

The Differentiation of Bone Marrow Stem Cells Into Cardiomyocytes: An Immunocytochemical Analysis

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SUMMARY

Bone marrow stem cells (BMSC) can be differentiated into cardiomyocytes (CMC), which is of value in myocardial infarction repair. Hence, the expression of muscle-specific protein Alpha Sarcomeric Actin (ASA) and heart specific protein Phospholamban (PLB) were assessed after BMSC exposure to different induction factors that were reported to induce myogenic differentiation. More than 80% (PLB=94.44±0.51%; ASA=84.44±3.78%) of cells after 5-azacytidine exposure expresses both PLB and ASA protein but cells viability were noted around 60.16 ±6.26%. In the basic Fibroblast Growth Factor (bFGF) and Hydrocortisone with 5-azacytidine incorporated group, there are about 75.16 ± 4.84% of PLB expression and 95.66 ± 1.89% of ASA expression. The cell viability of this group is around 79.42±0.93%. Hence, it is better to combine 5-azacytidine with bFGF and hydrocortisone for CMC differentiation.

INTRODUCTION

Cardiomyocytes is reported to be terminally differentiated, thus they could not regenerate sufficiently following myocardial infarction. BMSC were tested with several induction factors that include 5-azacytidine, bFGF and Hydrocortisone to determine the optimal induction factors in vitro.

MATERIAL AND METHODS

Sheep, *Ovis aries* bone marrow was aspirated from the illiac crest. Cells were cultured in Iscove's modified Dulbecco's medium supplemented with 10% Fetal Bovine Serum, 1% antibiotic-antimycotic, 1% Glutamax and 1% Vitamin C at 37°C with 5% CO₂ in 6-well plate at passage 0 (P₀). Cells were

divided into three different induction groups at passage 1(P₁) on chamber slides (5 X 10³ cells/cm²). All groups were exposed to induction factors for 24 hours. Monoclonal antibodies to Phospholamban (PLB) and Alpha Sarcomeric Actin (ASA) were purchased from Abcam, UK. Cells were washed with Phosphate Buffer Saline twice before fixing them with 4% Paraformaldehyde for 10 minutes. Permeabilization of cells was done using 0.1% Triton X-100 for 5 minutes. After blocking with 10% of Rabbit Serum for 30 minutes, cells were incubated with primary antibodies overnight at 4°C. Secondary antibody (FITC-conjugated anti-rabbit IgG) was added after serial washing the next day for 1 hour at room temperature. Observation and image analysis was done using epifluorescence microscope.

RESULTS

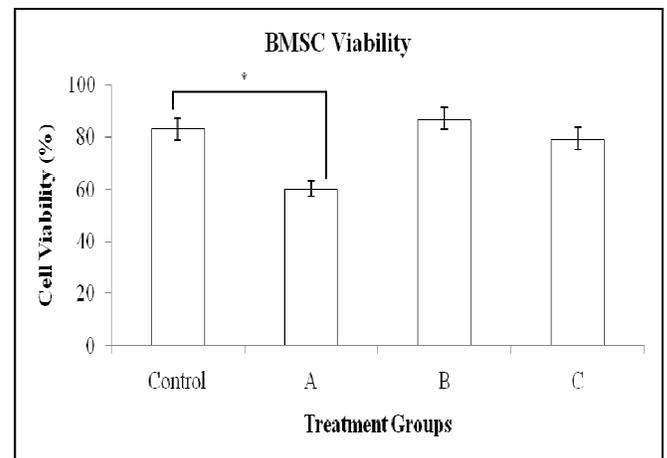


Fig 3: BMSC viability for all induction groups compared to control. Group (A) 5µM of 5-azacytidine; Group (B) 10nM bFGF + 50µM Hydrocortisone; and Group (C) 5µM of 5-azacytidine + 10nM bFGF + 50µM Hydrocortisone. Cell viability for 5-azacytidine (*) exposed group is significantly lower as compared to the control group (p<0.05).

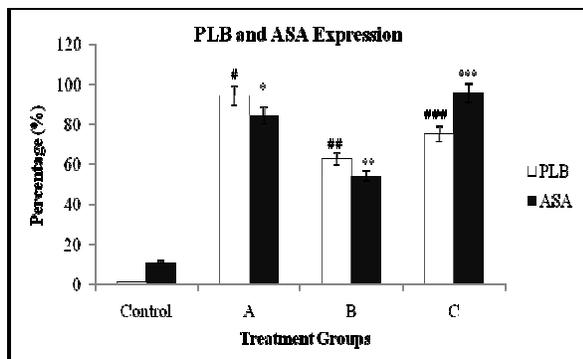


Fig 4: PLB and ASA protein expression for all induction groups compared to control. Group (A) 5 μ M of 5-azacytidine; Group (B) 10nM bFGF + 50 μ M Hydrocortisone; and Group (C) 5 μ M of 5-azacytidine + 10nM bFGF + 50 μ M Hydrocortisone. Expression of both proteins on all induction groups are significantly higher as compared to the control group which were cultured without any induction factors in the media.

*PLB expression for treatment group A vs. control group. (p<0.05)

**PLB expression for treatment group B vs. control group. (p<0.05)

***PLB expression for treatment group C vs. control group. (p<0.05)

#ASA expression for treatment group A vs. control group. (p<0.05)

##ASA expression for treatment group B vs. control group. (p<0.05)

###ASA expression for treatment group C vs. control group. (p<0.05)

In the 5-azacytidine exposed group (Group A), 94.44 \pm 0.52% of the cells expressed PLB protein and 84.44 \pm 3.78% of them were observed to express ASA but the cells have 60.16 \pm 6.26% cell viability. Whereas inclusion of bFGF and Hydrocortisone in the culture medium (Group B) increase the cells viability up to 86.94 \pm 1.83% but gave less than 60% (PLB=62.71 \pm 2.85%; ASA=54.28 \pm 2.69%) expression of both proteins. Incorporation of bFGF and differentiation to CMC without compromising the cell viability.

DISCUSSION

The most commonly utilized induction factor for myogenic lineages is 5-azacytidine [1]. The result correlates to the ability of bFGF and hydrocortisone as potent mitogen and proliferation agents [2, 3]. In conclusion, this study finds that 5-azacytidine and bFGF plus hydrocortisone are the best combination to induce BMSC towards myogenic lineages.

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Trypsinization of Human Keratinocytes And Fibroblasts using Animal Free Trypsin: Towards Clinical Application

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SUMMARY

The objective of this study was to compare the efficacy between trypsin extracted from animal (Trypsin EDTA) and recombinant trypsin (TrypLE Select). The skin biopsy was digested using collagenase type 1 and the epidermis was dissociated with Trypsin EDTA or TrypLE Select. The cells were cultured until passage 2 and trypsinized using respective trypsin. The cells yield was counted after trypsinization. The cells yield for both keratinocytes and fibroblasts were comparable between Trypsin EDTA and TrypLE Select. In conclusion, recombinant trypsin can be used as an alternative to the conventional animal-origin trypsin for clinical application.

INTRODUCTION

Skin regeneration is important in tissue engineering especially in cases of chronic wounds¹. With tissue engineering technology, skin equivalents have been used clinically to repair burns and chronic ulcers². In order to construct bilayered skin, *in vitro* expansion of keratinocytes and fibroblasts is a crucial process. Unfortunately, many skin substitutes use components derived from animal origin. Therefore it is a challenge to produce a skin substitute using animal free product. In this study, the commercial trypsin from animal origin was compared with recombinant trypsin for human dermal fibroblast and keratinocyte isolation and expansion.

MATERIALS AND METHODS

A 1x3 cm² skin biopsy was cleaned and swabbed with 70% alcohol. The skin was minced and digested in 0.4-0.8% collagenase Type I for 4-8 hours in a 37°C incubator-shaker to release the fibroblasts from dermis. After collagenase digestion, the fibroblasts and epidermis were pelleted down by centrifugation and the supernatant was discarded.

The epidermis layer was digested with two different methods:

- Animal Origin Trypsin:** 8-10ml of Trypsin EDTA was added to the pellet, incubate for 5-10 minutes at 37°C for keratinocytes cells

dissociation. Then, 8-10 ml of soybean trypsin inhibitor was added to stop the trypsin activity.

- Recombinant Trypsin:** 8-10ml of TrypLE Select was added to the pellet and incubated for 15-20 minutes at 37°C for keratinocytes cells dissociation.

The cell suspension was centrifuged before re-suspending in 6 ml of 1:1 DKSFM and F12:DMEM supplemented with 10% Fetal Bovine Serum (FD+10%FBS). Fibroblasts and keratinocytes were seeded into three 9.6cm² wells and co-cultured at 37°C in 5% CO₂. Fibroblasts were removed when cells reached confluence and expanded into a T75 flask with FD+10%FBS. The remaining keratinocytes were cultured in DKSFM. The keratinocytes and fibroblasts were subcultured after they reach 70% confluence. Keratinocytes were trypsinized using their respective trypsin: Trypsin EDTA for 5 minutes after 5 minutes incubation with Versene; TrypLE Select for 10-15 minutes. Fibroblasts were trypsinized using both trypsins for 5 minutes. Keratinocytes and fibroblasts were subcultured until passage 2 (P2) with seeding density of 1.0 x 10⁵ per well for keratinocyte and per flask for fibroblast and cultured in their respective medium.

RESULTS

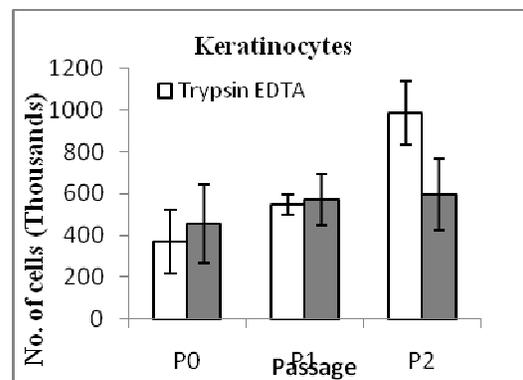


Fig. 1

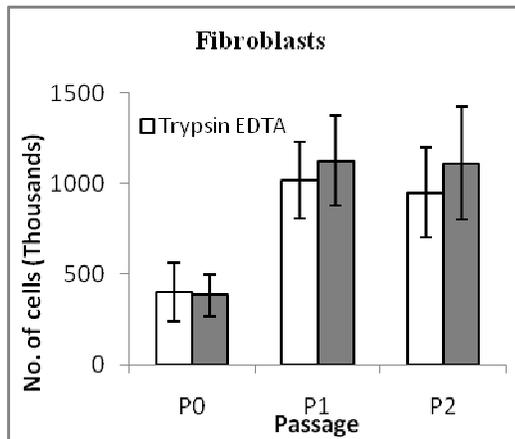


Fig. 2

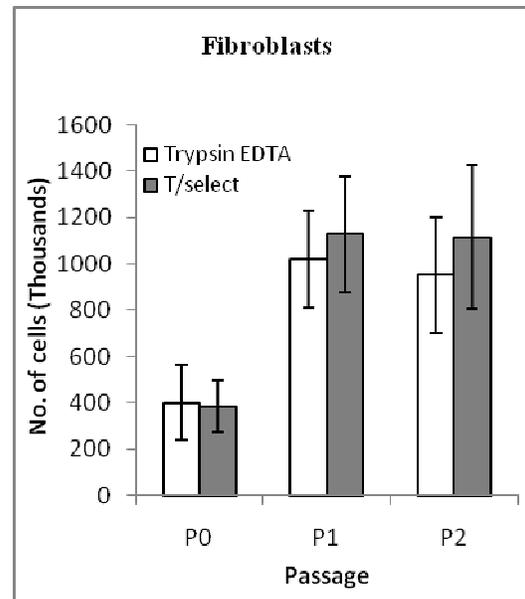


Figure 1& 2 showed the comparison of cell yield between Trypsin EDTA and TrypLE Select for keratinocytes and fibroblasts in various passages. For keratinocytes, the number of cells increased rapidly for each passages started with 4×10^5 cells at P0 and increased up to 1×10^6 at P2 by using Trypsin EDTA. On contrast, the number of fibroblasts cells increase rapidly in TrypLE Select from P0 up to P1 with total cells 4×10^5 cells to 1×10^6 cells. However, there are no significant differences between both enzymes for both keratinocytes and fibroblasts cells.

DISCUSSION

The manipulation of skin cells for clinical application requires standardized protocols to ensure the product is safe and effective to be used in human³. Study performed by Ellerström et al. (2006) also demonstrated that animal components are not necessary for the establishment and culture of human cells⁴. In this study, preliminary results obtained showed that TrypLE Select gives comparative result compared to Trypsin EDTA. Reagents containing animal products can be a source of contamination which can evoke immune reaction. Therefore, TrypLE Select would be a more suitable choice since it does not contain animal product. In conclusion, we have successfully trypsinize human keratinocytes and fibroblast using recombinant trypsin which can be used as an alternative to the conventional animal-origin trypsin for clinical application.

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Establishment and Characterisation of Malaysian Human Breast Cancer Cell Lines.

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SUMMARY

This project aimed to establish immortalised Malaysian primary breast cancer cell lines to facilitate the creation of an ideal Asian breast cancer model. Breast cancer cells were spontaneously immortalised from the malignant breast cancer specimens. After immortalisation, the cells were genotypically and phenotypically characterised. MCF7 cell line was used as control. After subsequent passages, two cell lines, C1 and C2 were created through spontaneous immortalisation. C1 was ER α and PR positive while C2 was ER α positive but PR negative. Both cells were HER2 positive. Wound healing assays confirmed the migratory capacity of these cells. The isolation and establishment of immortalised Malaysian primary breast cancer cell lines provide a new avenue in cancer research within an Asian perspective.

INTRODUCTION

Breast cancer is the most common cancer among women worldwide. In Malaysia, the National Cancer Registry (NCR) in 2003, reported breast cancer as the most frequent cancer among females in all age groups. It is more common in the Chinese ethnic group followed by Indians and Malays¹. Approximately 64.1% of cases were diagnosed in women between 40 and 60 years old and the lifetime risk of breast cancer among women is 1 in 16. In breast cancer research, cell lines are useful *in vitro* experimental models to study many aspects of carcinogenesis². They are used to determine key initiation and progression factors and obtain a better understanding of the disease, as well as the efficacy prediction of novel therapeutic compounds. However, breast cancer cell lines currently used are mostly derived from the Caucasian population³.

MATERIALS AND METHODS

Breast cancer cells were isolated from tissues by using the Panomics Cancer Cell Isolation Kit and then were immortalised by continuous cultures. Two newly immortalised breast cancer cell lines, designated as C1 and C2, were successfully characterised genotypically and phenotypically, and

compared with established breast cancer cell line, MCF7. In order to characterise the established breast

cancer cell lines, both cell lines were examined using DNA-Profiling, karyotyping, immunocytochemistry and phenotypic assays (clonogenic assay and wound healing assay).

RESULTS AND DISCUSSION

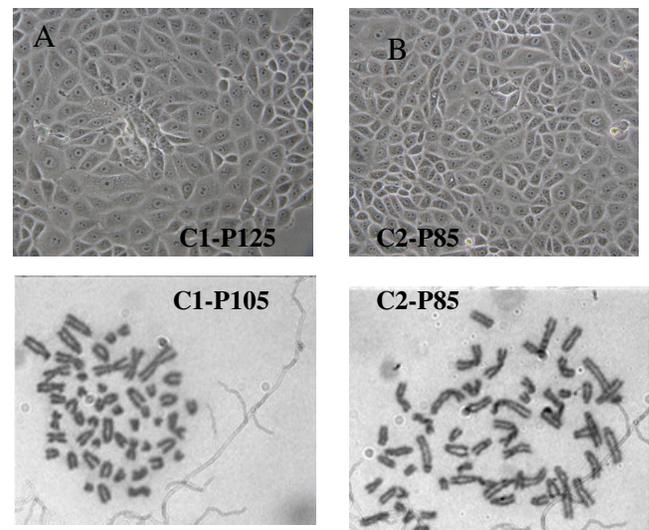
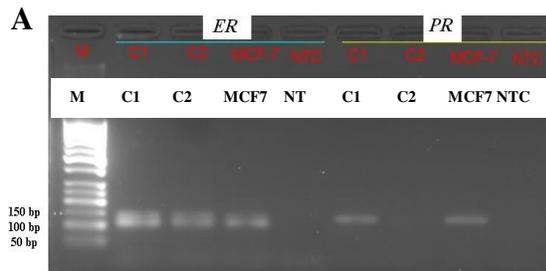


Fig 1 (A) C1 and C2 cellular morphology at passage 125 and 85 respectively. (B) Giemsa stained metaphase spread of C1 and C2 at passage 105 and 85 respectively.

Figure 1A illustrates the breast cancer cells of C1 and C2 that grow as adherent monolayers with characteristic epithelial morphology and maintained continuously *in vitro*. While in figure 1B, metaphase spreads of both cell lines demonstrate hyperploidy with gross chromosomal arrangements. This corroborates with the current paradigm that invasive cancer cells acquire genomic instability through the failure of DNA damage response and repair in these cells.



HER2 Expression

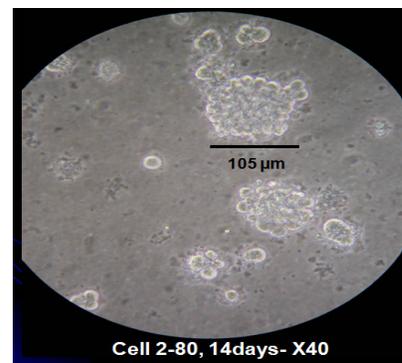
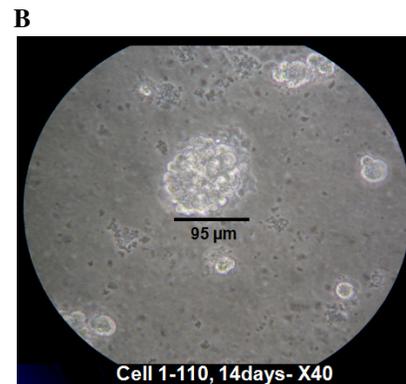
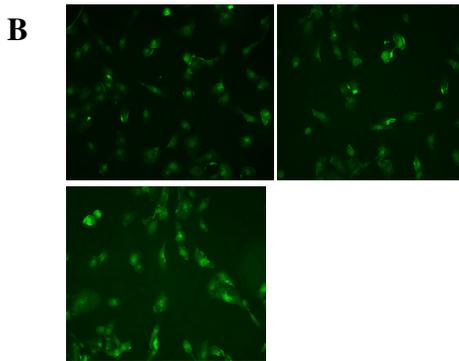


Fig 2 (A) Agarose gel electrophoresis of RT-PCR products of ER and PR in C1, C2 and MCF7 cell lines. (B) Fluorescent immunocytochemistry of HER2 in C1, C2 and MCF7 cell lines. C1 is ER+, PR+ and Her2+; while C2 is ER+, PR- and Her2+.

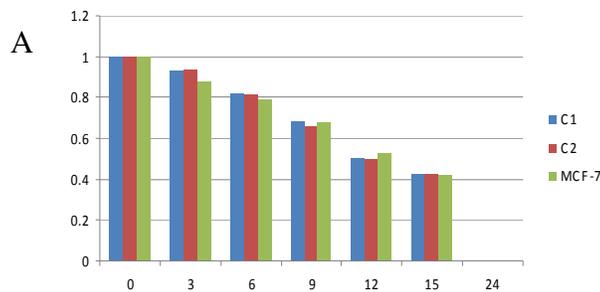


Fig 3 (A) Migration rates of C1, C2 and MCF7 (wound healing assay). (B) Clonogenic potential of C1 and C2 at 14 days (clonogenic assay). C1 and C2 demonstrated migratory and colony forming ability with very similar trends to that of MCF 7.

CONCLUSION

In conclusion, the two newly established and characterised breast cancer cell lines from Malaysian patients provide an ideal *in vitro* model of Asian breast cancers. These cell lines can be used as tools for the development of future diagnostic and therapeutic targets that take into account the Asian genotypic variation.

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Respiratory Epithelial Cells Cultured from Human Nasal Polyps Showed Evidence of Viral Infections

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SUMMARY

Respiratory epithelium cell monolayers derived from human nasal turbinate and nasal polyp have been developed and maintained *in-vitro* up to passage 2. In this study, the morphological changes of respiratory epithelium cell cultured from normal human nasal turbinate and nasal polyps have been observed and compared. Respiratory epithelium derived from nasal polyps showed intra-cytoplasmic inclusion bodies and cytopathic effect which is the evidence of virus infection, compared with the respiratory epithelial derived from nasal turbinate. Further studies need to be carried out to determine the pathogenesis of nasal polyps via tissue engineering technology.

INTRODUCTION

The nasal turbinate (NT) is a long, narrow and curled bone shelf which protrudes into the breathing passage of the nose. Nasal polyp (NP) is a chronic inflammatory disease of the upper airways, and the incidence rate is high in Southeast Asia. Polyps are not a separate growth, as is a tumor, and they consist of the same tissue as their surrounding areas. In general, NP is a multifactorial disease with several different etiological factors^{1, 2}. Histopathological examination of the removed tissue is the most common method for NP diagnosis and etiopathogenesis study^(1, 2). Unfortunately, polyps have a strong tendency to return after they have been surgically removed. Heman Chopra, 2008¹ found that 16 out of 41 cases of NP were caused by infection which included fungal, bacteria and virus. The presence of the virus often gives rise to morphological changes in the host cell. Any detectable changes in the host cell due to infection are known as a cytopathic effect (CPE). Cytopathic effect may consist of cell rounding, disorientation, swelling or shrinking, death, detachment from the surface and multinuclear giant cell formation. The objective of this study is to look for evidence of virus infection in respiratory epithelial cell cultured from nasal polyps. This would further bring new light into the mechanism of nasal polyp formation.

MATERIALS AND METHODS

Nasal turbinates are tissues discarded from patient during turbinectomy and nasal polyps discarded tissues after polypectomy. Usages of human NT and NP specimens have been approved by the Universiti Kebangsaan Malaysia Research and Ethical Committee. Six human nasal turbinate specimens and six nasal polyps were studied. The samples were processed as described by Noruddin *et al*, 2007³ using co-culture method. The monolayer respiratory epithelial cells (REC) morphologic features was examined and observed daily using the inverted light microscope (Olympus, Shinjuku-ku, Tokyo). The primary respiratory epithelial cultures were established by co-culture method until passage 2.

RESULTS

The REC had a typical and uniform polygonal morphology. Both the REC cultured derived from NT and NP was maintained up to passage 2. The morphology of the cells were observed and compared for any changes. Overall, the cells maintain polygonal morphology from passage 0 to 1. Binuclear cells were observed in the majority of cells which mean that the cells were dividing and growing normally. However, the REC derived from nasal polyps showed intracytoplasmic inclusion bodies (Figure 1A, black arrow) compared with the REC derived from nasal turbinates (Figure 1B). At passage 2, the RE derived from NP start to appear multiple shapes (Figure 2A) compared with RE derived from NT (Figure 2B) which still maintain the polygonal shape. Cytopathic effect was found in REC cultured from NP at passage 2 (Figure 3A&B), which is the evidence of virus infection.

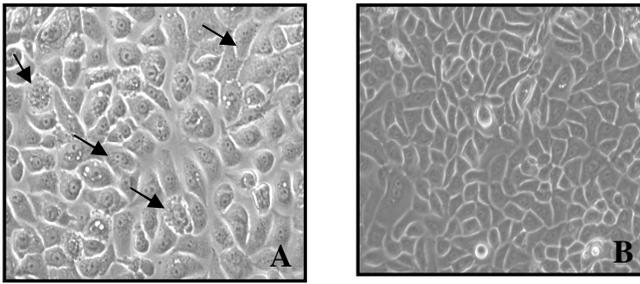


Fig1. Respiratory epithelium cells at passage 1. (A) Respiratory epithelium derived from human nasal polyp at day 5 post seeding, passage 1, (20X). Black arrow, showed the intracytoplasmic inclusion body. (B) Respiratory epithelium derived from human nasal turbinate at day 5 post seeding, passage 1, (20X).

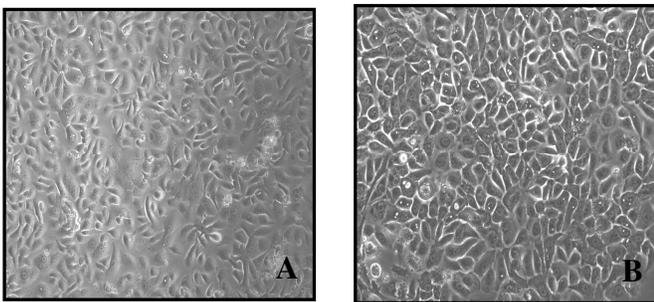


Fig 2. Respiratory epithelium cells at passage 2. (A) Respiratory epithelium derived from human nasal polyp at day 5 post seeding, passage 2 appears of multishape cells (10X). (B) Respiratory epithelium derived from human nasal turbinate at day 5 post seeding, passage 2, cells still maintain polygonal shape (10X).

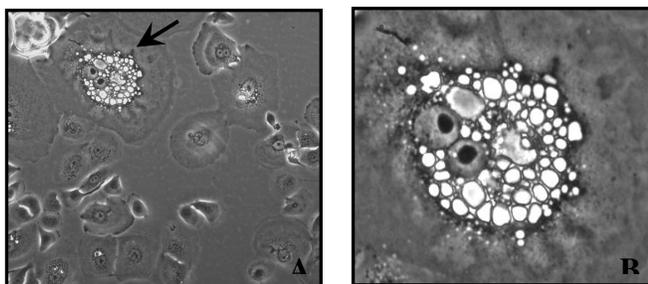


Figure 3. The cytopathic effect (CPE) of virus infected cell culture. (A) Giant multinucleated cells (black arrow) with peri-nuclear vacuolation was detected in P2, day 8 (20X). (B) The enlarge image for giant cell with cytopathic effect.

DISCUSSION AND CONCLUSION

Virat, 2005³ concluded that NP is a multifactorial disease with infectious, non infectious, inflammation, anatomic and genetic abnormalities. In clinical practice, the NP was characterized by histological findings of the removed tissue. To date, no REC has been cultured *in vitro* to study the etiology, pathology mechanism and treatment of the NP. The inclusion vacuoles in RE of NP may caused by the abnormal grow tendency or may caused by virus or bacteria invasion. Inclusion bodies are nuclear or cytoplasmic aggregates of stainable substances, usually proteins. They typically represent sites of viral multiplication in a bacterium or a eukaryotic cell and usually consist of viral capsid proteins. Inclusion bodies can also be hallmarks of genetic diseases. Protein inclusion bodies are classically thought to contain miss-folded protein. When genes from one organism are expressed in another the resulting protein sometimes forms inclusion bodies. Virus replication is often detected by the morphological changes, or cytopathic effect (CPE). CPE refers to degenerative changes in cells, especially cell culture, and may be associated with the multiplication of certain viruses. In spite of many recent advances in basic science, the etiology and pathogenesis of NP have still not been clearly clarified. In this study, we found evidence of virus infection in REC cultured from NP. Further study need to be carried out using tissue engineering technique to determine the pathogenesis and mechanisms of NP.

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Physical Model of a Dynamic Bioreactor Prototype for Skin Tissue Culture

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SUMMARY

For cases of burns, skin damage or diseases, there is a need for skin grafting. Conventional skin grafts are harvested from healthy part of the same patient. The drawbacks include, pain at or an additional injury at the donor site and limited availability of healthy skin in patients and major skin loss. This is where technology in tissue engineering is useful to produce biological skin substitute. To date the technology uses a static skin culturing technique which needs to be further improved. This study will focus on the development of a physical model of a dynamic bioreactor prototype for skin tissue culture to simulate the continuous air- liquid interface of skin *in vivo*. Statically cultured skin construct remains less than practical in terms of mechanical properties. This arises the need to culture tissue using dynamic bioreactor which is aim to provide efficient skin construct *in vitro* with the required properties. Method of bilayered skin equivalent culturing will be adopted to culture the fibrin-fibroblast and fibrin-keratinocyte skin tissue. This technique will initially involve static skin tissue culture and then advanced culture to be performed in the dynamic bioreactor to generate the equivalent skin tissue. Fibrin material will be used as a biomaterial scaffold for the regeneration of artificial skin construct.

INTRODUCTION

Bioreactors refer to systems in which conditions are closely controlled to permit or induce certain behaviour in living cells or tissues. The concept of bioreactors is neither new nor restricted to the field of tissue engineering. Microbiologists use bioreactors to grow culture of microorganism under defined condition. Bioreactors are also used in brewing, food, and pharmaceutical and biotechnology industry. The principle that physical stimuli can modulate cell function and tissue development has motivated the development of biomechanically active stimulation system to recellularise *in vitro* tissues by exposing them to physiological relevant mechanical stimulations. Tissue engineering bioreactors are intended for tissue engineering functions, for growing functional cells and tissue for transplantation. And also to control *in vitro* studies on the regulation effect

of biochemical and biomechanical factors on cells and tissue development⁴. Dynamic bioreactor for tissue culture should ideally be based on the good understanding of the native environment of the tissue of interest. Tissue engineering bioreactors are designed to precisely regulate the cellular environment, support cell viability and organization, as well provide control of signalling during culturing period. They are also designed to account for specific mechanism of nutrient transfer and specific physical factors which are inherent in the native tissue. Tissue culture grown in 3D tissue cultures, starting from isolated cells has been hindered by the complexity of the dynamic system and the interplay between the different culture parameters³. To obtain an efficient tissue construct, while culturing tissue in dynamic bioreactor. Culture can be carried out in a two or three dimensional scaffold that allows and induces the formation of new tissues after implantation. The scaffold provides structural templates for cell attachment and tissue growth, and this can be improved by applying certain stimuli that can elicit specific responses to the cells, which can be done either mechanically or chemically².

MATERIALS AND METHODS

The dynamic bioreactor prototype is designed from various design concepts and input specifications. The physical model is then developed from the original design.

RESULTS AND DISCUSSION

The conceptual design of the bioreactor for dynamic skin tissue culture is shown in Fig 1.

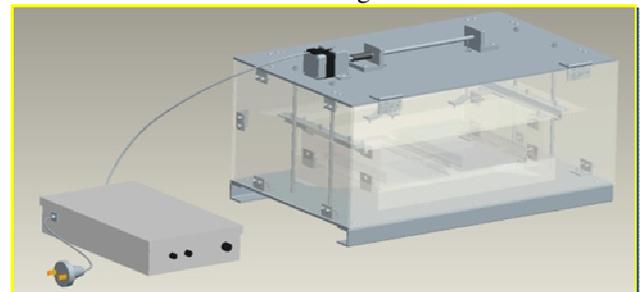


Fig 1: Computational model of the prototype

The physical model of the prototype bioreactor coupled with a motorized stage is shown Fig 2.

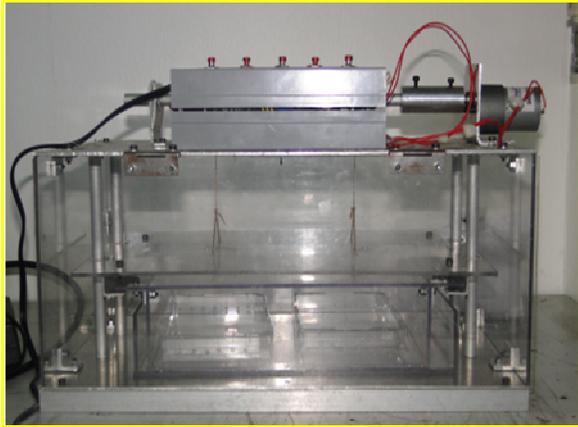


Fig 2: Developed physical model of the prototype

The developed dynamic bioreactor consists of a movable platform to which a tray holder is inserted which contains the culture trays. The platform is balanced horizontally by four guiding rods and is attached to a shaft using two strings (cables). The shaft itself is supported on two plain bearings and two supports. The shaft is connected to a motor shaft through a socket and its movement is set to be synchronous as that of the motor. A DC brush motor is used for the movement of the shaft and the resulting sequential vertical movement of the platform. The motor can change the direction of rotation allowing the platform to be moved upwards and downwards when the motor is switched on. Method of bilayered skin equivalent culturing will be adopted to culture the fibrin-fibroblast and fibrin-keratinocyte skin tissue under static condition¹. The constructed bilayered skin, will then be further cultured in the dynamic bioreactor that will be housed an incubator. This dynamic culture will enable the air- liquid interface of the skin, where the bottom skin layer will be immersed in media and the top layer comes in contact with the air. This air-liquid interface skin culture also permits the recognition of the skin layers during implantation period after growth. The needed dynamic condition is obtained when the bioreactor is constantly operated in sequential movement technique or motion in the incubator. During the culturing process of skin cell in the bioreactor, skin stratification and simulation as well formation of the dense epithelium skin layer can be formed due to the interaction between air and liquid forming the air-liquid interface media. The dynamic bioreactor will also increase the mechanical properties of the skin which can be similar to those found in human dermis and epidermis with good

prospect of clinical application⁵. Proof of concept will be conducted on bilayered skin construct. Control of the duration of construct submerges alternately in air and liquid will be further tested.

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Transfection of Human Bone Marrow-Derived Mesenchymal Stem Cells with hIFN γ Gene

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SUMMARY

Bone marrow-derived mesenchymal stem cells (BM-MS C) have been proposed as potential cellular vehicles in therapeutic gene delivery because of their high proliferative capacity, multilineage differentiation ability, low immunogenicity and capability on migration to injured sites and cancers. Isolated BM-MS C were characterized, and expanded BM-MS C were transfected with pORF-hIFN γ plasmid DNA by nucleofection. Transfection efficiency of around 64% was achieved. ELISA analysis revealed that the production of hIFN- γ protein in transfected BM-MS C reached the peak at 24.72 ng/mL after 48 h post transfection. Real Time PCR was carried out to determine the mRNA expression levels in transfected BM-MS C. The mRNA expression of hIFN γ in transfected BM-MS C after 72 h post transfection was 12.29 x 10³ fold higher when compared to control BM-MS C (Non-transfected BM-MS C).

INTRODUCTION

Human Mesenchymal Stem cells (hMSC) are multipotent progenitor cells that possess high proliferative capacity and are capable of multilineage differentiation¹⁻². MSC also exhibit direct immunosuppressive ability both *in vitro* and *in vivo*, suggesting that they are suitable vehicle for targeted delivery and local production of gene products for therapeutic purposes³⁻⁴. Interferon gamma (IFN γ), or type II interferon, is important in promoting host defense, inhibiting tumor generation, increasing tumor antigenicity and augmenting tumor immunity⁵. These suggest that IFN γ maybe a potential therapeutic agent for various tumor cells. In this study, isolated bone marrow-derived mesenchymal stem cells (BM-MS C) were tested for their capacity in carrying and delivering human interferon-gamma (hIFN γ) gene *in vitro*.

MATERIALS AND METHODS

Isolated BM-MS C were characterised through morphology, immunophenotyping, and differentiation

assay. Expanded BM-MS C were transfected with pORF-hIFN γ plasmid DNA by nucleofection using

Human MSC Nucleofector Kit (Amaxa GmbH). Non-transfected BM-MS C served as a negative control. Supernatants from BM-MS C were harvested at every 24 h of post transfection for three days. ELISA was used to measure the levels of hIFN γ protein expressed *in vitro*. Total RNA of BM-MS C were also extracted at every 24 h of post transfection. The mRNA expression of hIFN γ in BM-MS C was determined by Real Time PCR using Taqman universal PCR master mix (Applied Biosystems) and FAM-labelled Taqman probes (hIFN γ and β -actin). β -actin was used as endogenous control for normalisation.

RESULTS

Isolated BM-MS C showed typical morphology and were capable of differentiating into adipocytes and osteocytes upon chemical induction. Immunophenotyping via flow cytometry showed that cultured BM-MS C expressed the typical MSC surface markers: CD147, CD44, CD13, CD73, CD90 and CD166. Expanded BM-MS C were successfully nucleofected with PI-labelled pORF-hIFN γ plasmid DNA and the transfection efficiency of around 64% was achieved. ELISA analysis on different time points after transfection revealed that the hIFN- γ protein expressed in transfected BM-MS C was detectable at day 1 (0.17 ng/mL), peaked at day 2 (24.72 ng/mL), and decreased slightly to 19.28 ng/mL at day 3. Non-transfected BM-MS C (Control) showed no production of hIFN- γ protein (Figure 1). Real Time PCR was carried out and mRNA expression levels of hIFN γ in BM-MS C were calculated by comparative C_T method (Table 1). Real Time PCR data analysis showed up regulation of hIFN γ gene in transfected BM-MS C. The mRNA expression of hIFN γ in transfected BM-MS C at 24 h post transfection was 0.66 x 10³ fold higher when compared to the control BM-MS C (Non-transfected BM-MS C). This mRNA expression was 5.8 x 10³ fold higher at day 2 and raised up to 12.29 x 10³ fold

higher relative to control BM-MSC at day 3 (Figure 2).

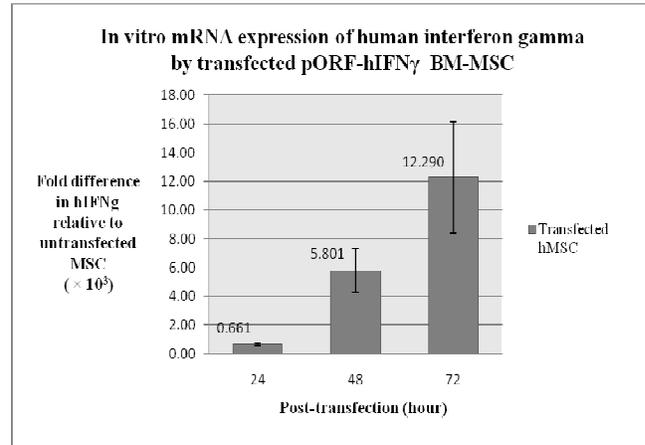
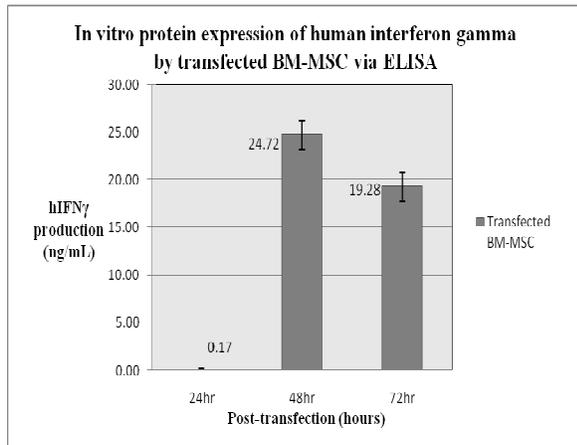


Fig 1: Protein expression of human interferon gamma by transfected BM-MSC with pORF-hIFN γ plasmid. Non-transfected BM-MSC served as negative control. *Data are expressed as mean \pm SD.

Fig 2: *In vitro* mRNA expression of human interferon gamma by transfected BM-MSC with plasmid pORF-hIFN γ . *Data are expressed as mean \pm SD.

Sample	hIFN γ Average C_T	ACTB Average C_T	$\Delta C_T = \frac{C_T \text{ hIFN}\gamma}{C_T \text{ ACTB}}$	$\Delta\Delta C_T = (\Delta C_T \text{ post transfected} - \Delta C_T \text{ untransfected})$	Fold difference in hIFN γ relative to untransfected BM-MSC
Untransfected BM-MSC	33.11 \pm 0.04	24.02 \pm 0.04	9.09 \pm 0.06	0.00 \pm 0.06	1 (0.96-1.04)
24hr Post-transfection	23.68 \pm 0.17	23.91 \pm 0.16	-0.23 \pm 0.23	-9.32 \pm 0.23	660.8 (572.05-749.61)
48hr Post-transfection	21.71 \pm 0.11	25.07 \pm 0.37	-3.36 \pm 0.39	-12.45 \pm 0.39	5801 (4269.94-7332.05)
72hr Post-transfection	21.83 \pm 0.14	26.25 \pm 0.45	-4.42 \pm 0.47	-13.51 \pm 0.47	12290.4 (8422.31-16158.44)

*Data are expressed as mean \pm SD.

Table 1: Fold change expression of hIFN γ in transfected BM-MSC, calculated by comparative C_T method ($\Delta\Delta C_T$)

DISCUSSION

In the present study, BM-MSC were successfully isolated and shown to display the biological properties of MSC. Successful transfection efficiency of 64% was obtained by nucleofection with plasmid expressing gene of interest, pORF-hIFN γ , in BM-MSC. Transfected BM-MSC were shown to express hIFN- γ protein and mRNA *in vitro*.

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Generation of Dendritic-Cell Based Cancer Vaccine for Mouse Acute Myeloid Leukemia

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SUMMARY

Dendritic cells (DCs) have the capacity to initiate immune responses, making DC-based vaccination a novel approach in cancer therapy. Bone marrow cells of C57BL/6 mice were cultured in the presence of granulocytes-macrophages colony-stimulating factor (GM-CSF), interleukin-4 (IL-4) and tumour necrosis factor- α (TNF- α) to generate DCs. Generated BMDCs were characterised by flow cytometry analysis. The functionality of DC-based vaccine in inducing T cell proliferation and exerting anti-leukaemic cytotoxicity were evaluated by using CellTiter-Glo Luminescence Cell Viability assay. T cell proliferation was best stimulated by pulsed-BMDCs at a ratio of 1:5. Preliminary studies also showed that murine C1498 AML cell line co-cultured with primed-T cells has lower cell viability.

INTRODUCTION

Dendritic cells (DCs) derived from bone marrow cells are attractive candidates for cancer immunotherapy by virtue of their capacity to initiate immune response. They are specialized antigen-presenting cells that play an essential role in the induction of T cell immunity¹. These cells possess the unique ability to activate naïve CD4+ and CD8+ T cells *in vitro* and *in vivo*. Their capacity in recognising, capturing, processing and presenting antigens to naïve T cells underscore the interest in exploiting DCs to develop immunotherapies for different types of malignancies. Cancer vaccine is developed based on the concept that the induction of tumour antigen-specific T cell immunity would lead to tumour elimination or rejection². Thus, the aim of this work was to develop a DC-based vaccine for murine acute myeloid leukaemia (AML).

MATERIALS AND METHODS

Bone marrow cells were harvested from femurs and tibiae of female C57BL/6 mice aged week 6 to 13 and cultured in the presence of granulocytes-macrophages colony-stimulating factor (GM-CSF), interleukin-4 (IL-4) and tumour necrosis factor- α (TNF- α) to generate the DCs. The generated BMDCs were characterised based on their cell surface markers by

flow cytometry analysis. AML DC-based vaccine was generated by pulsing of BMDCs with C1498 tumour lysate at the ratio of 3:1 (tumour lysate:DC). The DC-based vaccine functionality, including the capability to induce T cell proliferation and the *in vitro* anti-leukaemic cytotoxicity, were evaluated by using CellTiter-Glo Luminescence Cell Viability Assay (Promega).

RESULTS

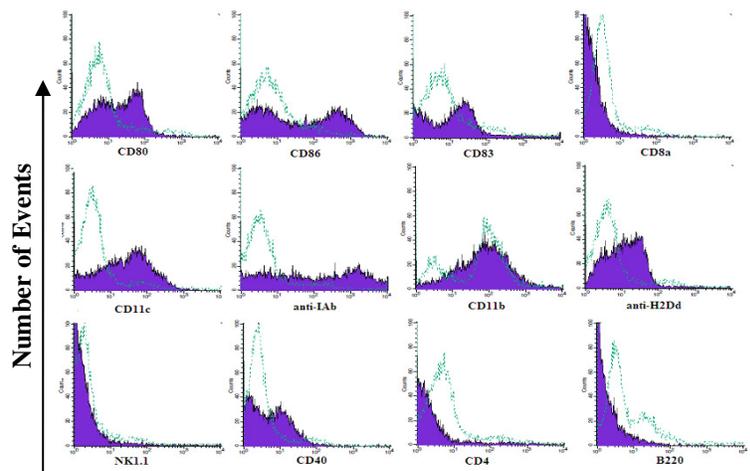


Figure 1: Cell surface expression profiles of mature BMDC from C57BL/6 mice analysed at Day 9. Fluorochrome-labelled mAbs against H-2d, I-A, CD11b, CD11c, CD83, CD86, CD80, CD40, NK1.1, B220, CD8a and CD4 molecules were utilised for FACS analysis.

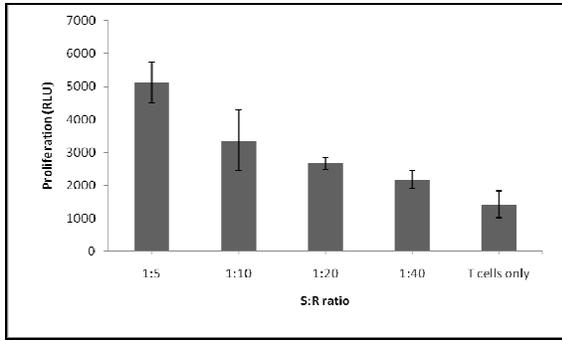


Figure 2: T-cell stimulatory capacity of tumour lysate-pulsed bone marrow derived dendritic cells (pulsed-BMDCs).

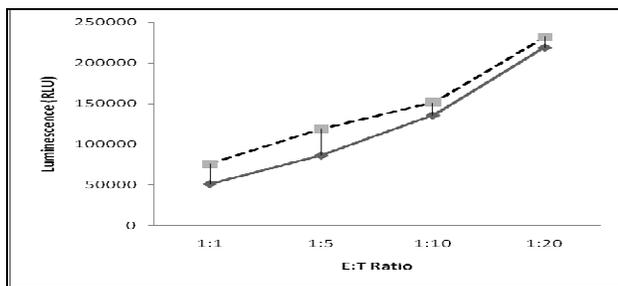


Figure 3: Preliminary data on cytotoxic effect of murine T cells primed by BMDC pulsed with C1498 tumour lysate. The dashed line represents the viability of C1498 murine AML cells co-cultured with unprimed-T cells. The solid line represents the viability of C1498 cells co-cultured with primed-T cells.

Phenotypic evaluation of DCs pulsed with tumour lysate showed that mature BMDC express more co-stimulatory molecules (CD80, CD86, and CD40), DC markers (CD11c and CD83), H-2D^d, I-A^b, and CD11b antigen. However, several antigens were not detected such as CD4, CD8a (T lymphocytes marker), B220 (B lymphocytes marker), and NK1.1 (Natural killer cell markers) surface marker. The capacity of T cell to proliferate was stimulated in the presence of pulsed-BMDCs as T cells tended to proliferate more when cultured with the different ratios of pulsed-BMDCs as compared to T cells alone. The result demonstrated that the S:R ratio of 1:5 was the optimal ratio for stimulating the T cells proliferation. This preliminary study shows that the C1498 cells that were co-cultured with unprimed-T cells possessed higher cell viability than C1498 cells co-cultured with primed-T cells at different effectors to target cell ratio. The cytolytic response of T cells on C1498 cells increased when the number of T cells used increased.

DISCUSSION

Mature murine DCs that expressed MHC-II surface marker were able to stimulate vigorous proliferation of T cells³. Our findings showed that the BMDCs that were generated were able to induce T cell proliferation and could effectively prime T-cells to modestly exert anti-tumour effects on murine AML cell line.

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