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Response to "Technical approaches to reduce interference of Fetal calf serum derived RNA in the analysis of extracellular vesicle RNA from cultured cells"

Dear Editor

We read with great interest the article by Driedonks and colleagues (2019) on "Technical approaches to reduce interference of fetal calf serum derived RNA in the analysis of extracellular vesicle RNA from cultured cells" [1]. We would like to comment on our experience utilizing FBS-EV "depletion" protocols, in the hope that such a dialogue might be constructive to others investigating EVs derived from cultured cells, and the future evolution of FBS-EV "depletion" protocols.

Foetal bovine serum (FBS) extracellular vesicle (EV) contamination in FBS-supplemented culture media is of great concern to the EV-field, especially those assessing *in vitro* EV (and exosome) cargo composition and characterization from cultured cells [2]. We recognize the valiant effort put forth by our colleagues, modifying existing EV "depletion" protocols [3] and maximizing reductions in residual FBS-EVs (and RNA species) within supplemented culture media. Our concern remains, however, that despite the authors' technical modifications (i.e. adjusting serial serum dilutions and supernatant removal techniques – decant versus pipette) of existing protocols, there remain significant quantities of FBS-EVs (and RNA species) present in the supernatant used for culture that is difficult to isolate and remove. Using nanoparticle tracking analysis with ZetaVIEW®, our group has shown that a large population (10^9 EVs/ml) of residual FBS-derived EVs persist in EV- "depleted" FBS using a similar 18-h ultracentrifugation protocol (20% FBS dilution, 100,000 ×*g* with an SW41 Ti Rotor, *k* factor = 124) [4]. Additionally, previous reports have demonstrated that FBS EVs may alter phenotype (and likely genotype) of cultured cells [5–8]. We have also shown that FBS-EV-"depleted" media provides suboptimal conditions for primary astrocyte growth and viability in culture [4].

Thus, these contaminant EVs not only influence the ability to characterize the EVs isolated from conditioned *in vitro* culture media but also may affect the underlying cellular processes of those cells, including EV release dynamics and EV cargos. More importantly, our field is at the mercy of existing technological capabilities. There remain relevant issues regarding limits of detection in available technologies that reportedly quantify differences at the nano (10^{-9}) scale [9,10]. Further, current technologies available for EV quantification (e.g. NanoSight, ZetaVIEW, qNano, High-Resolution Flow Cytometry) produce inconsistent and unreliable results, varying across institutions and laboratories [11,12]. Therefore, we contend that it remains impossible to confidently assert that using existing methodologies for FBS EV-depletion (i.e. ultracentrifugation [3], chemical-based precipitation, size-exclusion chromatography, ultrafiltration [13]) can reduce the number of FBS EVs present within the resultant supernatant used to supplement culture media to non-confoundable levels.

Based on our experience and perspective, we would ask the EV-research field to reconsider the continued use of FBS EV-"depletion" protocols for *in vitro* EV (and exosome) analyses, and increase the focus towards serum-free media alternatives [14,15]. Advancing towards utilizing serum-free media, supplemented with a necessary cocktail of growth factors to support *in vitro* cell growth, may result in a better indication of both physiologic and pathobiologic cellular processes, in the absence of influential exogenous EVs [16]. Such an approach may allow for more relevant understandings of differential EV cellular dynamics and cargos within specific cell types, leading towards novel diagnostic and/or therapeutic health-care options [17].

Disclosure statement

Dr. Fiandaca reports intellectual property related to exosomes as diagnostics. No additional potential conflicts of interest were reported by the authors.

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