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EDITORIAL

# Transfection reagents affect Extracellular Vesicle cargo transfer to recipient cells: The importance of appropriate controls in EV research

In this issue of Journal of Extracellular Vesicles McCann et al., 2022 as well as McConnell et al., 2022 have carefully dissected the effect of transfection complexes (TCs) on extracellular vesicle (EV) cargo transfer, and found TCs to be a culprit behind artefactual delivery results (McCann et al., 2022; McConnell et al., 2022). This methodological problem was identified both in a Cre-LoxP reporter cell system, as well as in the transfer of EV RNA cargo to recipient cells.

EVs have gained a lot of interest the last decade for their use as a potential system for nonviral intracellular delivery of advanced molecules. To generate modified EVs, transfection reagents are frequently used for endogenous engineering of the EVs (Gupta et al., 2021). However, up until today, transfection procedures have not been reported as a method that can influence engineered EVs and produce artifacts. However, since TCs are similar in size and reside in endosomes and the extracellular space, similar to EVs during their biogenesis, release and uptake, there is a clear possibility that TCs can influence the functionality of EVs as well as contaminate EVs isolated from transfected cells.

McConnel et al. attempted to recapitulate published data concerning Cre delivery to recipient Cre-LoxP reporter cells. Earlier reports have shown high (Yim et al., 2016) as well as low delivery of Cre protein by EVs (Sterzenbach et al., 2017). By fusing Cre to the EV loading domain of BASP-1, the authors generated Cre loaded EVs isolated by ultracentrifugation (UC), which were added to Cre reporter cells. The Cre loaded EVs produced few recombination events, but clearly over background, similar to previous reports (Sterzenbach et al., 2017). The reporter cells were additionally treated with chloroquine, which increased the number of positive cells 5-fold. Chloroquine has numerous effects on cells; however, one reported effect is the rupture of endosomes which leads to an increased endosomal escape of compounds from the endosomal-compartment. However, if the EVs remain intact inside the endosomes chloroquine most likely do not increase the delivery of EV associated Cre, due to the fact that Cre would still remain inside the EVs after endosome rupture. To increase EV mediated delivery a compound has to either induce membrane fusion or induce the rupture of both the endosome and the EV membrane simultaneously. Hence, this indicated that the Cre delivery was not EV mediated, however, since chloroquine has several effects no clear conclusions could be made.

To further elucidate if bona fide EVs delivered Cre to the recipient cells the authors applied an Optiprep gradient to separate any contaminations and Cre loaded EVs. Cre protein was found almost exclusively in the EV fraction, however, the recombination activity in the reporter cells was significantly higher in the non-EV fractions with no or trace amounts of Cre protein, indicating again that Cre is not delivered by the EVs. Moreover, the coisolation of TCs and EVs by UC was observed for several different transfection reagents, which highlights this to be a universal phenomenon for TCs. Lastly, McConnel et al. demonstrated that neither media change nor DNAse treatment removed the TCs.

McCann et al. on the other hand speculated that TCs can interfere with the function of small EVs (sEVs), since TCs reside in the same cellular compartments. To examine this the authors investigated the size of different TCs and found them to be of similar size as sEVs for all transfection reagents tested. The TCs were additionally isolated by UC corroborating the findings by McConnel et al. Since TCs and EVs reside in the same endolysosomal compartments as exosomes during their biogenesis, there is moreover a risk that the TCs and exosomes are fusing. However, the authors could conclude after several experiments that fusion did not occur or occurred at a level below the detection limit of their assays.

The authors then demonstrated that neither EV protein markers or the levels of miR-15b and miR-106a per particle changed after transfection demonstrating that the TCs are a minority of the isolated particles after isolation by UC. However, since each TC can contain large amounts of siRNA, they can still contribute to a large part of the siRNA found in the sEV pellet. By utilizing Optiprep gradients the majority of the siRNA found after UC was indeed shown to originate from the TCs, however, siRNA was additionally found in the sEV fraction, indicating loading into the EVs did occur.

Next McCann et al. investigated if the transfer of EV cargo to recipient cells is affected by TCs. First, sEVs derived from a cell source stably expressing superoxide dismutase 1 (SOD1) siRNA in the pre-miR-451 backbone was compared with sEVs from cells transfected with SOD1 siRNA. The pre-miR-451 backbone has been shown to mediate EV loading and here the authors changed the native targeting sequence of pre-miR-451 to instead targeting SOD1 (Reshke et al., 2020). The sEVs derived from stable cells induced knockdown of the SOD1 mRNA at doses 10-fold lower than the transfected counterpart in mixed motor neurons, despite normalizing the input to siRNA amounts. This could be due to the fact that contaminating TCs do not transfect motor neurons with a high efficiency. By contrast, the effect can alternatively be a result of reduced ability of the sEVs to deliver their cargo due to the TCs affecting their biogenesis and lipid content. To further examine whether the transfection procedure could reduce the delivery capabilities of isolated sEVs, the authors performed several clever control experiments. Again, the authors used sEVs loaded with siRNA by engineering of the pre-miR-451 backbone. However, here sEVs were harvested from stable cells expressing an alpha-synuclein siRNA in the pre-miR-451 backbone, and the cells were either transfected with a control siRNA or left untransfected. Surprisingly, the sEVs from transfected cells did not mediate any significant target knockdown in recipient cells, while the sEVs from untransfected cells promoted robust knockdown in all experiments. This was true for all three transfection reagents tested: RiboJuice, INTERFERin and RNAiMax. The reduced knockdown efficiency could not be explained by a reduced loading of siRNA into the vesicles, since the copy number of loaded siRNA into the EVs were not significantly reduced by transfection of control siRNA. In summary, the transfection procedure appears to affect the ability of sEVs to transfer their siRNA cargo. However, how the EVs are affected is not further elucidated in the article. If these findings can be substantiated by others, and with other types of cargos, it has vast implications for the EV field, since most studies where transfection has been utilized to manipulate EV source cells, have to be seen in a new light.

McConnel et al. took advantage of the Cre-LoxP-reporter system, which is highly sensitive since, in theory, one functional Cre molecule induces full expression of the reporter-gene after delivery to a cell. Hence, the dynamic range for a single cell is either on or off. The knockdown of mRNA targets by siRNA in recipient cells used by McCann et al. are likewise sensitive, however, not at all as sensitive as the on/off switch of the Cre-LoxP reporter cells. The Cre assay and similarly the CROSS-FIRE reporter system for CRISPR guide-RNA (de Jong et al., 2020) on/off nature detection needs to be considered when the assays are used. The reporter systems are likely more sensitive than most detection assays in the lab, thus it can be hard to rule out contaminants that cause artifacts in the reporter cells, if not the right controls are used.

Previous studies have shown high rates of transfer of Cre to recipient reporter cells contrary to the findings by McConnel et al. (Yim et al., 2016). However, in the studies showing high recombination rates after EV delivery, other loading motives and EV producing cells were used and it is therefore hard to make direct comparisons between the studies.

The observation by McCann et al. that transfection complexes appear to abolish the ability of EVs to transfer their cargo to recipient cells is a surprising and important finding. It will be essential to additionally investigate how TCs effect other EV cargos, such as protein and mRNA, as well as the role of the producing cells and what may happen in subpopulation of EVs. The diminished effect observed after transfection can be due to a disruption of the biogenesis of the sEVs by the TCs in the producer cells or an effect on the recipient cells by the coisolated TCs. It would have been interesting if the authors spiked in TCs into harvested sEVs to examine if the mere presence of TCs together with the sEVs abolished the transfer of the siRNA to recipient cells. The reduced EV transfer of cargo after transfection can moreover explain the lack of transfer of Cre protein by the EVs in McConnel et al., despite large amount of loaded Cre protein in the EVs. It should be highlighted that the effects observed by McCann et al. may not be generalized for any transfection reagent but appear valid for all transfection reagents tested in the study. Therefore, as the authors conclude in both articles, it is strongly recommended to use stable expression systems when studying EV biology and delivery when possible.

In summary, both of these studies clearly demonstrate that TCs and sEVs are coisolated by UC and that the isolated TCs have effects on EV function and EV uptake in recipient cells, thus further highlighting the importance of rigorous controls and characterization of the isolated EVs before drawing excessive conclusions from EV experiments.

## CONFLICT OF INTEREST

Joel Z. Nordin has stock interest and is a consultant for Evox Therapeutics.

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