Development of Hepatitis C Virus Production Reporter Assay Systems Using Two Different Hepatoma Cell Lines

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Summary

The hepatitis C virus (HCV) infection system was developed using the HCV JFH-1 strain (genotype 2a) and HuH-7 cells, and this cell culture is so far the only robust production system for HCV. In patients with chronic hepatitis C, the virological effects of pegylated interferon and ribavirin therapy differ depending on the HCV strains and genetic background of the host. Recently, we reported the hepatoma-derived Li23 cell line, in which the JFH-1 life cycle was reproduced at a level almost equal to that in the HuH-7-derived cell line. To monitor the HCV life cycle more easily, we here developed JFH-1 reporter assay systems using both the HuH-7- and Li23-derived cell lines.

To identify the genetic mutations by long-term cell culture, HCV RNAs in HuH-7 at 130 days after infection were amplified and subjected to sequence analysis to find the adaptive mutation for robust viral replication. We identified two mutations, H2505Q and V2995L, in the NS5B region. V2995L but not H2505Q enhanced JFH-1 RNA replication. However, we found that H2505Q but not V2995L enhanced HCV RNA

49 replication of the strain O (genotype 1b). We also selected highly permissive D7 cells
50 by serial subcloning of Li23 cells. The expression level of claudin-1 and Niemann-Pick
51 C1-like 1 in D7 cells is higher than that in parental Li23 cells.
52 In this study, we developed HCV JFH-1 reporter assay systems using two distinct
53 hepatoma cell lines, HuH-7 and Li23. The mutations in NS5B resulted in different
54 effects on strains O and JFH-1 HCV RNA replication.
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66 **Introduction**

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68 Hepatitis C virus (HCV) infection frequently causes chronic hepatitis and leads to
69 liver cirrhosis and hepatocellular carcinoma. The elimination of HCV by antiviral
70 reagents seems to be the most efficient therapy to prevent the fatal state of the disease.
71 HCV belongs to the *Flaviviridae* family and contains a positive single-stranded RNA
72 genome of 9.6 kb. The HCV genome encodes a single polyprotein precursor of
73 approximately 3000 amino acid residues, which is cleaved by host and viral protease
74 into at least 10 proteins in the following order: Core, envelope 1 (E1), E2, p7,
75 non-structural 2 (NS2), NS3, NS4A, NS4B, NS5A, and NS5B (Kato, 2001; Kato *et*
76 *al.*, 1990; Tanaka *et al.*, 1996).

77 The evaluation of anti-HCV reagents was difficult before the development of the
78 HCV replicon system by Lohmann et al. in 1999 (Lohmann *et al.*, 1999). The HCV
79 replicon system enabled investigation of the anti-HCV reagents and the cellular factors
80 involved in HCV RNA replication. Following the introduction of the replicon system,

81 the genome-length HCV RNA replication systems and reporter assay systems were also
82 developed (Ikeda *et al.*, 2005; Ikeda *et al.*, 2002; Lohmann *et al.*, 2001;
83 Pietschmann *et al.*, 2002). In 2005, an HCV infection system was developed using
84 the genotype 2a JFH-1 strain (Lindenbach *et al.*, 2005; Wakita *et al.*, 2005;
85 Zhong *et al.*, 2005). The JFH-1 infection system has been used to study not only the
86 viral RNA replication, but also viral infection and release. This HCV cell culture system
87 was developed using the human hepatoma cell line HuH-7 and thus far HuH-7 is the
88 only cell line to exhibit robust HCV production. Therefore, we intended to test the
89 susceptibility of various other cell lines to HCV RNA replication. Recently we
90 reported that the hepatoma cell line Li23 supported robust HCV RNA replication (Kato
91 *et al.*, 2009). Li23 was also susceptible to authentic JFH-1 infection (Kato *et al.*,
92 2009). Microarray analysis revealed that HuH-7 and Li23 cells exhibited distinct gene
93 expression profiles (Mori *et al.*, 2010). For example, we identified 3 genes (New York
94 esophageal squamous cell carcinoma 1, β-defensin-1, and galectin-3) showing
95 Li23-specific expression. Using HuH-7 and Li23 cells in combination with HCV strain
96 O (genotype 1b), we developed drug assay systems, OR6 and ORL8, respectively, by

97 introducing the renilla luciferase (RL) gene (Ikeda *et al.*, 2005; Kato *et al.*, 2009).
98 We found and reported that the sensitivities to anti-HCV reagents were different
99 between the HuH-7 and Li23 assay systems. For example, the Li23 assay system was 10
100 times more sensitive to ribavirin than the HuH-7 assay system (Mori *et al.*, 2011).
101 Methotrexate showed very strong anti-HCV activity in the Li23 assay system, although
102 it showed very weak anti-HCV activity in the HuH-7 assay system (Ueda *et al.*, 2011).
103 These results encouraged us to develop a JFH-1 reporter assay system using HuH-7 and
104 Li23 cells. This JFH-1 reporter assay system not only facilitated the monitoring of the
105 viral infection and release, but also provided us with new information that could be
106 missed in these steps when using only a HuH-7 assay system. However, the extension
107 of the size of the viral genome by introducing exogenous genes (RL and
108 encephalomyocarditis virus internal ribosomal site (EMCV-IRES)) reduced the
109 efficiency of HCV RNA replication. To overcome this issue, we tried to improve the
110 efficiency of HCV RNA replication by introducing adaptive mutations and by
111 subcloning the parental cells.
112 Here, we developed JFH-1 HCV production reporter assay systems in HuH-7- and

Li23-derived cells using adaptive mutations and subcloned cells, which monitor the life cycle of HCV with luciferase activity. We also tested the effect of the mutations in NS5B from JFH-1 strain on RNA replication of the specific genotype 1b O strain.

Results

HCV mutations caused by long-term cell culture. The efficiency of HCV RNA replication depends on viral genetic mutations, host cells, and viral genome size. For development of the HCV reporter assay system, use of a longer viral genome reduced the efficiency of viral replication. To compensate for this issue, we tried to introduce adaptive mutations into the JFH-1 genome. We examined the viral sequences of JFH-1 at 130 days after infection to HuH-7-derived RSc cells. We performed reverse transcription polymerase chain reaction (RT-PCR) for 3 parts of the viral genome, Core to NS2, NS3 to NS5A, and NS5B to 3'X. These 3 parts were separated by the *Age*I, *Spe*I, *BsrG*I, and *Xba*I sites on the viral genome. We introduced PCR products into the

129 cloning vector and 3 independent clones were subjected to sequencing analysis.

130 In the Core to NS2 region between the *Age*I and *Spe*I sites (designated as AS), there

131 were 8 common mutations with amino acid substitutions, Lysine to Glutamate at amino

132 acid position 78 (K78E) in Core, P251L and A351D in E1, V402A, I414T, K715N in

133 E2, Y771C in p7, and D962G in NS2 (Fig. 1a). In the NS3 to NS5A region between

134 *Spe*I and *BsrG*I sites (designated as SB), there were 8 common mutations with amino

135 acid substitutions, V1460I, M1611T in NS3, and I2270T, Q2307R, S2363L, M2392T,

136 S2426A, and C2441S in NS5A (Fig.1b). In the NS5B to 3'X region between the *BsrG*I

137 and *Xba*I sites (designated as BX), there was only 1 common mutation with an amino

138 acid substitution, V2995L in NS5B (Fig. 1c). The determined sequences were examined

139 for further study to enhance HCV RNA replication in the JFH-1 reporter assay.

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141 **The effect of genetic mutations on HCV RNA replication.** To monitor the viral

142 life cycle more easily, we constructed dicistronic JFH-1 with a reporter gene, pJR/C-5B.

143 The first cistron contained the RL gene and was translated by HCV IRES. The second

144 cistron contained the JFH-1 open reading frame and was translated by EMCV IRES.

This construct facilitated monitoring of all steps of the viral life cycle by the quantification of RL activity. However, the use of a longer viral genome resulted in less replication efficiency. We tested the effect of amino acid substitution caused during long-term cell culture on HCV RNA replication.

The amino acid substitution clusters from 3 independent clones in Core to NS2 (AS-1, AS-2, AS-3) were introduced into pJR/C-5B. The *in vitro*-transcribed HCV RNA was introduced into HuH-7-derived RSc cells, and the RL activities were monitored at 24, 48, and 72 hours after electroporation (Fig. 2a). AS-3 exhibited higher replication efficiency than the wild type (WT). However, the replication efficiency of AS-2 was almost equal to that of the WT and AS-1 exhibited lower replication efficiency than the WT. AS-3 possessed the highest replication efficiency among the tested JFH-1 mutants; at 72 hours the luciferase value of this clone was approximately 100 times that at 24 hours.

The three pJR/C-5B constructs with mutations in NS3 to NS5A (SB-2, SB-3, and SB-4) were transcribed and introduced into RSc cells to compare the efficiency of HCV RNA replication (Fig. 2b). Unexpectedly, RL activity was not increased over the 72 hours

161 after electroporation and exhibited a pattern similar to JFH-1 without the GDD motif.
162 This result indicates that the mutation in NS3 to NS5A exhibited negative efficiency on
163 HCV RNA replication.
164 Finally, we tested the effect of the mutations in the NS5B region on HCV RNA
165 replication. BX-2 contains 2 mutations with amino acid substitution (H2505Q and
166 V2995L) and BX-7 contains only V2995L (Fig. 2c). JFH-1 with mutation(s) of BX-2 or
167 BX-7 exhibited strong enhancement of HCV RNA replication. These results indicate
168 that V2995L works as a strong replication-enhancing mutation (REM) in JFH-1 HCV
169 RNA replication.
170
171 **The mutations in NS5B differently enhanced 1b and 2a HCV RNA replication.**
172 V2995L in NS5B is a common substitution in 3 clones and H2505Q is conserved in 2
173 clones (BX-2 and BX-10). We examined the corresponding amino acids at positions
174 2995 and 2505 in genotype 1b replication-competent HCV strains, O, 1B-4, and KAH5
175 (Fig. 3a) (Nishimura *et al.*, 2009). The histidine at amino acid position 2505 in
176 JFH-1 is conserved in O, 1B-4, and KAH5 at the corresponding amino acid position

177 2482. The valine at amino acid position 2995 in JFH-1 is an alanine in O, 1B-4, and
178 KAH5 at the corresponding amino acid position 2972 (Fig. 3a). It is not clear whether
179 or not the adaptive mutation found in genotype 2a is effective in genotype 1b HCV.
180 Therefore, we investigated the effect of V2995L and/or H2505Q substitution on
181 genotype 1b HCV RNA replication. We introduced V2995L and/or H2505Q
182 substitution into the subgenomic replicon, pOR/3-5B (HCV-O). In contrast to the case
183 of JFH-1, H2505Q but not V2995L enhanced HCV-O RNA replication (Fig. 3b). These
184 results indicate that the mutations in NS5B derived from JFH-1 functioned differently in
185 genotype 1b HCV-O RNA replication.
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188 **HCV infection in the HuH-7- and Li23-derived cell lines.** The choice of host
189 cells is important for the efficiency of HCV RNA replication as well as viral genetic
190 mutations. The cured cells such as HuH-7.5, HuH-7.5.1, and our RSc cells exhibit
191 higher replication efficiency than that of their parental HuH-7 cells (Ariumi *et al.*,
192 2007; Blight *et al.*, 2002; Zhong *et al.*, 2005). Therefore, we examined whether or

193 not subcloned Li23 cells may enhance HCV RNA replication. We performed serial
194 subcloning of Li23 cells from Li23-derived ORL8c cells by the limiting dilution method
195 (Fig. 4a). ORL8c cells are a cured cell line, in which genome-length HCV RNAs were
196 eliminated by interferon (IFN) treatment (Kato *et al.*, 2009). The subclonal
197 Li23-derived cell lines were selected from among 50-100 independent single cells in
198 96-well plates by 3-round limiting dilution from ORL8c cells (sFig. 1a). First, L8c15
199 cells were selected from their parental ORL8c cells by limiting dilution. Then, C22 cells
200 were selected from their parental L8c15 cells by limiting dilution. And finally, D7 cells
201 were selected from C22 cells by limiting dilution (sFig.1b). Together, these steps
202 resulted in the selection of 3 subclonal cell lines that respectively exhibited the strongest
203 replication efficiency in each round selection. The lineages of the selected cell lines
204 after 3 rounds of subcloning were L8c15, C22, and D7 cells, respectively.

205 We tested the subcloned cells for their HCV infectivities in comparison with HuH-7
206 and HuH-7-derived RSc cells. We previously reported that RSc cells could highly
207 support HCV replication and production (Kato *et al.*, 2009). Li23 and its derived
208 ORL8c, L8c15, C22, and D7 cells were infected using the supernatant from JR/C-5B

209 with BX-2 mutants replicating RSc cells at a moi of 0.2 (Fig. 4b and 4c). The RL
210 activities were determined at 24, 48, 72, and 96 hours after infection and focus-forming
211 units (FFUs)/ml were determined at 48 hours after infection. The efficiency of HCV
212 infectivity was highest in D7 cells, followed in order by C22, L8c15, and Li23 cells.
213 The HCV RNA replication in D7 cells was almost equal to that in RSc cells. These
214 results suggest that the subcloned cell lines exhibit higher susceptibility to HCV
215 infection than that of their parental cells.

216 Next, we further characterized the susceptibility of D7 cells to HCV infection in
217 comparison with RSc cells, because D7 cells exhibited the highest susceptibility to
218 HCV infection among the Li23-derived cell lines. D7 cells also exhibited the highest
219 production and release of Core into the supernatant among the parental
220 C22-derived subclonal cells (sFig. 1b). The susceptibility of the HCV reporter assay
221 system to HCV infection was examined using HuH-7- and Li23-derived cells.
222 Supernatants from JR/C-5B with AS-3 or with BX-2 mutations replicating RSc cells
223 were used as inocula. The supernatant from authentic JFH-1 replicating RSc cells was
224 used as a positive control. RSc and D7 cells were inoculated with each HCV containing

225 supernatant and the number of FFUs/ml was determined at 48 hours after infection. As
226 shown in Fig. 4(d), the values of FFUs/ml for AS-3 were 2.5 x 10^4 and 1.0 x 10^4 in RSc
227 and D7 cells, respectively. The values of FFUs/ml for BX-2 were 3.1 x 10^4 and 1.8 x
228 10^4 in RSc and D7 cells, respectively. The values of FFUs/ml for authentic JFH-1 were
229 2.9 x 10^5 and 1.2 x 10^5 in RSc and D7 cells, respectively. These results indicate that the
230 infectivities of these 3 inocula were almost equal between RSc and D7 cells.
231 Next we examined Core expression after infection of HCV to RSc and D7 cells, as
232 D7 cells exhibited the highest infectivity among the Li23-derived cell lines (Fig. 4e).
233 Core was detected at 2, 3, and 4 days after infection of the supernatant from JR/C-5B
234 with BX-2-infected RSc cells. Although the Core expression in D7 cells was slightly
235 weaker than that in RSc cells, the signal of Core in HCV-infected D7 cells was equal to
236 that in stable ORL8 cells. These results suggest that the JFH-1 reporter assay system in
237 Li23 cells is useful not only for the RL assay but also for Core expression.
238
239 **The expression of HCV receptors in parental and subclonal hepatoma cell lines.**
240 We further tested the expression of HCV receptors, CD81, scavenger receptor class B

241 member I (SR-BI), claudin-1 (CLDN1), and occludin (OCLN). We also examined the
242 expression of the recently reported HCV entry factor, Niemann-Pick C1-like 1
243 (NPC1L1) (Sainz *et al.* 2012). The expression levels of CD81 in Li23 and its subclonal
244 cells were higher than that in HuH-7 and RSc cells (Fig. 5a). Although, the expression
245 of CD81 in Li23-derived cell lines were lower than that in parental Li23 cells,
246 interestingly the expression levels of CD81 increased, when the rounds of selection
247 were going on. There is no tendency in the expression of SR-BI among the cell lines
248 tested (Fig. 5b). The expressions of CLDN1 in Li23-derived cells were higher than that
249 in parental Li23 cells (Fig. 5c). The expression levels of OCLN in Li23 and its
250 subclonal cells were higher than those in HuH-7 and RSc cells (Fig. 5d). Finally, the
251 expressions of NPC1L1 in Li23-derived cell lines were higher than that in parental Li23
252 cells (Fig. 5e). It is noteworthy that the expression level of NPC1L1 in RSc cells was
253 approximately 2 log (10) lower than that in parental HuH-7 cells. Taken these results
254 together, the expressions of OCLN and NPC1L1 in Li23-derived cells were higher than
255 that in parental Li23 cells.
256

Life cycle of the HCV reporter assay system in Li23-derived cells. We investigated whether or not D7 cells produce infectious HCV. First, D7 cells were inoculated using the supernatant from JR/C5B with BX-2 in RSc cells, and the supernatant was stocked at 17 days after infection. Then, the supernatant derived from the D7 cells was used as an inoculum for reinfection to naïve D7 cells. RL activities were determined at 2, 6, 10, and 14 days after reinfection (Fig. 6). The RL activity was increased after reinfection in D7 cells and reached a plateau at 10 days after reinfection. These data indicate that the JFH-1 reporter assay system is also useful for monitoring the HCV life cycle in Li23-derived cell lines.

Discussion

In this study, we developed an HCV production reporter assay system using 2 distinct hepatoma cell lines, HuH-7 and Li23. The robust HCV RNA replication and

273 viral production were achieved by the introduction of REMs into the structural
274 region or into the NS5B region. These REMs were obtained from JFH-1-infected
275 long-term cell culture. The two REMs in NS5B (H2505Q and V2995L substitutions)
276 derived from JFH-1 had different effects on genotype 1b HCV-O RNA replication, and
277 2a JFH-1 RNA replication. Furthermore, the subcloned Li23-derived D7 cells produced
278 by serial limiting dilution supported this HCV production reporter assay system.

279 Several groups have reported JFH-I reporter virus systems (Koutsoudakis *et al.*,
280 2006; Marcello *et al.*, 2006; Pietschmann *et al.*, 2002; Wakita *et al.*, 2005).
281 However, robust reporter virus production was limited within the study using
282 HuH-7-derived cells. Therefore, we attempted to developed a JFH-1 reporter virus assay
283 system using our previously reported line of Li23 cells (Kato *et al.*, 2009).

284 The introduction of RL and EMCV-IRES genes into the HCV gene lengthened the
285 genome size of HCV by approximately 1.9 kb and led to a reduction in the efficiency of
286 HCV RNA replication. To overcome this disadvantage, we adopted the following
287 strategies: (1) Introduce the REMs; (2) Select the cloned Li23-derived cells with a
288 highly permissive host condition by the serial limiting dilution method. For the first

289 purpose, we performed sequence analyses for HCV RNA from JFH-1-infected RSc
290 cells. The mutations from Core to the NS2 or NS5B region enhanced HCV RNA
291 replication. However, the combination of these mutations from two different regions
292 reduced HCV RNA replication (sFigs. 2a-c). The reason for this result may be that these
293 2 mutation clusters were obtained from distinct RT-PCR amplified clones and they were
294 not necessarily located on the same viral genome. It had been reported that the
295 combination of REMs exhibited an antagonistic effect on HCV RNA replication
296 (Lohmann *et al.*, 2001). For the second purpose, we selected the highly permissive
297 Li23-derived clonal cells by the limiting dilution method. We obtained 3 Li23-derived
298 subclonal cell lines, L8c15, C22, and D7, in order from parental Li23-derived ORL8c
299 cells. The efficiency of infectivity was highest in D7 cells, followed in order by C22,
300 L8c15, and Li23 cells. D7 cells were highly permissive for infection of HCV with
301 NS5B mutations.

302 As shown in Fig. 3(a), the histidine at amino acid position 2505 in JFH-1 was
303 conserved in the replication competent O, 1B-4, and KAH5 strains at the corresponding
304 amino acid position 2482. The valine at amino acid position 2995 in JFH-1 was alanine

305 in O, 1B-4, and KAH5 strains at the corresponding amino acid position 2972. The
306 REMs in genotype 1b HCV were usually obtained by the selection with neomycin after
307 HCV RNA electroporation. Pietschmann et al. reported that the REM impaired the
308 infectious viral production (Pietschmann *et al.*, 2009). Most of the REMs were
309 located in the NS3 and NS5A regions (Abe *et al.*, 2007; Blight *et al.*, 2002;
310 Lohmann *et al.*, 2001; Pietschmann *et al.*, 2002). NS5A is a key molecule for
311 viral production and REMs affect the phosphorylation status of NS5A and the
312 interaction with Core (Kato *et al.*, 2008; Masaki *et al.*, 2008; Tellinghuisen *et al.*,
313 2008). In contrast, our REMs in NS5B were obtained in JFH-1-infected long-term cell
314 culture without drug selection. Taking this information into account, we considered that
315 H2505Q in NS5B might not interfere with the genotype 1b viral production. We
316 attempted to apply this REM from genotype 2a to genotype 1b and found that H2505Q
317 enhanced replication of the genotype 1b HCV-O replicon. We are currently
318 investigating whether or not our NS5B REM could enhance genotype 1b HCV
319 production. As for the amino acid substitution at 2995 in JFH-1 (2972 in genotype 1b),
320 we should be careful in interpretation, because the backgrounds at this position are

different between genotypes 2 and 1. HCV database (http://s2as02.genes.nig.ac.jp/) analysis revealed that the consensus amino acid at 2995 in genotype 2 and at 2972 in genotype 1 was valine and alanine, respectively. Furthermore, alanine and valine are not found at 2995 in genotype 2 and at 2972 in genotype 1, respectively. These observations indicate that amino acid substitution between alanine and valine at these positions may be lethal for both genotype HCVs. Amino acid at 2995 in genotype 2 (2972 in genotype 1) is just upstream of cis-acting replication element in NS5B. Therefore, RNA at this position may affect the HCV RNA replication. To clarify this issue, further study will be needed.

A comparative study using HuH-7- and Li23-based JFH-1 reporter assay systems would be expected to reveal new information on the viral entry and release steps, because the backgrounds of these cells are different. Our recent study on these cells revealed the difference in sensitivities to anti-HCV reagents, including ribavirin and methotrexate (Mori *et al*., 2011; Ueda *et al*., 2011). Furthermore, the IL28B genotype was different between HuH-7 and Li23 cells. The IL28B genotype (rs8099917) of HuH-7 cells were resistant to pegylated IFN and ribavirin and that of Li23 cells was

337 sensitive to pegylated IFN and ribavirin (Ikeda et al. unpublished data).

338 Recently, it was reported that stable expression of miR122 enhanced JFH-1 HCV
339 production in Hep3B and HepG2 (Kambara *et al*., 2011; Narbus *et al*., 2011). It is
340 noteworthy that the expression of miR122 in Li23-derived cells was almost the same as
341 that in HuH-7 cells (sFig. 3). The high level expression of miR122 in Li23 cells may be
342 one of the reasons that Li23 cells can support HCV production as robust as that in
343 HuH-7 cells among the hepatocyte-derived cell lines. Interestingly, the expression
344 levels of miR122 are higher in ORL8c, L8c15, and D7 cells, but not in C22 cells than
345 that in parental Li23 cells (sFig. 3). This result suggests that the expression level of
346 miR122 may partly contribute to the fitness of HCV replication and production.

347 So far, we have only little information regarding the mechanism that subclonal
348 cells support HCV replication and production more efficiently than the parental cells. In
349 this study, we found that the expression levels of CLDN1 and NPC1L1 in Li23-derived
350 subclonal cells were higher than those in the parental Li23 cells. These results suggest
351 that high expression level of these entry factors in the Li23-derived subclonal cells may
352 contribute to enhance the viral entry. In the course of the experiment to determine the

353 expression levels of NPC1L1 in HuH-7- and Li23-derived cell lines, we found that RSc
354 cells expressed very low level of NPC1L1 compared with the parental HuH-7 cells. The
355 possible mechanisms for this are (1) very low expression of NPC1L1 is enough for
356 HCV entry, (2) unknown entry factor compensate for NPC1L1 in the entry step in RSc
357 cells. Further study will be needed to clarify this issue.

358 In summary, we have developed JHF-1 reporter assay systems using
359 HuH-7-derived RSc and Li23-derived D7 cells. The expression levels of CLDN1 and
360 NP1C1L were higher than those in the parental Li23 cells. We found different effects of
361 REMs (V2995L and H2505Q substitutions) in NS5B on genotypes 2a and 1b HCV
362 strains in viral RNA replication. These findings will become useful tools in the study of
363 the life cycle of HCV.

364

365

366 **Materials and Methods**

367

368 **Cell cultures.** RSc cells and ORL8c cells were derived from the cell lines HuH-7

369 and Li23 as previously described (Kato *et al.*, 2009). L8c15 cells were selected from
370 ORL8c cells by limiting dilution. C22 cells were selected from the L8c15 cell line by
371 limiting dilution. D7 cells were selected from C22 cells by limiting dilution. HuH-7 and
372 RSc cells were cultured in Dulbecco's modified Eagle's medium supplemented with
373 10% fetal bovine serum (FBS). Li23-derived cell lines were maintained in F12 medium
374 and DMEM (1:1 in volume) supplemented with 1% FBS and epidermal growth factor
375 (50 ng/ml) as described previously (Kato *et al.*, 2009).
376
377 **RT-PCR and sequencing analysis.** RSc cells were infected with cell
378 culture-grown HCV (HCVcc) and cultured for 130 days. Total RNAs from these cells
379 were prepared using an RNeasy extraction kit (Qiagen, GmbH, Germany). These
380 RNA samples were used for RT-PCR in order to amplify the Core to NS2 region (4.0
381 kb), NS3 to NS5A region (3.6 kb), and NS5B to 3'X region (1.9 kb). RT was performed
382 with the Oligo dA23 primer, 5'-AAAAAAAAAAAAAAAAAAAAAAA-3'. The
383 following primer pair was employed to amplify the Core to NS2 region: for JFH-1/*Age*I,
384 5'-CCCAAGCTTACCGGTGAGTACACCGGAATTGC-3'; and for JFH-1/*Spe*IR,

385 5’-TGCCATGTGCCTTGGATAGGTACG -3’. The primer pair JFH-1/*Spe*I:
386 5’-CCCAGGGGTACAAAGTACTAGTGC-3’ and JFH-1/*BsrG*IR:
387 5’-CCCAAGCTTTACCTTTTTAGCCCTCTGTGAGGC-3’ was employed to amplify
388 NS3 to NS5A region. The primer pair JFH-1/*BsrG*I:
389 5’-CCGCTCGAGACCCTTTGAGTAACTCGCTGTTGC-3’ and JFH-1/*Xba*IR:
390 5’-GCTCTAGACATGATCTGCAGAGAGACCAGTTAC-3’ was employed to amplify
391 NS5B to 3’X region. SuperScript III reverse transcriptase (Invitrogen, Carlsbad, CA)
392 and KOD-plus DNA polymerase (Toyobo, Osaka, Japan) were used for RT and PCR,
393 respectively. PCR products were ligated into pBluescript II and 3 independent clones
394 were subjected to sequencing analysis.
395
396 **Plasmid construction.** pJR/C-5B plasmid is a dicistronic HCV JFH-1 construct.
397 The RL genes and HCV open reading frame were introduced in the first cistron and in
398 the second cistron, respectively. To construct this plasmid, we fused the JFH-1 5’
399 5’untranslated region (UTR) region with the RL gene by overlap PCR, and the PCR
400 products were ligated into pFGR-JFH-1 (GenBank Accession No. AB237837) at the

401 *Age*I and *Pme*I sites. For the first PCR, the primer pair
402 5'-GCGCCTAGCCATGGCGTTAGTATG -3' (for J5dC) and
403 5'-AAGCCATGGCCGGCCCTGGGCGACGGTTGGTGTTTCTTTTGG-3' (for
404 J5dCR) was employed to amplify the 5'UTR, and the primer pair
405 5'-AACCGTCGCCCAGGGCCGGCCATGGCTTCCAAGGTGTACGACCCC-3' (for
406 JRL) and 5'-TCGAAATCTCGTGATGGCAGGTTGG-3' (for JRLR) was employed to
407 amplify the RL region. These first PCR products were used in the second PCR as the
408 templates. For the second PCR, the primer pair J5dC and JRLR was employed to
409 amplify 5'UTR and RL. KOD-plus DNA polymerase was used for PCR.
410 The H2505H and/or A2995 mutations were introduced into the HCV-O replicon by
411 QuickChange mutagenesis (Stratagene, La Jolla, CA) as described previously (Ikeda *et*
412 *al.*, 2002).
413
414 **Luciferase reporter assay.** For the luciferase assay, 1.0~1.5 × 10^4
415 HCV-harboring cells were plated onto 24-well plates in triplicate and were cultured for
416 24 to 96 hours after electroporation or infection, as described previously (Ikeda *et al.*,

417 2005). The cells were harvested with renilla lysis reagent (Promega, Madison, WI) and
418 subjected to RL assay according to the manufacturer's protocol.
419
420 **Western blot analysis.** The preparation of cell lysates, sodium dodecyl
421 sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and immunoblotting were
422 performed as described previously (Kato *et al.*, 2003). The antibodies used in this study
423 were Core- (CP11; Institute of Immunology, Tokyo, Japan) and β-actin antibody
424 (AC-15; Sigma, St. Louis, MO). Immunocomplexes were detected with a Renaissance
425 enhanced chemiluminescence assay (Perkin Elmer Life Science, Boston, MA).
426
427 **HCV infection and determination of FFU.** To determine FFU/ml, 6 x 10^3 cells
428 were plated onto a 96-well plate 24 hours before infection. The supernatant of HCV
429 RNA-replicating cells was serially diluted and was used as an inoculum. Forty-eight
430 hours after infection, the cells were fixed and Core was stained with anti-Core antibody
431 and HRP-conjugated mouse anti-IgG antibody. Then, the expression of Core was
432 visualized with a DAB substrate kit (DAKO). The culture supernatants and cells were

433 collected for quantification of the Core by enzyme-linked immunosorbent assay
434 (ELISA) (Mitsubishi Kagaku Bio-Clinical Laboratories, Tokyo, Japan).
435
436 **The quantitative RT-PCR analysis.** The quantitative RT-PCR analysis for HCV
437 receptors was performed using real-time LightCycle PCR (Roche Diagnostics, Basel,
438 Switzerland) as described previously (Ikeda *et al*., 2005). The primer pairs for CD81,
439 SR-BI, CLDN1, and OCLN were previously reported by Nakamuta et al. (Nakamuta *et*
440 *al*., 2011). The primer pair, NPC1L1: 5'-agatcttcttcttccgcctcca-3' and NPC1L1R:
441 5'-tgccagagccgggttaac-3' was used for NPC1L1.
442
443 **Statistical analysis.** The luciferase activities were statistically compared between
444 the various treatment groups using Student's *t*-test. *P* values of less than 0.05 were
445 considered statistically significant. The mean ± standard deviation is determined from at
446 least three independent experiments.
447
448 **Acknowledgements**

449 The authors would like to thank Masayo Takemoto, Takashi Nakamura and Keiko
450 Takeshita for their technical assistance. This work was supported by grants-in-aid for
451 research on hepatitis from the Ministry of Health, Labor, and Welfare of Japan.

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457 **References**

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Figure Legends

Fig. 1. The genetic mutations with amino acid substitutions during long-term HCV

577 infection. RT-PCR was performed for HCV RNAs from HuH-7 cells at 130 days after
578 JFH-1 infection. PCR products were subcloned into pBluescript II plasmid. (a) Three
579 clones of the Core to NS2 region between the *Age*I and *Spe*I sites (AS) were subjected
580 to sequencing analysis. (b) Three clones of the NS3 to NS5A region between the *Spe*I
581 and *BsrG*I sites (SB) were subjected to sequencing analysis. (c) Three clones of the
582 NS5B to 3'X region between the *BsrG*I and *Xba*I sites (BX) were subjected to
583 sequencing analysis. Closed and open triangles are conservative and nonconservative
584 amino acid substitutions, respectively.
585
586 **Fig. 2.** The effect of amino acid substitutions on HCV RNA replication. (a) The Core to
587 NS2 region. (b) The NS3 to NS5A region. (c) NS5B to 3'X region. Amino acid
588 substitutions were introduced into pJR/C5B and *in vitro*-synthesized RNAs were
589 electroporated into HuH-7-derived RSc cells. RL activity was determined at 24, 48, and
590 72 hours after electroporation. dGDD is a negative control without the GDD motif and
591 WT is a wild type.
592

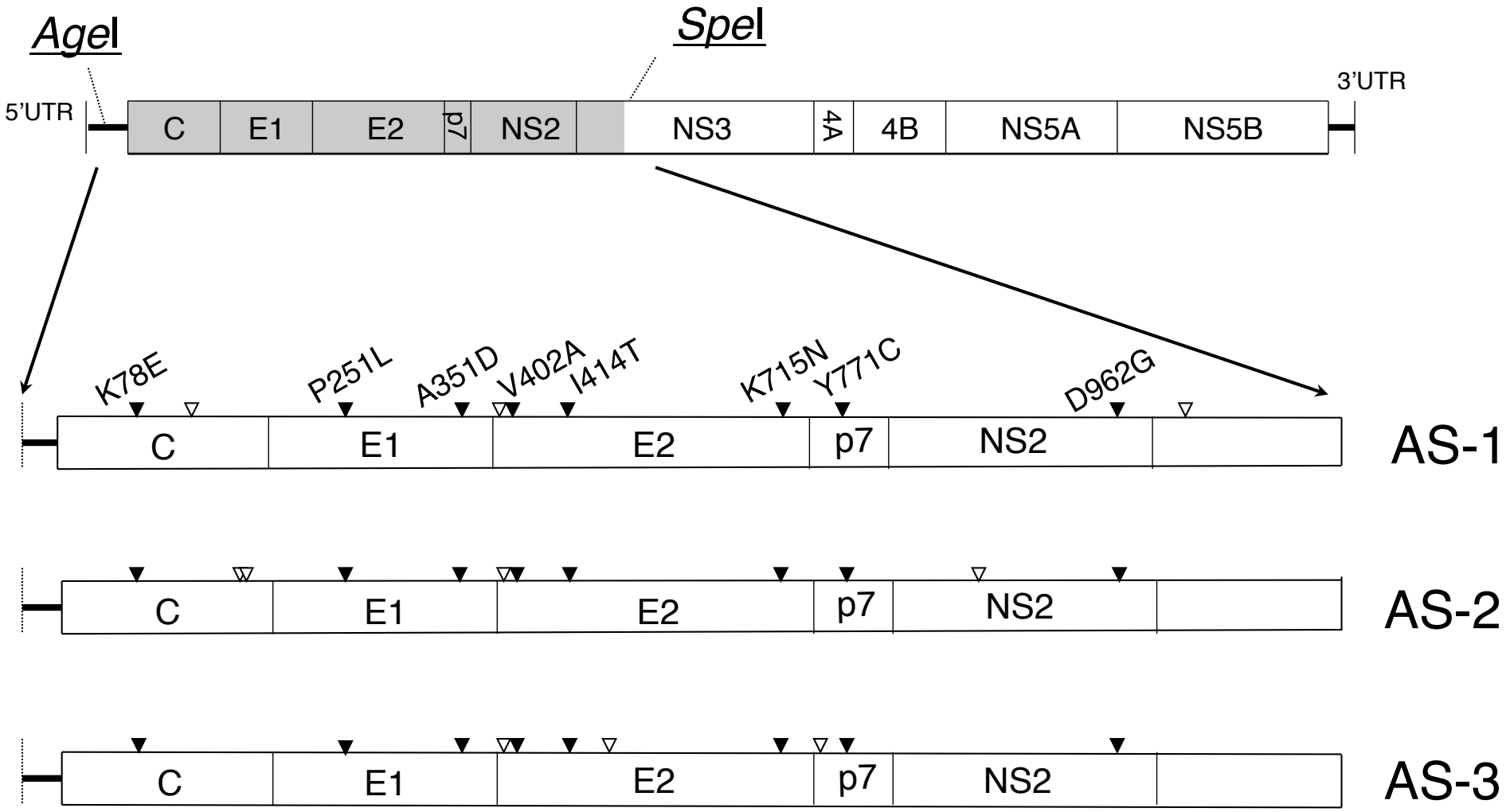
593 **Fig. 3.** The effect of amino acid substitutions in NS5B on genotype 1b and 2a HCV
594 RNA replication. (a) Alignment of amino acids at positions 2505 (JFH-1) and 2482
595 (genotype 1b) and around the adjacent region (upper panel). Alignment of amino acid at
596 positions 2995 (JFH-1) and 2972 (genotype 1b) and around the adjacent region (lower
597 panel). The HCV strains of O, KAH5, and 1B-4 belong to genotype 1b. (b) H2505Q
598 and/or V2995L were introduced into the HCV-O subgenomic replicon (pOR/3-5B), and
599 transcribed RNAs were electroporated into RSc cells. The RL activities were tested at
600 24, 48, and 72 hours after infection. dGDD is a negative control without a GDD motif
601 and WT is a wild type.
602
603 **Fig. 4.** HCV infection in HuH-7- and Li23-derived cell lines. (a) History of the selection
604 of subclonal Li23-derived cell lines. (b) HuH-7, HuH-7–derived RSc, Li23-derived
605 ORL8c, L8c15, C22, and D7 cells were inoculated with supernatant from JR/C5B/BX-2
606 replicating RSc cells. (c) FFUs were determined at 48 hour after infection of HCV to
607 HuH-7- and Li23-derived cells using the supernatant from JR/C5B/BX-2 replicating
608 RSc cells. (d) FFUs were determined at 48 hours after infection of HCV to RSc or D7

609 cells using the supernatant from JR/C5B/AS-3- or JR/C5B/BX-2-replicating RSc cells.
610 The supernatant from authentic JFH-1-replicating RSc cells was used as a positive
611 control. (e) The Core expressions in RSc or D7 cells were determined at 1, 2, 3, and 4
612 days after infection of JFH-1 with BX-2 mutations. Lanes 1 and 6 are mock-infected
613 cells. Lanes 2 and 7 are cells at 1 day after infection. Lanes 3 and 8 are cells at 2 days
614 after infection. Lanes 4 and 9 are cells at 3 days after infection. Lanes 5 and 10 are cells
615 at 4 days after infection. Lanes 11 and 12 are OR6c and OR6 cells, respectively. Lanes
616 13 and 14 are ORL8c and ORL8 cells, respectively. OR6 and ORL8 were used as
617 positive controls. OR6c and OR8c were used as negative controls.
618
619 **Fig. 5.** The expression levels of HCV receptors in HuH-7- and Li23-derived cells.
620 Quantitative RT-PCR was performed for CD81, SR-B1, CLDN1, OCLN, and NPC1L1
621 as described in Materials and Methods. Relative expression levels of mRNA were
622 shown, when the expression level of each receptor in HuH-7 was assigned to be 1.
623 GAPDH was used as an internal control. Experiments were done in triplicate.
624

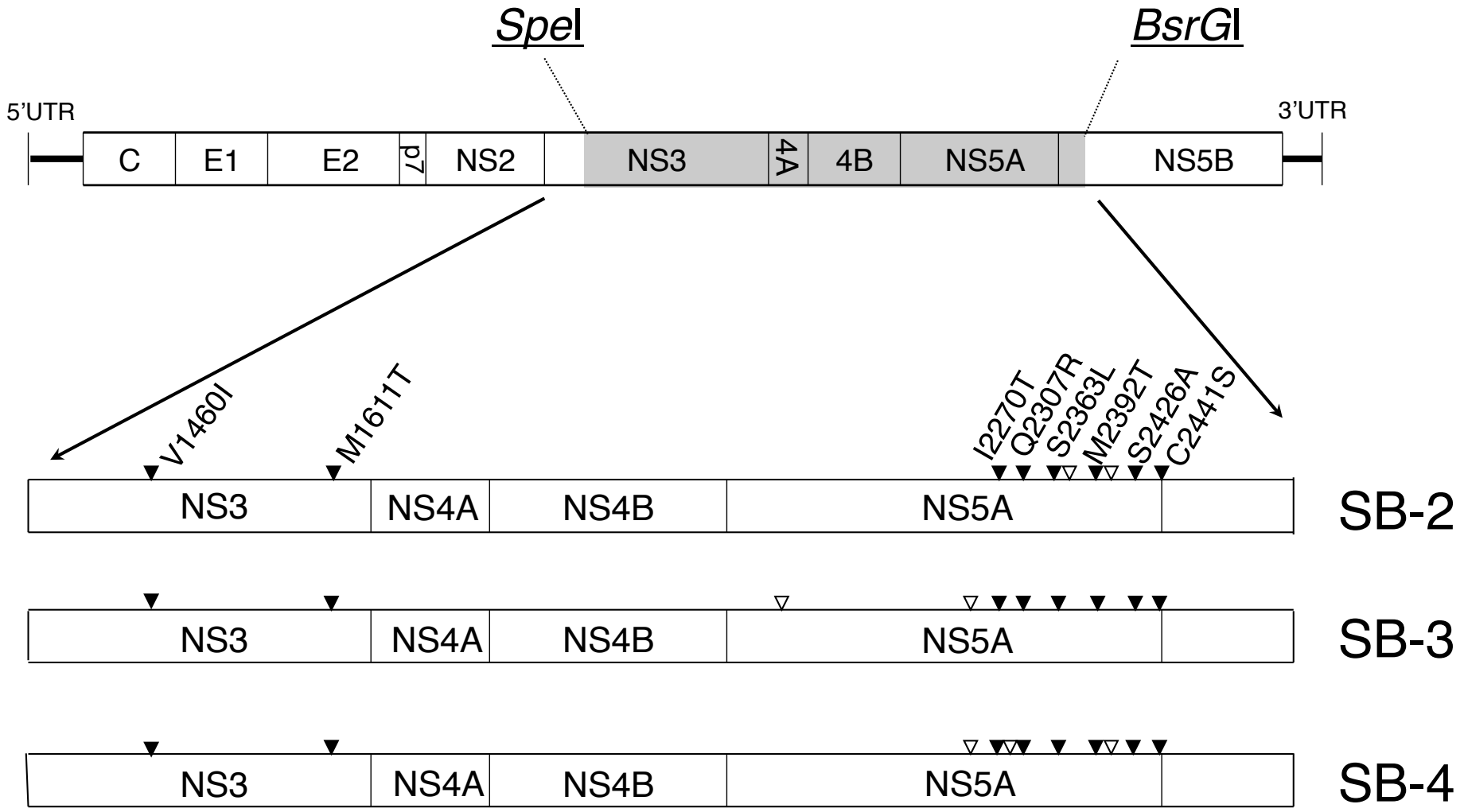
625 **Fig. 6.** HCV life cycle in Li23-derived D7 cells. D7 cells were inoculated with the

626 supernatant from D7 cells after infection of JFH-1 with BX-2 mutants. RL activities

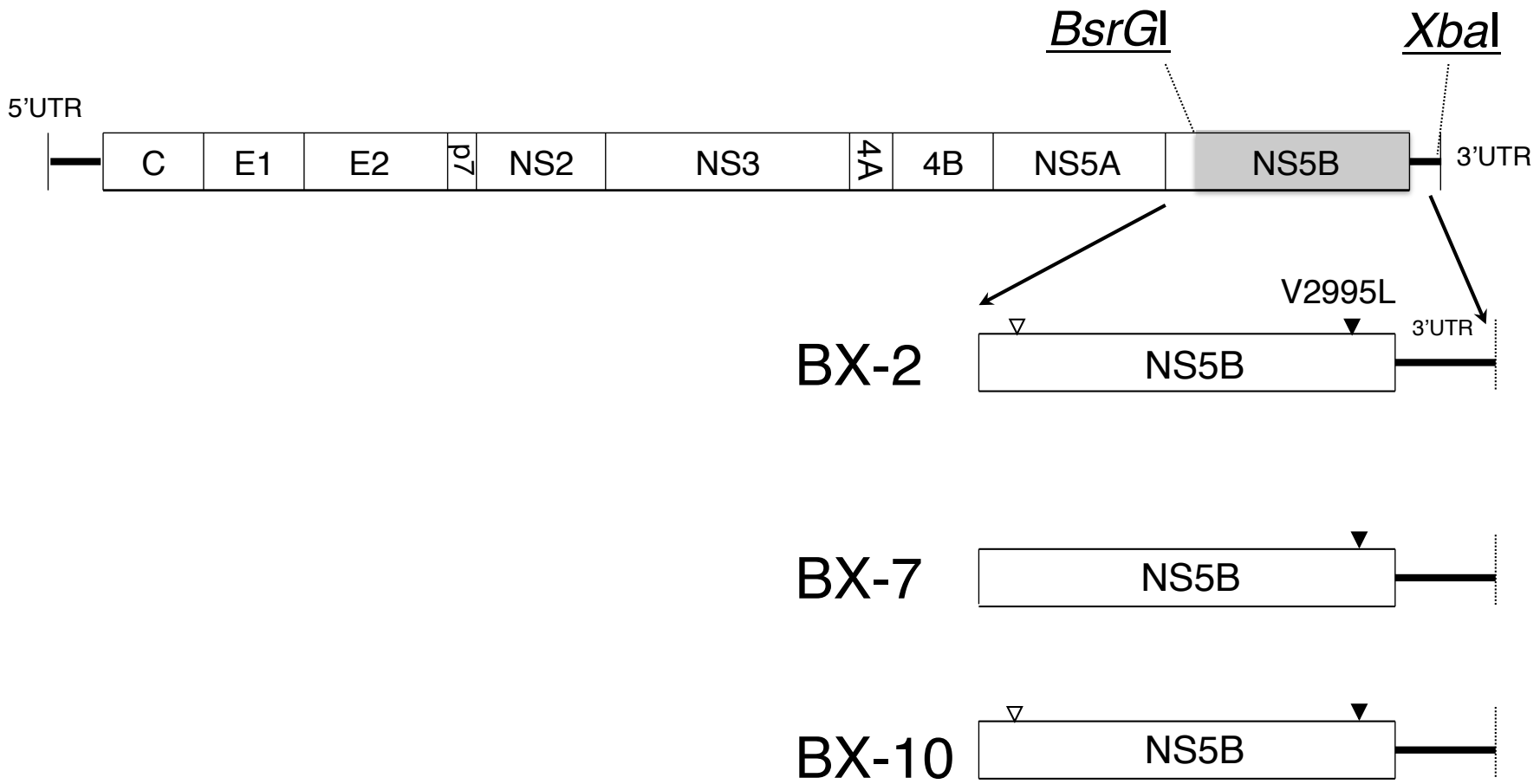
627 were tested at 2, 6, 10, and 14 days after infection.



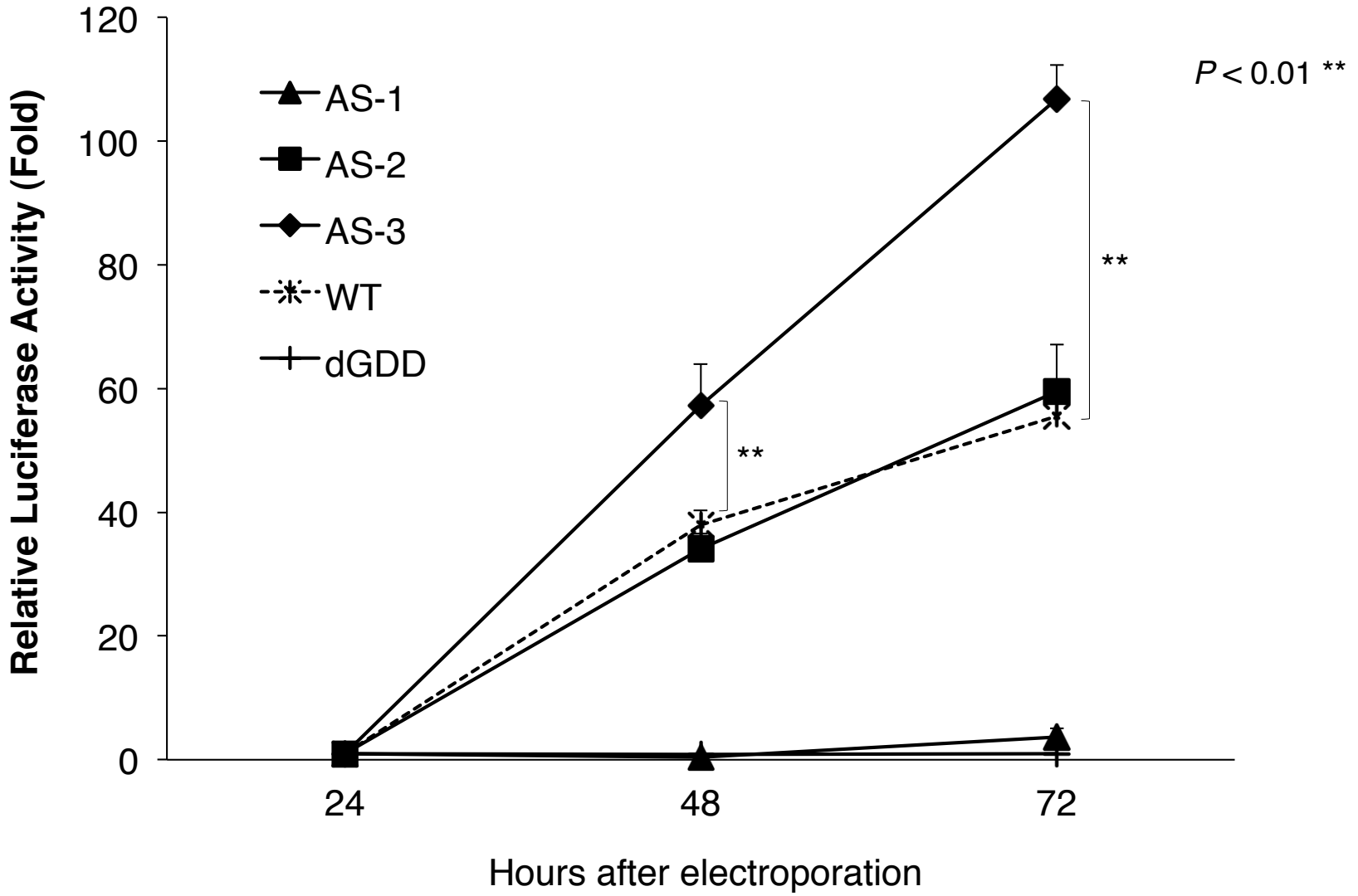
Takeda et al. Fig. 1a



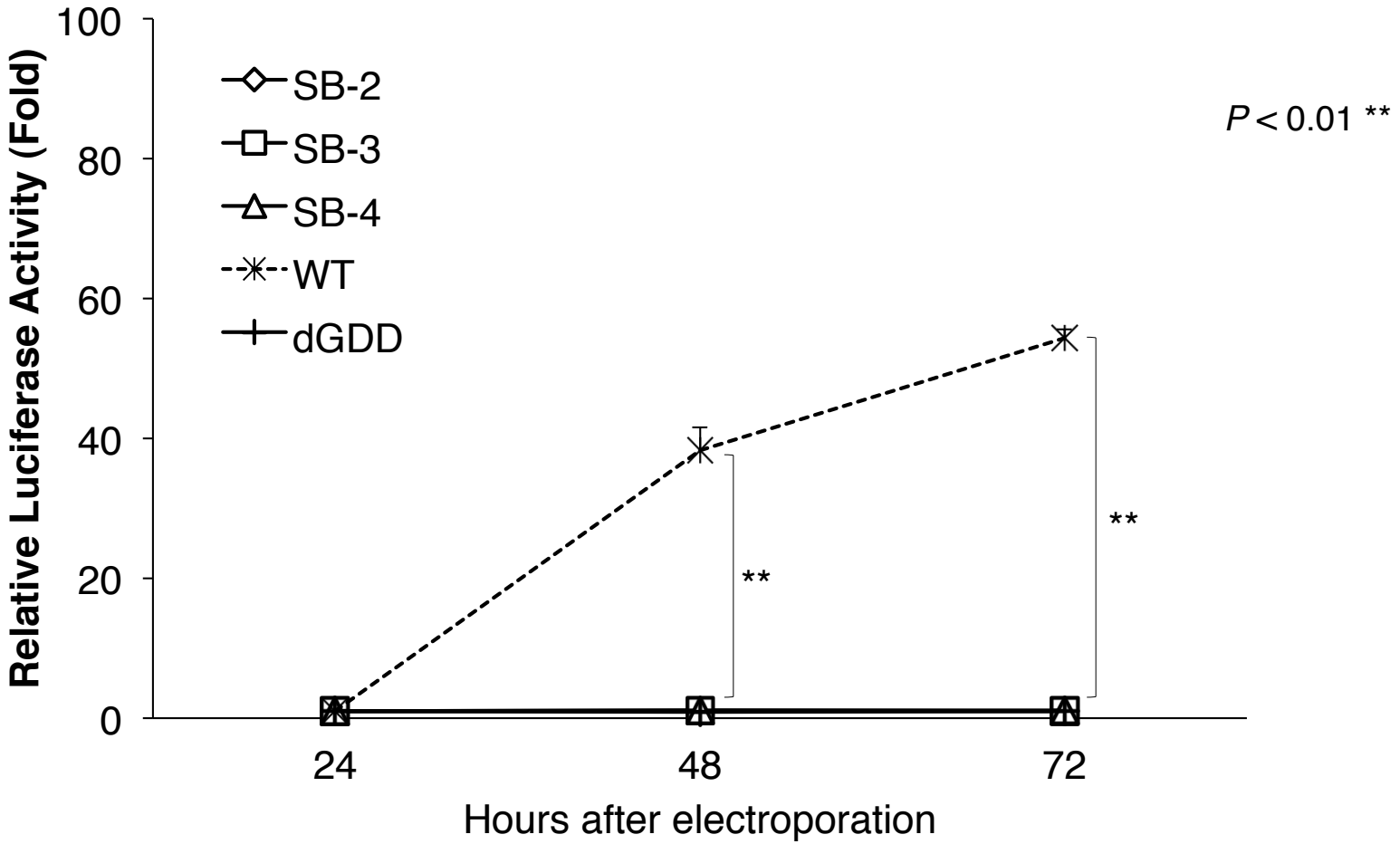
Takeda et al. Fig. 1b



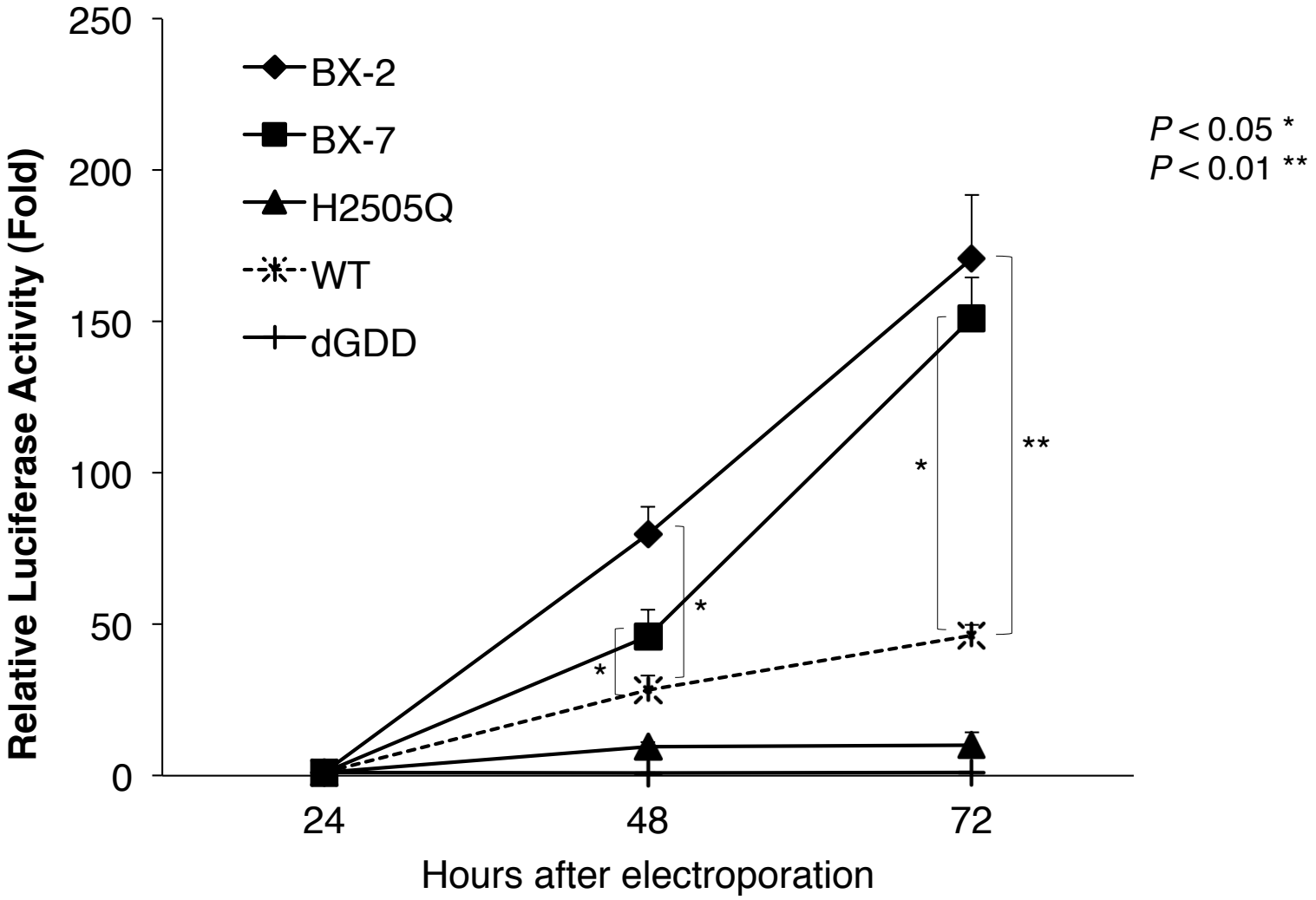
Takeda et al. Fig. 1c



Takeda et al. Fig. 2a



Takeda et al. Fig. 2b

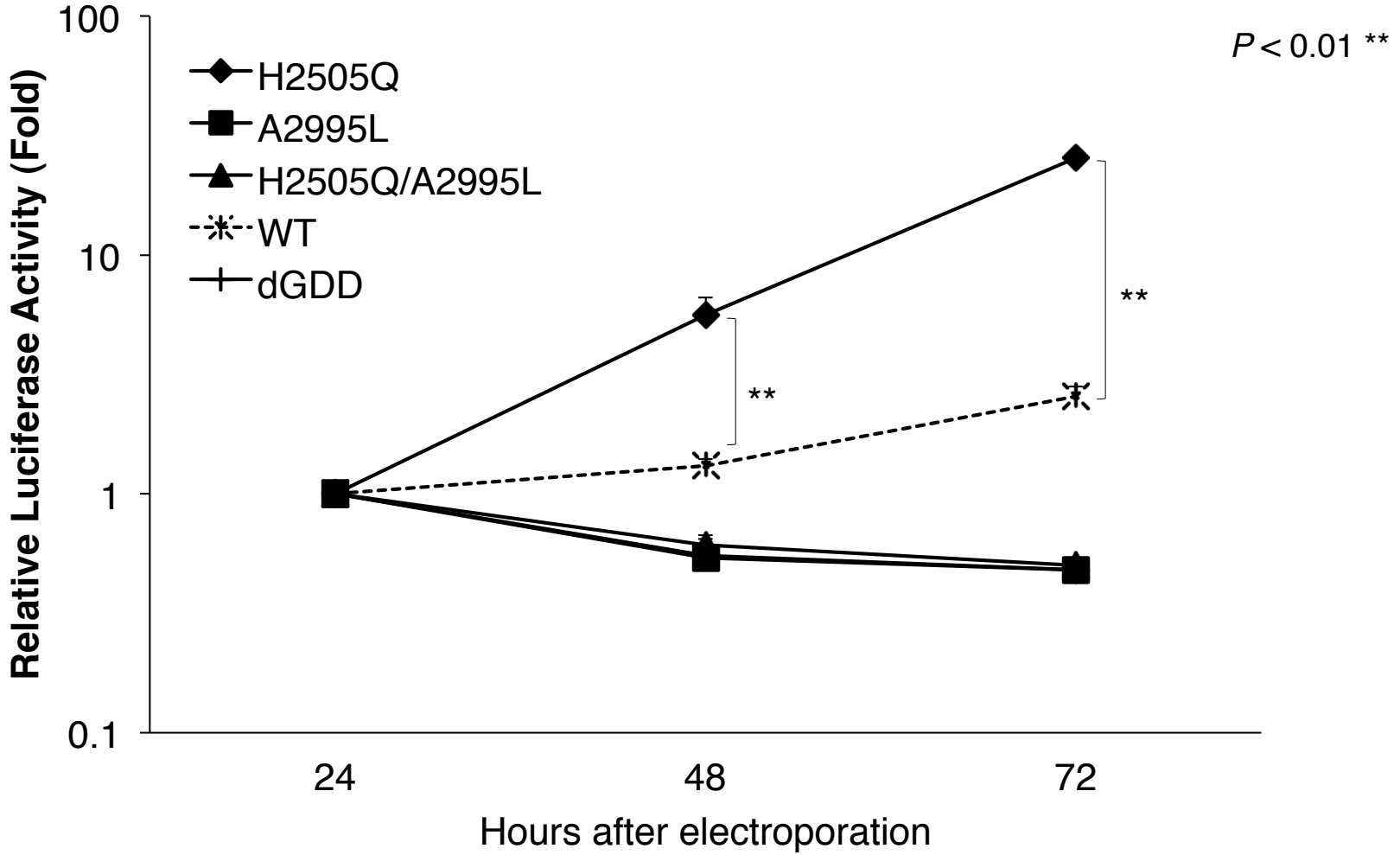


Takeda et al. Fig. 2c

consensus		TFDRLQVLDD**H**YRDVLKE	
JFH-1	2495	TFDRTQVLDA**H**YDSVLKD	2512
O	2472	TFDRLQVLDD**H**YRDVLKE	2489
KAH-5	2472	TFDRLQVLDD**H**YRDVLKE	2489
1B-4	2472	TFDRLQVLDD**H**YRDVLKE	2489

consensus		PAASRLDLSGWFV**A**GYSGGDIYHS	
JFH-1	2982	PEARLLDLSSWFT**V**GAGGGDIFHS	3005
O	2959	PAASRLDLSGWFV**A**GYSGGDIYHS	2982
KAH-5	2959	PAASRLDLSGWFV**A**GYSGGDIYHS	2982
1B-4	2959	PAASRLDLSGWFV**A**GYSGGDIYHS	2982

Takeda et al. Fig. 3a

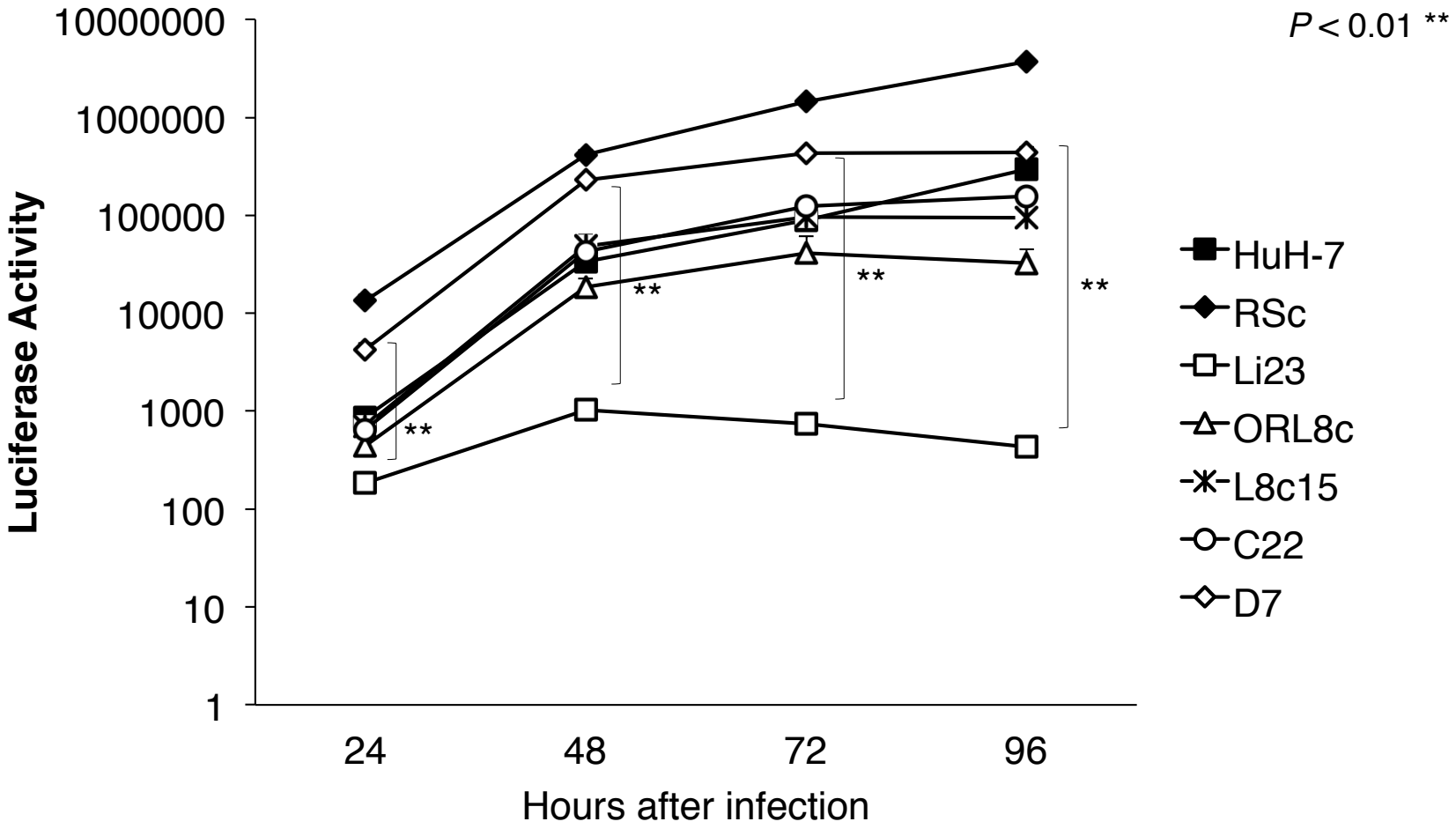


Takeda et al. Fig. 3b

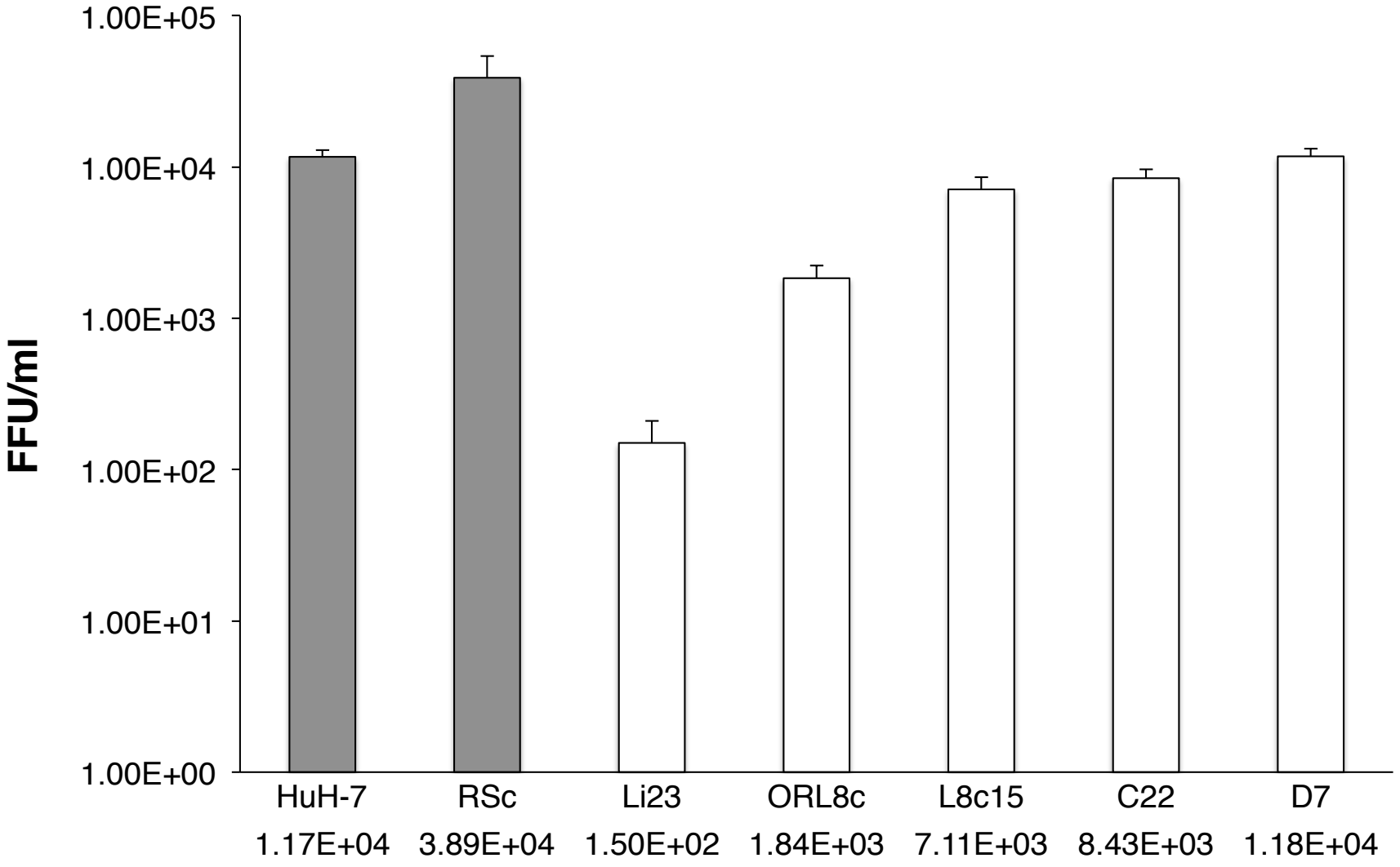
A

Li23 → ORL8c → L8c15 → C22 → D7

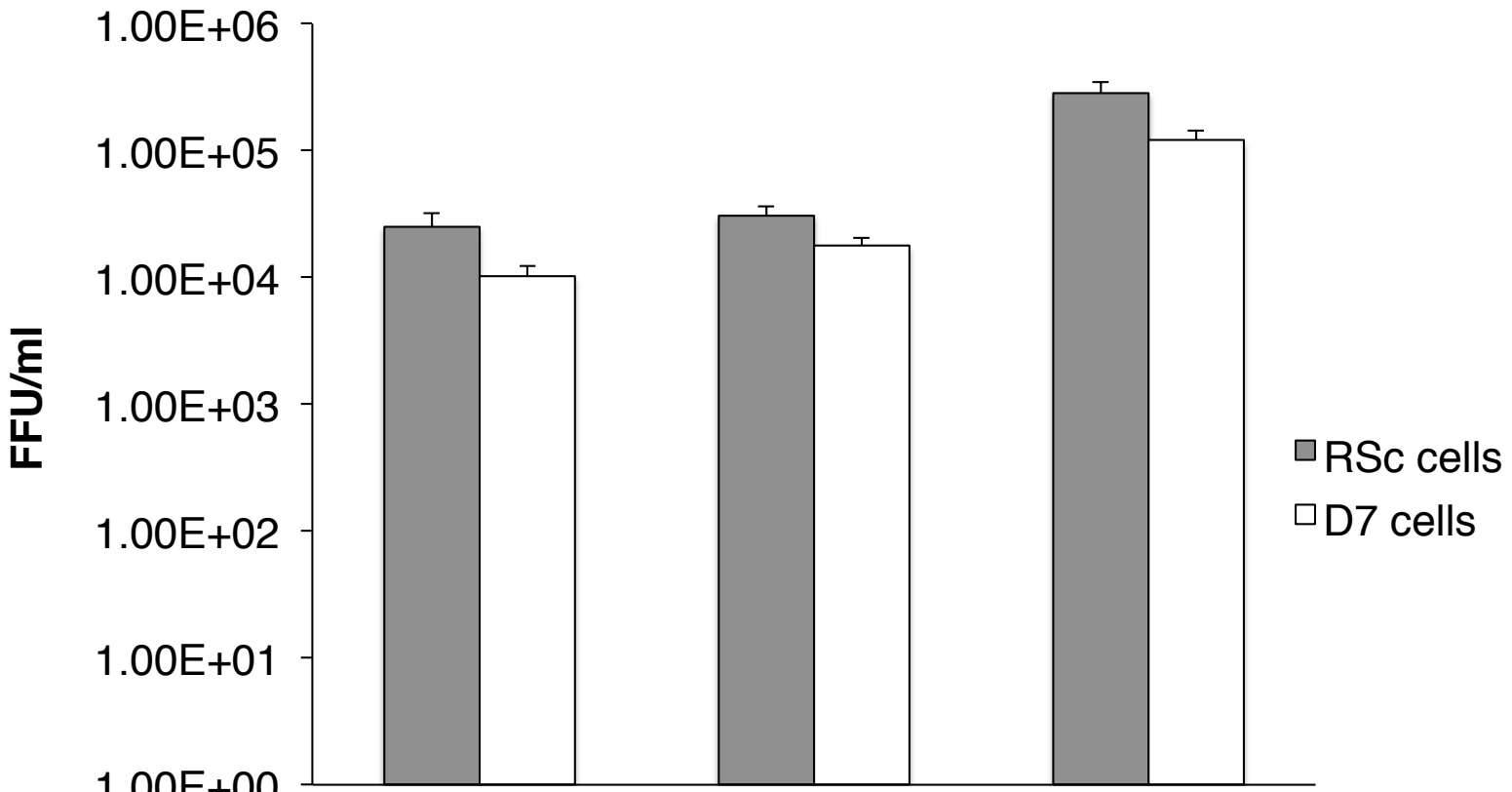
B



Takeda et al. Fig. 4a and 4b

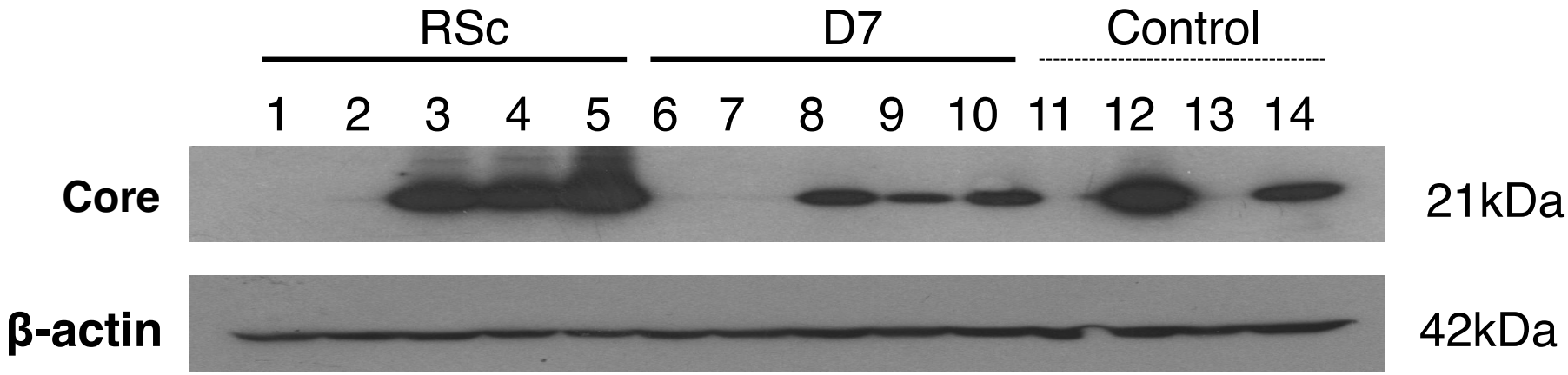


Takeda et al. Fig. 4c

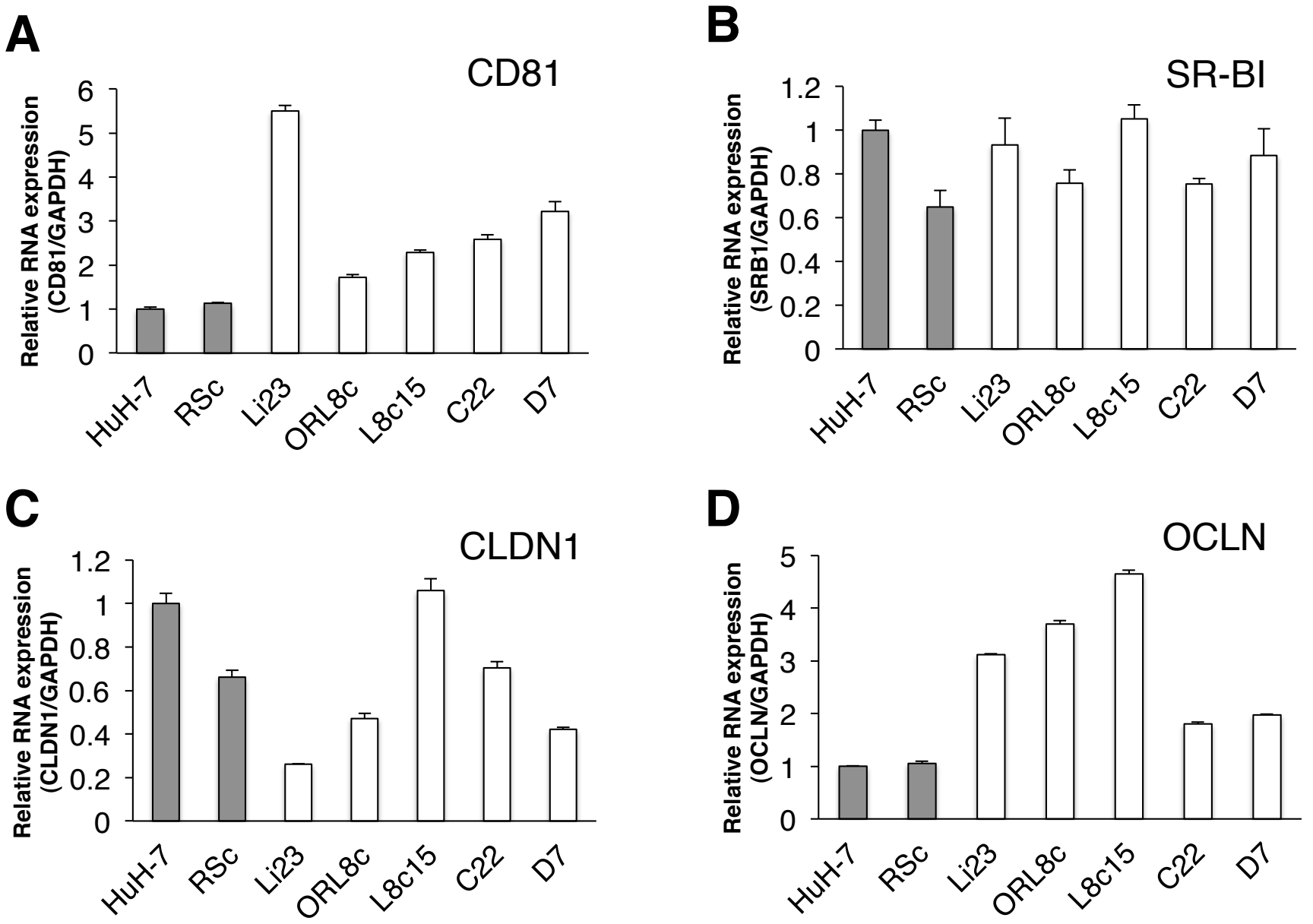


	AS-3	BX-2	JFH-1
RSc cells	2.50E+04	3.08E+04	2.85E+05
D7 cells	1.02E+04	1.77E+04	1.20E+05

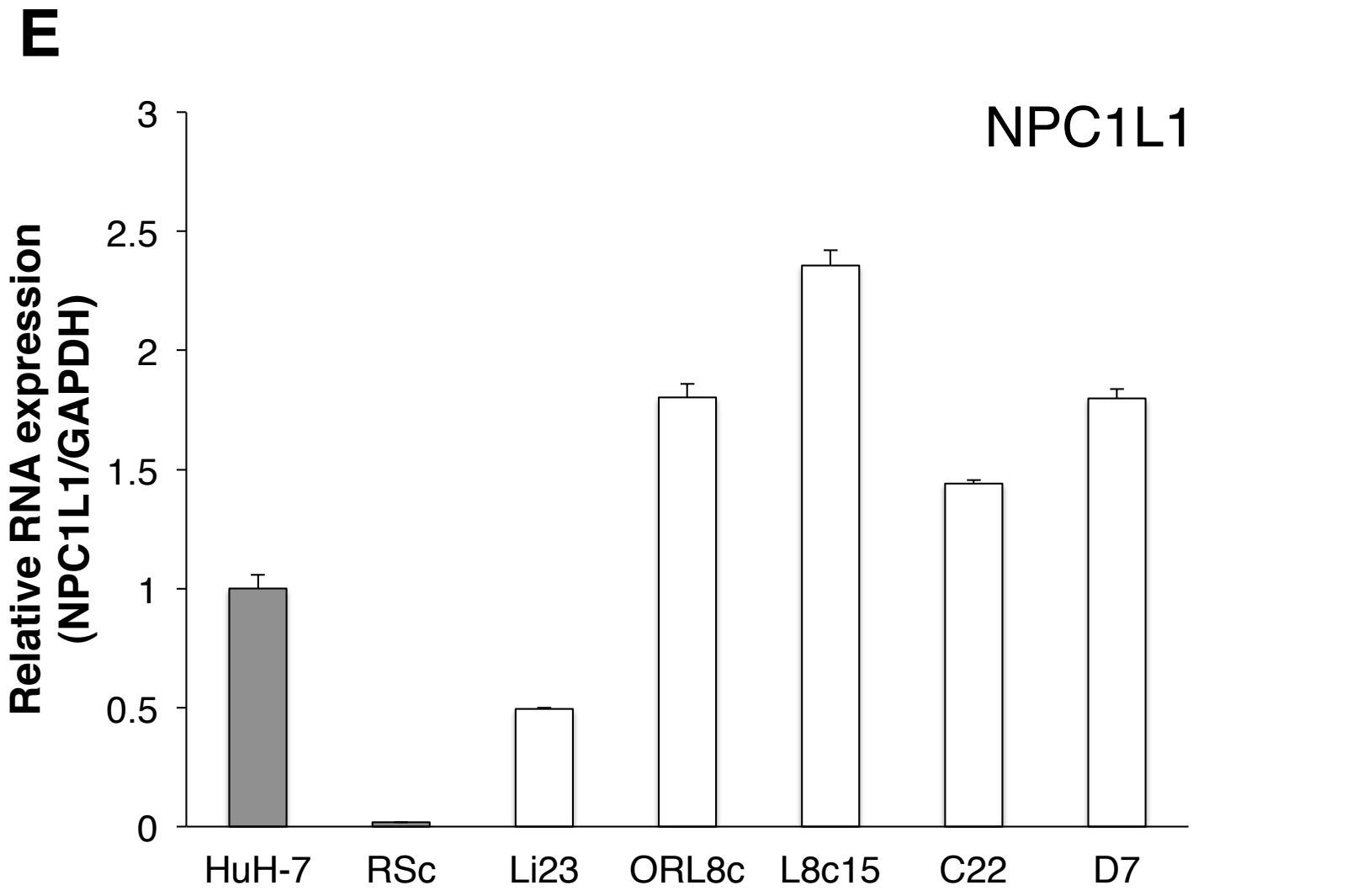
Takeda et al. Fig. 4d



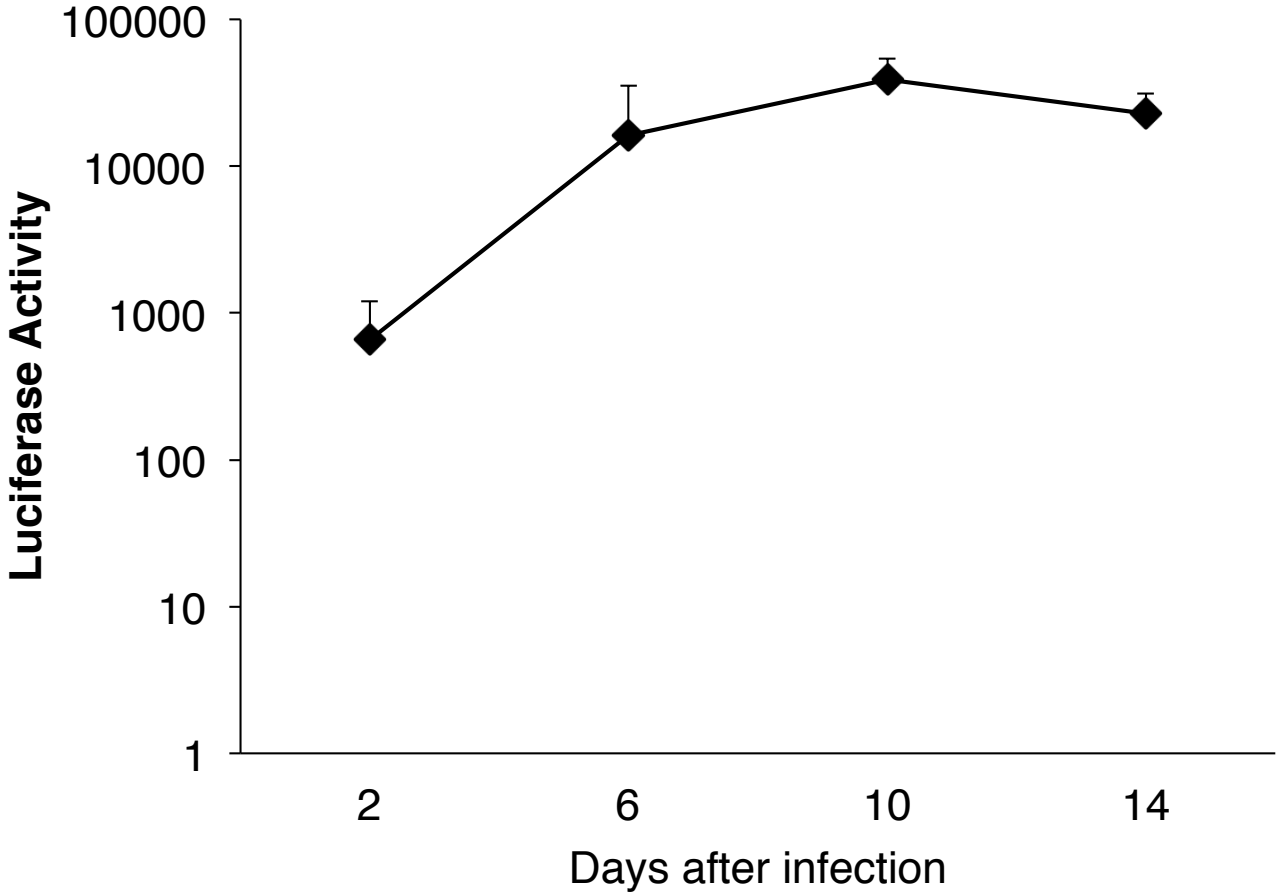
Takeda et al. Fig. 4e



Takeda et al. Fig. 5a-5d



Takeda et al. Fig. 5e



Takeda et al. Fig. 6

Supplementary Figure Legends

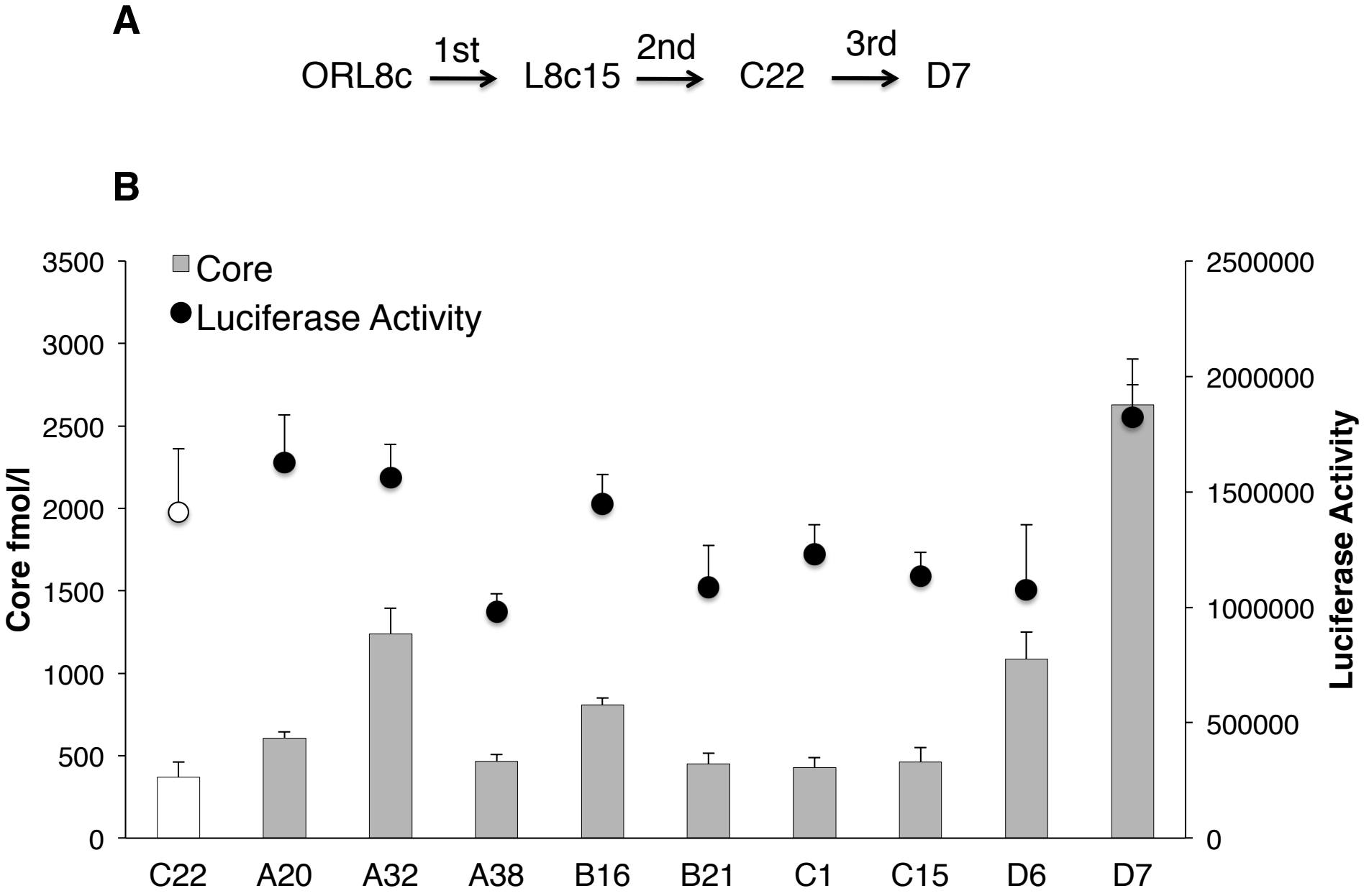
sFig. 1. Selection of subclonal cell lines derived from Li23 cells.

(a) Selection of subclonal cell lines from ORL8c by 3 round limiting dilutions. (b) Selection of D7 cells from parental C22 cells. Nine clonal cell lines derived from Li23-derived C22 cells were examined for their replication efficiency and Core production by RL activity and ELISA.

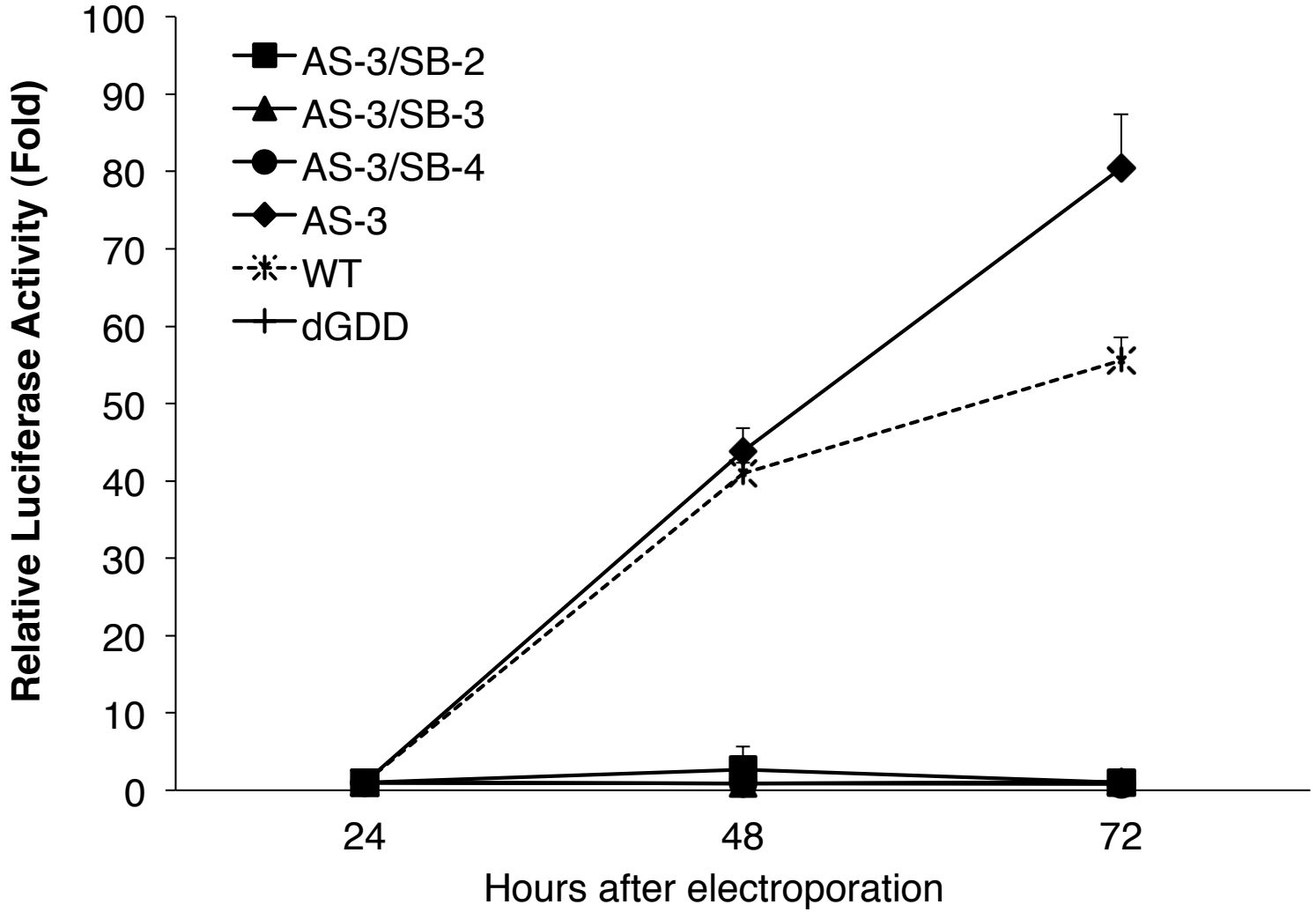
sFig. 2. The effect of amino acid substitutions on HCV RNA replication. (a) The mutations in the Core to NS2 region (AS-3) were tested for their replication efficiency in combination with those of the NS3 to NS5A region (SB-2, SB-3, and SB-4). RL activity was determined at 24, 48, and 72 hours. (b) The mutations in the NS5B to 3'X region (BX-2) were tested for their replication efficiency in combination with those of the NS3 to NS5A region (SB-2, SB-3, and SB-4). RL activity was determined at 24, 48, and 72 hours. (c) The mutations in the Core to NS2 region (AS-3) were tested for their replication efficiency in combination with that of the NS5B to 3'X region (BX-2). RL

activity was determined at 24, 48, and 72 hours. dGDD is a negative control without the GDD motif and WT is a wild type.

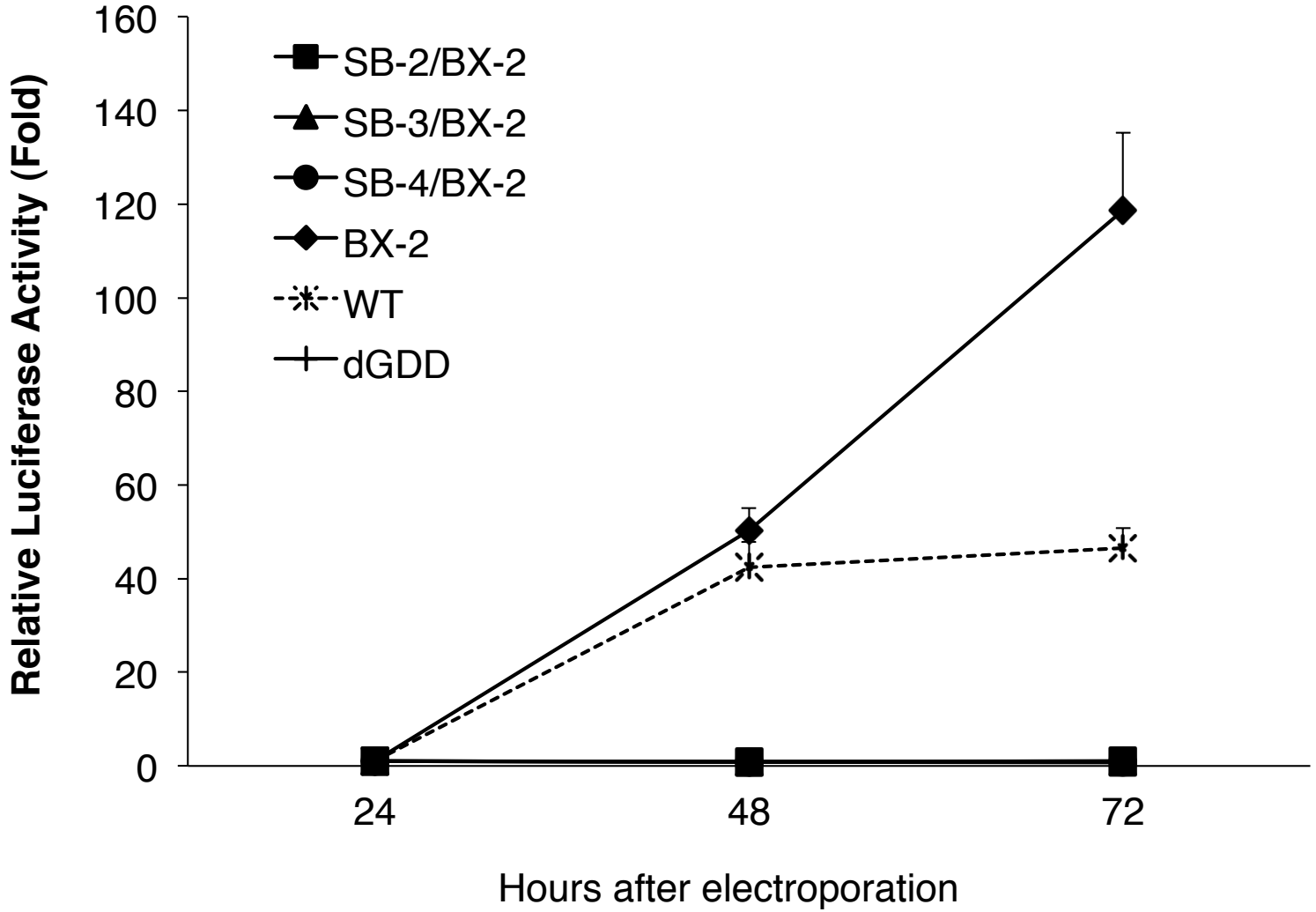
sFig. 3. Mir122 expression in HuH-7- and Li23-derived cell lines. RNA preparations for HuH-7, RSc, Li23, ORL8c, L8c15, C22, and D7 cells were performed with ISOGEN (Nippon Gene, Tokyo, Japan). The extracted RNAs were subjected to quantitative RT-PCR for miR122 using Mir-X^{TM} miRNA qRT-PCR SYBR$^{®}$ Kit (Takara Bio, Shiga, Japan) according to the manufacture's instruction. U6 was used as an internal control.



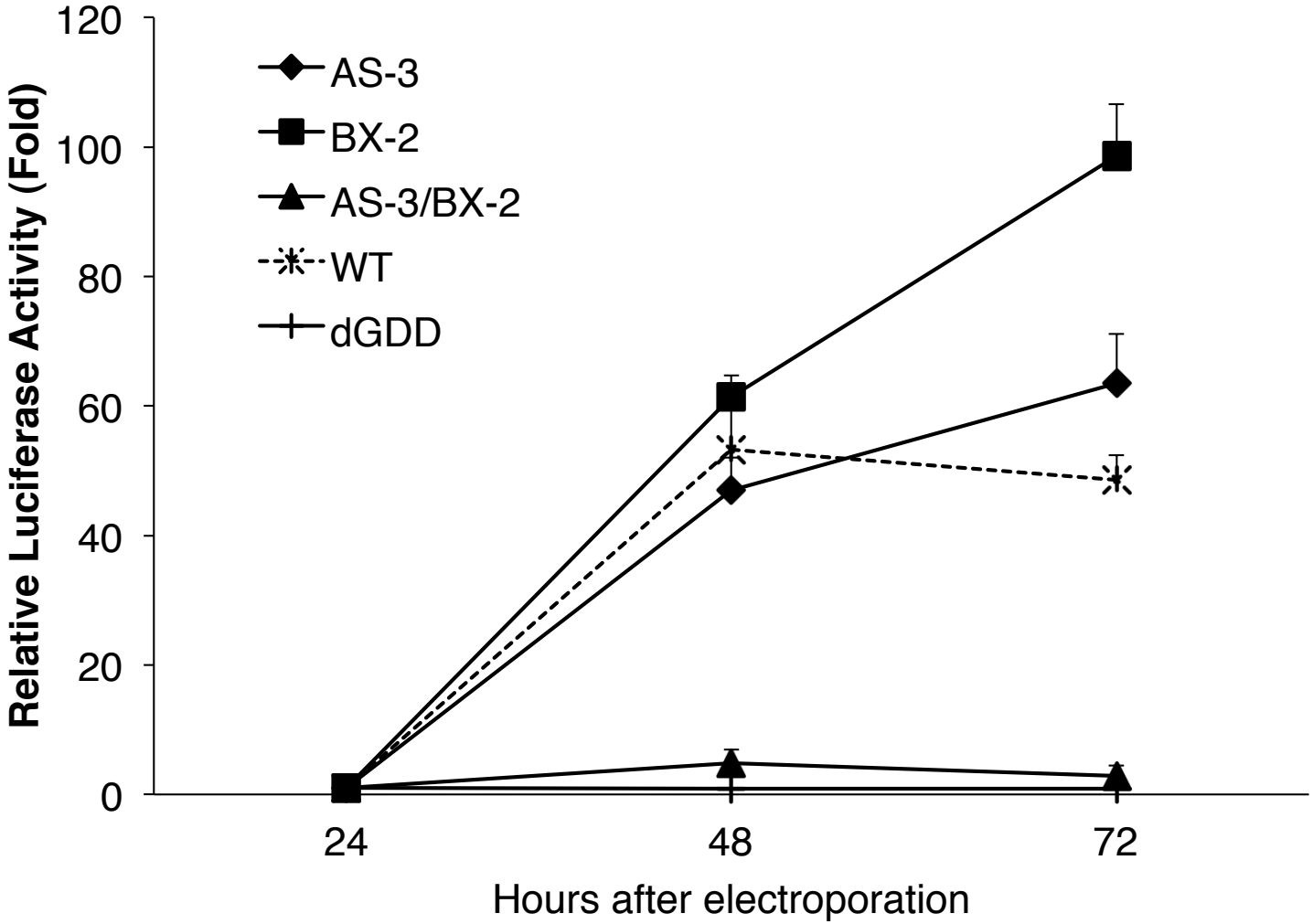
Takeda et al. sFig. 1a and 1b



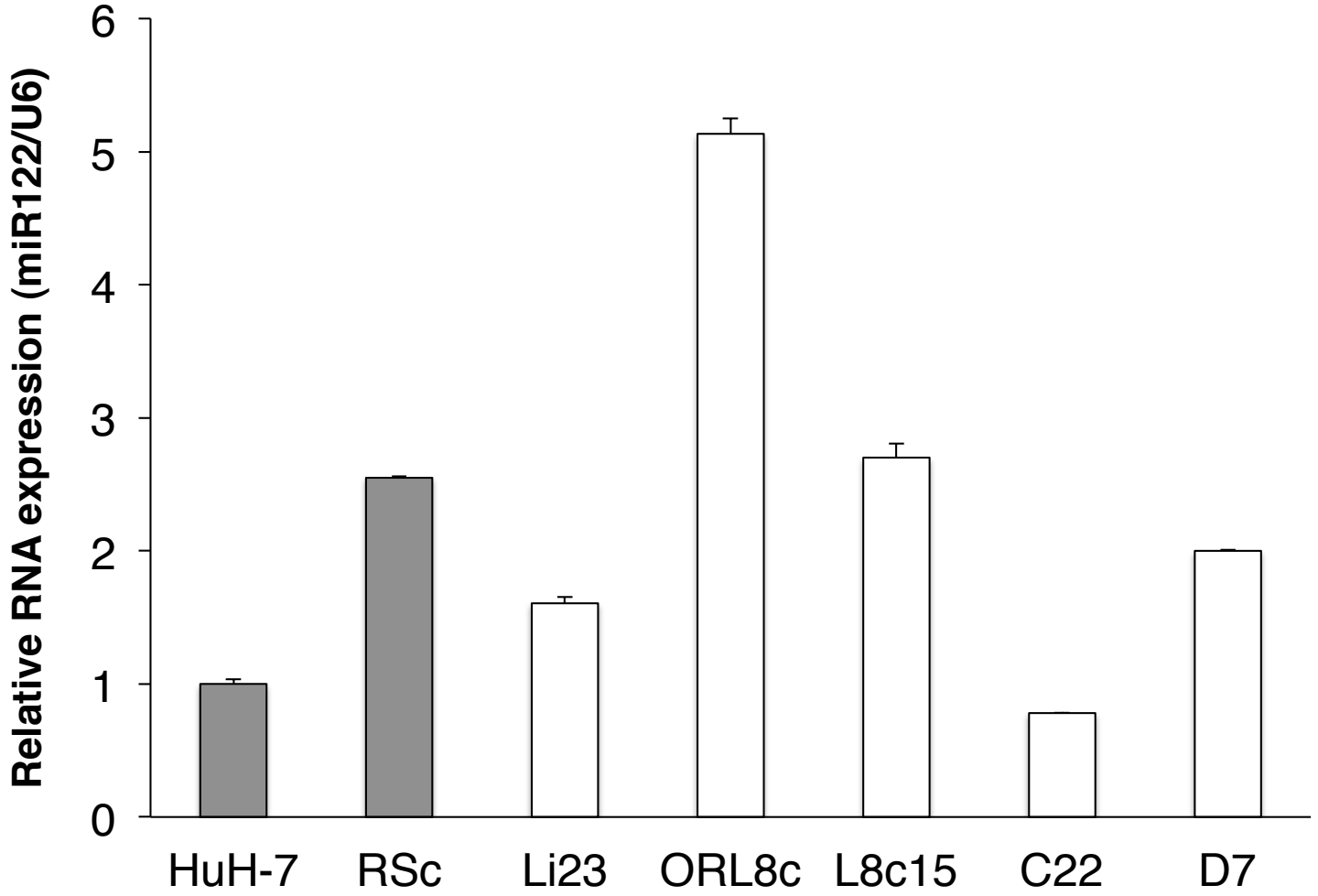
Takeda et al. sFig. 2a



Takeda et al. sFig. 2b



Takeda et al. sFig. 2c



Takeda et al. sFig. 3