Reply to the comments to the paper 'Over-expressing transient receptor potential cation channel 6 in podocytes induces cytoskeleton rearrangement through increases of intracellular Ca^{2+} and RhoA activation'

In the comment article kindly sent by the editor of *EMB*, Kistler *et al*. raised several questions regarding to the results in this paper. In review of the comments along with some of the published relevant papers, we would like to generously provide a point-by-point response seen below to Kistler *et al*.'s stated comments.

First, Kistler *et al*. state that cultured podocytes are notoriously difficult to transfect and a transfection efficiency exceeding 5% cannot usually be achieved, and the low transfection efficiency precludes meaningful analysis of cell lysates by Western blot. However, Okamura *et al*.[1] used the transfection reagent GeneJuice to transiently transfect podocytes with plasmid constructs and obtained significant luciferase activity changes after stimuli. In our laboratory, we successfully detected the alterations by Western blot in the podocytes transiently transfected with mutant podocin.[2] In addition, in previous studies from other groups, the protein expression could be detected in podocytes transfected with different related constructs.[1,3,4] In this paper, we only showed the endogenous TRPC6 (106 kDa) bands in podocytes transfected with the recombinant plasmid pReceiver-M29-TRPC6, leading to the misunderstanding of the results. Here we append a new figure (Figure 1) showing a typical Western blot profile of two bands, an endogenous TRPC6 band (106 kDa) and an eGFP-tagged recombinant TRPC6 band (133 kDa) from podocytes transiently transfected with TRPC6 plasmid by using lipofectamine 2000.

Second, Kistler *et al*. suggest that the 110 kDa band rather than the 44 kDa band of synaptopodin should be used to demonstrate a reduction in synaptopodin protein expression in TRPC6 over-expressed podocytes, because the intact full-length synaptopodin (the podocyte isoform) has an apparent molecular weight of ca. 110 kDa, and the 44 kDa band may represent a cleavage product of synaptopodin. In our study, synaptopodin also demonstrated a band of 110 kDa size in the Western blot (Figure 2).

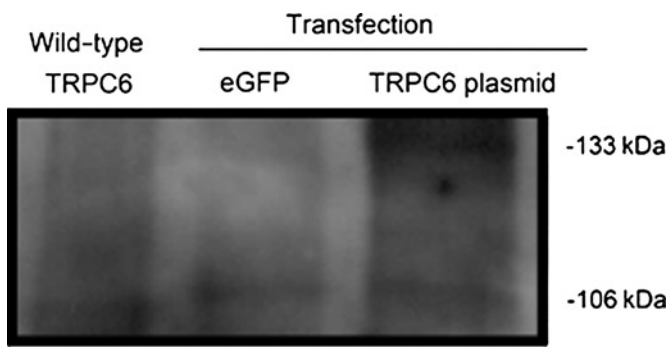

Figure 1 Mouse podocytes were transiently transfected with the recombinant plasmid pReceiver-M29-TRPC6 which expresses eGFP-TRPC6 recombinant fusion protein (the third lane) or the pReceiver-M29 vector which expresses eGFP (the second lane) as the control. Two bands can be seen in the third lane; the lower band is the endogenous TRPC6 (106 kDa) and the upper band is about 133 kDa representing the over-expressed eGFP-TRPC6 recombinant fusion protein

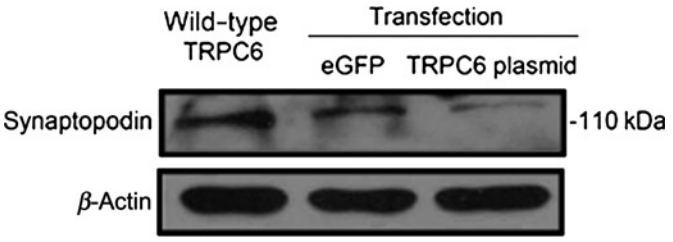

Figure 2 Mouse podocytes were transiently transfected with the pReceiver-M29 vector (lane 2) or the recombinant plasmid pReceiver-M29-TRPC6 (lane 3). Proteins on membrane were blotted by anti-synaptopodin antibody. Synaptopodin expression (110 kDa) was down-regulated in podocytes over-expressing TRPC6

Third, Kistler *et al*. doubt the significance of the experiment that TRPC6 over-expressing podocytes exhibit an increased response to the DAG analog OAG, which can be blocked by U73122. They denote that OAG acts downstream of PLC and U73122 should not have any effect on OAG-induced TRPC6 activation. Actually, U73122 has been shown to inhibit various changes relating to agonist-induced inositol 1,4,5-trisphosphate (IP3) production and the subsequent increase in cytosolic Ca^{2+}, being an inhibitor of PLC-dependent processes.[5–7] In addition, U73122 blocks TRPC6, of which the mechanism is unknown but is probably unrelated to its effects on PLC.[8] We demonstrated in this paper that U73122 could efficiently block the TRPC6 ion channel activated by OAG or carbachol (CCh). Previous studies from other groups have also found that U73122 inhibits the current induced by OAG.[9–13]

Fourth, Kistler *et al*. consider that the morphology of the wild-type podocytes we used is not a typical feature of differentiated podocytes, and may be a sign of cellular stress such as seen with mycoplasma infection. They then query the retraction of cell processes in TRPC6 over-expressing podocytes. We cultured the conditionally immortalized mouse podocytes at 33°C in RPMI-1640 and then shifted to 37°C for differentiation. This cell line growing under permissive conditions displays characteristics of undifferentiated cobblestone-type podocytes, whereas shifting to non-permissive conditions induces conversion into 'arborized' cells.[14] The reports from other research groups also obtained the same morphological characteristics of the podocytes.[3] In our laboratory, regular tests including fluorescence detection and morphological examinations are performed to rule out the possibility of mycoplasma infection.

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Additional comments on response of *Jiang et al.*

We appreciate the response to our concerns by Jiang *et al*. Some issues, however, were addressed inadequately or not at all.

(1) The authors have now provided a Western blot showing a 133 kDa band for EGFR-TRPC6. However, the strong bands at about 106 representing endogenous TRPC6 that are evident in the published paper are now hardly visible in the newly provided blot.

(2) The newly provided, uncropped blot for synaptopodin shows much reduced intensity of the 110 kDa band in the eGFP-transfected cells and a stronger band in the TRPC6-transfected cells. These data argue against the conclusion that synaptopodin expression is reduced by TRPC6. Furthermore, the uncropped Western blot shows no band at 44 kDa which is in contrast to the originally published data, where very strong bands at 44 kDA are shown in several figures.

(3) In contradiction to statements by Jiang *et al*. in their response, none of the cited references 10–14 used U73122 to inhibit OAG-induced TRPC6 activation. These studies used U73122 to inhibit Ca^{2+} responses to histamine or angiotensin II, which act upstream of PLC. Although the study by Estacion *et al*. suggested a potential direct effect of U73122 on TRPC6, the study by Ju *et al*. found that 'U73122-induced inhibition of anti-PIP2 antibody-evoked TRPC6/C7 channel activity was overcome by co-application of 10 μM OAG, which rules out the possibility that U73122 was acting as a direct channel blocker'.

(4) Although cultured, differentiated, conditionally immortalized podocytes were described as arborized, process-bearing cells, these do not usually display such extremely enlarged processes as shown in the publication by Jiang *et al*. The significance of such morphological characteristics remains to be determined.

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