Decreased expression of GPC1 in human skin keratinocytes and epidermis during ageing

G. Perrot, C. Colin-Pierre, L. Ramont, I. Proult, V. Bardey, C. Jeanmaire, S. Mine, L. Danoux, N. Berthelemy, F.X. Maquart, et al.

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3) Full Names of the Authors:

G. Perrot[1,4*], C. Colin-Pierre[1,4*], L. Ramont[1,2], I. Proult[1], C. Garbar[3], V. Bardey[4], C. Jeanmaire[4], S. Mine[4], L. Danoux[4], N. Berthélémy[4], FX. Maquart[1] , Y. Wegrowski[1], S. Brézillon[1]

* : these 2 authors contributed equally to the manuscript.

4) Complete Postal addresses of the affiliations of the authors :

[1] CNRS UMR 7369, Université de Reims Champagne-Ardenne, Faculté de Médecine, 51 rue Cognacq Jay, 51095 Reims, France

[2] CHU de Reims, Laboratoire Central de Biochimie, avenue du Général Koenig, 51092, Reims, France

[3] Institut Jean Godinot, 1 rue du Général Koenig, 51100 Reims, France

[4] BASF Beauty Care Solutions France S.A.S, 3 rue de seichamps, 54425 Pulnoy, France

5) Corresponding author : Stéphane Brézillon – Laboratoire de biochimie et biologie moléculaire, CNRS UMR 7369, Faculté de Médecine, 51, rue Cognacq-Jay, CS 30018, 51095 Reims, France – stephane.brezillon@univ-reims.fr, Tel. : +33 (0)3 26 91 37 34 ; Fax : +33 (0)3 26 91 80 55

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The expression of GPC1 protein was already described in epidermis. Our study shows that GPC1 expression decreases in primary keratinocytes and in skin with ageing and that it plays a role in keratinocytes response to FGF2.

Abbreviations

BSA bovine serum albumin

DAPI 4,6-Diamidino-2-phenylindole

FCS fetal calf serum

FGF fibroblast growth factor

GPC glypican

GPI glycosylphosphatidylinositol

KGF keratinocyte growth factor

PBS phosphate buffered saline

PLC phospholipase C

PPIA peptidylprolyl isomerase A

SDHA succinate deshydrogenase A

TBP TATA binding protein

TGF transforming growth factor

7) Abstract

Background: Glypicans (GPCs) are heparan sulfate cell membrane proteoglycans containing glycosylphosphatidylinositol (GPI) anchor. They play important role in cell behavior by activating/presenting numerous growth factors and cytokines. **Objectives:** the expression of GPCs was investigated in primary culture of skin keratinocytes sampled from healthy donors of different age. **Materials and Methods:** Primary keratinocytes from healthy female donors aged from 20 to 89 years old (n= 30) were either isolated from breast or abdominal skin samples (n= 27) or purchased (n= 3)

GPCs expression was examined by qPCR, immunohistochemistry and western blot. Its role in proliferation induced by fibroblast growth factor 2 (FGF2) was also studied. **Results:** glypican 1 (GPC1) was the major expressed GPC in human keratinocytes. Its expression was up to two orders of magnitude higher than other GPCs and was significantly decreased with the age of the donors. It was localized at the cell surface and associated with intracellular granules. In skin sections, GPC1 was mainly localized in basal layer of epidermis. Shedding of GPCs decreased the proliferative effect of FGF2, confirming their role of modulator of growth factor effects on keratinocytes. These results established GPC1 as an important player in epidermis biology and skin ageing.

Key words: ageing, epidermis, glypican 1, human skin, keratinocytes.

Introduction

Skin heparan sulfate cell membrane proteoglycans have a crucial role in cell response to growth factors, including keratinocyte growth factor (KGF) -1 and -2 (Yayon *et al*., 1991), fibroblast growth factors (FGFs), transforming growth factor beta (TGFβ), Wnt or Hedgehog. On the other hand, heparan sulfate supports cell attachment to the basement membrane laminins and influence keratinocyte migration (Utani *et al.*, 2001). In human, there are two families of proteoglycans bearing heparan sulfate chains: the syndecans, containing 4 genetically distinct members, and glypicans, containing six genetically distinct members.

Glypicans (GPCs) are fixed to the cell membrane *via* glycosylphosphatidylinositol (GPI) anchor. Through heparan sulfate chains, GPCs modulate the activity of FGFs in a long lasting way, especially when syndecans are displaced by shedding (Ding *et al*., 2005). For example, GPC1 but not syndecans was able to decrease the activity of FGF-7 (KGF-1) and to increase the activity of FGF-1 (acid FGF) on the BALB/MK mouse keratinocyte cell line (Bonneh-Barkay *et al.*, 1997; Reich-Slotky *et al.*, 1994).

It is well demonstrated that GPCs, as other HSPG are important for presenting growth factors to their receptor (Yayon *et al.*, 1991). This effect was demonstrated in a variety of cells. For instance, it increased the response to FGFs of keratinocytes (Bonneh-Barkay *et al.,* 1997), endothelial cells (Monteforte *et al.*, 2016), and fibroblasts (Zhang *et al*., 2001). It also increases the proliferative response to lymphatic cells *in vitro* (Bonneh-Barkay *et al.*, 1997).

The knowledge of the role of GPCs in human epidermis biology, particularly in keratinocytes, is very scanty. GPCs were shown to be present as a diffuse staining in the epidermis, and on the surface of keratinocytes (Lundqvist and Schmidtchen, 2001). In skin sections, glypican-1 was detected in the human epidermis in the cytoplasm and at the plasma membrane of the keratinocytes (Patterson *et al*, 2008).—Similar aspects were observed by Cheng and collaborators who described that recycling of glypican-1 takes place in caveolin-1 containing endosomes (Cheng *et al*, 2002). Glypican is synthesized in the endoplasmic reticulum and acquires its heparan sulfate chains in the Golgi and, when intact, is retained at the cell surface or in nearby vesicles. Glypican may also recycle while the HS chains are degraded and re-built as described by Fransson and collaborators (Fransson *et al*, 2000; Edgren *et al,* 1997; Fransson *et al*, 1995). In some cells (keratinocytes, but also endothelial cells), it was observed as perinuclear, which may reflect the recycling process of this proteoglycan (Fransson *et al.,*

1995). Among other GPC family members, the presence of GPC3 only was noted as a weak, punctuate staining of the basal layer of epidermis (Patterson *et al.*, 2008).

To maintain the skin integrity and function, keratinocytes are stimulated by different growth factors including KGF and TGF families (Beer *et al.,* 2000; Zavadil *et al.*, 2007). When the former controls cell proliferation and differentiation, the second plays a role in different, sometimes pathological, conditions like inflammation, including that arising after UV irradiation, or in wound healing. Keratinocytes from aged subjects respond less to the growth factors stimulation, which in turn results in increased fragility of epidermis to external insults.

During the senile atrophy of ageing skin, there are important changes of the proteoglycan structure and metabolism in the dermis and the epidermis (Maquart *et al.*, 2017). In the epidermis, the expression of heparan sulfate decreases, contributing to the changes of cell – growth factor cooperation (Oh *et al.*, 2011). Moreover, the activity of heparanase increases during skin photo-ageing (Kurdykowski *et al.*, 2012). There are some results showing glycosaminoglycan, including heparan sulfate, changes during epidermis ageing. However, to our knowledge, there are no data concerning the expression and abundance of heparan sulfate proteoglycans during this process.

The aim of this work was to study the expression of five known GPC genes in keratinocytes cultures obtained from donors of different ages. The expression of GPC2, also named cerebroglycan, was not considered because it was specifically expressed in neuronal differentiation (Stipp *et al.*, 1994). We have shown that *GPC1* is the most important transcript observed in keratinocytes and the abundance of this transcript decreases with age. GPC1 was detected at the cell surface but also as intracellular and perinuclear staining. The most important GPC staining was observed in the basal layer of epidermis. Elimination of GPCs from the cell surface decreased the cell response to FGF, contributing to the keratinocyte ageing.

Materials and methods

Reagents

TriReagent® was supplied by MRC Gene (Cincinnati, OH, USA). Collagen I coating matrix kit and recombinant human FGF2 (rhFGF2) were supplied by Gibco (Invitrogen, Carlsbad, CA, USA). Phosphate buffered saline (PBS) and Evans blue were purchased from BioMérieux (Marcy l'étoile, France). The GPC1 antibodies (#ab137604) and (#16700-1-AP) were provided by Abcam (Cambridge, UK) and Proteintech (Chicago, IL, USA), respectively. For double immunolabelling, the monoclonal anti-GPC1 (clone B485M, #OAMA02173, Aviva Systems Biology) and rabbit polyclonal antibody to caveolin-1 (#3238, Cell Signaling) were used. Monoclonal anti-actin I-19 was supplied by Santa Cruz biotechnology (Dallas, TX, USA).

The negative control was done using non immune immunoglobulin from rabbit (rabbit (DA1E) monoclonal antibody IgG isotype control, #3900, Cell Signaling).

Goat anti-mouse and goat anti-rabbit secondary antibodies coupled with Alexa Fluor® 488 or Alexa Fluor® 568 and ProLong® Gold mounting medium containing 4,6-Diamidino-2-phenylindole (DAPI) were supplied by Molecular Probes (Eugene, OR, USA). Phospholipase C (PLC), IgG1 isotype control (#M5284) from murine myeloma and cell proliferation reagent WST-1 were supplied by Sigma-Aldrich (Saint-Louis, MO, USA). Maxima first strand cDNA synthesis kit with dsDNAse, Maxima SyBr green/ROX kit, EZ-Link sulfo-NHS-LC-biotin, streptavidin magnetic beads and paraformaldehyde were purchased from ThermoFisher scientific (Waltham, MA, USA). Recombinant human GPC1 (#4519-GP) was purchased from R&D Systems (Minneapolis, MN, USA).

Isolation and culture of keratinocytes

Primary keratinocytes from healthy female donors (n= 30) were either isolated from breast or abdominal skin samples (n= 27) or purchased (n= 3) from Promocell (Heidelberg, Germany) or Lonza (Basel, Switzerland). The age of the healthy donors are listed (Table S1). For keratinocyte isolation, breast healthy skin samples were obtained after informed consent from Caucasian female donors undergoing breast surgery. Skin piece were sample near nipple and are not considered like photoexposed. Epidermis/dermis separation was performed using 1.25% trypsin-EDTA (Gibco) for 1h at 37°C. Then, epidermis was separated from dermis,

dipped in a solution containing 10% FCS and keratinocytes were detached by pipetting. After centrifugation at 270g for 7 min, keratinocytes were transferred to KGM-Gold-supplemented medium (Lonza) and seeded on collagen I-coated flasks (Corning Biocoat™, VWR Radnor, PA, US). They were cultured at 5% CO_2 and 37°C, with regular changes of the medium, 3-times a week, up to 80% of confluence. Then, they were detached with ReagentPack™ Subculture Reagents (Lonza) and used for experiments or freezed at -80°C in Synth-a-freeze® (Gibco).

RNA isolation and quantitative polymerase chain reaction

Primary keratinocytes isolated from n=10 (Fig. 1) or n=15 (Fig. 2) healthy donors seeded on collagen I plates were grown in complete medium up to reach confluency. Total RNA was extracted with TriReagent® according to the manufacturer's instructions. Chloroform was added at a 5:1 ratio and the mix was incubated for 3 min before centrifugation at 12,000g for 15 min at 4°C. Aqueous phase was collected and an equal volume of isopropanol was added to precipitate RNA. The mixture was incubated for 10 min at room temperature and centrifuged at 12,000g for 10 min at 4°C. The supernatant was discarded and the RNA pellet was washed twice with 1mL of 75% ethanol. Finally, it was dried and resuspended with 50µL of DEPC water. The amount of isolated RNA was quantified by measuring its absorbance at 260nm with Nanovue plus (GE Healthcare) and their quality was controlled by migration in 1% agarose gel.

Complementary DNA was synthetized with Maxima first strand cDNA synthesis kit with dsDNAse, using 250ng of total RNA, according to the manufacturer's instructions. Incubation conditions were 25°C for 10 min, 50°C for 30 min, then 85°C for 5 min.

Real-time PCR was performed in 25µL final volume using Maxima SyBr green/ROX kit in presence of 300nM of probes and 0.5µL of cDNA. Fluorescence detection was carried out with Agilent MX300P device and MxPro software (Santa Clara, CA, US). All reactions were made in duplicate and a no template control (NTC) was performed for each gene. Relative expression of different gene transcripts was calculated by the ΔCt method. The Ct (threshold cycle) of interest gene was compared with average of the Ct of three different reference genes: peptidylprolyl isomerase A (*PPIA*), succinate deshydrogenase A (*SDHA*) and TATA binding protein (*TBP*), according to the recommendations of the MIQE Guidelines (Bustin *et al.,* 2009), specific papers describing the use of housekeeping genes in real-time PCR (Kozera and Rapacz, 2013) and dedicated to human keratinocytes (Bar *et al.*, 2009).

These genes were chosen for their constant expression in primary keratinocytes from different donors (Balogh *et al.*, 2008; Bar *et al.,* 2009). Relative quantitative expression was determined as $2^{-\Delta Ct}$. Melting point determination and gel electrophoresis confirmed the expected size and identity of PCR products. Genes of interest and utilized primers are presented in supplementary Table S2.

Immunocytochemistry and immunohistochemistry

Primary keratinocytes from 5 heathy donors aged between 39 to 44 years old for the young group and from 5 healthy donors between 65 to 89 years old for the old group were seeded (50000 cells/well) in 24-well plates on glass coverslips coated with collagen I (coating matrix kit) for 1h. Excess of collagen I was washed with PBS before seeding cells. Cells were grown at least 24h before fixation with 4% paraformaldehyde for 5 min at room temperature. Then, saturation with 3% bovine serum albumin (BSA) was performed for 1h and cells were incubated overnight at 4°C with 10µg/mL GPC1 antibody (#ab137604, Abcam or #16700-1-AP, Proteintech or clone B485M, Aviva Systems Biology). Negative controls were performed using the rabbit or mouse IgG isotype control. The secondary antibody labelled with Alexa Fluor® 488 or Alexa Fluor® 568 was used at a dilution of 1:1000 for 45 min at room temperature. The coverslips were mounted using ProLong® Gold containing DAPI.

Skin samples were frozen in liquid nitrogen and tissue blocks were sliced (5µm thick). The sections were fixed with 4% paraformaldehyde for 5 min at room temperature. Saturation with 3% BSA was performed for 1h. Then, skin samples were incubated overnight at 4°C with 10µg/mL GPC1 antibody (#ab137604, Abcam or #16700-1-AP, Proteintech). Negative control was performed using the rabbit IgG isotype control. Samples were incubated with the secondary antibody labelled with Alexa Fluor® 488 for 60 min at room temperature. The sections were mounted using ProLong® Gold containing DAPI.

Fluorescence acquisition was realized with confocal laser scanning microscope LSM 710 NLO or Axio observer (Zeiss, Oberkochen, Germany). Immunofluorescence was quantified using Image J software to measure the number of fluorescent pixels normalized to the number of pixels of the total surface of the epidermis. Thus, the semi-quantitative fluorescence analysis took into account the decrease of the thickness of the epidermis with age. The fluorescent labelling observed in the stratum corneum is not specific and has not been taken into account thanks to a determined threshold.

Proliferation assay

Keratinocytes, isolated from four different healthy donors aged from 20 to 39 years old, were seeded on collagen I-coated 96-well plates (n= 7000 cells/well) and grown at least 24h with complete culture medium. Then, cells were depleted of supplements for 24h. The following steps were performed as previously described (Matsuda *et al.*, 2001). Briefly, cells were pre-incubated with 0.5u/mL of phospholipase C (PLC) for 1h and treated for 24h with supplement-free medium without or with 0.1u/mL of PLC and/or 20ng/mL of rhFGF2. Cells were quantified with colorimetric method using WST-1 dye, according to manufacturer instructions. The absorbance was measured at 450nm after 90 min of incubation with WST-1 substrate.

Membrane protein isolation by cell surface biotinylation

Membrane protein isolation was performed as previously described (Perrot *et al.*, 2012). Keratinocytes were washed twice with ice-cold PBS, and cell surface proteins were biotinylated with PBS containing 0.5 mg/ml of EZ-Link sulfo-NHS-LC-biotin for 30 min at 4°C. After three washes, cells were incubated with 100mM glycine in PBS for 15 min at 4°C to eliminated non-fixed biotin. Cells were washed three times before protein extraction in ice-cold RIPA buffer supplemented with protein cocktail inhibitor. Cell extracts were pelleted at 10000g for 10 min at 4°C, and protein quantification was performed. Solubilized biotinylated proteins (500µg) were then specifically affinity purified using 50µl of streptavidin magnetic beads. Incubation was performed 1h at room temperature with gentle orbital agitation, followed by two washes with Tris Buffered Saline-Tween (Tris/HCl 50mM pH7.5, NaCl 150mM, Tween® 20 0.1% v/v) to remove nonspecific binding. For immunoblotting experiments, 40µl of 2X SDS-containing Laemmli (Tris 0.5M, pH 6.8 ; bromophenol blue 0.5% p/v ; SDS 10% p/v ; saccharose 20% p/v ; β-mercaptoéthanol 10% v/v) was added, and samples were heated at 100°C during 5 min, and resolved by SDS-PAGE followed by immunoblotting analysis.

Western immunoblotting

Protein samples were prepared as described above. They were separated on 9% SDS-polyacrylamide gel, transferred to polyvinylidene difluoride membranes (Millipore, Billerica, MA, USA), and blocked with 5% Blotting-grade Blocker (Biorad, Hercules, CA, USA) in Tris Buffered Saline-Tween. The blots were incubated with appropriate primary antibodies

that recognize GPC1 (#16700-1-AP, Proteintech) or actin (#I-19). Bound primary antibodies were detected using secondary antibodies conjugated with horseradish peroxidase and ECL Prime kit (GE Healthcare, Little Chalfon, UK) and luminescence was spotted with Chemidoc MP (Biorad).

Statistical analysis

Results were expressed as mean +/- SEM. Statistical analyses were performed using non parametric Mann-Whitney and Wilcoxon tests.

Results

Effect of ageing on the expression of glypican genes in human keratinocytes.

To study the gene expression of the different GPCs, the RNA of ten different adult donor skins was extracted from keratinocytes in primary culture, followed by reverse transcription and qPCR. The relative expression of five GPC genes (*GPC1* and *GPC3* to *6*) was normalized by the expression of three reference genes (*PPIA*, *SDHA* and *TBP*), as shown in figure 1. Among all GPCs studied, *GPC1* was the most expressed gene in keratinocytes from human adults. The expression of *GPC4* and *GPC6* genes was about 70 times and 80 times lower, respectively, than those of *GPC1* and the expression of *GPC3* gene about 500 times lower than that of *GPC1* (Fig. 1). Only traces of *GPC5* mRNA were detected (see below).

The relative expression of GPC genes was measured in keratinocyte cultures from 15 patients of different ages (Fig. 2). Statistically significant decrease of GPC gene expression with age was observed for *GPC1* and *GPC3*. While the decrease of *GPC1* mRNA was linear with age, with a high R coefficient, that of *GPC3* better fits to the inverse logarithmic function. This suggests a minimal expression of *GPC3* in older subjects. Other GPCs did not show significant changes of their expression during ageing. Moreover, among the 15 subjects tested, the keratinocytes from 9 subjects did not express *GPC5* mRNA, independently of the age of the donors. These results indicate that *GPC1* is the major GPC expressed by human keratinocytes whatever the age of the donor.

Characterisation of GPC1 expression in human keratinocyte culture and epidermis.

As *GPC1* mRNA steady state level in keratinocytes was at least two orders of magnitude higher than other GPCs mRNA, we focused on the GPC1 protein expression. In keratinocytes primary cultures, immunocytochemistry observations indicated that GPC1 was present both in the cytoplasm and at the plasma membrane (Fig. 3b). In the cytoplasm, GPC1 seemed located in vesicular compartment.

The presence of GPC1 was also detected on human skin sections. As shown in Fig. 3d, GPC1 was expressed in skin, particularly in the epidermis, with a stronger expression of GPC1 in the epidermal basal layer as shown in the enlargement of dermal-epidermal junction (Fig. 3e).

The expression of GPC1 was also studied by immunoblotting in total cell extract of keratinocytes with a recombinant human GPC1 (rhGPC1) loaded as positive control. Western blot revealed a 62kDa protein corresponding to the heparan sulfate-deprived form of GPC1 (Fig. 3f).

To check that the decrease of *GPC1* gene expression observed in keratinocyte primary cultures was accompanied by a decrease of the GPC1 protein, we performed immuno-histochemical staining of GPC1 protein on skin samples from 10 different young or old patients (Figs. 4a-c). Then, we compared the ratio between the number of fluorescent pixels present in the epidermis and the total area of the epidermis for each patient. The results presented in figure 4d show that the immunostaining of GPC1 protein decreased by 23%, in the skin of old patients compared to young patients.

Effect of GPCs shedding on cell proliferation

GPCs are GPI-anchored proteoglycans undergoing a shedding process in physiological conditions (Zhang and others 2001). To study the influence of GPCs on cell proliferation, we took advantage on the fact that phospholipase C (PLC) sheds GPCs to the culture medium. As shown by western blot (Figs. 5a and b), incubation of the cells with PLC decreased GPC1 expression at the cell membrane and in the cytoplasm. In parallel, keratinocytes treated with PLC responded less than non-treated cells to FGF2 stimulation (Fig. 5c). These results suggest, that GPCs, and particularly GPC1 is an important contributor to the regulation of cell proliferation and cell response to growth factors, especially FGF2.

Discussion

One of the main factors of epidermis senile atrophy is the decrease of keratinocytes renewal, especially in women (Calleja-Agius et al., 2007). Keratinocytes, for their physiological function, need to be stimulated by growth factors. GPC1 is a main co-receptor for different growth factors, including FGF2 (Matsuo and Kimura-Yoshida, 2013).

Our results demonstrate that keratinocytes from adult donors express mainly GPC1. The protein was expressed on the cell membrane but it was also found in cytoplasmic vesicles and in perinuclear space. Glypican is synthesized in the endoplasmic reticulum and acquires its heparan sulfate chains in the Golgi and, when intact, is retained at the cell surface or in nearby vesicles. Glypican may also recycle while the HS chains are degraded and re-built, as described by Fransson and collaborators (Fransson *et al*, 1995; Edgren *et al,* 1997; Fransson *et al*, 2000). These results corroborate with previous studies done on other cell systems, e.g. fibroblasts and endothelial cells showing the rapid recycling of surface protein *via* caveolin coated pits (Cheng *et al.*, 2002). Double immunolabellings of primary cell culture of human keratinocytes for caveolin (a marker of caveolae and endosomes), and GPC1 were performed. Confocal Z-stack images of GPC1 and caveolin labellings showed clearly partially overlapping yellow color suggesting a co-localization and a distribution of GPC1 in caveolae (supplementary Fig. S1). Our western blot results, showing that the major part of GPC1 expressed by keratinocytes has a molecular mass of 62 kDa, which corresponds to the non glycosylated form.

The expression of other glypican genes in keratinocyte primary cultures is very weak in comparison of those of GPC1. Moreover, in normal conditions, keratinocytes express a small quantity of syndecan-1 (Le Bitoux *et al.*, 2009). This last is mainly expressed during wound healing and inflammatory activated cells. So, the regulation of growth factor activity on keratinocytes in physiological state may rely, at least partly, on GPC1 and its heparan sulfate chain.

It was shown that in different cancer systems the overexpression of GPC1 activates FGF2 pathway (Melo *et al.*, 2015; Whipple *et al.,* 2012). Interestingly, the *GPC1* gene contains the coding sequence of miR-149. This miRNA regulates not only *GPC1* expression but also *FGFR1* expression, coding the important receptor for FGF2 (Chamorro-Jorganes *et al.*, 2014). The same type of regulation may also concern the keratinocytes, as the shedding of GPC1 by PLC decreases FGF2-driven mitogenic activity of the cells.

The expression of *GPC1* gene in keratinocytes significantly decreases with age of the donors.

It is known that the expression of some genes may also decrease during ageing (Carrino et al., 2000) but it is not a general phenomenom. For instance, the expression of the small leucine rich proteoglycans, decorin (Vuillermoz *et al.,* 2005) and versican (Oh *et al.*, 2011), and of MMP-2 (Wang *et al.*, 2003) increases with age.

In skin, the expression of GPC1 protein in epidermis was poorly described; it was detected in all layers and particularly in pericellular region of keratinocytes (Harthan *et al.,* 2013; McKnight *et al.,* 2015). In our study, the expression of GPC1 protein was more pronounced in the basal layer of the epidermis as described by Patterson and collaborators (Patterson *et al*, 2008) and decreased with the age of the donor. GPC1 staining was not observed in the basement membrane but was predominantly expressed at the basal layer of the epidermis.

As GPC1 contributes to the regulation of cell proliferation in normal keratinocytes, decreased expression in older patients may influence different phenomena contributing to senile epidermal atrophy and dermatoporosis. In a turkey satellite cell model, changes in proliferation, FGF2 responsiveness and decreased *GPC1* expression were observed with age (Monteforte *et al*., 2016). Decreased *GPC1* expression with age and subsequent loss of FGF2 responsiveness might partially explain the decrease of skin renewal in old person. In our study, depletion of GPC1 was performed by incubation of keratinocytes with phospholipase C which is known to cleave glypicans of their GPI anchor at the cell surface membrane. A significant decrease of the immunolabelling of GPC1 was observed after 24h incubation of keratinocytes with PLC as compared to control keratinocytes. Altogether, the results of the present study suggest more a correlation than a cause-effect relationship which still needs to be demonstrated.

In addition, it would be interesting to study the role of sulfatase-2 since sulforaphane suppresses skin cancer *via* blocking sulfatase-2 with subsequent elevation in HSPGs and reduction in glypican-3 (Alyoussef *et al,* 2019). In pathological conditions, like senile purpura, the topical use of growth factors like FGF2 was proposed. It was also suggested for preventing dermatoporosis (McKnight *et al.*, 2015). On the other hand, nanoliposomes delivery of GPC1 was shown to increase angiogenesis induced by FGF2, compared to FGF2 alone (Lundqvist and Schmidtchen, 2001). This technology allowed to reverse the loss of

GPC1 expression observed in blood vessels and skin of diabetic mice. Increasing GPC1 expression in senile epidermis might be an important factor contributing to the improvement of skin quality in the ageing process.

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Figure legends

Fig. 1. Expression of GPCs mRNA in human adult keratinocytes in primary culture. Quantitative RT-PCR was normalized to three reference genes (PPIA, SDHA and TBP). GPC1 mRNA is the most expressed GPC in keratinocytes. Results are expressed as mean +/- SEM of 12 different keratinocytes from breast of 12 different females aged of 58 +/- 18 years.

Fig. 2. Expression of GPCs mRNA during ageing. Human keratinocytes in primary culture from 15 different female patients aged from 23 to 84 years old were analyzed by quantitative RT-PCR. RNAs were normalized to three reference genes (PPIA, SDHA and TBP). GPC1 and GPC3 mRNA expression significantly decreased with ageing. *p* values are shown on the figure.

Fig. 3. Characterisation of GPC1 expression in human keratinocytes and skin sections. Representative labelling of GPC1 in human keratinocytes from arm's skin of 23 years old female provided by Promocell. Cells were stained with rabbit control isotype (**a**) or anti-GPC1 ab137604 (**b**). GPC1 is located at the plasma membrane and in the cytoplasm of keratinocytes. Representative labelling in skin sections from abdominal skin of 25 years old female stained with rabbit control isotype (**c**) or anti-GPC1 (**d**). GPC1 is expressed in the epidermis and mainly present in the basal layer. (**e**) Enlargement of the frame drawn in (**d**). Samples were counterstained with DAPI for nuclei visualization (**a-e**). (**f**) Detection of GPC1 in human keratinocytes by Western immunoblotting. Recombinant human GPC1 (rhGPC1) was used as positive control. Scale bars : 20µm for (**a-b**), 30µm for (**c-d**) and 7µm for (**e**). E : epidermis, D : dermis, white arrows : epidermis basal layer, KCS : keratinocytes.

Fig. 4. Expression of GPC1 in skin during ageing. Representative GPC1 labelling in skin sections of a 39 years old (a) and a 69 years old (b) patients with B485M antibody. Negative control performed with mouse control isotype (c). Samples were counterstained with DAPI for nuclei visualization (a-c). (d) Graph representing the quantification of fluorescent pixels of the epidermis normalized to the total area of the epidermis. Results are expressed as mean +/- SEM (n=5), each group contained 5 patients aged between 39 to 44 years old (41,2 +/-2,6 y) for young group and between 65 to 89 years old (73,4 +/- 9,2 y) for old group. Fluorescent area decreases in skin sections of elders. * : $p<0.05$ (Mann-Whitney test). Scale bar : 20µm.

Fig. 5. Shedding of GPC1 decreases keratinocytes proliferation in response to FGF2. (a) Keratinocytes were treated with 0.5u/mL of PLC for 1h at 37°C. Cell surface and whole cell proteins were extracted and analyzed by Western immunoblotting. (b) Quantification of GPC1 protein expression presented in panel (a). The same quantity of protein was deposed in each conditions, membrane and total GPC1 protein expression was normalized with actin. Results are expressed as mean +/- SD of two different experiments. The PLC cleaved GPCs and decreased GPC1 expression at cell membrane. (c) Cells were incubated without or with rhFGF2 and/or PLC for 24h as described in the Materials and Methods section, and living cells were measured by colorimetric assay using wst-1 dye. The PLC treatment decreased keratinocytes response to FGF2. Each experiment was performed with 8 replicates and normalized with non-treated cells. Results are expressed as mean +/- SEM of four different experiments realized with different keratinocytes (isolated from 4 different patients aged from 20 to 39 years old). ns : non-significant, *** : $p<0.001$ with Mann-Whitney test. PLC, phospholipase C.

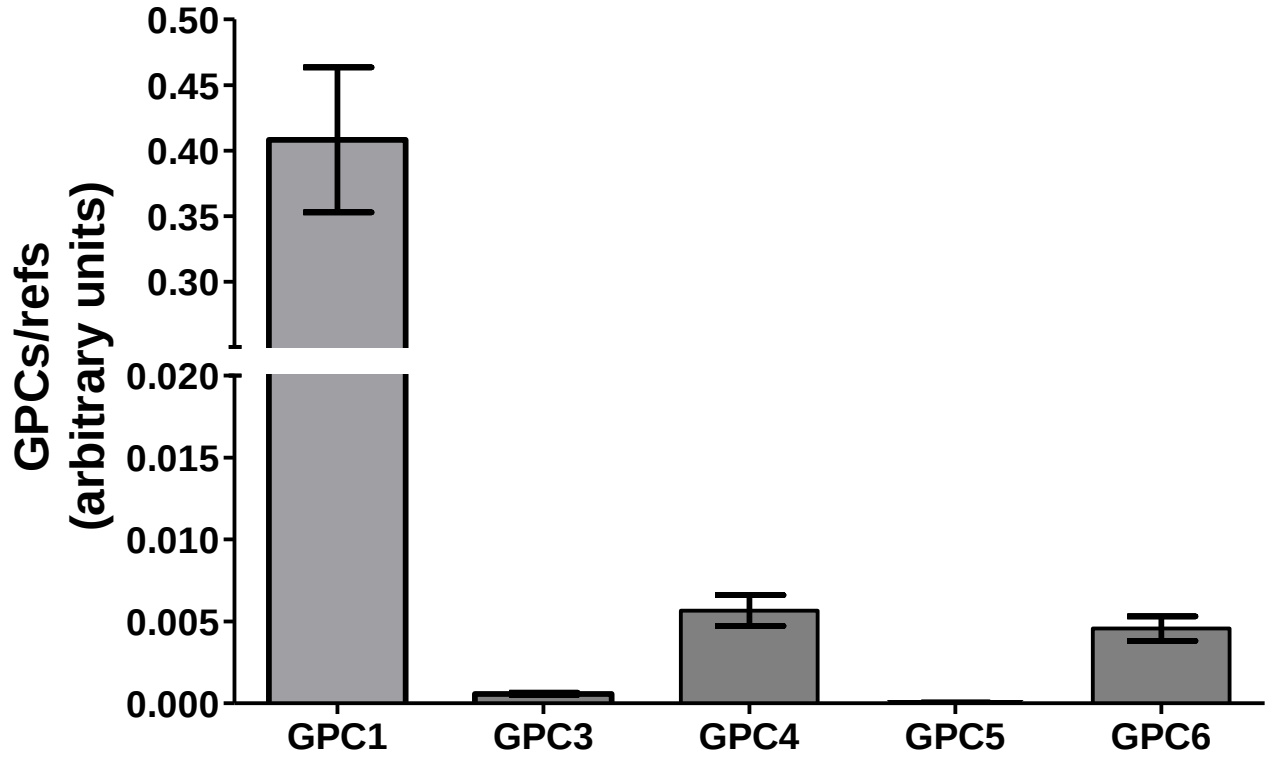
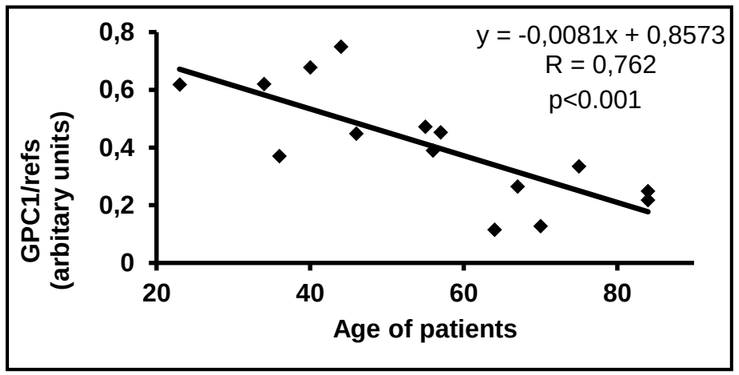
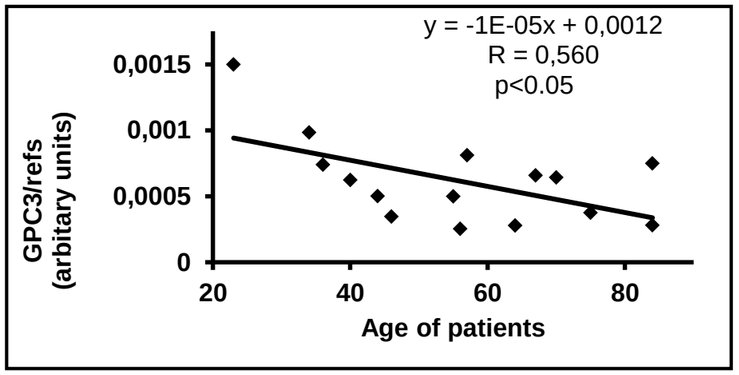
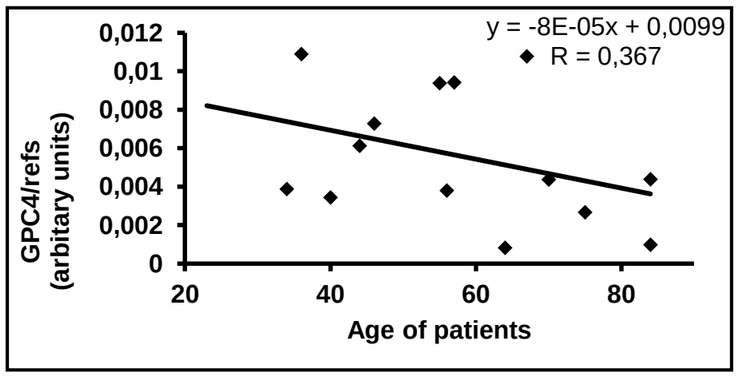
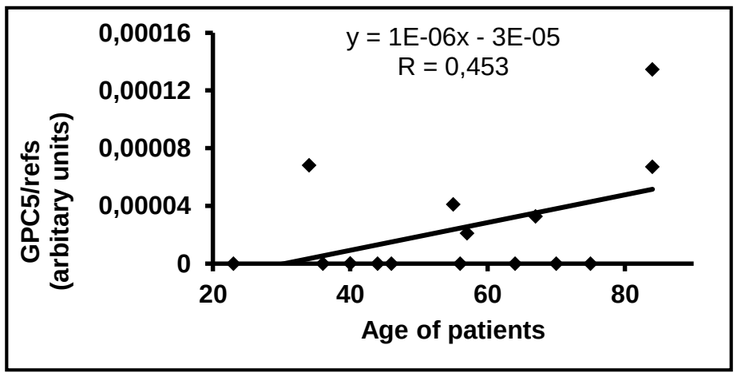

GPCs/refs
(arbitrary units)
0.50
0.45
0.40
0.35
0.30
0.020
0.015
0.010
0.005
0.000
GPC1
GPC3
GPC4
GPC5
GPC6

Figure 2


y = -0,0081x + 0,8573
R = 0,762
p<0.001
GPC1/refs (arbitary units)
0,8
0,6
0,4
0,2
0
20
40
60
80
Age of patients


y = -1E-05x + 0,0012
R = 0,560
p<0.05
GPC3/refs (arbitary units)
0,0015
0,001
0,0005
0
20
40
60
80
Age of patients


y = -8E-05x + 0,0099
R = 0,367
GPC4/refs (arbitary units)
0,012
0,01
0,008
0,006
0,004
0,002
0
20
40
60
80
Age of patients


y = 1E-06x - 3E-05
R = 0,453
GPC5/refs (arbitary units)
0,00016
0,00012
0,00008
0,00004
0
20
40
60
80
Age of patients

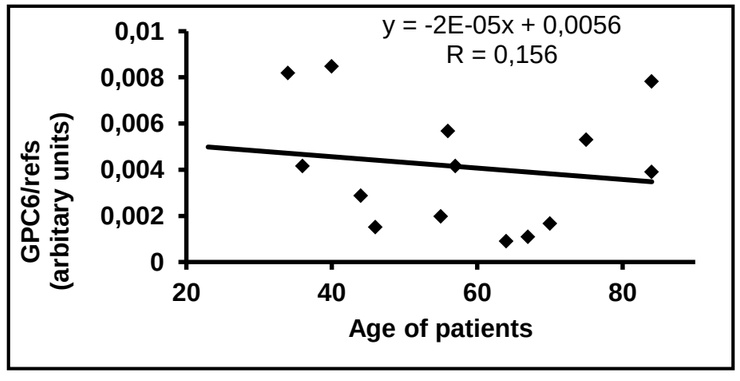
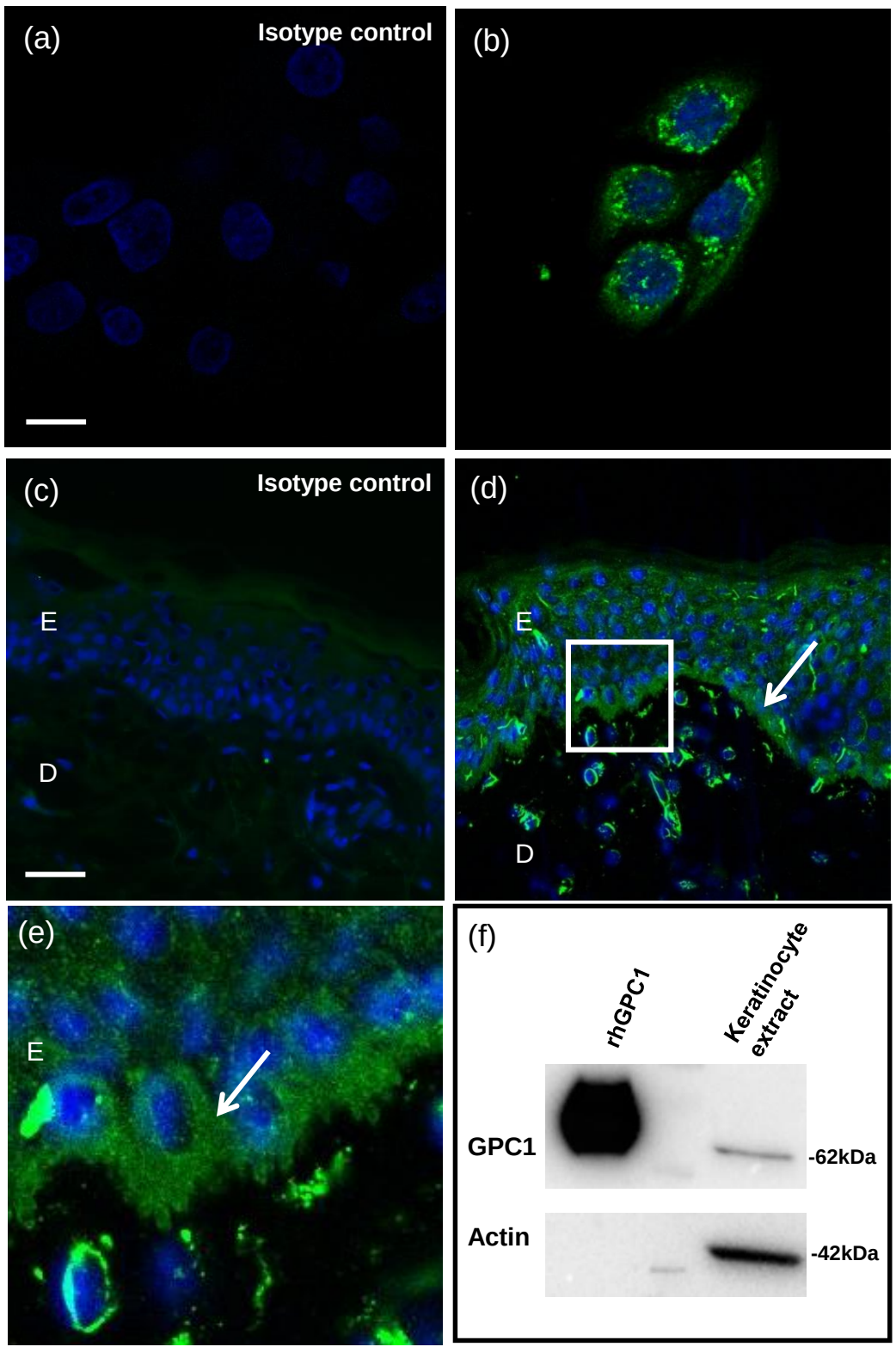

y = -2E-05x + 0,0056
R = 0,156
GPC6/refs (arbitary units)
0,01
0,008
0,006
0,004
0,002
0
20
40
60
80
Age of patients

Figure 3


(a)
Isotype control
(b)
(c)
Isotype control
E
D
(d)
E
D
(e)
E
(f)
rhGPC1
Keratinocyte extract
GPC1
-62kDa
Actin
-42kDa

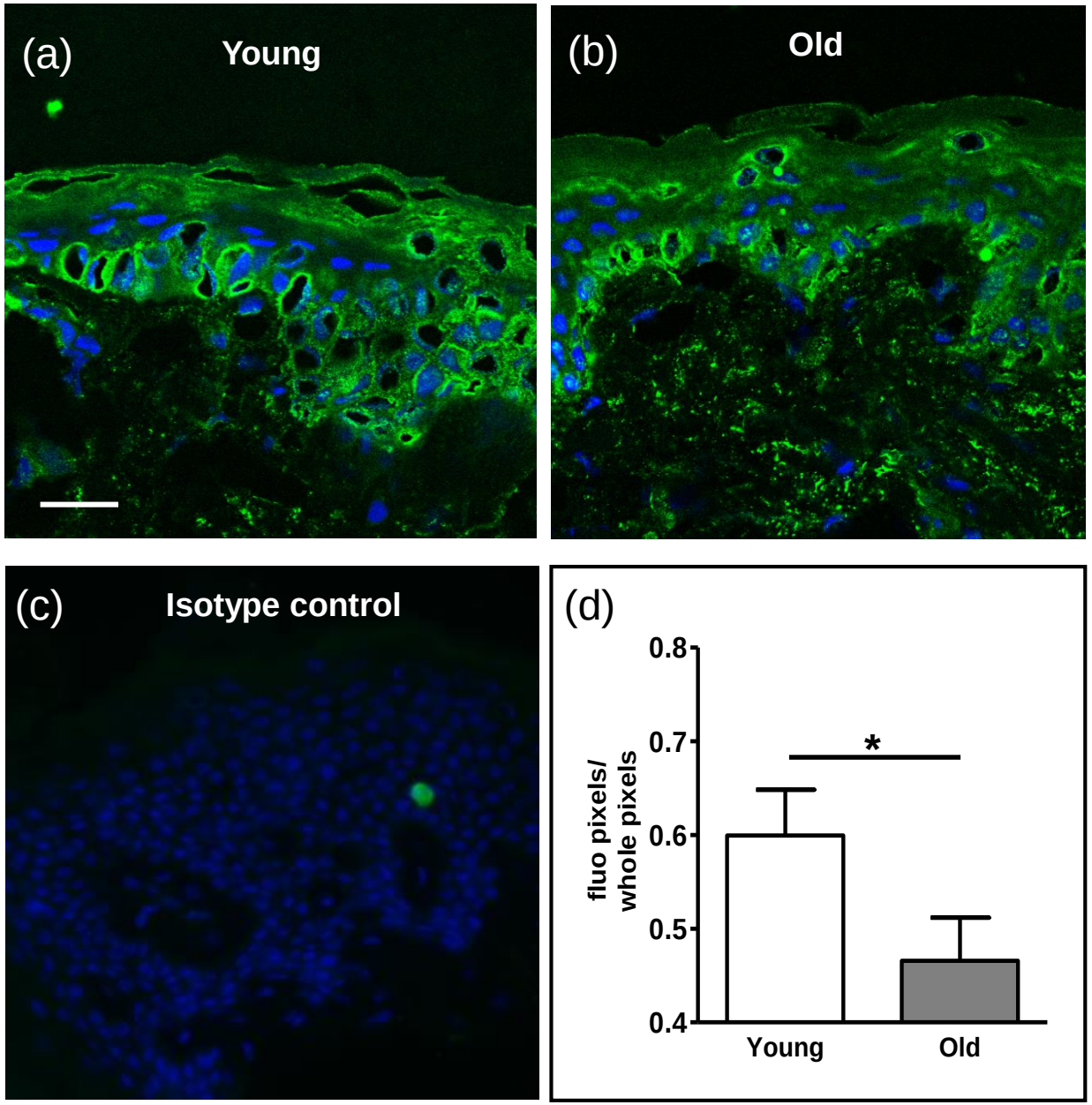
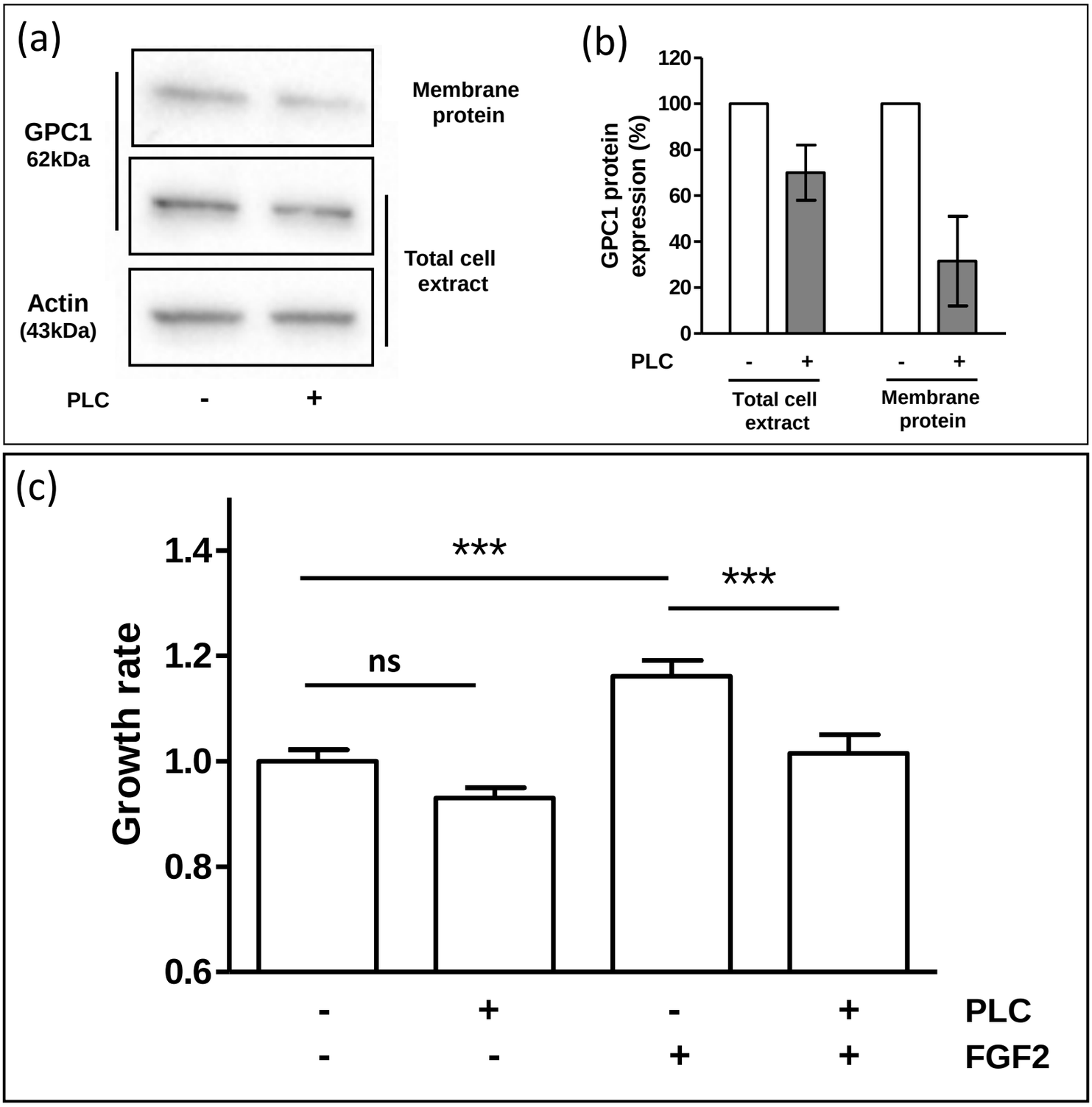

(a)
Young
(b)
Old
(c)
Isotype control
(d)
fluo pixels/
whole pixels
0.8
0.7
0.6
0.5
0.4
*
Young
Old

Figure 4

Figure 5


(a)
GPC1
62kDa
Actin
(43kDa)
Membrane protein
Total cell extract
PLC
-
+
(b)
GPC1 protein expression (%)
120
100
80
60
40
20
0
PLC
-
+
-
+
Total cell extract
Membrane protein
(c)
Growth rate
1.4
1.2
1.0
0.8
0.6
***
***
ns
-
+
-
+
PLC
-
-
+
+
FGF2