

27 **Abstract**

28 **Background:** Up-regulation of kallikreins (KLK) including KLK5 has been reported in atopic
29 dermatitis (AD). KLK5 has biological functions which include degrading desmosomal proteins and
30 inducing pro-inflammatory cytokine secretion through protease activated receptor 2 (PAR2).
31 However, due to the complex interactions between various cells in AD inflamed skin, it is difficult
32 to dissect the precise and multiple roles of up-regulated KLK5 in AD skin.

33 **Objective:** We investigated the effect of up-regulated KLK5 on the expression of epidermal related
34 proteins and cytokines in keratinocytes and on skin architecture.

35 **Methods:** Lesional and non-lesional AD skin biopsies were collected for analysis of morphology
36 and protein expression. The relationship between KLK5 and barrier related molecules was
37 investigated using an *ex-vivo* dermatitis skin model with transient KLK5 expression and a cell
38 model with persistent KLK5 expression. The influence of up-regulated KLK5 on epidermal
39 morphology was investigated using an *in vivo* skin graft model.

40 **Results:** Up-regulation of KLK5 and abnormal expression of desmoglein 1 (DSG1) and filaggrin
41 (FLG), but not PAR2 were identified in AD skin. PAR2 was increased in response to transient up-
42 regulation of KLK5, while persistently up-regulated KLK5 did not show this effect. Persistently up-
43 regulated KLK5 degraded DSG1 and stimulated secretion of IL-8, IL-10 and TSLP independent of
44 PAR2 activity. With control of higher KLK5 activity by the inhibitor SFTI-G, restoration of DSG1
45 expression and a reduction in AD-related cytokine IL-8, TLSP and IL-10 secretion were observed.
46 Furthermore, persistently elevated KLK5 could induce AD-like skin architecture in an *in vivo* skin
47 graft model. .

48 **Conclusion:** Persistently up-regulated KLK5 resulted in AD-like skin architecture and secretion of
49 AD-related cytokines from keratinocytes in a PAR-2 independent manner. Inhibition of KLK5-
50 mediated effects may offer potential as a therapeutic approach in AD.

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53 **Key messages**

- 54 • Persistently up-regulated KLK5 induces PAR2-independent IL-8, IL10 and TSLP secretion,
55 causing abnormal keratinocyte growth and AD-like skin architecture.
- 56 • Inhibition of KLK5-mediated effects restored DSG1 expression and decreased AD-related
57 cytokine expression, thus suggesting that KLK5 inhibition may be useful as a potential
58 treatment for AD.

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60 **Capsule summary**

61 Persistently increased serine protease kallikrein 5 modifies skin barrier proteins, upregulates AD-
62 related cytokine expression and induces AD-like skin architecture. Inhibition of KLK5 may offer
63 potential as a treatment strategy in AD.

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65 **Key word**

66 Kallikrein 5, atopic dermatitis, skin barrier, serine protease inhibitor, SFTI

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68 **abbreviations**

69 KLK5 (kallikrein), DSG1 (desmoglein 1), PAR2 (protease activated receptor 2), CAP18
70 (cathelicidin precursor cationic antimicrobial protein 18), AD (atopic dermatitis), NS (Netherton
71 syndrome), FLG (filaggrin), UT (untransduced keratinocytes), AP (PAR2 agonist), TSLP (thymic
72 stromal lymphopoietin), rKLK5 (recombinant KLK5).

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78 **Introduction**

79 Tissue kallikreins (KLKs) are a family of fifteen (chymo)trypsin-like serine proteases which
80 function through proteolytic cascades in the skin. Eight KLKs are expressed in the skin with KLK5
81 being one of the three most important, the others being KLK7 and KLK14¹. KLK5 is produced as
82 an inactive precursor from keratinocytes and activated by matriptase and KLK14^{2,3}, but can also
83 undergo self-activation. It is able to activate other KLKs, therefore, KLK5 has been considered to
84 be the initiator of KLK activation cascades within the skin⁴.

85 KLK5 is expressed in the outmost layers of the epidermis, and the importance of its biological
86 function in the epidermal barrier was initially discovered through studies on Netherton Syndrome
87 (NS), a rare severe autosomal recessive skin disorder caused by mutations in the *SPINK5* gene^{4,5}. In
88 NS, *SPINK5* mutations cause loss of function of its encoded protein LEKTI, a multi-domain serine
89 protease inhibitor, leading to elevated activity of KLK5. This results in cleavage of intercellular
90 adhesion protein desmoglein 1 (DSG1), causing excessive desquamation of corneocytes and leading
91 to a severely defective skin barrier, a major cause of early neonatal death in NS⁶. In addition to its
92 involvement in DSG1 degradation, KLK5 is able to activate protease activated receptor 2 (PAR2), a
93 subfamily of G protein-coupled receptors, and trigger expression of pro-inflammatory cytokines
94 such as IL-8^{7,8}. KLK5 is also involved in the innate immune system within the skin by cleaving the
95 cathelicidin precursor cationic antimicrobial protein 18 (CAP18) at its c-terminus to produce 37
96 amino acid peptide LL-37, a major antimicrobial peptide with broad-spectrum antimicrobial
97 activity⁹.

98 Up-regulation of kallikreins including KLK5 has been reported in many chronic inflammatory skin
99 diseases including atopic dermatitis (AD)^{10,11}. AD is a multifactorial disease caused by complex
100 interactions between genetic and environmental factors, with evidence that irritants (such as those
101 contained in soaps) can further damage the skin barrier and exacerbate the inflammation in AD
102 patients¹². In the past decade, significant progress has been made in the area of molecular genetics
103 with identification of several genes linked to AD including *SPINK5*, *KLK7* and *FLG*¹³⁻¹⁵. These

104 findings have led to the proposition that an impaired epidermal barrier is the primary event,
105 allowing percutaneous allergen penetration and causing an enhanced Th2-skewed immune-
106 response¹⁶. The induced inflammatory response further compromises barrier function, resulting in
107 abnormal expression, activity and assembly of skin barrier related proteins, enzymes and lipids.
108 Aberrant up-regulation of KLK5 in AD skin has been reported^{10,11} and increased KLK5 may play a
109 key role in the pathogenesis of the dysfunctional skin. However, due to the complex interactions
110 between various cells in AD inflamed skin, it is difficult to dissect the exact role of up-regulated
111 KLK5 in AD skin.

112 In this study, we confirmed up-regulation of KLK5 and abnormal expression of KLK5 down-stream
113 molecules DSG1 and filaggrin (FLG), but not PAR2 in AD skin. We also identified significantly
114 increased KLK5 and PAR2 expressions in an *ex-vivo* dermatitis model, but not in the cell model
115 with persistent over-expression of KLK5, illustrating different responses of PAR2 to transient or
116 persistent KLK5 stimulation. We also demonstrated that increased IL-8, IL-10 and TSLP in
117 keratinocytes with persistently expressed KLK5 was independent of PAR2 activity, and that
118 inhibition of KLK5 activity with the serine protease inhibitor SFTI-G reduced cytokine production
119 and normalised DSG1 protein expression. Furthermore, persistent KLK5 over-expression alters
120 keratinocyte behaviour *in vivo*, resulting in epidermal acanthosis similar to that observed in AD skin,
121 indicating a key role for KLK5 in AD pathology.

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129 **Materials and Methods**

130 Skin biopsies and haematoxylin and eosin staining (H&E)

131 Skin biopsies were taken from non-lesional and lesional skin from five AD patients. Five age-
132 matched healthy donors were also obtained. This study was approved by the local ethics committee
133 (LREC number 05/Q0508/106). Skin samples were formalin fixed paraffin embedded, and H&E
134 staining was performed on 6 µm thickness paraffin skin sections using standard histochemistry
135 techniques.

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137 Immunostaining and protein quantification

138 Immunofluorescence and immunohistochemistry staining were carried out on frozen or paraffin
139 embedded tissue sections using a purified anti-KLK5 mouse polyclonal antibody in 1:500 dilutions
140 (Novus Biologicals, Abingdon, UK), or an anti-DSG1 mouse monoclonal antibody recognizing N-
141 terminal extracellular domain (clone P124) in 1:100 dilutions (2B Scientific Ltd, Upper Heyford,
142 UK), or an anti-FLG monoclonal antibody in 1:100 dilutions (Leica biosystems, Newcastle, UK), or
143 an anti-involucrin mouse monoclonal antibody in 1:1000 (Sigma, Dorset, UK) or an anti-keratin 10
144 mouse monoclonal antibody (clone number LHP2, a gift from Royal London Hospital, UK).
145 MolecularProbes secondary antibodies conjugated with fluorescence dye were obtained from Life
146 Technologies (Paisley, UK). The detection of immunohistochemistry used biotinylated secondary
147 antibodies and DAB substrate kit for peroxidase (Vector laboratories, Peterborough, UK). The
148 staining procedures were as described by Di et al¹⁷, and negative controls were performed in each
149 staining with the secondary antibody alone.

150 The quantification of protein expression and activity in the epidermis was performed based on the
151 staining intensity using software Image-Pro Plus v6.0 (Media Cybernetics, Cambridge, UK). Briefly
152 images of three non-overlapped but adjacent regions in each section were recorded and saved
153 digitally. The epidermis in each image was then highlighted as an area of interest (AOI) and the
154 defined positive staining threshold was applied to the AOIs. The optical counts of positive staining

155 within AOIs were automatically counted based on the defined threshold and the expression or
156 activity in each AOI was calculated as mean staining intensity /area.

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158 Primary keratinocyte and keratinocyte cell line culture

159 Primary keratinocytes and fibroblasts were isolated from skin biopsies by incubation with 0.25%
160 trypsin-EDTA (Life Technologies, Paisley, UK) 3 hours for keratinocytes and Serva 50 U/ml
161 collagenase NB6 (Universal Biologicals, Cambridge, UK) 2 hours for fibroblasts. Isolated primary
162 keratinocytes were then co-cultured with lethally irradiated 3T3 mouse fibroblasts and grown in
163 keratinocyte culture media. The media contained equal amount of DMEM and DMEM/Ham F12
164 (Life Technologies, Paisley, UK) supplemented with 10% FCS (Labtech, East Sussex, UK), 100
165 IU/ml of penicillin and 100 µg/ml of streptomycin (Life Technologies, Paisley, UK). Human
166 keratinocyte growth supplement was then added to the media at final concentrations of 10 ng/ml of
167 EGF (Bio-Rad AbD Serotec, Oxford, UK), 0.4 µg/ml of hydrocortisone, 5 µg/ml of transferrin, 5
168 µg/ml of insulin, 2×10^{-11} M of liothyronine and 1×10^{-10} M of cholera toxin (Sigma, Dorset, UK).
169 Ntert¹⁸, a keratinocyte cell line was cultured in the same keratinocyte media. Fibroblasts were
170 cultured in DMEM supplemented with 10% FCS, 100 IU/ml of penicillin and 100 µg/ml of
171 streptomycin.

172

173 Ex-vivo dermatitis model and immunofluorescent staining

174 Skin from female breast tissue, obtained with informed consent and ethical approval (LREC
175 07/Q1704/59) following mastectomy was used as an *ex vivo* dermatitis model. The skin, which was
176 surplus to histopathology requirement, was placed in ice cold PBS immediately following removal
177 and stored on ice for a maximum of 2 hours, then placed into a 60 mm petri dish and the epidermal
178 surface carefully blotted dry. Rubber O-rings with a diameter of 8 mm were sealed on to the skin
179 using soft paraffin wax to create wells; care was taken to ensure that paraffin wax was applied only
180 to the area where the O-ring made contact with the skin in order to avoid altering the permeability

181 of the skin or the protease activity within the epidermis. To prevent the skin from drying out during
182 the incubation period, the remaining space within the petri dish was filled with DMEM, containing
183 10% FCS, at a depth of approximately 2 mm, ensuring no medium touched the epidermal surface of
184 the skin. Irritant substances and appropriate vehicle controls were applied carefully to separate
185 wells (50 µl solution each) and the skin incubated at 37°C with 5% CO₂ for the required length of
186 time. Following incubation, the entire irritant and vehicle control solutions were aspirated off the
187 epidermis and 6 mm punch biopsies were taken from the treated sites of the skin sample (without
188 removing the rubber O-rings) using sterile biopsy punches. A fresh biopsy punch was used for each
189 treatment to avoid cross-contamination, and the biopsy was removed from within the rubber O-ring
190 using forceps to ensure only treated skin was extracted. The biopsied tissue was placed in a 1.5 ml
191 eppendorf tube and snap frozen in liquid nitrogen. Samples were stored at -80°C until further
192 investigations.

193 Following removal of skin biopsies from -80°C storage and embedding in Tissue-Tek® optimal
194 cutting temperature medium (OCT) (Sakura Finetek, Thatchem, UK), 8 µm tissue sections were cut
195 on a cryostat, allowed to dry onto poly-L-lysine coated glass slides, then fixed for 10 minutes in ice
196 cold acetone at -20°C. Slides were washed with TBS, blocked for 30 minutes with blocking buffer
197 followed by incubation with primary antibody in TBS overnight at 4°C. Slides were washed for 3 x
198 5 minutes in TBS, subsequently incubated with secondary antibody for 1 hour in the dark, then
199 washed in TBS for 3 x 5 minutes and, where necessary, counterstained by incubation with DAPI for
200 10 minutes, followed by a final wash of 3 x 5 minutes in TBS. Coverslips were attached using
201 Mowiol and the samples then visualised by fluorescence microscopy (Axioskop 2 MOT, Zeiss).
202 Slides were magnified at 100x and 400x and images digitally recorded (Axiocam, Zeiss). Slides
203 stained in the absence of primary antibody were used to set the exposure levels to reduce
204 background staining. Images were analysed using ImageJ v1.46 software, with 10 vertical regions
205 of interest (ROI's) from outer to inner surface of the epidermis selected from 1 field of view from 3
206 consecutive sections for each sample and the contribution from each layer along this measurement

207 was recorded. The 10 measured ROIs were normalised to 100% and the mean pixel intensity
208 obtained for every 5% of the depth (i.e. from outer to inner surface) of the epidermis. The minimum
209 average pixel intensity for a 5% section in the PBS sample was set at 1 and all other readings for the
210 sample set calculated relative to this value.

211

212 Construction of lentiviral vectors and transduction of keratinocytes

213 Human KLK5 cDNA was cloned into the pCCL lentiviral vector containing upstream spleen focus-
214 forming virus (SFFV) promoter and downstream enhanced green fluorescent protein (eGFP)
215 reporter gene linked to KLK5 via an internal ribosomal entry sequence from the endomyocarditis
216 virus. The vector encoding eGFP alone was used as negative control. Lentiviruses were produced
217 by co-transfecting 293T cells. Infectious lentiviruses were harvested 48 and 72 hours post-
218 transfection, and the culture supernatants were concentrated by ultracentrifugation. The lentivirus
219 concentration were titrated by viral copy number using qPCR and flow cytometry and the titres of
220 eGFP viral vector and KLK5/eGFP viral vector were 8×10^8 and 4×10^8 TU/ml, respectively.

221 Human primary keratinocytes and cell line Ntert were transduced by one round of exposure to
222 eGFP or KLK5/eGFP vectors at an MOI of 10. Transduced cells were subcultured for further
223 experiments.

224

225 Intracellular calcium mobilization assay

226 Measurement of intracellular calcium mobilization was performed using FluoForte Calcium Assay
227 kit (Enzo Life Sciences, Exeter, UK). Mobilization of intracellular calcium was detected utilizing a
228 fluorogenic calcium-binding dye. Keratinocytes were plated in 96-well plates at the density of $1 \times$
229 10^4 cells per well. After 24 hours, the growth medium was removed and 100 μ l of dye-loading
230 solution was added. The cells were further incubated with the dye-loading solution for 45 min at
231 37°C and then 15 minutes at room temperature. The cells were then inoculated with 100 μ M of
232 PAR2 activating peptide SLIGKV-NH₂, (Bachem, Cambridge Bioscience, Cambridge, UK), and

233 intracellular calcium signal was recorded via real-time monitoring of fluorescence intensity at
234 excitation of 530 nm and emission of 570 nm using the microplate reader FLUOstar OPTIMA,
235 (BMG, Lutterworth, UK). Intracellular calcium mobilization was calculated as changes of
236 fluorescence intensity in relative fluorescence units (RFU) and the mobilisation curves were
237 generated by RFU values plotted against the time.

238

239 Western blotting

240 Cells were suspended in a cooled lysis buffer composed of 50 mM Tris-HCl pH 8.0, 150 mM NaCl,
241 5 mM EDTA, cocktail protease inhibitors and 1 mM PMSF. Samples were incubated for 15 minutes
242 at 4°C and then were centrifuged at 12,000 RPM for 15 minutes. The total protein concentration in
243 the supernatant of lysed sample was determined by Bio-Rad protein assay Kit (Bio-Rad,
244 Hertfordshire, UK). Samples were further diluted 5 times in 0.5 mM Tris-HCl pH 6.8 sample buffer
245 containing 100 mM DTT, 10% SDS, 30% glycerol, 0.001% bromphenol blue. Equal amounts of
246 total protein were loaded in SDS-PAGE. After electrophoresis, proteins were transferred to PVDF
247 membranes and incubated with primary antibody overnight. The following day, membranes were
248 incubated with secondary antibody conjugated with horseradish peroxidase (Sigma, Poole, UK),
249 and signals were detected using the ECL Prime system (GE Healthcare, Bucks, UK). Ponceau red
250 (Sigma-Aldrich, Poole, UK) staining was used as loading control for culture supernatants.

251

252 In situ zymography and casein gel zymography

253 *In situ* zymography assay using casein-derived substrate measured the total protease activity in the
254 skin. Briefly, the frozen skin sections were rinsed with PBS containing 0.1% Triton X-100 (Sigma-
255 Aldrich, Poole, UK) and incubated at 37°C with 10 µg/ml casein conjugated with BODIPY TR-X
256 (Life Technologies, Paisley, UK) in the buffer containing 10 mM Tris-HCl, pH7.8 in a humid
257 chamber for two hours,. The fluorescent intensity was detected under a fluorescence microscope
258 and quantified using Image-Pro.

259 Casein gel zymography was used for cells that were cultured in keratinocyte culture media without
260 FCS for 48 hours. The culture media were then collected and concentrated using Amicon
261 centrifugal filter devices (Millipore, Watford, UK). Samples were re-dissolved in non-reducing
262 Novex® Tris-Glycine SDS sample buffer (Life Technologies, Paisley, UK) and separated on 12%
263 polyacrylamide gels copolymerized with casein substrate (Life Technologies, Paisley, UK). After
264 electrophoresis, the gels were soaked in renaturing buffer containing 50 mM Tris, pH 8 and 2.5%
265 Triton X-100 for 1 hour. The gels were then incubated in developing buffer containing 50 mM Tris,
266 pH 8 (Life Technologies, Paisley, UK) at 37°C overnight. Casein degrading activity was visualized
267 when the gels were stained with 1% Coomassie Brilliant blue (Sigma-Aldrich, Poole, UK).

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269 Human cytokine antibody array

270 The cytokines in culture media collected from the different cell lines were assessed using The
271 Human Cytokine Antibody Array Panel A kit (R&D System, Oxfordshire, UK) according to the
272 manufactory's instruction. A total number of 36 cytokines were measured and the intensities of the
273 blots were quantified by densitometry. As each reference or target protein was blotted in duplicate,
274 mean pixel density from duplicate blots was calculated and normalised by reference blots.

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276 RT-PCR for IL-8

277 Following RNA extraction using the RNeasy® Plus Mini Kit (Qiagen), a second genomic DNA
278 elimination step was employed to prevent genomic DNA contamination, and cDNA was then
279 synthesised using the RT² first strand kit (SABioScience). Quantitative PCR was performed in
280 duplicate wells for each time point using a 7900HT Fast Real-Time PCR System (Applied
281 Biosystems, CA, USA) and the data collected using SDS 2.4 software (Applied Biosystems, CA,
282 USA). The PCR protocol consisted of an initial cDNA denaturation at 95°C for 10 min, followed
283 by 35 cycles of denaturation at 95°C for 15 seconds and annealing and data collection at 60°C for
284 60 seconds. Δ CT values were calculated using the Ct value for the housekeeping gene 26S and

285 analysis of fold change in gene regulation was performed using automated Microsoft Excel analysis
286 tools from SABioscience.

287

288 Enzyme linked immunosorbent assay (ELISA)

289 Cells were seeded in 24 well plates and cultured until confluence, then cultured in serum free media
290 for 48 hours before culture media were collected and concentrated using Amicon centrifugal filter
291 devices (Millipore, Watford, UK). The total protein concentration was quantified using Bio-Rad
292 protein assay kit. The level of IL-8 was measured using IL-8 ELISA kit (BD Biosciences, Oxford,
293 UK). TSLP and IL-10 were quantified using ELISA kits from eBioscience (eBioscience, Hatfield,
294 UK). All sample reads were normalized to the total protein concentration.

295

296 Bio-engineered skin sheet and grafting onto immunodeficient mice

297 The methods of generating bio-engineered skin sheet and grafting to mice were as described by Di
298 et al¹⁹. Briefly, primary human keratinocytes were seeded on the top of a fibrin matrix populated
299 with primary human fibroblasts. After keratinocytes reached confluence, the bioengineered skin
300 constructs were grafted onto the dorsum of 6 weeks old female immunodeficient mice (NMRI
301 strain, Charles River, UK). 8 weeks after grafting, skin samples from grafts were taken post-mortem
302 and formalin fixed and paraffin-embedded or OCT-embedded. H&E staining and immunostaining
303 for KLK5, FLG and DSG1, and zymographics were performed on these tissues.

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310 **Results**

311 **Increased expression and activity of KLK5 in AD skin**

312 Skin biopsies taken from lesional and non-lesional skin in five children with AD were examined for
313 epidermal morphology and KLK5 expression. Five age-matched normal donor skin biopsies were
314 used as controls. Compared to normal skin, AD lesional skin exhibited epidermal changes including
315 acanthosis, spongiosis, parakeratosis and elongated rete ridges. Non-lesional skin also showed
316 histopathological characteristics consistent with the disease, but far less prominent than those
317 observed in lesional skin (**Figure 1A. a-c**). The expression of KLK5, as detected by
318 immunostaining, was localised in the cornified layer in normal skin, whereas in AD skin, especially
319 in the lesional skin, KLK5 was present in the granular layer and upper stratum spinosum with high
320 staining intensity (**Figure 1A. d-f and Supplementary materials, Figure S1**). Quantification of
321 KLK5 based on mean optical staining intensity/area further demonstrated significant increased
322 expression of KLK5 in both lesional and non-lesional AD skin, compared to the normal skin
323 ($p < 0.05$) (**Figure 1B**). As DSG1 is the proteolytic substrate of KLK5, and FLG can be degraded by
324 elastase 2 which is a serine protease activated by KLK5 in the skin²⁰, DSG1 and FLG expressions
325 were also examined by immunostaining. Both FLG and DSG1 expression were significantly
326 reduced in lesional skin of AD ($p < 0.05$) (**Figure 1A. g-i & j-l, Figure 1B**). The protease activity in
327 the skin were further examined by *in situ* staining, and results showed a similar location and
328 staining pattern to KLK5 expression with more diffuse and enhanced fluorescence intensity in the
329 AD skin (**Figure 1A. m-o, Figure 1B and Supplementary materials, Figure S2**). Although the
330 caseinolytic serine protease assay detects total protease activity, as KLK5 is a major serine protease
331 in the skin² and the protease activity closely matched the extent and distribution of KLK5 protein
332 expression, it is likely that KLK5 is a significant contributor to the increased activity of serine
333 protease observed in the AD skin.

334

335 **Transient up-regulation of KLK5 stimulated PAR2, but persistent activated KLK5**
336 **desensitised PAR2**

337 The expression of the KLK5 targeted molecule PAR2 was further examined in the donor skin (n=5)
338 and AD non-lesional and lesional skin by immunostaining. No significant changes in PAR2
339 expression was noted in lesional, non-lesional AD skin and normal donor skin ($p>0.05$) (**Figure 1A.**
340 **p-r, Figure 1B**), although there was a fluctuation of PAR2 expression level among individuals.
341 This result suggests that up-regulated KLK5 does not modify PAR2 in AD skin. Considering that
342 the up-regulation of KLK5 was likely to be chronic and persistent in AD skin, we speculated that
343 the response of PAR2 to KLK5 might differ between AD skin and skin with transient KLK5 up-
344 regulation. To determine this, the influence of transiently increased KLK5 on PAR2 was tested
345 using an *ex-vivo* irritant dermatological model in which irritants were applied onto normal skin
346 cultured *in vitro*. Following the application of croton oil or SDS or acetone for 30 minutes on the
347 *ex-vivo* skin model, increased epidermal expression of KLK5 and PAR2 were detected in the
348 epidermis (**Figure 2. a**). Quantification using image analysis confirmed significantly higher
349 expression of both KLK5 and PAR2 across all layers of the epidermis (**Figure 2. b-e**).

350 The effect of persistently increased KLK5 on PAR2 was tested in the keratinocyte cell line Ntert
351 (KLK5-Ntert) or primary keratinocytes (KLK5-pKC) ectopically over-expressing KLK5. Cells
352 transduced with eGFP vector alone were used as control (GFP-Ntert or GFP-pKC). The
353 transduction efficiency in both KLK5 transduced cells and GFP transduced cells was nearly 60% as
354 determined by eGFP expression. Overexpression of KLK5 in KLK5-transduced cells and culture
355 media was confirmed by western blotting (**Figure 3. a&c**). The activity of KLK5 was further
356 assessed by zymography. Active KLK5 was detected in the culture media collected from KLK5
357 transduced cell culture (**Figure 3. d**), but not in cell lysates (**Figure 3. b**). KLK5 is synthesized as
358 inactive pre-pro-KLK5 (precursor or zymogene) which is then translocated into the endoplasmic
359 reticulum in cells (see review by Debela M *et al*)²¹. Following the removal of the signal peptide
360 (~30 amino acids), the pre-pro-KLK5 becomes pro-KLK5 that is secreted into the extracellular

361 space and subsequently becomes activated upon release of its 37 amino acids propeptide from the
362 N-terminus of KLK5. Thus, the KLK5 extracted from the cells would not contain the final activated
363 KLK5 and it is not surprising that there was no positive digested band detected in zymography
364 loaded with cell lysates. In contrast, a digested band was seen in zymography loaded with culture
365 medium from cells over expressing KLK5/eGFP as a result of the culture medium containing the
366 active form of KLK5. Although a ~70kDa band was present on both western blotting and
367 zymography, the intensity of the protein band remained unchanged among cell lysates from
368 untransduced cells, cells transduced with GFP alone and KLK5/GFP, therefore this was considered
369 to represent a nonspecific protein, but having a proteolytic activity on casein-derived substrate.

370 The durability of KLK5 expression in both KLK5-Ntert and KLK5-pKC was assessed following
371 propagation of transduced cells and there was no decline in KLK5 expression over this time as
372 determined by eGFP expression, indicating persistent KLK5 expression in the KLK5-cell model
373 (**Supplementary materials, Figure S3**). As a previous study showed that PAR2 was mainly
374 expressed in differentiated keratinocytes, the Ntert cell line was checked for differentiation markers
375 keratin 10 and involucrin and results showed positive expression for both proteins (**Supplementary**
376 **materials, Figure S4**). The activity of PAR2 and a KLK5 down-stream target was then examined
377 in untransfected Ntert (UT-Ntert), GFP-Ntert and KLK5-Ntert by a PAR2-dependent calcium
378 mobilisation assay. Mobilisation of calcium was observed in the untransfected keratinocytes after
379 addition of a PAR2 agonist (AP), and similarly following addition of recombinant KLK5 (rKLK5),
380 albeit slightly later than that induced by AP (**Figure 4. a**), confirming that KLK5 was able to
381 activate PAR2. In contrast, a decline in calcium mobilisation was detected in KLK5-Ntert cells
382 compared to that in GFP-Ntert (**Figure 4. b**). Thus, a different PAR2 dependent calcium
383 mobilisation response was observed in cells with persistent expression of KLK5 versus cells with
384 transient rKLK5 stimulation.

385 PAR2, like many other receptors, can be desensitized if continuously or repeatedly exposed to its
386 agonist^{22,23}, and these results suggest that persistent over-expression of KLK5 could desensitise the

387 PAR2 receptor resulting in a lower response of PAR2 to its agonist AP. Since PAR2 desensitisation
388 can be reversed by removal of PAR2 activators, we looked at calcium mobilisation when KLK5-
389 Ntert cells were treated with the serine protease inhibitor SFTI-G, an analogue derived from the
390 naturally occurring substance sunflower trypsin inhibitor 1²⁴. Following treatment with 100µM
391 SFTI-G overnight, the PAR2 dependent calcium mobilisation in KLK5-Ntert cells recovered to
392 levels similar to that in GFP-Ntert and untransfected keratinocytes (**Figure 4. c**).

393

394 **Persistently activated KLK5 induced cytokine expression/secretion despite PAR2** 395 **desensitisation**

396 Activated PAR2 induced pro-inflammatory cytokine elevation and secretion, including of IL-8, has
397 been reported²⁵. In the *ex-vivo* irritant dermatological skin model, increased IL-8 mRNA expression
398 was detected by RT-PCR within 12 hours following exposure to SDS and to a lesser extent at 12
399 hours following application of croton oil (**Figure 5. a**). In the KLK5-pKC cells with persistent
400 KLK5 expression, IL-8 protein, measured by cytokine antibody array, was also increased (**Figure**
401 **5.b&c**). In addition, IL-10 was elevated in KLK5-pKC cells (**Figure 5. b&c**), but other cytokines
402 including IL-1, IL-4, IL-6 and IFN-gamma were not (**Supplementary materials, Table S1**). TSLP,
403 a prominent pro-inflammatory cytokine in AD skin also showed increased expression in KLK5-
404 pKC cells as measured by ELISA (**Figure 5. c**). However, increased IL-10 and TSLP were not
405 detected in cells transiently challenged with rKLK5 (data not shown).

406

407 **Inhibition of persistent KLK5 activity reversed KLK5 effects on DSG1 and cytokine** 408 **production**

409 To examine the downstream effects of inhibition of persistently raised KLK5 activity, primary
410 keratinocytes transduced with KLK5 (KLK5-pKC) or eGFP (GFP-pKC) were cultured in serum
411 free media inoculated with 100 µM of serine protease inhibitor SFTI-G for twenty-four hours.
412 Although the level of secreted KLK5 in culture media, as determined by western blot, remained

413 elevated in the KLK5-pKC cells 24 hours post SFTI-G treatment, the expression of full length
414 DSG1 was restored in treated KLK5-cells compared to untreated cells, whereas there was no
415 significant change in the level of DSG1 in GFP-pKC cells before (-) and after SFTI-G (+)
416 treatment, indicating the suppression of KLK5 activity by SFTI-G in the KLK-5 culture (**Figure 6.**
417 **a**). It was noticed that in KLK5-pKC cells, there was no DSG1 detected. The DSG1 antibody used
418 in the study was a monoclonal antibody (clone P124) recognizing the N-terminal extracellular
419 domain of DSG1 and full length DSG1. Primary keratinocytes were used for untreated as well as
420 treated experiments, and treated cells showed a DSG1 band. Therefore, no DSG1 band in KLK5-
421 pKC cells without SFTI-G treatment was most likely due to DSG1 levels being too low to be
422 detected and/or the antibody does not recognize the cytoplasm domain of DSG1 alone following the
423 cleavage of the extracellular domain by over-expressed/activated KLK5. Cytokines IL-8, IL-10 and
424 TSLP were also significantly reduced in SFTI-G treated KLK5-pKC cells, compared to untreated
425 KLK5-pKC cells, and these changes were not observed in treated or untreated GFP-cells as
426 confirmed by both cytokine antibody array and ELISA (**Figure 5.b, 6.b&c, Supplementary**
427 **materials, Table S1**).

428

429 **Keratinocytes with persistent activated KLK5 exhibit an AD-like epidermal architecture**

430 To examine the influence of persistently up-regulated KLK5 activity on epidermal architecture *in*
431 *vivo*, primary keratinocytes from the non-lesional skin in a patient with AD (AD-pKC), or primary
432 normal keratinocytes ectopically over-expressing KLK5 (KLK5-pKC) or GFP (GFP-pKC) were
433 cultured *in vitro* as bio-engineered skin and grafted onto immuno-deficient mice. 8 weeks post-
434 grafting, the skin from the grafted area was harvested. Grafts generated from KLK5-pKCs and AD-
435 pKC showed AD-like morphology, with acanthosis, mild parakeratosis and enlarged intercellular
436 spaces compared to the GFP-pKC graft (**Figure 7. a-c**). Increased expression of KLK5 and protease
437 activity and decreased DSG1 were observed in both KLK5-pKC and AD-pKC grafts compared to
438 GFP-cell graft (**Figure 7. d-l**), which were analogous to findings in AD skin. Altered FLG

439 expression was also detected in KLK5-pKC and AD-pKC grafts, it was more evident in the upper
440 stratum spinosum similar to that seen in the AD skin (**Figure 7. m-o and Figure 1A. h&i**). As the
441 FLG antibody used for the study only detects FLG produced from human cells, the mouse-human
442 skin boundary was easily visible in the FLG stained skin, indicating that the keratinocytes within
443 the grafts were of human origin (**Figure 7. g-i**). In addition, the mouse-human skin boundary
444 images showed an increased thickness of mouse epidermis (acanthosis) next to the grafts generated
445 by KLK5-pKC and AD-pKC, but not by the GFP-pKC, which may have resulted from a paracrine
446 effect of activated KLK5 secreted from these grafts.

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463 **Discussion**

464 Up regulated KLK5 together with skin barrier defects in AD has been reported in previous
465 studies^{10,11,26}, which have shown that both genetic and environmental factors can cause aberrant
466 KLK5 activity. Indeed, as AD shares a number of clinical features with NS, it has been speculated
467 that AD might also share some pathological mechanisms of dysfunctional skin barrier with NS^{27,28}.
468 Genome-wide association studies have shown several single nucleotide polymorphisms (SNPs) in
469 *SPINK5* associated with AD, in particular Glu420Lys²⁸ and functional investigations have further
470 confirmed that Glu420Lys SNP alters *SPINK5* encoded protein LEKTI proteolytic activation and
471 results in dysregulation of proteases including the KLKs²⁹. Environmental factors that disrupt the
472 skin barrier, including irritants and infection, and trigger KLK5 up-regulation have also been
473 reported³⁰. In this study, we have demonstrated that the irritants croton oil and SDS increase KLK5
474 and PAR2 expression, but that transient KLK5 expression seems to have different effects on PAR2
475 expression/activity than that observed with persistent KLK5 expression.

476 KLK5 activation of PAR2 has been demonstrated previously³¹ and we also showed rKLK5
477 activated PAR2 in this study using an intracellular calcium mobilization fluorescence assay. The
478 fluorescence peak induced by rKLK5 was, however, delayed 40-50 seconds compared to the peak
479 induced by the PAR2 agonist (AP). This difference in peak time was likely to be due to the tethered
480 ligand mechanism with regards to maximum rate of PAR2 activation by KLK5. Oikonomopoulou
481 and colleagues³¹ have reported that KLK5 activation of PAR2 is a two-step process involving
482 cleavage and tethered ligand binding to the PAR2 receptor, whereas a one-step process is involved
483 in the PAR2 agonist directly binding to the receptor.

484 The signalling pathway of KLK5-PAR2-NFκB-cytokines has been recognised for more than a
485 decade^{32,33}, but most studies have been carried out in models with transient exposure to exogenous
486 rKLK5³¹. PAR2 can exhibit desensitization due to continuous or repeated stimulation by its agonist,
487 leading to reduced responsiveness³⁴. AD is a chronic skin condition, and up-regulated KLK5
488 activity in affected skin is most likely to be persistent than transient. However, the examination of

489 PAR2 activity in skin *in situ* is technically difficult. Currently, the activity of PAR2 is assessed by
490 intra-cellular calcium mobilization in live cells following stimulation/inhibition with its
491 agonist/antagonist. Tissues from AD skin or murine AD models are generally fixed/embedded or
492 snap frozen, and thus are not suitable for use in the calcium mobilization assay. There is an indirect
493 way to check PAR2 activity by examination of PAR receptor internalization, e.g. by tracking GFP-
494 tagged PAR2 fusion protein trafficking³⁵ or by analysing the distribution of activated (cytoplasmic)
495 and unactivated (cell membrane) PAR2 receptor³⁶, but these also require cell-culture models rather
496 than skin tissue. However, although the desensitisation of PAR2 in the skin *in situ* cannot be
497 measured directly, previous work by Moniaga and colleagues³⁷ supports our view that PAR2 is
498 desensitised in AD-like skin lesions. In their study³⁷, a PAR2 agonist could up-regulate TLSP in
499 murine keratinocytes following transient (one-off) stimulation, but only a marginal increase of
500 TSLP production was noted in the skin of flaky tail mice following repeated topical application of
501 dust mite for 7 weeks; this discrepancy of TLSP production between transient stimulation in cell
502 culture and repeated challenge in mouse skin was probably because the repeated challenge caused
503 PAR2 desensitisation, resulting in low PAR2 activity. Related to this, an *in vivo* study by Briot and
504 colleagues showed that TLSP production was independent of PAR2 activation and that PAR2 was
505 not central to the production of the skin inflammation when there was persistent KLK5 activity³⁸.
506 In mice with double knockout of SPINK5^{-/-} and PAR2^{-/-} and high KLK activity, the deletion of
507 PAR2 in the adult double knockout-grafted skin did not result in the reduction of TLSP and did not
508 suppress the skin inflammation³⁸. This result suggests that the inflammatory skin in Netherton
509 syndrome and AD is not solely caused by PAR2 activation.

510 Based on our observations in the AD skin with persistent KLK5 overexpression and the *ex-vivo*
511 irritant dermatological skin model mimicking a transiently increased KLK, we demonstrated that
512 PAR2 had a higher response to transient KLK5 stimulation, but had a weak response to persistent
513 KLK5 stimulation. Interestingly, despite the low activity of PAR2 in cells overexpressing KLK5,
514 these cells up-regulated and secreted pro-inflammatory and Th2-polarizing cytokines, including IL-

515 8, IL-10 and TSLP, indicating that persistent KLK5 induced IL-8, IL-10 and TSLP. The exact
516 pathway of persistent KLK5 expression/activity induced IL8, IL10 and TLSP secretion in KLK5-
517 pKC remains unclear, and further investigations will be required to elucidate this. The keratinocyte-
518 based nature of our KLK5 over-expressing model, which lacks immune cells, meant that it was not
519 possible to investigate cytokine secretion from immune cells following KLK5 activation, which
520 may explain why our cytokine antibody array data did not show elevation of other
521 cytokines/chemokines reported in AD patients (such as IL-6, IL-4, GM-CSF, IL-1 and TNF α).

522 The influence of activated KLK5 on epidermal architecture in the *in vivo* human: murine chimeric
523 skin graft model, which showed AD-like skin architecture in grafts generated using cells over-
524 expressing KLK5, further indicated that KLK5 plays a key role in this process. Similar observations
525 have also been detected using a transgenic mouse model over-expressing KLK5³⁹. Furthermore, as
526 the human: murine chimeric skin graft model was immunodeficient and maintained in specific
527 pathogen-free environment, our results suggest that the AD-like histopathological features and
528 abnormal barrier protein expression in the epidermis generated by AD-cells and KLK5-pKC cells
529 were a specific consequence of persistent up-regulation of KLK5 in the keratinocytes.

530 Our study also suggests that increased KLK5 in AD skin should not simply be viewed as a
531 ‘biomarker’ in this skin disorder, but as a protease which has significant functional impact in this
532 condition. In AD patients, environmental factors can trigger the cytokine cascade and stimulate a
533 Th2-skewed inflammatory infiltrate through the initial defective skin barrier, resulting in
534 susceptibility to allergy or ‘atopy’ (“outside-inside” aetiological mechanism)^{16,40}. The induced
535 inflammatory response further compromises barrier function, causing keratinocyte damage and
536 inducing upregulation of certain molecules, such as KLK5. The initial damage secondary to
537 increased KLK5 forms a vicious cycle of inflammation-induced barrier impairment in AD (outside-
538 inside-outside)¹⁶.

539 Amongst the currently known inhibitors of kallikreins⁴¹⁻⁴³, the naturally occurring cyclic peptide
540 SFTI has been extensively investigated due to it being amenable to chemical manipulation which

541 has allowed for the creation of synthetic variants⁴⁴⁻⁴⁶. We used the analogue SFTI-G derived from
542 SFTI²⁴ to control KLK5 activity and our *in vitro* results showed a normalised DSG1 expression,
543 depletion of depressed PAR2 dependent calcium mobilisation and reduction of IL-8, IL-10 and
544 TSLP. Thus, reducing KLK5 activity could offer a therapeutic option for the treatment of AD,
545 where control of higher KLK5 activity might help to reverse (at least part of) the AD phenotype in
546 patients with this disorder.

547

548 **Acknowledgements**

549 The authors are grateful to David Rew, University Hospital Southampton NHS Foundation Trust
550 for assistance in procuring skin samples.

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559 **Legends**

560 **Figure 1. Skin morphology and protein expression in AD**

561 A: Skin sections from normal donor (n=5) and AD skin (n=5) were examined by H&E (a-c),
562 immunostaining (d-l & p-r), and *in situ* zymography (m-o). Green/brown colour represents protein
563 expression or protease activity. Nuclei were stained in blue colour. Scale bar = 50 μ m.

564 B: Quantification of staining intensity (n=3 per sample) was measured by mean staining
565 intensity/area using ImagePro.

566

567 **Figure 2. Increased KLK5 and PAR2 expression in *ex-vivo* dermatitis skin model**

568 Immunofluorescence staining of epidermal KLK5 and PAR2 following application of 3% croton oil
569 and 5% SDS compared with acetone-treated and PBS-treated skin. Quantification of relative KLK5
570 (b,d) and PAR2 (c,e) expression from stratum corneum to basal layer (0%-100% depth
571 respectively). KLK5 (n=9, n=7), PAR2 (n=7, n=6) for 3% croton oil and 5% SDS respectively.

572

573 **Figure 3. Characterisation of keratinocyte over expressing KLK5**

574 The expression and activity of KLK5 in cell lysate (a,b) and culture media (c,d) from the cells
575 transfected with KLK5 gene were examined by Western blot (left panel) and gel zymography (right
576 panel). β -actin were used as loading controls. UT = untransduced cells; eGFP = cells transduced
577 with eGFP alone vector; KLK5 = cells transduced with KLK5/eGFP vector and rKLK = activated
578 recombinant KLK5 protein (where rKLK was added directly to the gel as a positive zymography
579 control).

580

581 **Figure 4. PAR2-dependent calcium mobilisation in keratinocytes**

582 PAR2-dependent calcium mobilisation was measured in untransfected Ntert cells challenged with
583 AP or rKLK5 (a); cells transfected with GFP or KLK5 challenged with AP (b); and cells transfected

584 with GFP or KLK5, treated with SFTI-G and then challenged with AP (c). PBS was used as
585 negative control.

586

587 **Figure 5. Cytokine expression in keratinocytes**

588 Cytokine levels were measured in the *ex-vivo* skin model with transiently up-regulated KLK5 using
589 RT-PCR for IL8 (a); KLK5-pKC cells with persistent KLK5 expression using antibody array blots
590 (b). The IL-8 and IL-10 levels detected by cytokine antibody array and quantified by mean pixel
591 density, and TSLP level measured by ELISA are shown in the bar chart (c). Data in (a) are shown
592 relative to PBS-treated skin.

593

594 **Figure 6. The inhibition of KLK5 by serine protease inhibitor SFTI-G**

595 Primary keratinocytes transduced with GFP or KLK5 gene were treated with 100 μ M of SFTI-G
596 overnight. KLK5 in culture media and DSG1 in cell lysates were measured by Western blot (a).
597 Cytokine secretions in the culture media following SFTI-G treatment were measured by cytokine
598 antibody array (b) and confirmed by ELISA (c). The symbol * is representative of statistical
599 significance ($p < 0.05$) and NS stands for non-significance.

600

601 **Figure 7. Persistent KLK5 activity induced AD-like skin changes**

602 A: Skin graft sections from human: murine skin graft mice were examined for morphology by H&E
603 (a-c), KLK5 (d-f) expression by immunohistochemistry, DSG1 (j-l) and FLG (m-o) expression by
604 immunofluorescence, and protease activity (g-i) by *in situ* zymography. Brown and green colour
605 show protein expression/protease activity and purple and blue colour show stained nuclei. Scale
606 Bar= 50 μ m.

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Supplementary materials

Table S1. Quantification of dots by densitometry for cytokine antibody array

Coordinate of the plate	Target/control	Mean pixel density			
		GFP-pKC		KLK5-pKC	
		SFTI (-)	SFTI (+)	SFTI (-)	SFTI (+)
A1,A2	Reference spot	17896	17221	17701	17193
A3,A4	C5/C5a	232	294	245	302
A5,A6	CD40 ligand	216	259	241	226
A7,A8	G-CSF	308	299	327	302
A9,A10	GM-CSF	341	367	319	298
A11,A12	GRO-alpha	18958	19020	19110	19212
A13,A14	I-309	327	381	338	306
A15,A16	sICAM-1	404	394	420	441
A17,A18	IFN-gamma	336	385	312	397
A19,A20	Reference spot	16350	16361	16222	16119
B3,B4	IL-1a	188	226	209	198
B5,B6	IL-1b	217	275	232	202
B7,B8	IL-1ra	15933	15659	15899	16021
B9,B10	IL-2	297	310	268	289
B11,B12	IL-4	322	338	301	297
B13,B14	IL-5	275	311	291	298
B15,B16	IL-6	397	344	380	393
B17,B18	IL-8	15922	15273	22598	16276
C3,C4	IL-10	224	243	8236	268
C5,C6	IL-12 p70	313	289	326	301
C7,C8	IL-13	406	484	415	461
C9,C10	IL-16	359	331	360	314
C11,C12	IL-17	448	429	465	438
C13,C14	IL-17E	385	375	397	408
C15,C16	IL-23	535	521	519	569
C17,C18	IL-27	449	425	456	406

D3,D4	IL-32a	398	331	386	350
D5,D6	IP-10	421	452	409	398
D7,D8	ITAC	415	429	406	466
D9,D10	MCP-1	399	411	394	429
D11,D12	MIF	12868	12525	13310	12806
D13,D14	MIP-1a	275	310	298	322
D15,D16	MIP-1b	393	404	415	438
D17,D18	Serpin E1	18256	18010	18166	18125
E1,E2	Reference spot	17510	17725	17566	17621
E3,E4	RANTES	435	509	466	428
E5,E6	SDF-1	449	399	425	462
E7,E8	TNF-alpha	398	439	412	402
E9,E10	sTERM-1	421	461	435	489
E19,E20	Negative control	275	211	261	293

Legends

Figure S1. KLK5 expression in non-lesional and lesional skin from five AD patients

Skin sections from normal donor (n=5, control 1-5, left panel) and AD patients (n=5, patient 1-5) from non-lesional (middle panel) and lesional (right panel) skin were examined for KLK5 expression using immunohistochemistry. Brown colour represents protein expression and blue colour shows nuclei stain. Scale bar = 100 µm.

Figure S2. *In situ* protease activity in non-lesional and lesional skin from AD patients

Skin sections from normal donor (n=4, control 1-4, left panel) and AD patients (n=4, patient 1-4) from non-lesional (middle panel) and lesional (right panel) skin were examined for total protease activity by *in situ* zymography. Green colour represents protease activity, whereas nuclei are stained blue. Scale bar = 50 µm.

Figure S3. Stability of transgene KLK5 expression in keratinocytes

Primary keratinocytes and Ntert keratinocyte cell line were transduced with KLK5/eGFP transgene and the stability of transgene in cells was assessed by GFP positive cells (GFP+) using flow cytometry. Primary keratinocytes were only monitored for a period of 12 days due to proliferative lifespan of primary cells in *in vitro* culture.

Figure S4. Differentiation markers in Ntert keratinocytes

Keratin 10 and involucrin expression in cell lysates from untransduced Ntert keratinocytes (UT), or transduced with KLK5 (KLK5) or eGFP vector alone (eGFP) were assayed by Western blot. Positive expressions of both proteins indicated a proportion of differentiated cells in the Ntert cell line.

Figure 1A
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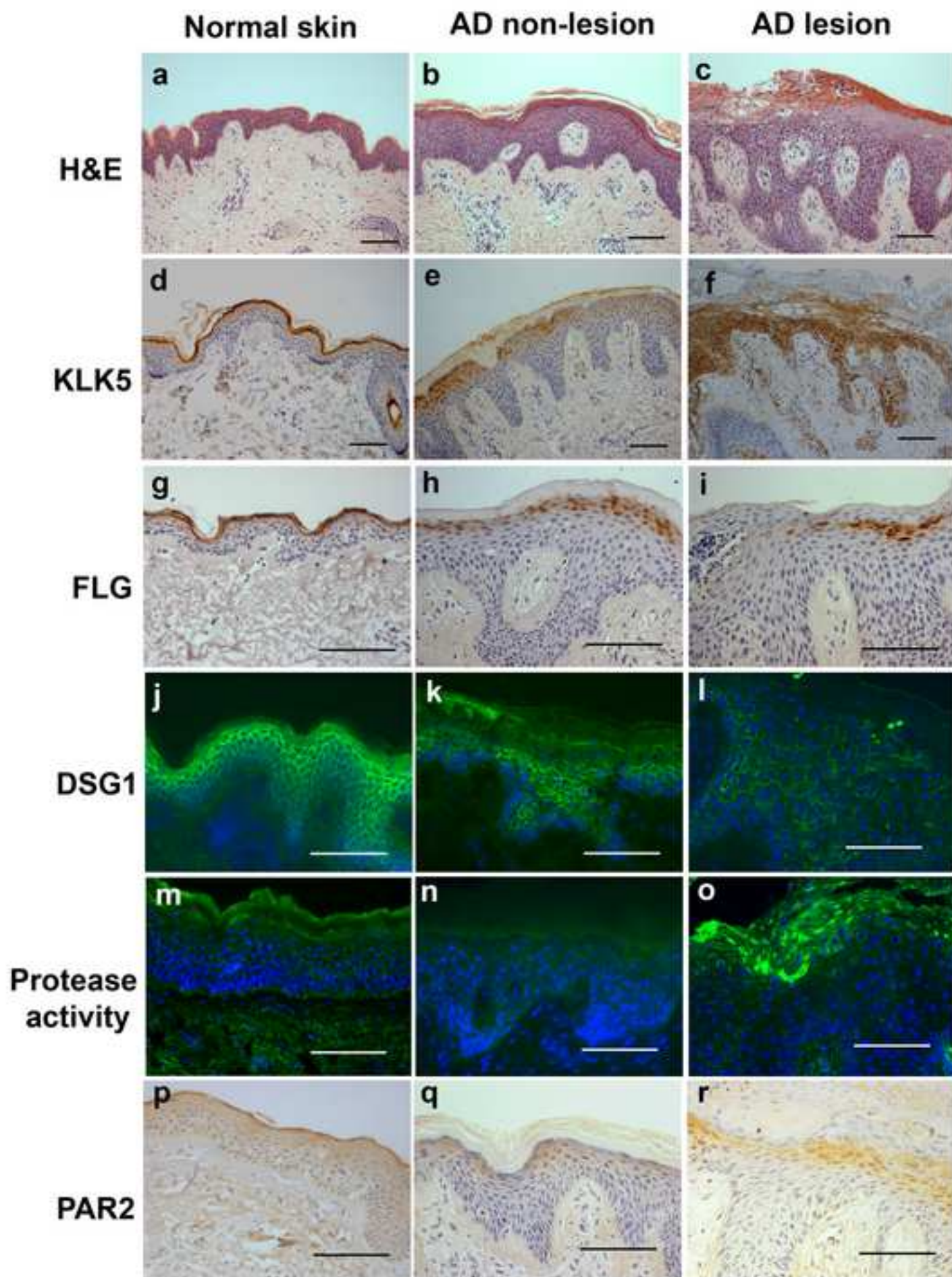


Figure 1B
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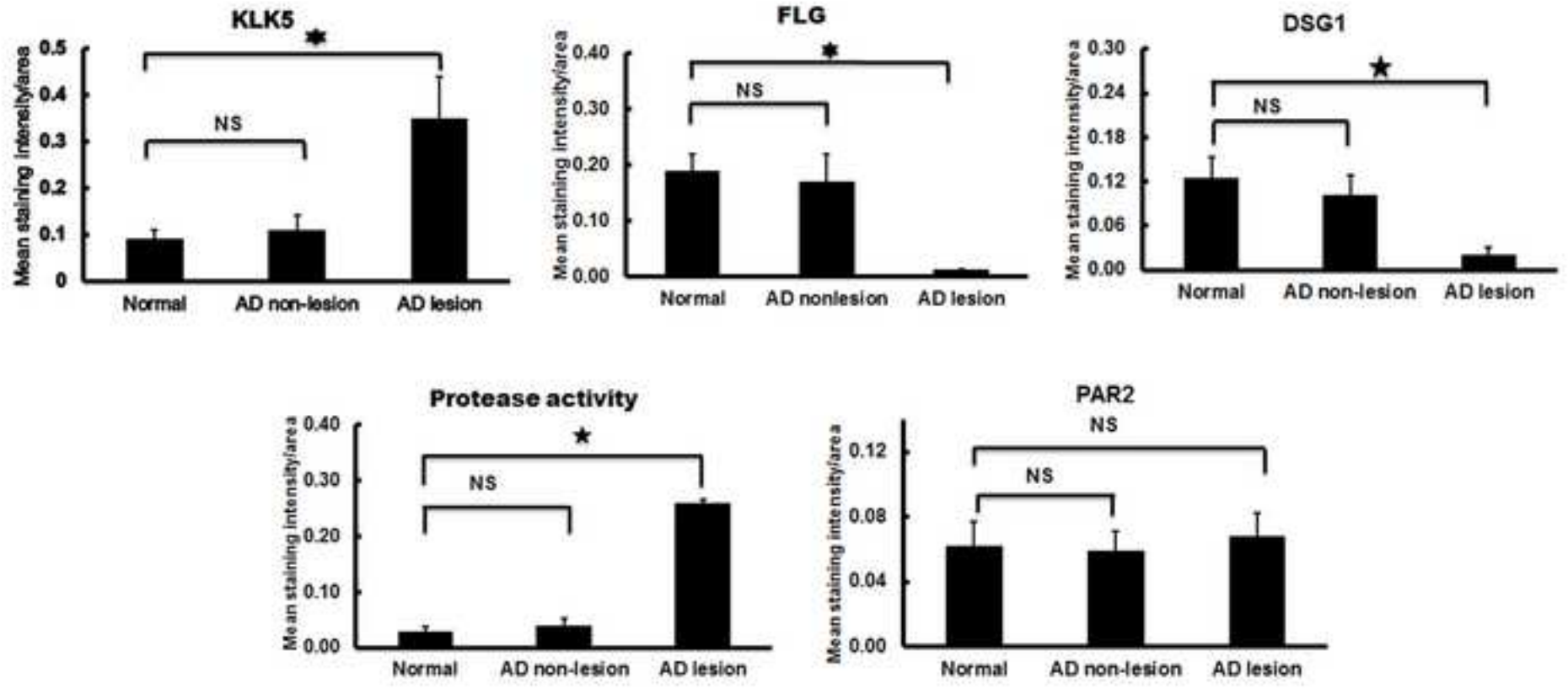


Figure 2

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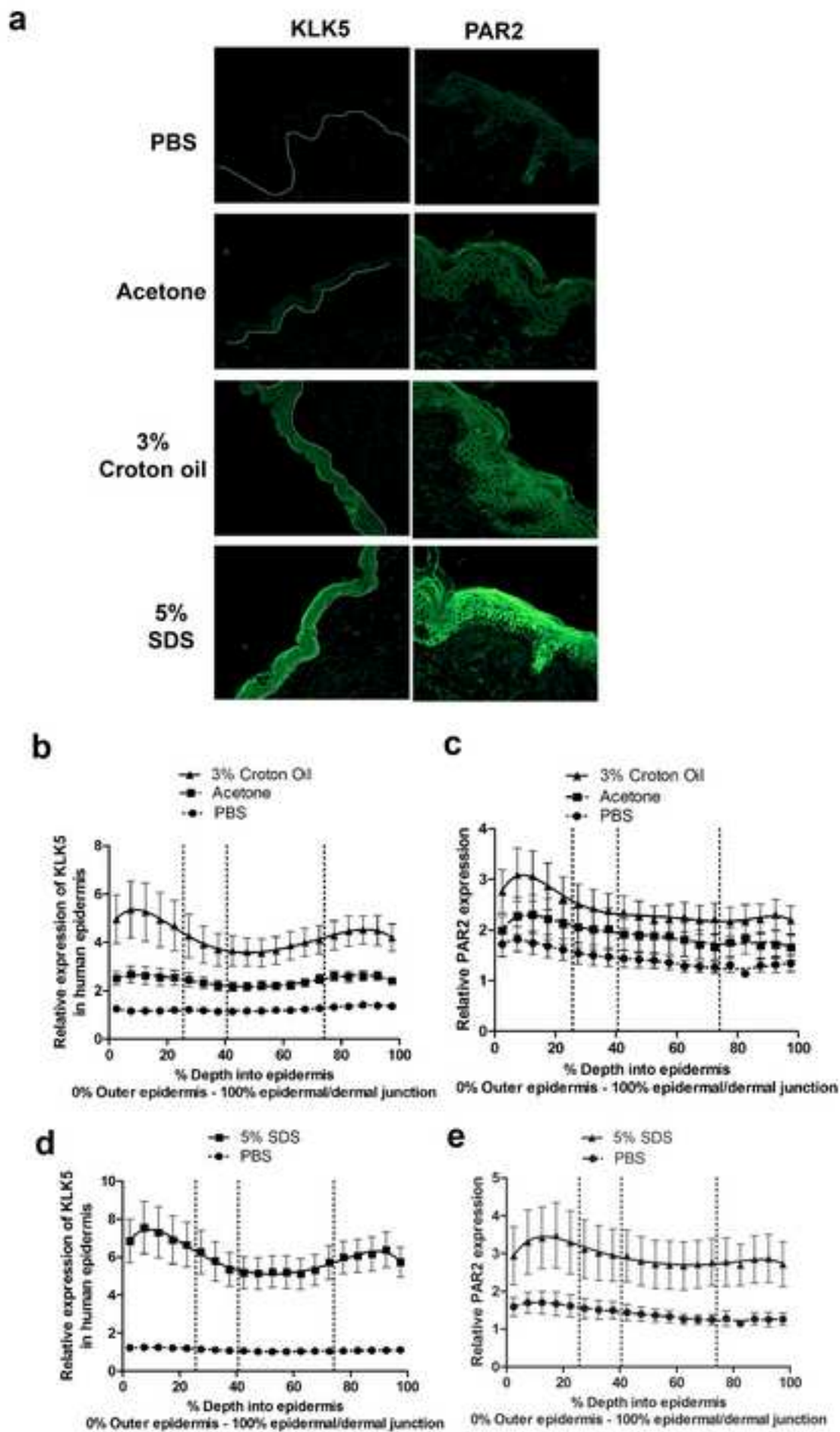


Figure 3
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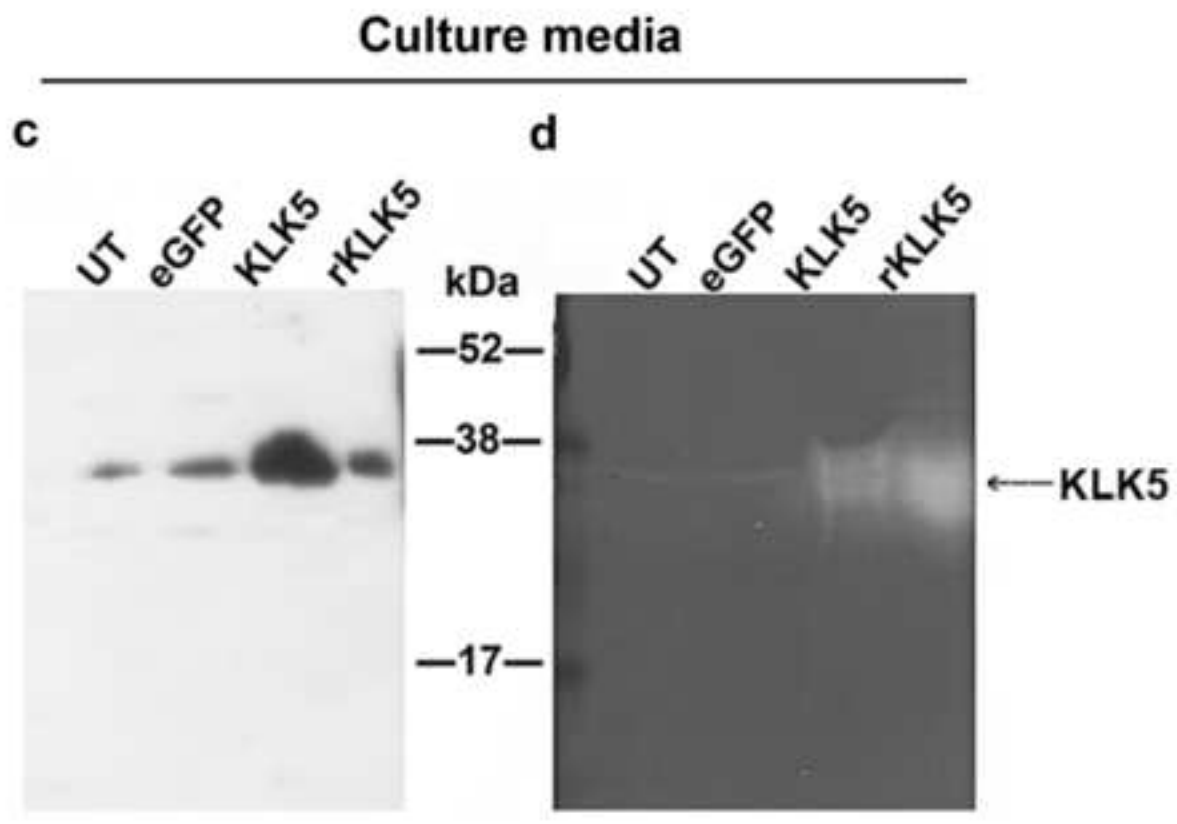
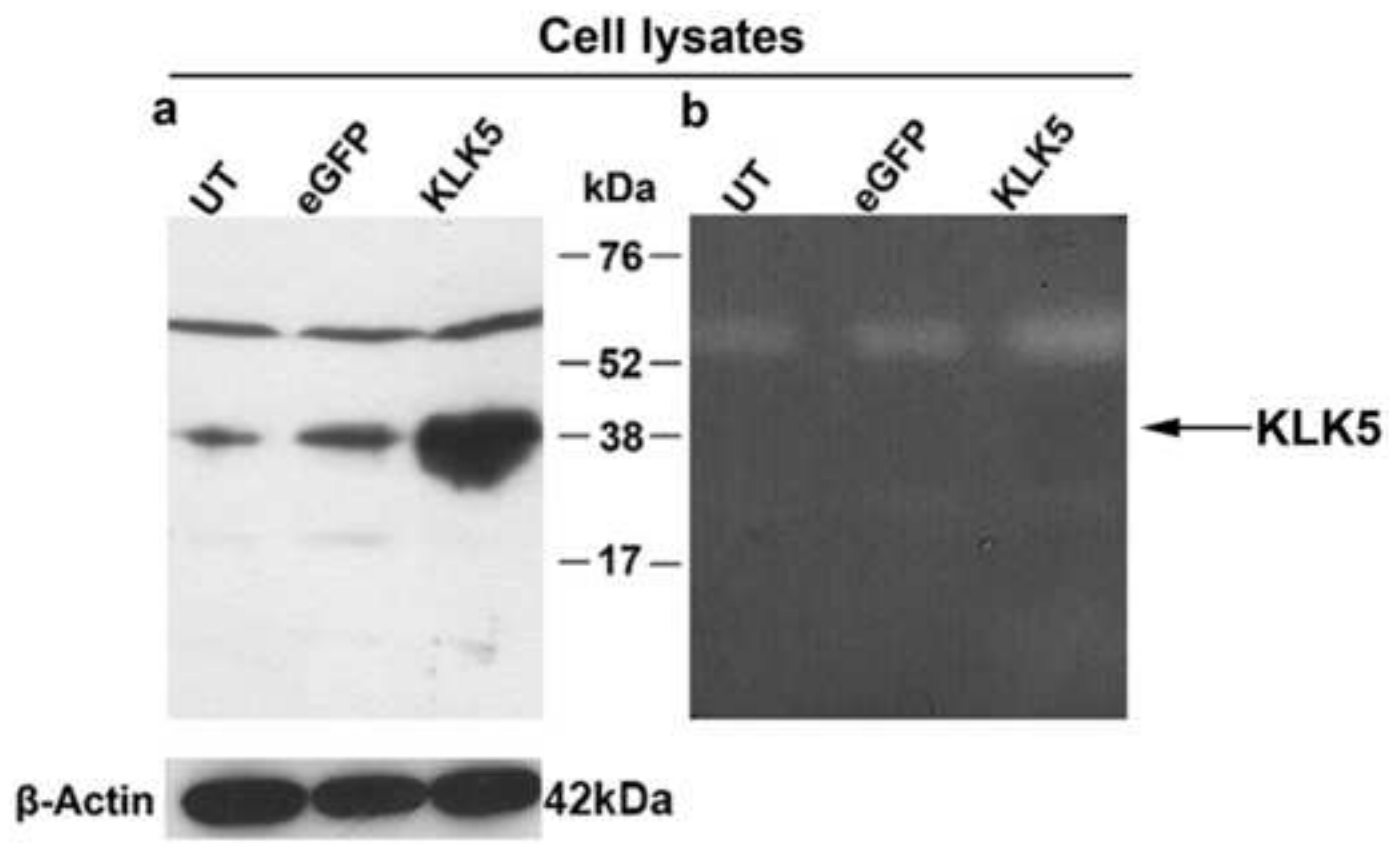
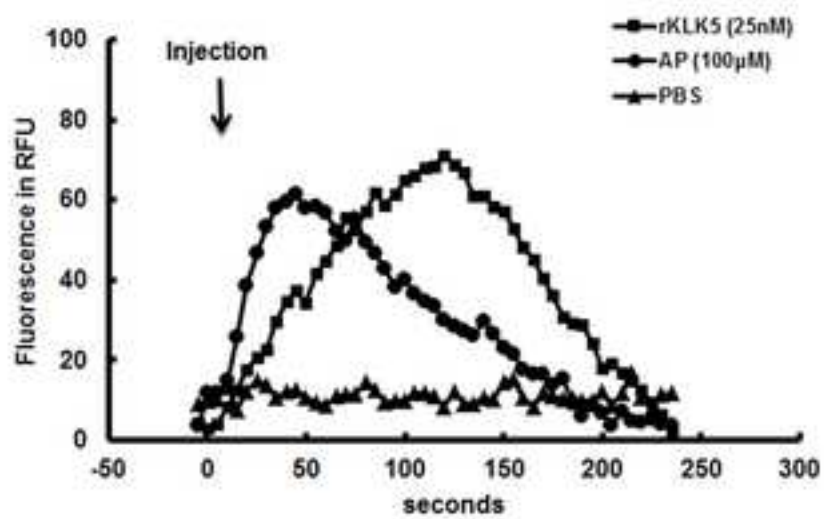


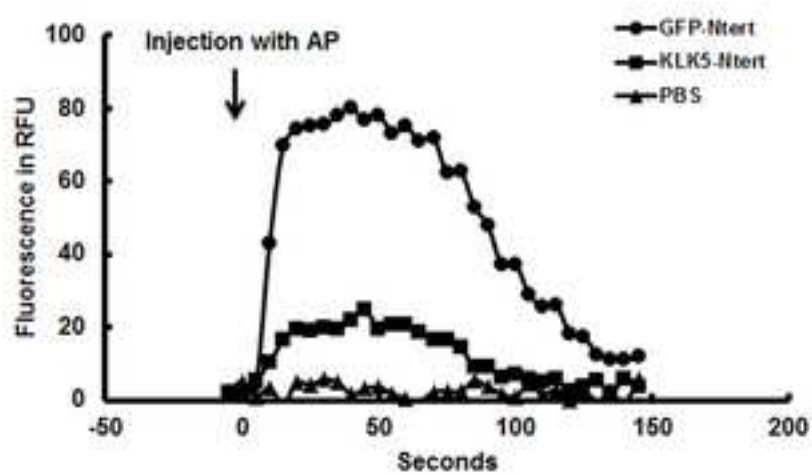
Figure 4

[Click here to download high resolution image](#)

a



b



c

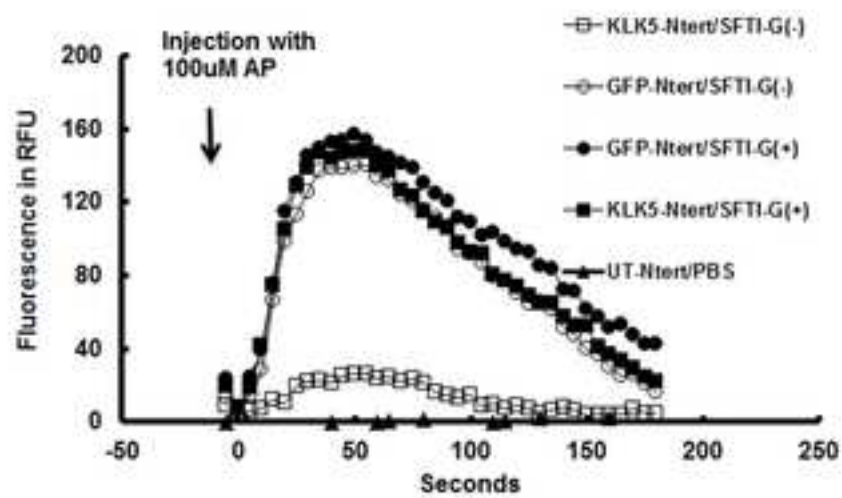
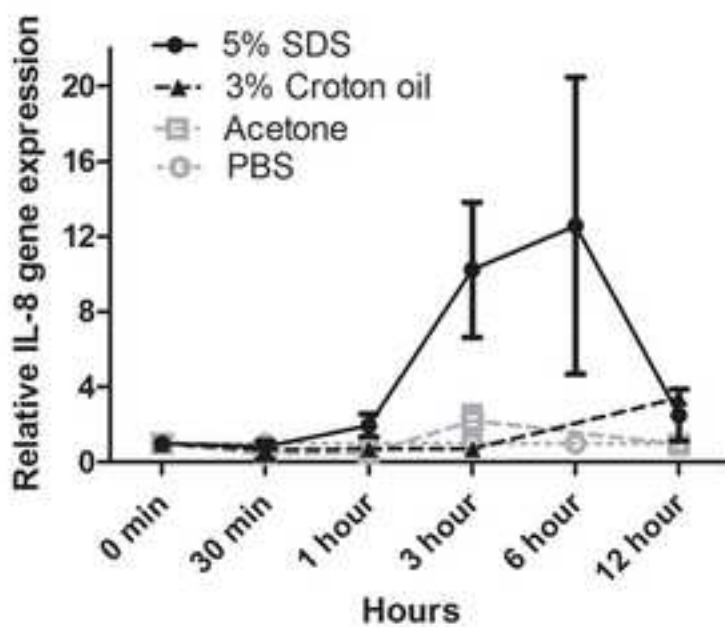
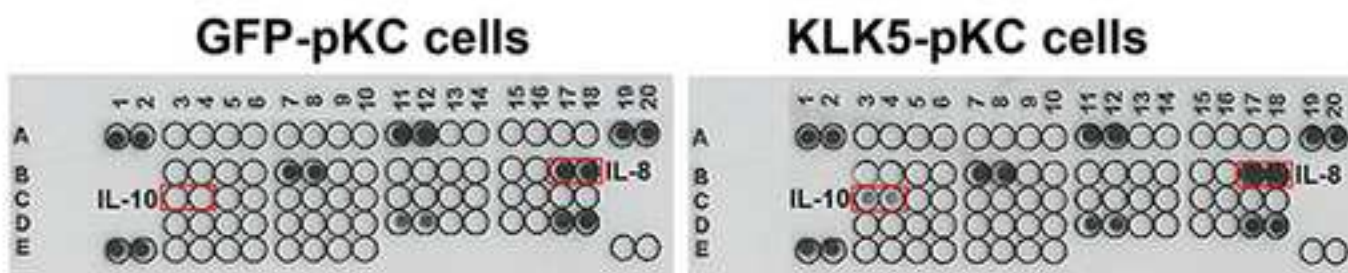


Figure 5
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a



b



c

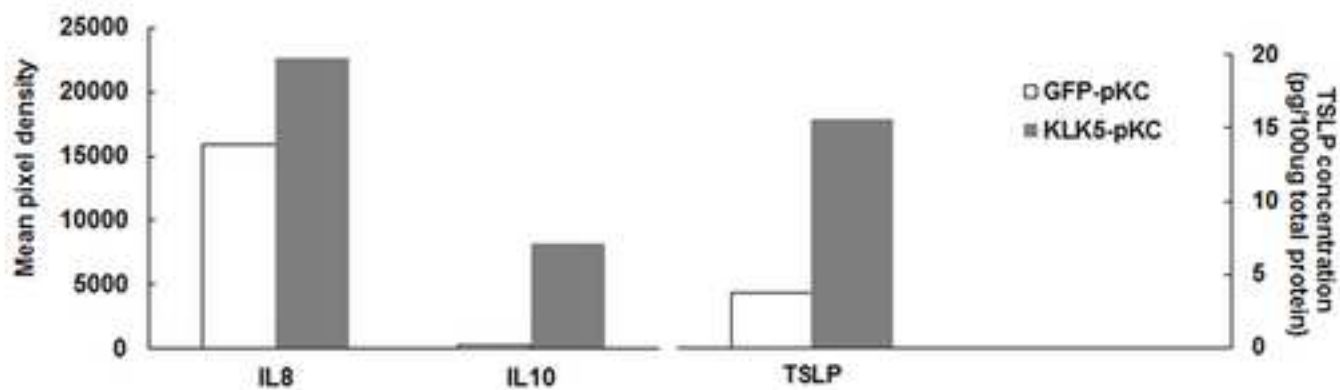


Figure 7
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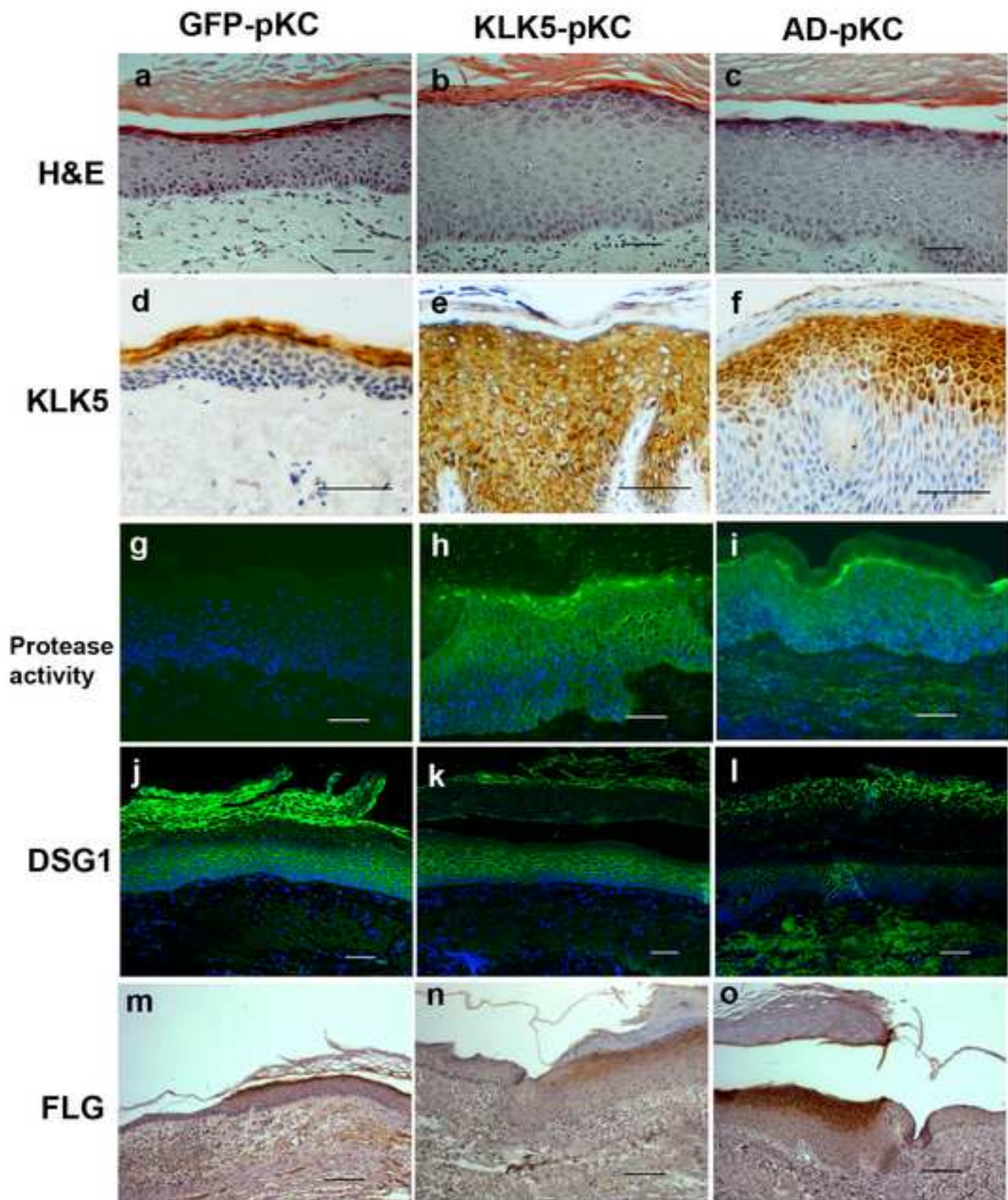


Figure S1

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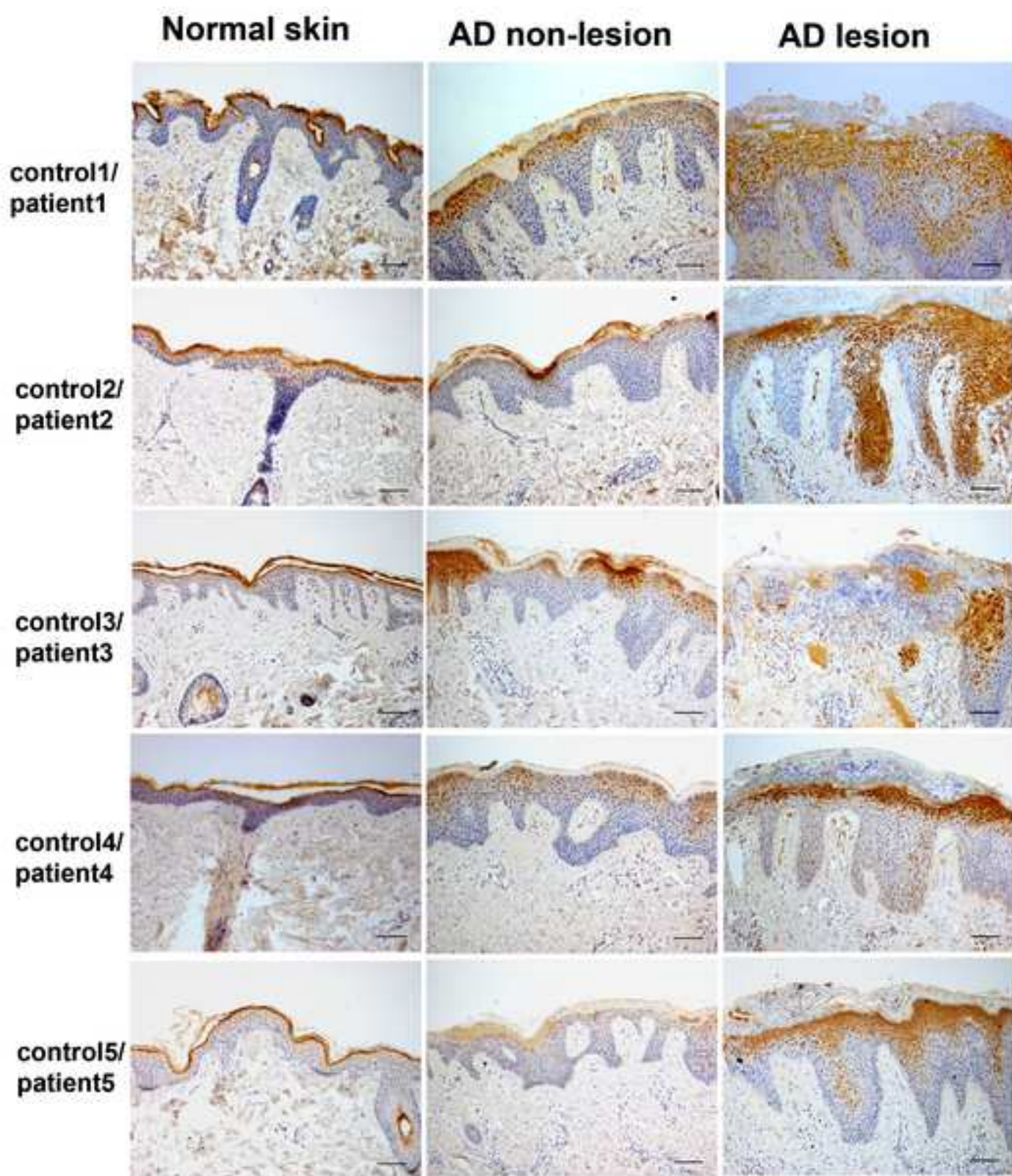


Figure S2

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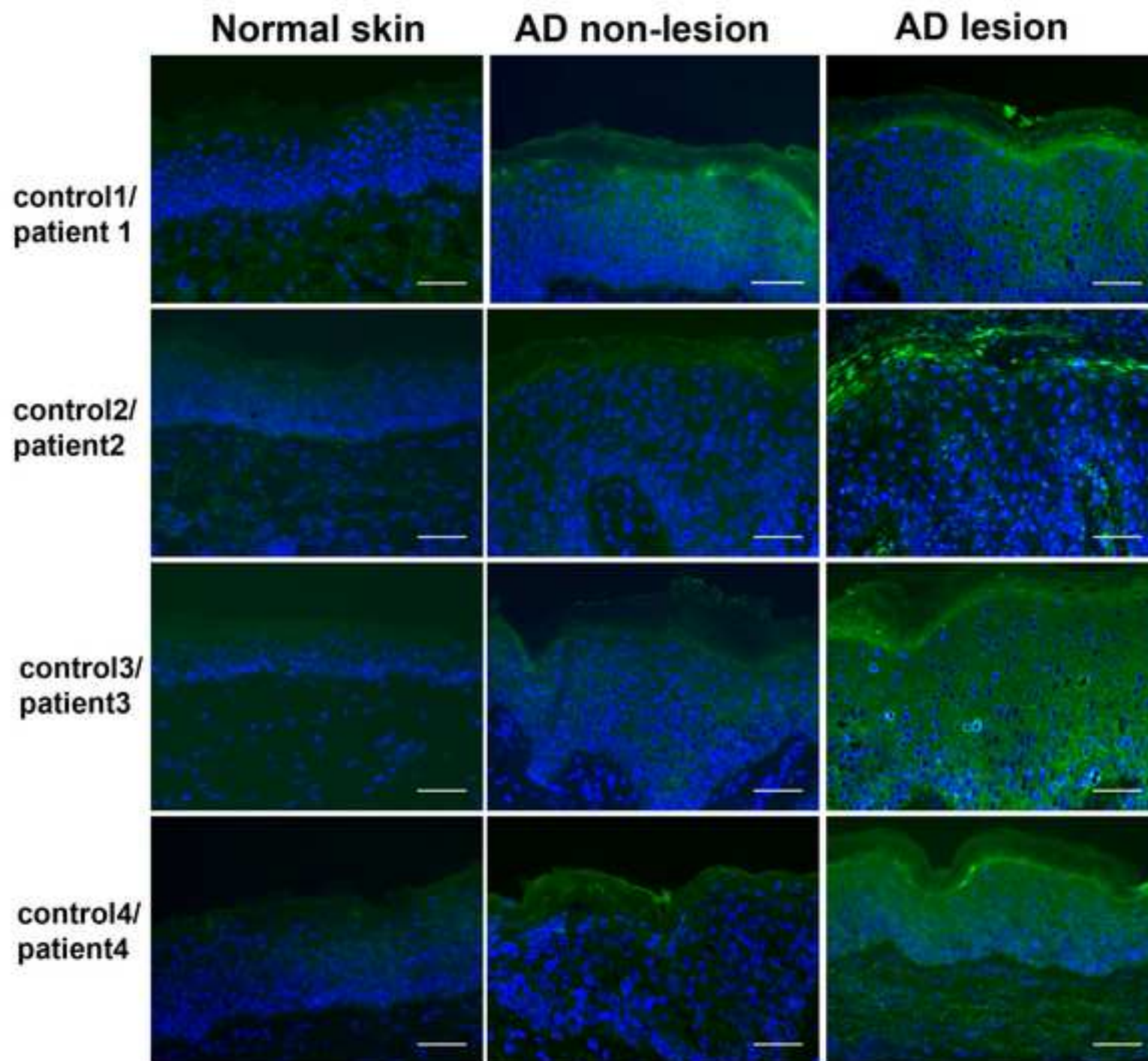


Figure S3
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