Imaging Analysis of 3D Culture of Human Hepatoma Cells

Samuel Wong, Nicole Wong, Shwe Hlaing Win, Christina Kuemerle, Sona Avanesyan, Pujan Dobaria, Tin Aung Than

Dept of Medicine, University of Southern California, Los Angeles, CA , USA

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USC University of Southern California

Abstract

3D culture of hepatocytes in extra cellular matrix forms polarized spheroid or organoid structures. Clear differences are seen in the cellular morphology and the localization and distribution of cell markers between monolayer and organoid cultures. Furthermore, hepatocyte spheroid or organoids can adopt either a proliferative or a metabolic state depending on the culture conditions. The long-term organoid culture of murine and human primary hepatocytes method has been described before. We used human hepatoma cells HepG2 and mouse hepatocyte AML-12 cells to culture 3D spheroid. Cells were maintained in conventional 2D culture and transferred to spheroid culture condition. AML-12 cells well formed spheroids on day 3 but lost the 3D spheroid morphology on day 7. Morphology of spheroids were examined by nuclear staining with hematoxylin. We found that 3D spheroid formed consistently similar size and shape in low-attachment petri dish, and the method delivered sufficient number of spheroids for experimental set up such as mitochondrial respiration (Seahorse) measurement, hepatocyte toxicity and hepatic stress response studies in future.

Objective

The objective of this study is to define the suitable method for spheroid culture of mouse and human hepatocytes in liver disease studies.

Background

The 3D tissue/cell culture is a technique utilized in embryology and microbiology to allow the growth that would otherwise be restricted by the 2D flat plane of culture dishes and also to minimize the surface area to volume ratio, slowing evaporation. Hanging drop culture to generate 3D spheroid cells was first adapted to culture nerve cells and first to observe the development of growth cones 100 years ago. Recently the 3D spheroid culture has been adapted to hepatocyte culture for drug screening and discovery. Drug toxicity testing on primary mouse and human hepatocytes in spheroid culture predicts better than conventional flat plane culture methods. Therefore, to establish the spheroid hepatocyte culture, we used human hepatoma cell, HepG2 and mouse hepatocyte cell, AML-12. Here we demonstrated the establishment of spheroid AML-12 cell culture in hanging drop method and low attachment petri dishes.

Hanging Drop Method

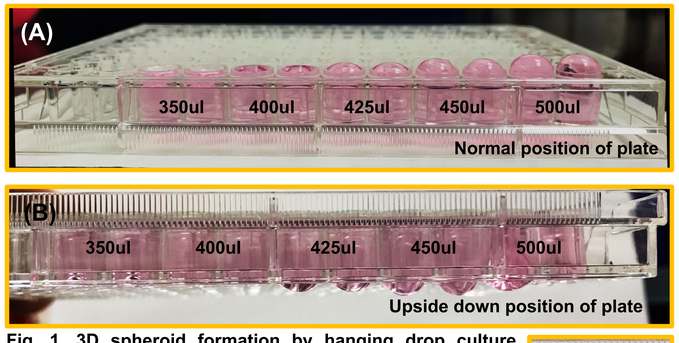

Fig. 1. 3D spheroid formation by hanging drop culture method. AML-12 cells in growth medium DMEM/F12 with supplements of fetal bovine serum, ITS, dexamethasone and penicillin/streptomycin were cultured in cell culture dishes. Cell were detached and collected using TE buffer. Single cell suspension in low concentration were transferred to 96 well assay plates at different volumes 350-500ul **(A)**. Plate was quickly inverted upside down **(B)** and continue in upside down plate position for 24 hours. Hanging drops (arrow pointed) are formed in 450ul group **(C)** and then plate was returned to normal position after 24hrs culture. Cells accumulated in the drops forms spheroids and continue to grow as spheroids **(D)** which is fixed in 1% neutral buffer formalin and nucleus was stained by hematoxylin (dense stained purple color).

3D Low Attachment Method

In addition to hanging drop method, AML-12 cells in growth culture medium were cultured in low attachment petri dishes which we expected that cell-attachment to the dish was prevented, and spheroid formation was facilitated.

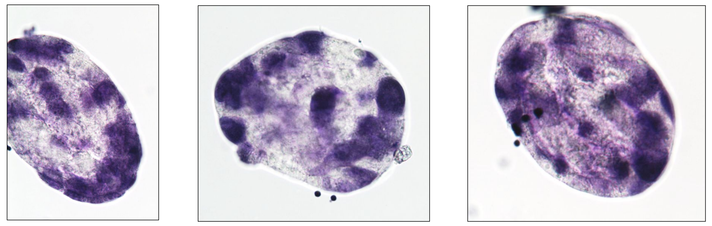

Fig. 2. 3D spheroid formation in low-Attachment Petri Dishes. AML-12 cells in growth medium DMEM/F12 with supplements of fetal bovine serum, ITS, dexamethasone and penicillin/streptomycin were cultured in cell culture dishes. Cell were detached and collected using TE buffer. Single cell suspension in low concentration were transferred to petri dishes. Cell growth continue from single cell to multiple cells and forms spheroids on day 3 (shown above). Spheroids tethered to the dish culture surface, but spheroids were easily detachable from the dish and were rolling in the medium. Spheroids were collected and fixed in 1% neutral buffer formalin and nucleus was stained by hematoxylin (dense stained purple color).

Spheroid size correlates with cell concentration

Next, to determine the culture condition generating spheroids, AML-12 cells in cell culture dishes were detached and collected using TE buffer, and different volume (0.5, 1, 3 ml) of single cell suspension was transferred to petri dishes to culture the cells at different cell concentration. As shown below different size of spheroids were formed on day 3.

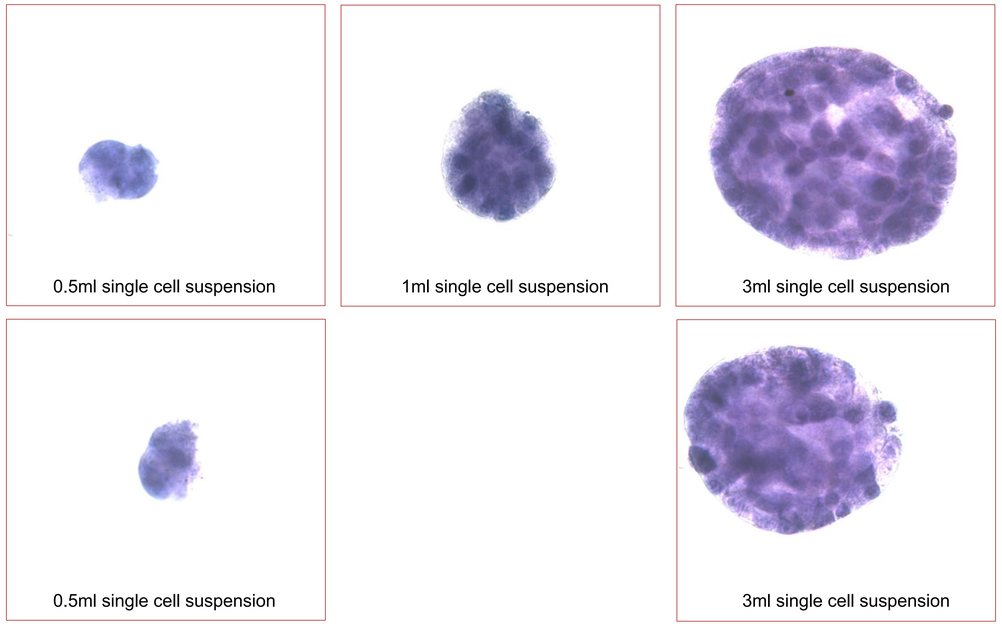

Fig. 3. Spheroid size varies with seeded cell concentration. AML-12 cells in growth medium DMEM/F12 with supplements of fetal bovine serum, ITS, dexamethasone and penicillin/streptomycin were cultured in cell culture dishes. Cell were detached and collected using TE buffer. Various volume (0.5, 1, 3 ml) of single cell suspension were transferred to petri dishes to culture the cells at different cell concentration. Spheroids were collected and fixed in 1% neutral buffer formalin and nucleus was stained by hematoxylin (dense stained purple color). Images were taken at same magnification.

Spheroid microenvironment enhances attachment

Spheroids were continued to culture and on day7 spheroid formation was lost and cells grew on flat plane. The number of spheroids lost is highest in high-cell density dish (3ml dish) and lowest in low-cell density dish (0.5ml dish). The number of 2D transformed spheroids were highest in high-cell density dish (3ml dish) and lowest in low-cell density dish (0.5ml dish).

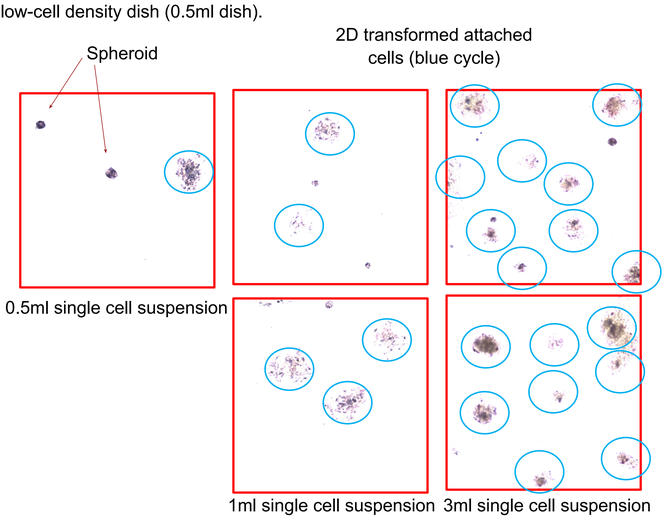

Fig. 4. Spheroid size determines the spheroid microenvironment which enhances cell attachment on petri dish and 2D transformation of spheroid. AML-12 cells in growth medium DMEM/F12 with supplements of fetal bovine serum, ITS, dexamethasone and penicillin/streptomycin were cultured in cell culture dishes. Cell were detached and collected using TE buffer. Various volume (0.5, 1, 3 ml) of single cell suspension were transferred to petri dishes to culture the cells at different cell concentration. On day 7 cells were fixed in 1% neutral buffer formalin and nucleus was stained by hematoxylin (dense stained purple color). Images were taken at same magnification. Spheroids were arrow pointed. 2D transformed cells are circled blue.

Summary

- Spheroids formed in both hanging drop and low-attachment petri dish methods.
- Culturing in Low-attachment petri dish methods delivered sufficient number of spheroids for experimental set up such as mitochondrial respiration (Seahorse) measurement, hepatocyte toxicity and hepatic stress response studies during spheroid formation period day 3 to day 7.
- AML-12 spheroid created the spheroid microenvironment which changed the spheroid 3D to 2D formation in longer culture.

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CONTACT US

samw3223@gmail.com, tthan@usc.edu