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Human Mesenchymal Stem Cell Derived from Bone Marrow and Umbilical Cord Display Anti-Cancer Activity in Cancer Cell Lines *in vitro*

(Sel Stem Mesenkima Manusia Diambil daripada Sum-sum Tulang dan Tali Pusat menunjukkan Aktiviti Anti-Kanser dalam Titisan Sel Kanser secara *in vitro*)

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ABSTRACT

The anti-tumour efficacy of engineered mesenchymal stem cell (MSCs) in cancers have been well documented by several reports. However, the impact of MSCs on the pathogenesis of solid cancers remains elusive. The study aims to elucidate the role of MSCs from bone marrow (BMMSCs) and umbilical cord (UCMSCs) on the proliferation, apoptosis and clonogenicity of cancer cell including H2170 (squamous cell carcinoma), LN18 (glioblastoma) and MCF7 (breast cancer) *in vitro*. Highest concentration of conditioned medium derived from the UCMSCs was significantly ($p<0.001$) effective to inhibit the proliferation of H2170 (25.8 ± 3.5%), LN18 (17.6 ± 6.5%) and MCF7 (33.2 ± 6.8%) as compared to 100% viability in basal. Both MSCs and its conditioned medium were able to significantly ($p<0.001$) induce apoptosis (early and late) to the H2170 and LN18 cells. However, for MCF7 cells, co-cultured with both MSCs had higher impact on the apoptosis as compared to their condition medium. Furthermore, conditioned medium from UCMSCs were able to significantly reduced the number of colonies in H2170 (609.5 ± 4.9) and LN18 (171.3 ± 12.6) as compared to control (H2170; 1196.3 ±12.8 and LN18; 253.3 ± 12.3), suggesting that these two cancer cells are sensitive to the MSCs. Notably, by co-culturing of all three cancer cell lines with the MSCs' conditioned medium, we found that there was an increased expression of more than two-fold in BAX, BAD, and APAF1 genes showing the ability of MSCs' conditioned medium to induce the intrinsic apoptosis pathway in the cancer cells. Collectively, our findings demonstrated that the MSCs could induce apoptosis and inhibit both H2170 and LN18 cancer cell proliferation. Furthermore, this study did not find evidence of MSCs in enhancing tumorigenic characteristics of these cancer cells, and thus we postulate that MSCs are basically safe as a cell-based therapy in cancer treatment.

Keywords: Anti-cancer; cancer cell lines; *in vitro*; mesenchymal stem cell

ABSTRAK

Efikasi kejuruteraan sel stem mesenkima (MSC) dalam menangani beberapa jenis kanser telah pun dilaporkan. Namun demikian, impak MSC terhadap patogenesis kanser pepejal masih kurang diketahui. Kajian ini bertujuan untuk menjelaskan peranan MSC daripada sum-sum tulang (BMMSC) dan tali pusat (UCMSC) terhadap pertumbuhan, apoptosis dan fungsi klonogenisiti sel kanser H2170 (karsinoma sel skuamosa), LN18 (glioblastoma) dan MCF7 (kanser payudara). Media pertumbuhan berpekatan tertinggi yang diperoleh daripada UCMSC adalah berkesan ($p<0.001$) untuk menghalang pertumbuhan sel H2170 (25.8 ± 3.5%), LN18 (17.6 ± 6.5%) dan MCF7 (33.2 ± 6.8%) apabila dibandingkan dengan 100% keviabelan asas. Kedua-dua BMMSC dan UCMSC serta media pertumbuhan mereka mendorong apoptosis (peringkat awal dan akhir) bererti ($p<0.001$) terhadap sel-sel H2170 dan LN18. Walau bagaimanapun, bagi sel-sel MCF7, pengkulturan bersama kedua-dua MSC menunjukkan kesan apoptosis yang lebih tinggi berbanding dengan media pertumbuhan mereka. Selain itu, media pertumbuhan daripada UCMSC nyata dapat mengurangkan bilangan koloni sel H2170 (609.5 ± 4.9) dan LN18 (171.3 ± 12.6) berbanding dengan kawalan (H2170; 1196.3 ±12.8 dan LN18; 253.3 ± 12.3), mencadangkan bahawa kedua-dua sel kanser adalah sensitif terhadap MSC. Secara ketara, kami mendapati bahawa pengkulturan ketiga-tiga titisan sel kanser tersebut bersama dengan media pertumbuhan MSC dapat meninggikan lebih dua kali ganda eskpresi gen BAX, BAD dan APAF1,

menunjukkan bahawa media pertumbuhan MSC dapat mendorong laluan apoptosis intrinsik pada sel kanser tersebut. Secara keseluruhan, kajian ini telah menunjukkan bahawa MSC dapat mendorong apoptosis dan menyekat pertumbuhan sel kanser H2170 dan LN18. Di samping itu, kajian ini tidak menunjukkan bahawa MSC meningkatkan ciri tumorigenik pada sel kanser, maka kami mempostulatkan bahawa MSC adalah selamat bagi terapi berasaskan sel bagi rawatan kanser.

Kata kunci: Anti-kanser; *in vitro*; sel kanser; sel stem mesenkima

Introduction

Human mesenchymal stem cells or MSCs are among a group of adult stem cells present in the bone marrow and capable of multilineage differentiation into osteoblast, chondrocyte and adipocytes (Peng et al. 2008). MSCs can be isolated from several sources including umbilical cord, adipose tissue and dental pulp (Kern et al. 2006; Pierdomenico et al. 2005). MSCs have attracted great interest recently in the context of cell therapy as studies have reported the therapeutic efficacy of MSCs in several diseases including liver cirrhosis (Wu, Chen & Tang 2018), diabetes (Wu, Chen & Tang 2018), cardiac ischemia (Chang et al. 2021), muscle atrophy (Piao et al. 2022), and cancer (Stagg 2008). Furthermore, MSCs also exhibit low immunogenicity, which means that these cells can be expanded ex vivo and stored as biobank for allogeneic transplant (Jacobs et al. 2013).

Factors that contribute to the hallmarks of cancer including cellular proliferation, tumour growth, chemoresistance, and metastasis have been the subjects of intense investigation (Hanahan & Weinberg 2000). Studies have shown that these characteristics are the results of compounding genetic mutations that accumulated over time (Jang, Kim & Lee 2013; Takeshima & Ushijima 2019), while others have suggested the involvement of tumour microenvironment or TME during tumorigenesis (Gunaydin 2021; Rodrigues et al. 2021). Tumour microenvironment comprises an intricate network of cells and extracellular matrix such as fibroblast, immune cells, stroma cells including MSCs and blood vessels which regulate tumour development through a complex networking between tumour cells and its environment (Ribeiro Franco et al. 2020). MSCs have been known to produce vast arrays of chemokines, cytokines and factors through paracrine release and as MSCs are also part of the TME, it is expected that these cells could also contribute to the development or inhibition of the tumour (Li et al. 2022). MSCs are known to affect the proliferation and differentiation of dendritic cells (Lu et al. 2019), T cells (Laing et al. 2019) and natural killer (NK) cells (Spaggiari et al. 2006), which are cells involved during tumorigenesis.

The tumour-homing characteristics of MSCs shown by several reports have paved the way of using these cells beyond degenerative diseases (Koseki et al. 2016; Nieddu et al. 2019; Senthilkumar et al. 2018). Studies have demonstrated that engineered MSCs are capable of migrating and home into the tumour environment and induced in situ targeted drug delivery of several cytokines and anti-cancer agents including interleukin-2 (Chulpanova et al. 2021), interferon-beta (Studeny et al. 2002) and TRAIL (tumour necrosis factor related apoptosis inducing ligand) (Kolluri, Laurent & Janes 2013; Sasportas et al. 2009). Engineered MSCs expressing pro-drug converting enzymes (Ho et al. 2021; Ho et al. 2020) and oncolytic viruses (Keshavarz et al. 2020; Mahasa et al. 2020) have also been reported to exert a strong anti-cancer activities in pre-clinical models. Furthermore, the anti-tumour characteristics of native MSCs in several cancer models including leukaemia (Malini et al. 2012) cervical cancer (Meng et al. 2021) and hepatoma (Qiao et al. 2008a) have made MSCs' as an attractive candidate for cell-based gene therapy in cancer. However, others have demonstrated that MSCs could also induce epithelial to mesenchymal transition (EMT) (Martin et al. 2010) in breast cancer and can enhance tumorigenic characteristics of several other cancers (Chen et al. 2019; Sai et al. 2019). These factors have raised the question of both safety and potential risk of using these cells for cancer treatment. Moreover, the ambiguous role of MSCs in cancer might also be owed to several factors including the heterogeneity of MSCs due to its sources, culture conditions and the initial growth environment that contribute to both pro- and anti-cancer characteristics of these cells (Kidd et al. 2008).

Although bone marrow was first described as the source of MSCs (Kemp, Hows & Donaldson 2005), other sources such as umbilical cord is now being commonly used for clinical investigations due to its availability and greater proliferation rate (Kern et al. 2006). Nonetheless,

as both BMMSCs and UCMSCs are the most common MSCs being investigated for cell-based gene therapy in cancer, we seek to understand further the impact of these two types of MSCs on the regulation of cancer cell lines in vitro. Both umbilical cord (UCMSCs) and bone marrow (BMMSCs) were grown in the same culture condition to reduce the heterogeneity associated with different culture conditions. Furthermore, corresponding to the current studies of MSCs application in targeting solid tumours such as lung cancer (Cortes-Dericks & Galetta 2019; Li et al. 2011; Liu et al. 2017), glioma (Deng et al. 2018; Kazimirsky et al. 2016; Wang et al. 2017) and breast cancer (Liu et al. 2018; Liu et al. 2015); cellular regulation of BMMSCs and UCMSCs on these solid tumours were evaluated in the study by direct co-culture with H2170, LN18 and MCF7 cancer cell lines, representing model of lung cancer, glioma and breast cancer, respectively. Apoptosis induced by these MSCs on cancer cells was further assayed using Annexin-V and PI staining and subsequently quantified using flow cytometry. Finally, gene expression analysis using quantitative PCR (qPCR) was performed to further identified genes involved in intrinsic apoptosis.

Materials and Methods

Mesenchymal stem cells lines

Human umbilical cord derived mesenchymal stem cells (UCMSCs) at passage 2 were purchased from the American Type Culture Collection (ATCC, Manassas, USA) and human bone marrow derived mesenchymal stem cells (BMMSCs) was purchased from Lonza (Lonza Group, US). The MSCs were cultured in specific growth medium containing knockout DMEM, 1% penicillin/streptomycin, 2 mM L-glutamine (200 mM stock), 10% fetal bovine serum (FBS), 5 ng/mL fibroblast growth factor (FGF) basic and 5 ng/mL recombinant epidermal growth factor (rhEGF) (Gibco). The cells were incubated in a humidified incubator at 37 ºC supplied with 5% carbon dioxide, routinely maintained in 75 cm^2 tissue culture flasks and harvested when cells reached 80-90% confluence using 0.05% trypsin-EDTA (Gibco) for subculture and expansion.

Cancer cell lines

Cancer cell lines [H2170 (lung squamous cell carcinoma), LN18 (glioblastoma) and MCF7 (breast adenocarcinoma)] were purchased from ATCC and grown until they reached 80%-90% confluence. These cells were harvested with 0.25% trypsin-EDTA (Gibco) and sub-cultured for expansion. All of the cell lines were cultured in RPMI-1640 supplemented with 10% FBS and 100 IU/mL penicillin, 100 µg/mL streptomycin, 1% non-essential amino acids (NEAA) and 1% L-Glutamine (all from Invitrogen Corporation, Carlsbad, CA). The cultures were maintained in 5% CO2 in a humidified incubator at 37 °C.

Immunophenotyping analysis of MSCs

The MSCs were stained with multiple fluorescence conjugated antibodies against a panel of mesenchymal markers (CD44, CD73, CD106, and CD90). All antibodies were purchased from Becton Dickinson (BD), Heidelberg, Germany. Briefly, 0.5 - 1.0 × 10^6 cells at passage 2 was suspended in 100 µL of PBS supplemented with 2% fetal bovine serum (FBS; Invitrogen Corporation) and incubated with 10 µL of fluorescein conjugated antibodies for 20 min in dark at room temperature. After two washes, cells were suspended again in 0.5 mL of PBS supplemented with 2% FBS. Stained cells were subjected to flow cytometric acquisition using FACS (fluorescence activated cell sorting) Calibur instrument (Becton Dickinson [BD]) and a total of 10,000 events were acquired for data analysis by using Cell Quest software (BD, San Jose, CA). An isotype control was included in each experiment to exclude data from non-specific binding.

Adipogenic and osteogenic differentiation

The MSCs were induced to adipogenic and osteogenic differentiation using specific lineage differentiation medium (ATCC, Manassas, USA). Passage 3 MSCs were seeded at 1.7 × 10^5 cells/well in 6 wells tissue culture plates for 24 h. The next day, medium was replaced with a specific adipogenic and osteogenic differentiation medium. Cells were induced for 21 days with the medium being changed once every alternate day. After 21 days, the adipogenic and osteogenic cultures were fixed and stained with oil red o (0.3%) and alizarin red solution, respectively. The stained cells were examined under an inverted microscope immediately after staining.

Generation of MSCs conditioned medium

Human MSCs were seeded with complete media at a density of 1.0 × 10^5 cells/mL in T75 flask at 37 ºC and 5% CO_2 for 48 h. After 48 h, conditioned medium was harvested and centrifuge at at 4000 r.p.m. for 5 min to

eliminate cells and cellular debris and supernatant was stored at -80 ºC until further used. The conditioned medium was diluted with fresh prepared complete medium to prepare different concentrations of MSCs conditioned medium (20%, 60% and 100%).

VIABILITY ASSAY/MTS ASSAY

To investigate whether MSCs exhibit tumour supportive or inhibitory effect on cancer cells *in vitro*, we performed a direct co-culture experiment. The MTS assay (3-(4,5-dimethylthiazol-2-yl)-2H-tetrazolium, inner salt) purchased from Promega was used to evaluate the cellular proliferation of H2170, LN18 and MCF7 cells. During the execution of the experiments, all the cells (MSCs and Cancer) were grown in MSCs complete medium for culture standardization. Briefly, cancer cells were seeded in 96 wells plate at a density of 1.0×10^4 cells/well in 50 µL MSCs complete media on and left to grow overnight. The next day, 50 µL of increased number of BMMSCs, and UCMSCs or fresh MSCs complete medium (control) was added into the wells containing the cancer cell at a different ratio (the number of MSCs to cancer was 1:500, 1:20 and 1:1, respectively) and incubated for 48 h. For this purpose, various ratios ranging from low to high concentration of MSCs or MSCs conditioned media are tested in order to obtain an optimal result (Goers, Freemont & Polizzi 2014). Four hours before the end of the experiment, 20 µL of MTS solution was added into each well and the absorbance at 490 nm was measured using Odyssey ® SA Imaging System (Li-Cor), using wells without cells as blank. Calculation on the percentage of cancer cells viability as follows: cell viability (% of control) = ((OD_{490} of cancer cells with MSCs – OD_{490} of MSCs only)/ OD_{490} of cancer cells only) × 100%. Direct co culture of cancer cells with different concentrations of MSCs conditioned medium was also performed to further elucidate the paracrine regulation of MSCs on the proliferation of cancer cells. During the execution of the experiments, all of the cells (MSCs and Cancer) were also grown in MSCs complete medium for culture standardization. Initially, 1.0×10^4 cancer cells were pellet and suspended in 100 µL of different percentage of BMMSCs and UCMSCs conditioned medium (stimulated cells) and incubated for 48 h. Cancer cells suspended in 100% fresh MSCs complete medium were used as control (unstimulated cells). 20 µL of MTS was added 4 h prior to absorbance reading at 490 nm. Proliferation of stimulated cells was referred to the activity of unstimulated cells and calculated as follows; Cell viability (% of control) = (OD_{490} of stimulated cancer cells/OD_{490} of unstimulated cells) × 100%.

APOPTOSIS ASSAY (ANNEXIN V AND PROPIDIUM IODIDE/PI) STAINING

Apoptotic induction of MSCs conditioned medium from various sources to the cancer cells was performed using the Annexin V/ PI double staining kit purchased from (Becton Dickinson, BD, Heidelberg, Germany). Cancer cells were incubated with 60% MSCs conditioned medium or 60% MSCs complete medium (control) for 48 h. Direct co-culture between both MSCs and cancer cells were also performed using 24 wells plate, 0.4 µm trans-well system for physical separation of both cancer cells and MSCs (SPL Life Sciences). Sixty percent of MSCs conditioned medium was selected for the experiment based on the data taken from the proliferation assay. Briefly, 1.0×10^5 cells/well of MSCs were seeded on the insert and the same number of cancer cells (1.0×10^5 cells/well) were seeded on the bottom wells and co cultured directly for 48 h. Treated cancer cells by MSCs conditioned medium were harvested by trypsinization and collected by centrifugation. Cell palette was suspended in 100 µL of 1X Annexin V binding buffer (BD Bioscience) and 5 µL of Annexin-V-FITC was added. Antibody incubation was performed at 4 ºC for 20 min and 1 µL of propidium iodide (PI) was later added before FACS acquisition. Stained cells were subjected to flow cytometric analysis using FACS Calibur instrument (Becton Dickinson, BD) and a total of 10,000 events were acquired and analyzed using Cell Quest software (BD, San Jose, CA). Cells positive only for PI or annexin v were gated as dead or apoptotic cells, respectively. However, cells positive for both annexin v and PI were considered as late apoptotic.

CLONOGENIC ASSAY

Briefly, 1.0×10^5 cells/well of MSCs were seeded on the insert and cancer cells were seeded in triplicates (1000 cells/mL) in 6 well plates and co cultured directly for 14 days. At the end of 14 days, cells were washed with PBS (2×) and fixed with 3.7% formaldehyde for 2 min at room temperature. Then, cells were permeabilized with 100% methanol for 20 min at room temperature. Next, giemsa (Sigma-Aldrich (St.Louis, MO, USA)) staining was added (1 mL/well) to the colonies and incubated in the dark or using aluminium foil at room temperature approximately 15 min. Once dried, colonies on each plate were counted.

QUANTITATIVE REVERSE TRANSCRIPTION POLYMERASE CHAIN REACTION (qRT-PCR)

To evaluate the apoptotic induction of cancer cells, the mRNA expression levels of intrinsic apoptotic genes

(APAF1, BAD, BAX and CASP 9) were measured by qRT-PCR. Cancer cells were cultured with 60% MSCs conditioned medium or 60% fresh MSCs complete medium as basal. Sixty percent of MSCs conditioned medium was selected for the experiment based on a previous study (Wahyu et al. 2021). Initially, total RNA was extracted from cells after 48 h incubation with MSCs conditioned medium using RNAeasy kit (Qiagen Hamburg GmbH, Germany) according to the manufacturer's protocol. The extracted RNA was quantified at 260 nm absorbance, and purity of RNA was evaluated from the 260/280-absorbance ratio using the spectrophotometer (BioPhotometer Plus, Eppendorf, Hamburg, Germany). First strand cDNA was synthesized using the Transcriptor First Strand cDNA synthesis kit (Roche Applied Science, Nonnenwald, Penzberg, Germany) according to manufacturer protocol. The qRT-PCR reaction was prepared using SYBR 1 master mix (Roche Applied Science, Nonnenwald, Penzberg, Germany) and primers as stated (Table 1). Quantitative RT-PCR was performed using the LightCycler 480 (Roche) under the following cycle conditions; Pre-denaturation for 4 min at 95 ºC followed by 40 cycles consisting of denaturation at 95 ºC for 15 s, annealing at 60 ºC for 30 s and extension at 72 ºC for 30 s followed by dissociation curve. The basic relative gene expression (RQ) was calculated manually and the efficiency (E) of primer binding was standardized to 2.

$$\textbf{Relative expression (RQ)} = \frac{E_{\text{Target}}^{\Delta Cp\ \text{Target (Mean Cp Control} - \text{Mean Cp Sample)}}}{E_{\text{Reference}}^{\Delta Cp\ \text{Reference (Mean Cp Control} - \text{Mean Cp Sample)}}}$$

Target: Target genes analyzed

where Reference is the Reference Genes/Endogenous Control; Sample is the tumors cultured with 60% MSCs conditioned medium; and Control is the Tumors cultured with 60% MSCs complete medium.

STATISTICAL ANALYSIS

Statistical analysis was performed using the IBM SPSS statistic, version 21. Comparison between any two groups was analysed by the two-tailed Student's t-test. Values of $P < 0.05$ were considered statistically significant.

RESULTS

CHARACTERISATION OF BONE MARROW AND UMBILICAL CORD DERIVED MSCs

Characterisations of MSCs were performed based on mesodermal lineage differentiation (adipogenic and osteogenic) and surface marker expression (CD90, CD44, CD73 and CD105). Both BMMSCs and UCMSCs were positive for adipogenesis and osteogenesis differentiation (Figure 1(A)). Analysis of MSCs surface marker expression have shown that both BMMSCs and UCMSCs expressed CD90, CD44, CD73 and CD105 (Figure 1(B)). However, slightly lower expressions of CD44 and CD105 were noticed in UCMSCs as compared to BMMSCs.

VIABILITY OF CANCER CELLS TREATED WITH MSCS

Cancer cells (H2170, LN18 and MCF7) were cultured in different concentrations of MSCs' conditioned medium or MSCs (different Cancer to MSC ratio) in a co-culture assay for 48 h and subsequently evaluated using MTS assay (Figure 2). Significant inhibitions ($p<0.001$) with 32.5 ± 1.5%, 34.9 ± 5.3% and 68.3 ± 6.5% of cancer cell viabilities were noticed for all three cancer cell lines, H2170, LN18 and MCF7 when cultured with the highest concentration of BMMSCs conditioned medium (100%) (Figure 3). High concentration of UCMSCs conditioned medium (100%) was also able to significantly inhibit ($p<0.001$) the viability of H2170 (25.8 ± 3.5%), LN18 (17.6 ± 6.5%) and MCF7 (33.2 ± 6.8%) as compared to basal (Figure 3(B)). Analysis of co-culture between cancer cell and MSCs showed that the MSCs (UCMSCs and BMMSCs) were able to inhibit the proliferation of both H2170 and LN18 between 1:20 and 1:1, cancer to MSC ratio (Figure 3(A) and 3(B)). However, inhibition of MCF7 proliferation was only detected when cancer cells were co-cultured with UCMSCs at 1:1 ratio (Cancer to MSC) (Figure 3(C)).

INDUCTION OF CANCER CELL APOPTOSIS BY MSCs

Analysis of apoptosis (early and late) and dead cells were performed using annexin-v and propidium iodide (PI) staining. Cancer cells (H2170, LN18 and MCF7) were grown with 60% MSCs conditioned medium or co-cultured with MSCs in a trans-well assay for 48 h and subjected to flow cytometry analysis. As shown by Figure 4(A), both MSCs and its conditioned mediums were able to induce early and late apoptosis to the H2170 cell line

as compared to basal (control) (Figure 4(A)). MSCs and its conditioned medium from both bone marrow and umbilical cord were also able to induce late apoptosis and cell death to the LN18 (Figure 4(B)), indicating that these two cancel cell lines, H2170 and LN18 were sensitive to the apoptotic effect induced by MSCs (Figure 4(A) and 4(B)). Although the MSCs from both BMMSCs and UCMSCs were able to induce apoptosis and cell death to the MCF7 using the trans-well assay, there was no substantial effect of MSCs'conditioned medium against the MCF7 (Figure 4(C)).

INHIBITION OF CLONOGENICITY IN CANCER CELLS CULTURED WITH MSCs

Cancer cells (H2170, LN18 and MCF7) were seeded at a low density with MSCs (on insert) in a 24-well plate. After 14 days, formed colonies were stained using crystal violet and manually counted. Lower number of colonies were noticed for H2170 and LN18 when these cancer cells were cultured with the MSCs. However, not much difference in the number of colonies were observed for MCF7, between the control and co-cultured with the MSCs (Figure 5(A)). Analyses of H2170 cancer cell line indicated that there was a significant reduction in the number of colonies when these cancer cells were cultured with UCMSCs (609.5 ± 4.9) as compared to basal (1196.3 ± 12.8) (Figure 5(B)). Similar observation was noticed for the LN18 where reduction in the number of colonies from 253.3 ± 12.3 (basal) to 171.33 ± 12.6 when cultured with UCMSCs (Figure 5(C)). However, there were no differences in the number of colonies observed for MCF7, when these cancer cells were co-cultured with the MSCs (Figure 5(D)).

GENE EXPRESSION ANALYSIS OF CANCER CELLS CULTURED WITH MSCs' CONDITIONED MEDIUM

Gene expression analysis using quantitative PCR have shown that genes involve in the intrinsic apoptotic pathways were upregulated in cancer cells cultured with the MSCs conditioned medium. In this regard, the expression of BAD gene was highly upregulated in both LN18 (Figure 6(B)) and MCF7 at about 10.4 and 8.1 folds, respectively (Figure 6(C)) when cultured with the conditioned medium of both BMMSC and UCMCS. Other genes, such as BAX was particularly upregulated by about 4.1-fold in H2170 (Figure 6(A)), while APAF1 and CASP 9 were slightly increased in LN18 by about 3.8-fold and MCF7 by about 3.6-fold, respectively (Figure 6(B) and 6(C)).

DISCUSSION

Despite several studies that have shown the anti-cancer efficacies of engineered MSCs and its by-products in several tumour models (Fazileh et al. 2018; Han et al. 2019; Rossignoli et al. 2019; Zahra et al. 2020), more studies are still needed to fully understand the role of native MSCs during tumour development. Although studies have reported that MSCs could inhibit cancer cell proliferation (Li et al. 2021; Liu et al. 2020; Meng et al. 2021), others have also suggested that MSCs could promote tumour progression and metastasis (Karnoub et al. 2007; Zhang et al. 2018). Due to this ambiguity, we seek to understand further the role of MSCs from different sources on the carcinogenesis of three cancer cell lines, H2170, LN18 and MCF7. We believe that the study has demonstrated for the first time the anti-cancer activity of native MSCs derived from bone marrow and umbilical cord on several cancer cell lines as a model of solid tumours.

Reports have shown that the mesodermal lineage differentiation between MSCs may vary depending on its sources (Brown et al. 2019; Pittenger et al. 1999). However, our findings indicated that both bone marrow and umbilical cord exhibited the same level of differentiation into adipogenesis, osteogenesis and chondrogenesis. Analysis on the MSCs marker expression indicated a lower expression of CD44 and CD105 were noticed in UCMSCs compared to BMMSCs as shown in Figure 1. This observation could be due to the cellular mechanistic properties of MSCs that may contribute to the degree of MSC markers expression. UCMSCs which were described as 'hardest' cells by a report due to its mechanical properties have been shown to express a slightly lower MSC marker compared to MSCs derived from other sources such as the bone marrow and adipose tissue (Han et al. 2017).

Although the effects of conditioned medium harvested from both BMMSCs and UCMSCs to inhibit the proliferation of all three cancer cell lines were observed in the study, the inhibition of all three cancer cell lines were mildly seen in the co-culture assay (Figure 3). This observation may suggests that though MSCs' could produce factors that can inhibit tumour growth, the direct co-culture between MSCs and cancer cells may have produced growth benefit which can artificially support one cell to the other rather than a true anti-cancer effects that come from the MSCs (Kalamegam et al. 2012; Zhang et al. 2013). This can be seen from the viability assay that has shown the efficacy of conditioned medium derived

from BMMSCs and UCMSCs to inhibit the growth of all three cancer cell lines (Figure 3(A), 3(B) and 3(C)). This finding is consistent with a recent study by Wahyu et al. (2021), suggesting that factors released by MSCs may have a significant impact on inhibiting tumour development.

In this study, the efficacies of BMMSC and UCMSC in inducing apoptosis and cell death to the H2170, LN18, and MCF7 were also evaluated. From our analyses, we have found that the MSCs and its conditioned medium from both bone marrow and umbilical cord were able to induce apoptosis and cell death to both the H2170 and LN18 cancer cell lines (Figure 4(A) and 4(B)). This finding is in line with reports that have shown the ability of MSCs to exert anti-tumour activity in several type of cancers in including lung adenocarcinoma through the secretion of soluble factors (Ciavarella et al. 2015; Cortes-Dericks & Galetta 2019; Li et al. 2011). Our analyses have found that the conditioned medium from both BMMSCs and UCMSCs were also capable to induce apoptosis and cell death to the H2170, which is a cell line derived from lung carcinoma (Figure 4(A)). The secretion of soluble factors by MSCs could be the possible reason for the induction of apoptosis in H2170 as several apoptotic factors such as interferon-beta (IFN)-ß and tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) are some of the proteins detected in MSCs' conditioned medium as shown by several reports (Jung et al. 2019; Kamal Shaik et al. 2019). In addition, recent study has also described that exosomes secreted by MSC may contain miRNA that can modulate proliferation and induced apoptosis in tumour cells (Zhou et al. 2018).

We have also noticed that the co-culture seems to promote more apoptosis in MCF7 (Figure 4(C)) while in viability test, conditioned medium caused greater cell death (Figure 3(C)). These results could suggest that conditioned medium derived from the MSCs have greater effect on cell death (necrosis) as compared to the co-cultured system as shown by the MTS assay. While the co-culture system was more effective in causing early and late apoptosis in MCF7 (Figure 4(C)), the dead cells population remained low and was consistent with the results obtained from the MTS assay. The discrepancy of results between the two assays, Annexin V/PI and MTS assay for the treatment with conditioned medium might be due to the different sensitivity between the two applied methods toward apoptosis and cell death, respectively. Propidium iodide/PI and MTS assay for example have different sensitivity to cell death due to its different target site (i.e., MTS to mitochondria and PI to nucleus) (Husniza et al. 2019). Previous research showed that upon apoptosis and necrosis, disintegration in nucleus happens earlier, followed by mitochondria and necrosis will then occur when cells are void of ATP (Crowley et al. 2016). Therefore, we postulate that the disparity in results as noticed for the MCF7 between the two assays (MTS assay and Annexin V/PI) might be due to the different target site of each assay which occur at different time points leading to different outcome.

The role of MSCs either to suppress or support tumour growth is based on multiple factors. MSCs can interfere with the extracellular mechanisms of the TME through varying mechanisms including secretion of paracrine factors and immune suppression which can affect tumour growth (Praveen Kumar et al. 2019). In addressing this issue, we assess the effect of BMMSCs and UCMSCs on the clonogenicity of cancer cells using *in vitro* trans-well assay. The data presented in the study indicates that UCMSCs have an inhibitory effect on the clonogenicity of both H2170 and LN18 cancer cell lines. However, BMMSCs have been shown to neither inhibit nor enhance the clonogenicity of all three cancer cell lines as depicted in Figure 5. This observation could suggest that the inherent characteristics of BMMSCs that were derived from an immunosuppressive marrow environment may have an effect to favours cellular growth (Ciavarella et al. 2015). On the other hand, the clonogenic assay showed that MSCs derived from sites other that marrow such as the UCMSCs were capable of inhibiting tumour proliferation. These results suggest that, each of these MSCs, depending on its source and physiological role may produce peculiar set of signals generating both pro- or anti-tumour effects to the cancer cells particularly the TME.

Increasing evidence has reported that MSCs can exerts potent anti-tumour effects by down-regulating essential protein crucial for cancer progression including the AKT (Serine/Threonine Kinase 1) and WNT (Wingless-Type MMTV Integration Site Family) signalling pathways (Qiao et al. 2008b). MSCs are also capable of inhibiting the WNT/β-catenin signalling pathway through DKK-1 (Dickkopf-related protein 1) activation which in turn block the expression of WNT downstream targets such as c-Myc (MYC Proto-Oncogene) and BCL-2 (B-cell lymphoma 2) that are crucial for tumour survivals (Zhu et al. 2009). Gene expression analysis using quantitative PCR has shown that the conditioned medium derived from MSCs were able to increase the expression of apoptotic genes, such as BAX (Bcl-2 Associated

X-protein), BAD (Bcl-2-associated death promoter) and APAF1 (Apoptotic protease activating factor 1) in both H2170 and LN18 as shown in Figure 6. Some of these genes, such as APAF1, BAD and BAX are precursor genes that activate the intrinsic apoptosis through mitochondrial release of cytochrome C and apoptosome that subsequently leads to cell death (Fulda & Debatin 2006). Tumours that harbour p53 mutation such as H2170 and LN19 are mostly resistance to the intrinsic apoptosis upon chemotherapy induction due to its p53-dependency. However, both H2170 and LN18 might be sensitive to the intrinsic apoptosis induced by MSCs as our findings have shown that the conditioned medium derived from MSCs were able to activate precursor genes involve in the intrinsic apoptosis in these two cancer cell lines.

TABLE 1. Primers for the qRT-PCR analysis. Primers are selected based on the genes involve in the apoptotic pathways

Gene	Accession	Sense primer	Antisense primer	Product Size (bp)
APAF 1	NM_013229.2	CACGTTCAAAGGTGGCTGAT	TGGTCAACTGCAAGGACCAT	214
BAX	XM_047439168.1	ACCAAGCTGAGCGAGTGTC	ACAAAGATGGTCACGGTCTGCC	411
BAD	NM_032989.3	CCCAGAGTTTGAGCCGAGTG	GCTGTGCTGCCCAGAGGTT	314
CASP 9	XM_011542273.4	TGTGGTGGTCATCCTCTCTCA	GTCACTGGGGGTAGGCAAACT	331
GAPDH	NM_001289746.1	TGAAGGTCGGAGTCAACGGATT	CATGTGGGCCATGAGGTCCACCAC	530

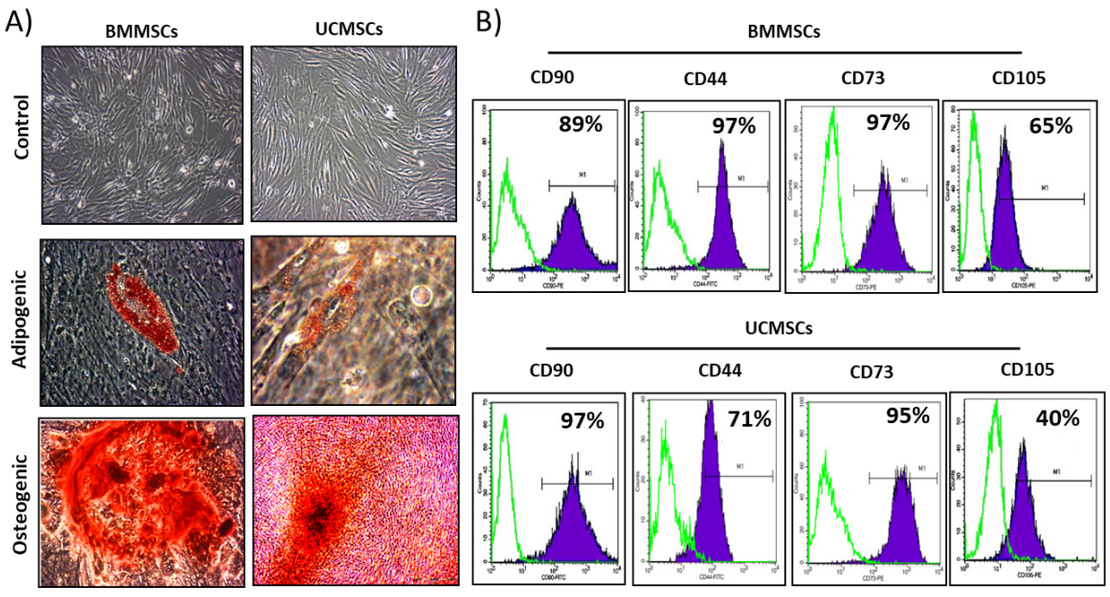

FIGURE 1. Characterisation of MSCs. (A) Both MSCs (BMMSCs and UCMSCs) display mesodermal lineage differentiation (adipogenesis and osteogenesis) based on histological staining (Oil Red-O and Alizarin Red), respectively. (B) Analysis of MSCs surface marker expression between both USMSCs and UCMSCs

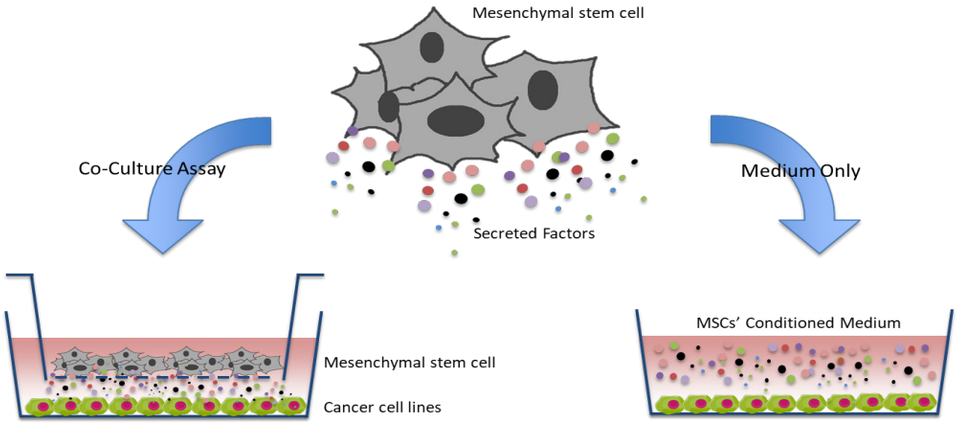

FIGURE 2. Diagram illustrating how the assays was performed. Cancer cell lines (H2170, LN18 and MCF7) were cultured with either MSCs (trans-well) or its conditioned medium and subsequently analysed for proliferation, apoptosis and clonogenicity

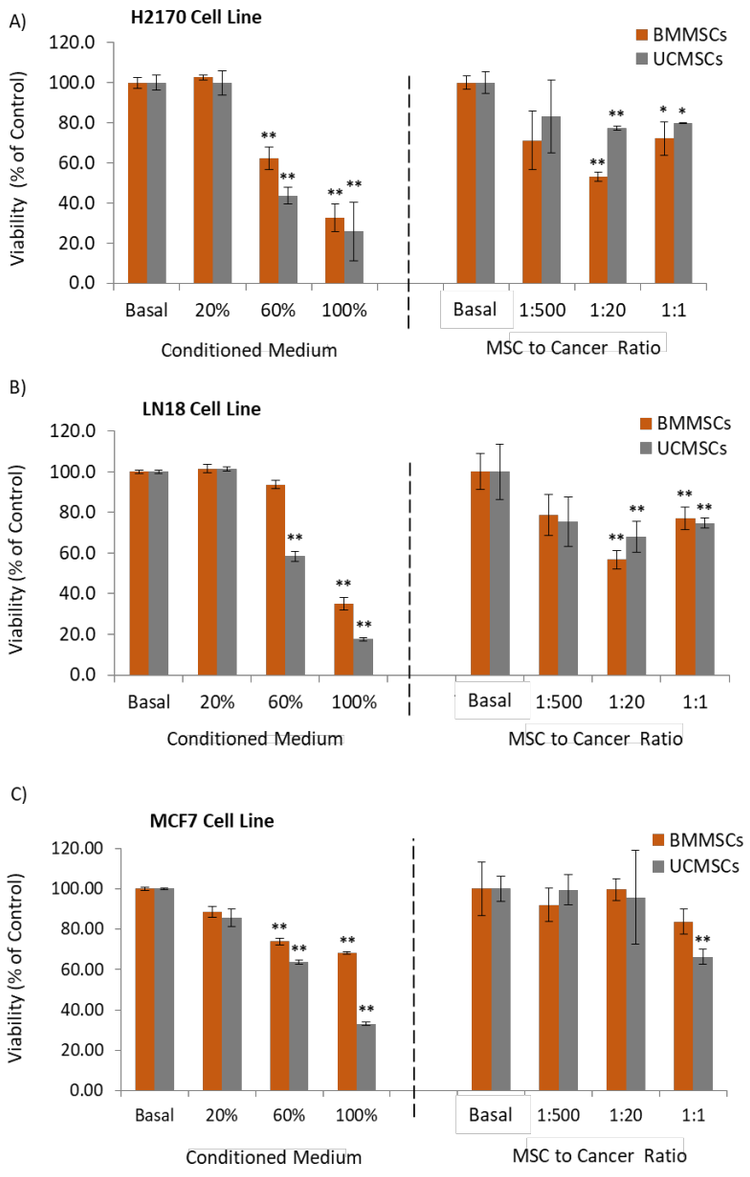

FIGURE 3. Inhibition of Cancer Cell Viability by MSCs. (A, B and C) Cells (H2170, LN18 and MCF7) were cultured in different concentrations of MSCs' conditioned medium and in several cancer cells to MSCs ratio for 48 hours and subsequently analysed for viability assay (**p<0.001, *p<0.01; t-test)

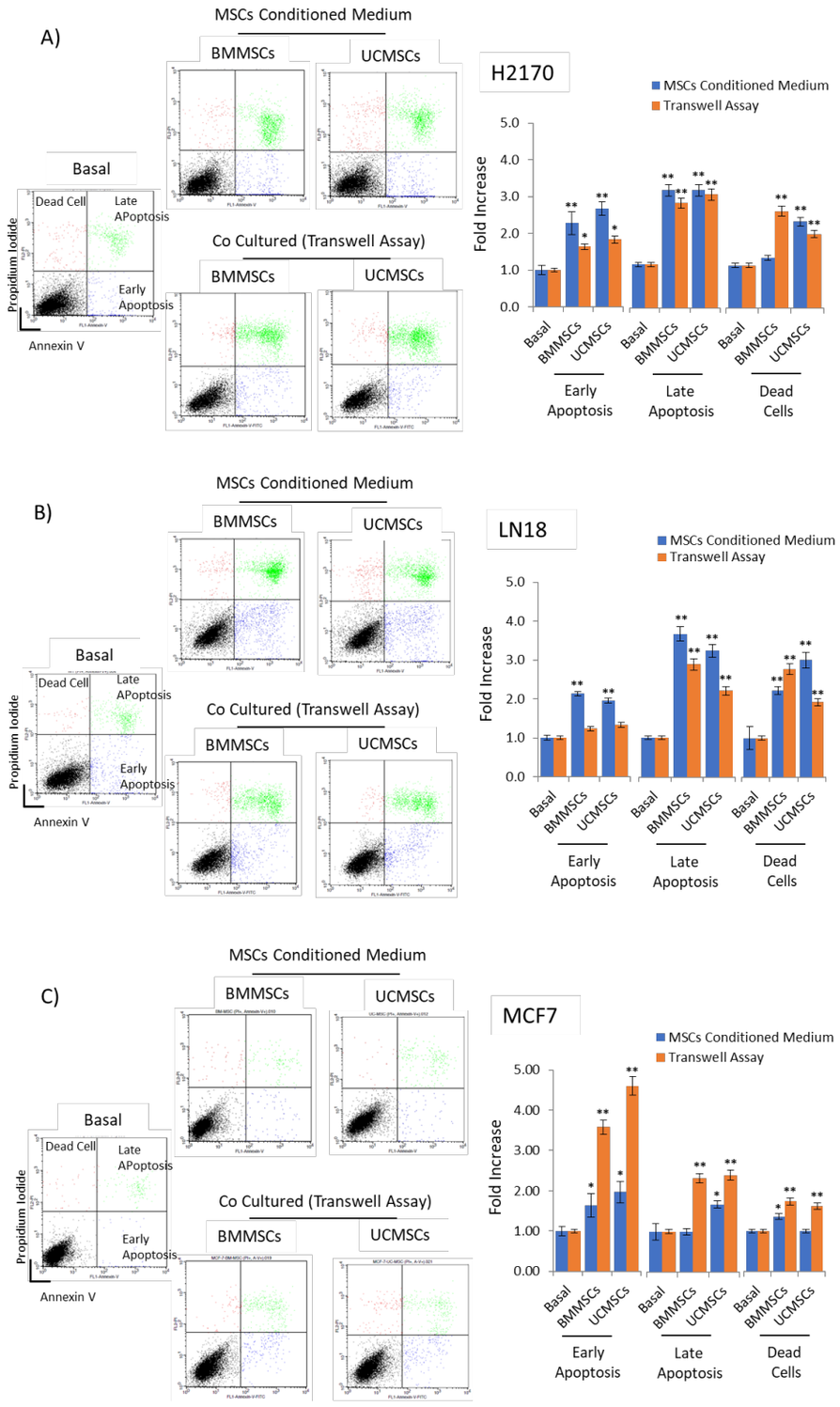

FIGURE 4. Analysis of apoptosis and cell death in cancer cells by MSCs (A, B and C) Dot plots and bar chart depicting the gating used for the analysis of apoptosis (early and late) and dead cells in cancer cells cultured with MSCs and its conditioned medium for 48 h (**p<0.001, *p<0.01; t-test)

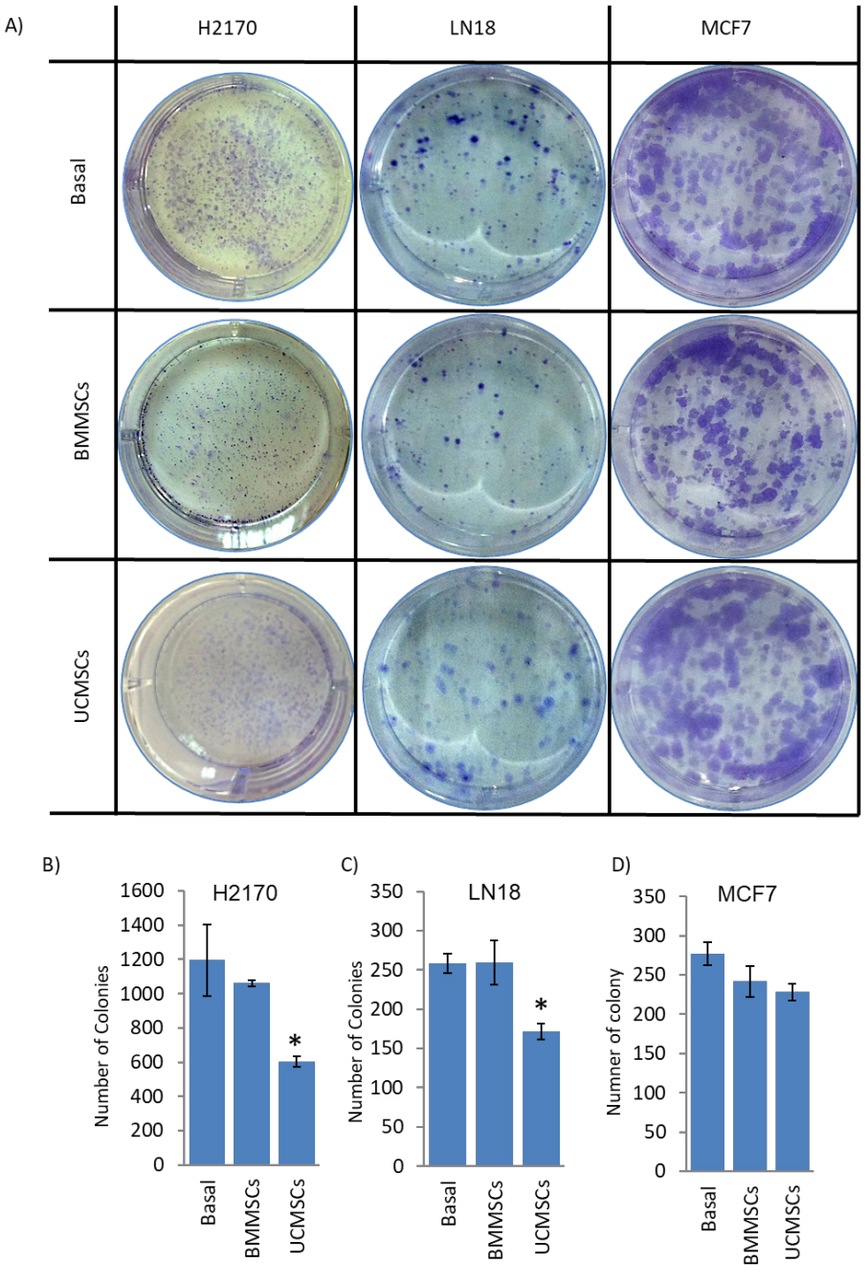

FIGURE 5. Clonogenicity of cancer cells co-cultured with MSCs (A) Photomicrograph showing colonies of cells formed when co-cultured with different MSCs. (B) number of colonies for each cancer cells were counted in triplicates and presented as bar chart (*p<0.01; t-test)

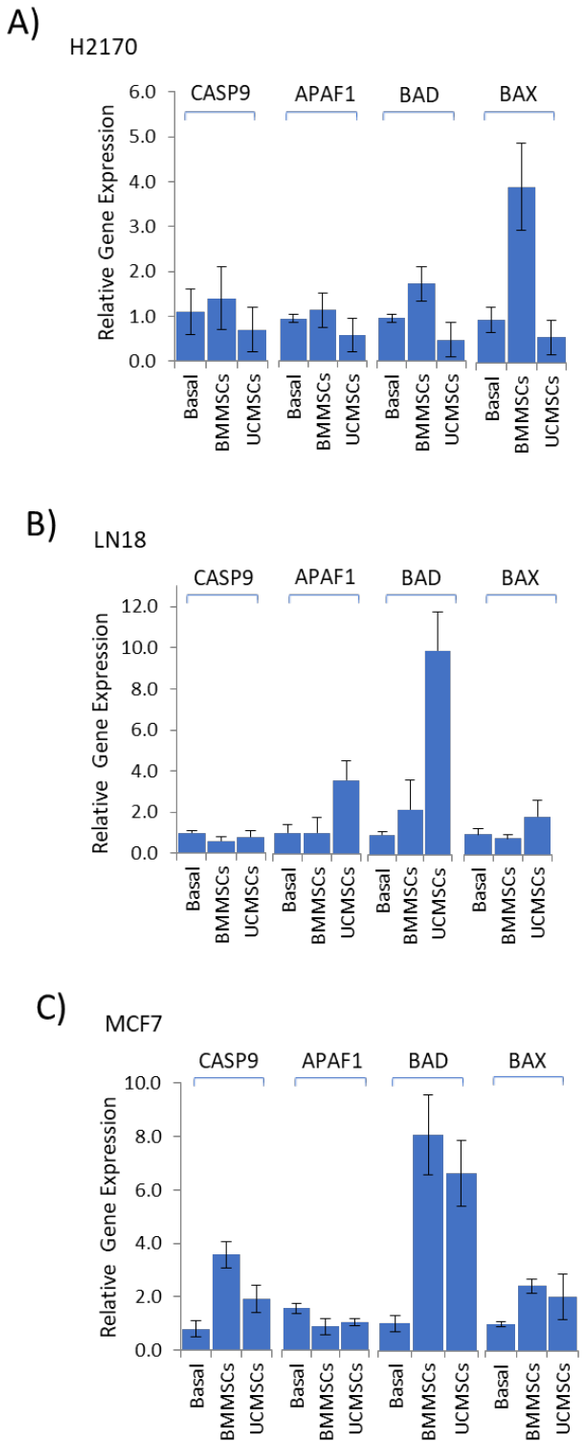

FIGURE 6. Quantitative PCR analysis of intrinsic apoptotic genes. Cancer cell lines, H2170, LN18 and MCF7 cultured with MSCs conditioned medium for 48 h and subsequently analysed for qRT-PCR analysis

CONCLUSION

Despite MSCs have been reported to exert bidirectional effects that regulate several hallmarks of cancer, our findings have shown no indication that these cells can enhance or supporting cancer growth. Although the study has found that these MSCs are capable of inducing cancer cell inhibition and apoptosis, due to the limitation in the number of cancer cells used in the study and assays performed, it is not possible to determine conclusively that these MSCs exhibit an anti-tumour activity. Further studies are therefore necessary in a larger sample using MSCs from greater sources including several types of cancer in order to fully understand the role of MSCs in tumour development. Nonetheless, based on our findings, we concluded that the anti-tumorigenic features of native MSCs are a therapeutic characteristic which favours these cells as an attractive candidate for cell-based gene therapy in cancer.

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