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LETTER TO THE EDITOR

Knockdown of flotillin-2 inhibits lung surfactant secretion by alveolar type II cells

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Dear Editor,

Lung surfactant is stored in lamellar bodies and exocytosed following fusion of the lamellar bodies with the plasma membrane of alveolar type II (AT2) cells [1]. A number of proteins have been shown to be involved in surfactant secretion including SNAREs, NSF, α-SNAP and annexin A2 [2, 3]. Lipid rafts enriched in SNAREs are crucial for surfactant secretion [4].

Flotillins are lipid raft marker proteins. There are two isoforms, flotillin-1 and flotillin-2. Both are expressed in many cell types including AT2 cells [4]. Several functions of flotillins have been reported including interaction with protein kinases and the cytoskeleton; formation of filopodia; insulin signaling; axonal regeneration and cell-cell contacts and membrane trafficking [5, 6]. The evidence for a role of flotillins in exocytosis is lacking except for a recent study which indicated the participation of flotillin-1 in mast cell exocytosis [7].

We investigated the functional role of flotillins in surfactant secretion by decreasing the expression of flotillin-1 and -2 using RNA interference. We used an adenoviral vector to express shRNAs because of its high transduction efficiency in primary cells. This vector contained a shRNA sequence under the control of the mouse U6 promoter with EGFP expression under the control of the CMV promoter (Supplementary information, Materials and Methods). We chose 3 siRNA sequences targeted to different regions for each isoform: 118-137, 522-540 and 584-603 of flotillin-1 [F-1 shRNA (A), (B) and (C)] and 53-73, 602-620 and 1163-1182 of flotillin-2 [F-2 shRNA (A), (B) and (C)]. The construct with irrelevant sequences served as a virus control (VC). The cells which were not transduced with any virus were used as blank controls (BC).

We cultured AT2 cells with an air-liquid culture system to maintain their phenotype and transduced them with adenoviruses for 5 days. F-1 shRNA (A) almost completely silenced flotillin-1 expression (Figure 1A). There was also a decrease in flotillin-2 expression although a significant amount of flotillin-2 remained in the F-1 shRNA (A)-treated AT2 cells (Figure 1B). Among F-2 shRNAs, F-2 shRNA (C) was the most effective in silencing flotillin-2 expression. This shRNA also reduced flotillin-1 expression (Figure 1A and 1B), probably because the stability of flotillin-1 is dependent on flotillin-2 [8]. Using F-1 shRNA (A) and F-2 shRNA (C), we determined the effect of flotillin-1 and -2 silencing on surfactant secretion. There were no statistically significant differences in the basal and stimulated secretions between control and F-1 shRNA (A) - treated cells (Figure 1C). However, F-2 shRNA (C) reduced stimulated surfactant secretion. The basal secretion appears to be decreased in the F-2 shRNA (C)-treated cells but the difference did not reach a significant level. The control virus had no effect on surfactant secretion.

We next examined whether the loss of flotillin affects lipid raft formation. Because large amounts of cells were needed for this experiment, we used L2 cells, a rat lung epithelial cell line that expresses flotillin-1 and -2 [4]. We transduced L2 cells with F-1 shRNA (A) or F-2 shRNA (C). Infection efficiencies increased with the multiplicity of infection (MOI) of the virus as revealed by EGFP fluorescence (Supplementary information, Figure S1A). Western blot analysis indicated a significant decrease in protein expression of flotillin-1 or -2 in a dose-dependent manner (Supplementary information, Figure S1B). When compared with blank and virus controls, there were no obvious differences in the amounts of protein and cholesterol of the raft fractions between control and flotillin-1 or -2 knockdown cells (Supplementary information, Figure S1C and S1D). When cholesterol concentration was expressed

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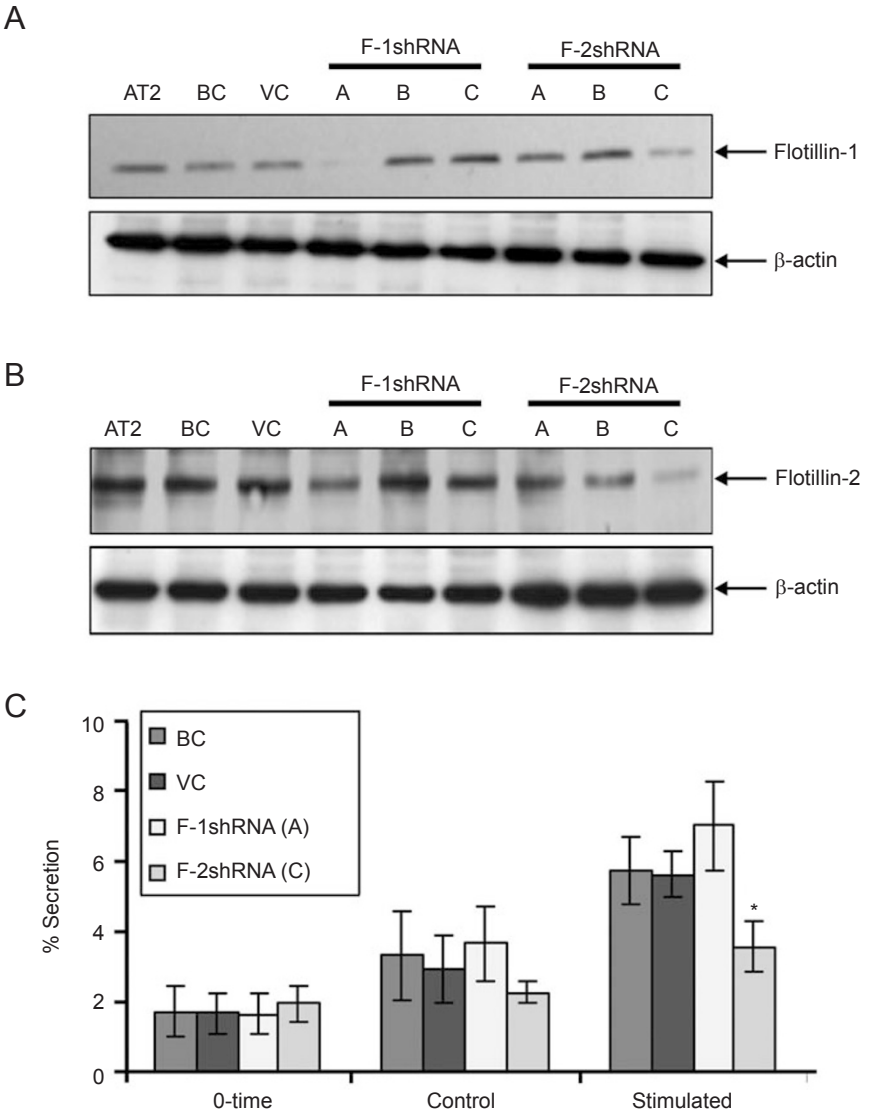

Figure 1 Effect of flotillin silencing on surfactant secretion. Alveolar type II (AT2) cells were transduced with different shRNA adenoviruses at an equal dose of 100 multiplicity of infection (MOI) and cultured for 5 days. Equal amounts of proteins were immunoblotted for flotillin-1 **(A)** and flotillin-2 **(B)**. The same membranes were re-probed for β-actin to confirm equal loading. **(C)** Surfactant secretion. The control and flotillin knockdown cells were washed and equilibrated 30 min in serum-free medium (0-time). The cells were incubated 2.5 h in the absence (control) or presence of 100 μM ATP, 0.1 μM phorbol 12-myristate 13-acetate (PMA) and 10 μM terbutaline (stimulated). The results were expressed as means ± SE. **P* < 0.05 versus BC ($n \geq 3$ independent cell preparations, each assay in duplicates). BC: blank control, VC: virus control.

as μg per μg protein, cholesterol content in the lipid raft fraction was enriched 3-, 10-, and 20- fold in comparison with non-raft fractions 3, 4, and 5.

SNARE proteins were enriched in lipid rafts of AT2 cells [4]. We examined whether knockdown of flotillin leads to dissociation of SNAREs with lipid rafts. Following silencing, little flotillin-1 and -2 proteins were detected in any of the fractions, indicating an effective silencing. When raft fractions were probed for two plasma membrane SNARE proteins, syntaxin-2 and SNAP-23 [2] following silencing, there was no change in the association of these proteins with lipid rafts (Supplementary information, Figure S1E and S1F).

Lipid rafts are also reported to be involved in cholesterol homeostasis. We further investigated the effects of flotillin-1 and -2 silencing on cholesterol levels in L2 cells. Following silencing of flotillin-1 and -2, cell lysates were analyzed for total cholesterol levels. Our results indicated

that there were no statistical significant differences in cholesterol levels under silencing conditions when compared with controls (Supplementary information, Figure S1G).

We conclude that flotillin-2 is required for surfactant secretion but not via influencing the association of SNAREs with the rafts or cholesterol homeostasis. The mechanisms by which flotillin-2 regulates surfactant secretion remain to be determined, but could be due to its effects on signaling pathways or cytosketetal rearrangement because flotillin-2 knockdown blocks the G-protein coupled receptor (GPCR)-mediated activation of p38 MAPK [9] and trans-negative mutant flotillin-2 leads to faulty localization of Vav, resulting in altered cytoskeletal rearrangements [10].

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(**Supplementary Information** is linked to the online version of the paper on the Cell Research website.)