

**Figure 1** Myrcludex B does not inhibit either <sup>3</sup>H-TCA or Tauro-NOR-THCA-24-DBD uptake radiolabelled TCA (A) or fluorescent Tauro-NOR-THCA-24-DBD (B) uptake in LX2 cells with or without TGFβ activation with U2OS\_HA-hNTCP cells as a positive control. DPM, desintegrations per minute; TGF, transforming growth factor.

transporter NTCP in primary human hepatic stellate and LX2 cells and suggest a role for NTCP-mediated bile salt uptake and consequent activation of stellate cells in the pathogenesis of liver fibrosis.

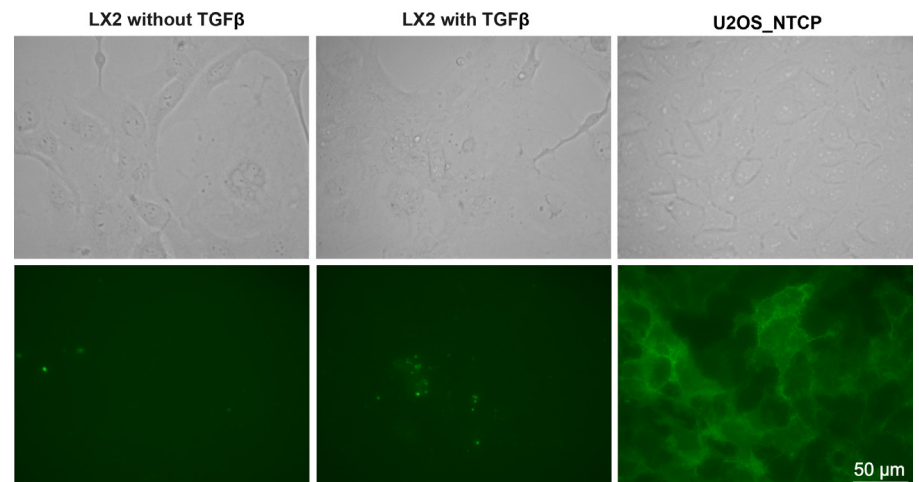
The authors used a commercially available antibody against human NTCP for immunofluorescent stainings and Western blots, fluorescence activated cell sorting (FACS) analysis and as a neutralising antibody in bile salt uptake experiments. Remarkably, despite high immunopositive signals, no cell surface signal was observed in the immunofluorescent stainings. Using this antibody, the authors show complete inhibition of NTCP-mediated bile salt uptake in LX2 cells, whereas the epitope to which the antibody was raised is mostly intracellular. In addition, the NTCP migration pattern shown on Western blot deviates from the characteristic diffuse

NTCP migration pattern seen in hepatocellular lysates as well as in NTCP-overexpressing cell lines.<sup>2</sup> NTCP is a heavily glycosylated protein and we have previously shown that this glycosylation is essential for its plasma membrane trafficking and activity.<sup>2</sup>

A highly interesting though puzzling observation is the beneficial effect of NTCP neutralisation on fibrosis development in a CCL<sub>4</sub> mouse model. The antibody used to neutralise mouse NTCP was raised in rabbits and targets a synthetic peptide corresponding to the intracellular C terminal region of mouse NTCP. Therefore, it is unclear how this antibody could operate as a blocking antibody. The effects observed in the mouse model of fibrosis may be related to an acute inflammatory response elicited by the use of rabbit antibodies in mice. No antibody control was used in this experiment. We and others have previously demonstrated that both

### Insufficient evidence for NTCP activity in stellate cells

We read with interest the study of Salhab postulating the sodium+/taurocholate cotransporting polypeptide (NTCP) as novel therapeutic target against liver fibrosis.<sup>1</sup> The authors report functional expression of the bile acid uptake



**Figure 2** Myrcludex B binds to the cell surface of NTCP-positive U2OS cells, but not LX2 cells. Bright field (top) and fluorescent (bottom) microscopy images showing strong plasma membrane binding of Myrcludex B-FITC in NTCP positive U2OS but not LX2 cells. The latter only shows non-specific intracellular signal. FITC, fluorescein isothiocyanate; NTCP, sodium+/taurocholate cotransporting polypeptide,

the NTCP-mediated as well as Oatp1a/1b-mediated bile salt uptake machinery is rapidly downregulated in inflammatory conditions, which may explain the modestly elevated taurocholic acid (TCA) elevation seen in CCL<sub>4</sub>/anti-NTCP injected mice.

Because our research interests centre around hepatobiliary transport processes, including NTCP function, we studied NTCP cell surface presence and activity in LX2 cells without relying on antibodies (online supplemental material). We performed bile salt uptake assays in the presence and absence of the specific and well-characterised NTCP inhibitory peptide Myrcludex B (also called bulevirtide).<sup>3</sup> In contrast to U2OS cells overexpressing human NTCP, <sup>3</sup>H-TCA as well as Tauro-NOR-THCA-24-DBD uptake was at background levels in LX2 cells, independent of TGFβ-induced activation, and could not be further blocked with Myrcludex B (figure 1A,B). TGFβ-activated LX2 cells did not show any plasma membrane labelling on incubation with FITC-labelled Myrcludex B (figure 2), in contrast to NTCP-positive U2OS cells. This suggests that no, also no low-abundance subsets of, NTCP-positive cells were present in this cell population. Our bile salt uptake data are in sharp contrast to the data provided by the authors, where the culture medium containing 100 μM TCA is completely depleted from TCA after 45 min, supposedly due to uptake activity of NTCP. If we (safely) assume that the height of the cells is <1% of the total height of the culture medium, the intracellular TCA concentration would need to reach at least 10 mM inside these NTCP-positive stellate cells, an unlikely scenario as this is above the critical micelle concentration of TCA.<sup>4</sup>

In conclusion, we believe that a direct role of NTCP activity in stellate cells contributing to fibrosis is not proven by the experiments presented by Salhab *et al.* More investigations using specific inhibitors and better controlled conditions are necessary before we can pursue NTCP in stellate cells as a promising antifibrotic target.

Roni F Kunst,<sup>1,2</sup> Coen C Paulusma,<sup>1,2</sup>  
Stan F J van de Graaf<sup>1,2</sup>

<sup>1</sup>Amsterdam Gastroenterology, Endocrinology, Metabolism, Amsterdam University Medical Centers, Amsterdam, The Netherlands

<sup>2</sup>Tytgat Institute and Department of Gastroenterology and Hepatology, Amsterdam UMC Locatie AMC, Amsterdam, North Holland, The Netherlands

**Correspondence to** Dr Stan F J van de Graaf, Tytgat Institute for Liver and Intestinal Research, Amsterdam UMC Locatie AMC, Amsterdam 1105 AZ, North Holland, The Netherlands; k.f.vandegraaf@amsterdamumc.nl

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**ORCID iD**

Stan F J van de Graaf <http://orcid.org/0000-0003-4238-4359>

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## Supplemental material

### Methods

Both LX2 and U2OS\_HA-hNTCP<sup>1</sup> cells were grown until confluence in 24-well plates and LX2 cells were activated by a 48h 10 ng/ml TGF $\beta$  pre-treatment before start of the experiment. Cells were incubated for 5 min with 1 $\mu$ M MyrB before incubation with 100 $\mu$ M TCA with trace amounts of [<sup>3</sup>H]TCA for 15min and lysed/counted. For the Tauro-nor-THCA-24-DBD uptake, cells were incubated for 30min at 37°C with 10 $\mu$ M N-(24-[7-(4-N,N-dimethylaminosulfonyl-2,1,3-benzoxadiazole)]-amino-3 $\alpha$ ,7 $\alpha$ ,12 $\alpha$ -trihydroxy-27-nor-5 $\beta$ -cholestan-26-oyl)-2'-aminoethanesulfonate (Tauro-nor-THCA-24-DBD, Genomembrane Company, Ltd., Yokohama Japan) in the presence or absence of 400nM Myrcludex B. Cells were washed in PBS and lysed in 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% (v/v) Nonidet-40, 0,1% (w/v) sodium dodecyl sulfate (RIPA) before fluorescence was quantified by the Clariostar (BMG Labtech). Finally, for the Myrcludex-FITC staining, cells were imaged after incubation for 1h with 400nM MyrB-FITC at 37 °C.

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