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# NO ANSWER TO THE LACK OF SPECIFICITY: Mouse monoclonal antibody targeting the angiotensin II type 1 receptor $AT_1$ fails to recognize its target.

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**Number of figures:** 2

## ABSTRACT

**Purpose.** Numerous antibodies targeting G protein-coupled receptors, GPCRs, have been described as non-specific among them polyclonal antibodies against angiotensin II type 1 receptor ($AT_1$). We have tested the newly developed $AT_1$ receptor mouse monoclonal antibody for its specificity.

**Methods.** Human Embryonic Kidney HEK293 cells, which do not endogenously express $AT_1$ receptor, were transfected in order to overexpress a fluorescently labelled EGFP-tagged human $AT_1$ receptor. Western blot and immunofluorescence assays were performed to test the specificity of the Santa Cruz monoclonal antibody sc-57036. These results were compared to the ones obtained with the polyclonal sc-1173 anti-$AT_1$ receptor antibodies that have already been described as non-specific.

**Results**. While the positive controls using GFP antibodies detected the EGFP-tagged $AT_1$ receptor, both polyclonal and monoclonal anti-$AT_1$ receptor antibodies failed to specifically recognize the corresponding band by western blot, as similar bands were revealed in either transfected or non-transfected cells. It also failed to detect $AT_1$ receptor in immunofluorescence experiments.

**Conclusion.** The lack of target recognition of the monoclonal $AT_1$ receptor antibody in our experimental conditions suggests that this antibody could give misleading results such as misidentification of the protein. To our knowledge, no specific antibodies targeting $AT_1$ receptors have been developed so far and the field is thus in need of new technical developments.



**Key words:** G protein-coupled receptors – Angiotensin II type 1 receptor –antibody – specificity

## INTRODUCTION

G protein-coupled receptors (GPCR) antibodies raise a lot of questions concerning offtarget detections (Michel et al. 2009). Numerous publications criticize the non-specificity of different GPCR antibodies: histamine H4-receptors (Beermann et al. 2012) ; P2Y6 receptors (Yu and Hill 2013) ; cannabinoid receptors (Cécyre et al. 2014) ; dopamine receptors (Bodei et al. 2009).

Polyclonal antibodies targeting both angiotensin II (AngII) receptors type 1 ($AT_1$) and type 2 ($AT_2$) have been described as non-specific (Benicky et al. 2012; Herrera et al. 2013; Hafko et al. 2013). Despite these findings, antibodies directed against $AT_1$ receptors are still widely used in the literature with more than twenty publications in 2017 using the Santa Cruz aspecific sc-1173 polyclonal antibodies (Chao et al. 2017; Young et al. 2017; Liu et al. 2017) and already five publications in 2018 (Ahad et al. 2018; Ishikane et al. 2018; Rizzetti et al. 2018; Zhao et al. 2018; Ribeiro et al. 2018). In these papers, authors assume that they proved the specificity of these antibodies. However, in most cases, the control consisted of using the blocking peptide provided by the supplier to highlight the disappearance of the detected bands (Oestreicher et al. 2006). This type of experiment proves that the antibodies recognize the peptide used for immunization, however it is not sufficient to confirm that they recognize the targeted full-length protein. To overcome this issue, companies have developed monoclonal anti-$AT_1$ receptor antibodies presented as more specific.

In the present work, we evaluated the specificity of the Santa Cruz sc-57036 monoclonal anti-$AT_1$ receptor antibody.

Human Embryonic Kidney (HEK293) cells, that lacks endogenous $AT_1$ receptor expression, were transfected with a plasmid containing the human $AT_1$ receptor tagged at its extracellular N-terminus with the enhanced green fluorescent protein (EGFP-

$AT_1$). First, we assessed the robustness of our model by verifying the functionality of the fluorescent receptor and its localization. Second, we tested the specificity of the monoclonal sc-57036 antibody by Western blot and immunofluorescence. We compared our data to the ones obtained using the polyclonal sc-1173 antibodies that have already been described as non-specific. Our results indicate that the Santa Cruz anti-$AT_1$ receptor monoclonal antibody sc-57036 does not label human $AT_1$ receptor. Moreover we confirmed the aspecificity of the polyclonal sc-1173 antibodies.

## MATERIALS AND METHODS

### ***Cell culture and transfection***

HEK 293 (Human Embryonic Kidney) cells were cultured to ~80% confluence in T-75-$cm^2$ flasks in MEM with Earle's salt supplemented with 10% fetal calf serum, 2 mM glutamine and 1% antibiotics (penicillin/streptomycin) and re-plated twice a week. The expression vector pIRES (Clontech) encoding for the fluorescent EGFP-human $AT_1$ receptor was a gift from Prof Haiech and Prof Kilhoffer (University of Strasbourg) and PCBIS UMS3286 CNRS-University of Strasbourg.

Cells stably expressing human EGFP-$AT_1$ receptors were, in addition, cultured with 600µg/ml G418 (Invitrogen)

### ***Antibodies***

EGFP was detected with mouse monoclonal anti-GFP (A11120 Life technologies) and goat polyclonal anti-GFP directly coupled to Horse Radish Peroxidase HRP (AB6663, ABCAM). Tested antibodies against $AT_1$ receptors were the anti-$AT_1$ N-10 (Santa Cruz sc-1173 batch number C2415) and the anti-$AT_1$ TONI-1 (Santa Cruz sc-57036 batch number I2111). The anti-$AT_1$ N-10 is a rabbit polyclonal IgG with an epitope mapping within the N-terminal extracellular domain of $AT_1$ receptor of human origin. It has just been discontinued. The anti-$AT_1$ TONI-1 is a mouse monoclonal antibody raised against a GST fusion protein corresponding to amino acids 297-356 of $AT_1$ receptor of human origin. Both antibodies are recommended by the manufacturer for detection of $AT_1$ receptor of mouse, rat and human origin by Western blot, immunoprecipitation, immunofluorescence and ELISA.

***Calcium mobilization assay***

Cells were cultured for three days in T10, then loaded with 2.5 µM Fluo-4 AM (Molecular Probes) for 1 h. Cells were rinsed in PBS, collected in PBS-5mM EDTA and suspended in 9 mL Hepes-BSA buffer (10 mM HEPES pH 7.4, 137.5 mM NaCl, 1.25 mM MgCl2, 1.25 mM CaCl2, 6 mM KCl, 5.6 mM glucose, 0.4 mM NaH2PO4, 0,1% Bovine Serum Albumine). Cells were loaded in 96-well plates (Greiner 655090, 100 µL/well) and centrifuged for 3 min at 800 rpm. AngII-evoked increases in intracellular calcium were recorded over time using a Flexstation III (Molecular Devices, Sunnyvale, CA, USA) (3 sec intervals over 220 sec) at 37°C through fluorescence emission at 520 nm (excitation at 485 nm). Peak response amplitudes were normalized to basal fluorescence levels. For each concentration of the agonist, we determined the maximal value of the increase in calcium concentration in order to build concentration response curves to AngII.

***Cell surface expression of the fluorescent EGFP-$AT_1$ receptor***

Cells were cultured for two days in T10, then rinsed in PBS, collected in PBS-5mM EDTA. Immunolabelling of EGFP-$AT_1$ receptors expressed at the cell surface was done on intact cells (approximately 1 million cells) pre-incubated in 1 mL PBS-1% BSA on ice for 1 hour, then labelled using mouse monoclonal anti-GFP (A11120 Life technologies 1/100 dilution in PBS 1% BSA) for 30 min on ice and secondary labelling done using a R-phycoerythrin-conjugated AffiniPure F(ab')2 fragment goat anti-mouse IgG (1/100 dilution). EGFP fluorescence and phycoerythrin (PE) staining were detected by flow cytometric analysis (20,000 cells per sample) on a cytometer (FACSCalibur, Becton-Dickinson).

130 ***Western blotting to detect the EGFP-tagged $AT_1$ receptor***

131 Cells were cultured for two days in 6-well plates. Cell lysates were performed in 250
132 µL RIPA buffer (20 mM de Tris-HCl pH 7.4, 150 mM de NaCl, 1% de NP40, 0.25% de
133 Na-deoxycholate, 0.1% SDS, Complete protease inhibitor EDTA-free (Roche)
134 according to Lecat et al. (2015). In order to concentrate the receptors, in some
135 experiments, cell lysates underwent immunoprecipitation. Briefly 450 µg of proteins
136 were exposed to mouse monoclonal anti-GFP (A11120 Life technologies, 1:100)
137 overnight on a wheel at 4°C before coupling to Protein A sepharose 4B (Life
138 technologies) for 2 hours at 4°C the following day. Protein A / antibodies complexes
139 were washed 3 times in 1 mL RIPA buffer by successive centrifugations at 2000 rpm
140 for 1 min at 4°C before being suspended in 2X Laemmli buffer.
141 Proteins were not heated and directly separated on 10 % acrylamide SDS-PAGE gel
142 electrophoresis. Proteins were then transferred onto PVDF polyvinyl difluoride
143 membranes for 2 hours and western blotting was performed according to Lecat et al.
144 (2015). Concentrations of antibodies were: polyclonal rabbit anti-$AT_1$ receptor (Santa
145 Cruz sc-1173) 1:500 and monoclonal mouse anti-$AT_1$ receptor (Santa Cruz sc-57036)
146 1:500. PVDF membranes were washed in acid buffer for desorbing the antibodies and
147 reprobed with goat polyclonal anti-GFP directly coupled to Horse Radish Peroxidase
148 HRP (AB6663, ABCAM) 1:25 0000. Secondary anti-rabbit HRP and anti-mouse HRP
149 antibodies were used at a dilution of 1:10 000 and 1:5 000 respectively (Immuno
150 Research Laboratories).
151
152 ***Immunofluorescence***

153 Cells were cultured for 24 hours in 24-well plates on 12-mm in diameter coverslips
154 coated with rat type I collagen. Cells were fixed in PBS containing 4%

paraformaldehyde, rinsed twice with PBS and exposed 15 min to 50 mM ammonium chloride in order to quench remaining paraformaldehyde fluorescence. Cells were then rinsed three additional times in PBS. Cells exposed to the monoclonal sc-57036 antibody were permeabilized 3 min in PBS containing 0,1% Triton X-100. Immunolabelling was performed in PBS-0.2% BSA with either polyclonal anti-$AT_1$ receptor (sc-1173, 1:50), monoclonal anti-$AT_1$ receptor (sc-57036, 1:50) or monoclonal anti-GFP (A11120, 1:100). Cells were washed 3 times in PBS-0.2% BSA and incubated further with either secondary goat anti-rabbit or goat anti-mouse antibodies coupled to Alexa 594 (Invitrogen ;1:200). After further washes in PBS, coverslips were mounted onto slides using an anti-fading agent (Mowiol; Calbiochem). Image acquisition was performed with a fluorescent microscope LEICA (motorized microscope Leica DM5500B together with cold-camera Leica CTR 6000). Figures were created with FigureJ.

## RESULTS

### *Validation of the experimental model: in transfected cells, fluorescently-labelled $AT_1$ receptors are functional and localized at the plasma membrane*

In cells expressing the fluorescently labelled EGFP-$AT_1$, the receptor is fused at its extracellular N-terminus with the fluorophore (Fig. 1A). We verified that the receptor was active in these cells by measuring calcium responses to AngII. While the parental HEK293 cells did not respond to the agonist, showing that the receptor is not endogenously expressed, EGFP-$AT_1$ cells gave a nice AngII dose-response with an EC50 around $2.10^{-8}$ mol/L AngII (Fig. 1B). Using fluorescence cell sorting analysis, we have controlled that all the cells were expressing the fluorescent receptor (as detected by the peak of EGFP fluorescence as compared to non-transfected cells, Fig. 1C, left) and that the receptor was properly localized at the cell surface (as detected by surface labelling with anti-GFP antibodies followed by red fluorescent secondary antibody labelling, Fig. 1C, right).

### *Polyclonal and monoclonal anti-$AT_1$ antibodies detected bands in Western blot whether the receptor was expressed or not*

The expected size of the fusion protein, EGFP-$AT_1$ receptor, estimated from the amino-acid sequence, is 73 kDa. The estimation does not take into account post-translational modifications such as *N*-glycosylation. In Western blot of total cell extracts (40 µg of proteins), both monoclonal sc-57036 and polyclonal sc-1173 antibodies detected bands whether the fluorescently labelled receptor was expressed or not (Fig. 2A; total lysates). Reprobing of the membrane with monoclonal anti-GFP antibody specifically revealed a major band in HEK-EGFP-$AT_1$ transfected cells (but not in parental cells) around 90 kDa corresponding to the tagged-receptor. A smaller band around 40 kDa

193 is also detected with the anti-GFP, that could correspond to degraded receptors mainly
194 with the EGFP tag left.
195 In a second attempt to detect the receptors with anti-$AT_1$ receptor antibodies, we
196 performed immunoprecipitations of 450 µg of protein extracts with monoclonal anti-
197 GFP antibody in order to concentrate the receptors prior to Western blotting. Again,
198 polyclonal sc-1173 and monoclonal sc-57036 revealed unspecific bands in both non-
199 transfected and HEK-EGFP-$AT_1$ transfected cell lysates (Fig. 2A; anti-GFP-
200 immunoprecipitated lysates). Reprobing the membrane with anti-GFP-HRP antibody
201 specifically revealed the EGFP-$AT_1$ receptor bands only in cells expressing the
202 fluorescently-labelled receptor (Fig. 2A; immunoprecipitated lysates).
203

204 ***Both anti-$AT_1$ receptor antibodies failed to recognize the receptor by***
205 ***immunofluorescence***

206 Next, we tested the antibodies in immunofluorescence labelling experiments on EGFP-
207 tagged $AT_1$ receptor expressing cells. Cells cultured on coverslips were labelled either
208 with anti-GFP or anti-$AT_1$ receptors antibodies, followed by a red fluorescent
209 secondary antibody detection. Using epifluorescence microscopy, images collected in
210 the green channel to directly visualize the fluorescence of EGFP showed a localization
211 of the receptor principally at the cell plasma membrane and in some intracellular
212 perinuclear compartments (Fig. 2B-*a*, 2B-*d*, 2B-*g*).
213 Staining with the monoclonal GFP antibody on non-permeabilized cells revealed only
214 the plasma membrane localization of the receptor (Fig. 2B-*b)*.
215 When using the polyclonal anti-$AT_1$ receptor antibodies, no plasma membrane staining
216 was detected (Fig. 2B-*e*). We obtained similar results with the monoclonal anti-$AT_1$
217 receptor antibody. For this antibody, we performed permeabilization of the cells due to

218 the localization of the epitope in the C-terminus tail of the receptor. While, in the green
219 channel, the receptor could still be detected by the direct fluorescence of EGFP (Fig.
220 2B-*g*), no specific labelling with the monoclonal anti-$AT_1$ receptor antibody in the red
221 channel (Fig. 2B-*h*) and no colocalization in the overlay channel were visible (Fig. 2B-
222 *i*).
223 Therefore, in accordance with our results obtained by Western blot, monoclonal and
224 polyclonal anti-$AT_1$ receptors antibodies also failed to recognize $AT_1$ receptors by
225 immunofluorescence.

## DISCUSSION

Here we bring the first evidence of the lack of specificity of the Santa Cruz monoclonal anti-$AT_1$ antibody sc-57036 as it failed to specifically recognize the $AT_1$ receptor. In the present work we used an heterologous model of human $AT_1$ receptors expression in which the receptor is tagged with the EGFP fluorescent protein at its extracellular N-terminal domain. Before assessing the specificity of anti-$AT_1$ receptor antibodies, we first evaluated the robustness of our model. HEK cells stably expressed the EGFP-$AT_1$ receptors homogeneously in the cell population. The receptors were properly localized at the cell surface and functional.

We confirm here the aspecificity of the $AT_1$ receptor polyclonal antibodies sc-1173, since bands were revealed by Western blot in all lysates whether $AT_1$ receptor was present or not. We obtained the same results with the $AT_1$ receptor monoclonal antibody sc-57036. Moreover, both $AT_1$ receptor antibodies failed to recognize the receptor in immunofluorescence labelling assays. To our knowledge, this is the first report questioning the specificity of Santa Cruz monoclonal $AT_1$ receptor antibody sc-57036. Already used in 10 publications (including three in 2017 and one in 2018) (Filice et al. 2017; Yim et al. 2017; Ferrão et al. 2017; Sinphitukkul et al. 2018), this antibody that lacks specificity could lead to false positive staining on tissue or primary cell culture and therefore misinterpretations.

Concerning the sc-1173 antibodies, our results are in accordance with the ones obtained by Benicky et al. (2012). They tested 6 commercially available anti-$AT_1$ receptor antibodies (including sc-1173) by Western blot and immunofluorescence. All of them failed to recognize specifically the receptor. First, they assessed the

robustness of their models ($AT_{1A}$ knock-out mice and rat hypothalamic 4B cells) by verifying the lack of expression of $AT_1$ receptor in both models using radioligand binding assays. Second, they compared the immunoreactivity of the antibodies in knock-out tissues and in cells transfected or not with $AT_1$ receptor. The six antibodies (including sc-1173) displayed very different pattern and intensity of immunoreactivity. But all tested antibodies gave signals in tissues from $AT_{1A}$ knock-out mice and non-transfected 4B cells. Despite this first report of aspecificity, the sc-1173 antibodies were still widely used in the literature up to now (Rizzetti et al. 2018; Zhao et al. 2018; Ribeiro et al. 2018) and it has just been discontinued.

Since antibodies directed against GPCR have been widely criticized for their lack of specificity, a correct validation of the used antibodies should be provided by the authors and requested by journals editors. Use of blocking peptide is a weak proof of specificity and using tagged receptors is a more reliable alternative method. Authors could also rely on one of the four criteria proposed by Michel et al. (2009) to demonstrate specific immunolabelling of receptors in tissues: a) disappearance of staining in knock-out animals of the target receptor, b) reduction of staining upon knock-down approaches such as siRNA treatment, c) selectivity of staining in immunoblots or immunocytochemistry for the target receptor *vs* related subtypes when expressed in the same cell line and/or d) antibodies raised against multiple distinct epitopes of a receptor yielding very similar staining patterns.

Given the issues concerning GPCR antibody specificity, the use of new tools to detect these membrane proteins is thus essential. Single-domain antibody (or nanobody) arouse great interest in the scientific community. Indeed, they show a promising

potential for research or therapeutic (Rasmussen et al. 2011; Maussang et al. 2013). They have smaller sizes (around 15 kDa *vs* 150 kDa for a classical antibody), they can be expressed intracellularly, they can display higher affinity and specificity and they have lower cost of production. Hence, the emergence of nanobodies is likely to replace actual existing polyclonal and monoclonal antibodies to ensure a reliable detection of GPCR proteins.

In conclusion, we demonstrate here that the monoclonal $AT_1$ receptor antibody sc-57036 should be added to the long list of non-specific antibodies targeting GPCRs.

## ABBREVIATIONS

AngII: angiotensin II

$AT_1$: angiotensin II type 1 receptor

$AT_2$: angiotensin II type 2 receptor

EGFP: enhanced green fluorescent protein

HEK293: human embryonic kidney cell

GPCR: G protein-coupled receptor

PE: phycoerythrin

PBS: phosphate buffered saline

## AUTHORS CONTRIBUTION

SL conceived and designed the experiments. MLB and SL conducted the experiments. MLB, IL, FD, SL analyzed data and wrote the manuscript. All the authors read and approved the manuscript.

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## FIGURES

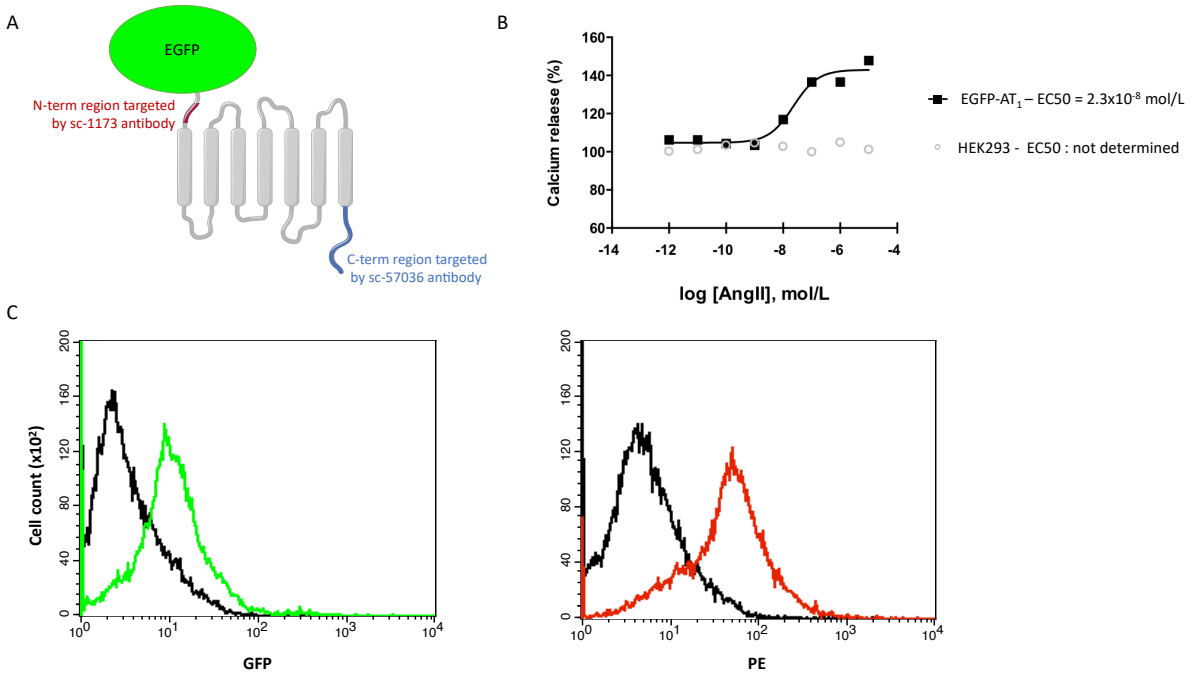


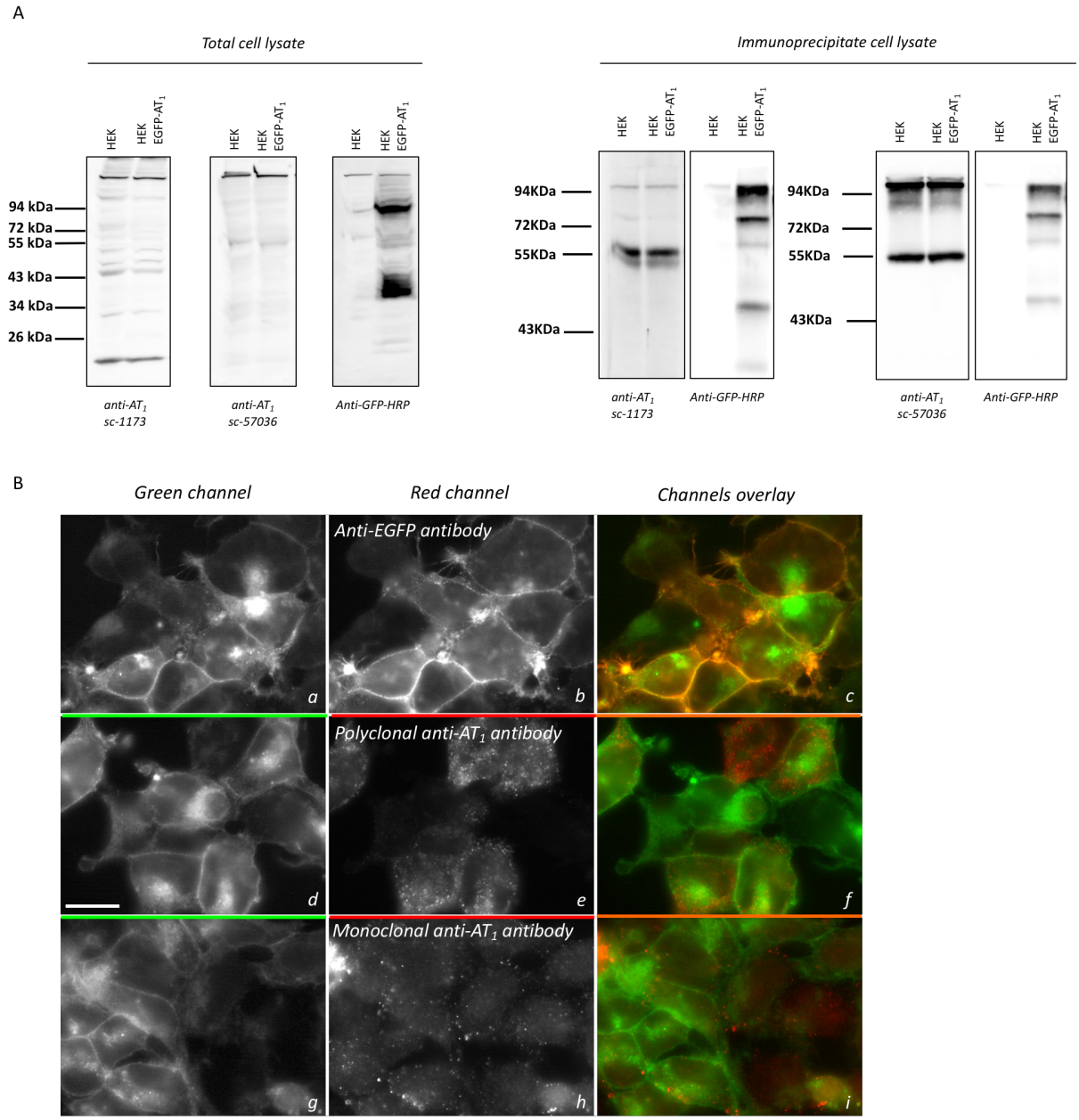

**Figure 1. In EGFP-$AT_1$ transfected cells, $AT_1$ receptor is properly localized at the cell surface**

A - Schematic view of the fusion protein EGFP-$AT_1$ receptor. The EGFP fluorophore is localized in the extracellular N-terminal part of the receptor. The sc-1173 polyclonal anti-$AT_1$ receptor targets the short N-terminal domain of the receptor while the epitope for the sc-57036 monoclonal anti-$AT_1$ receptor encompassed the whole C-terminal tail of the receptor.

B – HEK293 and EGFP-$AT_1$ transfected cells were exposed to Angiotensin II (AngII ; $10^{-12}$ to $10^{-4}$ mol/L). AngII-evoked increases in intracellular calcium were recorded over time using a Flexstation III (3 sec intervals over 220 sec) at 37°C through fluorescence emission at 520 nm (excitation at 485 nm). Peak response amplitudes were normalized to basal fluorescence levels. Each point represents the maximal value at 520 nm for a given AngII concentration.

C - The cell population expressing the EGFP-$AT_1$ receptors was compared to non-

transfected cells by fluorescence-activated cell sorting. Left panel: Histogram of cells expressing the GFP fluorescence (green line: cells expressing stably the EGFP-$AT_1$ receptors, black line: non-transfected HEK293 cells). Right panel: Histogram of cells labelled with phycoerythrin (PE): intact cells were stained with anti-GFP antibodies and secondary phycoerythrin-labeled antibodies (PE). Fluorescence intensities of non-transfected HEK cells (black line) and cells expressing stably the EGFP-$AT_1$ receptors (red line).




398

399 **Figure 2. Monoclonal and polyclonal anti-$AT_1$ receptor antibodies failed to**
400 **recognize EGFP-$AT_1$ receptor in both Western blot and immunofluorescence**
401 **assays**

402 A - Immunoblot of total cell lysates (left – 40 µg of proteins) or of anti-GFP
403 immunoprecipitated material (right – 450 µg of proteins) of non-transfected HEK293
404 cells and of cells expressing stably the EGFP-$AT_1$ receptors. Proteins were revealed
405 with either polyclonal anti-$AT_1$ sc-1173, monoclonal anti-$AT_1$ sc-57036 or anti-GFP-

HRP antibodies. Anti-GFP antibody revealed bands only in cells expressing the EGFP-$AT_1$ receptor whereas sc-1173 and sc-57036 anti-$AT_1$ antibodies revealed unspecific bands whether the receptor had been transfected or not.

B - Antibodies immunolabelling on EGFP-tagged $AT_1$ cells. (*a*, *d*, *g*) Images collected in the green channel using epifluorescence microscopy showing the fluorescence of the EGFP-tagged receptor which is localized principally at the cell plasma membrane and in some perinuclear compartments. (*b*, *e*, *h*) Images collected in the red channel, stained with primary antibodies directed against GFP epitope in *b*, or $AT_1$ receptor (*e*, sc-1173, *h*, sc-57036) followed by a red secondary antibody. Plasma membrane localization of the receptor and colocalization with the GFP signal (displayed in the channel overlay) is only detected with the anti-GFP labelling (*b* and *c* respectively), whereas no plasma membrane signal (*e* and *h*) or colocalization (*g* and *i*) could be detected when using anti-$AT_1$ antibodies. Scale bar is 10 µM.