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Characterization of woodchuck apolipoprotein A-I: a new tool for drug delivery and identification of altered isoforms in the woodchuck chronic hepatitis model

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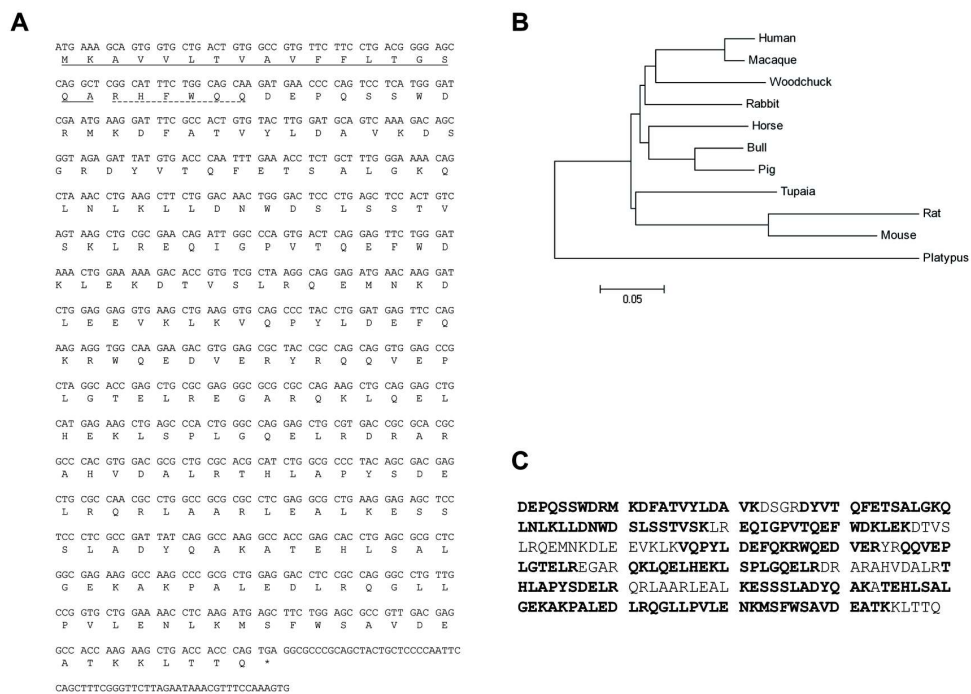


Fig. 1 A) Nucleotide sequence of the woodchuck apolipoprotein A-I and the putative amino acid sequence of the encoded protein. Putative "pre" segment and "pro" segment are underlined with a solid line and dashed line respectively. B) Phylogenetic (Neighbor-joining) analysis of coding sequences of ApoA-I using the MEGA4.0 program. Accession numbers of sequences used are human (Homo sapiens), NM_000039.1; macaque (Macaca fascicularis), XM_001090774.1; rabbit (Oryctolagus cuniculus), X15908.1; horse (Equus caballus), XM_001502469.1; bull (Bos taurus), NM_174242.3; pig (Sus scrofa), NM_214398.1; tupaia (Tupaia glis), AF005638.1; rat (Rattus norvegicus), NM_012738.1; mouse (Mus musculus), X64262.1; platypus (Ornithorhynchus anatinus), XM_001517296.1. C) Sequence of the predicted mature woodchuck apolipoprotein A-I. Matched peptides used to identify the protein with mass spectrometry data are shown in bold.

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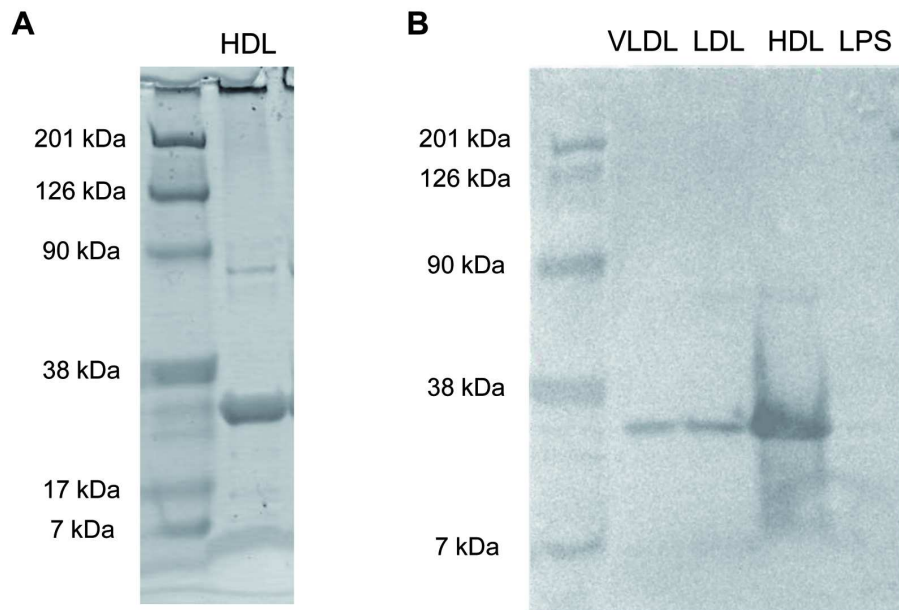


Fig. 2 A) Coomassie staining of purified woodchuck HDLs separated on 4-20% SDS-PAGE. B) Western blot of the different fractions obtained by differential ultracentrifugation in sodium bromide gradient using a commercial anti-ApoA-I (E-20) mAb. Secondary antibody was anti-goat IgG-HRP and detection was by chemoluminescence. VLDL: Very Low Density Lipoprotein; LDL: Low Density Lipoprotein; HDL: High Density Lipoprotein; LPS: Lipoprotein Depleted Serum.
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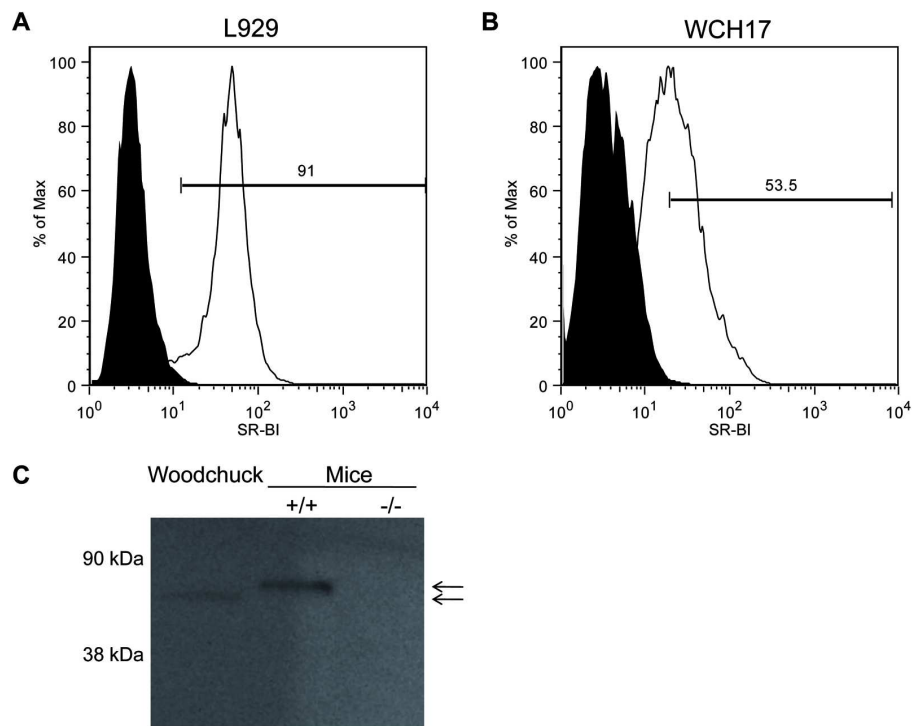
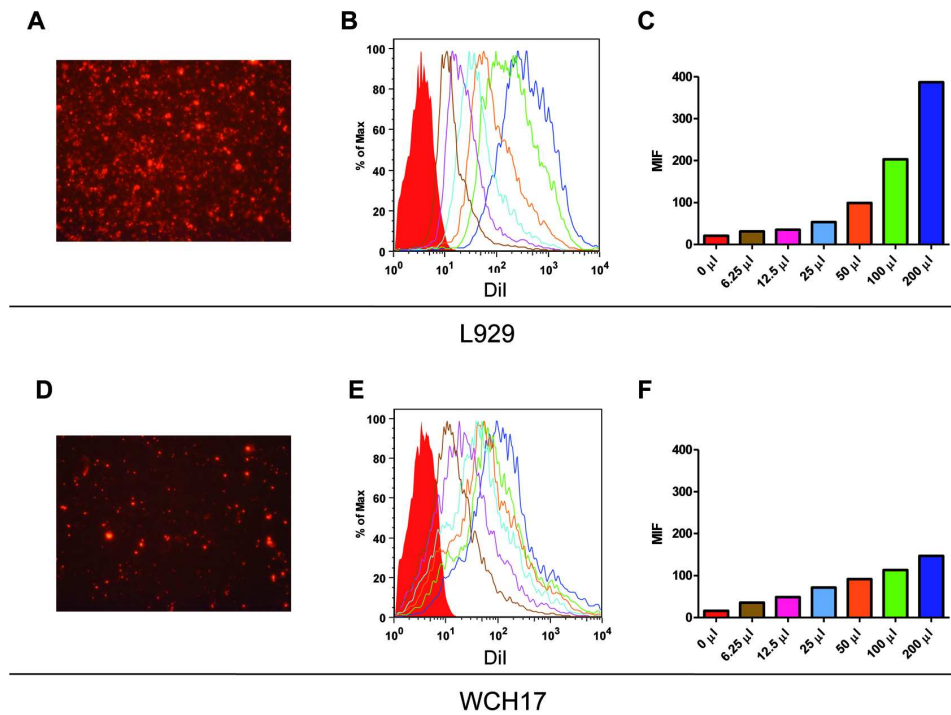
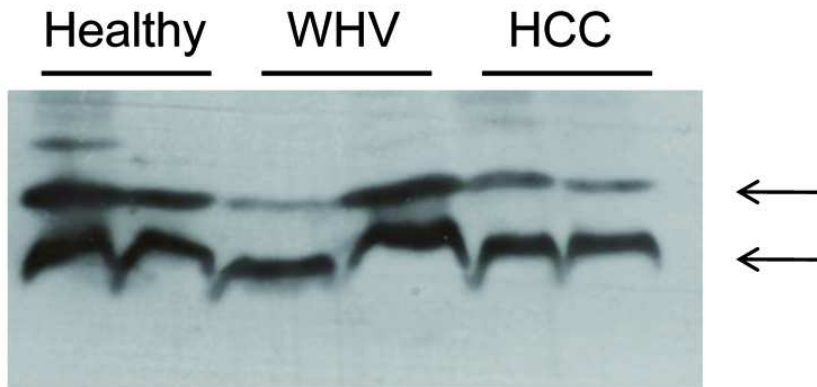


Fig. 3 Flow cytometry analysis of SR-BI expression on A) L929, control cells and on B) WCH17, woodchuck hepatocarcinoma cell line. Cells were labeled with anti-SR-BI polyclonal antibody (EP1556Y) and a secondary FITC Anti-Rabbit IgG Antibody. C) SR-BI western blot analysis in proteins extracted from livers obtained from woodchuck, SR-BI wild type or knock out mice as an antibody specificity control.
150x112mm (300 x 300 DPI)



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Fig. 4 DiI-HDL uptake. Control cells L929 or woodchuck WCH17 hepatoma cell line, were incubated with DiI-HDL and visualized in fluorescent microscopy (A and D), or incorporated DiI-HDL at different concentrations that were quantified by flow cytometry: histograms showing percentage of DiI+ cells (B and E) and mean fluorescence intensity (C and F).
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Fig. 5 Isoelectrofocusing and western blotting against woodchuck ApoA-I. Serum samples from healthy or chronically infected, or woodchucks with HCC were analyzed by isoelectrofocusing and western blotting using a commercial anti-ApoA-I (E-20) mAb. The secondary antibody was anti-goat IgG-HRP and detection was performed by chemoluminescence. Arrows indicate the three ApoA-I isoforms that can be detected.
75x39mm (300 x 300 DPI)

1 **Characterization of woodchuck apolipoprotein A-I: a new tool for**
2 **drug delivery and identification of altered isoforms in the**
3 **woodchuck chronic hepatitis model**

4
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21
22 Short Title: woodchuck apolipoprotein A-I description

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6 **23 Abstract**
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8 Apolipoprotein A-I (ApoA-I) is the major protein component of high density
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10 lipoprotein (HDL) particles in serum, and participates in the reverse transport of
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12 cholesterol from tissues to the liver for excretion. The natural HDL tropism to the
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14 liver and cancer cells has been used extensively to target encapsulated drugs. The
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17 alteration of the plasmatic isoforms of ApoA-I is a hallmark of chronic hepatitis
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19 and hepatocarcinoma in mice and humans. Woodchucks infected with the
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21 woodchuck hepatitis virus (WHV) represent the best animal model for the study
22
23 of chronic viral hepatitis B and viral induced hepatocarcinoma (HCC). WHV-
24
25 infected woodchuck represents a clinically relevant animal model under which
26
27 new treatment strategies can be evaluated and optimized. Therapeutic efficacy in
28
29 this model is likely to be translated into a successful therapy for patients infected
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31 with HBV. The present study describes, for the first time, the cloning and
32
33 characterization of woodchuck ApoA-I. The open reading frame (ORF) of the
34
35 woodchuck ApoA-I is 795 bp long, coding for 264 amino acids. Unexpectedly,
36
37 phylogenetic analysis revealed that the closest sequences are those of human and
38
39 macaque. Woodchuck HDLs were isolated successfully from sera by density
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41 gradient ultracentrifugation. A commercial antibody that recognized the
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43 woodchuck ApoA-I was also identified. Finally, taking advantage of the
44
45 techniques and tools developed in this study, two potential applications of
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47 woodchuck HDLs are illustrated: drug delivery to a woodchuck hepatocarcinoma
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49 cell line and the use of isoelectrofocusing to identify ApoA-I isoforms.
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6 45 **Keywords: High density lipoprotein / Scavenger receptor class B type 1/**

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8 46 **Hepatitis B virus.**

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11 47 Conflicts of interest: The authors state that they do not have any financial or other
12 conflict of interest that might be construed to influence the contents of the
13 manuscript, including the results or interpretation of this publication.
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20 50 Experimental Ethics: All experiments were performed in compliance with
21 relevant laws and institutional guidelines and in accordance with the ethical
22 standards of the Declaration of Helsinki. The study was approved by the Ethics
23 and Biosafety Committee according to guidelines from the University of Navarra
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25
26
27 53 and Government of Navarra.
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6 **56 Introduction**
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8 Infection with hepatitis B virus (HBV) is a major public health problem and is
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10 responsible for about 1.2 million deaths per year worldwide. More than 350
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12 million people throughout the world are infected chronically with HBV and are at
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14 high risk of developing chronic hepatitis, cirrhosis, and hepatocellular carcinoma
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17 (HCC) [Villeneuve, 2005; Yim and Lok, 2006]
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20 Infection of eastern woodchucks (*Marmota monax*) with woodchuck hepatitis
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22 virus (WHV) can progress to chronicity, which then leads ultimately to the
23
24 development of HCC [Roggendorf and Tolle, 1995; Tennant et al., 2004]. Chronic
25
26 WHV infection in woodchucks is considered the best animal model for studying
27
28 pathogenesis, prevention and treatment of human HBV infection [Roggendorf and
29
30 Tolle, 1995; Tennant and Gerin, 2001]. Recent studies also have indicated the
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32 usefulness of this model for the development of new strategies for the prevention
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34 and treatment of HCC [Tennant, 2001; Tennant et al., 2004]. Efforts to expand the
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36 arsenal of experimental tools open constantly doors to new findings in the
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38 woodchuck model. One of these desirable tools is the characterization of
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40 woodchuck high density lipoproteins (HDLs), which will allow evaluation of
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42 HDLs as drug delivery vehicles in the woodchuck model and proteomic analysis
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44 of altered ApoA-I isoforms in woodchucks with chronic hepatitis or
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46 hepatocarcinoma.
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53 HDLs are formed by an external amphipathic layer with free cholesterol,
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55 phospholipids and several apolipoproteins, and an internal hydrophobic core with
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57 triglycerides and cholesterol esters [Kim et al., 2005]. Apolipoprotein A-I (ApoA-
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6 79 I) represents 80% of the total HDL protein content. HDLs are produced in the
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8 80 liver to transport cholesterol from tissues to the liver [Hovorka et al., 2006;
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10 81 Schmidt et al., 1997; Thompson et al., 2004]. The ApoA-I protein is synthesized
11
12 82 in the liver and intestine as a pre-pro-protein which is cleaved to pro-ApoA-I
13
14 83 inside the cell before secretion [Bojanovski et al., 1985; Edelstein et al., 1983;
15
16 84 Saku et al., 1994; Saku et al., 1993]. ApoA-I is believed to be controlled primarily
17
18 85 at the post-translational level [Panduro et al., 1990]. These post-translational
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20 86 modifications produce several isoforms. Oxidative stress induced by liver diseases
21
22 87 such as chronic hepatitis and/or HCC alters the ApoA-I isoform pattern.
23
24 88 [Fernandez-Irigoyen et al., 2005; He et al., 2003; Steel et al., 2003; Yang et al.,
25
26 89 2010]. In addition, changes in the circulating levels of ApoA-I have been
27
28 90 correlated with alterations in hepatocellular function in cirrhosis of the liver
29
30 91 [Nayak et al., 1988], acute viral hepatitis [Geiss et al., 1996], and chronic liver
31
32 92 disease [Selimoglu et al., 2002]. Once secreted, pro-ApoA-I is cleaved quickly to
33
34 93 the mature form [Bojanovski et al., 1985; Edelstein et al., 1983; Saku et al., 1994;
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36 94 Saku et al., 1993]. In serum, most of the ApoA-I circulating is complexed into
37
38 95 HDLs. Only 3.7%-8.1% of total ApoA-I circulates as free protein [Neary and
39
40 96 Gowland, 1987]. The half life of Apo A-I is 15- 54 h [Nanjee et al., 1996], and the
41
42 97 renal clearance is 0.4-1.8%/hour [Brinton et al., 1990; Brinton et al., 1991;
43
44 98 Brinton et al., 1994; Gylling et al., 1992; Magill et al., 1982; Schaefer et al.,
45
46 99 1982]. Cholesterol-loaded HDLs reach the liver by the circulating blood, where
47
48 100 cholesterol is internalized by hepatocytes. It has also been demonstrated that after
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50 101 systemic administration of ApoA-I, this exogenous ApoA-I is accumulated in the
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6 102 liver [Kim et al., 2007]. These two properties, long half-life and liver targeting,
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8 103 make HDL and other ApoA-I decorated particles ideal candidates as vehicles for
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10 104 liver-targeted drug delivery [Feng et al., 2008a; Feng et al., 2008b]. Encapsulated
11
12 105 drugs are hydrophobic or amphiphilic compounds incorporated into the lipid core
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14
15 106 of lipoproteins [Counsell and Pohland, 1982] that can be taken up by HDL
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17 107 receptor-mediated mechanisms [Balazs et al., 2004; Connelly and Williams, 2004;
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19 108 Krieger, 2001; Reboul et al., 2006; Vishnyakova et al., 2003]. In addition to the
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21
22 109 liver, many tumor cells capture avidly HDLs and therefore HDLs complexes or
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25 110 HDLs modifications have proved to be useful for antitumor drug targeting [Cao
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27 111 et al., 2004; Lacko et al., 2002; Lacko et al., 2006; Lou et al., 2005; McConathy et
28
29 112 al., 2008; Zhang and Chen, 2010]. Recently, interference RNA has also been
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31 113 loaded successfully into HDLs and targeted at the liver [Kim et al., 2007]. HDLs
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33 114 present three main advantages over other drug nanocarriers such as polymer
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35 115 microspheres, liposomes and microemulsions. First, the hydrophilic-hydrophobic
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37 116 structure of lipoproteins provides an ideal domain for transporting amphiphilic
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39 117 drugs, which is a challenge with other drug delivery systems [Hamidi et al.,
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41 118 2006]. Secondly, since they are natural nanoparticles within the human body, they
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43
44 119 are not trapped avidly by dendritic cells and macrophages and they are not prone
45
46 120 to induce an immune response [Hamidi et al., 2006]. Finally, the uptake into cells
47
48 121 is mediated by specific high-affinity receptors avoiding sophisticated targeting
49
50 122 strategies [Fidge, 1999]. The main potential disadvantage of HDLs, that is, the
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52 123 availability of a large amount of natural HDLs, and the safety issues due to the use
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55 124 of a human-derived material, are circumvented by the development of a form of
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6 125 HDL derived synthetically and termed 'reconstituted HDL' [McConathy et al.,
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8 126 2008].
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11 127 In the present study, woodchuck apolipoprotein A-I gene was cloned, and new
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13 128 tools and methods have been developed that will allow the use of woodchuck
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15 129 HDLs. Two of the most interesting potential applications of woodchuck HDLs are
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17 130 illustrated in this study: drug targeting of the hepatocarcinoma cell line and the
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19 131 identification of woodchuck Apo A-I isoforms.
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6 133 **Materials and Methods**
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8 134 Animals and cells
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10 135 Woodchucks infected chronically with the virus of family *Hepadnaviridae*, genus
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12 136 *Orthohepadnavirus*, species *Woodchuck hepatitis virus* (purchased from
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15 137 Northeastern Wildlife, Ithaca, NY, USA), were handled according to the
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17 138 guidelines of the institution (Centro de Investigación Farmacobiológica Aplicada,
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19 139 Pamplona, Spain). The manipulation of animals, including ultrasound-guided liver
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21 140 biopsies, was performed under general anesthesia. Blood samples were obtained
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23 141 from the saphenous vein of the hind legs, and serum was recovered by
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25 142 centrifugation at 9300 x g for 10 min and stored at -20°C. HCC positive
26
27 143 woodchucks were confirmed by direct observation after laparotomy on
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29 144 anesthetized woodchucks. B6;129S2-Srb1^{tm1Kri} (003379) were purchased from the
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31 145 Jackson Laboratory, and treated in accordance with the guidelines of the Center
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33 146 for Applied Medical Research (CIMA, Pamplona, Spain). Woodchuck hepatoma
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35 147 cells WCH-17 were obtained from the ATCC (ATCC No. CLR-2082). Mouse
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37 148 fibroblast L929 cells were obtained from Sigma (Tres Cantos, Spain). Both cell
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39 149 lines were cultured and maintained in DMEM medium supplemented with 100
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41 150 U/mL penicillin, 100 µg/mL streptomycin and 10% fetal bovine serum (all from
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43 151 Gibco, Invitrogen, CA, USA) under standard conditions.
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53 153 Cloning of 5'-end of woodchuck apolipoprotein A-I
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55 154 RNA was extracted from a woodchuck liver biopsy using TRI reagent (Sigma,
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57 155 Madrid, Spain). 1 µg of total RNA was treated with 0.1 U/µl of DNase I
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6 156 (Invitrogen, Carlsbad, CA) and 1.6 U/ μ l RNaseOUT (Invitrogen, Carlsbad, CA)
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8 157 in 1X DNase I reaction buffer at room temperature for 15 min, and the DNase I
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10 158 was inactivated by addition of 1 μ l of 25 mM EDTA solution and heat 10 min at
11
12 159 65°C. A reverse transcription (RT) reaction was performed in a final volume of 20
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14 160 μ l containing the DNaseI-treated RNA and 1X RT buffer, 1 mM dNTPs, 5 mM
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16 161 DTT, 12 ng/ μ l random hexamers, 1.2 U/mL RNaseOUT and 6 U/ μ l MuLV
17
18 162 reverse transcriptase (Invitrogen, Carlsbad, CA). The reaction was performed at
19
20 163 37°C for 60 min and stopped at 95°C 1 min. PCR was undertaken in 50 μ l with 1
21
22 164 μ l RT reaction (containing cDNA corresponding to 50 ng of the original RNA),
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24 165 1X PCR buffer, 50 μ M dNTPs, 2.5 mM MgCl₂, 1 U/mL of BioTaq DNA
25
26 166 polymerase (Bioline GmbH, Luckenwalde, Germany) and 200 nM of each primer
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28 167 (Fw 5'- GTCACCCACACCCTTCAGGATG-3' and Rv 5'-
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30 168 TTGCAGCTCCTGCACCTT CTG -3'). PCR conditions were as follows: one
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32 169 initial denaturation step at 95°C for 5 min, followed by 35 cycles at 95°C for 40
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34 170 sec, 57°C for 30 sec, and 72°C for 45 sec, and a single final extension at 72°C for
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36 171 1 min. PCR product was purified and cloned into the vector pcDNA™3.1/V5-His
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38 172 TOPO® TA (Invitrogen, Carlsbad, CA). Ten clones were selected, and plasmids
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40 173 were isolated and sequenced.
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51 Cloning of full open reading frame by 3' rapid amplification cDNA end (RACE)

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53 176 Liver biopsies obtained from four woodchucks were homogenized and RNA was
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55 177 extracted using the TRI reagent (Sigma, Madrid, Spain). 5 μ g of total RNA were
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57 178 incubated 2 min at 70°C with 1 μ l of 12 μ M 3'-RACE primer (5'-
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6 179 AAGCAGTGGTATCAACGCAGAGTACTTTTTTTTTTTTTTTTTTTTTTTTTTTTTT
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8 180 TTTTVN -3' where N = A, C, G, T and V = A, C, G). A reverse transcription
9
10 181 (RT) reaction without random hexamers and PCR was carried out as described
11
12 182 above. The primers used in the PCR were 200 nM of primer forward (5'-
13 183 ATGAAAGCAGTGGTGCTGACTG -3'), 40 nM of the reverse primer (5'
14 184 CTAATACGACTCACTATAGGGCAAGCAGTGGTATCAACGCAGAGT -3')
15 185 and 200 nM of the second reverse primer (5'- CTAATACGACTCA
16 186 CTATAGGGC -3'). PCR conditions were as follows: one initial denaturation step
17 187 at 95°C for 2 min, followed by 20 cycles at 95°C for 30 sec, 58°C for 30 sec, and
18 188 72°C for 45 sec, and a single final extension at 72°C for 2 min. PCR product was
19 189 purified and cloned into the vector pcDNA™3.1/V5-His TOPO® TA (Invitrogen,
20 190 Carlsbed, CA). Ten clones of each woodchuck were selected, and plasmids were
21 191 isolated and sequenced. The sequence was submitted to GenBank (accession
22 192 number GU562343)
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41 Isolation of HDLs by differential ultracentrifugation in sodium bromide gradient

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43 195 The sodium bromide (NaBr) solutions at different densities were prepared in a
44 196 final volume of 25 mL in distilled water. NaBr (Fluka) was added to obtain the
45 197 solutions: 0.225 g ($\rho= 1.006\text{g/mL}$), 1.431 g ($\rho= 1.04\text{ g/mL}$), 7.085 g ($\rho= 1.21$
46 198 g/mL) and 13.573 g ($\rho= 1.4\text{ g/mL}$). EDTA at a final concentration of 0.05% (w/v)
47 199 was added. Since NaBr is a highly hygroscopic salt, the density was verified and
48 200 corrected by adding distilled water when necessary. The method of sequential
49 201 separation of lipoproteins by flotation after ultracentrifugation was performed
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6 202 following the protocol described previously with minor modifications [Rodriguez-
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8 203 Sureda et al., 2002]. All centrifugation steps were performed in an Optima MAX
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10 204 ultracentrifuge, using a TLA100.4 rotor (Beckman Coulter) and the following
11
12 205 solution densities: VLDL < 1.006 g/mL, LDL 1.006-1.04 g/mL and HDL 1.04-
13
14 206 1.21 g/mL. i) Isolation of VLDL: 400 μ l of woodchuck plasma were transferred to
15
16 207 3 mL polycarbonate tubes and 1100 μ l of a $\rho=1.006$ g/mL NaBr solution were
17
18 208 added and centrifuged for 2 hours, 4°C, 336000g. Approximately 650 μ l of
19
20 209 supernatant were collected and stored at -20°C. ii) Isolation of LDL: the remaining
21
22 210 volume and sediment were adjusted to a density of 1.04 g/mL in 1.5 mL by
23
24 211 adding appropriate volume of the $\rho=1.4$ g/mL NaBr solution and centrifuged for
25
26 212 2.5 hours, 4°C, 336000 x g. 300-400 μ l of supernatant were collected and stored at
27
28 213 -20°C. iii) Isolation of HDL: approximately 800 μ l of sediment were transferred to
29
30 214 a new tube, and the density was adjusted to 1.21 g/mL, and taken to a volume of
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32 215 1.5 mL with the $\rho= 1.21$ g/ml NaBr solution. The samples were centrifuged for 3.5
33
34 216 hours, 4°C, 336000 x g, and a supernatant fraction of approximately 400 μ l
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36 217 corresponding to HDL, and the sediment fraction corresponding to the
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38 218 lipoprotein-depleted plasma (LDP), were collected and stored at -20°C.
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220 Electrophoresis, isoelectrofocusing and immunoblotting against wApo A-I

221 25 μ L of each fraction were separated in 4-20% Tris-hepes PAGE LongLife iGels
222 (Nusep, Lawrenceville, GA) gradient gels. Samples were boiled for 3 min in the
223 presence of protein loading buffer containing 1% β -mercaptoethanol, were loaded

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6 224 in pre-cast gels and then run at 100 V for 1 h. Finally, bands were visualized using
7
8 225 the GelCode Blue safe Protein Stain (Thermo Scientific, Rockford, IL).

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10 226 For immunoblotting, after electrophoresis, samples were transferred to
11
12 227 nitrocellulose membranes (Whatman, Maidstone, UK). The woodchuck
13
14 228 apolipoprotein A-I protein was detected with the goat polyclonal antibody against
15
16 229 mApoA-I, 1:200 dilution (Goat polyclonal anti-Apolipoprotein A1 (E-20), Santa
17
18 230 Cruz Biotechnology, Santa Cruz, CA) and antibody against goat IgG, 1:20000
19
20 231 dilution (Anti-goat IgG (whole molecule)- HRP conjugated, Sigma-Aldrich, St.
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22 232 Louis, MO). The membrane was developed with ECL Plus Western Blotting
23
24 233 Detection Reagent (Amersham, Chalfont St. Giles, UK).

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27 234 For isoelectrofocusing, 10 µg of total serum protein concentration was loaded into
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29 235 Ready Gel IEF pH5-8. The lower buffer chamber was filled with 1X IEF Anode
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31 236 buffer and the upper buffer chamber with 1X IEF Cathode buffer. All reagents
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33 237 were from BioRad (Hercules, CA). The gel was run at 4°C, 5 mA and 100V for
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35 238 1hr, then 250V for 1 hr and 500V for 30 min. Samples were transferred to
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37 239 methanol-incubated nitrocellulose membranes at 4°C for 1 hr in 0.7% acetic acid.
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39 240 The woodchuck apolipoprotein A-I was then detected as described above.
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48 242 Sequence analysis

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51 243 The cleavage site of signal peptides was predicted by the SignalP 3.0 program
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53 244 (<http://protfun.net/services/SignalP/>). Multiple alignment and phylogenetic
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55 245 analyses were conducted using MEGA version 4 (Tamura, Dudley, Nei, and
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57 246 Kumar 2007). For protein identification, a coomassie stained gel with purified
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6 247 woodchuck HDLs was used, and the protein was degraded with sequencing grade
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8 248 trypsin. Mass fingerprinting of the digests was done by MALDI-TOF mass
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10 249 spectrometry and data were analyzed with the Mascot search engine
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12 250 (www.matrixscience.com).

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16 17 252 Measurement of SR-BI expression

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20 253 L929 cells and WCH17 were detached using citrate saline and 8×10^5 cells were
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22 254 incubated in 96-well plates for 10 minutes with 1: 200 rabbit anti-SR-BI
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24 255 polyclonal antibody (EP1556Y) (Novus Biologicals Littleton, CO). The negative
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26 256 control cells were incubated without anti-SR-BI. Then, cells were washed in PBS
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28 257 and 5% fetal bovine serum and were incubated for 10 minutes with 1:200 FITC-
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30 258 Conjugated Donkey Anti-Rabbit IgG Antibody (Jackson ImmunoResearch, West
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32 259 Grove, PA). The stained samples were studied by flow cytometry using
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34 260 FACScalibur (Becton Dickinson, Mountain View, CA). Flow cytometry data were
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36 261 analyzed using FlowJo software (Tree Star). For SR-BI Western blot analysis,
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38 262 woodchuck or mice livers were frozen, homogenized in 250 μ l of RIPA buffer
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40 263 with 1% protease inhibitor (protease inhibitor cocktail tablets; Roche, Basel,
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42 264 Switzerland), and centrifuged for 30 min at 9300 x g. The supernatant protein
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44 265 content was measured by Bradford, and 20 μ g of total protein content was
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46 266 immunoblotted as described previously with anti-SR-BI polyclonal (EP1556Y)
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48 267 and secondary donkey anti-rabbit antibody (SouthernBiotech).

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52 53 269 DiI-HDL incorporation

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6 270 900µl of woodchuck HDLs were incubated with 10 µl of Vybrant DiI cell-
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8 271 labeling solution (Invitrogen, Carlsbad, CA) for 30 min at 37°C. Non incorporated
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10 272 DiI was removed by overnight dialysis against PBS at 4 °C.
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12 273 WCH17 and L929 cells were incubated with different concentrations of HDL-DiI
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15 274 for 3 hr at 37°C. Then, cells were washed twice with PBS and analyzed by
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17 275 fluorescence microscopy or FACS. To analyze the inhibitory effect of fucoidan,
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19 276 WCH17 cells were pretreated with fucoidan (Sigma, Madrid, Spain) 2mg/ml for
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22 277 30 min.
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6 279 **Results**
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8 280 Woodchuck apolipoprotein A-I description
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10 281 Apolipoprotein A-I gene is highly conserved among species. Taking advantage of
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12 282 conserved regions, two primers were designed which amplified the 5'-end of
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14 283 woodchuck ApoA-I coding sequence using liver cDNA as template. The rest of
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16 284 the full coding sequence and 3'-UTR was cloned successfully by 3' Rapid
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18 285 Amplification of cDNA ends (RACE) using a woodchuck specific primer.
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20 286 Sequence analysis showed that wApoA-I is highly homologous to ApoA-I gene
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22 287 from other species with more than 75% nucleotide identities when aligned with
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24 288 other mammalian apolipoproteins A-I. The coding sequence extends to 792 bp
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26 289 and the 3'-UTR is 68 bp long (Figure. 1A). Surprisingly, the phylogenetic analysis
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28 290 revealed that the sequence was more related to human and macaque sequences
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30 291 than to other rodent species (Figure. 1B). However, woodchuck ApoA-I is 264
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32 292 aminoacids long, three aminoacids shorter than human and macaque ApoA-I.
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34 293 Woodchuck sequence lacks a characteristic proline of primate sequences at
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36 294 position +29 and two glycines at position +209. These deletions are also observed
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38 295 in mouse ApoA-I. The "pre" segment of apolipoprotein A-I is composed by the
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40 296 signal peptide. The software SignalP3.0 predicted a signal peptide of 18
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42 297 aminoacids. The "pro" segment that is cleaved upon secretion is composed of the
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44 298 consensus mammalian sequence RHFQQ. The predicted mature protein is 240
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46 299 aminoacid long, with MW 27.7 kDa and pI 5.14.
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57 301 Isolation of woodchuck HDL
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6 302 Differential ultracentrifugation in sodium bromide gradient allows the isolation of
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8 303 the different serum lipoproteins of multiple species. This method was applied to
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10 304 woodchuck sera using the following solution densities: for VLDL < 1.006 g/mL,
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12 305 for LDL 1.006-1.04 g/mL and for HDL 1.04-1.21 g/mL. Coomassie staining of
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14 306 the SDS-PAGE gel revealed that the fraction corresponding to HDL contained a
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16 307 major band with a similar MW of woodchuck ApoA-I (Figure 2A). This band was
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18 308 identified by MALDI-TOF-MS as apolipoprotein A-I (Figure 1C and
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20 309 Supplementary Table 1). A 75% of sequence coverage was achieved. To further
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22 310 confirm the identification of this band, a Western blot against apolipoprotein A-I
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24 311 was performed using a commercial antibody raised against a peptide mapping
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26 312 within an internal region of apoA-I of mouse origin. This antibody recognizes
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28 313 ApoA-I from mouse, rat and human, indicating that this antibody was raised
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30 314 against a conserved epitope. As seen in figure 2B, the antibody recognized the
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32 315 expected ApoA-I band in the HDL fraction. Minor HDL contamination was
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34 316 observed in the fractions corresponding to VLDL or LDL, but was absent in the
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36 317 lipoprotein-depleted fraction (Figure 2B). Therefore, HDL from woodchuck sera
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38 318 can be isolated using a gradient ultracentrifugation, and a commercial antibody
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40 319 against woodchuck ApoA-I was identified allowing the detection of this protein
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42 320 by Western blot.
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52 53 322 FACS analysis of woodchuck SR-BI

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55 323 Scavenger receptor class B I (SR-BI) constitutes the main HDL receptor in the
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57 324 organism [Connelly and Williams, 2004; Krieger, 2001; Vishnyakova et al.,
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6 325 2003]. The interaction of HDL and SR-BI is required for reverse cholesterol
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8 326 transport and cholesterol efflux. To analyze the expression of this key molecule in
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10 327 the HDL biology, a commercial antibody was tested against SR-BI that is
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12 328 described to cross-react with SR-BI from mouse, rat and human. The woodchuck
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15 329 hepatocarcinoma cell line WCH17 was used, and as positive control cells
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17 330 expressing SR-BI, the murine fibroblast cell line L929. Cells were incubated with
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19 331 the anti-SR-BI antibody and a secondary fluorescent antibody was used to
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21 332 visualize antibody-cell complexes by flow cytometry. As shown in figure 3A, the
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23 333 L929 cell line expressed high levels of SR-BI. WCH17 also was recognized by
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25 334 the anti-SR-BI antibody but the intensity of expression was lower than in L929
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27 335 (Figure 3B). To test the specificity of this antibody to SR-BI, livers from
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29 336 woodchuck, SR-BI wild type or SR-BI null mice were analyzed by Western blot
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31 337 using this antibody. A specific band was observed in woodchuck or SR-BI^{+/+}
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33 338 mice, while no band could be detected in SR-BI^{-/-} mice, as expected.
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41 DiI-HDL uptake

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43 341 HDLs have been used to target lipophilic drugs loaded inside the lipoproteins to
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45 342 the liver [Feng et al., 2008a; Feng et al., 2008b] or tumor cells [Cao et al., 2004;
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47 343 Lacko et al., 2002; Lou et al., 2005; McConathy et al., 2008; Zhang and Chen,
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49 344 2010] . To test the potential use of HDL particles in the woodchuck model, we
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51 345 labeled woodchuck HDLs with the lipophilic fluorescent dye DiI (1,1'-
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53 346 dioctadecyl-3,3',3'-tetramethylindocarbocyanine perchlorate). After incubation
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57 347 of L929 cells as control, and the woodchuck WCH17 cell line with DiI-HDL,
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6 348 fluorescent cells were visualized by microscopy and the fluorescent intensity was
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8 349 quantified by flow cytometry. As shown in figure 4A and 4D, both cell lines
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10 350 internalized the fluorescent HDL. In correlation with the intensity of SR-BI
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12 351 expression, L929 cells were able to capture more HDLs than the WCH17 cell line
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15 352 (Figure 4B, 4C, 4E and 4F). To analyze whether HDL-DiI uptake was mediated
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17 353 by a scavenger receptor, WCH17 was pretreated with fucoidan. This compound is
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19 354 a non-specific inhibitor of scavenger-type receptors including SR-BI [Barth et al.,
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21 355 2008; Husemann et al., 2001]. Non-treated cells were able to capture DiI whereas
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23 356 cells treated with fucoidan did not. Therefore, the uptake HDL-DiI in WCH-17
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25 357 involves a scavenger receptor. (Supplementary Figure 1).
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32 359 Altered Pattern of Apolipoprotein A-I Isoforms

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34 360 Recently, the pattern of apolipoprotein A-I isoforms in the sera and in the liver
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36 361 has been shown to be altered in chronic hepatitis and in HCC patients [Fernandez-
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38 362 Irigoyen et al., 2005; He et al., 2003; Steel et al., 2003; Yang et al., 2010]. Several
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40 363 patterns have been described depending on the model or the gender, and a
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42 364 proteomic analysis of woodchuck sample could be very informative. To analyze
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44 365 whether these techniques could be feasible, woodchuck ApoA-I isoforms from
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46 366 sera were resolved by isoelectrofocusing and visualized by Western blot using the
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48 367 commercial antibody described above. Blood from two healthy animals, two
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50 368 infected chronically and two woodchucks with HCC were analyzed. As shown in
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52 369 figure 5, two different isoforms were identified. One of the isoforms
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54 370 corresponding to the upper band, was reduced clearly in sera from one out of two
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6 371 woodchucks with chronic infection and in two out of two woodchucks with HCC.

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8 372 Thus, ApoA-I isoelectrofocusing may represent a new method for HCC screening

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10 373 in woodchucks. Further studies are required to validate this technique.

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For Peer Review

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6 375 **Discussion**
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8 376 Apolipoprotein A-I is the main protein component of the high density
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10 377 lipoproteins. Its biology has been studied extensively due to its crucial function in
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12 378 cholesterol reverse transport and in the pathogenesis of atherosclerosis. In
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15 379 addition, the highly efficacious mechanism of cholesterol transfer of HDLs
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17 380 constitutes an ideal targeting system to deliver therapeutic molecules to the liver.
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19 381 HDLs have also been shown to deliver cholesterol-conjugated siRNAs to the liver
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22 382 and silence gene expression *in vivo* in mice. The evaluation of HDLs as a vehicle
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24 383 for targeting the liver in animal models which are relevant clinically would be of
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26
27 384 great interest. Woodchucks infected chronically with WHV represent the best
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29 385 animal model for the development of new antiviral and antitumor strategies.
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31 386 The evaluation of the safety and efficacy of these applications in the woodchuck
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33 387 model requires the characterization of woodchuck ApoA-I and the development of
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35 388 tools to work with woodchuck ApoA-I-based drug delivery systems. In the
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37 389 present study, the full open reading frame and the 3' untranslated region of
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39 390 woodchuck ApoA-I (wApoA-I) were cloned. The identity of long fragments
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41 391 among different species allowed us to find primers which amplified half of the
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43
44 392 full sequence. With this partial woodchuck sequence, it was possible to design a
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46 393 woodchuck specific primer that amplified the full sequence by the 3'-RACE
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49 394 technique. The new cloned sequence shares characteristics of primate and rodent
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51 395 sequences. It is highly homologous to human and macaque but presents three
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54 396 deletions, resembling the rodent sequences.
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6 397 The good conservation of several peptides among different ApoA-Is facilitated the
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8 398 finding of a commercial antibody that cross-reacted with woodchuck ApoA-I.
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10 399 This antibody represents an important tool, since it permits the detection of the
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12 400 wApoA-I by immunoblot. In this study, the wApoA-I immunoblot was used to
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14 401 evaluate the HDL purification by ultracentrifugation and to analyze the different
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16 402 serum isoforms.

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19 403 The ultracentrifugal procedure described in this study provides an easy technique
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21 404 to obtain large amounts of woodchuck HDL from peripheral blood. More
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23 405 importantly, it was shown that purified woodchuck HDL particles can be used to
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25 406 deliver hydrophobic molecules to a woodchuck hepatocarcinoma cell line. Thus,
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27 407 experiments designed to target antiviral drugs or iRNA at the liver with HDLs in
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29 408 the woodchuck model are now viable.

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32 409 Chronic infection with HBV is a significant risk factor for the development of
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34 410 HCC. Monitoring of disease progression and prediction of the outcome depends
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36 411 currently on a combination of physical and serological assessments [Lisi et al.,
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38 412 2003]. Unfortunately, these methods often lack the sensitivity for detecting HCC
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40 413 at an early stage when therapeutic options will be the most effective. Several
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42 414 investigators have reported that significant decreases in ApoA-I levels were
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44 415 observed in patients with liver diseases. Recently, variations on the amount of
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46 416 ApoA-I isoforms in serum have been described in patients infected chronically
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48 417 with HBV or HCC [Fernandez-Irigoyen et al., 2005; He et al., 2003; Steel et al.,
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50 418 2003]. The oxidative status of the liver, induced by different physiopathological
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52 419 conditions, alters the post-transcriptional modification of the nascent ApoA-I
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6 420 [Fernandez-Irigoyen et al., 2005]. For this reason ApoA-I is also a matter of great
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8 421 interest for the diagnosis and treatment of chronic hepatitis and HCC. ApoA-I is
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10 422 therefore an abundant sensor of liver function in serum.

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12
13 423 In the study reported above, high resolution isoelectrofocusing and Western blot
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15 424 were used to analyze the number of ApoA-I isoforms present in the serum of
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17 425 woodchucks infected chronically with WHV with or without HCC. It was shown
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19 426 that there is a significant reduction in the ApoA-I isoform with the lowest pI, in
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21 427 the serum of a woodchucks with chronic hepatitis and in two animals which
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23 428 developed HCC. The experiment performed in the present study exemplifies this
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25 429 application, but further work is required to validate this technique. In addition, the
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27 430 identification of the precise post-translational modifications that are altered may
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29 431 improve our knowledge of liver pathogenesis.

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32 432 Therefore, the characterization of wApoA-I paves the way to numerous studies for
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34 433 the treatment and understanding of chronic viral hepatitis and hepatocarcinoma,
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36 434 expanding the possibilities of the use of the woodchuck model.
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7
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26 602 **Figure legends**

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29 603 **Fig. 1** A) Nucleotide sequence of the woodchuck apolipoprotein A-I and the
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31 604 putative amino acid sequence of the encoded protein. Putative “pre” segment and
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33 605 “pro” segment are underlined with a solid line and dashed line respectively. B)
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35 606 Phylogenetic (Neighbor-joining) analysis of coding sequences of ApoA-I using
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37 607 the MEGA4.0 program. Accession numbers of sequences used are human (*Homo*
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39 608 *sapiens*), NM_000039.1; macaque (*Macaca fascicularis*), XM_001090774.1;
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41 609 rabbit (*Oryctolagus cuniculus*), X15908.1; horse (*Equus caballus*),
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43 610 XM_001502469.1; bull (*Bos taurus*), NM_174242.3; pig (*Sus scrofa*),
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45 611 NM_214398.1; tupaia (*Tupaia glis*), AF005638.1; rat (*Rattus norvegicus*),
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47 612 NM_012738.1; mouse (*Mus musculus*), X64262.1; platypus (*Ornithorhynchus*
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49 613 *anatinus*), XM_001517296.1. C) Sequence of the predicted mature woodchuck
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51 614 apolipoprotein A-I. Matched peptides used to identify the protein with mass
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53 615 spectrometry data are shown in bold.

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8 617 **Fig. 2** A) Coomassie staining of purified woodchuck HDLs separated on 4-20%
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10 618 SDS-PAGE. B) Western blot of the different fractions obtained by differential
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12 619 ultracentrifugation in sodium bromide gradient using a commercial anti-ApoA-I
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15 620 (E-20) mAb. Secondary antibody was anti-goat IgG-HRP and detection was by
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17 621 chemoluminescence. VLDL: Very Low Density Lipoprotein; LDL: Low Density
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19 622 Lipoprotein; HDL: High Density Lipoprotein; LPS: Lipoprotein Depleted Serum.
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24 624 **Fig. 3** Flow cytometry analysis of SR-BI expression on A) L929, control cells and
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26 625 on B) WCH17, woodchuck hepatocarcinoma cell line. Cells were labeled with
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28 626 anti-SR-BI polyclonal antibody (EP1556Y) and a secondary FITC Anti-Rabbit
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30 627 IgG Antibody. C) SR-BI western blot analysis in proteins extracted from livers
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32 628 obtained from woodchuck, SR-BI wild type or knock out mice as an antibody
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34 629 specificity control.
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41 631 **Fig. 4** DiI-HDL uptake. Control cells L929 or woodchuck WCH17 hepatoma cell
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43 632 line, were incubated with DiI-HDL and visualized in fluorescent microscopy (A
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45 633 and D), or incorporated DiI-HDL at different concentrations that were quantified
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47 634 by flow cytometry: histograms showing percentage of DiI⁺ cells (B and E) and
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49 635 mean fluorescence intensity (C and F).
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55 637 **Fig. 5** Isoelectrofocusing and western blotting against woodchuck ApoA-I. Serum
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57 638 samples from healthy or chronically infected, or woodchucks with HCC were
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6 639 analyzed by isoelectrofocusing and Western blotting using a commercial anti-
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8 640 ApoA-I (E-20) mAb. The secondary antibody was anti-goat IgG-HRP and
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10 641 detection was performed by chemoluminescence. Arrows indicate the three ApoA-
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12 642 I isoforms that can be detected.

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