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► To cite this version:

Isabel Fofana, Laetitia Zona, Christine Thumann, Laura Heydmann, Sarah C. Durand, et al.. Functional analysis of claudin-6 and claudin-9 as entry factors for hepatitis C virus infection of human hepatocytes using monoclonal antibodies.. *Journal of Virology*, 2013, epub ahead of print. 10.1128/JVI.01691-13 . inserm-00850891

HAL Id: inserm-00850891

<https://inserm.hal.science/inserm-00850891>

Submitted on 31 Jan 2014

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1 **Functional analysis of claudin-6 and claudin-9 as entry factors for**
2 **hepatitis C virus infection of human hepatocytes using monoclonal**
3 **antibodies**

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19 **Word count**

20 Abstract: 75 words; Main manuscript: 1,595 words; Figures: 5

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26 **The relevance of claudin-6 and claudin-9 in hepatitis C virus (HCV) entry**
27 **remains elusive. We produced claudin-6 or claudin-9 specific monoclonal**
28 **antibodies that inhibit HCV entry into non-hepatic cells expressing exogenous**
29 **claudin-6 or claudin-9. These antibodies had no effect on HCV infection of**
30 **hepatoma cells or primary hepatocytes. Thus although claudin-6 and claudin-9**
31 **can serve as entry factors in cell lines, HCV infection into human hepatocytes**
32 **is not dependent on claudin-6 and claudin-9.**

33

34 Hepatitis C virus (HCV) enters cells via a multi-step process that requires viral and
35 host cell factors including the viral envelope glycoproteins E1 and E2, tetraspanin
36 CD81, scavenger receptor class B type I (SR-BI), tight junction proteins claudin-1
37 (CLDN1) and occludin (OCLN), as well as co-entry factors such as epidermal growth
38 factor receptor (EGFR), ephrin receptor A2 (EphA2), Niemann-Pick C1-Like1
39 (NPC1L1) and transferrin receptor 1 (1-3). Understanding the mechanisms of viral
40 entry is a prerequisite to defining anti-viral therapies targeting early step(s) in the viral
41 life cycle.

42 CLDNs are critical components of tight junctions (TJ) and regulate paracellular
43 permeability and polarity. The CLDN superfamily comprises more than 20 members
44 that are expressed in a tissue-specific manner. CLDN1 is an essential host factor
45 defining HCV entry (4) and CLDN1-specific antibodies inhibit HCV infection of human
46 hepatocytes *in vitro* (5, 6). CLDN1 associates with CD81 in a variety of cell types and
47 the resulting receptor complex is essential for HCV infection (5, 7-9). CLDN6 and
48 CLDN9 have been reported to mediate HCV entry in CLDN1-deficient Bel7402
49 hepatoma cells (10) and CLDN null 293T embryonic kidney-derived cells (11-13).
50 State-of-the-art cell culture models that support HCV replication include human

51 hepatoma Huh7-derived cell lines and primary human hepatocytes (PHHs). However,
52 the role of CLDN6 and CLDN9 in mediating HCV infection of these cells and their
53 potential as antiviral targets are unknown.

54 To investigate the functional role of CLDN6 and CLDN9 in HCV entry we
55 generated monoclonal antibodies (mAbs) by genetic immunization using full-length
56 human CLDN6 or CLDN9 cDNA expression vectors as previously described (6, 14).
57 Following lymphocyte fusion, 10 96-well plates were screened for each target using
58 transiently transfected cells expressing specific CLDNs on their cell surface and 54
59 positive clones were subsequently amplified and subcloned. We selected five
60 CLDN6- (WU-8F5-E7, WU-9E1-G2, WU-5H6-D6, WU-10A4-B9, WU-3C9-B11) and
61 three CLDN9- (YD-4E9-A2, YD-6F9-H2, YD-1C4-A4) specific mAbs that bind specific
62 CLDNs expressed on 293T cells without any cross-reactivity for further studies (6)
63 (Fig. 1A). To characterize these antibodies, we first used well characterized 293T
64 cells that do not endogenously express CLDNs (12, 13, 15) (Fig. 1B) and thus allow
65 to express each target CLDN individually (Fig. 1C). A previously described CLDN1-
66 specific mAb (OM-7D3-B3) was used as control (6). We confirmed that, in contrast to
67 naive 293T cells, HCV pseudoparticles (HCVpp) expressing a diverse panel of
68 glycoproteins (strains H77 (1a), HCV-J (1b), JFH1 (2a), UKN3A1.28 (3a),
69 UKN4.21.16 (4) described in reference (16)) infect 293T cells engineered to express
70 CLDN1, CLDN6 or CLDN9 (Fig. 1D). In contrast to previous reports (10, 11), we
71 noted that HCV-JFH1 only infected 293T cells expressing CLDN1, suggesting that
72 this strain cannot utilize CLDN6 or CLDN9. Interestingly, a recent study also reported
73 that Huh6 cells - expressing CLDN6 but devoid CLDN1 - are resistant to HCVpp
74 expressing genotype 2a glycoproteins in contrast to HCVpp of genotype 1 (17). Next,
75 we assessed the ability of CLDN-specific mAbs to inhibit HCVpp entry into these cells

76 using CD81-specific antibody as a positive control (18). All of the CLDN6- and
77 CLDN9-specific mAbs inhibited the entry of HCVpp expressing representative
78 genotype 1a and 1b glycoproteins into CLDN-expressing 293T cells (Fig. 1E). The
79 two most potent mAbs were further characterized for their dose-dependent inhibition
80 of entry HCVpp genotype 1a and 1b (Fig. 2A-B) and cross-reactivity to inhibit a panel
81 of HCVpp expressing diverse glycoproteins. In contrast to mouse leukemia virus
82 pseudoparticle (MLVpp) entry (19), CLDN6- and CLDN9-specific mAbs inhibited the
83 entry of HCVpp expressing glycoproteins of genotypes 3a and 4 into 293T-derived
84 cells (Fig. 2C). These data demonstrate that CLDN6- and CLDN9-specific mAbs
85 inhibit HCV entry of 293T cells in a genotype independent manner.

86 CLDN6 and CLDN9 mRNA have been reported to be expressed in human
87 liver, albeit at low levels (10). All of the CLDN6-specific mAbs bound Huh7.5.1 cells
88 with comparable values to CLDN1-specific mAb, whereas we failed to detect binding
89 of the anti-CLDN9 mAbs (Fig. 3A). These data suggest that CLDN6 is expressed in
90 this hepatoma cell line and that CLDN9 is either not or weakly expressed on
91 Huh7.5.1 cells. This is consistent with CLDN6 and CLDN9 mRNA expression in these
92 cells (Fig. 1B). Then, to investigate whether the antibodies inhibit HCV entry into
93 human hepatoma cells, Huh7.5.1 cells were pre-incubated with CLDN-specific mAbs
94 before infection with HCVpp from different genotypes (strains H77 (1a), HCV-J (1b),
95 JFH1 (2a)). Surprisingly, in contrast to CLDN1-specific mAb and to results obtained
96 with CLDN-expressing 293T cells (Fig. 1-2), none of the CLDN6- or CLDN9-specific
97 mAbs inhibited HCVpp entry into Huh7.5.1 cells even at high concentrations (Fig.
98 3B). Furthermore, none of the CLDN6- or CLDN9-specific mAbs inhibited infection of
99 cell culture-derived HCV (HCVcc; strains Luc-Con1, genotype 1b/2a and Luc-Jc1,
100 genotype 2a/2a described in reference (20)) in these cells (Fig. 3C).

101 Exogenous expression of CLDN6 or CLDN9 in CLDN1-silenced Huh7.5 has
102 been reported to confer a small but statistically significant level of HCVpp genotype
103 1b entry, but to have no effect on HCVcc infection (11). To investigate whether
104 CLDN6 or CLDN9 may be used as a substitute for CLDN1 in Huh7.5.1 cells, we
105 assessed the ability of CLDN-specific mAbs to inhibit HCVpp entry in CLDN1-
106 silenced Huh7.5.1 cells. In contrast to CLDN1-specific mAb, neither CLDN6- nor
107 CLDN9-specific mAbs were able to further decrease HCV entry into CLDN1-silenced
108 Huh7.5.1 cells (Fig. 3D). To further investigate a potential interplay between CLDN1
109 and other CLDNs during HCV infection, we studied whether the combination of
110 CLDN6- or CLDN9- and CLDN1-specific mAbs provided additive or synergistic
111 inhibitory effect(s) on HCVpp entry (6). Combining CLDN6- or CLDN9-specific mAbs
112 with CLDN1-specific mAb did not result in any additive effect on inhibition of HCVpp
113 entry of different genotypes (Fig. 4). Taken together, these data suggest a limited role
114 of CLDN6 and CLDN9 in HCV entry into Huh7-derived cell lines.

115 Finally, to further investigate the *in vivo* relevance of CLDN6 and CLDN9 as
116 potential HCV entry factors and antiviral targets, we performed similar experiments
117 on the natural and most relevant target cell of HCV, the human hepatocyte. In
118 comparison to CLDN1-specific mAb, we noted low to negligible binding of CLDN6 or
119 CLDN9-specific mAbs to PHHs (Fig. 5A). These data suggest that CLDN6 and
120 CLDN9 are either not or weakly expressed in PHHs. Most importantly, in contrast to
121 CLDN1-specific mAb, CLDN6- and CLDN9-specific mAbs had minimal or absent
122 effect(s) on HCVpp infection of PHHs (Fig. 5B). These data are consistent with the
123 limited detection of CLDN6 and CLDN9 mRNA in hepatocytes isolated from a range
124 of donors, in contrast to the high level expression of CD81, SR-BI and CLDN1 (Fig.
125 5C). Another study recently reported high levels of the main HCV entry factors but

126 highly variable CLDN6 mRNA levels in liver biopsies from HCV patients (17).
127 Immunostaining of liver sections also demonstrated that, in contrast to CLDN6-
128 positive seminoma, CLDN6 was not detected in human liver sections (Fig. 5D).

129 The aim of this study was to explore the functional role of CLDN6 and CLDN9
130 in the HCV entry process in PHHs and hepatoma cells. Understanding the
131 mechanism of HCV entry in the most relevant cell culture model will allow the
132 development of more efficient antivirals. We generated novel CLDN6- and CLDN9-
133 specific mAbs that specifically interact with CLDN6 or CLDN9 (Fig. 1A) and inhibit
134 HCVpp entry into human embryonic 293T kidney cells expressing defined CLDN
135 receptors that have been classically used to assess the role of CLDNs in the HCV
136 entry process (Fig. 1-2). These data demonstrate that the antibodies are functional
137 with respect to binding cognate receptors and inhibiting HCV internalization. In line
138 with mRNA expression data (Fig. 1B), antibody binding studies demonstrate
139 expression of CLDN6 on Huh7.5.1 cells (Fig. 3A), albeit with lower binding values
140 than observed with 293T cells. However, CLDN6- or CLDN9-specific mAbs showed
141 low or absent binding to PHHs (Fig. 5A), suggesting minimal expression, a
142 conclusion further supported by the low mRNA levels of these CLDNs in PHHs
143 isolated from multiple donors and absent detection of CLDN6 in liver sections (Fig.
144 5C-D). Noteworthy, functional studies demonstrate that CLDN6- and CLDN9-specific
145 mAbs had no effect on HCV infection of Huh7.5.1 cells or PHHs (Fig. 3-5).
146 Interestingly, silencing of CLDN6 in Huh7.5 cells also did not affect HCV infection
147 (17). Furthermore, these mAbs were unable to further decrease HCVpp entry into
148 CLDN1-silenced Huh7.5.1 cells or when added simultaneously with CLDN1-specific
149 mAb to naive Huh7.5.1 cells, suggesting that CLDN6 and CLDN9 are not able to
150 complement absence of CLDN1 expression or blockage by anti-CLDN1 mAb (Fig. 4).

151 Given their low expression (Fig. 5C-D), other CLDN members may not be able to
152 compensate CLDN1-specific inhibition of HCV entry into PHHs. In contrast, a recent
153 study showed that silencing CLDN6 expression in Huh7.5 cells further reduced
154 CLDN1-specific antibody-mediated inhibition of HCV infection of genotype 1b and 3a
155 (17). Differences of CLDN6 expression in Huh7-based cell lines or experimental
156 design may account for the different findings. Given the very low levels of CLDN6
157 protein expression in the human liver *in vivo* (Fig. 5) and its absent or limited
158 functional role in human hepatocytes (Fig. 5), the relevance of CLDN6 escape
159 observed in Huh7.5 cells (17) remains to be determined in human hepatocytes or *in*
160 *vivo*. If relevant, the CLDN6-specific mAbs (Fig. 1,2) could address this issue.

161 In summary, this study demonstrates that although CLDN6 and CLDN9 can
162 serve as entry factors upon exogenous expression in certain cell culture models such
163 as CLDN-deficient 293T cells, they do not play a major role for HCV entry into human
164 hepatocytes. Yet, given their functional activity, the novel antibodies described in this
165 study may represent interesting tools to investigate the role of CLDN6 and CLDN9 in
166 physiology and disease.

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Acknowledgements

169 This work was supported by Inserm, University of Strasbourg, the Zentrales
170 Innovationsprogramm Mittelstand, the European Union (ERC-2008-AdG-233130-
171 HEPACENT, INTERREG-IV-Rhin Supérieur-FEDER-Hepato-Regio-Net 2009 and
172 2012, EU FP7 HEPAMAB), ANRS (2011/132, 2012/239, 2012/318, 2013/108) and
173 Laboratoire d'excellence LabEx HepSYS (Investissement d'Avenir; ANR-10-LAB-28).
174 Research in McKeating lab is funded by Medical Research Council and NIHR Liver
175 Biomedical Research Unit.

176 The authors thank R. Bartenschlager (University of Heidelberg, Germany), F.-L.
177 Cosset (Inserm U1111, ENS Lyon, France) and J. Ball (University of Nottingham, UK)
178 for providing plasmids for production of HCVcc and HCVpp, C. M. Rice (The
179 Rockefeller University) and F. V. Chisari (The Scripps Research Institute, La Jolla,
180 CA) for Huh7.5 and Huh7.5.1 cells, respectively, P. Bachellier (Service de Chirurgie
181 Générale, Hépatique, Endocrinienne et Transplantation, Hôpitaux Universitaires de
182 Strasbourg, France) for providing primary human hepatocytes, D. Trono (Ecole
183 Polytechnique Fédérale de Lausanne, Switzerland) for lentiviral expression
184 constructs and T. Pietschmann (TWINCORE, Centre for Experimental and Clinical
185 Infection Research, Germany) for helpful discussions. The authors acknowledge P.
186 Balfe (University of Birmingham, UK) for RT-PCR data as well as excellent technical
187 assistance of S. Glauben (Aldevron GmbH, Germany) and C. Bach (Inserm U1110,
188 France).

189 T. F. B. designed research. I.F., L.Z., C.T., L.H., S.C.D., J. L., P.P., H.E.B., G.M.R.,
190 J.A.McK., F.G., J.T., M.B.Z and T.F.B. performed research. I.F., L.Z., C.T., L.H.,
191 S.C.D., J.L., P.P., H.E.B., G.M.R., J.A.McK., F.G., J.T., M.B.Z and T.F.B. analyzed
192 data. C.G. contributed essential reagents. I.F., M. B. Z. and T.F.B. wrote the paper.

193 The authors declare no conflict of interest. Inserm and Aldevron have previously filed
194 a patent application on the use of anti-Claudin-1 antibodies for inhibition of HCV
195 infection (WO2010034812).

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FIGURE LEGENDS278 **Figure 1. CLDN6- and CLDN9-specific mAbs inhibit HCVpp infection of 293T**279 **cells engineered to express CLDNs.** (A) Reactivity of anti-CLDN mAbs for 293T

280 cells transfected with different human CLDNs. CLDN-deficient 293T cells were

281 transfected to express *Aequorea coerulescens* green fluorescent protein (AcGFP)

282 tagged human CLDN1, CLDN6 or CLDN9 as described (6) before detachment and

283 staining with CLDN-specific mAbs or control isotype-matched irrelevant rat IgG at 20

284 $\mu\text{g/ml}$. Primary bound antibodies were detected with goat anti-rat PE conjugated285 F(ab')_2 fragment (PN IM 1623, Beckman Coulter). After washing, the cells were fixed

286 with 2% PFA and analyzed by flow cytometry (FACscan). AcGFP tagged CLDN

287 protein expression was confirmed by flow cytometric quantification of AcGFP

288 expression (data not shown). Specific binding of CLDN1 (OM-7D3-B3) (6), CLDN6

289 (WU-8F5-E7, WU-9E1-G2, WU-5H6-D6, WU-10A4-B9, WU-3C9-B11) and CLDN9

290 (YD-4E9-A2, YD-6F9-H2, YD-1C4-A4) mAbs to AcGFP tagged CLDNs is shown as

291 the difference of the mean fluorescence intensity of cells stained with CLDN-specific

292 mAb and cells stained with control rat mAb isotype. (B) Expression of HCV entry

293 factors in 293T, Hela, HepG2, Bcl-2 and Huh-7.5 cells. RNA was purified from these

294 cell lysates using a Qiagen miniRNA kit following the manufacturer's instructions.

295 Samples were amplified using a Cells Direct RT-PCR kit (Invitrogen). A primer-limited

296 GAPDH referent primer set was included in all PCRs and used to compare

297 expression. (C) Entry factor expression in CLDN1-, CLDN6- or CLDN9-transfected

298 293T cells. The relative expression of each entry factors was determined by flow

299 cytometry and is indicated as fold expression compared to parental 293T cells. (D)

300 HCVpp (strains H77 (1a), HCV-J (1b), JFH1 (2a), UKN3A1.28 (3a), UKN4.21.16 (4);

301 produced as described in reference (16)) were allowed to infect CLDN1-, CLDN6- or

302 CLDN9-293T cells, and infection assessed by luciferase activity in cell lysates 72 h
303 post-infection. Results are expressed in relative light units (RLU). The threshold for a
304 detectable infection in this system is indicated by a dashed line, corresponding to the
305 mean \pm 3 SD of background levels, i.e., luciferase activity of naive non-infected cells
306 or cells infected with pseudotypes without HCV envelopes. Means \pm SD from three
307 independent experiments performed in triplicate are shown. (E) 293T cells
308 engineered to express CLDN1 (left panel), CLDN6 (middle panel) or CLDN9 (right
309 panel) were pre-incubated for 1 h at 37°C with CD81-specific (10 μ g/mL), CLDN-
310 specific or control (50 μ g/ml) mAbs before infecting with HCVpp (strains H77,
311 genotype 1a; HCV-J, genotype 1b) for 4 h at 37°C. HCVpp entry was assessed as
312 described in (D). Means \pm SD from three independent experiments performed
313 triplicate are shown. Statistical analysis relative to the control mAb was performed
314 using the Student's *t* test, **p*<0.05.

315

316 **Figure 2. Anti-CLDN genotype independent inhibition of HCVpp infection of**
317 **293T cells engineered to express CLDN receptors.** CLDN1-, CLDN6- and CLDN9-
318 293T cells were pre-incubated for 1 h at 37°C with (A-B) serial dilutions of or (C) a
319 fixed dose (100 μ g/ml) of CLDN1-, CLDN6- (WU-9E1-G2), CLDN9- (YD-4E9-A2)
320 specific or control mAbs before infecting with HCVpp (strains H77 (1a), HCV-J (1b),
321 UKN3A1.28 (3a), UKN4.21.16 (4)) or MLVpp for 4 h at 37°C. Pseudoparticle entry
322 was assessed as described in Fig. 1. Means \pm SD from three independent
323 experiments performed in triplicate are shown. Statistical analysis relative to the
324 control mAb was performed using the Student's *t* test, **p*<0.05

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326

327 **Figure 3. CLDN6- and CLDN9-specific mAbs do not inhibit HCV infection in**
328 **Huh7.5.1 cells.** (A) Binding of CLDN-specific mAbs to Huh7.5.1 cells. Huh7.5.1 cells
329 were detached and incubated with CLDN-specific mAbs. MAb binding (4 µg/ml) was
330 revealed by flow cytometry using CLDN1-specific mAb (OM-7D3-B3) as a positive
331 control. Inhibition of (B) HCVpp entry or (C) HCVcc infection by CLDN-specific mAbs.
332 Huh7.5.1 cells were pre-incubated for 1 h at 37°C with CLDN-specific or control
333 mAbs (100 µg/ml) before infection with HCVpp (strains H77 (1a), HCV-J (1b), JFH1
334 (2a)) or HCVcc (strains Luc-Con1, genotype 1b/2a and Luc-Jc1, genotype 2a/2a) for
335 4 h at 37°C. HCV infection was assessed by luciferase activity in cell lysates 72 h
336 post-infection. (D) Inhibition of HCVpp entry in CLDN1-silenced Huh7.5.1 cells.
337 CLDN1-silenced Huh7.5.1 (4) were pre-incubated for 1 h at 37°C with CLDN-specific
338 or control mAbs (100 µg/ml) before infection with HCVpp (strains H77 (1a), HCV-J
339 (1b), JFH1 (2a), UKN3A1.28 (3a), UKN4.21.16 (4)). Western blot demonstrating
340 CLDN1 silencing is indicated below. Means ± SD from three independent
341 experiments performed in triplicate are shown. Statistical analysis relative to the
342 control mAb was performed using the Student's *t* test, **p*<0.05

343

344 **Figure 4. Combining CLDN6- or CLDN9-specific mAbs with anti-CLDN1 mAb**
345 **does not result in an additive neutralizing effect.** Huh7.5.1 cells were pre-
346 incubated with serial concentrations of CLDN1-specific or respective isotype control
347 mAb for 1 h at 37°C and either (A) CLDN6- (WU-9E1-G2, upper panel) or (B)
348 CLDN9- (YD-4E9-G2, lower panel) specific mAbs (10 or 100 µg/ml) before infection
349 with HCVpp (strains H77 (1a), HCV-J (1b), JFH1 (2a), UKN3A1.28 (3a)) in the
350 presence of both compounds. HCVpp infection was analyzed as described in Fig. 3.
351 Means ± SD from one representative experiment performed in triplicate are shown.

352 **Figure 5. CLDN6- and CLDN9-specific mAbs do not inhibit HCVpp infection of**
353 **PHHs.** (A) Binding of CLDN-specific mAbs to PHHs. Following PHH isolation, cells
354 were incubated with CLDN-specific mAbs. MAb binding (20 µg/ml) was assessed as
355 described in Fig. 3. (B) Inhibition of HCVpp entry into PHHs by CLDN-specific mAbs
356 (100 µg/ml) was performed as described in Fig. 3. Means ± SD from three
357 independent experiments performed in triplicate are shown. Statistical analysis
358 relative to the control mAb was performed using the Student's *t* test, **p*<0.05. (C)
359 Expression of HCV entry factors in PHHs. Total RNA was extracted from hepatocyte
360 samples (260-282), Hela, Huh7.5 and Caco-2 cells and mRNA copies determined by
361 RTqPCR. Absolute quantities were normalized to GAPDH and data are displayed as
362 % expression relative to CD81 and means ± SD of one experiment performed in
363 quadruplicate are shown. Any signal <0.1 % of CD81 (1,000 fold lower) is negligible.
364 (D) CLDN6 expression in normal liver. CLDN6 expression was analysed by
365 immunohistochemistry on formalin fixed paraffin embedded normal donor and normal
366 liver (right panel), distal to tumour resection tissues (21). A Seminoma case was used
367 as a positive control (middle panel) and a control IgG performed as negative control
368 (left panel). Positive staining is shown in red. Microscopic analysis of tissues showed
369 strong membranous staining of the tumour cells for CLDN6 in the positive Seminoma
370 control section. Normal donor and resection liver tissue demonstrated no detectable
371 CLND6 expression and IgG controls on all sections were negative.

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