ORIGINAL ARTICLE

Establishment of Serum Free Chemically Defined Culture Medium to Study the Interactions Between Colonic Myofibroblasts and Colorectal Cancer Cells

Marahaini Musa[1], Djamila Ouaret[2], Walter F. Bodmer[2]

[1] Human Genome Centre, School of Medical Sciences, Universiti Sains Malaysia, 16150 Kubang Kerian, Kelantan, Malaysia
[2] Cancer and Immunogenetics Laboratory (CIL), Weatherall Institute of Molecular Medicine (WIMM), Department of Oncology, University of Oxford, OX3 9DS Oxford, United Kingdom

ABSTRACT

Introduction: A crucial factor in cell culture technology is the use of appropriate culture medium which can promote cell growth and cellular functions. Development of serum free chemically defined medium enables the researchers to conduct the experiment in a more controlled manner. Myofibroblasts of the tumour microenvironment drive the colorectal carcinogenesis. *In vitro* study of the tumour-myofibroblast interaction using serum free medium may give a better insight into potential treatment for colorectal cancer (CRC) in the future. This study aims to establish serum free chemically defined medium to study the interplay between myofibroblast and CRC cells. **Methods:** A myofibroblast-specific serum free culture medium named as M-CIL, was developed to study the interactions between myofibroblasts and CRC cell lines *in vitro*. The influence of substrate (collagen type I) and subculturing of cells under incubation with M-CIL medium were also analysed. The effect of M-CIL medium on CRC cell growth also was studied. Gene expression analysis using quantitative real time polymerase chain reaction on amine oxidase, copper containing 3 (*AOC3*) was conducted to investigate the effect of individual components of the medium on myofibroblasts. **Results:** M-CIL medium supports the proliferation of myofibroblasts and produce minimal effect on CRC cells' growth. Our data also shows the influence of M-CIL components on gene expression in myofibroblasts. **Conclusion:** M-CIL culture medium, which was designed with known and defined components, proved to be a suitable alternative to complete medium (DMEM + 10% FBS) for co-culture experiments of myofibroblasts and CRC cell lines.

Keywords: Culture medium, Serum free, Myofibroblast, Cancer, Colon

Corresponding Author:
Walter F. Bodmer, PhD
Email: walter.bodmer@hertford.ox.ac.uk
Tel: +4401865 222356

INTRODUCTION

An appropriate culture medium is one of the most important factors in cell culture technology. It supports cell survival and proliferation, as well as cellular functions, that directly determine the research outcome (1). Fetal bovine serum (FBS) is normally included in cell culture media as it contains many essential components that can support cell growth *in vitro*. Its components include a cocktail of growth factors, detected at various concentrations and provide essential supplements for cells growth. To date, Dulbecco's Modified Eagle Medium (DMEM) with addition of FBS is usually used to culture human fibroblasts (2). Despite its routine application in tissue culture, there is a lack of publications on specific constituents of FBS as they may vary between sources and batches of serum (3). Moreover, the usage of serum is associated with high possibility of viral, bacterial and endotoxin contamination as it is a by-product of livestock beef industry (4). Besides scientific issues, FBS application in cell culture also has been associated with ethical concerns particularly related to its collection method (5).

The invention of serum free, chemically-defined medium with addition of specific, known components which includes growth factors like platelet derived growth factor (PDGF), epidermal growth factor (EGF) and transforming growth factor beta 1 (TGFβ1) has enabled the study of cells in culture under more controlled conditions. Different formulations of serum free media have been suggested to culture various types of cells (6-8). These discoveries have significantly facilitated research of many disease models including cancer.

Colorectal cancer (CRC) is the third most commonly diagnosed malignancy globally (9). Rather than focusing on the cancer cells alone, many has started to investigate the role of tumour microenvironment component such as myofibroblasts in driving the CRC progression. Myofibroblast-cancer cell interplay drives

colorectal carcinogenesis (10). Myofibroblasts can be found in cancerous and normal tissues including lymph nodes, blood vessels, uterine submucosa and intestinal villous core (11, 12). These cells are characterized by positive expression of amine oxidase copper containing 3 (*AOC3*) (13). Accumulation of myofibroblasts in CRC cases is often associated with poor prognosis and recurrence of the cancer (14, 15).

Investigation into the nature of the tumour-myofibroblast interplay may therefore provide better insight on potential therapeutic avenues to eradicate the tumour (16). To ensure a more controlled experimental setup to study the interactions between myofibroblasts and CRC cells, a serum free medium would be a great advantage for use in such functional tests. It is essential to select an appropriate medium with defined components that is suitable to culture myofibroblasts as the presence of numerous unidentified growth factors in FBS may influence the properties of myofibroblasts.

In this paper, the establishment of a serum free chemically defined medium, known as M-CIL medium is described. The final formulation of M-CIL medium was optimized from modified Pharmaceutical Production Research Facility (PPRF) medium, which was designed based on PPRF-msc6 medium formulation, initially described in the literature as a medium that can support mesenchymal cell growth (17).

MATERIALS AND METHODS

Reagents and chemicals

Reagents and chemicals were purchased from Sigma-Aldrich (UK) unless stated otherwise. Cell culture flasks and plates were purchased from Corning (USA). All the cell lines were maintained in Gibco® cell culture media by Life Technologies.

Cell lines

Three different cell types namely CRC epithelial, myofibroblasts and skin fibroblast lines were assessed for their growth in the serum free medium. All cells were obtained from cryogenic storage of the Cancer and Immunogenetics laboratory (CIL) at the Weatherall Institute of Molecular Medicine (WIMM), Oxford, UK. Selected CRC cell lines used in the study were HT29, SW1222, SKCO1, HDC9, HCT116 and Colo320DM. Skin fibroblasts were selected for this study to investigate and compare the effect of the medium on two different cell types namely normal fibroblasts from skin and myofibroblasts.

CCD-18Co, a myofibroblast line from neonatal colonic mucosa, was acquired from American Type Culture Collection (ATCC, no. CRL1459). Skin fibroblasts were derived from a healthy donor. The isolation of these skin fibroblasts was conducted in the CIL using the conventional enzymatic method. CCD-18Co and skin fibroblasts of passage number between 14 to 18, and 9 were used in this study, respectively.

All cell lines were maintained in DMEM medium supplemented with 10% FBS, 100 U/mL penicillin and 100 µg/mL streptomycin and 2 mM L-glutamine, unless stated otherwise. The cell lines selected in this study were examined for mycoplasma contamination.

Primary myofibroblast culture

Colon tissues (cancer and adjacent normal colon) from patients, who underwent surgery for colorectal tumours (Oxford University Hospital, UK) were acquired. The samples were collected with informed consent after ethical approval (OCHRe Biobank approval no. 09/H0606/5+5) for the study was attained. Primary myofibroblasts were isolated from these tissues using collagenase enzymatic treatment (13) and cultured in DMEM medium supplemented with 10% FBS, 100 U/mL penicillin and 100 µg/mL streptomycin, 2 mM L-glutamine, 1:100 of 250 µg/mL of amphotericin and 1:100 of 10 mg/mL of neomycin. The established myofibroblast lines used in this study were Myo 8873 and Myo 0164, derived from normal and cancerous colon tissues respectively. Primary myofibroblast lines of passage 4 were tested in this publication.

Optimization of the serum free chemically defined medium (M-CIL), culture condition and substrate

To study the specific components supporting the growth and attachment of myofibroblasts, initial formulation of serum free chemically defined medium was designed based on the medium formulation of PPRF-msc6 medium (17). Several components from the PPRF-msc6 medium formulation were selected to make a modified version of the original medium which was called modified PPRF medium.

In the early stage of medium development, the influence of modified PPRF medium on the growth of myofibroblasts was tested. A total of 5 x 10^3 CCD-18Co were seeded in complete DMEM (DMEM + 10% FBS) in a 24-well plate and left in the humidified incubator for overnight to allow cell attachment. The medium was then discarded and replaced with either modified PPRF medium, complete medium with addition of serum or basal medium alone (control group). CRC cell line (HT29) and skin fibroblasts were also included in the comparison to analyse the effect of the modified PPRF medium on cancer cells and normal fibroblasts' growth respectively. Different FBS concentrations (2 and 10%) and two types of basal media (DMEM and DMEM/F12 with GlutaMAX) also were tested to compare their effects on myofibroblast growth. For the rest of this article, DMEM/F12 with GlutaMAX would be denoted by DMEM/F12.

The second stage of the experiment involved testing collagen type I as a substrate for direct seeding and

culture of myofibroblasts using modified PPRF medium. The effect of collagen coating was studied by using collagen type I from rat tail (stock concentration: 3.6 mg/mL). The collagen was diluted using 17.5 mM acetic acid according to calculated concentration:

$$C_{collagen}\ [\mu g/mL] = (A_{coating}\ [cm^2] \times 5\ \mu g/\ cm^2)/V\ [mL]$$

A 24-well plate was pre-coated with 1 mL of 20 µg/mL of collagen type I and incubated at 37°C for 1 hour. The plate was rinsed once with phosphate buffered saline (PBS) and air-dried in the culture hood. CCD-18Co was then seeded in different seeding medium formulations either on uncoated or pre-coated wells (Day 0). After one day, the medium was changed into a new culture medium (serum free – DMEM/F12 alone, DMEM/F12 + 10% FBS or modified PPRF medium) (Day 1). Culture medium was changed every three days. Cell viability was assessed after six days of incubation (Day 7 of experiment) using calcein-acetoxymethyl ester (calcein AM) staining.

Modified PPRF medium was further optimized by omitting TGFβ1 from its formulation as previous data had shown its effect on the expression of *AOC3* in myofibroblasts (13). The final formulation of this optimized medium was then renamed as M-CIL medium (DMEM/F12 with GlutaMAX in addition of chemically defined lipid concentrate, FGFβ, L-ascorbic acid-2-phosphate magnesium salt, fetuin and hydrocortisone). Components of the initial medium formulation (PPRF-msc6), modified PPRF medium and the final formulation (M-CIL) of serum free chemically defined media are listed in Table I. For the rest of this article, serum free, 10% FBS and M-CIL correspond to DMEM/F12 alone, DMEM/F12 + 10% FBS and M-CIL medium respectively, unless stated otherwise.

Cell viability and proliferation assays - Calcein AM staining and sulforhodamine B (SRB) assay

Calcein AM staining was performed to analyse the viability of the cells at the end of each experiment. For viability assay, cells were incubated for 30 minutes at 37°C with serum free medium containing calcein AM (stock concentration of 1 mM) (Biolegend) at 1:1000 dilution. The cells were then observed using a fluorescence microscope (Axio Observer.Z1, Zeiss) and representative images were taken at 50x magnification.

Cell growth in the current work was analyzed using SRB assay. This assay is a widely used method to assess cell cytotoxicity and proliferation. SRB assay of myofibroblasts grown in either serum free medium (DMEM/F12 alone), DMEM/F12 + 10% FBS and M-CIL medium was done according to the protocol by Vichai and Kirtikara (2006) (18). Higher optical density (OD) value from this assay corresponds to greater cell growth.

Maintenance of myofibroblast in serum free chemically defined medium

The M-CIL medium also was evaluated for the maintenance of myofibroblasts (CCD-18Co) after several cell passages. CCD-18Co were seeded on collagen type I coated plates and sub-cultured into different groups (Passage 1) where these cells were incubated with either DMEM/F12 alone (serum free), DMEM/F12 with serum (10% FBS) or M-CIL medium. Subsequently, these cells were then split again into three groups (Passage 2) and grown in either DMEM/F12 alone (serum free), DMEM/F12 with serum (10% FBS) or M-CIL medium in a similar manner with the previous passage. The cells were maintained in these media for six days before stained with Calcein AM staining.

Screening of M-CIL effect on CRC cell lines' growth

Besides HT29, several other CRC cell lines (SW1222, SKCO1, HDC9, HCT116 and Colo320DM) were selected to screen the effect of M-CIL on their growth. In this experiment, 1 x 10^3 CRC cells were seeded directly in M-CIL medium on collagen type I coated 24-well plate (except for 10% FBS treatment groups) and left overnight to attach before medium was changed to either DMEM/F12 alone (serum free), M-CIL medium or DMEM/F12 + 10% FBS. The cell lines were maintained in those respective media for six days before stained with Calcein AM.

Myofibroblasts and CRC cells' growth in co-culture setting in M-CIL medium

The M-CIL medium also was tested for the co-culture of myofibroblasts and CRC cell lines. In this experiment, CCD-18Co and CRC cell line (HT29) were mixed together in M-CIL medium and seeded on top of collagen coated plates. CRC cells and myofibroblasts were maintained for six days before stained for AUA1 (anti-EpCAM antibody) and vimentin (Vim) respectively. Prior to the immunofluorescence staining, the cells were washed twice with PBS. For AUA1 and vimentin staining, the cells were fixed with 4% (v/v) PFA for 10 minutes at room temperature (RT) and permeabilized with 0.2% Triton in PBS solution for 10 minutes at RT before incubated with blocking solution (PBS + 2% FBS) for 30 minutes. The primary antibody containing solution diluted with washing buffer (PBS + 2% FBS) was added to the wells and the cells were kept at 4°C for overnight. Next, the cells were incubated with secondary antibody for 1 h at RT and stained with DAPI (1: 10000) for 5 minutes. The staining was examined with a fluorescence microscope (Axio Observer.Z1, Zeiss) and representative images of cells were taken at 50x magnification. Control of HT29 monoculture was included in the experiment.

Quantitative real time polymerase chain reaction (qRT-PCR)

Earlier publication has reported *AOC3* to be the marker

for myofibroblasts and its expression was significant downregulated after treatment with 10% FBS and TGFβ1 in comparison to serum free condition (13). The effect of M-CIL medium on *AOC3* expression of CCD-18Co maintained in this medium was studied using qRT-PCR. Total RNA was extracted from cells using the RNeasy (Qiagen) kit and synthesis of the complementary DNA (cDNA) from RNA was performed using High Capacity cDNA Reverse Transcription kits (Applied Biosystems Inc.) according the manufacturer's instructions. Reverse transcription was performed by using the thermal cycler and the optimized protocol; 25°C for 10 minutes, 37°C for 120 minutes and 85°C for 5 minutes. TaqMan® Gene Expression Assay, 20X FAM dye-labeled kit for *AOC3* (Hs02560271_s1) from Applied Biosystems was used for the quantification of mRNA.

Statistical analysis

The data is represented by the mean values ± standard error mean (SEM), assuming a normal distribution for the data. The p-values were calculated using an independent t-test, using three biological replicates (SPSS® version 22, IBM®, USA). For statistical comparisons, p-value of less than 0.05 ($p<0.05$) indicates significant difference between two independent groups.

RESULTS

Early optimization of serum free defined medium formulation - Modified PPRF medium

The M-CIL medium was established from modified PPRF medium, which was optimized based on PPRF-msc6 medium formulation, previously reported as a medium that can support the growth of mesenchymal cells (17). For the current study, we chose to omit several of the elements from the PPRF-msc6 medium such as transferrin and sodium selenite after preliminary test showed that these components inhibited growth of CCD-18Co (data not shown). This altered PPRF-msc6, which is referred to as modified PPRF medium in the next section, was used in early optimization of our serum free chemically defined medium.

For the selection of basal medium, two different culture media namely DMEM and DMEM/F12 (1:1 mixture of DMEM and Ham's F12) with GlutaMAX were compared. DMEM/F12 contains more components compared to DMEM, which is commonly used in cell culture practice. Both CCD-18Co and skin fibroblasts maintained in DMEM/F12 (addition of 10% FBS) demonstrated better growth compared to those cells incubated in DMEM under similar experimental conditions (Fig. 1a and b). Hence, DMEM/F12 was chosen as basal medium for development of serum free defined medium.

Three cell types (myofibroblasts, skin fibroblasts and CRC cell lines) maintained in the modified PPRF medium displayed different growth rates. After the cells were seeded in 10% FBS and allow to attach overnight, CCD-18Co, skin fibroblasts and HT29 (CRC cell line) were grown in different medium formulations for six days. Different percentages of FBS (2 and 10%) were included in the experiment to assess the influence of serum concentration on the proliferation of myofibroblasts. As expected, better cell growth was observed with higher serum concentrations. Both myofibroblasts and skin fibroblasts displayed better growth after incubation with modified PPRF medium (Fig. 1a and b) in comparison to basal medium (DMEM/F12) alone. This observation was more striking in skin fibroblasts when compared to CCD-18Co. A minimal effect of modified PPRF medium on HT29 proliferation was seen (Fig. 1c).

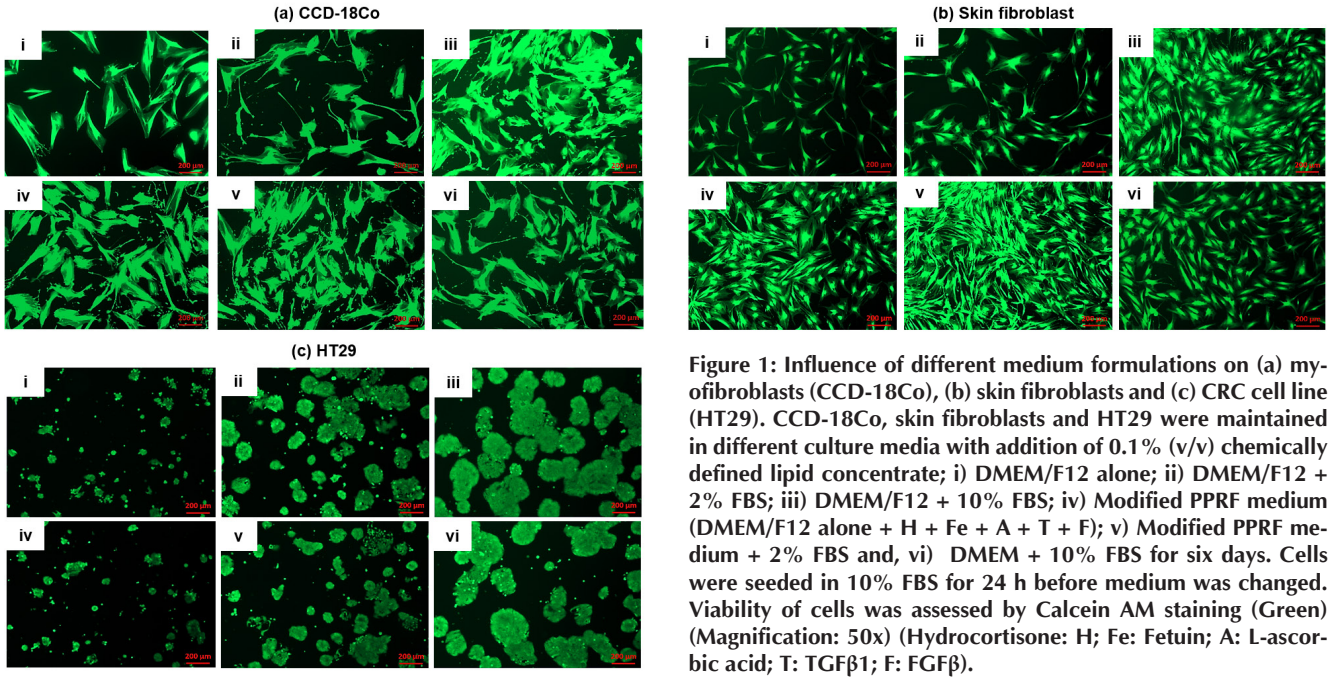


Figure 1: Influence of different medium formulations on (a) myofibroblasts (CCD-18Co), (b) skin fibroblasts and (c) CRC cell line (HT29). CCD-18Co, skin fibroblasts and HT29 were maintained in different culture media with addition of 0.1% (v/v) chemically defined lipid concentrate; i) DMEM/F12 alone; ii) DMEM/F12 + 2% FBS; iii) DMEM/F12 + 10% FBS; iv) Modified PPRF medium (DMEM/F12 alone + H + Fe + A + T + F); v) Modified PPRF medium + 2% FBS and, vi) DMEM + 10% FBS for six days. Cells were seeded in 10% FBS for 24 h before medium was changed. Viability of cells was assessed by Calcein AM staining (Green) (Magnification: 50x) (Hydrocortisone: H; Fe: Fetuin; A: L-ascorbic acid; T: TGFβ1; F: FGFβ).

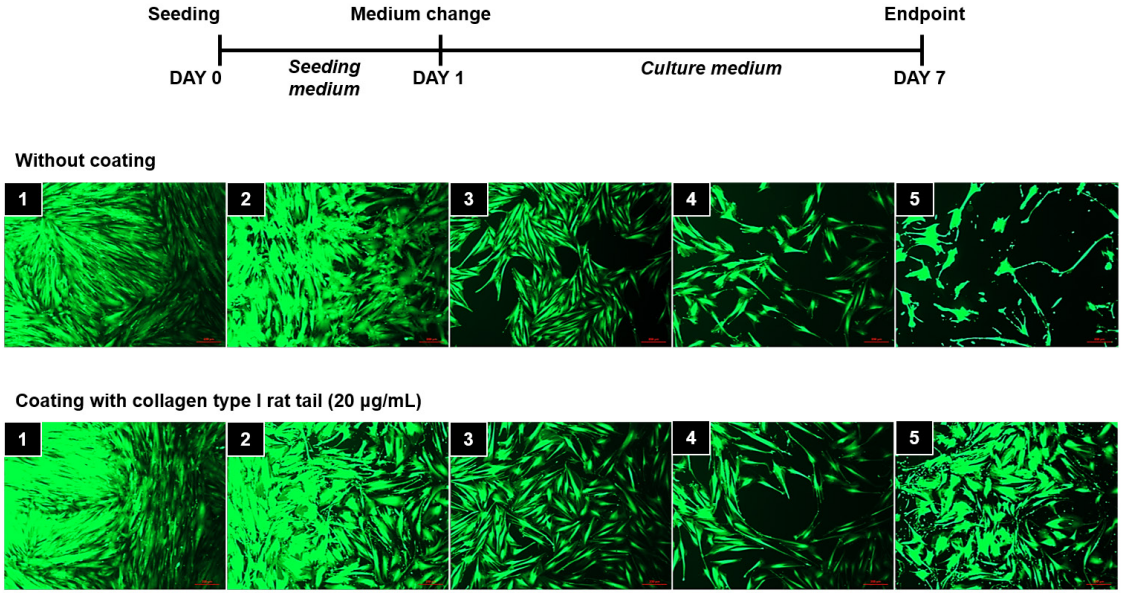
Collagen as tissue culture substrate coating material

One of the factors that needs to be considered for an optimal *in vitro* culture environment is cell attachment. Certain cell types do not attach to standard tissue culture plastic or glass unless an appropriate element such as extracellular matrix (ECM) which mimics the environment is used to coat its surface (19). We performed a preliminary test to determine whether modified PPRF medium supports the attachment and growth of CCD-18Co by either plating the cells directly into modified PPRF medium or in DMEM/F12 + 10% FBS and replacing the medium with modified PPRF medium after overnight incubation. We found that direct seeding and culture with modified PPRF medium led to adverse effects on their growth. Better cell survival was observed in CCD-18Co seeded in 10% FBS and maintained in modified PPRF medium. This observation indicates the need for a substrate which may be essential for cell attachment and FBS most likely contains elements that help cell to attach to the cell culture flask or plate. Thus, collagen type I rat tail was tested as a possible substrate.

In this experiment, CCD-18Co was seeded in a seeding medium, on either collagen type I coated or uncoated plates, and this medium was replaced with fresh culture medium after overnight incubation, which allow cells to adhere to surface. Different medium formulations were tested (serum free – DMEM/F12 alone, DMEM/F12 + 10% FBS and modified PPRF medium). The results demonstrated that CCD-18Co survived better when seeded and cultured in 10% FBS (Fig. 2). The absence of collagen coating in CCD-18Co cultured in modified PPRF medium led to a very minimal cell growth. Interestingly, CCD-18Co thrived when maintained in modified PPRF medium on collagen-coated plates, as shown by the Calcein AM staining. This observation illustrates the importance of collagen for the attachment and growth of myofibroblasts in serum free condition.

M-CIL medium formulation optimized from modified PPRF medium

The influence of individual components of modified PPRF medium on the growth of CCD-18Co was tested. The absence of chemically defined lipid concentrate, FGFβ, fetuin or L-ascorbic acid from modified PPRF medium resulted in slower cell growth and adverse effects on cell viability, in comparison to DMEM/F12 alone (data not shown). Considering the previous finding on the influence of TGFβ1 on the regulation of *AOC3* expression (13), we opted to remove this growth factor from the final formulation of our serum free defined medium. We found that CCD-18Co managed to sustain their growth rate when TGFβ1 were omitted from the culture medium (data not shown). This medium was later renamed as M-CIL medium which consists of DMEM/F12 with GlutaMAX, 2 ng/mL FGFβ, 50 µg/mL L-ascorbic acid-2-phosphate magnesium salt, 100 nM hydrocortisone, 1.0 g/L fetuin and 0.1% v/v chemically defined lipid concentrate. It is worth noting that we found an increase of cell growth when combination of fetuin and hydrocortisone were used, when compared to the addition of fetuin or hydrocortisone alone (data



Medium type/ Group	1	2	3	4	5
Seeding medium	10% FBS	10% FBS	10% FBS	SF	Modified PPRF
Culture medium	10% FBS	Modified PPRF	SF	SF	Modified PPRF

Figure 2: Maintenance of CCD-18Co in various medium formulations, either with or without collagen coating. Experimental layout as shown in the figure. Viability of cells is indicated by Calcein AM staining (Magnification: 50x) (Serum free: DMEM/F12 alone).

not shown). Thus, both components were included in the final formulation of the M-CIL medium.

Table I summarizes the individual components of serum free chemically defined medium (M-CIL medium) which started from alteration of PPRF-msc6 medium formulation, which was renamed as modified PPRF medium. By default, cells maintained in M-CIL medium were seeded on plates with a thin layer of collagen type I coating and this experimental setup was used throughout the rest of this paper.

M-CIL medium supports the growth of myofibroblasts

The effect of M-CIL medium on primary myofibroblast proliferation was tested and compared to CCD-18Co. Fig. 3a shows the SRB assay result of CCD-18Co incubated with serum free medium (DMEM/F12 only), DMEM/F12 + 10% FBS (without collagen coating) and M-CIL medium for six days. CCD-18Co grown in 10% FBS exhibited significantly better growth (higher OD value) compared to serum free condition. Interestingly, no significant difference was found in cell proliferation between 10% FBS and M-CIL groups although lower growth of CCD-18Co in M-CIL medium compared to 10% FBS was noted. Fig. 3a also illustrates distribution and morphology (stained with vimentin and DAPI) of CCD-18Co when cultured in three different media formulations. Parallel with the SRB assay result, CCD-18Co exhibited a good growth rate when maintained in M-CIL medium. Better cell proliferation was observed in the 10% FBS group, while cells treated with serum free medium clearly grew least well. This experiment was repeated with Myo 8873 and Myo 0164C (Fig. 3b) where both primary myofibroblasts, isolated from normal and cancerous colon tissues respectively, displayed

Table I: Development of M-CIL medium from modified PPRF medium which was optimized from PPRF-msc6

Medium	Component	Concentration
PPRF-msc6	DMEM/Ham's F12 with glutamine	1x
	Chemically defined lipid concentrate	0.1% v/v
	Sodium bicarbonate	20.5 mM
	HEPES	4.9 mM
	Bovine insulin	4.01 µM
	Human transferrin	0.318 mM
	Putrescine dihydrochloride	55.9 µM
	Sodium selenite	27 nM
	Progesterone	0.018 µM
	Heparin	0.7 U/mL
	FGFβ	2 ng/mL
	TGFβ1	1 ng/mL
	Ascorbic acid	50 µg/mL
	Fetuin	1 g/L
	Hydrocorstisone	100 nM
Modified PPRF	DMEM/F12 with GlutaMAX	1X
	Chemically defined lipid concentrate	0.1% v/v
	FGFβ	2 ng/mL
	TGFβ1	1 ng/mL
	Ascorbic acid	50 µg/mL
	Fetuin	1 g/L
	Hydrocorstisone	100 nM
M-CIL (final formulation)	DMEM/F12 with GlutaMAX	1X
	Chemically defined lipid concentrate	0.1% v/v
	FGFβ	2 ng/mL
	Ascorbic acid	50 µg/mL
	Fetuin	1 g/L
	Hydrocorstisone	100 nM

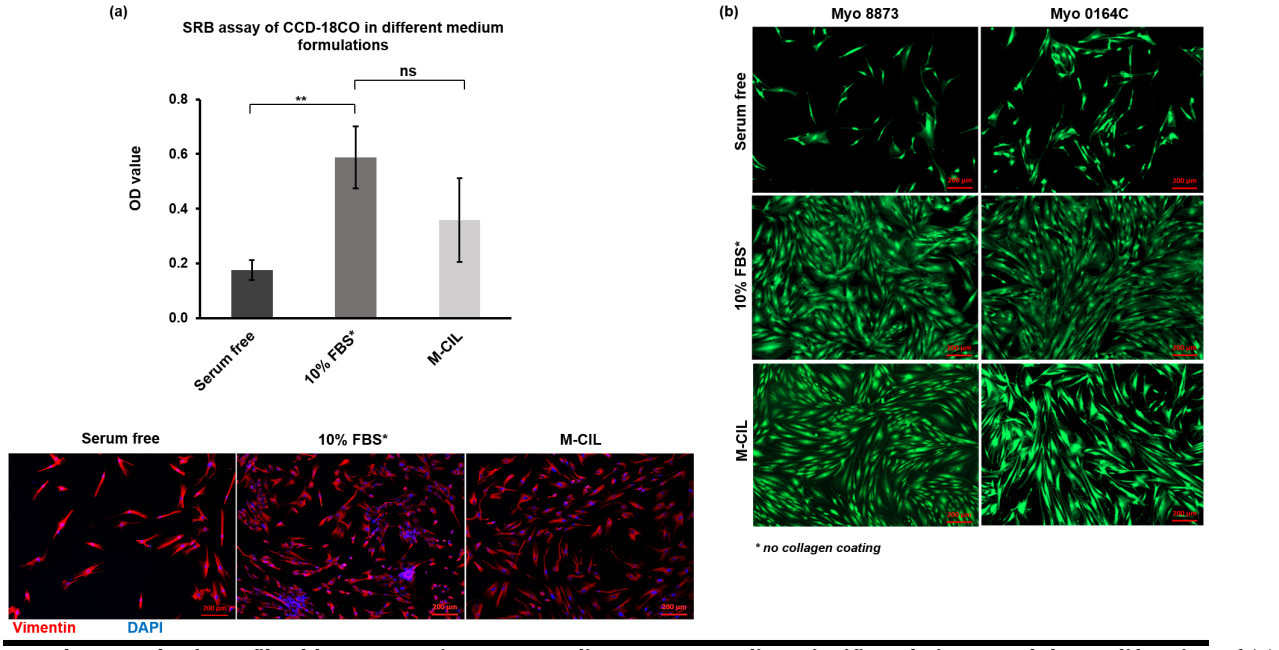


Figure 3: The growth of myofibroblasts grown in M-CIL medium. M-CIL medium significantly increased the proliferation of (a) CCD-18Co (stained with vimentin and DAPI) as compared to serum free medium. Similar effects of different medium formulations on (b) primary myofibroblasts' growth (Myo 8873 and Myo 0164C) also were found. Viability of primary myofibroblasts was confirmed using Calcein AM staining (*No collagen coating) (Magnification: 50x).

better growth when incubated with M-CIL medium in comparison to serum free condition, although at lower levels compared to DMEM/F12 + 10% FBS.

Maintenance of myofibroblasts in M-CIL medium

A sub-confluence flask of CCD-18Co, maintained in 10% FBS was sub-cultured into different plates (passage ratio of 1:3) where these cells were maintained in either DMEM/F12 alone, DMEM/F12 + 10% FBS and M-CIL medium for six days (Passage 1 – P1). Those cells were then again split (passage ratio of 1:3) and grown in their respective media namely DMEM/F12 alone (serum free), DMEM/F12 + 10% FBS or M-CIL medium for another six days (Passage 2 – P2). The representative images of CCD-18Co maintained in various medium formulations are shown in Fig. 4. At P1, CCD-18Co grown in M-CIL medium survived better compared to serum free condition, which agrees with our previous observation, although the cell density is lower compared to DMEM/F12 + 10% FBS group (Fig. 4a). As expected, at P2, cells incubated with 10% serum managed to survive better as compared to the other groups (serum free and M-CIL medium-treated CCD-18Co) (Fig. 4b). Very little growth was observed in the cells sub-cultured from CCD-18Co maintained in serum free medium at P1. It is worth noting that cells exhibited better proliferative activity in M-CIL medium in comparison to DMEM/F12 alone even after two cell passages. Optimal growth of CCD-18Co was found in cells sub-cultured from the group previously maintained in 10% FBS at P1. M-CIL medium seems to support extended *in vitro* culture of CCD-18Co as both cells at P1 and P2 show sufficient growth and better proliferation rate compared to those in serum free (DMEM/F12 alone) conditions.

Effect of M-CIL medium on CRC cell lines' growth

Various CRC cell lines with different characteristics were selected to investigate the proliferative effect of M-CIL medium on epithelial cells. Screening using SW1222, SKCO1, HDC9, HCT116 and Colo320DM revealed that these epithelial cell lines exhibited different proliferative activity under the same treatment. Both SKCO1 and HDC9 show minor, insignificant increment in the cell number after incubation with M-CIL medium (Fig. 5a). Strikingly, a significant increase in cell proliferation was observed in HCT116 maintained in M-CIL medium as compared to serum free medium (DMEM/F12 alone). A control of CCD-18Co maintained in M-CIL medium clearly shows better growth rate in comparison to DMEM/F12 (serum free) alone, as shown before. These data collectively demonstrate the difficulty in establishing a completely serum free medium that would satisfy both the requirements of the growth of myofibroblasts and, separately, all the CRC cell lines.

Co-culture of myofibroblasts and CRC cells in M-CIL medium

As most cells require the presence of serum or supplementation to survive well, a more defined medium without serum, which is able to promote cell proliferation would be a better alternative to be used in the *in vitro* assays to study the interaction between myofibroblasts and cancer cell lines as it would ensure an optimal cell growth and experimental outcome.

To assess the response of CRC cells and myofibroblasts when co-cultured in M-CIL medium, HT29, a CRC cell line which do not grow in M-CIL medium,was mixed and maintained with CCD-18Co for seven days in M-CIL

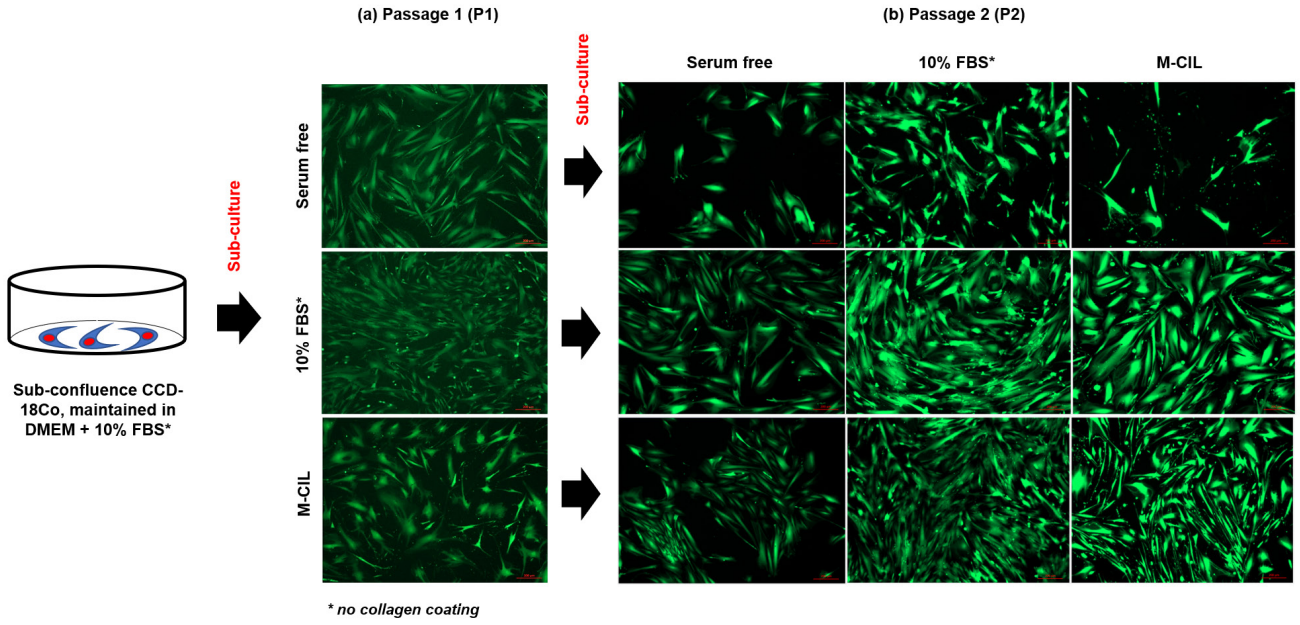


Figure 4: Maintenance of myofibroblasts (CCD-18Co) cultured in M-CIL medium. The cell distribution after first sub-culture from sub-confluence CCD-18Co, grown in three medium formulations (serum free, 10% FBS and M-CIL medium) for six days are shown in (a)(P1). These cells were then split and incubated with different culture media for another six consecutive days (P2) (*No collagen coating) (Magnification: 50x).

medium on collagen type I coated 24-well plate . After that, HT29 and CCD-18Co were stained for AUA1 (anti-EpCAM antibody) and vimentin (Vim), respectively to distinguish those two cell types. CRC cells possess positive expression of EpCAM, while CCD-18Co stained for vimentin. Positive control of DMEM/F12 + 10% FBS was included.

HT29 formed clusters of "islands" consisting of tightly packed cells in co-culture with CCD-18Co whereas more heterogenous spreading of cells on the culture plate was observed in monoculture. Noticeably, we observed that CCD-18Co grew and surrounded HT29 colonies when incubated in DMEM/F12 + 10% FBS, which was not seen as clearly in serum free condition (DMEM/F12 alone), most likely due to fewer myofibroblast cells and less prominent formation of HT29 colonies . Comparison of monoculture between serum free (DMEM/F12 alone) and M-CIL medium shows that higher number of HT29 cells were observed in M-CIL medium although this increment may or may not be significant, as was observed with HCT116 response to M-CIL medium. HT29 maintained in serum free medium (DMEM/F12 alone) + CCD-18Co was also found to grow at the centre of the well, and at a much lower cell concentration than the 10% FBS co-culture group. HT29 cultured in M-CIL medium grew evenly across the surface and produced greater cell density as compared to serum free conditions (DMEM/F12 alone). Interestingly, CCD-18Co supports the growth of HT29 as a higher cell number was seen in co-culture (HT29 + CCD-18Co), most notably in those incubated with M-CIL medium. Representative and enlarged images of HT29 both in monoculture and co-culture conditions are shown in Fig. 5b.

Influence of M-CIL medium on *AOC3* expression in CCD-18Co

Although M-CIL medium supports the growth of myofibroblasts, its influence on specific gene expression in myofibroblasts needs to be tested for. The effect of M-CIL medium and its individual components on *AOC3* expression in CCD-18Co was analysed using qRT-PCR. Fig. 6 shows the experimental layout and result of the experiment. There was a significant downregulation of *AOC3* expression in CCD-18Co after treatment with M-CIL medium, fetuin alone, hydrocortisone alone, and combination of fetuin and hydrocortisone when compared to DMEM/F12 alone. No significant differences in *AOC3* expression between DMEM/F12 alone and other groups (chemically defined lipid concentrate, ascorbic acid and FGF2 alone) was found. These data demonstrated that gene expression in myofibroblasts is

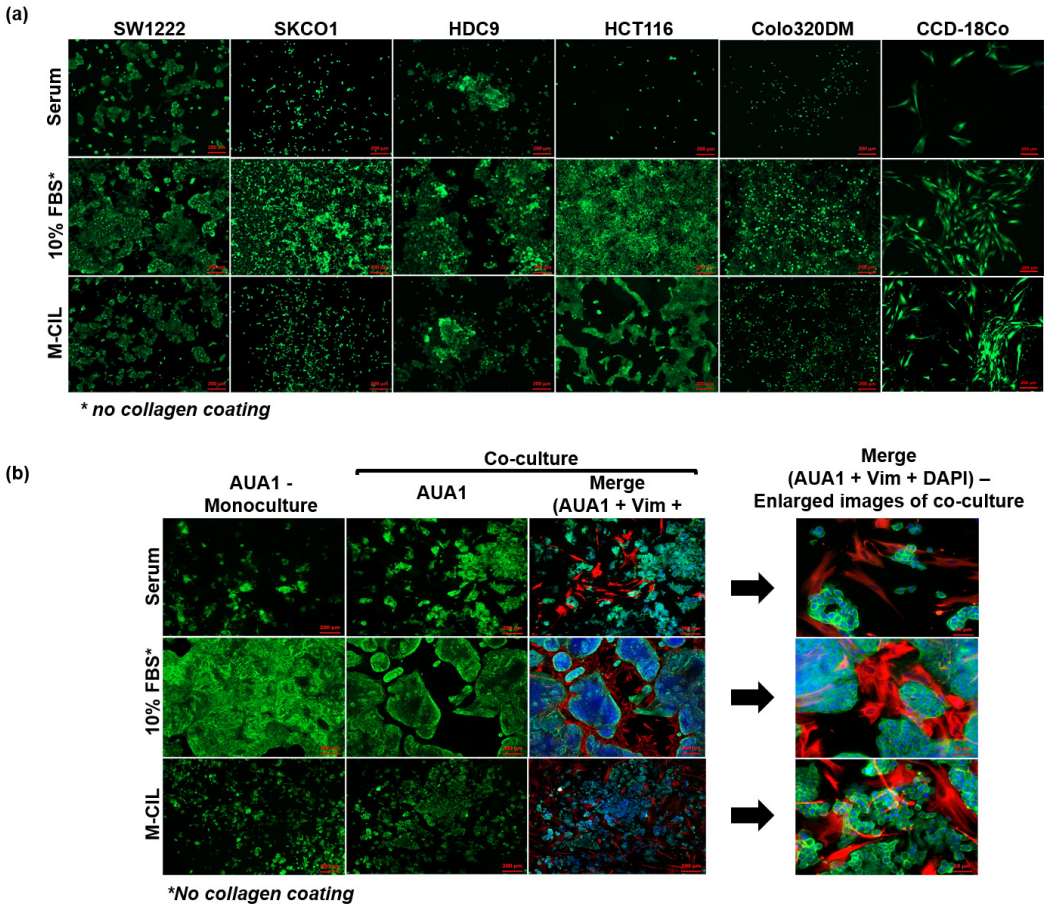


Figure 5: Representative images of a) various CRC cell lines incubated with serum free (DMEM/F12 alone), M-CIL medium or DMEM/F12+10% FBS and b) Co-culture of HT29 and CCD-18Co in M-CIL medium. The growth of CCD-18Co and HT29 were illustrated in the representative (a) (Magnification: 50x) and enlarged images (b) (Magnification: 200x) of both cells in co-culture and monoculture conditions.

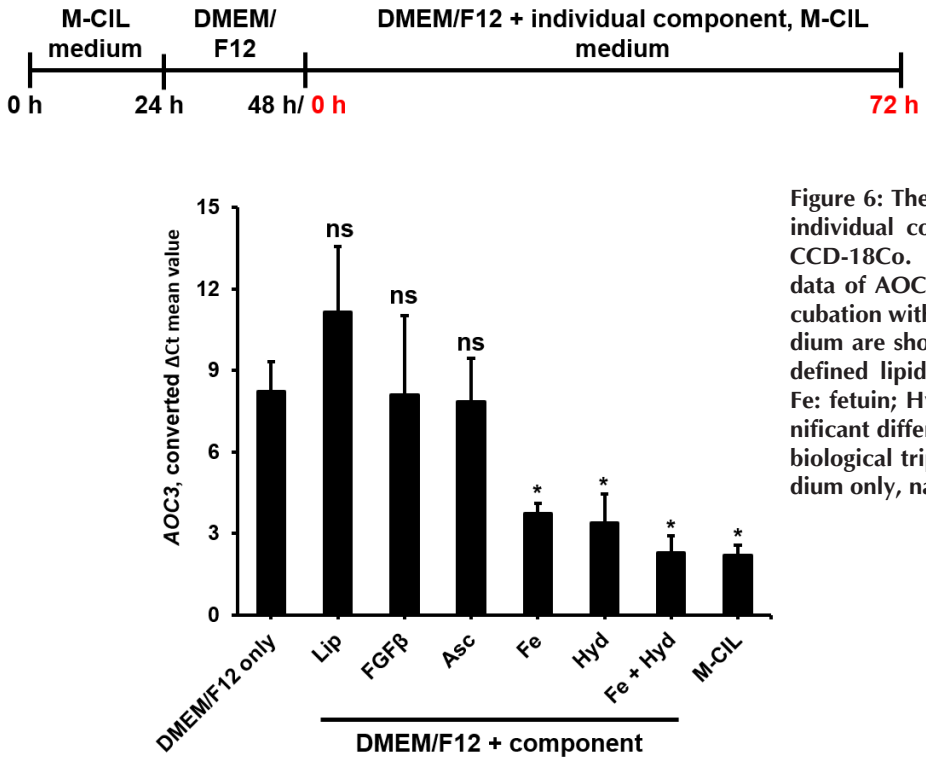


Figure 6: The influence of M-CIL medium and its individual components on *AOC3* expression in CCD-18Co. Experimental layout and qRT-PCR data of AOC3 expression in CCD-18Co after incubation with different components of M-CIL medium are shown as in the figure (Lip: chemically defined lipid concentrate; Asc: L-ascorbic acid, Fe: fetuin; Hyd: hydrocortisone) (*p<0.05 of significant difference; ns: not significant, from three biological triplicates, in comparison to basal medium only, namely DMEM/F12.

strongly affected by two of the components of M-CIL medium, namely fetuin and hydrocortisone.

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DISCUSSION

The addition of serum in cell culture medium to recapitulate the *in vivo* condition is highly debatable. Various studies employ different concentrations of serum for the experimental purposes, ranging from low or minimal serum (0-2% FBS) to high serum (10-20% FBS). DMEM with addition of FBS is usually applied to culture fibroblasts (2). Similar DMEM medium formulation was applied to grow myofibroblasts and skin fibroblasts in this study. Fibroblasts have a reasonably high proliferation rate when maintained in culture medium supplemented with serum. However, the exact composition of serum is poorly defined, and different lots of serum may lead to variability in the experimental outcome. Thus, many researchers have opted to use serum free defined medium for a more controlled experimental setting.

DMEM/F12 with GlutaMAX was chosen as the basal medium for the M-CIL medium as better cell growth was observed when myofibroblasts were maintained in this medium as compared to DMEM. This may be due to the additional components in DMEM/F12 that provide better supplementation for myofibroblasts. The comparison of the composition between DMEM/F12 and DMEM is listed in Supplementary information S1.

Our data revealed that several components of M-CIL medium (FGFβ, chemically defined lipid concentrate, L-ascorbic acid and fetuin) are crucial for myofibroblast growth as compared to others. The properties of those individual components are discussed below:

a) FGFβ - FGFβ was suggested as a supplement in cell culture medium (20). This growth factor promotes survival and protects the cells through resistance to radiation-induced-programmed cell death (21) via modulation of apoptosis pathway (22).

b) Chemically defined lipid concentrate - Studies have shown that the chemically defined lipid concentrate supported growth of different types of cells (23, 24). This mixture consists of a combination of unsaturated and saturated free fatty acids (FFA). The importance of lipids in cell growth has been documented by Hang et al. (2012) (25).

c) L-ascorbic acid-2-phosphate magnesium salt (APM) - APM is a more stable form of ascorbic acid. L-Ascorbic acid also known as vitamin C is sometimes used as an additive in culture medium and it possesses different biochemical functions including promotion of collagen synthesis in skin fibroblasts (26), phagocytosis of polymorphonuclear leukocytes (27) and differentiation of various mesenchymal cell types (28). L-ascorbic acid-2-phosphate also was reported to stimulate proliferation of skin fibroblasts (29). Moreover, APM promotes the synthesis of type 1 collagen synthesis and reduces cell damage by suppressing H2O2-induced intracellular reactive oxygen species (ROS) (30). Ascorbic acid also has been reported to elevate the expression of TGFβ1-induced genes, including discoidin domain receptor 1 *(DDR1)* and connective tissue growth factor *(CCN2)* (31).

d) Fetuin - Fetuin family protein members include fetuin A and B which can form dimers that are thought

to contribute to their functional effects. They are carrier proteins rather like albumin and found in the serum (32). Fetuin-A homologues have been identified as a major protein in bovine, sheep, pig, goat, human and rodent sera (33). Fetuin-A, also called α2-Heremans or Schmid glycoprotein (α2-HS glycoprotein/Ahsg) has been used in tissue culture for its promotion of cellular attachment and its functions probably depend on its carrier properties (34).

e) Hydrocortisone - Hydrocortisone is a glucocorticoid. It has been reported to be able to induce changes in the regulation of various genes involved in intestinal epithelial cell maturation, cell-cell and cell-ECM communication, metabolism, and promote cell differentiation (35, 36).

f) Collagen I as substrate - Our finding strongly suggests that myofibroblasts requires a substrate such as collagen for their attachment. Collagen type I has been proposed to be the basis of a 3D matrix which is a better representative of physiological conditions of tissue rather than using plastic dishes for fibroblast and myofibroblast culture (37). Collagen type I supports the attachment and growth of fibroblasts without the transient use of FBS. It is clear from the present study that FBS provides components that help cells to attach and proliferate as the omission of serum (in uncoated plate) leads to minimal cell survival.

Two of the M-CIL medium components, namely fetuin and hydrocortisone downregulated *AOC3* expression in CCD-18Co. This observation mimics the effect of TGFβ1 on expression of *AOC3*, prompted us to investigate association between fetuin and hydrocortisone with TGFβ1 activity. The fetuin A protein structure composed of a) two cystatin-like domains (38); b) a calcium phosphate-binding site near the N-terminus (39) and c) a TGFβ cytokine-binding motif (40). The later shares homology in sequence with extracellular domain of TGFβ receptor type II (TGβRII). These peptides bind to TGFβ and BMP cytokines. The TGFβ/BMP antagonistic property of fetuin A (41) may suggest that fetuin acts in a similar mechanism as TGFβ1 which leads to the downregulation of *AOC3* expression observed in fetuin-treated CCD-18Co). As for hydrocortisone, it was reported to supress the production of TGFβ mRNA in human fetal lung fibroblasts (42).

Hydrocortisone treatment also affects gene regulation (e.g. downregulating genes involve in the innate immune/inflammatory responses such as IL-1, IL-6, Toll-like receptor and TNF-α signalling) (35, 43). Thus, we speculated that hydrocortisone induces modulation of gene(s) that may influence the *AOC3* expression in CCD-18Co, in a similar manner to TGFβ1. Our data shows that fetuin and hydrocortisone were able to downregulate *AOC3* expression without the presence of TGFβ. This may indicate the independence of fetuin and hydrocortisone activity from TGFβ. The nature and mechanisms by which fetuin and hydrocortisone influence *AOC3* will require further investigation. As stated in the previous section, besides TGFβ1, 10% FBS also downregulated *AOC3* expression in CCD-18Co (13). Despite the effects of fetuin and hydrocortisone on CCD-18Co gene modulation, M-CIL medium presents as a better alternative to grow myofibroblasts compared to DMEM with serum as its components are well defined and known.

Several CRC cell lines and myofibroblasts were tested to analyse the influence of M-CIL medium on the growth of different cell types. Various epithelial cells responded differently when incubated with M-CIL medium. Some CRC cell lines seem to need minimal requirements to proliferate, whereas the others are more stringent and selective with respect to their growth requirements (44, 45). Myofibroblasts (CCD-18Co) and HT29, survived well under co-culture condition using M-CIL medium as shown in Fig. 5 which suggests the potential for this medium to be used for future work involving *in vitro* co-culture of those two cell types. Higher cell growth of HT29 was observed when co-cultured with CCD-18Co in M-CIL medium. This observation indicates the release of growth factors and chemokine from CCD-18Co which promote the proliferation of HT29. Sufficient growth of CCD-18Co was seen when maintained in M-CIL medium, better than in DMEM/F12 alone (basal medium only).

CONCLUSION

Our results demonstrated that serum free chemically defined medium named as M-CIL medium enables good growth of myofibroblasts in contrast to basal medium alone which only supported a much lower rate of cell proliferation. The experimental setup involving co-culture myofibroblasts and CRC cells in M-CIL medium will enable future studies on the influence of specific growth factors and cytokines, and their inhibitors on both cancer cells and myofibroblasts, when they are incubated together.

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