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TITLE PAGE

Short Communication

Co-expression of functional human Heme Oxygenase 1, Ecto-5'-Nucleotidase and Ecto-Nucleoside Triphosphate Diphosphohydrolase-1 by "self-cleaving" 2A peptide system

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ABSTRACT

We developed an F2A-based multicistronic system to evaluate functional effects of co-expression of three proteins important for xenotransplantation: heme oxygenase 1 (HO1), ecto-5'-nucleotidase (E5NT) and ecto-nucleoside triphosphate diphosphohydrolase-1 (ENTPD1). The tricistronic p2A plasmid that we constructed was able to efficiently drive concurrent expression of HO1, E5NT and ENTDP1 in HEK293T cells. All three overexpressed proteins possessed relevant enzymatic activities, while addition of Furin site interfered with protein expression and activity. We conclude that our tricistronic p2A construct is effective and optimal to test the combined protective effects of HO1, E5NT and ENTDP1 against xeno-rejection mechanisms.

Keywords: 2A peptide; Heme Oxygenase 1; Ecto 5' Nucleotidase; Ecto Nucleoside Triphosphate Diphosphohydrolase 1; Multicistronic vector; Xenotransplantation.

Abbreviations: **bp**: base pairs; **CO**: carbon monoxide; **E5NT**: ecto-5'-nucleotidase; **ENTPD1**: ecto-nucleoside triphosphate diphosphohydrolase 1; **FMDV**: foot and mouth disease virus; **GAPDH**: glyceraldehyde 3-phosphate dehydrogenase; **GFP**: green fluorescent protein; **HO1**: heme oxygenase 1; **HPLC**: high performance liquid chromatography; **mCher**: mCherry; **RAKR**: arginine alanine lysine arginine; **SGSG**: serine glycine serine glycine; **wt**: wild type.

56 **HIGHLIGHTS:**

- 57 1. We produced an F2A-based tricistronic plasmid encoding the human *HO1*, *E5NT* and *ENTPD1*
58 genes.
- 59 2. The three proteins were functionally expressed and possessed relevant enzymatic activities.
- 60 3. The addition of Furin and spacer sites upstream of F2A sequences decreased proteins expression
61 and activity.
- 62 4. This new combination of genes could be used in a pre-clinical model of xenotransplantation to
63 investigate their protection against xenograft rejection mechanisms.

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1 Introduction

Clinical xenotransplantation, which involves the transplantation of cells or organs from pigs to humans, could resolve the issue of limited availability and complications derived by the use of human organs and cells currently available for allotransplantation. However, all the immunological and species-related barriers, as well as the multiple required genetic modifications of the donor pigs are still major hurdles to overcome [1]. Several transgenic pigs overexpressing human genes have been created and their organs have shown to be protected against hyperacute [2] and acute [3] xenograft rejection mechanisms. It has been estimated that at least 10 genetic modifications are required in order to achieve acceptable protection of the xenograft [4] making it necessary to test different combinations of protective genes in relevant pre-clinical models.

The F2A sequence from the foot-and-mouth disease virus (FMDV) is a well-established method for co-expressing multiple proteins starting from a single open reading frame [5]. Briefly, the peptide bond formation between the glycine and the proline at the F2A site is prevented, which causes the ribosome to skip and begin to translate the next protein from the **next** codon. The F2A sequence remains as a tail at the C-terminus of the upstream protein while the ribosome starts translating the following coding sequence [6]. **To date other multigene expression strategies have been developed, such as co-transfection with several vectors or co-infection with multiple lentiviruses and also the use of Internal Ribosomal Entry Sites (IRES) [7].** For the purpose of xenotransplantation, the F2A technology has been proposed as the tool to solve the issue of combining multiple genetic modifications in the pig genome instead of time-consuming breeding strategies [4].

Our aim was to test the feasibility of a new combination of three xenotransplantation relevant human genes, Heme Oxygenase 1 (HO1), Ecto 5' Nucleotidase (E5NT) and Ecto Nucleoside Triphosphate Diphosphohydrolase 1 (ENTPD1) using F2A technology.

HO1 is a stress inducible enzyme that catabolizes heme to Carbon Monoxide (CO), free iron and biliverdin, which have antioxidant, anti-inflammatory and anti-apoptotic effects [8]. Among its known protective effects, HO1 was reported to inhibit the expression of proinflammatory genes

106 associated with endothelial cells activation [9] and protect the heart from transplant associated
107 ischemia-reperfusion injury [10].

108 ENTPD1 and E5NT are ectonucleotidases involved in the modulation of purinergic signaling by the
109 conversion of extracellular pro-inflammatory ATP/ADP to AMP and AMP to anti-inflammatory
110 and immunosuppressive Adenosine, respectively [11]. ENTPD1 exerts a vascular protective
111 function against platelet aggregation and vessel occlusion by terminating the prothrombotic and
112 proinflammatory effects of ATP and ADP [12]. E5NT- mediated Adenosine production protects
113 against vascular inflammation and neointima formation [13], diminishes trans-endothelial leukocyte
114 trafficking and mitigates inflammatory and immune sequelae of cardiac transplantation [14].

115 The protective role for each of these genes alone has been previously demonstrated in different
116 xenotransplantation models [3, 15, 16], moreover, the combined overexpression of E5NT and
117 ENTPD1 has been recently suggested to promote the protective roles of E5NT-mediated Adenosine
118 pathway to the ENTPD1-based beneficial thromboregulatory effects [17]. However, the feasibility
119 of expressing HO1, ENTPD1 and E5NT as a new combination of genes has not been investigated.

120 In this work we developed two versions of F2A-based multicistronic constructs and we analyzed
121 their functionality in an easily transfectable cell line. We demonstrated that p2A plasmid mediated
122 the correct expression of the three exogenous proteins. Moreover, HO1, ENTPD1 and E5NT were
123 functional as shown by appropriate enzymatic activity assays.

124

125 **2 Materials and Methods**

126 **2.1 Construction of tricistronic expression cassettes**

127 Primers and oligonucleotides used for plasmids construction were purchased from Sigma-Aldrich.
128 The 2A fragments were obtained by annealing the two complementary single strand DNA
129 molecules of FMDV 2A sequences (Tab. 1 Supporting Information). In a total volume of 50 µl of
130 annealing buffer (10 mM Tris-HCl pH 8, 50 mM NaCl, 1 mM EDTA), 2 µg of each complementary

131 oligonucleotide were mixed; this solution was incubated for three minutes at 95°C and then allowed
 132 to slowly cool down for two hours at 37°C. After annealing, the first F2A sequence (F2A1 or
 133 FurF2A1) was ligated by directional cloning into pcDNA3.1+ (Invitrogen) digested with *NheI/AflIII*.
 134 The second F2A sequence (F2A2 or FurF2A2) was ligated into pcDNA3.1 F2A1/FurF2A1 digested
 135 with *BamHI/XhoI*, obtaining pcDNA3.1-F2A1/FurF2A1-F2A2/FurF2A2. An Eppendorf
 136 Mastercycler EP silver block thermocycler was used for synthesizing DNA by PCR reaction.
 137 Primers used for PCR are listed in Tab.1 (Supporting information). Two rounds of recombinant
 138 PCR were performed to amplify human Heme Oxygenase 1 (HO1, [NCBI: NM_002133](#)). Firstly,
 139 HO1 was PCR-amplified from pcDNA3.1-HO1 plasmid (a gift of Prof. Fritz Bach lab, Harvard
 140 Medical School, Boston) without the stop codon using primers *EcoRI* Kozak HO1 fw/*NheI* HO1 rev
 141 and the corresponding amplicon was cloned into pGEM T-easy vector (Promega). This intermediate
 142 vector was used as template for the second PCR performed using primers *NheI* HO1 fw/HO1 rev
 143 and the corresponding product was cloned again into pGEM T-easy vector. The entire coding
 144 sequence was excised by *NheI* digestion and cloned into *NheI*-linearized pcDNA3.1-
 145 F2A1/FurF2A1-F2A2/FurF2A2 upstream of F2A1/FurF2A1, obtaining pcDNA3.1-HO1-
 146 F2A1/FurF2A1-F2A2/FurF2A2. Human Ecto-5'-Nucleotidase (E5NT or CD73, [NCBI:](#)
 147 [NM_002526.3](#)) was PCR-amplified from pcDNA3-E5NT plasmid (received from Dr. Jozef
 148 Spychala, University of Michigan, Ann Arbor) without the stop codon using primers *AflIII* E5NT
 149 fw/*BamHI* E5NT rev and the product was cloned into pGEM T-easy vector. Then the entire coding
 150 sequence was excised by *AflIII/BamHI* double digestion and ligated into *AflIII/BamHI*-linearized
 151 pcDNA3.1-HO1- F2A1/FurF2A1-F2A2/FurF2A2, in frame between F2A1/FurF2A1 and
 152 F2A2/FurF2A2, obtaining pcDNA3.1-HO1-F2A1/FurF2A1-E5NT-F2A2/FurF2A2. Two rounds of
 153 recombinant PCR were performed to amplify human ecto-nucleoside triphosphate
 154 diphosphohydrolase 1 (ENTPD1 or CD39, [NCBI: NM_001776.5](#)). Firstly, ENTDP1 was PCR-
 155 amplified from pcDNA3-ENTPD1 plasmid (received from Dr. Simon Robson, Harvard Medical
 156 School, Boston) including the stop codon using primers *XhoI* ENTDP1 fw/*EcoRI* ENTDP1 rev and

157 the corresponding product was cloned into pGEM T-easy vector. This intermediate vector was used
158 as a template for the second PCR performed using primers ENTPD1 fw/*XbaI* ENTPD1 rev and the
159 corresponding product was cloned into pGEM T-easy vector. Then the entire coding sequence was
160 excised by *XhoI/XbaI* double digestion and ligated into *XhoI/XbaI*-linearized pcDNA3.1-HO1-
161 F2A1/FurF2A1-E5NT-F2A2/FurF2A2 downstream of F2A2/FurF2A2, obtaining the final
162 constructs pcDNA3.1-HO1-F2A1-E5NT-F2A2-ENTPD1 and pcDNA3.1-HO1-FurF2A1-E5NT-
163 FurF2A2-ENTPD1, which were named p2A and pFur2A respectively.
164 Restriction and sequencing analyses were performed on all the intermediate and final constructs
165 (data not shown).

166 2.2 Cell culture and transfections

167 HEK293T cells were cultured in High glucose DMEM (EuroClone) supplemented with 10% heat
168 inactivated fetal bovine serum (EuroClone) and 1X Penicillin/Streptomycin (EuroClone), at 37°C
169 and 5% CO₂. Cells were transiently transfected using TurboFect *in vitro* Transfection Reagent
170 (Fermentas) following the manufacturer instructions and incubated for 48 hours prior to performing
171 expression or activity assays.

172 2.3 Immunoblotting analyses

173 30 µg of total protein extracted in RIPA buffer were separated on 10% NuPAGE BT gel (Life
174 Technologies) and then transferred onto nitrocellulose membranes using the iBlot system (Life
175 Technologies). After blocking in TBST with 5% w/v nonfat dry milk, membranes were probed with
176 rabbit anti-hHO1 (1:2000, EP1391Y, Epitomics), mouse anti-hE5NT (1:500, LS-C138754,
177 LifeSpan BioSciences), mouse anti-hENTPD1 (1:500, HPA014067, Sigma Aldrich), and anti-β-
178 actin (1:5000, AC-15, Sigma Aldrich) primary antibodies. Anti-mouse IgG (H+L) HRP-conjugate
179 (Alpha Diagnostic Intl. Inc.) and ECL anti-rabbit IgG HRP linked (GE Healthcare) secondary
180 antibodies were used at 1:5000 dilutions. Immunoreactive proteins were visualized by enhanced
181 chemiluminescence (SuperSignal West Dura, Thermo Scientific) and digitally acquired using

182 G:BOX (Syngene) instrument.

183

184 **2.4 Immunofluorescence analyses**

185 HEK293T cells were washed with PBS and fixed with methanol-acetone (1:1) for 10 min at -20°C.
186 Following fixation, cells were blocked in 1% BSA (w/v) PBS for 30 min. Fixed cells were co-
187 incubated with rabbit anti-hHO1 (1:200, EPR1390Y, Epitomics) and mouse anti-hE5NT (1:200,
188 4G4, Novus Biologicals) or mouse anti-hENTPD1 (1:200, BU61, Santa Cruz) primary antibodies
189 for 1h. All primary antibody were diluted in 1% BSA (w/v) PBS. After three washes in PBS, cells
190 were incubated for 30 minutes with the appropriate secondary antibodies diluted 1:5000 in 1% BSA
191 (w/v) PBS (Alexa Fluor 488-conjugated anti rabbit and Alexa Fluor 594-conjugated anti mouse,
192 Life Technologies). Cells were washed twice with PBS and counterstained with DAPI. The stained
193 cells were mounted with mounting medium (Fluoromount; Sigma Aldrich) and analyzed by Eclipse
194 80i microscope (Nikon). Images were acquired by Genikon software (Nikon).

195

196 **2.5 E5NT and ENTPD1 activity assay**

197 Ectonucleotidases activity assay was performed as previously described [18]. Briefly, HEK293T
198 cells were pre-incubated for 15 minutes in HBSS supplemented with glucose (1 mg/ml) and
199 Adenosine Deaminase inhibitor, Erythro-9-(2-Hydroxy-3-Nonyl) Adenine, EHNA (5 µM). Cells
200 were incubated with 50 µM of AMP or ATP and supernatant samples were collected after 0, 5, 15,
201 30 min and analyzed for nucleotide metabolite content by reverse phase HPLC [19].

202 **2.6 Heme Oxygenase activity assay**

203 Heme oxygenase activity assay was performed as previously described [20]. 300 µg of crude lysate
204 were incubated with 15 µM hemin and 10 U/ml recombinant biliverdin reductase A. Bilirubin
205 fluorescence was detected every 2 min for 2 h in a fluorescence reader (Infinite M200; Tecan) at
206 37°C (excitation/emission wavelengths: 441/528 nm).

207 **2.7 Statistical analysis**

208 Values are presented as mean $\pm$ standard error of mean (SEM). Statistical analyses were performed
209 using SPSS v.19 for Mac. One-way analysis of variance (ANOVA) with Tukey's post hoc test was
210 used to compare between cell experimental groups. A *p*-value of < 0.05 was considered as
211 significant difference.

212

213 **3 Results and Discussion**

214 **3.1 Plasmids construction**

215 In this study we developed two F2A-based plasmid systems and evaluated functionality of co-
216 expression of three proteins that are important for xenotransplantation: heme oxygenase 1 (HO1),
217 ecto-5'-nucleotidase (E5NT) and ecto-nucleoside triphosphate diphosphohydrolase-1 (ENTPD1).
218 We produced p2A plasmid by cloning HO1 as the first coding sequence linked to the first F2A
219 sequence (F2A1) in frame with the E5NT coding sequence, which was then followed by the second
220 F2A sequence (F2A2) and finally the ENTPD1 coding sequence (Fig. 1). HO1 has a cytoplasmic
221 localization [21] while active E5NT and ENTPD1 are localized at the plasma membrane [22, 23].
222 Moreover, E5NT encodes a N-terminal signal peptide, which directs it through the endoplasmic
223 reticulum before reaching the plasma membrane [24]. The selected gene order was chosen to
224 maximize the likelihood of correct subcellular processing of each of the three proteins and to avoid
225 HO1 (lacking any signal sequence) being "slipstreamed" through the translocon formed by the
226 (signal-bearing) E5NT [25]. In an attempt to reduce the possible negative effects of the C-terminal
227 F2A tail on HO1 and E5NT expression and activity [26], we generated a second F2A-based
228 construct (named pFur2A (Fig. 1B)) by adding a Furin recognition site (RAKR) followed by a
229 spacer (SGSG) upstream of each F2A sequence (FurF2A1 and FurF2A2), according to a previously
230 described strategy [27, 28].

231 **3.2 Expression analyses**

232 We tested the functionality of the two tricistronic constructs (p2A and pFur2A) by transiently
233 transfecting HEK293T cells for 48 h. Appropriate mock- and single gene-transfected cells were
234 used as controls. We investigated expression of each the three proteins by performing immunoblot
235 analyses (Fig.2). Immunodetection with the indicated antibodies in p2A-transfected cells, clearly
236 detected bands corresponding to the exogenously expressed HO1 (Fig. 2A), E5NT (Fig. 2B), and
237 ENTPD1 (Fig. 2C). Lower expression levels of each exogenous protein was observed in pFur2A- as
238 compared to p2A-transfected cells (Fig. 2A, 2B, 2C), suggesting that the Furin/spacer sites had a
239 negative impact on F2A-mediated cleavage. The molecular weight of HO1 exogenously expressed
240 from either p2A- or pFur2A-transfected cells was larger than those cells transfected with HO1-
241 alone (Fig. 2A), resulting from the presence of the F2A-derived or Furin + Spacer + F2A-derived
242 C-terminal extension, respectively, indicating an absence of any Furin-mediated cleavage of 2A tail
243 in pFur2A-transfected cells. No difference in size was observed between the exogenously expressed
244 E5NT in either p2A- and pFur2A-transfected cells as compared to the corresponding protein in
245 E5NT-transfected cells, suggesting the absence of F2A sequence at the C-terminus of E5NT
246 expressed from both of the tricistronic plasmids (Fig. 3B). It was reported that E5NT undergoes
247 proteolytic cleavage of its C-terminal signal and simultaneous replacement with
248 glycosylphosphatidylinositol, which functions as the plasma membrane anchor of the mature
249 protein [24]. We speculated that this cleavage may be responsible for the release of the F2A tail at
250 the C-terminus of exogenous E5NT. Transfection of each the three genes alone resulted in very
251 high protein expression levels (Fig. 2A, 2B, 2C) as compared to expression from the tricistronic
252 plasmids, probably due to the reduction in transfection efficiency and consequently amount of
253 translated proteins, which has been reported in larger sizes plasmids as compared to small plasmids
254 [29]. On the contrary, pFur2A was only 48 base pairs (bp) larger than p2A, not enough in our
255 opinion to be cause of the observed proteins expression differences. A slight band of 130 kDa
256 uncleaved product, probably corresponding to E5NT-2A-ENTPD1, has been observed both in p2A-
257 and pFur2A-transfected cells lines of ENTPD1 immunoblot, however this band was more intense in

258 pFur2A- as compared to p2A-group (Fig. 2C). Additionally, the correct subcellular localization of
259 HO1, E5NT and ENTPD1 was found to be the same in both p2A- and pFur2A-transfected cells, as
260 detected by immunofluorescence. HO1 had a main perinuclear localization, while both E5NT and
261 ENTPD1 showed a plasma-membrane staining pattern (Fig. 2D), accordingly with the literature
262 [21, 22, 23].

263 We observed more protein expression in the p2A-transfected cells as compared to pFur2A-
264 transfected cells (Fig. 2A, 2B, 2C) with more uncleaved product in the latter group (Fig. 2C),
265 suggesting that the added Furin cleavage sites and spacers in pFur2A may interfere with the F2A-
266 mediated cleavage. Taken together, these analyses indicated that p2A results in a more efficient
267 expression of HO1, E5NT and ENTPD1 than pFur2A. Although an increase in expression levels
268 derived by the addition of Furin recognition site and spacers [28] as well as the versatile usage of
269 the Furin-2A system in the production of multi-transgenic constructs for xenotransplantation
270 purposes [30] were reported, we did not obtain the same outcomes in our system. Verrier et al.
271 showed that the addition of a Furin recognition site upstream of 2A sequence in their GFP-2A-
272 mCher plasmid interfered with 2A cleavage [31] and suggested that the inclusion of an additional
273 amino acid spacer between the Furin and 2A sites would improve cleavage efficiency of exogenous
274 proteins [31]. Whereas the addition of both Furin recognition site and spacer upstream of F2A
275 sequences in our system resulted in an inefficient production of our transgenes. To improve the 2A
276 efficiency, recent efforts are aimed to identify the optimal length of the F2A sequence itself for
277 successful co-expression strategies [32].

278 The presence of the F2A derived C-terminal extension could potentially induce an immune
279 response in xenotransplantation settings. Several F2A-based multi-transgenic pigs have already
280 been produced [33, 34] but no 2A-mediated immune responses have been reported so far. Recently,
281 2A sequences have been successfully incorporated in vectors used in human gene therapy studies
282 without eliciting discernible immune responses [35]. Moreover, the exposure of human PBMCs to
283 viral-derived 2A sequences did not produce T cell responses in an ex-vivo model [36]. These last

reports strengthen the use of F2A technology even in xenotransplantation research.

3.3 Enzymatic activity analyses

Next, we tested the enzymatic activity of ENTPD1 and E5NT produced from the two tricistronic plasmids using an extracellular nucleotides metabolism assay. Confluent untransfected (wt) and transiently transfected cells were incubated with ATP or AMP and supernatant samples were collected at different time points and analyzed for their nucleotide content by HPLC [18, 19]. During incubation with ATP, extracellular ADP concentration in ENTPD1-transfected cells reached a maximal concentration after 5 min ($4.9 \pm 0.1 \mu\text{M}$) after which it was completely catabolized (Fig. 3A). A similar but delayed trend was observed in p2A-transfected cells, where the ADP concentration decreased to $4.9 \pm 1.6 \mu\text{M}$ after reaching a maximal level at 15 min ($5.8 \pm 0.1 \mu\text{M}$, Fig. 3A). In pFur2A-, E5NT-transfected cells and controls an increasing formation of ADP over time was observed with the highest concentration being observed in the pFur2A-transfected cells ($8.3 \pm 0.6 \mu\text{M}$, Fig. 3A). Conversion of AMP in ENTPD1-transfected cells increased to $50.9 \pm 0.4 \mu\text{M}$ while the concentration remained below $2 \mu\text{M}$ in untransfected, mock- and E5NT-transfected cells ($p < 0.05$), suggesting a very low basal ATP hydrolyzing activity under these controls conditions (Fig. 3B). In p2A- and pFur2A-transfected cells the extracellular AMP increased to 18.7 ± 1.9 and $19.3 \pm 1.5 \mu\text{M}$, respectively, with no statistical difference being observed between the two tricistronic plasmids (Fig. 3B). A substantial increase in Adenosine formation from ATP was also observed only for the p2A- and pFur2A-transfected cells (34.7 ± 3.3 and $22.8 \pm 2.9 \mu\text{M}$ respectively, Fig. 3C) with Adenosine production being higher in p2A- as compared to pFur2A-transfected cells at all time points ($p < 0.05$, Fig. 3C). No Adenosine production in the time of incubation has been observed in ENTPD1- and E5NT-transfected cells as well as in controls (Fig. 3C). During incubation with AMP, the concentration of Adenosine in the medium of p2A-, pFur2A- and E5NT-transfected cells increased to 40.2 ± 1.7 , 20.6 ± 1.9 and $41.9 \pm 2.1 \mu\text{M}$, respectively, while it

310 remained below 2 μ M in ENTPD1-transfected and controls cells (Fig. 3D). At every time points the
 311 concentration of Adenosine in p2A-transfected cells was significantly higher than in pFur2A-
 312 transfected cells (Fig. 3D), correlating with a more efficient production of active E5NT in p2A-
 313 transfected cells (Fig. 2B). The Adenosine levels were also similar between p2A- and E5NT-
 314 transfected cells at every measured interval (Fig. 3D). No evident Adenosine production in the
 315 period of incubation has been observed in control cells as well as in ENTPD1-transfected cells
 316 suggesting a very low basal AMP hydrolyzing activity (Fig. 3D).

317 The activity of HO1 was investigated by measuring the increase of bilirubin fluorescence during the
 318 incubation of total cellular lysates with hemin [20]. As shown in Fig. 4, the activity of HO1 in p2A-
 319 transfected cells was 5-fold higher than the basal activity observed in untransfected (wt) and mock-
 320 transfected control cells (1.54 ± 0.12 nmol/h/mg versus 0.31 ± 0.04 and 0.33 ± 0.07 nmol/h/mg,
 321 respectively, in wt and mock-transfected cells, $p < 0.05$). No difference in activity was observed
 322 between wt and mock-transfected cells ($p > 0.05$, Fig. 4). Moreover a slight activity increase was
 323 observed in pFur2A-transfected cells (0.74 ± 0.05 nmol/h/mg) as compared to untransfected (wt) and
 324 mock-transfected control cells ($p > 0.05$, Fig. 4). This result correlated with the lower protein
 325 expression of HO1 in pFur2A- as respect to p2A-transfected cells (Fig. 2A). A substantial increase
 326 in activity was observed in HO1-transfected cells (4.94 ± 0.4 nmol/h/mg, Fig. 4), which correlated
 327 with a markedly higher expression level of exogenous HO1 in HO1-transfected cells than in p2A-
 328 transfected cells (Fig. 2A).

329 All together, our findings on enzymatic activity suggested a more efficient production of active
 330 enzymes driven by p2A than those driven by pFur2A. The significantly higher production of
 331 Adenosine in p2A-transfected cells following incubation with ATP (Fig. 3C), suggested a better
 332 coordination of both ectonucleotidases activity in p2A-transfected cells. We hypothesize that the
 333 lower ENTPD1- AMP production following ATP incubation in both p2A- and pFur2A-transfected
 334 cells as compared to ENTPD1-transfected cells was due to the simultaneous E5NT-mediated AMP
 335 catabolism to Adenosine (Fig. 3B). Although the ENTPD1-mediated AMP production in p2A- and

336 pFur2A-transfected cells was apparently similar (Fig. 3B), we observed a more efficient production
337 of Adenosine in the former group (Fig. 3C) which let we hypothesize a higher ENTPD1-mediated
338 production of AMP substrate for E5NT in p2A- as compared to pFur2A-transfected cells. However,
339 an initial increase in ADP concentration during incubation with ATP in p2A-transfected cells (Fig.
340 3A) highlights the need for careful and complex analysis of consequences of such manipulations on
341 coagulation and inflammation. A higher HO1 activity in p2A- as compared to pFur2A-transfected
342 cells has been also showed (Fig. 4).
343 We reported that F2A sequences alone gave an efficient expression of the transgenes with no
344 interference with their enzymatic activity regardless a slight amount of uncleaved E5NT/ENTPD1
345 dimer (Fig. 2C).

346

347 3.4 Conclusions

348 To translate xenotransplantation to the clinic, major immunologic and biochemical barriers need to
349 be overcome [1]. Multiple genetic modifications in donor pigs are necessary and different
350 combinations of these will need to be tested in relevant pre-clinical models. The 2A technology has
351 been proposed as an important tool with which to resolve the issue of combining genetic
352 modifications [4]. We previously demonstrated that F2A-mediated co-expression was functional for
353 expression of E5NT and ENTPD1 in an appropriate endothelial cell line [18] and here we now
354 demonstrated the correct and functional co-expression of three proteins, HO1, E5NT and ENTPD1.
355 We showed that p2A allowed the correct expression of three proteins that were differentially
356 processed (Fig. 2). More importantly, these proteins were found to be functional as demonstrated by
357 the increase of Heme Oxygenase 1 and both Ectonucleotidases products in their respective activity
358 assays (Fig. 3 and 4). Therefore the catabolism of pro-inflammatory heme and ATP/ADP may be
359 enhanced together with the production of anti-inflammatory products. We observed less efficient
360 protein expression levels together with more uncleaved product (Fig. 2) and decreased activities
361 (Fig. 3 and 4) in pFur2A-transfected cells as compared to p2A-transfected cells.

362 In conclusion, having demonstrated its correct functionality, we propose p2A construct to be tested
363 in a relevant pre-clinical model of xenotransplantation to investigate if the combining protective
364 effects of HO1, E5NT and ENTPD1 may be able to attenuate a larger spectrum of xeno-rejection
365 mechanisms. The p2A plasmid could be used to produce human HO1, E5NT and ENTPD1-triple
366 transgenic pigs by a single round of Somatic Cell Nuclear Transfer (SCNT) [34] or by Sperm
367 Mediated Gene Transfer (SMGT) [37].

368

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375

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497 **Figure Legends**

498 Fig. 1. Schematic representation of p2A (A) and pFur2A (B) constructs. Two F2A (87 bp) or
499 FurF2A (111 bp) sequences have been cloned in pcDNA3.1 commercial vector (Life Technology).
500 FurF2A sequences contain a Furin recognition site and a spacer site upstream of the F2A sequences.
501 PCR-amplified HO1 (867 bp), E5NT (1725 bp) and ENTPD1 (1533 bp) have been respectively
502 cloned as first, second and third coding sequence. Plasmids representations are not in scale.

503

504 **Fig. 2. Expression analyses in wt and transfected HEK293T cells.** Immunoblotting analyses of HO1
505 **(A)**, E5NT **(B)** and ENTPD1 **(C)** on 30 µg of total lysates from wt, mock-, p2A- and pFur2A-
506 transfected cells. HO1-, E5NT- and ENTPD1-transfected cells lysates have been used as positive
507 controls in (A), (B) and (C) respectively. β-actin has been used as loading control. HO1 band: ≈32
508 KDa; E5NT band: ≈63 KDa; ENTPD1 band: ≈70 KDa; β-Actin band: 42 KDa. An uncleaved
509 product of ≈130 KDa has been detected in p2A and pFur2A lanes. [Ab anti HO1: Rabbit
510 monoclonal (EP1391Y, Epitomics) 1:2000; Ab anti E5NT: Mouse monoclonal (LS-C138754,
511 LifeSpan BioSciences) 1:500; Ab anti ENTPD1: Mouse monoclonal (HPA014067, Sigma Aldrich)
512 1:500; Ab anti β-Actin: Mouse monoclonal (AC-15, Sigma Aldrich) 1:5000] **(D)**

513 **Immunofluorescence analysis with double stainings of HO1 (green) and E5NT or ENTPD1 (red) in**
514 **mock- (left), p2A- (middle) and pFur2A- (right) transfected cells. Nuclei were counterstained with**
515 **DAPI (blue). [Ab anti HO1: Rabbit monoclonal (EPR1390Y, Epitomics) 1:200; Ab anti E5NT:**
516 **Mouse monoclonal (4G4, Novus Biologicals) 1:200; Ab anti ENTPD1: Mouse monoclonal (BU61,**
517 **Santa Cruz) 1:200].**

518

519 Fig. 3. ENTPD1 and E5NT activity assays in wt and transfected HEK293T cells. (A) ADP
520 production during incubation with 50 µM ATP in wt, mock-, E5NT-, ENTPD1-, p2A- and pFur2A-
521 transfected cell groups. * $p < 0.05$ ENTPD1- vs. all groups; ** $p < 0.05$ pFur2A-, p2A-, ENTPD1- vs.
522 wt, mock- and E5NT-groups; *** $p < 0.05$ p2A- and pFur2A- vs. wt, mock-, E5NT- and ENTPD1-

523 groups; # $p < 0.05$ pFur2A- vs. p2A-, E5NT- and ENTPD1-groups; § $p < 0.05$ p2A- and E5NT- vs.
524 pFur2A- and ENTPD1-groups. (B) AMP production during incubation with 50 μ M ATP. * $p < 0.05$
525 ENTPD1- vs. all groups; ** $p < 0.05$ p2A- vs. all groups except pFur2A-group. (C) Adenosine
526 production during incubation with 50 μ M ATP. * $p < 0.05$ vs. all groups. (D) Adenosine production
527 during incubation with 50 μ M AMP. * $p < 0.05$ vs. all groups; ** $p < 0.05$ E5NT vs. all groups except
528 p2A-group. Values at each time points represent mean $\pm$ SEM (n=3).

529

530 Fig. 4. Heme Oxygenase activity assay on wild type and transfected HEK293T cells. Bilirubin
531 production during a 2 h incubation of wt, mock-, HO1-, p2A- and pFur2A-transfected cells with 15
532 μ M hemin in reaction buffer containing 300 μ M BSA and 10 U/ml of recombinant biliverdin
533 reductase. Bilirubin production is expressed as nanomoles of bilirubin produced per hour and per
534 milligram of proteins. * $p < 0.05$ vs. all groups. Values represent mean $\pm$ SEM (n=3).

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538 Figure 1

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Figure 2

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544 Figure 3

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547 Figure 4