Development of a 3D HepG2 suspension culture system to enable reducible investigations into 2D vs. 3D HepG2 culture-derived sEV biophysical properties and cancer pathway-related miRNA content

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Background & Innovation

Small extracellular vesicles (sEVs), otherwise known as exosomes, are nanoscale vesicles that cells release to communicate and affect their local and remote microenvironments. Cancer cells specifically use their sEVs to enable tumor survival, including inactivation of anti-tumor macrophage immune responses. For sEV studies *in vitro*, 2D cells grown in adherent culture are typically used to manufacture sEVs. To more closely simulate *in vivo* tissues, human hepatocellular carcinoma cells (HCCs) can be grown in a 3D ECM as spheroids. However, the ECM-based method is technically challenging as well as time-consuming since it is incredibly difficult to grow individual spheroids for the purpose of extracting scalable numbers of sEVs for downstream applications. To address this issue, we developed a unique 3D suspension spheroid culture system. 3D suspended spheroid culture is expected to more accurately recapitulate the *in vivo* 3D scenario minimizing the negative aspects of using 3D matrix-based cell culture for sEV production.

Objective

The objective was to evaluate biophysical and cancer pathway-focused miRNA content differences between HepG2 sEVs produced by 2D adherent vs. 3D spheroid suspension culture.

Methods

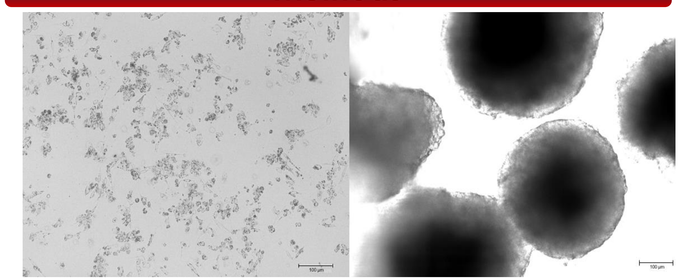

Figure 1. HCC HepG2 culture cells. Traditional 2D adherent cells (right) and 3D spheroid suspension cells (left) grown for 7 days. Scale bar = 100 µm

- sEVs were isolated from 2D adherent and 3D suspension cell culture using differential ultracentrifugation.
- Images of 2D and 3D cells were obtained using a ZOE Cell Imager after culture in DMEM with 10% FBS media at 37°C and 5% CO2.
- BCA protein assay was performed on sEVs extracted from 2D and 3D cells and compared to BSA standards to determine sEV yield in terms of protein concentration (µg/ml).
- Nanoparticle tracking analysis was used to assess sEV biophysical characteristics, including size and zeta potential, a stability indicator.
- RT-qPCR was performed to determine fold regulation of 2D and 3D derived sEV miRNAs compared to their source cells and each other.

Results

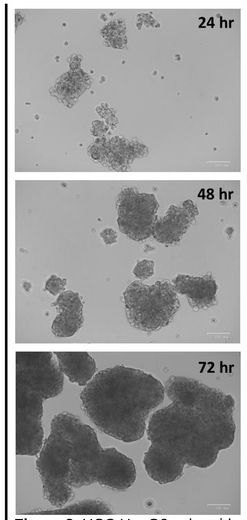

Figure 2. HCC HepG2 spheroid suspension culture cell growth over 72 hrs. Scale bars = 100 µm

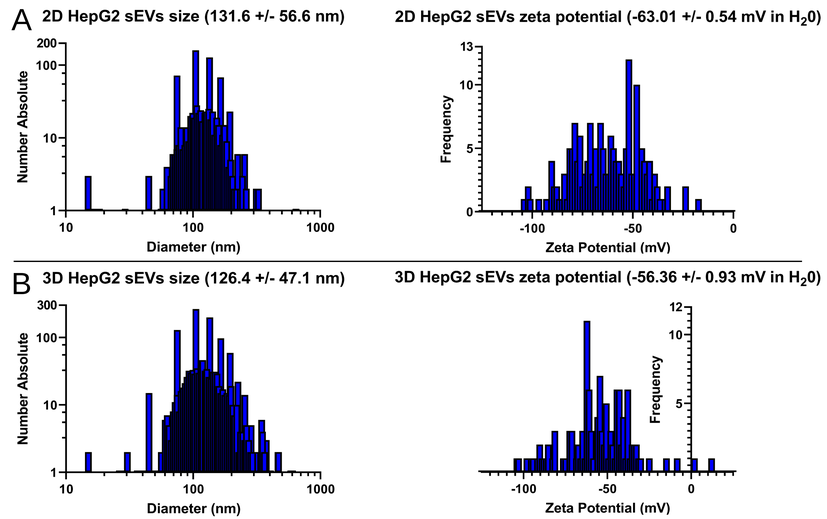

Figure 3. Assessment of 2D adherent vs. 3D spheroid suspension culture derived HepG2 sEV biophysical characteristics. (A) 2D adherent cell culture derived sEV size and zeta potential distributions. (B) 3D spheroid suspension cell culture derived sEV size and zeta potential distributions.

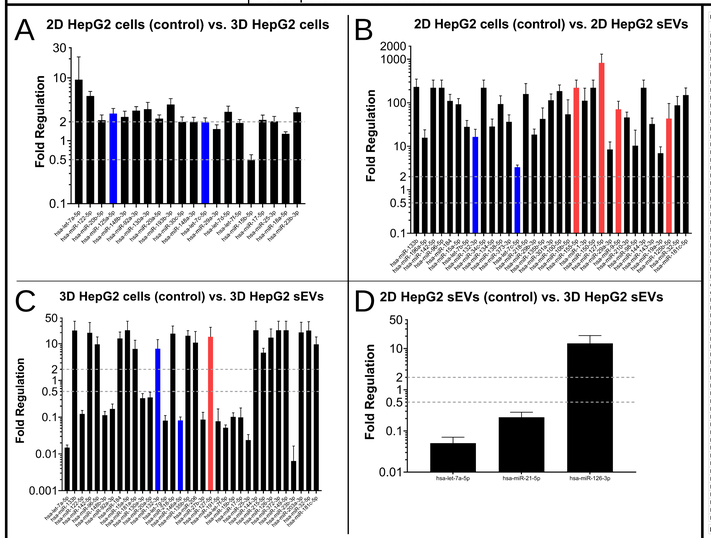

Figure 4. A comparison of cancer relevant pathway miRNA content found within sEVs obtained from 2D adherent versus 3D spheroid suspension cultured HepG2 cells. miRNA content was determined by RT-qPCR for (A) 2D HepG2 cells (control) vs. 3D HepG2 cells, (B) 2D HepG2 Cells (control) vs. 2D HepG2 sEVs, (C) 3D HepG2 cells (control) vs. 3D HepG2 sEVs, and (D) 2D HepG2 sEVs (control) vs. 3D HepG2 sEVs. Blue and red shaded bars denote known immunosuppressive (M2) and proinflammatory (M1) miRNA inducers of macrophage function, respectively. n = 3 replicate arrays for 3 pooled batches of sEVs. Error bars = SD, * = p </= 0.05, and was considered statistically significant. Student's t-test was used to compare groups. Only statistically significant (p </= 0.05) miRNAs upregulated or downregulated approximately 2-fold are shown.

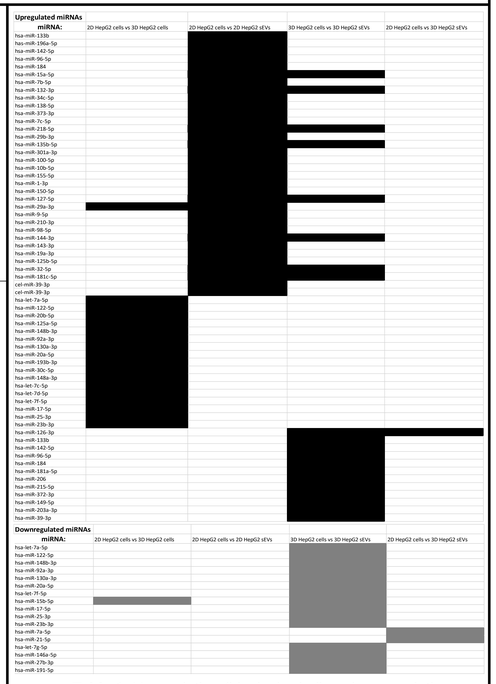

Table 1. Upregulation (black shaded) or downregulation (gray shaded) of miRNAs identified in Figure 4.

Summary & Conclusions

Cell culture brightfield images demonstrated that 3D suspension cultured HepG2s have a markedly different morphological appearance than 2D cells. The 3D cells are clustered in spheroids ranging from 300 to 500 µm.

2D adherent and 3D spheroid suspension culture-derived HepG2 sEVs do not differ significantly in terms of size and zeta potential. With zeta potentials < -30 mV, both sEV types exhibit moderate resistance to vesicle aggregation.

2D adherent and 3D suspension cultured HepG2 sEVs were found to be differentially enriched in various cancer pathway-related miRNAs vs. their source cells. For example, four proinflammatory M1 macrophage inducing miRNAs (miR-155-5p, miR-127-5p, miR-9-5p, miR-125b-5p) vs. two immunosuppressive M2 macrophage inducing miRNAs (miR-132-3p, let-7c-5p) were upregulated in 2D HepG2 sEVs vs. their source cells. In contrast, only M1 miRNA, miR-127-5p, and M2 inducing miR-132-3p were upregulated while M2 inducing miR-146a-5p was downregulated in 3D HepG2 sEVs vs. their parent cells.

A comparison of 2D vs. 3D sEVs revealed differences in the enrichment of let-7a-5p (decreased in HCC *in vivo*), miR-21-5p (enables HCC drug resistance), and miR-126-3p (impairs HCC tumor volume *in vivo*). let-7a-5p and miR-21-5p were downregulated, while miR-126-3p was upregulated in 3D sEVs compared to 2D sEVs (control) respectively.

Overall, similarities and differences between 2D and 3D sEV miRNA content were observed relevant to HCC pathogenesis. The 3D HepG2 spheroid suspension model provides an additional reducible level of HCC sEV investigation to augment traditional 2D sEV studies while better simulating an *in vivo* 3D source for HCC sEVs.

Significance and Impact

One significant aspect of this study is that our 3D spheroid suspension model used in conjunction with typical 2D culture, might be used to streamline the identification of candidate HCC sEV-based biomarkers and therapeutic targets for HCC and other cancers. This will be achieved by 1. better simulating the 3D tumor mass microenvironment for sEV production and functional studies, and 2. more accurately predicting functional miRNA and other sEV content. This 3D model can further be adapted for the exploration of other sEV types or multi-omics approaches to facilitate the study of cancer and other diseases.

Acknowledgements

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