The effect of fibroblast co-culture on HepG2 growth

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Introduction

Traditionally, in vitro cell cultures are monocultures of a single cell type. However, in vivo, cells interact with many different cell types either via direct cell-cell contact or via chemical signaling (via e.g. cytokines or growth factors). In order to mimic natural interactions between cell populations or to improve culturing success for certain cell types, co-culture setups can be used. In co-culture setups, two or more different cell types are grown with some degree of (physical or chemical) contact between them.

Hepatocytes, for example, are cells that can be difficult to culture in monocultures, as they often have limited viability or lose their liver-specific functions[1]. Co-culture with nonparenchymal cells like fibroblasts can prolong hepatocyte viability and improve liver-specific functions such as urea production and albumin secretion[2–5]. Bhatia et. al. (1998) have shown that liver-specific functions were significantly improved at co-culture rates with twice or more the amount of fibroblasts compared to hepatocytes[2]. Cho and colleagues have confirmed this in 2010 when using a hepatocyte to fibroblast ratio of 1:3[3]. When comparing the secretion of liver-specific molecules, hepatocyte cell growth should also be taken into account as an increase in hepatocyte proliferation equals in a higher total secretion.

Cell growth can be determined in co-culture setups by simply counting the different cell types at the end of the experiment. However, when certain functionalities of the co-culture setup (such as molecule secretion) are measured multiple times throughout the culture period it is more accurate to determine cell growth in parallel with the measurement. Fluorescence live-cell imaging of the cells labeled with different fluorophores can be an easy solution to monitor cell growth in real-time inside co-culture setups.

One of the current challenges with fluorescent live-cell imaging is to maintain the cells at proper culture conditions (e.g. temperature, CO_2-level, humidity). The CytoSMART Lux3 FL is a small fluorescent live-cell imaging microscope that can be placed inside a cell culture incubator to ensure optimal culture conditions. In this proof-of-concept study, we demonstrate the use of fluorescent live-cell imaging to determine the effect of different ratios of fibroblasts on hepatocyte growth. The CytoSMART Lux3 FL was used to determine confluence of HepG2 cells (hepatocytes) and 3T3 cells (fibroblasts) in time-lapse movies of co-culture setups with different ratios of HepG2 cells to 3T3 cells.

Material and methods

HepG2 cells were cultured in HepG2 medium consisting of Advanced DMEM (Invitrogen) supplemented with 10% FBS (Gibco), 1% penicillin-streptomycin (Gibco) and 1% non-essential amino acids (Gibco). 3T3 cells were cultured in 3T3 medium consisting of Advanced DMEM supplemented with 10% FBS and 1% penicillin-streptomycin.

After culture, cells were washed with PBS (Gibco) and stained with 8 µM CellTracker Orange (HepG2; Invitrogen) or 8 µM CellTracker Green (3T3; Invitrogen) in serum-free medium by incubation for 30 minutes at 37°C. Cells were washed again with PBS, trypsinized and counted using the Corning Cell Counter. Cells were subsequently seeded in a 6-well plate at a ratio of 1:1 with a density of respectively $2.1 \cdot 10^4$ cells/cm^2 HepG2 and $2.1 \cdot 10^4$ cells/cm^2 3T3 cells or at a ratio of 1:3 with a density of respectively $2.1 \cdot 10^4$ cells/cm^2 HepG2 and $6.3 \cdot 10^4$ cells/cm^2 3T3 cells. The co-cultures were cultured in HepG2 medium to accommodate the culture requirements of both HepG2 and 3T3 cells.

Approximately 2 hours after seeding, the samples were placed on the CytoSMART Lux3 FL inside a cell culture incubator (37°C, 5% CO_2). Brightfield, green and red fluorescent images were taken every 30 minutes for 24 hours. The images were uploaded to the CytoSMART Cloud were the confluence (percentage area covered by cells) was determined for all three channels. The data was downloaded from the CytoSMART Cloud to normalize the confluence compared to the confluence at the first timepoint. This resulted in a relative increase in confluence over time.

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Results

At the start of the time-lapse experiment, both HepG2 and 3T3 cells were attached and started to spread (Fig. 1). Over time, 3T3 cells steadily proliferated with a similar rate in both the 1:1 (HepG2 : 3T3) co-culture ratio sample and the 1:3 co-culture ratio sample as seen by the similar trend in confluence increase (Fig. 2A). Normalized HepG2 confluence showed that the presence of three times the amount of 3T3 cells compared to HepG2 cells had a positive effect on HepG2 proliferation (Fig. 2B). The normalized confluence of the 1:3 co-culture ratio sample immediately increased from the start of the experiment until it reached a 1.64 fold increase after 24 hours. The normalized confluence of the 1:1 co-culture ratio sample, on the other hand, remained approximately stable for the first 6 hours. Thereafter it steadily increased to a 1.24 fold increase after 24 hours.

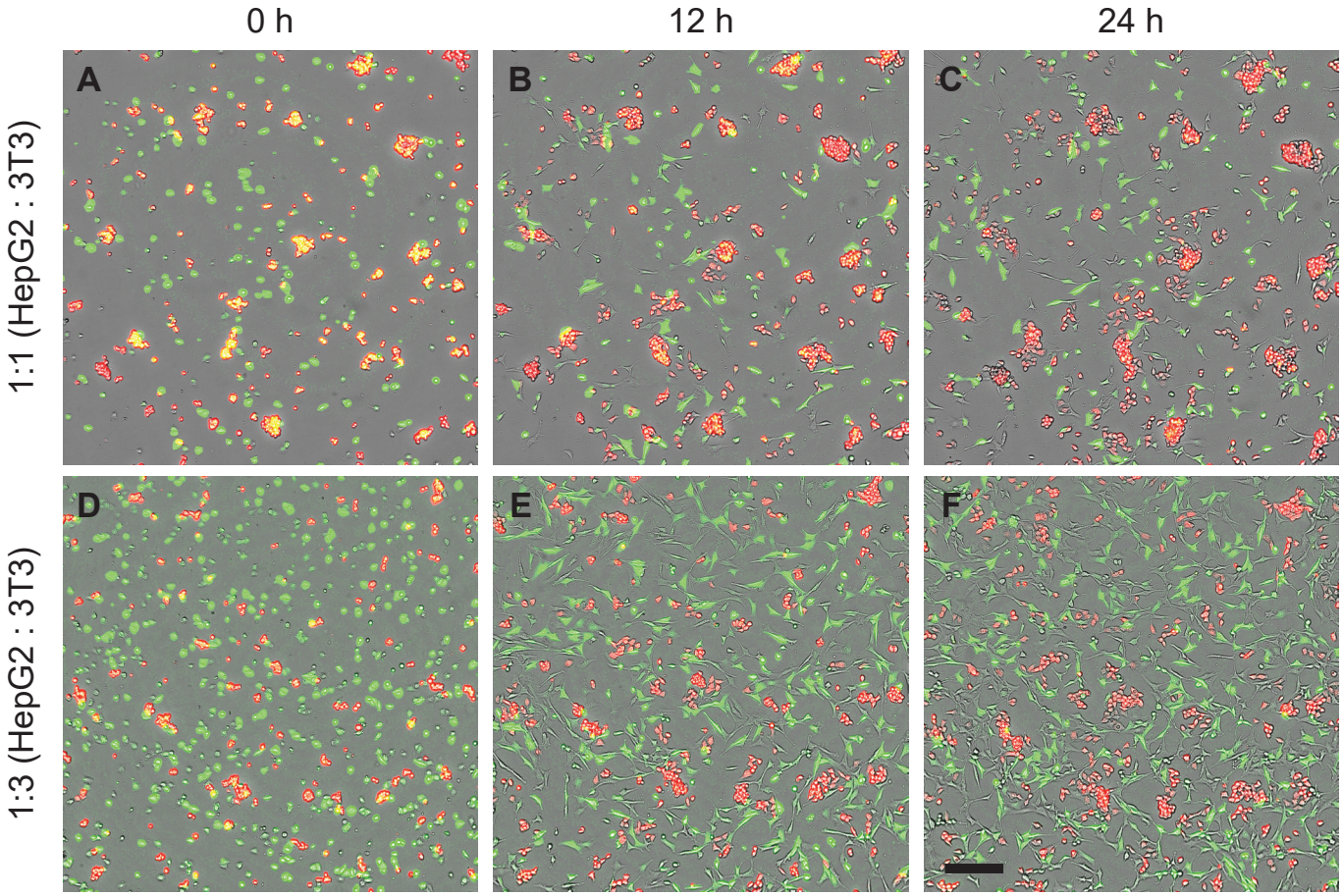

Figure 1. Overlay of brightfield, red and green fluorescent images of HepG2 and 3T3 co-cultures at 0 (A, D), 12 (B, E) and 24 h (C, F). A-C) Co-culture at 1:1 ratio of HepG2 (CellTracker Orange; red fluorescence) to 3T3 cells (CellTracker Green; green fluorescence). D-F) Co-culture at 1:3 ratio of HepG2 to 3T3 cells. Scalebar represents 200 µm and applies for all images.

Discussion

In co-culture setups, cell proliferation is often determined at the end of the experiment. However, measuring cell proliferation throughout the entire co-culture experiment can provide valuable insights into the effect of cell-cell interactions on cell growth. Fluorescent labelling of the different cell types used in a co-culture experiment can help to easily distinguish the cell types from each other. When combined with automated time-lapse imaging, the change in confluence of each cell type can be determined throughout the entire experiment. In this proof-of-concept study, the CytoSMART Lux3 FL was used to investigate the effect of the amount of 3T3 cells co-cultured with HepG2 cells on HepG2 cell proliferation.

We have shown that the 3T3 cells had a similar rate in confluence increase in both the 1:3 and 1:1 co-culture samples. On the other hand, when HepG2 cells were cultured with 3T3 cells at a 1:3 ratio, the confluence of HepG2 cells increased more rapidly in 24 hours compared to the confluence of HepG2 cells at a co-

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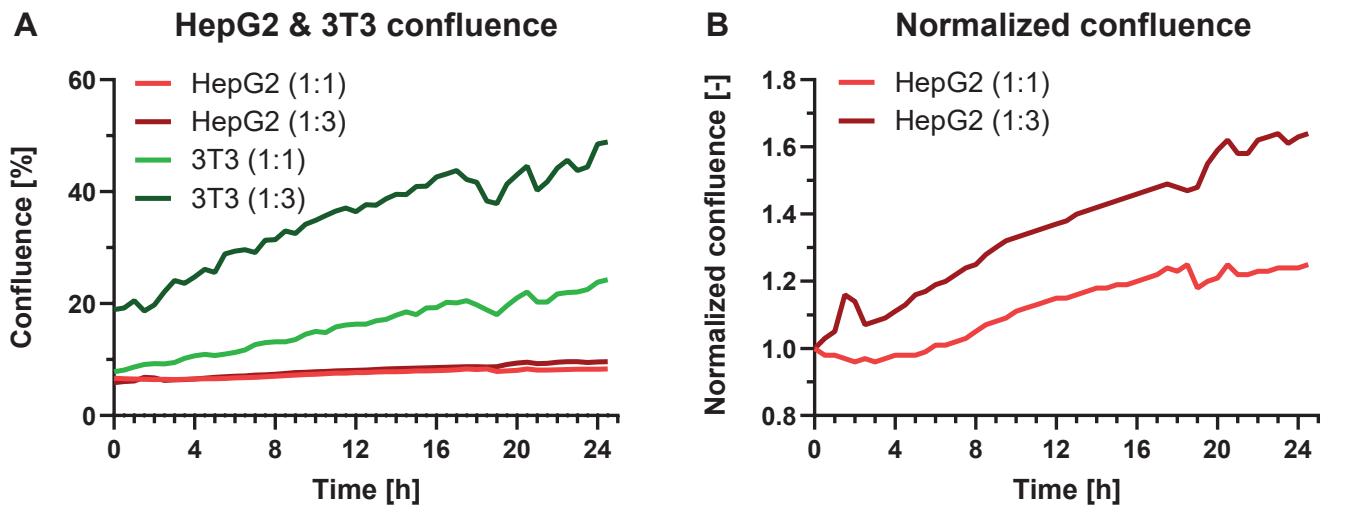

Figure 2. A) Confluence increase of the red fluorescence channel (HepG2 cells) and green fluorescence channel (3T3 cells) at a co-culture ratio of 1:1 (HepG2 : 3T3; light colored lines) and 1:3 (HepG2 : 3T3; dark colored lines). B) Normalized increase in confluence of HepG2 cells seeded at a co-culture ratio of 1:1 (HepG2 : 3T3) and 1:3 (HepG2 : 3T3).

culture ratio of 1:1. This higher increase in HepG2 confluence is likely related to the increased cell-cell interactions with 3T3 cells compared to the 1:1 co-culture ratio sample. Previously it has been shown that the loss of liver-specific functions over time in monocultures was related to a decrease in hepatocyte number[4]. While the number of hepatocytes, and thus also the liver-specific functions, were maintained over time in the co-culture setup[4]. This supports the hypothesis that the increase in HepG2 confluence is related to the number of 3T3 cells they can interact with.

The findings in this study stress the importance of monitoring cell confluence throughout co-culture experiments since improvements in cell-specific functionalities can not only be caused by culture together with a different cell type, but also by improved viability or proliferation.

Conclusion

In this study, we have shown a proof-of-concept of monitoring changes in cell confluence throughout an entire co-culture experiment using fluorescent live-cell imaging. By labelling the different cell types with different fluorophores, the cells could be easily distinguished. Next to this, the integrated image analysis of the CytoSMART Lux3 FL enabled automated confluence measurements for all channels (brightfield, red, green) simultaneously.

References

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