***In Vitro* Analyses of Interactions Between Colonic Myofibroblasts and Colorectal Cancer Cells for Anticancer Study**

MARAHAINI MUSA¹, DJAMILA OUARET² and WALTER F. BODMER²

¹Human Genome Centre, School of Medical Sciences, Universiti Sains Malaysia, Kubang Kerian, Malaysia;

²Cancer and Immunogenetics Laboratory (CIL), Weatherall Institute of Molecular Medicine (WIMM), Department of Oncology, University of Oxford, Oxford, U.K.

Abstract. *Background/Aim: Interactions between colorectal cancer (CRC) cells and myofibroblasts govern many processes such as cell growth, migration, invasion and differentiation, and contribute to CRC progression. Robust experimental tests are needed to investigate the nature of these interactions for future anticancer studies. The purpose of the study was to design and validate in vitro assays for studying the communication between myofibroblasts and CRC epithelial cell lines. Materials and Methods: The influence of co-culture of myofibroblasts and CRC cell lines is discussed using various in vitro assays including direct co-culture, transwell assays, Matrigel-based differentiation and cell invasion experiments. Results: The results from these in vitro assays clearly demonstrated various aspects of the crosstalk between myofibroblasts and CRC cell lines, which include cell growth, differentiation, migration and invasion. Conclusion: The reported in vitro assays provide a basis for investigating the factors that control the myofibroblast-epithelial cell interactions in CRC in vivo.*

Colorectal cancer (CRC) is the third most commonly diagnosed malignancy globally (1). The approach to studying the treatment and related molecular basis of CRC has evolved over the years. Rather than focusing only on neoplastic cells, there has been more emphasis on studying the role of the tumour microenvironment in driving CRC progression (2-4).

This article is freely accessible online.

Correspondence to: Marahaini Musa, Human Genome Centre, School of Medical Sciences, Universiti Sains Malaysia, Health Campus, 16150 Kubang Kerian, Kelantan, Malaysia. Tel: +60 97676794, Fax: +60 97673370, e-mail: marahaini.musa@usm.my

Key Words: Neoplasms, colon, tumour microenvironment, tumour heterogeneity.

This stems from widely reported definition of tumour as a combination of heterogeneous and interdependent populations of cancer cells and tumour microenvironment (known as stroma) (5). The tumour microenvironment drives the progression of most solid tumours, especially the epithelial-based carcinomas. Major cellular components of tumour microenvironment include myofibroblasts, cancer-associated fibroblasts (CAFs), macrophages and endothelial cells, that may interact with the extracellular matrix (ECM) (6, 7).

Myofibroblasts have been described as a phenotypic variant of many mesenchymal, fibroblastic cell types (8). They can be characterized by specific markers, in particular amine oxidase, copper containing 3 (AOC3) and NK2 homeobox 3 (NKX2-3) (9). Accumulation of myofibroblasts around adenomatous colorectal polyps and primary CRC tumour sites have been associated with a higher rate of recurrence and advanced stage of cancer (10, 11). These activated myofibroblasts, characterized by positive expression of alpha smooth muscle actin (α -SMA) make up CAFs which are abundantly found in solid tumours (12). The prominent role of CAFs in carcinogenesis is evidently indicated by the up-regulated expression of genes associated with poor prognosis for CRC predominantly found within the CAF population, not in tumour cells (13).

Myofibroblasts drive tumour progression through interplay with cancer cells, facilitated by various secretomes. Activated forms of myofibroblasts are reported to promote tumour invasion and migration, stimulate cancer cell proliferation, suppress cancer killing function of T-cells and support the stemness of cancer stem cells (14-17).

Despite many studies conducted on the bi-directional communication between myofibroblasts and CRC tumour cells, there is poor understanding on the nature and exact mechanisms involved in this myofibroblast-cancer cell interaction. This is significantly attributed to lack of robust *in vitro* experimental methods to recapitulate the biological processes that occur in cancer niche *in vivo*.

Better understanding of the interplay between myofibroblasts and CRC epithelial cells would lead to discovery of novel therapeutic avenues for CRC (18). The overall aim of this study was to design and validate *in vitro* assays for studying the various modes of communication between myofibroblasts and CRC-derived epithelial cells including cell proliferation, differentiation, migration and invasion.

Materials and Methods

Cell lines. All CRC epithelial cell lines, myofibroblasts and skin fibroblasts used in this study were obtained from cryogenic storage of the Cancer and Immunogenetics laboratory (CIL) at the Weatherall Institute of Molecular Medicine (WIMM), Oxford, UK. A subset of CRC cell lines was selected for these studies (RKO, HT29, LS180, SW480 and SW620). Human foreskin fibroblasts were included to enable comparisons between myofibroblasts and normal skin fibroblasts. CCD-18Co (no. CRL1459), a myofibroblast line from neonatal colonic mucosa, was obtained from the American Type Culture Collection (ATCC). Skin fibroblasts were derived from a healthy subject and isolated using conventional enzymatic methods. Primary myofibroblasts were derived from colon tissue from patients who underwent surgery for CRC (Oxford University Hospital, Oxford, UK). The samples were collected with informed consent (ethically approved under the OCHRe Biobank approval no. 09/H0606/5+5). Primary myofibroblast cultures were isolated using collagenase enzymatic treatment (9). The established primary myofibroblasts used in this study were Myo 6526, Myo 7395 and Myo 6550.

CRC, myofibroblast and fibroblast lines were grown in DMEM culture medium supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100 µg/ml streptomycin antibiotic solution and 2 mM L-glutamine. The cells were maintained in a humidified incubator at 37°C with 10% CO₂.

Cell growth assay. This assay was conducted to study the influence of co-culture of myofibroblasts and CRC cell line (HT29) on cell growth. For this experiment, 100 µl of 50% Matrigel® Growth Factor Reduced (GFR) Basement Membrane Matrix, Phenol Red-Free (BD Biosciences, San Jose, CA, USA), diluted with an equal volume of serum free culture medium (DMEM) was added to a 24-well plate and left to harden at 37°C for 30 min in a humidified incubator. Then, a total of 4×10⁴ CCD-18Co cells (myofibroblasts) suspended in serum free DMEM (DMEM alone) were seeded on the Matrigel and incubated for 48 h at 37°C, 10% CO₂ until 80% confluence. Next, 8×10⁴ HT29 cells mixed with 100 µl of serum free DMEM were added on top of the layer of myofibroblasts. Control groups of HT29 or CCD-18Co alone on Matrigel layer were included. CRC cells and myofibroblasts were respectively pre-labelled with cell linker; PKH67 (red) and PKH26 (green) (Sigma-Aldrich, St. Louis, MO, USA). The morphology and growth of the HT29 after one week of co-culture was observed using a fluorescence microscope. Parallel experiments without Matrigel were also performed to assess its role as a substrate.

Cell differentiation assay. Ashley *et al.* have demonstrated that a subset of CRC cell lines when grown in Matrigel is able to form either (i) large crypt-like structures consisting of polarised cells surrounding a cell-free lumen that can be identified using F-actin staining (lumen forming cells), or (ii) small non-lumen colonies (19). Different types

of differentiated colorectal epithelial cells can be found in the lumen forming colonies. Hence, formation of lumen has been considered as an indication for CRC cell line differentiation. Two *in vitro* Matrigel-based assays were designed to study the differentiation of CRC cells when co-cultured with myofibroblasts. Matrigel was used to mimic the ECM in *in vivo* condition. LS180 was used for this assay due its well-established ability to form lumens in Matrigel. The experimental layouts of these assays are described below:

a) Differentiation assay A: Involved combination of both CRC cells (1×10³ of LS180) and myofibroblasts (1×10⁴ of CCD-18Co) in 40 µl of 50% Matrigel (diluted serum free DMEM) co-cultured in a 96-well plate. The Matrigel and cell suspension was left to harden at 37°C for 30 min, before serum free culture medium (DMEM alone) was added to the wells. Both cells were cultured at 37°C, 10% CO₂ for 14 days, with serum free DMEM being changed every 3 days.

b) Differentiation assay B: A total of 1×10³ LS180 cells were mixed with 40 µl of 50% Matrigel (diluted with serum free DMEM) and cultured on top of a CCD-18Co lawn. The myofibroblast lawn consists of 2×10⁴ CCD-18Co seeded in a 96-well plate, 48 h prior to the addition of LS180 and the Matrigel layer. The plate was incubated at 37°C, 10% CO₂ for 14 days, as above, before analysis.

For both assays, cells were stained with 10 µg/ml 4'-diamidino-2-phenylindole (DAPI) and F-actin. For the staining of F-actin, the cells were washed with phosphate-buffered saline (PBS) twice with PBS before fixing with 100 µl of 4% (v/v) paraformaldehyde (PFA) (VWR Chemicals, Lyon, France) in PBS for 20 min. The plate was then washed with PBS and then 100 µl of 0.2% Triton-X in PBS was added for 10 min. Next, the wells were washed 4 times with 50 mmol/l glycine in PBS for and 100 µl TRITC-phalloidin (stock concentration of 200 U/ml) in PBS (1:1,000) was added. The plate was then incubated at 4°C overnight. After washing 3 times with PBS, 100 µl of DAPI in PBS was added to each well. The formation of lumens in LS180, which is indicated by the F-actin staining was visualized using a fluorescence microscope.

A total of three respective fields each for co-culture and monoculture groups were selected for analysis. Lumen size which is represented by diameter of the lumens (length) from 45-50 cells and number of lumen-forming cells in each field was quantitated using ImageJ software.

Migration assay. A transwell assay, also known as modified Boyden chamber is a widely accepted technique for analysing migratory activity of cells *in vitro* (20). To study the migration of CCD-18Co (myofibroblasts) under co-culture conditions with CRC cell lines, a total of 2×10⁴ epithelial cells (RKO, HT29, or LS180) were seeded in a 24-well plate in DMEM+ 10% FBS and left for 48 h at 37°C, 10% CO₂. The old medium was discarded and replaced with 800 µl of DMEM + 0.5% FBS. A total of 1×10⁴ CCD-18Co cells were added into a transwell insert (pore size: 8.0 µm) (Greiner Bio-One, Frickenhausen, Germany). Next, the insert was transferred to the wells containing epithelial cells or to a control well (DMEM + 0.5% FBS only).

The insert was removed after 48 h of co-culture and non-migrated CCD-18Co on the inside of the insert were scraped off gently using a cotton swab. Then, the insert was fixed and stained with a Kwik-Diff kit (Thermo Fisher Scientific, UK) according to the manufacturer's protocol. Cell migration was assessed using the EVOS® XL core microscope (Life Technologies, Austin, TX, USA). The total of migrated cells from five randomly selected fields from an insert was

Figure 1. Experimental layout of the Matrigel-based blob invasion assay (A) and an example of the quantification for the invasion assay (B). The maximum distance covered by invaded myofibroblasts and fibroblasts into the Matrigel layer when co-cultured with CRC cells from triplicate experiments was quantified using the ImageJ software and compared to the control (empty blob) after 6 days of experiments (magnification: 50x). MFs: Myofibroblasts; Fs: fibroblasts.

quantified using ImageJ software. Experiments were performed in triplicate. A similar method was used to study the migration of CRC cell lines in co-culture condition with CCD-18Co. In this experiment, CCD-18Co were cultured in the bottom chamber and CRC cell lines were seeded in the upper chamber (transwell insert).

Matrigel-based blob invasion assay. A Matrigel-based blob assay was designed to study the myofibroblast invasion when co-culture

with CRC cell lines. In this assay, a total of 1×10^4 CRC cells and 5×10^3 myofibroblasts or skin fibroblasts were separately mixed with a cold 50% Matrigel solution. Then, 3 μl of the Matrigel and cell suspension was added to a 48-well plate, forming a blob of cells. For co-culture groups, two individual blobs of CRC cell line cells and either myofibroblasts or skin fibroblasts are seeded apart from each other in the same well. For monoculture (control), empty Matrigel blobs (50% Matrigel without any cells) were included in

Figure 2. Co-culture with CCD-18Co (PKH67 – Red) supports the growth of HT29 (PKH26 – Green). (A) Matrigel acts as a substrate and facilitates the proliferation of HT29 cells. (B) The cells grew poorly after 8 days without serum, as seen for HT29 seeded on plastic alone (magnification: 200 $\times$).

the same well with either CRC cell, myofibroblast or fibroblast blobs. The plate was incubated for 30 min at 37°C, 10% CO₂ for the gel to harden. After that, undiluted Matrigel was added to the well, to cover the area surrounding the blobs followed by 30 min incubation at 37°C, 10% CO₂ for solidification of the Matrigel layer. Next, 300 μ l of serum free DMEM were added to the well and the plate was incubated for 6 days at 37°C, 10% CO₂ with medium (serum-free DMEM) being changed every 3 days. Images of the blobs at the start (Day 0) and end of the experiment (Day 6) were recorded. The cells were visualized using a light microscope.

The experimental layout and quantification method of this assay is shown in Figure 1. The boundary of each blob (either an empty blob or blob containing cells) is indicated by the black curved line. On day 0, the cells in the blob appear rounder and no invasion of cells out of the blob was found. After 6 days of experiment, myofibroblasts invaded the Matrigel layer which surrounds the Matrigel blobs. The red dotted line indicates the maximum distance of myofibroblast invasion (μ M) from the edge of the Matrigel blob containing cells at the end of the experiment. The average (Av) of maximum distance of myofibroblast invasion from the Matrigel blob in co-culture and monoculture groups were recorded for analysis.

Statistical analysis. The data in this study are represented by the mean values $\pm$ standard error mean (SEM), assuming a normal distribution for the data. Independent t-tests were used to calculate the *p*-values from the biological replicates (SPSS® version 22, IBM®, Armonk, NY, USA). **p*<0.05 and ***p*<0.001.

Results

Myofibroblasts promote the growth of CRC cells. PKH26-labelled HT29 (green) shows better growth in co-culture conditions with myofibroblasts (PKH67-labelled CCD-18Co, red) where formation of larger colonies was observed in comparison to HT29 alone (Figure 2). Matrigel proved to be an efficient substrate that supports the growth of cells as better cell proliferation was found when both cells were grown on 50% Matrigel layer than on uncoated plates (plastic), where significantly smaller colonies of HT29 and lower CCD-18Co numbers were observed.

Myofibroblasts support differentiation of CRC cells. Two different types of Matrigel-based experiments were conducted in order to study the influence of co-culture with myofibroblasts on CRC cell differentiation. The layout and results from both assays are illustrated in Figure 3A and B. A larger number of LS180 cells was found in the co-culture group, which suggests the ability of myofibroblasts to support CRC cell proliferation. Moreover, LS180 colonies in Figure 3A were also found to be more elongated and grew in close contact with CCD-18Co, differently from monoculture (LS180 alone) where LS180 cells formed

(A) Differentiation assay A

(B) Differentiation assay B

Figure 3. Co-culture with CCD-18Co supports LS180 growth and lumen formation. In both Figure 3A and 3B, the lumens formed in the LS180 co-culture group appeared to be more elongated and larger compared to the control group (LS180 alone). Significant differences in lumen size, but not colony count were found between monoculture and co-culture groups (Figure 3B) (* $p < 0.05$ from triplicate experiments). It is worth noting that, in both assays, LS180 cells were able to grow at an optimal rate in co-culture with myofibroblasts without the addition of serum, where both cell lines were maintained in serum-free DMEM (DMEM alone) (magnification: 50 $\times$).

Figure 4. Epithelial cells support the migration of myofibroblasts. Significant migration of CCD-18Co was found when co-cultured with CRC cells in comparison to control (no CRC cell lines at the bottom) after 48 h of co-incubation (** $p<0.001$, * $p<0.05$ from triplicates). Both cells were maintained in DMEM + 0.5% FBS (minimal serum) for this experiment.

Figure 5. The presence of CCD-18Co influences the migration of CRC cells. Different CRC cells exhibit different abilities to migrate when co-incubated with CCD-18Co. Significant migration of CRC cells was observed in co-culture for RKO & HT29 (* $p<0.05$ from triplicates). Both myofibroblasts and CRC cells were cultured in DMEM + 0.5% FBS for this experiment. Representative images from the assay show significantly higher numbers of migrated RKO compared to HT29 and LS180 in the presence of CCD-18Co. Migrated CRC cells stained with Kwik-Diff. Magnification: 200x. MFs: myofibroblasts.

Figure 6. CRC cells support myofibroblast invasion. A Matrigel-based invasion assay was conducted where either myofibroblasts (CCD-18Co, Myo 6526, Myo 7395, Myo 6550) or CRC cell lines (RKO, HT29, LS180, SW480 and SW620) were seeded in a Matrigel blob separated from each other by a Matrigel layer and cultured for 6 days. Selected CRC cells possess different capacities to induce invasion of various myofibroblasts in co-culture. In contrast to the observation with myofibroblasts, no CRC cell lines induced invasion of skin fibroblasts (* $p < 0.05$ from triplicate experiments, significantly different from control).

rounder and more uniform colonies. Interestingly in both assays, LS180 cells in co-culture gave rise to significantly larger lumens than LS180 alone, more prominently seen in the second assay where both LS180 and CCD-18Co were mixed and cultured together in a Matrigel layer. No significant difference was found in the colony number between the two co-culture and monoculture conditions.

Positive influence of myofibroblasts on CRC cell migration. A transwell assay was used to study the migration of myofibroblasts (Figure 4) where CRC cell lines and CCD-18Co were seeded in the bottom and top chamber (transwell insert), respectively. Significant migration of CCD-18Co through the transwell pores was found after 48 h of co-culture with CRC cell lines (RKO, HT29 and LS180) under minimal serum conditions (0.5% FBS) in comparison to the control (without CRC cell lines). The largest number of migrated CCD-18Co cells was in co-culture with RKO, followed by HT29 and LS180.

The chemoattractant ability of CCD-18Co to induce the CRC cell line migration was also studied using another similar experimental approach, by reversing the positions of the CRC cell lines and CCD-18Co. The number and

representative images of migrated CRC cells with and without CCD-18Co after 48 h post-incubation is shown Figure 5. Significant migration of RKO and HT29 through the transwell pores was found in comparison to the control (CRC cell lines only, absence of CCD-18Co). This demonstrated that RKO cells were found to have significantly higher migratory capacity when compared to HT29, whereas no migration of LS180 cells was observed. The number of migrated RKO was largest followed by HT29 cells, as was found in the co-culture groups.

CRC cells promote the invasion of myofibroblast but not fibroblasts. The invasive property of myofibroblasts and fibroblasts was studied using a Matrigel-based blob assay. Selected myofibroblast lines showed different abilities to invade Matrigel when co-cultured with CRC cell lines. Significant invasion of CCD-18Co was observed with CRC cell lines (RKO, HT29, LS180, SW480 and SW620) after 6 days of co-incubation when compared to monoculture (myofibroblasts alone) (Figure 6). SW620 induced the most significant effect on CCD-18Co invasion among other CRC cell lines. The experiments were repeated using two primary myofibroblasts (Myo 6526 and Myo 7395) from normal

colon tissues as well as a pair of myofibroblasts isolated from cancer and adjacent normal colon of a same patient (Myo 6551C and Myo 6550, respectively). As illustrated in Figure 6, all selected CRC cell lines induced invasion of both Myo 6526 and Myo 7395, except for RKO. Notably, no significant invasion of foreskin fibroblasts was observed in co-culture with CRC cell lines. Altogether, our findings strengthen the evidence for invasive properties of myofibroblasts and not skin fibroblasts.

Note that different basal levels of invasion (monoculture control, without presence of CRC cell lines) in both myofibroblasts and fibroblasts were found. Skin fibroblasts showed higher basal levels of invasion in comparison to myofibroblasts. There was no CRC cells invasion observed in the Matrigel layer, although the cells were clearly visible.

Discussion

In this paper we described several *in vitro* assays for studying the effects of interaction between myofibroblasts and CRC cell lines namely cell growth, migration, invasion and differentiation. All the assays were performed either under serum-free (DMEM alone) or minimal serum (DMEM + 0.5% FBS) conditions to minimise the effect of serum on our experimental output and ensure the specificity of the results to the effects of co-culture. This exclusion of serum was important as it has been found that essential components such as various growth factors present in the serum influence cell proliferation, propagation and attachment (21).

Our data showed that the HT29 CRC cell line growth was poor in the absence of serum, but greatly improved in co-culture with myofibroblasts (CCD-18Co). The proliferation of HT29 and CCD-18Co was also boosted in the presence of Matrigel. This is most likely due to the constituents of Matrigel including ECM components (laminin, collagens, fibronectin) as well as growth factors and cytokines such as transforming growth factor beta-1 (TGF β 1) that promote the growth and attachment of HT29 and CCD-18Co (22). The concentration of Matrigel used is also an important factor that can influence the results, as both cell types did not grow well on 100% Matrigel (data not shown). Thus, it is important to adjust the Matrigel concentration to ensure optimal proliferation and adhesion of cells (23).

In contrast to our data, Vermeulen *et al.* (2010) suggested that myofibroblasts inhibited the differentiation of cancer stem cells (CSCs) (24). Greicius *et al.* reported that secreted R-spondins from pericryptal myofibroblasts, particularly R-spondin3 (RSPO3), have a positive influence on cancer cell proliferation and support the stemness of epithelial cells through stimulation of Wnt/ β -catenin signaling (25). Our microarray data, however revealed low expression of RSPO3 in all tested myofibroblasts which may explain the lack of a negative effect of co-culture on LS180 differentiation seen

in our work. This highlights the variability in phenotype between different sources of myofibroblasts with respect to their effects on the growth and differentiation of CRC cells.

The transwell assay showed a pro-migratory effect of myofibroblasts on CRC cell line migration. It is also apparent from this assay that cancer cells secrete components that are able to induce the migration of myofibroblasts. The myofibroblast migration assay also showed that the extent of CCD-18Co's migratory influence was CRC cell line-dependent. This migration assay also suggests the production of growth factors or cytokines by RKO that promote the migration of CCD-18Co more than any produced by HT29 or LS180 as greater migratory ability of CCD-18Co was found under co-culture with RKO as compared to the other two cell lines. The data clearly demonstrate the heterogeneity in the panel of CRC cell lines. This is not surprising as CRC in particular is known to harbour considerable heterogeneity (26). It has also been reported that different morphologies in various CRC cells do not necessarily act as an indicator for genetic differences, and *vice versa* (27).

The Matrigel-based blob invasion assay showed strong pro-invasive effects of CRC cell lines (RKO, HT29, LS180, SW480 and SW620) on patient-derived myofibroblasts. However, RKO was the only of these CRC lines that had no effect on CCD-18Co. CCD-18Co, although not an immortalized cell line was derived from neonatal tissue and so may possess characteristics that distinguish it from myofibroblasts that were established from adult patient tissues. One of the properties that differentiates the patient-derived myofibroblasts from CCD-18Co is the population doubling level (PDL) which is significantly higher for CCD-18Co than for the other primary myofibroblasts used in the study. Thus, CCD-18Co can be maintained in culture till passage 20 before the cells start to senesce, as opposed to our primary myofibroblasts which can only be maintained in culture up to a maximum passage number of 10. This is probably explained by the fact that CCD-18Co was isolated from the colon of a neonate individual aged 2.5 months, while our primary myofibroblasts were isolated from colon of cancer, and so much older patients.

Collectively, our data show significant effects of myofibroblasts on the growth, migration, invasion and differentiation of CRC cells. Our results support many previous findings on the prominent role of myofibroblasts on colorectal carcinogenesis (28-30). Albeit, it is worth noting that several of these assays (*i.e.* proliferation and transwell assays) were performed in a 2-dimensional setting which could infer diversity of experimental outcome in 3-dimensional architecture such as in organoid structure.

In vitro assays performed in the present study could be applied as precursors for confirmatory *in vivo* study on the relationship between stromal cells of the tumour microenvironment and CRC cells. Our results also corroborate

with previous studies where various functional assays performed *in vitro* provided insight on potential treatment avenues of cancer by targeting the myofibroblast–cancer cell interaction (31, 32). In addition, the ability to test different types and sources of myofibroblasts and tumour cells (primary cells or cell lines) through *in vitro* analysis gives liberty to the researchers to explore different molecular pathways that may be involved in colorectal carcinogenesis and potential anticancer activity of various components or drugs on tumour development. This further highlights the importance to design experimental tests or analyses that could address the variation in genetic profile between individuals or subjects.

CRC is a heterogeneous and complex disease. Our genetic makeup significantly influences the progression of cancer that involves different mechanisms such as cell proliferation, differentiation, migration and invasion. *In vitro* study allows the researchers to investigate further the fundamental processes that occur physiologically and dictate carcinogenesis in different subjects or populations. The establishment and use of primary cells isolated from various CRC patients *in vitro* in particular have significantly contributed to cancer research. This is clearly reported in a study by van de Wetering *et al.* (2015) where they performed drug screening (a total of 83 target-known inhibitors and chemotherapy drugs) on 19 organoids derived from CRC patients. It was postulated that organoid technology will serve as a foundation for precision medicine by bridging the gap between cancer genetics and patient trials and complement cell line- and xenograft-based drug studies (33).

It is also worth noting that there are several advantages and disadvantages in the present study. The advantages include; a) the convenience of *in vitro* assays to be conducted in the laboratory; b) well-designed *in vitro* assays which allow an unbiased analysis of the results by the evaluators as they are performed under a controlled experimental setting and c) the ability to test and assess different properties (proliferation, migration, invasion) of multiple cell types under the same controlled environment. As with many *in vitro* studies, the current work also has several disadvantages and limitations such as a) inability to assess various cell properties in a single experiment; b) experiments conducted in a 2-dimensional setting that could affect the cell property (*in vitro* vs. *in vivo*) and c) assessment of physical properties of cells was performed, rather than analysis on the molecular mechanism at gene/protein level (*e.g.* changes in the cell migratory ability and its effect on expression of associated genes with cell migration).

In conclusion, although these *in vitro* assays have certain limitations, most notably that they do not properly recreate the 3-dimensional architecture of the tumour and its microenvironment, they nevertheless provide valuable clues on the bi-directional communications between myofibroblasts and CRC cells *in vivo*. These analyses also highlighting the

heterogeneity in both myofibroblast and epithelial cancer cell lines. Thus, they will be very useful for investigating the secreted factors and their receptors that mediate the myofibroblast-epithelial cancer cell interactions which may promote to the CRC progression. These *in vitro* assays would also serve as valid experimental methods to determine anticancer properties of different compounds, blocking antibodies and therapeutic drugs against CRC.

Conflicts of Interest

None declared.

Authors' Contributions

Marahaini Musa (MM), Djamila Ouaret (DO) and Walter F. Bodmer (WFB) conceived the study. MM, DO and WFB participated in the design of the study. MM performed the experiments. MM, DO and WFB analysed and interpreted the data. MM wrote the manuscript with the help of DO, under the supervision of WFB with input from all Authors.

Acknowledgements

The study was supported financially by the Oncology Department, University of Oxford, UK.

References

- 1 Bray F, Ferlay J, Soerjomataram I, Soerjomataram I, Siegel RL, Torre LA and Jemal A: Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA Cancer J Clin* 68(6): 394-424, 2018. PMID: 30207593. DOI: 10.3322/caac.21492
- 2 Halama N, Zoernig I, Grabe N and Jaeger D: The local immunological microenvironment in colorectal cancer as a prognostic factor for treatment decisions in the clinic: The way ahead. *Oncoimmunology* 1(1): 62-66, 2012. PMID: 22720213. DOI: 10.4161/onci.1.1.18460
- 3 Giordano G, Febbraro A, Venditti M, Campidoglio S, Olivieri N, Raieta K, Pietro Parcesepe P, Imbriani GC, Remo A and Pancione M: Targeting angiogenesis and tumor microenvironment in metastatic colorectal cancer: Role of aflibercept. *Gastroenterol Res Pract* 2014: 13, 2014. PMID: 25136356. DOI: 10.1155/2014/526178
- 4 Zhang R, Qi F, Shao S, Li G and Feng Y: Human colorectal cancer-derived carcinoma associated fibroblasts promote CD44-mediated adhesion of colorectal cancer cells to endothelial cells by secretion of HGF. *Cancer Cell Int* 19: 192, 2019. PMID: 31367190. DOI: 10.1186/s12935-019-0914-y
- 5 Drovak HF: Tumors: wounds that do not heal. Similarities between tumor stroma generation and wound healing. *N Engl J Med* 315(26): 1650-1659, 1986. PMID: 3537791. DOI: 10.1056/NEJM198612253152606
- 6 Simon-Assmann P, Kedinger M, De Arcangelis A, Rousseau V and Simo P: Extracellular matrix components in intestinal development. *Experientia* 51(9): 883-900, 1995. PMID: 7556570. DOI: 10.1007/BF01921739

- 7 Pattabiraman DR and Weinberg RA: Tackling the cancer stem cells - what challenges do they pose? *Nat Rev Drug Discov* 13(7): 497-512, 2014. PMID: 24981363. DOI: 10.1038/nrd4253
- 8 Bochaton-Piallat ML, Gabbiani G and Hinz B: The myofibroblast in wound healing and fibrosis: answered and unanswered questions. *F1000Res* 5: F1000 Faculty Rev-752, 2016. PMID: 27158462. DOI: 10.12688/f1000research.8190.1
- 9 Hsia LT, Ashley N, Ouaret D, Wang LM, Wilding J and Bodmer WF: Myofibroblasts are distinguished from activated skin fibroblasts by the expression of AOC3 and other associated markers. *Proc Natl Acad Sci USA* 113(15): E2162-E2171, 2016. PMID: 27036009. DOI: 10.1073/pnas.1603534113
- 10 Adegboyega PA, Mifflin RC, DiMari JF, Saada JI and Powell DW: Immunohistochemical study of myofibroblasts in normal colonic mucosa, hyperplastic polyps, and adenomatous colorectal polyps. *Arch Pathol Lab Med* 126(7): 829-836, 2002. PMID: 12088453. DOI: 10.1043/0003-9985(2002)126<0829:ISOMIN>2.0.CO;2
- 11 Tsujino T, Seshimo I, Yamamoto H, Ngan CY, Ezumi K, Takemasa I, Ikeda M, Sekimoto M, Matsuura N and Monden M: Stromal myofibroblasts predict disease recurrence for colorectal cancer. *Clin Cancer Res* 13(7): 2082-2090, 2007. PMID: 17404090. DOI: 10.1158/1078-0432.CCR-06-2191
- 12 Orimo, A Gupta PB, Sgroi DC, Arenzana-Seisdedos F, Delaunay T, Naeem R, Carey VJ, Richardson AL and Weinberg RA: Stromal fibroblasts present in invasive human breast carcinomas promote tumor growth and angiogenesis through elevated SDF-1/CXCL12 secretion. *Cell* 121(3): 335-348, 2005. PMID: 15882617. DOI: 10.1016/j.cell.2005.02.034
- 13 Calon A, Lonardo E, Berenguer-Llargo A, Espinet E, Hernando-Mombona X, Iglesias M, Sevillano M, Palomo-Ponce S, Tauriello DVF, Byrom D, Cortina C, Morral C, Barceló C, Tosi S, Riera A, Attolini CS, Rossell D, Sancho E and Batlle E: Stromal gene expression defines poor-prognosis subtypes in colorectal cancer. *Nat Genet* 47(4): 320-329, 2015. PMID: 25706628. DOI: 10.1038/ng.3225
- 14 Powell DW, Mifflin RC, Valentich JD, Crowe SE, Saada JI and West AB: Myofibroblasts. I. Paracrine cells important in health and disease. *Am J Physiol* 277(1): C1-9, 1999. PMID: 10409103. DOI: 10.1152/ajpcell.1999.277.1.C1
- 15 De Wever O, Demetter P, Mareel M and Bracke M: Stromal myofibroblasts are drivers of invasive cancer growth. *Int J Cancer* 123(10): 2229-2238, 2008. PMID: 18777559. DOI: 10.1002/ijc.23925
- 16 Labernadie A, Kato T, Bragues A, Serra-Picamal X, Derzsi S, Alwert E, Weston A, González-Tarragó V, Elosegui-Artola A, L, Alcaraz J, Roca-Cusachs P, Sahai E and Trepas X: A mechanically active heterotypic E-cadherin/N-cadherin adhesion enables fibroblasts to drive cancer cell invasion. *Nat Cell Biol* 19(3): 224-237, 2017. PMID: 28218910. DOI: 10.1038/ncb3478
- 17 Sahai E, Aatsaturov I, Cukierman E, DeNardo DG, Egeblad M, Evans RM, Fearon D, Gretchen FR, Hingorani SR, Hunter T, Hynes RO, Jain RK, Janowitz T, Jorgensen C, Kimmelman AC, Kolonin MG, Maki RG, Powers RS, Puré E, Ramirez DC, Scherz-Shouval R, Sherman MH, Stewart S, Tlsty TD, Tuveson DA, Watt FM, Weaver V, Weeraratna AT and Werb Z: A framework for advancing our understanding of cancer-associated fibroblasts. *Nat Rev Cancer* 20(3): 174-186, 2020. PMID: 31980749. DOI: 10.1038/s41568-019-0238-1
- 18 Eberlein C, Rooney C, Ross SJ, Farren M, Weir HM and Barry ST: E-Cadherin and EpCAM expression by NSCLC tumour cells associate with normal fibroblast activation through a pathway initiated by integrin α 5 β 1 and maintained through TGF β signalling. *Oncogene* 34(6): 704-716, 2015. PMID: 24488011. DOI: 10.1038/onc.2013.600
- 19 Ashley N, Yeung TM and Bodmer WF: Stem cell differentiation and lumen formation in colorectal cancer cell lines and primary tumors. *Cancer Res* 73(18): 5798-5809, 2013. PMID: 23867471. DOI: 10.1158/0008-5472.CAN-13-0454
- 20 Justus CR, Leffler N, Ruiz-Echevarria M and Yang LV: *In vitro* cell migration and invasion assays. *J Vis Exp* (88): 51046, 2014. PMID: 24962652. DOI: 10.3791/51046
- 21 Gstraunthaler G: Alternatives to the use of fetal bovine serum: serum-free cell culture. *ALTEX* 20(4): 275-281, 2003. PMID: 14671707.
- 22 Vukicevic S, Kleinman HK, Luyten FP, Roberts AB, Roche NS and Reddi AH: Identification of multiple active growth factors in basement membrane matrigel suggests caution in interpretation of cellular activity related to extracellular matrix components. *Exp Cell Res* 202(1): 1-8, 1992. PMID: 1511725. DOI: 10.1016/0014-4827(92)90397-q
- 23 Fischer RS, Myers KA, Gardel ML and Waterman CM: Stiffness-controlled three-dimensional extracellular matrices for high-resolution imaging of cell behavior. *Nat Protoc* 7(11): 2056-2066, 2012. PMID: 23099487. DOI: 10.1038/nprot.2012.127
- 24 Vermeulen L, De Sousa E Melo F, van der Heijden M, Cameron K, de Jong JH, Borovski T, Tuynman JB, Todaro M, Merz C, Rodermond H, Sprick MR, Kemper K, Richel DJ, Stassi G and Medema JP: Wnt activity defines colon cancer stem cells and is regulated by the microenvironment. *Nat Cell Biol* 12(5): 468-476, 2010. PMID: 20418870. DOI: 10.1038/ncb2048
- 25 Greicius G, Kabiri Z, Sigmundsson K, Liang C, Bunte R, Singh MK and Virshup DM: PDGFR α pericryptal stromal cells are the critical source of Wnts and RSPO3 for murine intestinal stem cells *in vivo*. *Proc Natl Acad Sci USA* 115(14): E3173-E3181, 2018. PMID: 29559533. DOI: 10.1073/pnas.1713510115
- 26 McGranahan N and Swanton C: Clonal heterogeneity and tumor evolution: past, present, and the future. *Cell* 168(4): 613-628, 2017. PMID: 28187284. DOI: 10.1016/j.cell.2017.01.018
- 27 Navin N, Krasnitz A, Rodgers L, Cook K, Meth J, Kendall J, Riggs M, Eberling Y, Troge J, Grubor V, Levy D, Lundin P, Månér S, Zetterberg A, Hicks J and Wigler M: Inferring tumor progression from genomic heterogeneity. *Genome Res* 20(1): 68-80, 2010. PMID: 19903760. DOI: 10.1101/gr.099622.109
- 28 Hanley CJ, Noble F, Ward M, Bullock M, Drifka C, Mellone M, Manousopoulou A, Johnston HE, Hayden A, Thirdborough S, Liu Y, Smith DM, Mellows T, Kao WJ, Garbis SD, Mirnezami A, Underwood TJ, Eliceiri KW and Thomas GJ: A subset of myofibroblastic cancer-associated fibroblasts regulate collagen fiber elongation, which is prognostic in multiple cancers. *Oncotarget* 7(5): 6159-6174, 2016. PMID: 26716418. DOI: 10.18632/oncotarget.6740
- 29 Sugai T, Uesugi N, Kitada Y, Yamada N, Osakabe M, Eizuka M, Sugimoto R, Fujita Y, Kawasaki K, Yamamoto E, Yamano H, Suzuki H and Matsumoto T: Analysis of the expression of cancer-associated fibroblast- and EMT-related proteins in submucosal invasive colorectal cancer. *J Cancer* 9(15): 2702-2712, 2018. PMID: 30087711. DOI: 10.7150/jca.25646
- 30 Bauer J, Emon MAB, Staudacher JJ, Thomas AL, Zessner-Spitzenberg J, Mancinelli G, Krett N, Saif MT and Jung B: Increased stiffness of the tumor microenvironment in colon cancer stimulates cancer associated fibroblast-mediated prometastatic

- activin A signaling. *Sci Rep* *10*(1): 50, 2020. PMID: 31919369. DOI: 10.1038/s41598-019-55687-6
- 31 Dai G, Yao X, Zhang Y, Gu J, Geng Y, Xue F and Zhang J: Colorectal cancer cell-derived exosomes containing miR-10b regulate fibroblast cells *via* the PI3K/Akt pathway. *Bull Cancer* *105*(4): 336-349, 2018. PMID: 29496262. DOI: 10.1016/j.bulcan.2017.12.009
- 32 Hanley CJ, Mellone M, Ford K, Thirdborough SM, Mellows T, Frampton SJ, Smith DM, Harden E, Szyndralewicz C, Bullock M, Noble F, Moutasim KA, King EV, Vijayanand P, Mirnezami AH, Underwood TJ, Ottensmeier CH and Thomas GJ: Targeting the myofibroblastic cancer-associated fibroblast phenotype through inhibition of NOX4. *J Natl Cancer Inst* *110*(1): 109-120, 2018. PMID: 28922779. DOI: 10.1093/jnci/djx121
- 33 van de Wetering M, Francies HE, Francis JM, Bounova G, Iorio F, Pronk A, van Houdt W, van Gorp J, Taylor-Weiner A, Kester L, McLaren-Douglas A, Blokker J, Jaksani S, Bartfeld S, Volckman R, van Sluis P, Li VSW, Seepo S, Pedamallu CS, Cibulskis K, Carter SL, McKenna A, Lawrence MS, Lichtenstein L, Stewart C, Koster J, Versteeg R, van Oudenaardeen A, Saez-Rodriguez J, Vries RGJ, Getz G, Wessels L, Stratton MR, McDermott U, Meyerson M, Garnett MJ and Clevers H: Prospective derivation of a living organoid biobank of colorectal cancer patients. *Cell* *161*(4): 933-945, 2015. PMID: 25957691. DOI:10.1016/j.cell.2015.03.053

Received September 6, 2020

Revised September 21, 2020

Accepted September 25, 2020