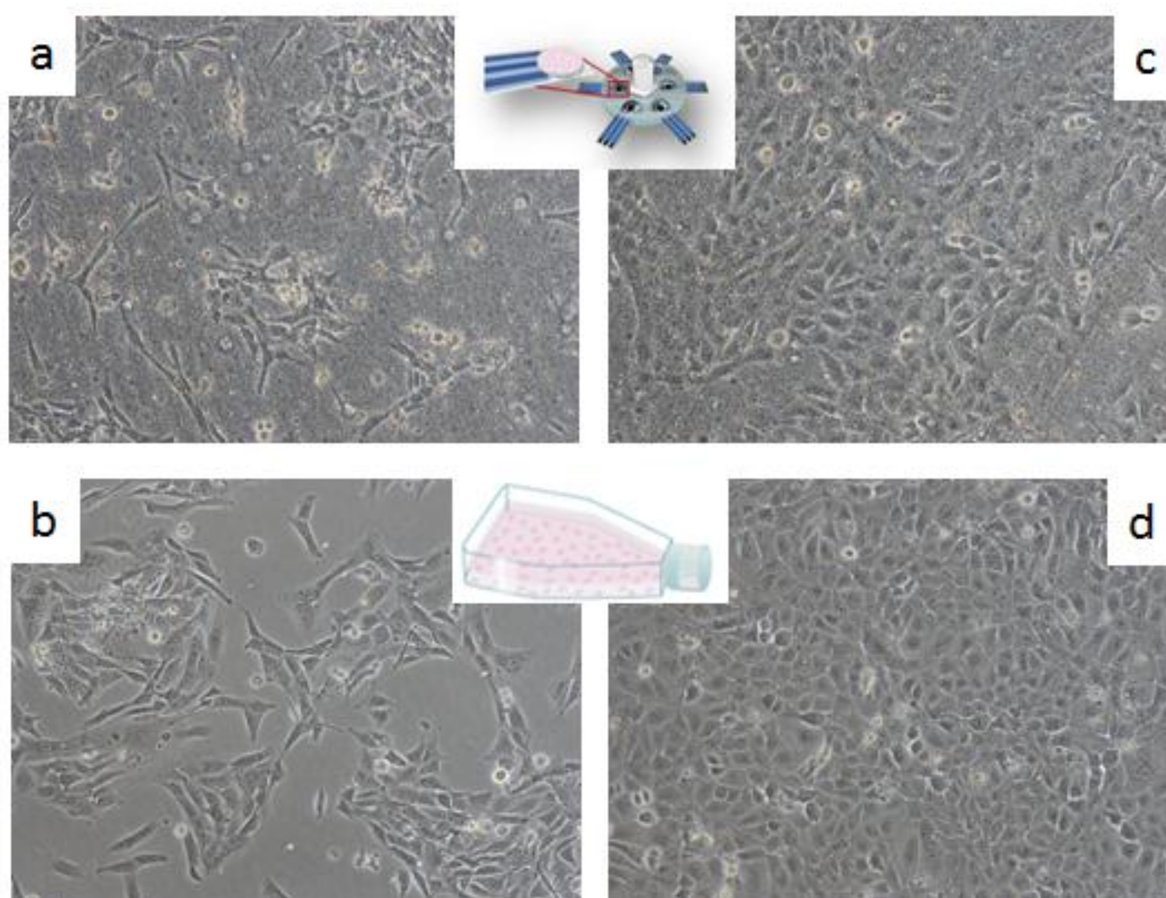

Figure 2. Optical microscope images (top view) of SK-N-AS (a,b) and HaCaT (c,d) cells seeded onto AAO nanoporous membranes (20 nm pore sized) (up) or in regular cell culture flasks (down) and allowed to grow for 48h.

Scanning electron microscopy (SEM) analysis was also used for a more accurate evaluation of the cultured cells on the membranes. As shown in **Figure 3**, cells adhered well onto the membranes, keeping the nanochannels free. This allows for the secreted PTHLH to enter the channels, being captured by the immobilized specific antibodies, and electrochemically detected.

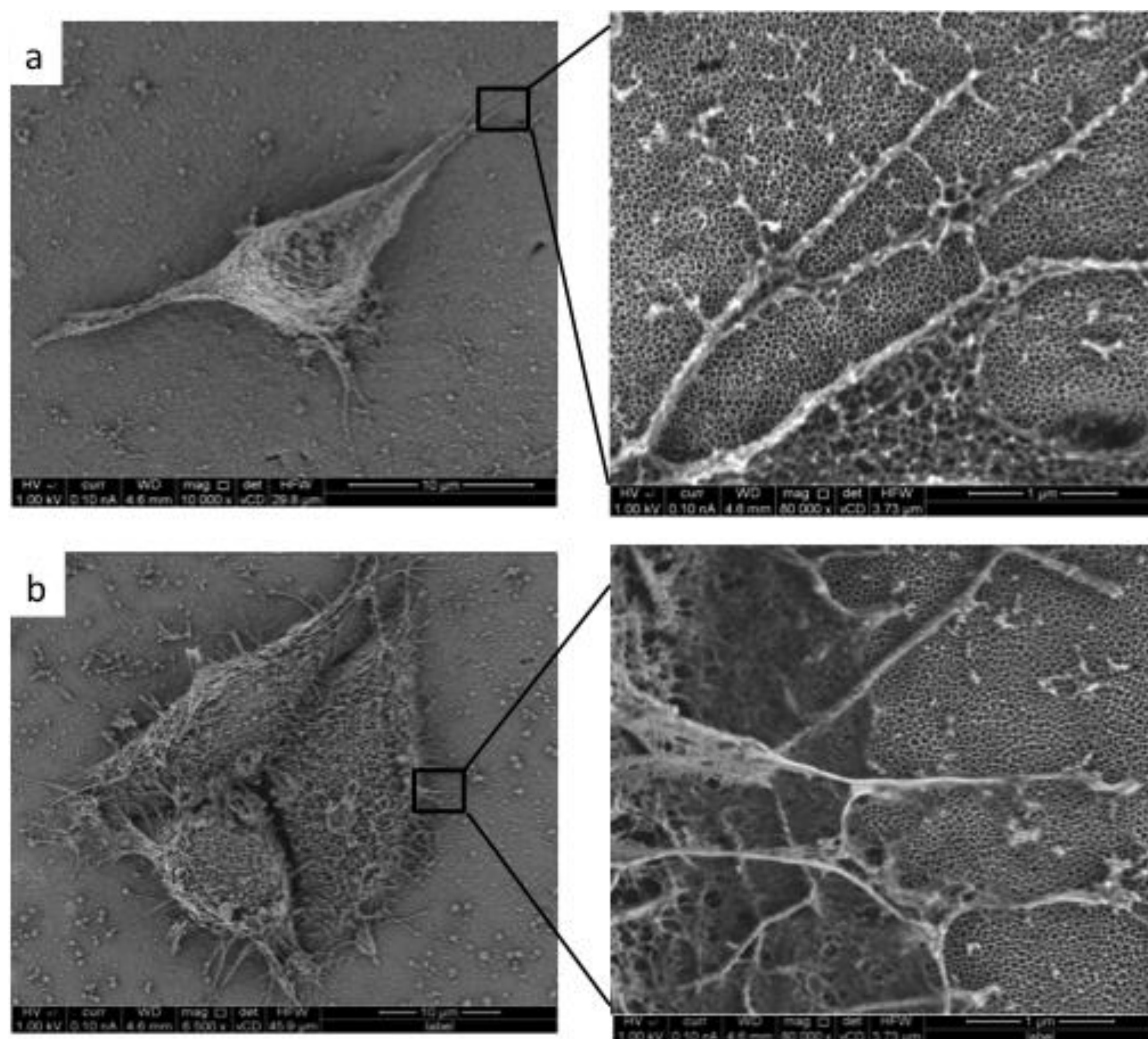

Figure 3. Scanning electrochemical microscope (SEM) images (top view) of SK-N-AS (a) and HaCaT (b) cells cultured on AAO nanoporous membranes (20 nm pore sized) for 48h.

3.2. Electrochemical detection of PTHLH in cell culture medium

The ability of our system to specifically detect and quantify PTHLH in the complex matrix of the cell culture medium was first evaluated. Standard solutions of PTHLH were spiked in such a medium as detailed in the *Experimental Section*. The sensing principle, previously optimized by our group (Espinoza-Castañeda et al. 2015), is schematized at **Figure 1 (right)** and in the cartoon shown at **Figure 4a**. The immunocomplex formation inside the pores produces a partial blockage in the diffusion of the Prussian blue nanoparticles (PBNPs) (red-ox indicator) through the nanoporous membranes to the electrochemical transducer surface, which results in a decrease in the voltammetric signal of oxidation of Prussian blue to Berlin green at approx. +500 mV which is chosen as analytical signal. As shown in **Figure 4a**, the analytical signal decreases by increasing spiked PTHLH concentration in the range 0 - 500 ng/mL. Both parameters are adjusted to a logarithmic relationship within that range, with a correlation coefficient of 0.993, according to the following equation:

$$ip\ (nA) = -0.569\ [\text{spiked PTHLH}]\ (ng/mL) + 423 \quad (\textbf{Equation 1})$$

The method shows a reproducibility (RSD) of 7 % (n= 3) for a PTHLH concentration of 100 ng/mL. A limit of detection (LOD, calculated as the analyte concentration giving a signal equal to the blank signal + three times its standard deviation) of 55 ng/mL of PTHLH is estimated. These results are similar to those previously obtained in PBS buffer medium (Espinoza-Castañeda et al., 2015), suggesting that the matrix of the cell culture medium is not affecting the biosensing response, probably thanks to the filtering effect of the membranes which allows removing of interferences that could affect the analytical signal. These results are also in agreement with the obtained by lateral-flow immunoassays and Western blot analysis (Chamorro-García et al., 2016). Although immunoradiometric assays (IRMA) showed better sensitivity for PTHLH detection (LOD at pg/mL levels) (Fraser et al., 1993; Pandian et al., 1992), our method is able to monitor the levels secreted by cells at the basal state (typically ranging from ng/mL to μg/mL) avoiding the problems related to the half-life

of the isotope, the potential hazard of radioactivity and the strict requirements needed to implement these procedures.

The linear relationship (calibration plot) obtained was later used for the estimation of PTHLH secreted by cells directly cultured onto AAO nanoporous membranes.

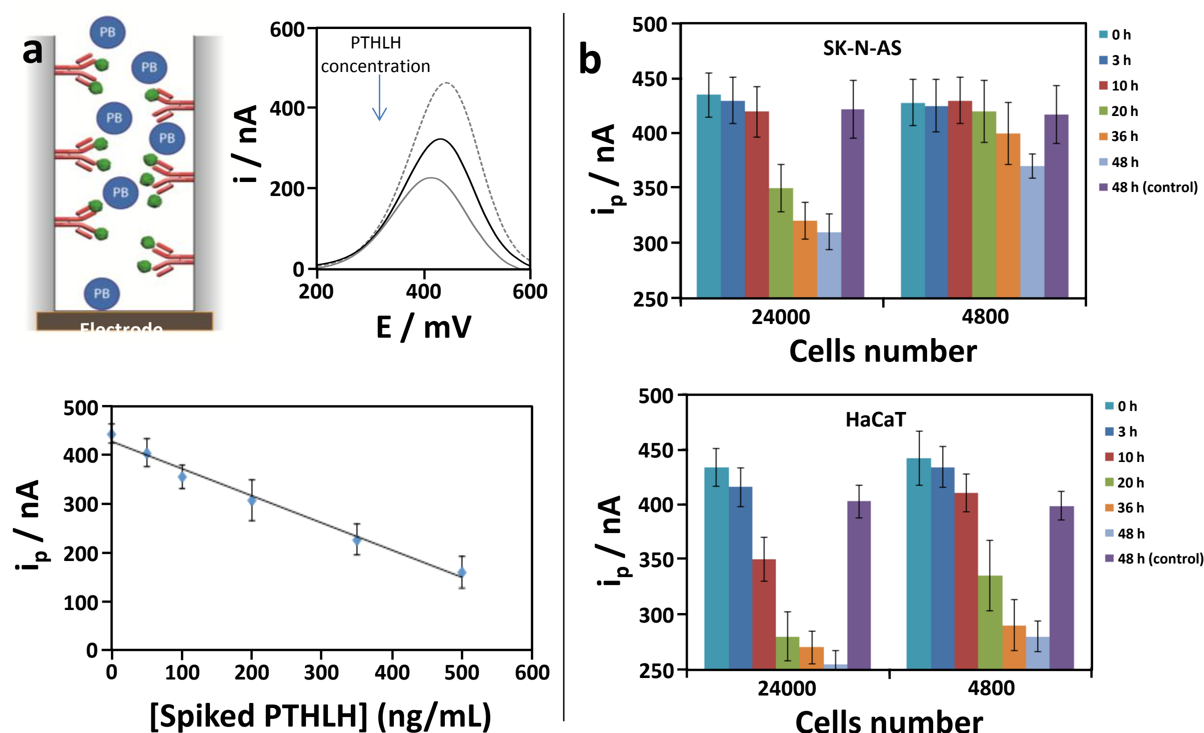

Figure 4. *In situ* quantitation of PTHLH in cell culture media obtained from human cells grown onto AAO nanoporous membranes. (a) Scheme of the sensing principle together with differential pulse voltammograms (DPVs) obtained following the experimental procedure detailed in the *Experimental Section* for increasing concentrations of PTHLH (0, 200 and 500 ng/mL, from up to down). PBNPs concentration: 10 µg/mL, Step potential: 10 mV, modulation amplitude 50 mV. The graph shows the analytical signals obtained for increasing spiked PTHLH concentrations (0 - 500 ng/mL) in cell culture medium. (b) Effect of total number of seeded cells and incubation time on the analytical signal for SK-N-AS (up) and HaCaT cells (down). "Control" assays were carried out by using membranes modified with antibodies non-specific to PTHLH.

3.3. *In situ* PTHLH secretion evaluation in cell cultures on AAO nanoporous membranes

As stated before, two human cell lines, a neuroblastoma cell line (SK-N-AS) and immortalized keratinocytes (HaCaT) as a control of high PTHLH production, were cultured onto AAO nanoporous membranes using the set-up described at the S*upplementary Material*. Secreted PTHLH was electrically monitored through the nanochannel blockage upon the immunocomplex formation inside the channels (**Figure 1, right**), following the procedure detailed in the *Experimental Section*.

The effect of the total number of cells seeded and incubation time on the secreted PTHLH levels were evaluated. Two different cell numbers (24000 and 4800) and incubation times ranging from 3h to 48h were evaluated. "0h" time corresponds to measurements performed right after plating the cells, without allowing them to adhere and grow. "Control" assays were carried out by using membranes modified with unspecific anti-human IgG. As shown in **Figure 4b**, secreted PTHLH is detected in both cell lines with higher values being observed in the presence of higher number of seeded cells and upon longer incubation times. The results obtained for the "Control" assays demonstrate the specificity of the system, ruling out unspecific signals.

Values of secreted PTHLH (extrapolated from **Equation 1**) are shown at **Table 1**. Levels range from approximately 60 to 400 ng/mL depending on cell type, number of seeded cells and incubation time.

Table 1. Estimated values of secreted PTHLH (extrapolated from Equation 1) for different cells amount and incubation time.

Cell line	Cells number	Incubation time (h)	Estimated [PTHLH] (ng/mL)
HaCaT	4800	3	<55
		10	94±30
		20	224±55
		36	276±27
		48	295±32
	24000	3	<55
		10	199±35
		20	295±38
		36	365±28
		48	398±34
SK-N-AS	4800	3	<55
		10	<55
		20	<55
		36	82±19
		48	95±27
	24000	3	<55
		10	58±33
		20	220±41
		36	260±37
		48	271±33

3.4. Cell transfection studies: inhibition of PTHLH secretion by siRNA

In order to further evaluate the specificity of our method, PTHLH secretion was quantified following transient silencing of *PTHLH* gene expression. This was conducted by means of small interfering RNA (siRNA). Transfection of eukaryotic cells is the process of inserting plasmid DNA or RNA into them. As pure genetic material would not naturally be transported into cells, an efficient transfection method with a transfection reagent is used as an easy, cost-effective, and efficient method for introducing foreign genetic material into cells. In our experiments, HaCaT cells (chosen for the demonstration of the proof-of-concept) were transfected with a small interfering RNA (siRNA) that specifically downregulates *PTHLH* messenger RNA (**Figure 5a**). Two different amounts of transfection reagent were evaluated, while no siRNA and non-targeting siRNA (siRNA-NT) were also assayed as blank and control respectively. As shown at the *Supplementary Material* (Supplementary Fig SM6),

upon transient transfection with a specific siRNA, a significant reduction of *PHTLH* mRNA was achieved in both SK-N-AS and HaCaT cells when compared to a siRNA-NT. Then, the inhibition of PTHLH production was *in situ* electrically monitored in cells showing normal or decreased levels of *PTHLH* mRNA expression. Voltammetric signals obtained under the different conditions assayed are shown in **Figure 5b.** The graph shows a summary of results in terms of % of signal inhibition, taking as reference the signal obtained for 24000 HaCaT cells cultured for 48h. A great inhibition of the analytical signal (close to 80%) was observed in cells with decreased expression of PTHLH mRNA following transfection with a high concentration of the transfection reagent. However, under these experimental conditions, a quite relevant signal inhibition was also observed for the blank (no siRNA) and for the control (siRNA-NT) (blue columns), thus suggesting that high doses of transfection reagent are likely toxic and reduce cell viability. Conversely, in the presence of lower doses of transfection reagent (red columns), an important signal inhibition of PTHLH production is recorded in cells transfected with the specific siRNA (approx. 70%), while such inhibition is not significant for neither the blank nor the control. These results show that, under such conditions, we are able to successfully monitor the specific inhibition of PTHLH secretion upon PTHLH downregulation promoted by a specific PTHLH siRNA. It has been previously described that inhibition of PTHLH secretion can reduce tumor cell proliferation, survival and invasiveness. Thus, our system provides a potentially useful tool for the evaluation of therapies aimed at reducing PTHLH secretion. Moreover, our method might be useful as well in the screening of drugs that regulate PTHLH production, and could be further applied to other secreted proteins.

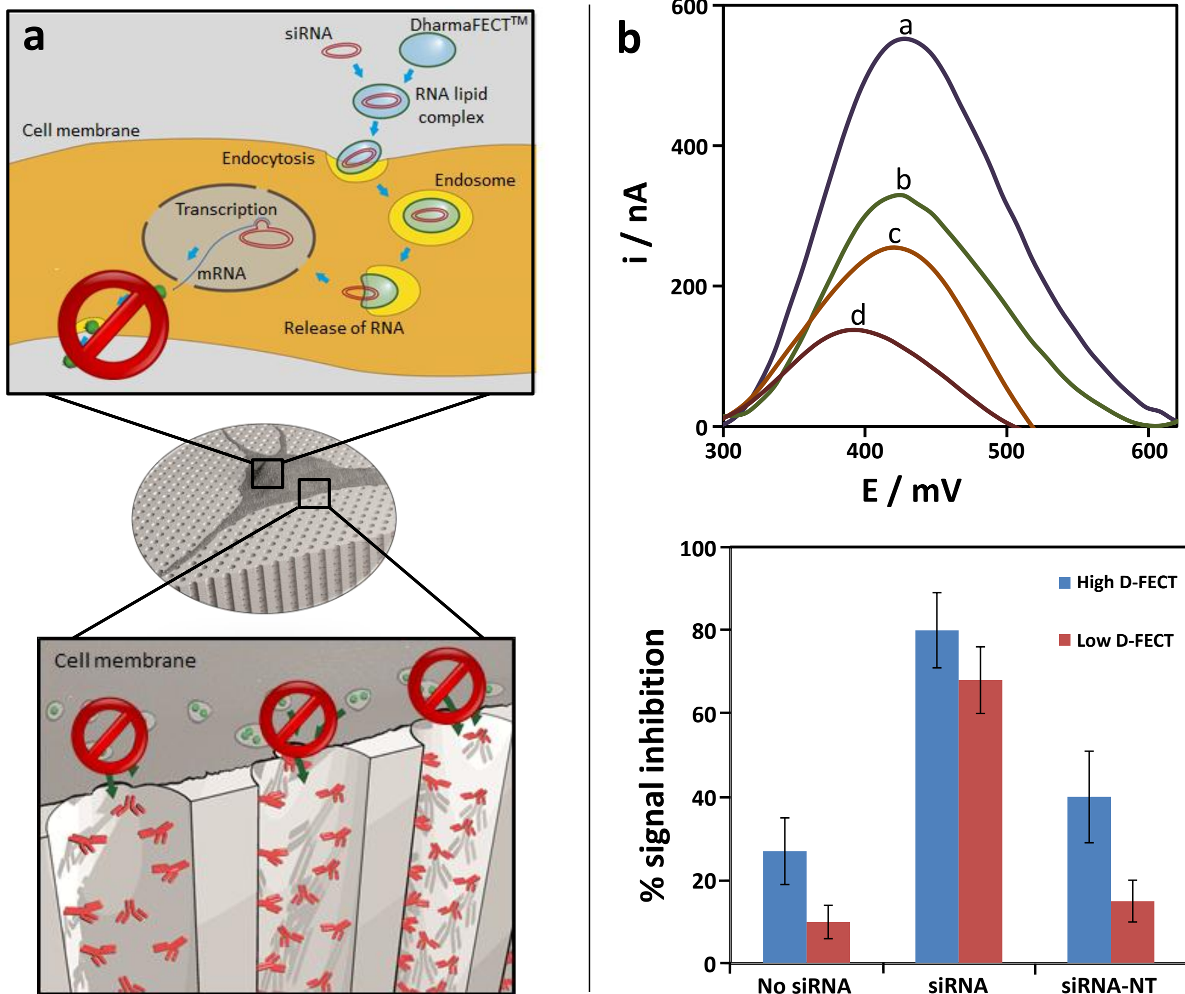

Figure 5. Inhibition of PTHLH secretion by siRNA. (a) Schematic illustration of cell transfection process with small interfering RNA (siRNA) and a transfection reagent (DharmaFECT™) which inhibits *PTHLH* mRNA expression. (b) DPVs obtained for HaCaT cells (24000 cells, 20h growth) incubated for 28h with siRNA combined with a high (-a-) and low (-b-) amount of DharmaFECT™ and with a non-targeting siRNA (siRNA-NT) combined with a high (-c-) and low (-d-) amount of DharmaFECT™. Graph shows a summary of results in terms of % of signal inhibition, considering as reference the signal obtained for 24000 HaCaT cells cultured during 48h. "No siRNA" corresponds to assays performed without siRNA (blank). DharmaFECT™ concentrations: 0.6 µL/well (high, blue columns) and 0.3 µL/well (low, red columns). siRNA and siRNA-NT concentration: 25 nM.

4. CONCLUSIONS

Our present data show that anodic aluminum oxide (AAO) nanoporous membranes can be successfully used to *in situ* quantify PTHLH secretion from neuroblastoma (SK-N-AS) and keratinocytes (HaCaT) cell lines. Normal cell morphology, viability and growth onto these membranes have been shown by means of optical and electronic microscopy. PTHLH has been detected at levels as low as 55 ng/mL by the electrical monitoring of nanochannels blockage upon the formation of the immunocomplex with specific antibodies, using PBNPs as red-ox indicators. These results are in agreement with those previously obtained in buffer medium (Espinoza-Castañeda et al., 2015), suggesting that the matrix of the cell culture medium is not affecting the biosensing response. Although not reaching the high sensitivity of immunoradiometric assays (IRMA) for PTHLH detection (LOD at pg/mL levels) (Fraser et al., 1993; Pandian et al., 1992), our method is able to monitor the levels secreted by cells at the basal state (typically ranging from ng/mL to μg/mL) overcoming the drawbacks of IRMA related to the half-life of the isotope, the potential hazard of radioactivity and the strict requirements needed to implement such procedures. Secreted PTHLH levels ranging from approximately 60 to 400 ng/mL have been detected depending on cell type, number of seeded cells and incubation time. These findings are also in line with the obtained by lateral-flow immunoassays and Western blot analysis (Chamorro-García et al., 2016).

Therefore, in all, these results show that our method is a valid tool for *in situ* monitoring of PTHLH secretion, thus providing a new tool to evaluate PTHLH function and regulation *in vitro*. The proof-of-concept of such potentiality was provided by detecting reduced PTHLH secretion upon cell transfection with a specific small interfering RNA (siRNA). Such siRNA is able to interfere in the expression of the gene responsible for PTHLH production, being the inhibition of PTHLH production *in situ* electrically monitored. This method holds great potential for the screening of drugs that regulate PTHLH secretion and could be extended to other secreted protein as well.

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