Regulation of serine protease inhibitor Kazal type-5 (SPINK5) gene expression in the keratinocytes

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Keywords

Serine protease inhibitor Kazal type-5 (SPINK5), Human keratinocyte, GATA3, Transcription regulation

Abstract

Objectives Serine protease inhibitor Kazal type-5 (SPINK5) plays a crucial role in deciding the timing of desquamation of the skin. Its gene expression is limited at the very surface of the stratum granulosum (SG), whereas expression of kallikreins (KLKs) encoding proteases is usually found throughout the stratum spinosum (SS) and SG.

Methods In order to explore the difference in expression regulation of these proteases/inhibitor, the function of SPINK5 promoter was examined using Luciferase assay.

Results Luciferase assay targeting the SPINK5 promoters (nucleotide -676/-532 and -318/-146 from the major transcription start site) showed high intensity in NHEK human keratinocyte. These two sites had neither common cis elements nor GATA3 element but electrophoretic mobility shift assay showed similar retardation bands. Moreover, DNA footprinting did not display specific protected bands. Thus, we could not identify cis-element(s) that controlled these elements. Differentiation induced by high Ca2+ medium failed to alter their luciferase activities. Transfection of GATA3 expressing vector significantly but slightly increased them and that of vector expressing its dominant negative form decreased.

Conclusions Although GATA3 is reportedly important for inhibition of proliferation and induction of differentiation of keratinocytes, its effect on SPINK5 expression was indirect and GATA3 alone was insufficient for final differentiation of keratinocytes where full SPINK5 expression was observed.

39 **Introduction**

40 To maintain a steady number of the stratum corneum (SC) layers is an important factor
41 for the solid skin barrier function. In the SC adjacent to the stratum granulosum (SG),
42 corneocytes are tightly bound to each other through the corneodesmosomes consisted of
43 extracellular corneodesmosin, transmembrane desmoglein-1 and desmocollin-1, and
44 cytoplasmic desmosomal plaque proteins (plakoglobin) [1, 2]. These firm contacts
45 reduce toward the surface and finally disappear on the surface where desquamation of
46 the corneocytes occurs [3]. This reduction is due to corneodesmosome degradation
47 caused by serine protease activities supplied by the tissue kallikrein gene (KLK) family
48 proteins consisting of fifteen members known as KLK1 to KLK15 [4]. Their activity is
49 regulated by serine protease inhibitor Kazal type-5 (SPINK5) [5, 6] in order to match
50 the shedding schedule to the proliferation rate of keratinocytes [7, 8]. This turnover is
51 very steady and maintained regardless of age [9, 10] on behalf of serine
52 protease-SPINK5 balance. In fact, the loss of function mutation of SPINK5 gene causes
53 hyperdesquamation and loss of the SC known as Netherton syndrome [11-13].
54 Thus, KLK and SPINK5 proteins co-localize and interact each other at the SC [14].
55 Nonetheless, expression of KLKs is usually found throughout the stratum spinosum
56 (SS) and SG [5] and, on the other hand, expression of SPINK5 is limited at the very
57 surface of SG [12]. For the ubiquitous expression of KLKs, GATA3 plays a very
58 important role [15], whereas the major regulators of local expression of SPINK5 are yet
59 known. Limited expression of SPINK5 plays a crucial role on avoidance of premature-
60 and/or hyper-desquamation. In order to clarify the basis of such limited expression of

61 SPINK5, the function of its promoter was explored in the present study.

62

63 **Materials and methods**

64 Keratinocyte culture

65

66 Normal human keratinocyte (NHEK; Kurabo, Osaka, Japan, several different single
67 donors) was inoculated at a concentration of 2,500 cells/cm^2 in culture media,
68 HuMedia-KG2 (Kurabo) supplemented with insulin 10 μg/ml, human epidermal growth
69 factor 0.1 ng/ml, hydrocortisone 0.5 μg/ml, bovine pituitary extract (BPE) 0.4% v/v,
70 gentamycin 50 μg/ml and amphotericin B 50 ng/ml. The culture was maintained at 37˚C
71 in a 95% O2/5% CO2 humidified chamber. The cells were subcultured twice in the
72 media of which calcium concentration was as low as 0.15 mM in order to avoid
73 differentiation. In vivo differentiation was induced as described elsewhere [16]. Briefly,
74 at the second passage when it became 40-60% confluent, the media was changed to
75 normal Ca^{2+} medium (0.15 mM Ca^{2+}; low Ca^{2+}) or high Ca^{2+} medium (1.5 mM Ca^{2+}) in
76 the absence of BPE and cultured for 2 days.

77

78 Reporter vectors

79

80 The proximal promoter of the human SPINK5 gene [nucleotide -1 to -1141 from the
81 major transcriptional start site (Accession No; Chromosome 5 - NC_000005.9)] was
82 PCR amplified using Sac I and Nhe I tailed primers, respectively -1141 and -1 (Table 1),

from the genome of NHEK as a template. Evolutional conservation search between human and Rhesus macaque (rheMac2) using ECR browser (http://ecrbrowser. dcode.org) displayed that -1 to -1214 was a putative promoter enhancer. Unfortunately, the primer search program (DNasis-Mac v3.6) selected a primer start with -1141 instead of -1214 was suitable for PCR amplification. After digestion with Sac I and Nhe I, the amplified products were initially cloned into pGL4.10[luc2] (Promega, Madison, WI, USA), designated as -1141/-1. To generate clones with different 5' terminal length, PCR was performed using different 5' primers for deletion and the 3' primer to conserve pGL4 sequence (Table 1). After digestion with Sac I, the products were re-ligated to obtain deletion clones. All cloned promoters in the reporter construct were sequenced to confirm their sequence was conserved after cloning.

Other vectors used

pGL-954/+40; among reporter vectors containing KLK1 promoter, the highest luciferase activity possessed [15], pcDNA3.1 (Invitrogen, Carlsbad, CA, USA), pcGATA3; the coding region of GATA3 [the open reading frame with 119 bp upstream to the start codon and 33 bp downstream from the stop codon] was cloned into pcDNA3.1, and pcGATA3mut; to obtain dominant negative mutant of pcGATA3, its sequence corresponding to 305KRR was changed to AAA [15].

SPINK5 promoter activity in NHEK cells

105

106 NHEK cells cultured on 24-well plates were transfected with 0.75 μg of reporter vectors
107 pGL4.10[luc2] cloned with different fragments from the proximal promoter of the
108 human SPINK5 using Tfx™-20 (Promega, Madison, WI, USA). After 48 hours, the
109 cells were lysed using a luciferase reporter assay system (Promega, Madison, WI, USA)
110 and the firefly luciferase activities were measured with a Lumat LB9507 (Berthold
111 Technologies, Tokyo, Japan). All experiments were performed at least three times for
112 each reporter plasmid and the relative luciferase activity was calculated. Results were
113 presented as the mean ± SD. In order to confirm that the putative sequence was really
114 active, -676/-532 or -318/-146 was PCR amplified using a set of primers (Table 1), used
115 for electrophoretic mobility shift assay (EMSA) and cloned into pGL4.10[luc2] for the
116 luciferase assay.

117

118 Search for putative cis elements

119

120 In order to find putative cis elements, sequence of -1141/-1 was subjected to
121 TFSEARCH (www.cbrc.jp/research/db/TFSEARCH.html), ALGGEN PROMO [17, 18]
122 and PhysBinder (http://bioit.dmbr.ugent.be/ physbinder/ [19]).

123

124 Electrophoretic mobility shift assay (EMSA)

125

126 According to the results of luciferase assay, -676/-532, and -318/-146 supposed to

127 contain activating element, were PCR amplified using primers listed in Table 1. Nuclear
128 protein was obtained from 1×10^6 NHEK cells cultured with KG2, or 0.15 and 1.5 mM
129 Ca^{2+} media without BPE, based on the protocol [20]. Briefly, after washed with ice cold
130 PBS (Ca^{2+} and Mg^{2+} free), cells were resuspended with 5 volumes of ice-cold cell
131 homogenization buffer (10 mM HEPES-KOH, pH7.8, 1.5 mM $MgCl_2$, 10 mMKCl,
132 0.5 mM DTT, 0.5 mM PMSF, and 1× proteinase inhibitor cocktail (Nakalai tesque,
133 Kyoto, Japan) to stand on ice for 10 min, and collected by centrifuging at 250 g for
134 10 min. The cell pellet was resuspended in 3 volumes of ice-cold cell homogenization
135 buffer containing 0.05% (v/v) Nonidet P-40, and homogenized on ice with 20 strokes of
136 a tight-fitting Dounce homogenizer. The nuclei were collected by centrifugation at
137 250 g for 10 min at 4 °C. After removing the supernatant, the nuclei pellet was
138 resuspended with 20 ml cell suspension buffer (40 mM HEPES-KOH, pH 7.8, 0.4 M
139 KCl, 10% glycerol, 1 mM DTT, 0.1 mM PMSF, and 1× proteinase inhibitor cocktail).
140 NaCl was added to a final concentration of 300 mM, and the resuspended nuclei was
141 incubated on ice for 30 min then centrifuged at 15,093 g at 4 °C for 15 min. The
142 supernatant containing nuclear protein was divided into aliquots and stored at -80 °C
143 until use. Protein concentration was determined by the Bradford method on Model 680
144 Microplate Reader (Bio-Rad, Hercules, CA, USA). After 100 ng of each DNA were
145 mixed with 6 μg of nuclear protein in a final volume of 20 μl binding buffer containing
146 10 mM Tris/HCl (pH 8.0), 50 mM KCl, 3.5 mM DTT, 0.25% Tween 20, and 0.025 μg
147 salmon sperm DNA and allowed to stand for 25 min at room temperature,
148 electrophoretic mobility shift assay (EMSA) was performed on 1% agarose/ethidium

149 bromide gel. Poly(dI-dC) as DNA and BSA as protein were served as negative control.

150

151

152 DNA footprinting

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154 In order to clarify the location that nuclear protein was attached, DNA footprinting
155 using DNase I protection was performed on pGLSP-676/-532 and pGLSP-318/-146 that
156 showed high luciferase activities. pGL-954/+40 was served as positive control. These
157 DNAs were bound with nuclear protein as described above. To this mixture, 1 U RQ1
158 RNase-free DNase I (Promega) and supplied DNase I buffer were added and allowed to
159 stand at room temperature for 1 minute. The digested DNAs were phenol/chloroform
160 extracted and ethanol precipitated. Footprinting ladder was PCR synthesized using PCR
161 primer, RVprimer3 by BigDye (Applied Biosystems, CA). The sequence was analyzed
162 by ABI Prism 3100 (Applied Biosystems).

163

164 Statistical analysis

165

166 In order to clarify statistical significance among luciferase activities, one-way ANOVA
167 with Tukey's HSD test as post hoc test was used.

168

169

170

171 **Results**

172 To examine the location of putative activating and/or inhibitory cis-elements, reporter
173 vectors were transfected into NHEK cells and luciferase assay was performed.
174 Transcriptional activity was almost null when pGLSP-1141/-1 and pGLSP-1076/-1 were
175 transfected to NHEK (Fig.1). When a clone deleted -1141 to -932, i.e. pGLSP-932/-1,
176 was transfected, the significantly higher intensity than pGLSP-1141/-1 and
177 pGLSP-1076/-1 was observed. Moreover, transfection of a clone deleted -1141 to -676,
178 i.e. pGLSP-676/-1, and a clone deleted -1141 to -318, i.e., pGLSP-318/-1 gave the
179 significantly higher intensity than other clones. The intensities of these two clones did
180 not show significant difference. The intensity of pGL-954/+40 containing KLK1
181 promoter was more than three times higher than that of pGLSP-318/-1.

182

183 Effect of high Ca^{2+} medium on transcriptional activity of SPINK5 putative promoter

184

185 Intensities of pGLSP-676/-1 and pGLSP-318/-1 were significantly higher than that of
186 pGLSP-932/-1, and those of pGLSP-318/-1 and pGLSP-318/-146 were almost the same
187 (Fig.2). Thus, pGLSP-676/-1, pGLSP-318/-1 and pGLSP-318/-146 were subjected to
188 examine the effects of high Ca^{2+} medium. Removal of BPE from Humedia-KG showed
189 no significant differences in the intensities of pGLSP-676/-532, pGLSP-318/-1 and
190 pGLSP-318/-146 (Fig.2). High Ca^{2+} media-cultured NHEK also did not show any
191 differences in their intensities.

192

193 TFSEARCH, ALGGEN PROMO and PhysBinder to search putative cis elements

194

195 Sequence of -1141/-1 was subjected to TFSEARCH, ALGGEN PROMO and
196 PhysBinder for searching putative cis elements. Among elements that hit by these
197 programs, those did not exist in -1141/-933, -860/-677, -554/-319, -169/-1 were listed
198 (Table 2). Excluding SRY, no common cis-elements existed in the activating regions
199 -932/-837, -676/-532 and -318/-146. A point mutation on -206 G to A reportedly
200 produce GATA3 binding site [21], but our -206 was G. Thus, no GATA3 binding site
201 was extracted in any of the activating regions.

202

203 Electrophoretic mobility shift assay (EMSA)

204

205 -676/-532 and -318/-146 supposed to contain activating element were PCR amplified
206 using primers listed in Table 1 and subjected to EMSA (Fig.3). Mobility of both
207 fragments was delayed by all of nuclear extracts from normal HuMedia-KG-, low Ca^{2+}
208 medium-, and high Ca^{2+} medium-cultured NHEK. Location of retarded bands was
209 almost identical regardless of -676/-532 or -318/-146 and regardless of culture media.

210

211 Effect of pcGATA3, and pcGATA3mut on SPINK5 promoter

212

213 Co-transfection of pcGATA3 with pGLSP-676/-532, -318/-146 and -318/-1 increased
214 the intensities significantly (Fig.4). However, their intensities were still less than 1/3 of

215 that of pGL-954/+40. On the other hand, pcGATA3mut co-transfection decreased the
216 activities significantly in any of the plasmids.
217
218 DNA foot printing
219
220 When pGLSP-676/-532 and pGLSP-318/-146 were subjected to DNA footprinting, no
221 specific sequence was protected (pGLSP-676/-532; data not shown and
222 pGLSP-318/-146, Fig.5). However, in case of pGL-954/+40, protected band was found
223 at -941/-937 where STAT4, c-Ets-1, c-Ets-2, Elk-1, Pax-5 and p53 could be putative
224 transcription factors. Thus, we could not specify cis-elements for pGLSP-676/-532 and
225 pGLSP-318/-146 and EMSA using oligo-DNA was currently unavailable.
226
227 **Discussion**
228 It was surprising that pGLSP-676/-1 and pGLSP-318/-1 displayed high luciferase
229 activities in NHEK cells. NHEK cells were cultured in culture media containing
230 0.15mM Ca^{2+} to avoid differentiation. Expression of SPINK5 is limited at the very
231 surface of SG, namely SPINK5 expression is limited at the very last step of
232 differentiation just before denucleation [5]. Thus, in NHEK cells without induction of
233 differentiation, no luciferase activity was expected in any of reporter constructs.
234 However, their intensities were only less than 1/3 of that of pGL-954/+40 containing
235 KLK1 promoter. Expression of KLK1 is observed throughout the SS and SG [5]. It is
236 not surprising that, independent of 0.15 mM Ca^{2+}, KLK1 promoter appeared to have an

237 activity in NHEK cells with very high intensity. Therefore, some NHEK seemed to
238 differentiate to the final step in this medium even when Ca^{2+} concentration was
239 maintained at 0.15mM and growth factors like BPE were included. Growth factors are
240 considered to suppress keratinocyte differentiation [22, 23]. However, neither removal
241 of growth factors alone (low Ca^{2+} medium) nor removal of growth factors and an
242 increase in Ca^{2+} concentration (high Ca^{2+} medium) altered the intensity of luciferase and
243 the movability of EMSA bands, indicating that such stimulation is not suitable for the
244 final differentiation. On the other hand, high cell density reportedly promotes
245 keratinocyte differentiation [23]. It is conceivable that several colonial clones may
246 differentiate further.

247 TFSEARCH, ALGGEN PROMO and PhysBinder did not find common transcription
248 factors in the activating regions -932/-837, -676/-532 a3d -318/-146. Only SRY
249 (Sex-determining region Y) was common to -932/-837 and -318/-146. However, SRY
250 was encoded on Y chromosome and regulated sex differentiation [24], indicating that
251 this transcription factor was not responsible for skin function. Judging from the results
252 of EMSA, the size of transcription factor binding to -676/-532 and -318/-146 was
253 almost the same. It is possible that the same transcription factor which could not be
254 detected by these programs occupied -932/-837 and -318/-146. Otherwise, the results of
255 DNA footprinting might indicate that more than one protein occupied these sequences.

256 An epidemiological research with case-control design in Chinese Han population
257 indicated that -206 G>A mutation produced GATA3 binding site in SPINK5 promoter
258 and that the G allele was significantly more common among patients with asthma than

259 among the controls [21]. Existence of GATA3 site might directly increase SPINK5
260 expression. However, our -206 was G, hence no GATA3 binding site was extracted in
261 neither -676/-532 nor -318/-146. Therefore, GATA3 unlikely affected directly on these
262 sites. Although intensity was still less than 1/3 of pGL-954/+40, induction of GATA3 in
263 NHEK increased their luciferase activity. On the other hand, transfection of dominant
264 negative GATA3mut suppressed it. The effect of GATA3 was limited and indirect. This
265 may be via transcription factor(s) downstream to GATA3. For example, GATA3
266 reportedly react with Smads [25] but none of TFSEARCH, ALGGEN PROMO, and
267 PhysBinder found any Smads site in -676/-532 and -318/-146. It seems necessary to
268 explore transcription factors downstream to GATA3 that could regulate these sites.
269 However, unfortunately, we could not yet identify the transcription factor(s) that
270 regulated SPINK5 expression in keratinocytes. Otherwise, inhibition of proliferation
271 and induction of differentiation of keratinocytes by GATA3 [26] might trigger the next
272 step of differentiation of keratinocytes and SPINK5 expression that was regulated by
273 transcription factor(s)/cis-element(s) different from GATA3 system. That is, GATA3 is a
274 very important factor for the differentiation of keratinocytes, but GATA3 itself seems to
275 be insufficient for the final differentiation and/or SPINK5 expression.
276 It is very difficult to obtain sufficient amount of nuclear protein from human skin where
277 SPINK5 expresses. It seems also very difficult to induce final differentiation to
278 keratinocyte culture. High Ca^{2+} stimulation in the present study was insufficient for the
279 final differentiation. Further examination on expression regulation mechanism of
280 -676/-532 and -318/-146 will supply important information related to a key regulator of

281 SPINK5 expression and final differentiation of keratinocytes.

282

283 Conflict of interest

284 We acknowledge that we have no conflict of interest.

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362

363 **Figure legends**

364 Fig.1.

365 Relative luciferase activities of reporter plasmids containing different lengths of
366 putative SPINK5 promoter in NHEK. Each column represents the mean of three
367 independent experiments, each done in triplicate; bars, ± SD. Inset; Significant
368 differences in the intensity between the groups ($P < 0.05$ One-way ANOVA and post hoc
369 test using Tukey's HSD test). When pGL-954/+40 was transfected, the intensity was
370 higher than $450x10^3$ RLU.

371

372 Fig.2.

373 Effect of high Ca^{2+} medium on relative luciferase activities of SPINK5 putative
374 promoters. pGLSP-676/-532, pGLSP-318/-1 and pGLSP-318/-146 were transfected to
375 normal HuMedia-KG2-, low Ca^{2+} medium-, and high Ca^{2+} medium-cultured NHEK.
376 Each column represents the mean of three independent experiments, each done in
377 triplicate; bars, ± SD. No significant difference was observed in any of pGLSP-932/-1,
378 pGLSP-676/-1 and pGLSP-318/-1 among different culture media (One-way ANOVA
379 and post hoc test using Tukey's HSD test).

380

381 Fig.3.

382 EMSA for fragments -676/-532 and -318/-146. In order to avoid non specific binding,
383 poly(dI-dC) was utilized. Aside from nuclear proteins, bovine serum albumin (BSA)
384 was served as negative control.

385

386 Fig.4.

387 Effects of co-transfection of pcGATA3 and pcGATA3mut with pGLSP-676/-532,

388 pGLSP-318/-1 and pGLSP-318/-146. Each column represents the mean of three

389 independent experiments, each done in triplicate; bars, ± SD. *Significant difference

390 (One-way ANOVA and post hoc test using Tukey's HSD test).

391 Fig. 5.

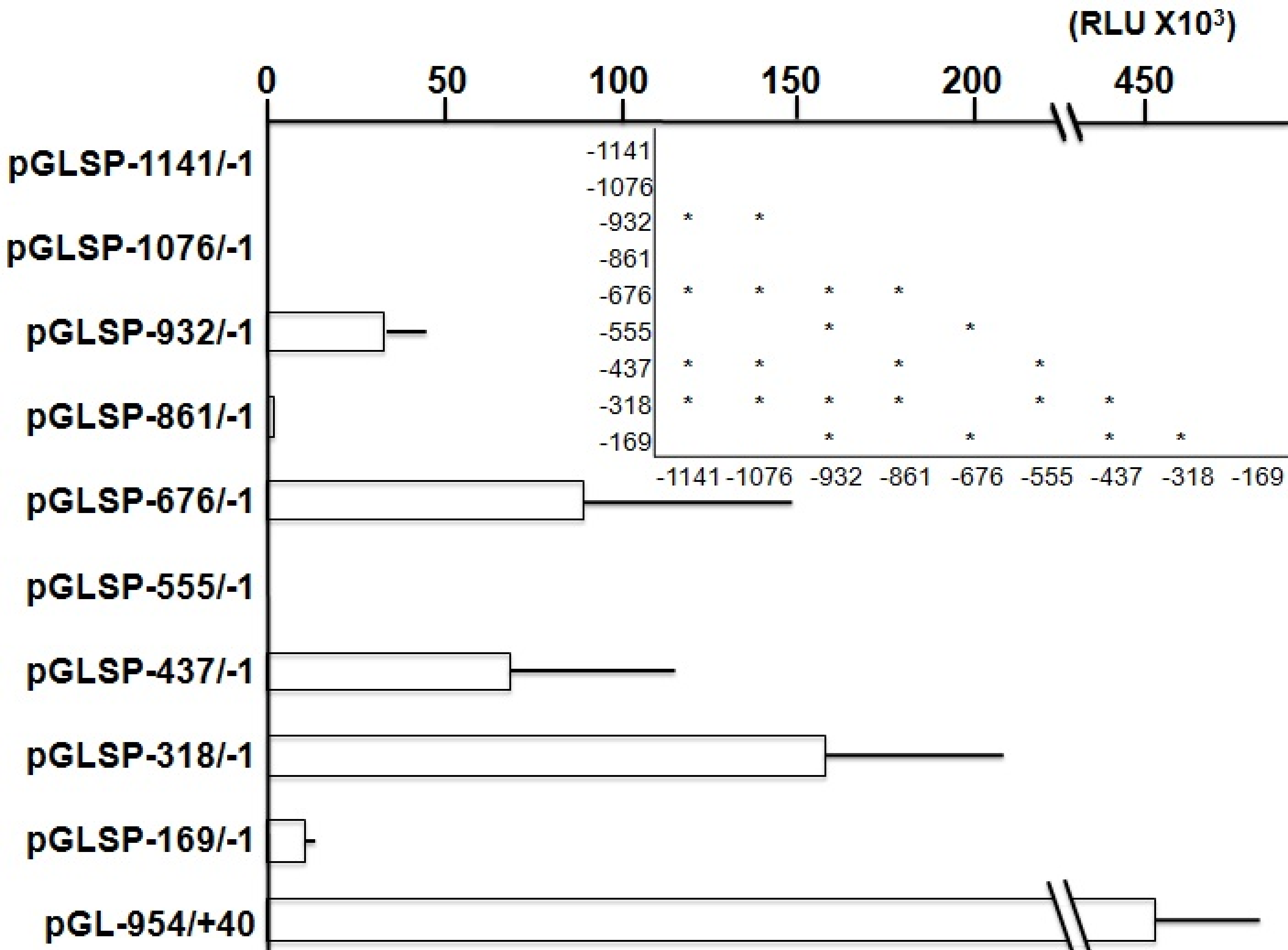
392 Sequence ladder of pGSP-318/-146 (Upper) and pGL-954/+40 (Lower; positive control)

393 after digestion by DNaseI along with nuclear protein. Sequence ladder was made by the

394 primer, RV3 (supplied for pGL4.10[luc2]). Underline; protected sequence where

395 TFSEARCH, ALGGEN PROMO, and PhysBinder selected STAT4, c-Ets-1, c-Ets-2,

396 Elk-1, Pax-5 and p53 as putative cis-elements.


(RLU X10³)
0
50
100
150
200
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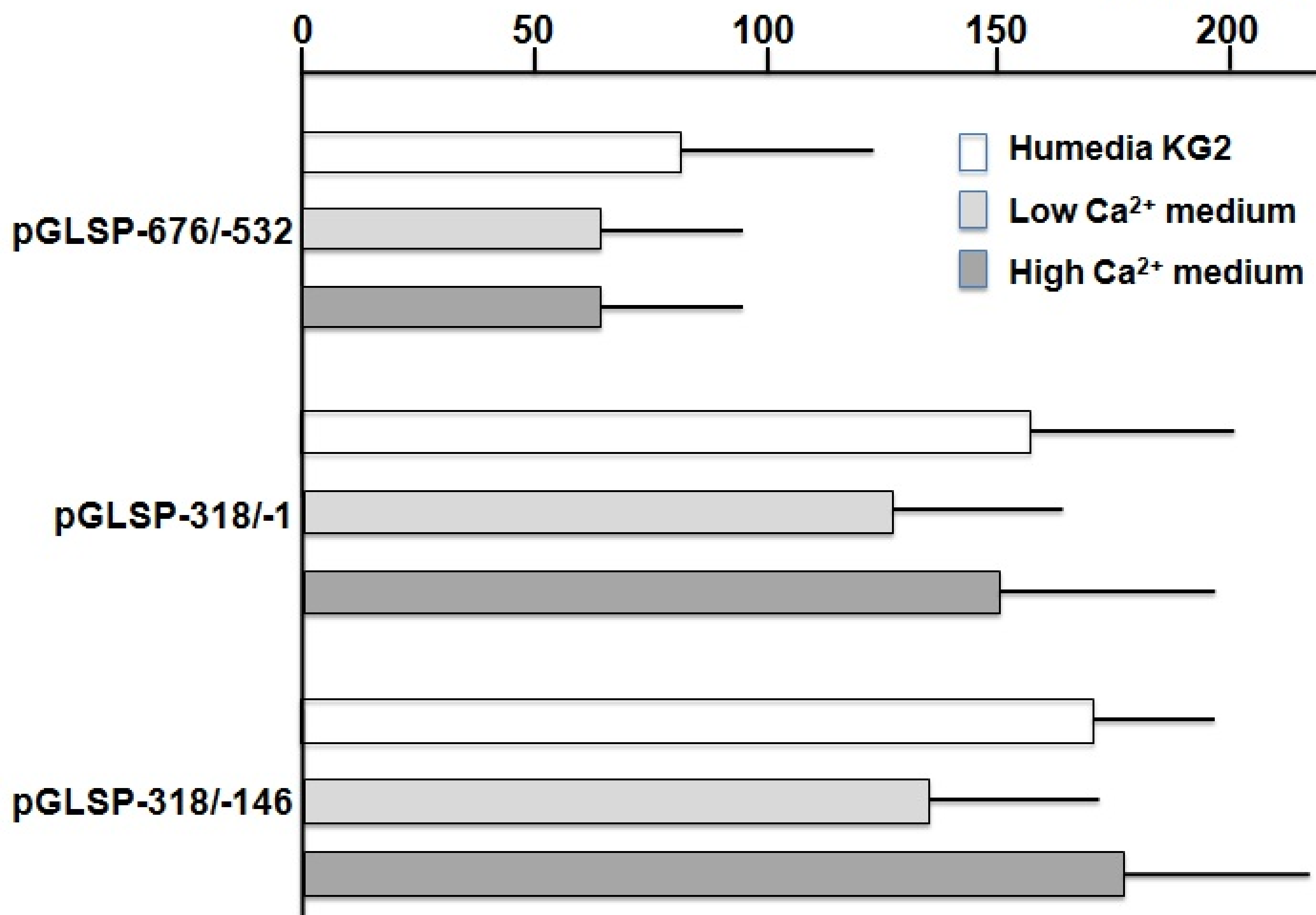
pGLSP-1141/-1
pGLSP-1076/-1
pGLSP-932/-1
pGLSP-861/-1
pGLSP-676/-1
pGLSP-555/-1
pGLSP-437/-1
pGLSP-318/-1
pGLSP-169/-1
pGL-954/+40
-1141
-1076
-932
-861
-676
-555
-437
-318
-169
-1141 -1076 -932 -861 -676 -555 -437 -318 -169

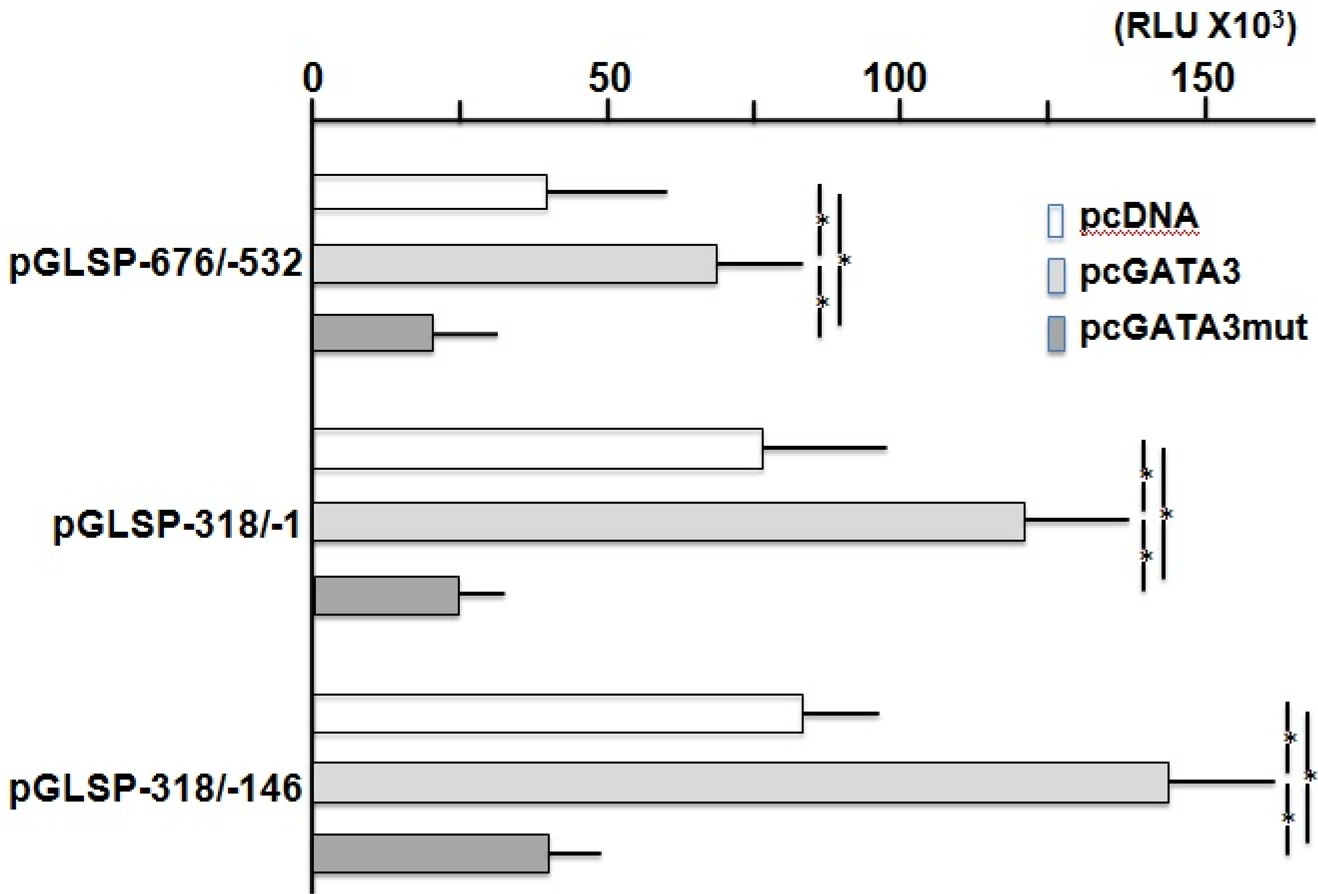

(RLU X10³)
0
50
100
150
200
Humedia KG2
Low Ca²⁺ medium
High Ca²⁺ medium
pGLSP-676/-532
pGLSP-318/-1
pGLSP-318/-146

Shifted DNA

Free DNA

100bp ladder

-	-	-318/-146	-676/-532				-318/-146					DNA
KG2	KG2 (dI-dC)	BSA	-	KG2	0.15mM Ca^{2+}	1.5mM Ca^{2+}	-	KG2	0.15mM Ca^{2+}	1.5mM Ca^{2+}	KG2 (dI-dC)	Nuclear protein



(RLU X10³)
0
50
100
150
pGLSP-676/-532
pGLSP-318/-1
pGLSP-318/-146
pcDNA
pcGATA3
pcGATA3mut
*

SPINK5 promoter (pGSP-318/-146)

GTCTACGAGGTAGTATATCTGGTATTTCTGAGGAATTCGGTTATT

ATATAGGATTATAGAATATTCAACTTTGAGTAGGGTCTGTGGACT

CCATCAATTGCGTTCTGGGGGAGGTACCATGAAAGAACATTCCTA

CTTTAGAAATAGATATTCTCTGTTCAAACCTGACACC

KLK1 promoter (pGL-954/+40)