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SHORT COMMUNICATION

Infection of primary hepatocytes with adenoviral vectors alters biliary lipid metabolism

Yuri Rueda · Itsaso Garcia-Arcos · Patricia Aspichueta · Begoña Ochoa · Lourdes Palacios · Olatz Fresnedo

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Abstract In the context of a study of the involvement of SND1 (also known as coactivator p100) in biliary lipid secretion by primary rat hepatocytes, first-generation adenoviral vectors were used to promote the overexpression and underexpression of the protein SND1. Although differential expression of SND1 did not result in significant changes in the processes studied, some effects of the adenoviral infection itself were observed. In particular, infected hepatocytes showed a higher intracellular taurocholate accumulation capacity. Additionally, small heterodimer partner (SHP) and farnesoid X receptor (FXR), which are nuclear receptors essential for the regulation of bile salt metabolism and transport, were underregulated at the mRNA level. Our results suggest that adenoviral vectors could be altering some important control mechanism and indicate that adenoviral vectors should be used with caution as transfection vectors for hepatocytes when biliary lipid metabolism is to be studied.

Y. Rueda · I. Garcia-Arcos · P. Aspichueta · B. Ochoa · L. Palacios · O. Fresnedo (✉)
Department of Physiology, Faculty of Medicine and Dentistry of the University of the Basque Country UPV/EHU, B° Sarriena s/n 48940 Leioa, Bizkaia, Spain
e-mail: olatz.fresnedo@ehu.es

Present Address:
I. Garcia-Arcos
Division of Preventive Medicine and Nutrition, College of Physicians and Surgeons, Columbia University School of Medicine, New York, USA

Present Address:
L. Palacios
Progenika Biopharma S.A., Technologic Park of Bizkaia, Derio, Spain

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Findings

Recombinant adenoviruses have been extensively used in research applications to induce transient transgene expression due to their ability to infect quiescent cells. Even though helper-dependent vectors which minimize the host response and prolong the transgene expression when used *in vivo* [1] have been developed, first-generation vectors are useful when employed in cell cultures, where there is no immune response against the virus or the infected cells. First-generation adenoviral vectors are derived by deletion of the E1 region of the viral genome, which ablates the replicative ability of the virus, enabling insertion of an expression cassette for the gene of interest.

In a previous work [2] we constructed first-generation adenoviral vectors able to induce differential expression of staphylococcal nuclease and tudor domain containing 1 (SND1, also known as p100 coactivator or SND p102), a protein broadly conserved in eukaryotes [3]. SND1 has been reported to participate in rather dissimilar processes: it was initially described as a transcriptional coactivator [4] but has also been identified as a component of the spliceosome [5] and of the RNA-induced silencing complex [6], and it has been reported to be related to lipid metabolism [7].

We produced a sense adenovirus (AdSS) carrying the cDNA sequence of the rat SND1 gene and an antisense adenovirus (AdAS) carrying an antisense fragment of the same sequence; we demonstrated that they induced overexpression and underexpression, respectively, of the protein in primary rat hepatocytes. Using those vectors we

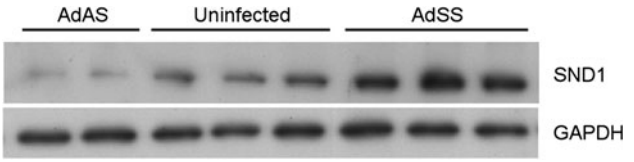
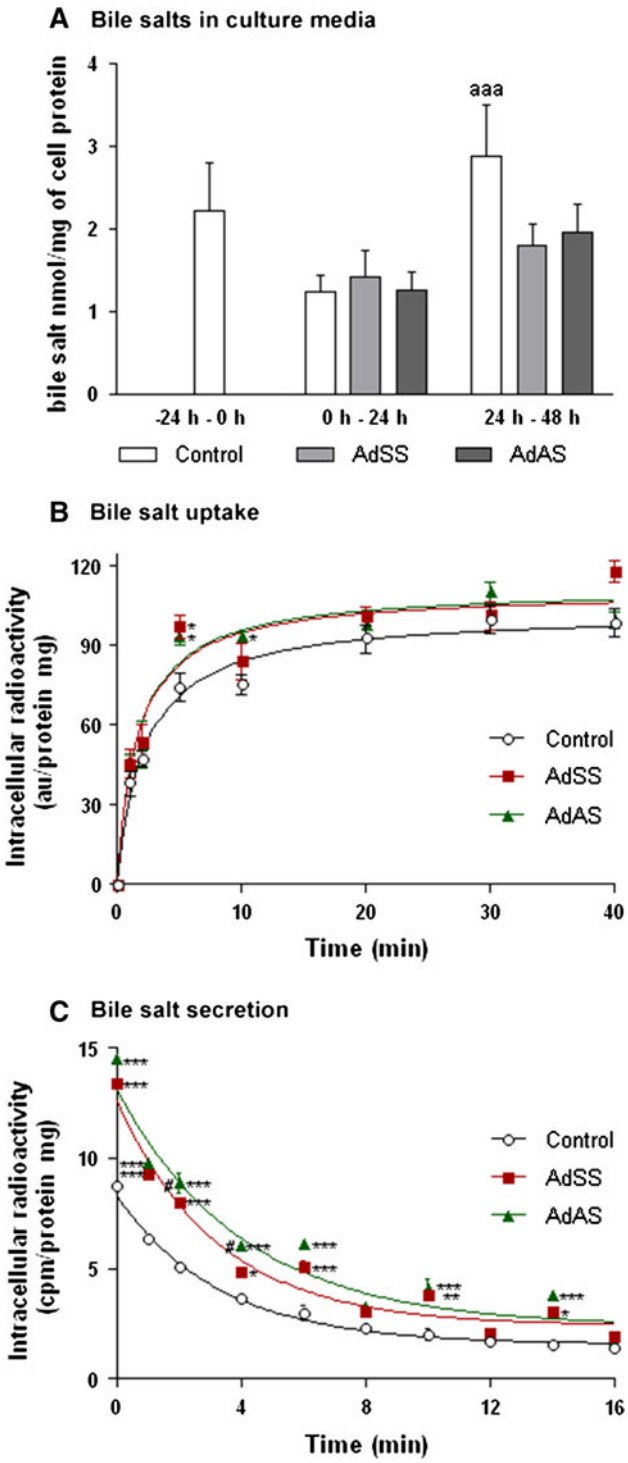


Fig. 1 Effect of adenoviral vectors on SND1 expression. Cells infected with sense (AdSS) or antisense (AdAS) adenoviral vectors or uninfected control cells were collected and lysed in a standard lysis buffer and denatured. 8 % SDS-PAGE was performed with 10 μg of total protein. Western blotting was performed as described elsewhere for SND1 [2]. SDS-PAGE and protein electrotransfer was done in the same way for GAPDH but immunodetection was performed with monoclonal mouse anti-rat GAPDH IgG from AbCam and HRP-linked anti-mouse IgG antibody from Cell Signaling Technology as primary and secondary antibodies, respectively, following manufacturers' instructions. Optic densitometry measurement with Quantity One software was used for quantification

observed that the SND1 rat homologue had an effect on the phospholipid (PL) content of secreted lipoproteins [2]. In that context, our next objective was to verify whether SND1 could have any role in biliary lipid secretion, which is the other main lipid-secreting process carried out by hepatocytes. For that purpose we applied the same methodology used previously. First, rat hepatocytes were incubated with 10 m.o.i. of one of the two adenoviruses for 90 min, and cells were harvested 24 h later. Whole-cell lysate protein was analysed by standard Western blotting and AdSS- and AdAS-infected cells presented an average SND1 expression of 312 and 23 % compared to uninfected controls, respectively (Fig. 1). It should be noted that such viral doses do not trigger cytotoxicity or apoptosis in hepatocytes [2].

Liver parenchymal bile secretion occurs by sequential transport of biliary lipids. According to the widely accepted Small model [8], bile salts (BS) are initially secreted by Bsep (Bile salt export pump, AbcB11), which promotes PL secretion by multidrug resistance protein 2 (Mdr2, AbcB4); BS and PL form mixed micelles, protecting the bile duct epithelium from the detergent action of BS. Finally, BS/PL micelles solubilise free cholesterol transported, in turn, by a heterodimer composed of AbcG5 and AbcG8; secretion of PL has been proven to be a requirement for subsequent biliary cholesterol secretion [9].

To determine the involvement of SND1 in this process we analysed the influence of its differential expression on the levels of BS in culture media of primary rat hepatocytes, cultured as described elsewhere [10]. We measured BS accumulated during the first 24 h of culture and in two 24-h periods after infection (Fig. 2a). The BS were extracted from culture media by reverse phase chromatography by the method described elsewhere [11], although greater volumes were needed in the washes of the columns in order to completely remove the phenol red present in the



media; BS quantification was performed following a fluorometric method based on the sequential action of the enzymes 3α-hydroxysteroid dehydrogenase and diaphorase, described in the same paper.

While SND1 differential expression did not seem to have any effect on accumulated BS levels, cells infected with either of the adenoviruses both seemed to respond and

◀ **Fig. 2** Adenoviral infection of primary hepatocytes in culture alters bile salt cellular utilization. **a** Bile salts accumulated over 24-h periods in culture media. Infection was performed with 10 m.o.i. at 0 h and culture media were collected over 24-h periods, including the period prior to infection. Data represent the mean ± SE of nine samples from three different preparations. Bonferroni's test: $^{aaa}P < 0.001$ compared with the 0–24 h period. **b** Time-course analysis of bile salt uptake. 24 h after infection cells were incubated with a [^{14}C]taurocholate-containing medium (50 μM, 3 Ci mol^{-1}) and intracellular radioactivity was measured at eight time points. Results from each experiment were normalized internally prior to being combined and are presented in arbitrary units (au); data represent the mean ± SE of nine samples from three different preparations. Two-way ANOVA analysis: $P < 0.01$ due to the treatments. Bonferroni's test: $^{*}P < 0.05$ compared with control cells. **c** Time-course analysis of bile salt secretion. 24 h after infection cells were incubated with the same medium as in **b** for 30 min, after which it was replaced with fresh medium and intracellular radioactivity was measured at eight time points. Data represent the mean ± SE of nine samples from three different preparations. Two-way ANOVA analysis: $P < 0.001$ due to the treatments. Bonferroni's test: $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ compared with control cells; $^{\#}P < 0.05$ compared with AdSS-infected cells

in a similar way: namely, infected cultures showed lower BS accumulation compared with control cell media in the second 24-h period after infection.

In vivo, hepatocytes not only synthesise and secrete bile components into bile canaliculi through the canalicular membrane, but also are in contact with sinusoidal blood and transport various compounds across the basolateral membrane, including BS returning from the intestine. That is why the transport of bile components across liver parenchyma is said to be vectorial. Specific transporters are needed in the basolateral membrane to mediate the uptake of those compounds.

When cultured, hepatocytes lose their polarity and the differentiated plasma membrane domains are in contact with the same culture medium. Therefore, the extracellular accumulation of BS is the result of a balance between their secretion and uptake. In order to monitor BS uptake, a time-course analysis of taurocholate (TC; 50 mM, 3 Ci mol^{-1}) accumulation was performed 24 h after infection. Maximum intracellular BS levels were achieved in less than 30 min (Fig. 2b). Although SND1 differential expression did not modify TC uptake, an effect caused by adenoviral vectors was observed: intracellular BS levels were higher through the whole studied period in infected cells. Data sets from AdSS and AdAS were merged (data not shown), and uninfected and infected cells were compared; statistical analysis showed that the difference was significant (ANOVA, $P < 0.01$), especially at the 5 min time point (Bonferroni's test, $P < 0.01$).

BS secretion measurement was made after a 30-min pulse using the radioactive TC-containing medium from the uptake assay. After the pulse, TC-free medium was added and the intracellular radioactivity decay was measured (Fig. 2c). ANOVA analysis found a significant difference ($P < 0.001$) between control and infected cells: from time 0 of the chase period to the steady state (reached at 12–16 min) infected cells showed higher levels of intracellular radioactivity.

Data adjusted to one phase decay equations from which rate constants could be calculated: 0.302 min^{-1} in control cells, 0.310 min^{-1} in AdSS-infected cells and 0.248 min^{-1} in AdAS-infected cells. So, even if the initial slope of the curves seemed to indicate that secretion rates in infected cells were higher, the rate constants were similar.

To complete the analysis of bile lipid secretion we assessed the influence of SND1 differential expression and adenoviral infection itself on secreted non-lipoprotein PL levels. Culture media adjusted to a density of 1.24 g ml^{-1} were subjected to ultracentrifugation as described elsewhere [2] to isolate the non-lipoprotein fraction. Lipids extracted by the Folch method [12] were separated using thin layer chromatography and quantified [13]. No significant differences were found in the PL levels (Fig. 3).

The mRNA levels of several different proteins involved in BS transport and its regulation were measured using quantitative reverse transcription PCR (Fig. 4a); expression data from three genes were used as normalizers (GAPDH, A cyclophilin and 18S rRNA) with the software of geNorm [14]. Once again, no differences were observed between AdSS and AdAS infected cells, which rules out any possible role of SND1 in the regulation of their transcription. To assess the effect of infection, data from all the infected cells were combined and compared with those from uninfected cells (Fig. 4b). It was then observed that genes involved in BS synthesis and export, for example

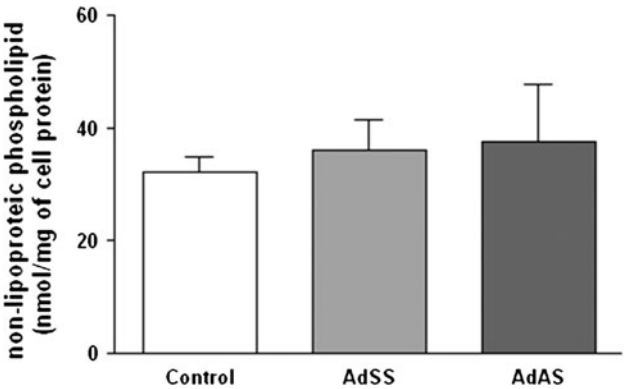


Fig. 3 Non-lipoprotein phospholipids secreted by hepatocytes infected with adenoviral vectors. 24 h after infection culture media were collected, lipoproteins were separated by ultracentrifugation and lipids from the isolated non-lipoprotein fraction were extracted and separated by thin layer chromatography; phospholipids were quantified by optical densitometry. Data represent the mean ± SE of nine samples from three different preparations

A

	Control	AdSS	AdAS	ANOVA
Bsep	1.00 ± 0.06	0.67 ± 0.18	0.46 ± 0.23**	##
Cyp7a1	1.00 ± 0.08	0.55 ± 0.15	0.62 ± 0.20	
FXR	1.00 ± 0.10	0.64 ± 0.09	0.67 ± 0.08	#
LRH-1	1.00 ± 0.26	0.46 ± 0.23	0.37 ± 0.19	
SHP	1.00 ± 0.29	0.43 ± 0.14	0.35 ± 0.01	

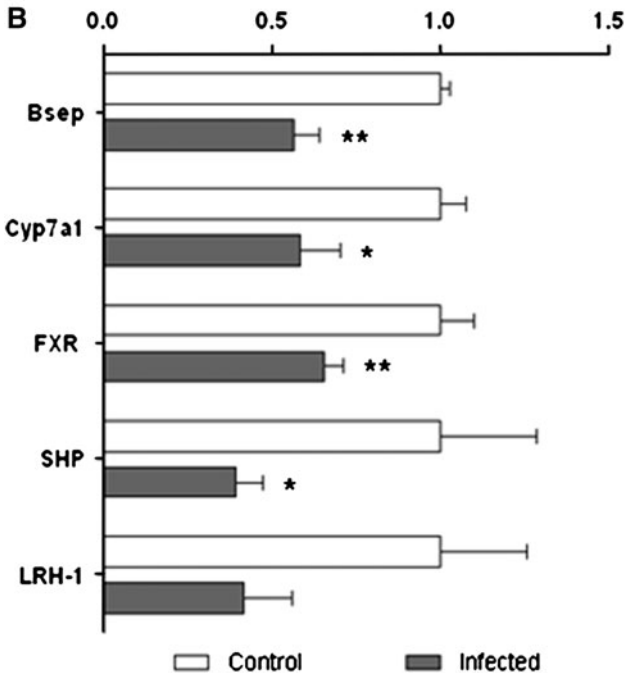


Fig. 4 Effect of adenoviral infection on the mRNA expression levels of genes relevant in hepatocyte bile salt metabolism. Total RNA was extracted from cells 24 h after infection and reverse transcribed, and quantitative PCRs were performed. Results were normalized with data from three control genes (GAPDH, A cyclophilin and 18S rRNA), using geNorm. GenBank accession numbers of sequences used for designing primers for PCRs and forward (f) and reverse (r) primers: Bsep (NM_031760), f: gccattgtgcgagatcctaaa, r: tgcaggtccgaccctctct; Cyp 7a1 (NM_012942), f: attgccgtgttggtgagctg, r: gaatcaacccgttctccaaagg; FXR (U18374), f: gtgacaaagaagccgcgaat, r: gcaggtgagcgcgttgtaat; SHP (D86580), f: ctcggtttgcatacagtgtttga, r: gcatattggcctggaggtttt; LRH-1 (NM_021742), f: tggtgagactccgttccctt, r: tggacgccttccaccag; GAPDH (NM_017008), f: gtgccagcctcgtctgatagac. r: aaggcagccctggtaaccag; A cyclophilin (NM_017101), f: ccaagactgagtggctggatg, r: gctccatggcttccacaatg; 18S rRNA (X01117), f: ttcgaacgtctgccctatcaac, r: gaaccctgattccccgtcac. **a** mRNA levels in AdSS- and AdAS-infected cultures, referred to control cells. Data represent the mean ± SE of nine samples from three different preparations. ANOVA analysis: $^{\#}P < 0.05$, $^{\#\#}P < 0.01$. Bonferroni's test: $^{**}P < 0.01$ compared with control cells. **b** Data from infected cells were merged and compared with those from control cells to assess the effect of infection itself. Student's *t* test: $^{*}P < 0.05$ and $^{**}P < 0.01$ compared with control cells

Bsep or cholesterol 7 α-hydroxylase (Cyp7a1), the rate-limiting enzyme in the main synthesis pathway of BS, were underexpressed in infected cells.

Most interestingly, farnesoid X receptor (FXR, NR1H4), which is the main intracellular sensor for BS, was also underexpressed. FXR is a type II nuclear receptor, and physiological BS are its agonists; when activated FXR induces the expression of several proteins involved in BS synthesis and transport, some of its final effects being the inhibition of BS uptake from blood [15], the inhibition of BS synthesis [16] and the induction of BS canalicular secretion [17, 18], thereby keeping the intracellular BS concentration at appropriate levels. We observed that FXR expression was reduced by 35 % in infected cells; given FXR is the main control mechanism of BS levels, should its expression be also reduced at protein level that would possibly explain the higher BS levels observed in infected cells.

Other evidence suggests that the FXR function may be reduced in adenovirus-infected cells. Small heterodimer partner (SHP, NR0B2) is a nuclear receptor family member; its expression is induced by FXR and it is responsible for the inhibitory effects of FXR by inhibiting the function of several nuclear receptors and transcription factors [19]. We found that SHP mRNA levels were reduced by 60 % in parallel with FXR underexpression in infected hepatocytes. Surprisingly, SHP underexpression did not correlate with an overexpression of Cyp7a1. SHP is known to repress Cyp7a1 expression by inhibiting the transcriptional activity of liver receptor homolog-1 (LRH, NR5A2); however, Cyp7a1 expression is regulated by many other factors such as cellular cholesterol levels or several nuclear factors (reviewed in [20]), which might be causing Cyp7a1 underexpression in our experimental model in an SHP-independent manner.

In summary, adenoviral infection itself seems to enhance intracellular BS levels. BS are synthesised by hepatocytes and secreted into bile, which drains into the duodenum where BS aid in lipid digestion thanks to their detergent properties. However, being detergents, BS are potentially harmful to cells and so their intracellular concentration and their transport must be kept under tight control. According to the data presented in this paper, adenoviral infection enhances intracellular BS in hepatocytes when they have to deal with increased extracellular levels, which could be due to a reduced sensitivity of the cell's control mechanisms, as might be caused by a reduction in FXR expression. Our findings suggest that adenoviral vector-based gene delivery systems should be used with caution in hepatocytes, especially when bile physiology is to be studied.

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Conflict of interest The authors declare that they have no conflict of interest.

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