Biliary exosomes influence cholangiocyte regulatory mechanisms and proliferation through interaction with primary cilia

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Biliary exosomes influence cholangiocyte regulatory mechanisms and proliferation through interaction with primary cilia. Am J Physiol Gastrointest Liver Physiol 299: G990–G999, 2010. First published July 15, 2010; doi:10.1152/ajpgi.00093.2010.—Exosomes are small extracellular vesicles that are thought to participate in intercellular communication. Recent work from our laboratory suggests that, in normal and cystic liver, exosome-like vesicles accumulate in the lumen of intrahepatic bile ducts, presumably interacting with cholangiocyte cilia. However, direct evidence for exosome-ciliary interaction is limited and the physiological relevance of such interaction remains unknown. Thus, in this study, we tested the hypothesis that biliary exosomes are involved in intercellular communication by interacting with cholangiocyte cilia and inducing intracellular signaling and functional responses. Exosomes were isolated from rat bile by differential ultracentrifugation and characterized by scanning, transmission, and immunoelectron microscopy. The exosome-ciliary interaction and its effects on ERK1/2 signaling, expression of the microRNA, miR-15A, and cholangiocyte proliferation were studied on ciliated and deciliated normal rat cholangiocytes. Our results show that bile contains vesicles identified as exosomes by their size, characteristic “saucer-shaped” morphology, and specific markers, CD63 and Tsg101. When NRCs were exposed to isolated biliary exosomes, the exosomes attached to cilia, inducing a decrease of the phosphorylated-to-total ERK1/2 ratio, an increase of miR-15A expression, and a decrease of cholangiocyte proliferation. All these effects of biliary exosomes were abolished by the pharmacological removal of cholangiocyte cilia. Our findings suggest that bile contains exosomes functioning as signaling nanovesicles and influencing intracellular regulatory mechanisms and cholangiocyte proliferation through interaction with primary cilia.

Both types of liver epithelia (i.e., hepatocytes and cholangiocytes) are exosome-releasing cells in culture (5, 9, 21, 45). Exosome-like vesicles were also found in a population of “microparticles” isolated from bile of bile-duct ligated rats (45), suggesting the existence of biliary exosomes in vivo.

Although the functional significance of exosomes in general remains largely obscure, these vesicles have been implicated in a number of physiological and pathological processes (15, 34, 35, 39). For example, exosomes released by platelets are involved in regulation of coagulation events (8). Exosomes derived from professional antigen-presenting cells stimulate T cell activation (33). Exosomes released by intestinal epithelia activate the mucosal immune system (19). Exosomes also participate in pathological processes by spreading pathogens such as prions from one cell to the other (16) or transferring oncogenic receptors from tumor cells (2). Thus recent studies suggest that the primary function of exosomes is likely associated with intercellular communication (12, 27, 35).

The mechanisms through which exosomes participate in intercellular communication remain unclear. It was hypothesized that exosomes can interact with target cells via unknown receptors and then can be endocytosed, or they can directly fuse with the cell membrane delivering the exosomal content to the recipient cell (27, 34, 35). Our observations on clustering of exosome-like vesicles around cholangiocyte cilia in the lumen of intrahepatic bile ducts (46) and on interaction of isolated urinary exosomes with renal and cholangiocyte primary cilia in vitro (13) strongly suggest that the exosome-ciliary interaction might be essential for intercellular communication.

Cholangiocyte primary cilia are sensory organelles detecting and transmitting extracellular stimuli (mechano-, chemo-, and osmo-) into intracellular signaling responses (11, 22–24). Hypothetically, chemostimuli can be delivered to cilia not only by single small or large signaling molecules, but also by signaling vesicles such as exosomes. In fact, it has been recently shown that “signaling vesicles” interact with primary cilia in the mouse embryonic node acting as paracrine messengers and inducing an intracellular Ca2+ signaling response (37).

To address whether biliary exosomes participate in intercellular communication by interacting with cholangiocyte cilia, we assessed the effects of exosomes isolated from bile of normal rats on ERK signaling, microRNA (miRNA) expression, and cholangiocyte proliferation (i.e., processes known to be associated with regulatory functions of exosomes in other cell types) in ciliated and deciliated rat cholangiocytes in culture. Our data show that biliary exosomes influence intracellular signaling, miRNA expression, and cholangiocyte proliferation.
proliferation through interaction with cholangiocyte cilia. Taken together, these data support the notion that interaction of biliary exosomes with cholangiocyte cilia represents a novel mechanism of intercellular communication in the liver.

MATERIALS AND METHODS

All chemicals were of highest purity commercially available and were purchased from Sigma Chemical (St. Louis, MO) unless otherwise indicated.

Animals and models. Male Sprague-Dawley rats (Harlan Sprague Dawley, Indianapolis, IN) (n = 18), PCK rats (n = 5) (26), wild-type mice (n = 5), Pkd1+/−/Pkd2+/−/ mice (n = 5) (46), and Pkd2−/− mice (n = 5) (36) were housed in a temperature-controlled room (22°C) with 12-h light-dark cycles and maintained on a standard diet with free access to water. All experimental procedures were approved by the Mayo Clinic Institutional Animal Care and Use Committee. Animals were anesthetized with pentobarbital (50 mg/kg body wt intraperitoneally) for in vivo procedures. For functional studies, cholangiocytes were isolated from rat liver and cultured for several passages [abbreviated NRCs (normal rat cholangiocytes)] as we previously described (31, 41). To address whether cilia are involved in the exosome-induced cholangiocyte functional responses, NRCs were treated with chloral hydrate (4 mM) for 24 h. Chloral hydrate deciliates cells presumably by destabilizing the junction between the ciliary axoneme and the basal body but does not cause other structural and/or functional cell alterations. This “deciliation” technique was previously validated by us for both freshly isolated and cultured rat and mouse cholangiocytes (11, 24).

Isolation of biliary exosomes. Bile was collected for 4 h from the cannulated common bile duct of normal rats (n = 18) into Eppendorf tubes on ice containing Complete protease inhibitor cocktail (Hoffmann-La Roche, Nutley, NJ) to prevent protein degradation. Biliary exosomes were isolated and purified by differential ultracentrifugation of collected bile by using a modified protocol that we previously employed for isolation of exosomes from human urine (13). According to a standard protocol for isolation of exosomes from viscous bodily fluids (38), bile was diluted with an equal volume of phosphate-buffered saline (PBS) and centrifuged at 2,000 g for 30 min at 4°C to remove cellular debris. Supernatant was carefully centrifuged to 38-nl polycarbonate thick wall ultracentrifuge tubes (Beckman 355631) without pellet contamination and centrifuged in a 70 Ti rotor at 15,000 g for 30 min at 4°C. To eliminate large vesicles and aggregates, supernatant was filtered through a 0.22-μm filter (Millipore GF filter unit, Millipore, Billerica, MA) into fresh ultracentrifuge tubes and centrifuged in a 70 Ti rotor at 110,000 g for 2 h at 4°C. The pellet was resuspended in 1 ml of PBS containing Complete and centrifuged in a 70 Ti rotor at 110,000 g for 90 min at 4°C. Although this protocol provides reasonably pure exosomes (38), we refer this material to as “crude exosomes.” The crude exosomes were resuspended in 50–100 μl of PBS and further fractionated and purified. They were layered on top of a 5–30% sucrose gradient generated by a BioComp Gradient Master Station (BioComp Instruments, Fredericton, NB, Canada) with D2O as the solvent. The gradient was centrifuged in a Sorvall TH-641 rotor at 200,000 g for 24 h at 4°C, and then 12 6-mm length fractions were harvested from the 12-ml (Sorvall 06752) tube by use of a BioComp Gradient Master station. Harvested gradient fractions had refractive indexes from 1.350 ± 0.0003 to 1.3760 ± 0.0017 (n = 5) representing a sucrose density of 1.12–1.23 g/ml in D2O. Since exosomes float at a sucrose density of 1.10–1.21 g/ml (27), fractions with a sucrose density of 1.12–1.21 g/ml were considered as fractions containing purified biliary exosomes. These fractions were diluted five-fold in PBS, combined, and centrifuged in a 70 Ti rotor at 110,000 g for 90 min at 4°C to recover purified biliary exosomes. Exosomes were resuspended in 50–100 μl of PBS, characterized by transmission (TEM) and scanning (SEM) electron microscopy, and used for functional studies or stored at −80°C for further experiments.

TEM and SEM of liver tissue. Rat and mouse livers (n = 3 animals from each group) were perfused through the portal vein with 4% paraformaldehyde-2% glutaraldehyde in phosphate buffer (pH 7.4). Then the liver was removed and cut into small pieces (2–3 mm3), which were fixed in 4% paraformaldehyde-2% glutaraldehyde for 2 h at room temperature or at 4°C overnight, then postfixed for 1 h in 1% osmium tetroxide. For TEM, the samples were dehydrated, embedded in Spurr’s resin, sectioned at 80 nm, and examined with a JEOL 1400 electron microscope (JEOL USA, Peabody, MA). SEM samples were dehydrated, dried in a critical point drying device, mounted, sputter coated, and examined with a Hitachi S-4700 microscope (Hitachi, Pleasanton, CA).

Immunoelectron microscopy of liver tissue. Rat and mouse livers (n = 3 animals from each group) were fixed in 4% paraformaldehyde-0.2% glutaraldehyde for 1 h at room temperature or at 4°C overnight, transferred in 20 mmol/l glycine-PBS for 15 min, permeabilized with 0.05% Triton X-100 in 0.1 M phosphate buffer for 30 min, and blocked with 10% fetal calf serum (FCS)-PBS. Then the liver tissues were incubated for 2 h at room temperature or overnight at 4°C with antibodies to CD63 (sc-51662, Santa Cruz Biotechnology, Santa Cruz, CA) and polycystin-1 (PC-1) [7e12 antibody (44)] diluted with blocking solution 1:20, then a secondary goat anti-mouse conjugated antibody with ultra small gold (Electron Microscopy Sciences) was applied (1:100 dilution with blocking solution) for 1 h. Samples were postfixed with 2.5% glutaraldehyde in phosphate buffer for 2 h, then enhanced with silver enhancement mixture (R-Gent SE-EM) for 30 min and osmicated with 1% osmium tetroxide for 30 min. In corresponding controls, the incubation of samples with primary antibodies was omitted.

Negative staining TEM of liver tissue. The isolated exosomes were resuspended in 2.5% glutaraldehyde-0.1 M phosphate buffer and fixed overnight at 4°C. The samples were then placed on Formvar-carbon-coated grid and air dried for 1 h. After being rinsed five times with 0.1 M phosphate buffer and distilled water, the grids were contrasted and embedded with a mixture of 4% uranyl acetate and 2% methylcellulose (1:9 ratio). The grids were air dried and observed with a JEOL 1400 electron microscope (JEOL USA).

Negative staining immunogold TEM of liver tissue. Exosomes isolated from rat bile were fixed in 4% paraformaldehyde-0.1 M phosphate buffer for 1 h or overnight at 4°C. The samples were then placed on Formvar-carbon-coated grid and air dried for 1 h. After saturation of free aldehyde group by 2% FCS-PBS containing 20 mmol/l glycine for 10 min and blocked with 10% FCS-PBS for 20 min, the grids were incubated overnight at 4°C with antibodies to CD63 (sc-51662, Santa Cruz Biotechnology) and Tsg101 (ab83, AbCam, Cambridge, MA) diluted with blocking solution 1:20, then incubated with rabbit anti-mouse IgG diluted with blocking solution 1:1,500 for 1 h. In corresponding controls, the incubation of samples with primary antibodies was omitted. Samples were labeled with protein A-10-nm gold for 1 h. The grids were contrasted and embedded with a mixture of 4% uranyl acetate and 2% methylcellulose (1:9 ratio). The grids were air dried and observed with a JEOL 1400 electron microscope (JEOL USA).

SEM of biliary exosomes. For SEM, isolated biliary exosomes were fixed immediately in 2.5% phosphate-buffered glutaraldehyde for 1 h, rinsed with phosphate buffer three times, and postfixed in 1% osmium tetroxide for 1 h on ice. SEM samples were dehydrated, dried in a critical point drying device, mounted, sputter coated, and examined with a Hitachi S-4700 microscope (Hitachi).

ERK signaling. We seeded 2 × 104 NRCs in 96-well collagen-coated microplate and incubated them in a serum-free medium for 3 days to promote cilia growth. When indicated, NRCs were treated for 24 h with the deciliating agent chloral hydrate (4 mM). To eliminate serum exosomes and exosomes potentially released by cholangiocytes in culture media, NRCs were washed several times with a serum-free medium. Then, ciliated and deciliated cholangiocytes were further incubated for 24 h in a serum-free medium containing 0.5 μg
exosomes isolated from bile of normal rats. Then cells were harvested with RIPA lysis buffer and the ratio of phosphorylated ERK1/2 to total ERK1/2 was determined by Western blot analysis using antibodies to phosphorylated ERK1/2, dilution 1:1,000 (AbCam) and to ERK 1/2, dilution 1:2,000 (BD Biosciences) as we previously described (4).

miR-15A expression. NRCs were treated with isolated biliary exosomes as described (see ERK signaling). RNA was isolated from NRC with TRIzol Reagent (Invitrogen). To measure miR-15A levels, a quantitative PCR approach using a LightCycler (Roche) was employed as we previously described (17). Expression of miR-15A was assessed according to the TaqMan MicroRNA Assay protocol (Applied Biosystems). cDNA was synthesized from 2 μg of total RNA by use of miR-15A-specific primers and the TaqMan MicroRNA Reverse Transcription Kit (both from Applied Biosystems). PCR for miR-15A was performed for 45 cycles at 95°C for 15 s, and annealing and extension steps were performed at 60°C. The data are shown as fold changes of average signals in untreated cholangiocytes and in cholangiocytes treated with exosomes.

Cholangiocyte proliferation. Ciliated and deciliated NRCs were grown as described (see ERK signaling), then washed several times and further incubated for 72 h in a serum-free medium containing 0.5 μg of exosomes isolated from bile of normal rats. A medium containing biliary exosomes was changed every 24 h. To address the involvement of miR-15A in the effects of exosomes on cholangiocyte proliferation, ciliated NRCs were treated with 50 nM of either anti-miR-15A inhibitor or pre-miR negative control (Ambion, Applied Biosystems) at day 0 and day 2 and then harvested at day 4. miRNA-transfected NRCs were treated with biliary exosomes at days 1, 2, and 3. Cholangiocyte proliferation was assessed by using a MTS-based kit assay (CellTiter 96 Aqueous One Solution Cell Proliferation Assay, Promega) as we previously described (4). The rates of cholangiocyte proliferation after exosome treatment are shown as the percentage of the proliferation rates of nontreated cholangiocytes (100%).

Statistical analysis. All values are expressed as means ± SE. Statistical analysis was performed by the Student’s t-test, and results were considered statistically different at P < 0.05.

RESULTS

Exosome-like vesicles are present in the lumen of intrahepatic bile ducts interacting with cholangiocyte cilia. The presence of exosome-like vesicles in the lumen of intrahepatic bile ducts and their potential interaction with cholangiocyte cilia were originally suggested by our TEM study of ciliary morphology in an animal model of autosomal recessive polycystic kidney disease (ARPKD), the Pkd1del2/del2 mouse (46). In the present study, we further confirmed and extended this observation showing the presence of vesicular structures of 50–80 nm in diameter (i.e., the size of exosomes) in the lumen of intrahepatic bile ducts of wild-type and Pkd1del2/del2 mice (Fig. 1). By TEM, these vesicles surround cholangiocyte cilia and some appear to attach to the ciliary membrane and microvilli (Fig. 1, A and B). The SEM images strongly suggest that exosome-like vesicles in fact attach to cilia (Fig. 1, C and D). By SEM, exosome-like vesicles attached to cholangiocyte
Biliary exosomes were also observed in normal and PCK (an animal model of ARPKD) rats and in Pkd2<sup>ws25/−</sup> mice (an animal model of ADPKD) (Fig. 2).

On the basis of quantitation of exosome-like vesicles that are seen in two-dimensional TEM images (Fig. 1), the number of vesicles surrounding cholangiocyte cilia in cystic liver was ten times greater compared with the number of vesicles located in a close proximity to cilia in normal liver (i.e., 105.7 ± 11.9 vesicles vs. 10.8 ± 2.9 vesicles; n = 10, P < 0.0001). By SEM, exosome-like vesicles do not attach to all observed cilia in both normal and cystic cholangiocytes; however, the number of cholangiocyte cilia to which vesicles attach was greater in cystic liver compared with normal liver. Quantitatively, exosome-like vesicles attached to 41 of 110 observed normal cholangiocyte cilia (i.e., to one-third of cilia) whereas in cystic liver exosome-like vesicles attached to 144 of 180 observed cholangiocyte cilia (i.e., to more than two-thirds of cilia). The pathophysiological significance of an increased number of exosome-like vesicles in the lumen of intrahepatic bile ducts of cystic liver and a number of cholangiocyte cilia to which exosome-like vesicles attach remains unknown.

Rat bile contains exosomes. To identify the vesicular structures seen in the lumen of intrahepatic bile ducts, we isolated biliary vesicles from rat bile using a protocol that we previously successfully employed for isolation of exosomes from human urine (13). Bile was differentially ultracentrifuged to pellet biliary vesicles that were further identified as exosomes. By using this technique, we were able to isolate about 10 μg of exosomes per 1 ml of rat bile.

By SEM, the pelleted biliary vesicles were observed as clusters of vesicles or single vesicles (Fig. 3, A and B). The self-clustering of exosomes shown in Fig. 3A is likely a result of their adhesive properties. A common feature of exosomes released by different cell types is the expression of adhesion molecules that include not limited to members of the IgG superfamily and the integrin family. Although equipped with machinery for adhering to a range noncellular and cellular components, the adhesive properties of exosomes are not well studied (7).

Isolated biliary vesicles float at a wide range of a sucrose density (i.e., 1.12–1.21 g/ml), suggesting that they should be heterogeneous in size. Indeed, by TEM, the biliary vesicles are 50–90 nm in diameter and have “saucer-shaped” or “deflated football-shaped” morphology (Fig. 3, C and D). Isolated biliary vesicles are also positive for exosomal markers, CD63 and Tsg101 (Fig. 4).

Exosomes have specific features that distinguish them from other plasma membrane-derived vesicles. In contrast to other vesicles that bud directly from the plasma membrane and are heterogeneous in size (i.e., 100 to >1,000 nm in diameter) and shape, exosomes are from 30 to 100 nm in diameter, have a unique flotation density (1.10–1.21 g/ml) on continuous sucrose gradients, and, following a negative staining, display a unique saucer-shaped or deflated football-shaped morphology by electron microscopy. In contrast, nonexosomal vesicles have irregular shape and float at higher sucrose densities (i.e., >1.23 g/ml) (27). Exosomes are positive for markers such as CD63 and Tsg101, demonstrating that they originate from MVBs (27, 33–35). Vesicles with a flotation density of 1.12–1.21 g/ml that we isolated from bile of normal rats were characterized by all of these criteria, providing strong evidence that rat bile contains exosomes.

Both hepatocytes and cholangiocytes release exosomes in bile. Given that both hepatocytes and cholangiocytes are exosome-releasing cells in culture (9, 45), bile obtained from whole animals may contain exosomes derived from both types of liver epithelial cells, i.e., hepatocytes and cholangiocytes. Indeed, our immunogold-TEM studies (Figs. 5 and 6) show that hepatocyte and cholangiocyte MVBs fuse with the canalicular and cholangiocyte apical plasma membrane, respectively, and release their content, i.e., exosomes, into the bile canalicular and into the lumen of intrahepatic bile ducts, respectively. The images in Fig. 5 show CD63-positive MVBs and vesicles accumulating in the subapical compartment of rat hepatocytes and in the canaliculus, suggesting that hepatocytes are exosome-releasing cells in vivo.

Figure 6 shows the accumulation of PC-1-positive MVBs and vesicles in the subapical compartment of mouse cholangiocyte cilia in vivo. Exosome-like vesicles (white arrows) attach to cholangiocyte cilia of normal (A) and PCK (B) rats, and wild-type (C) and Pkd2<sup>ws25/−</sup> (D) mice.
giocytes (Fig. 6, A and B) and in the lumen of intrahepatic bile ducts (Fig. 6, C and D). PC-1 is considered as an exosomal marker in epithelial cells (13), and in the liver it is strongly expressed in cholangiocytes (24). The accumulation of PC-1-positive MVBs and vesicles in the cholangiocyte subapical compartment and in the bile duct lumen suggests that a certain amount of biliary exosomes is derived from cholangiocytes.

Importantly, PC-1-positive exosomes are seen in a proximity to the cholangiocyte cilium (Fig. 6D), supporting our initial observation (Fig. 1) on exosome-ciliary interaction.

Biliary exosomes interact with cholangiocyte cilia. To provide direct evidence that biliary exosomes interact with cholangiocyte cilia, isolated exosomes were added to ciliated NRCs in culture and their interaction with cilia studied by SEM.

Fig. 3. Morphological characterization of vesicles isolated from bile of normal rats. Clusters of vesicles and single vesicles of 50–90 nm in diameter were observed by SEM (A and B) and TEM (C and D). Biliary vesicles have characteristic “deflated football-shaped” or “saucer-shaped” morphology (C and D), suggesting they are exosomes.

Fig. 4. Characterization of biliary exosomes by immunogold-TEM. Vesicles isolated from rat bile are positive for exosomal markers, CD63 (left) and Tsg101 (middle). In control (right), no primary antibodies to CD63 or Tsg101 were applied to isolated biliary exosomes.
Figure 7 shows that after the exposure of NRCs to isolated biliary exosomes they attach to cholangiocyte cilia; however, in the control (i.e., no biliary exosomes in culture medium) no exosomes are seen attached to cilia. A quantitative analysis shows that five to seven exosomes were attached to a cilium after their incubation with cholangiocytes, whereas in the control only an occasional single exosome attached to a cilium was observed (Fig. 7). Thus these experiments directly demonstrate the interaction between biliary exosomes and cholangiocyte cilia.

**Biliary exosomes alter ERK signaling in cholangiocytes in a ciliary-dependent manner.** To ascertain whether the interaction of biliary exosomes with cholangiocyte cilia has physiological consequences, we studied the effects of isolated biliary exosomes on ERK signaling in NRCs. The ERK signaling pathway was chosen based on its importance in cholangiocyte functions (4, 20) and on the work of others demonstrating that exosomes derived from different cell types influence ERK in the target cells (28, 29). Data in Fig. 8 show that the phospho-ERK-to-total ERK ratio in exosome-treated cholangiocytes...
was decreased by 61.8 ± 5.9%. However, this exosomal effect was not observed in NRCs deciliated with chloral hydrate, suggesting that the inhibition of ERK signaling by exosomes is a result of exosome-ciliary interaction.

**Biliary exosomes alter the expression of mir-15A in cholangiocytes in a ciliary-dependent manner.** To determine whether biliary exosomes influence intracellular regulatory mechanisms other than ERK signaling, the effect of exosomes on the expression of miR-15A (a miRNA associated with cholangiocyte proliferation) (17) was studied. The expression of miR-15A was evaluated by quantitative RT-PCR. The incubation of ciliated cholangiocytes with isolated biliary exosomes resulted in an eightfold increase in the amount of miR-15A (Fig. 9). However, the effects of exosomes on miR-15A expression were not observed in cholangiocytes deciliated with chloral hydrate (Fig. 9), indicating that interaction of biliary exosomes with cholangiocyte cilia are necessary for an exosome-induced increase in miR-15A expression.

**Biliary exosomes affect cholangiocyte proliferation in a ciliary- and mir-15A-dependent manner.** To address whether the exosome-induced responses of intracellular regulatory mechanisms are associated with other cholangiocyte functional changes, we tested the effects of biliary exosomes on cholangiocyte proliferation based on our previous observations that ERK signaling and miR-15A are critically involved in this process (4, 17). The effects of exosomes derived from different cell types on cell proliferation have also been previously reported (1, 40).

Data in Fig. 10A show that biliary exosomes induced a 19.0 ± 2.1% decrease in cholangiocyte proliferation. To address the involvement of cilia in the effects of exosomes on cholangiocyte proliferation, cells were deciliated with chloral hydrate and then treated with biliary exosomes at similar conditions. As shown in Fig. 10A, the inhibitory effect of biliary exosomes was not observed in deciliated cholangiocytes, supporting the important role of exosome-ciliary interaction in regulation of cholangiocyte proliferation.

To address whether an exosome-induced decrease in cholangiocyte proliferation (Fig. 10A) is associated with an exosome-induced increase in miR-15A expression (Fig. 8), NRCs were transfected with anti-miR negative control (scrambled miRNA) by 24.8 ± 2.6%, i.e., similarly to that observed in nontransfected NRCs (Fig. 10A). In contrast, the inhibitory
The effect of biliary exosomes on proliferation of NRCs transfected with anti-miR-15A was reduced by twofold, i.e., exosomes inhibited cholangiocyte proliferation by only 11.6/110061.7% (Fig. 10B). Thus the effect of biliary exosomes on proliferation of NRCs was partially abolished by anti-miR-15A, suggesting the functional involvement of miR-15A in an exosome-induced decrease in cholangiocyte proliferation.

**DISCUSSION**

The key findings of this study relate to biliary exosomes and their potential physiological significance. We demonstrated that 1) exosome-like vesicles surround and attach to cholangiocyte cilia in intrahepatic bile ducts in vivo; 2) vesicles isolated from rat bile by differential ultracentrifugation have characteristic features of exosomes; 3) isolated biliary exosomes interact with cholangiocyte cilia in vitro; and 4) by interacting with cholangiocyte cilia, biliary exosomes alter ERK signaling, miR-15A expression, and cholangiocyte proliferation. These findings indicate that bile contains exosomes that are involved in intercellular communication within the liver by interacting with cholangiocyte cilia.

The involvement in intercellular communication is likely the major physiological function of exosomes (27). This conclusion is based on several observations. First, exosomes are specifically targeted to recipient cells (33, 34). Second, they deliver functional proteins, lipids, and nucleic acids, including mRNAs and miRNAs, to neighboring or distant cells (33, 34). Third, they trigger downstream signaling events in targeted cells (30). The mechanisms by which exosomes are processed in recipient cells are currently unknown.

In the present work, we demonstrate for the first time that in ciliated cells, exosomes can be involved in intercellular communication by interacting with primary cilia. Signaling vesicles that interact with cilia inducing intracellular signaling response have been previously described in the mouse embryonic node (37). However, these vesicles, termed “nodal vesicular parcels,” may represent a different type of signaling vesicle because their size was 10–100 times larger compared with the size of exosomes. Thus our observations are the first direct evidence that exosome-ciliary interactions may affect cholangiocyte intracellular signaling and functional responses. More broadly, our data taken together with observations of others (34) suggest that exosomes perform their signaling functions by different cell type-specific, ciliary-dependent, and ciliary-independent mechanisms. In ciliated cells, the exosome-ciliary interaction appears to be essential for signaling functions of exosomes.

The signaling effects of exosomes depend on their molecular constituents and on the type of target cells. Our data show that ERK signaling in normal rat cholangiocytes in culture is inhibited by biliary exosomes. An inhibition of ERK signaling was also observed in a subclone of the breast cancer cell line BT-549Gal-3 when bovine exosomes were added to the culture medium (28). In contrast, exosomes released by human gastric cancer SGC7901 cells increased 3.2 times the phosphorylated-to-total ERK1/2 ratio in these cells (29). Thus our study...
together with published observations (28, 29), suggests that exosomes possess the capability to alter ERK signaling in different cell types. On the basis of our data and the work of others (28, 29), we speculate that exosomes that are released by tumor cells activate ERK, whereas exosomes of nontumor cell origin primarily inhibit this pathway. It is also important to note that exosomal effects on the ERK pathway are thought to be specific, because only PI3K/Akt and MAPK/ERK but not NF-κB signaling were activated in the SGC7901 cells by tumor-derived exosomes (29).

In this work, we also demonstrated for the first time that biliary exosomes influence cholangiocyte miR-15A expression. miRNAs are 22-nucleotide-long noncoding RNAs that regulate protein-encoding genes primarily at the posttranscriptional level (3). miRNAs have a profound impact on a variety of biological processes including cell proliferation in general (6, 43) and cholangiocyte proliferation in particular (17). We have recently reported that overexpression of miR-15A in rat cholangiocytes was associated with inhibition of proliferation. In contrast, suppression of miR-15A accelerated cholangiocyte proliferation (17).

The functional involvement of miR-15A in cholangiocyte proliferation is complex. By an in silico approach, we found 950 target proteins for miR-15A (25), including components of the ERK pathway, that plays an important role in cholangiocyte proliferation (4, 20, 32). Taking into account a recent study indicating that the expression ratio of ERK isoforms correlates with their activation ratio (18), our data signify the functional involvement of miR-15A in exosome-induced, ERK1/2-dependent inhibition of cholangiocyte proliferation. From our perspective, the decrease in the phospho-ERK-to-total ERK ratio and the increase in miR-15A expression that we observed in exosome-treated cholangiocytes are likely functionally associated. As we previously reported, cholangiocyte proliferation is inhibited when ERK signaling is suppressed (4) or when miR-15A is overexpressed (17). The results of our present study show a similar outcome; that is, exosome-induced inhibition of cholangiocyte proliferation is associated with inhibition of ERK signaling and with activation of miR-15A expression. Moreover, our study on NRCs transfected with anti-miR-15A directly supports the functional involvement of miR-15A in an exosome-induced decrease in cholangiocyte proliferation.

The effects of exosomes on cell proliferation have been previously reported in several studies. For example, mouse and human dendritic cell-derived exosomes triggered proliferation of natural killer cells (40). In contrast, exosomes released from cells of the thymus suppressed the proliferation of CD4+CD25-T cells (42). Exosomes derived from breast adenocarcinoma cells BT-474 increased proliferation of the releasing cells (14). Exosomes derived from human gastric cancer SGC7901 cells promoted proliferation of the releasing cells and another human gastric cancer cell line, BGC823, in a time- and dose-dependent manner (29). Our data extend these observations by demonstrating for the first time that biliary exosomes influence proliferation of normal biliary epithelial cells and that exosomal effects depend on their interaction with cholangiocyte cilia.

The mechanisms through which biliary exosomes influence ERK signaling, miR-15A expression, and cholangiocyte proliferation remain to be understood. Given the molecular and biochemical complexity of exosomes, structural and functional properties of cholangiocyte cilia, numerous miRNA targets, and the existence of multiple intracellular signaling pathways, the mechanisms through which biliary exosomes participate in intercellular communication within the liver appear to be intricate. Further molecular and biochemical characterization of biliary exosomes, accurate discrimination of hepatocyte- and cholangiocyte-derived exosomes, studies of the mechanisms of exosome-ciliary interaction and exosome-induced ciliary signaling, are among the priority tasks that will allow elucidation of physiological and pathophysiological relevance of biliary exosomes.

It has been shown by numerous studies that exosomes released by healthy and diseased cells are different with regard of their specific molecular and biochemical constituents and functions (33–35). On the basis of these observations, we can hypothesize that exosomes accumulating in bile of healthy individuals and in individuals with liver disease possess different molecular, biochemical, and functional characteristics that may contribute to disease development and/or progression. This speculation is supported by our data that exosome-like vesicles accumulate in the lumen of intrahepatic bile ducts of cystic liver and by observations of others (45) that exosomes containing components of Hedgehog signaling accumulate in bile of bile duct-ligated rats. In addition, our preliminary data also suggest that bile of PCK rats contains a larger amount of exosomes compared with bile of normal rats.

In conclusion, our data show that bile contains exosomes that function as signaling nanovesicles influencing intracellular regulatory mechanisms (i.e., ERK signaling, miR-15A expression) and cholangiocyte proliferation through interaction with primary cilia. In addition, these data suggest that chemosignals are delivered to cholangiocyte cilia not only as single small or large signaling molecules (22) but also by vesicles such as exosomes. The precise physiological and pathophysiological consequences of biliary exosomes and exosome-ciliary interaction remain to be elucidated. One of the most provocative interpretations of our present findings is that biliary exosomes and their interaction with cholangiocyte cilia may represent a novel mechanism of intercellular communication within the liver.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

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