Isolation of a Clonal Mesenchymal Stem Cell Line from Rat Bone Marrow and Its Application for Preventing Acute Fatal and Chronic Liver Failure

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SUMMARY

The use of a clonal mesenchymal stem cell as a source of hepatocytes has not been extensively studied. Here we report the isolation and establishment of several clonal mesenchymal stem cell lines from rat bone marrow. One of these cell lines, rBM25/S3 cells, small and asteroid in shape, were maintained in the presence of epidermal growth factor (EGF) and platelet-derived growth factor (PDGF)-BB on a fibronectin substratum. The cells expressed CD29, CD44, CD49b, CD90, vimentin and fibronectin but not CD45, indicating that they are of mesenchymal cell origin. When cultured in the presence of HGF and FGF-4 on Matrigel, the cells efficiently (~100%) differentiated within 7 days into hepatocyte-like cells (HLC) that expressed albumin.

Intrasplenic transplantation of these differentiated cells prevented fatal liver failure in 90%-hepatectomized rats, while the undifferentiated cells most effectively suppressed liver fibrosis. In conclusion, a clonal MSC line derived from adult rat bone marrow could differentiated into HLC, and transplantation of the cells could prevented fatal liver failure in 90%-hepatectomized rats and suppressed carbon tetrachloride-induced liver fibrosis. The present results indicate a promising strategy for treating human fatal liver disease.

Dermal Fibroblasts Conditioned Medium (DFCM) Enhanced the Expansion of Keratinocytes

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SUMMARY

The current study aimed to enhance *in vitro* expansion of keratinocytes by supplementing dermal fibroblasts conditioned medium (DFCM), and compare the effect of two different DFCM acquired by culturing fibroblasts in defined keratinocytes serum free medium (DKSFM) and F12:DMEM (FD) without serum. Keratinocytes cultured in DFCM-DKSFM assist significant increase in attachment efficiency (at 20 h of culture) and final cell concentration (at 120h of culture) compare to that in DFCM-FD and DKSFM (control), suggesting the enhancement in keratinocytes expansion.

INTRODUCTION

Tissue engineered skin is very useful in the treatment of burn, chronic ulcers, venous leg ulcers, pressure ulcers, limb amputations and in reconstructive surgery. Skin graft composite by incorporating a layer of fibroblasts seeded in biomaterials under the keratinocyte sheet provided better outcome such as higher skin graft uptake rate, quicker healing process and better cosmetics features (1-2). However, to reduce the transplantation time, the overall yield of keratinocytes needed to be enhanced. Previous studies shows that keratinocytes co-culture with dermal fibroblasts enhance the expansion process (3), which could be due to the factors secreted by fibroblast. Therefore, the aim of the current study was to compare the effect of dermal fibroblasts conditioned medium (DFCM) on the expansion of keratinocytes.

MATERIALS AND METHODS

Redundant skin tissue was obtained from the consented patient during surgery. Harvested skin sample was digested with collagenase type I. Resulting keratinocytes and fibroblasts were co-cultured in a mix-medium containing DKSFM with F12:DMEM+10% FBS (1:1). After reaching 80% confluency, fibroblasts were removed by partial trypsinization keeping keratinocytes on the culture surface, and continue expansion using DKSFM. Fibroblasts then cultured in FD+10% FBS until confluent, changed in either DKSFM or FD (without serum), and cultured for 2 days to prepare DFCM. Upon reaching 80% confluency, keratinocytes were trypsinized and seeded for next culture at 5000 cells/cm^2, supplementing either DFCM-DKSFM or DFCM-FD (25%) along with the control condition (DKSFM only). For analyzing growth profile, the adherent cell numbers were estimated by visual cell counting on captured images. All data was recorded as an average of triplicate determinations. The statistical analysis was performed using unpaired student *t*-test.

RESULTS AND DISCUSSION

The efficiency of keratinocytes attachment was found to increase significantly (p<0.05) in DFCM-DKSFM, giving an average value of 0.97±0.04, which was 1.5 and 2.1 times higher than that in DFCM-FD and DKSFM, respectively (Fig. 1). At 120 h of culture, the keratinocytes concentration in DFCM-DKSFM reached $12.4\times10^3\pm1.6\times10^3$ cells/cm^2, which was 1.3 and 2.3 times higher than that in DFCM-FD and DKSFM, respectively (Fig. 2), although apparent specific growth rate was almost similar (data not shown). These results suggested that DFCM-DKSFM facilitate the expansion of keratinocytes by increasing cell attachment. Previous study on DFCM demonstrated the presence of ECM components including collagen type I, one of the major factors required for keratinocytes attachment (4). However, further experiment is required to realize the mechanism of action of DFCM-DKSFM on keratinocytes culture.

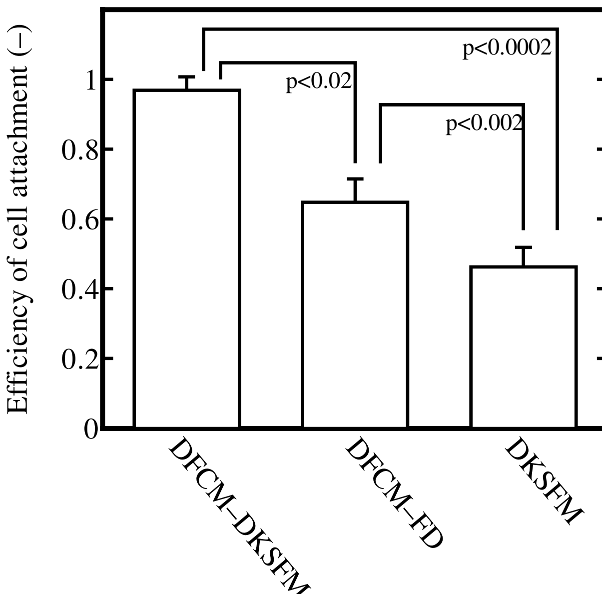

Fig. 1. Efficiency of keratinocytes attachment in different culture medium.

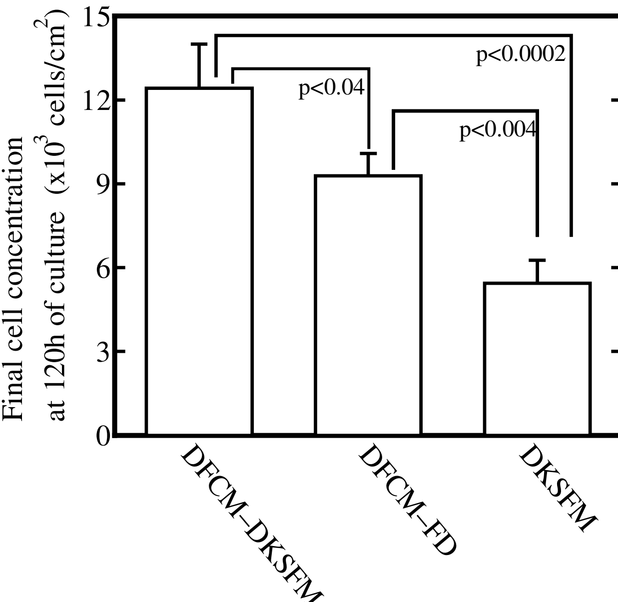

Fig. 2. Final cell concentration of keratinocytes (at 120 h of culture) in different culture medium

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Air-Dried Amniotic Membrane versus Autologous Fibrin Transplantation for Large Conjunctival Epithelial Defects in Rabbits

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SUMMARY

This study was conducted to compare the wound healing rate between air-dried human amniotic membrane (AM) and autologous fibrin transplantation after creating large conjunctiva epithelial defect in a rabbit model. The results showed that AM transplantation significantly enhanced conjunctiva epithelium restoration compared to bare sclera (control) and autologous fibrin with shorter days taken to completely epithelised. Hematoxylin and eosin staining on the harvested conjunctiva after 6 weeks showed multilayer epithelium formation with goblet cells in all groups. Such results are very encouraging for future clinical application to offer immediate tissue replacement of the ocular surface.

INTRODUCTION

There are many conditions that may cause conjunctiva epithelial defect such as in cases of ocular surface disorders including Steven-Johnson syndrome, chemical and thermal burns. Conjunctiva damage may also occur iatrogenically through ophthalmic surgical procedures that require manipulation and removal of the conjunctiva, such as in pterygium surgery, glaucoma surgery and oculoplastic procedures [1,2]. These ocular defects with conservation of limbal corneal junction are usually cured by spontaneous re-epithelisation. However, in cases of bigger conjunctiva epithelial defect, re-epithelisation may need the aid of other support materials such as human amniotic membrane (AM) and fibrin [3]. Hence, the purpose of this study was to evaluate the restoration of the conjunctiva epithelium by grafting onto bare sclera's air-dried human amniotic membrane and autologous fibrin gel.

MATERIALS AND METHODS

An experimental prospective animal trial was performed using 18 adult New Zealand White strain rabbits which were divided into 3 groups. Each group consist of 6 rabbits. The conjunctiva on the temporal site was excised 8mm x 8mm mimicking a pterygium surgery. In the first group, the excised area was left bare; the second group was transplanted air-dried AM and the third group was transplanted with autologous fibrin gel. At day 3, 5, 7, 10, week 4 and week 6 postoperative, grafted and control rabbit conjunctiva were compared in vivo with an anterior segment photograph and blue light fluorescein staining to measure the epithelium defect. Histological appearances of harvested biopsies were analyzed after 6 weeks transplantation.

RESULTS

Conjunctiva epithelisation was completed after a mean period of 30.33 + 2.33 days in the bare sclera group. Mean period for the AM group was 7.17 + 0.65 days. While mean period for the autologous fibrin group was 15.00 + 4.15 days Conjunctiva epithelisation was earlier in the AM group compared to the bare sclera and autologous fibrin.

The mean epithelisation rate (Fig. 2) was 4.78 + 0.68 mm2/day for the bare sclera group, 8.92 + 1.23 mm2/day for the AM group and 4.84 + 0.83 mm2/day for the autologous fibrin group. The AM group has the fastest epithelisation rate and it was statistically significant at $p < 0.05$ compared to bare sclera ($p < 0.05$) and autologous fibrin ($p < 0.05$). Six weeks after grafting, a normal conjunctiva phenotype was observed on the ocular surface of control rabbits and grafted rabbits (ungrafted or grafted with fibrin gel or AM) where histological signs of conjunctivalization were found (Fig. 3). The presences of goblet cells were also detected.

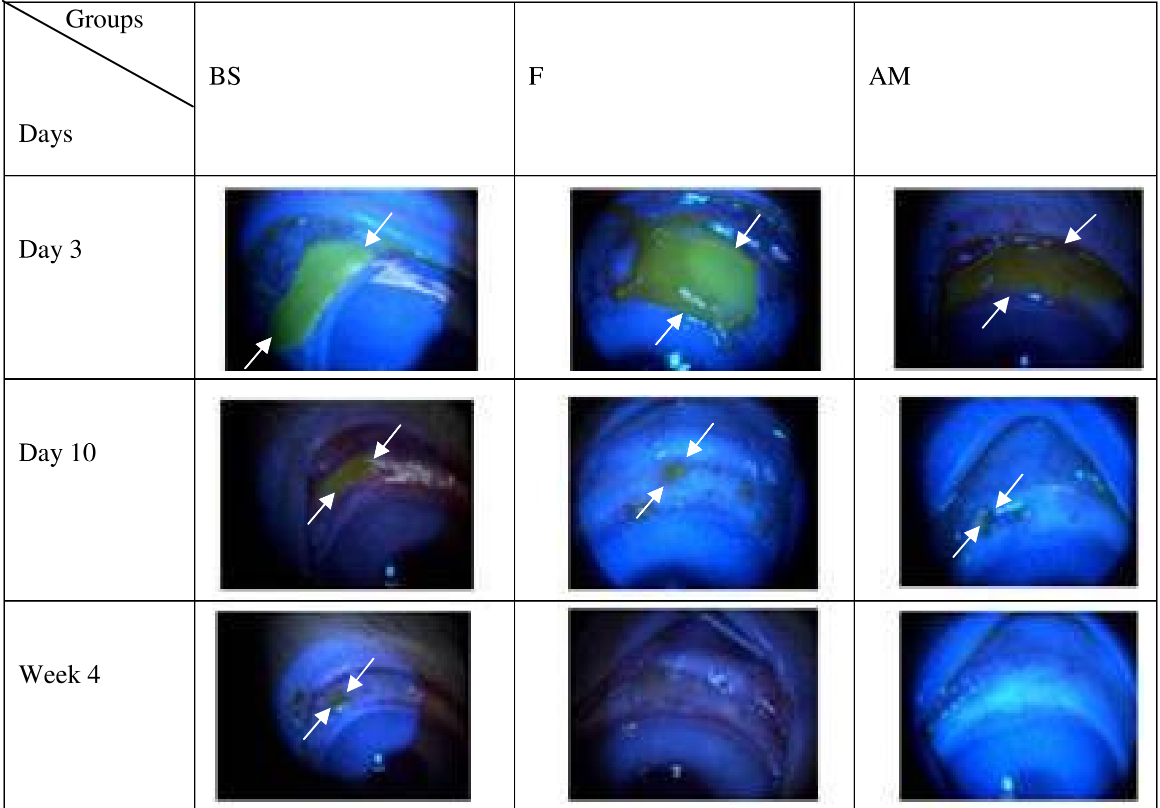

Fig. 1 - Examination of epithelial defect using fluorescein staining viewed under blue filter at day 3, 10 and week 4 for bare sclera (BS), autologous fibrin (F) and amniotic membrane (AM) group. Epithelial defect was detected by the yellowish appearance on the ocular surface (white arrow). The fluorescein stained areas were prominently visible at the earlier stage and getting smaller later in all groups. It showed the re-epithelisation took place where the desmosomes and hemidesmosomes had developed causing the fluorescein unable to seep through the epithelium.

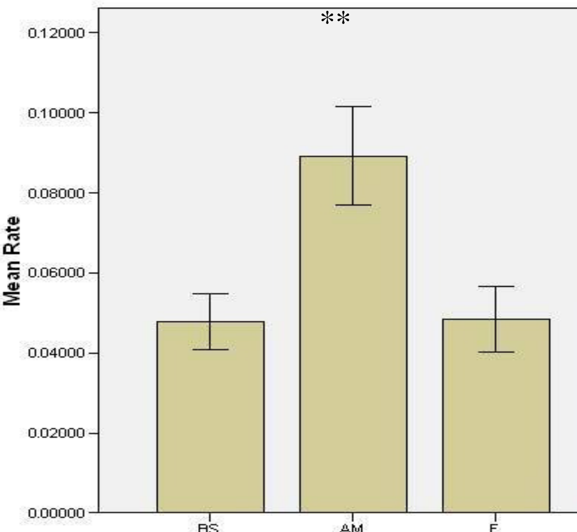

Fig. 2 – The rate of conjunctival epithelisation in bare sclera (BS), amniotic membrane (AM) and fibrin (F) groups. AM showed the fastest rate of epithelisation.

* AM vs BS ($p < 0.05$)
** AM vs F ($p < 0.05$)

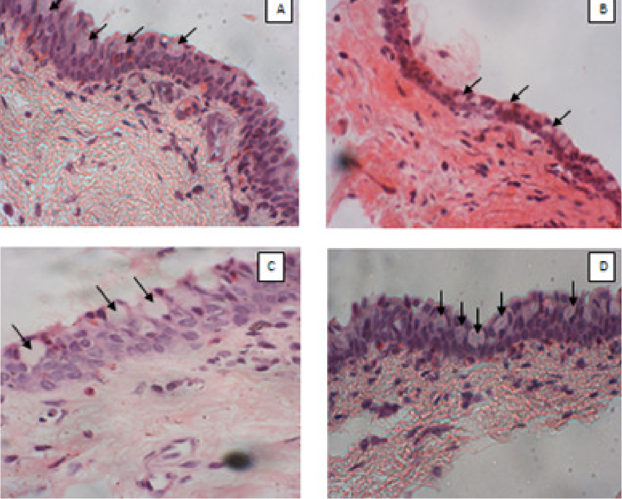

Fig. 3 – Hematoxylin and eosin staining on the harvested conjunctiva after 6 weeks showed multilayer epithelium formation with goblet cells of bare sclera (B), autologous fibrin (C) and AM (D) group. Bare sclera group showed fewer layer of epithelium compared to native conjunctiva (A). The presence of goblet cells were detected within the epithelium layer (black arrow).

DISCUSSION

Our results demonstrate that a physiologically biodegradable matrix such as amniotic membrane and autologous fibrin grafted on bare sclera enhanced conjunctiva epithelialisation. Amniotic membrane transplantation showed a faster epithelialisation rate. Such results are very encouraging for future clinical application to offer immediate tissue replacement on the ocular surface.

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Autologous Tissue Engineered Cornea Sustitute Preserve Corneal Suface Epithelial Morphology

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SUMMARY
The present work was to determine the structural epithelial surface features with the presence of microvilli of the autologous transplanted tissue engineered cornea by scanning electron microscopy and transmission electron microscopy. After 90 days, the corneas were harvested for analysis. The result indicates that the engineered cornea transplanted with bilayered *in vitro* cornea construct (BICC) showed good wound healing and preserve the corneal surface epithelial layer features similar to the normal cornea.

INTRODUCTION
The emergence of tissue engineering as a potential alternative to tissue or organ transplantation paved the way for new type of treatment in tissue loss and malfunction [1]. The cornea is an avascular and transparent tissue in the eye that is responsible for refracting incoming light [2]. Transparency of the cornea as the anterior surface of the eye is essential for vision. However, the maintenance of corneal transparency can be compromised through breakdown of the integrity of the outer limiting epithelial layer. If this layer is compromised, it can lead to corneal opacification, visual impairment, and even blindness [3]. The outer cell layer of the corneal epithelial surface is packaged into membrane folds called microvilli. These small projections serve to increase the surface area of the cells. This allow for oxygen absorbance to increase [4]. The main objective is to determine the structural and ultrastructural features of the surface epithelial of tissue engineered cornea.

MATERIALS AND METHODS
Limbal biopsy specimens were obtained from the rabbit, epithelial cells and keratocytes were cultured in different media until propagated. Rabbit cornea undergo lamellar keratectomy and autologous tissue engineered cornea substituted were transplanted into the lamellar keratectomized cornea. Transplant and control cornea were assessed by slit lamp microscopy to assess the wound healing progress and also to inspect the transparency, clearness, surface smoothness, vascularisation and rejection mark at day 1 and day 90. The corneal haze was scored in a masked fashion according to Opacity Clinical Scoring System [5]. For further investigation of surface epithelial morphology, scanning electron microscopy and transmission electron microscopy were performed.

RESULTS
Slit lamp microscopy revealed that, both corneas in engineered cornea (EC), which transplanted with BICC and defect cornea (DC), the lamellar keratectomy cornea, showed a good gross healing coverage, after 90 days. The corneas exhibited a clear and transparent cornea with visible pupil. Meanwhile, the fibrin cornea (FC) group, which was transplanted with fibrin without cell construct (FWCC), exhibited severe opacity, uneven surface cornea and no visible pupil. The score for corneal opacity among the four groups were significantly different, showed in Table 1. Through scanning electron microscopy, the appearance of microvilli and epithelial surface morphology were graded using the lesion score ranging from 0 to 10, and it showed significant differences between experimental groups (Table 2). The anterior surface of the epithelial cells of EC were significantly fully covered with microvilli without any pathological changes. On the other hand, the surface morphology of the epithelial cells for FC was almost covered by stubby microvilli with frequently observed multifocal absence of microvilli. The same pathological conditions were also observed in DC. TEM analysis further confirmed that the anterior portion of EC was covered with microvilli at the apical surface of the superficial squamous cells. In contrast, the FC and DC ultrastructural morphologies of stratified epithelial cells, showed some apparent differences with both experimental groups displayed an electron lucent with sparsely distributed organelles inside for both corneas, FC and DC in the superficial squamous epithelial cells of the cornea. In addition, the microvilli in both of FC and DC corneas were scattered and less distributed when compared to NC.

Table 1: Mean corneal opacity score at day 1 and day 90 post-transplantation

Days / Group	*Day 1* *(mean ± SD)*	*Day 90* *(mean ± SD)*	*Corneal opacity differences (mean ± SD)*
Normal cornea	0.00 ± 0.00 [a]	0.00 ± 0.00 [d]	0.00 ± 0.00 [g]
Engineered cornea	4.00 ± 0.00 [b]	0.33 ± 0.58 [df]	3.67 ± 0.58 [h]
Fibrin cornea	4.67 ± 0.58 [c]	4.33 ± 0.58 [e]	0.33 ± 0.58 [g]
Defect cornea	3.33 ± 0.58 [b]	0.67 ± 0.58 [f]	2.67 ± 0.58 [h]

Note: Figure indicated the average score of corneal opacity. [a,b,c,d,e,f,g,h] comparison of means across treatment at $p<0.05$.

Table 2: Mean corneal epithelial surface lesion score 90 days post-transplantation

Group	**Microvilli score**
Normal cornea	0.50 ± 0.26 [a]
Engineered cornea	0.56 ± 0.19 [a]
Fibrin cornea	4.72 ± 2.22 [b]
Defect cornea	5.67 ± 0.58 [b]

Note: Figure indicated the average score of corneal epithelial surface cells. All the data recorded were mean score ± SD. [a,b] comparison of means across treatment at $p<0.05$.

DISCUSSION

The BICC transplanted cornea appearance mimicked the normal rabbit cornea and demonstrated good wound healing coverage. This investigation proved that the stroma layer was supported by the regeneration of the epithelial layer during wound healing process. The survival and the persistent recovery demonstrated that BICC, consisted of limbal cells, possesses the ability to regenerate the epithelial layer and stroma layer. These findings corresponded with the previous study by [6] and [7] that reported the same features of improved corneal clarity with smooth and wettable corneal surface after corneal equivalent transplantation. The evidence that BICC promoted good healing coverage were by helping to increase the protective barrier through regeneration of microvilli, the first complex protective barrier of the cornea [8] as the distribution of microvilli was fully covering the epithelial cells anterior surface. These findings corresponded, that the presence of microvilli enhanced the stability of the tear film by increasing the surface area of the plasma membrane while simultaneously enhanced the epithelial integrity. The absence of microvilli could thus impair the balance of tear film stability and indirectly, impaired the physiological process of the cornea in maintaining a clear vision. The reepithelialisation of the corneal surface is an important process to restore the imaging properties of this tissue. The transplantation of autologous tissue engineered cornea substituted can successfully restore the corneal transparency after lamellar keratectomy with restoration of structural and ultrastructural features of surface epithelial cell a-like to normal cornea.

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Inflammatory Cytokines and Osteogenic Potential of Stem Cells from Human Exfoliated Deciduous Teeth (SHED)

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SUMMARY
Stem cells from human exfoliated deciduous teeth (SHED) have significant potential in renewal and regeneration of a variety of cells. This preliminary research studies the potential of SHED for stem cell bone regeneration therapy, in terms of the interaction between osteogenic proteins expressed in SHED and human immune system. Results of gene expression analysis showed that SHED has potentially improved immunomodulatory properties due to generally low expression levels of IL-1β, IL-6, IL-8 and TGF-β genes, as well as high OPG/RANKL ratio that characterized the osteogenic potential of SHED.

INTRODUCTION
SHED are highly proliferative, clonogenic cells capable of inducing bone formation [1] and directly differentiating into osteoblasts [2]. It is a potential alternative for stem cell bone regeneration therapy. However, stem cell therapy carries the risk of immune rejection mediated by inflammatory cytokines of human defense system. Inflammatory cytokines including interleukin-1β (IL-1β), interleukin-6 (IL-6) and tumour necrosis factors (TNF), as well as proteins like osteoprotegerin (OPG) and receptor activator of NF-kB ligand (RANKL), are mediators that regulate bone metabolism [3-6]. This research studies the interaction between SHED and immune system by determining the cytokines involved in the regulation of bone formation, remodeling and resorption expressed in SHED.

MATERIALS AND METHODS
Human fetal osteoblasts (hFOB) and isolated SHED from deciduous molars and incisors were cultured and harvested in triplicate. Extraction of total RNA was done, followed by reverse transcription cDNA synthesis of hFOB and SHED. Semi-quantitative reverse transcription PCR was done to detect expression levels of OPG and RANKL, and Multiplex PCR was performed using Human Inflammatory Cytokines Genes Set to detect the expression levels of TNF-α, IL-1β, GM-CSF, IL-6, IL-8 and TGF-β in both cell types.

RESULTS
Analysis showed that SHED expressed significantly lower amounts of cytokines IL-1β, IL-6, and IL-8 compared to hFOB. TNF-α and GM-CSF was not detected in both SHED and hFOB, and SHED expressed similar levels of TGF-β with hFOb. Furthermore, SHED also demonstrated high OPG/RANKL ratio in a same pattern as hFOB.

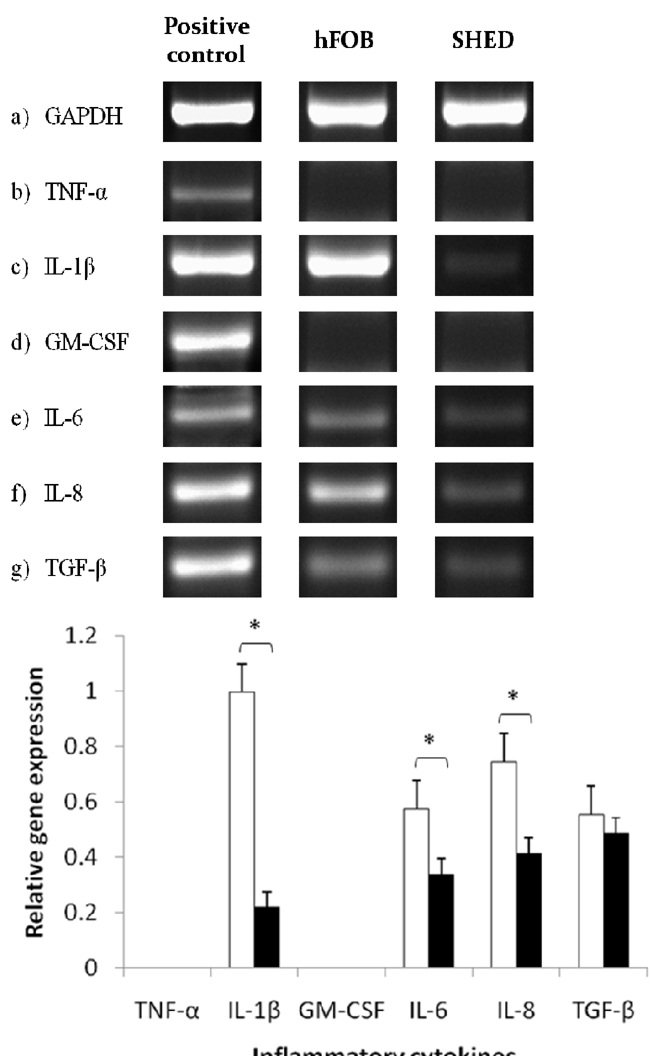

Fig. 1. Analysis of MPCR products of inflammatory cytokines genes. (□ hFOB, ■ SHED, *shows significance at P < 0.05).

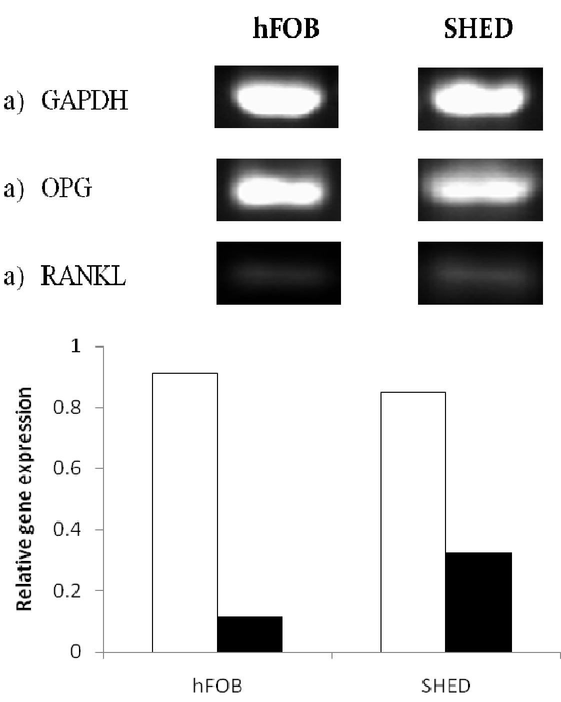

Fig. 2. Analysis of RT-PCR products of a) GAPDH, b) OPG and c) RANKL. (□ OPG, ■ RANKL)

DISCUSSION AND CONCLUSION

IL-1β is a potent bone-resorbing factor, while IL-6 and IL-8 induce osteoclastogenesis and osteolysis respectively [3, 4]. TNF-α stimulates osteoclastic activity, and conversely, TGF-β promotes osteoblastic proliferation [5]. Our findings suggest that osteoblasts derived from SHED may have improved immunomodulatory profile in terms of promoting relatively lower inflammatory reaction during transplant. Furthermore, in contrast with previous studies of undifferentiated marrow stem cells which show low OPG/RANKL ratio [7], SHED express high OPG/RANKL ratio, which prevents osteoclastogenesis and enhances bone regeneration [6]. SHED is an ideal source of osteoblasts to be used in bone regeneration, transplant and cellular therapy in human. Further studies on the immunomodulatory properties of SHED-derived osteoblasts are necessary to enable future stem cell therapy in immunocompetent hosts.

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Human Bone Marrow Mesenchymal Stem Cells Can Be Differentiated to Corneal Lineages proven by Presence of β-Intergrin and CEBPδ

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SUMMARY

Interest in developing tissue-engineered cornea has increased with the decrease in the supply of donor tissue. The aim of this study was to differentiate bone marrow mesenchymal stem cells (BMSC) to corneal epithelial lineages. Induced bone marrow was characterized by quantitative Real Time Polymerase Time Reaction for β-integrin and CEBPδ. β-integrin is a specific marker for cell adhesion regulation in corneal epithelial cells while CEBPδ acts as a regulator of differentiated corneal cells. Hence, we conclude that induced bone marrow by limbal medium has the potential to differentiate to corneal lineages because this culture resembles corneal epithelial cells by expressing β-integrin and CEBPδ.

INTRODUCTION

Corneal epithelium is maintained by limbal stem cells. Loss of limbal stem cells can lead to the painful and blinding conditions where limbal epithelium fails to function normally and was replaced by conjuctival epithelium and blood vessels. Bone marrow is recognized as a source of progenitor cells for epithelial and endothelial cells. Transplanted bone marrow can be engrafted to an injured cornea and promote wound healing by proliferation and differentiation. Integrins are transmembrane receptor proteins critical for growth and stabilization of vessels. CEBPδ is a regulator in self-renewal and cell cycle length of limbal stem cells and it is expressed in a variety of tissues and cell types and involved in the control of cellular proliferation and differentiation, metabolism and inflammation.

MATERIAL AND METHODS

Discarded corneal rings were obtained from tissue donor during corneal transplant surgery with written consent. It was treated with Trypsin EDTA and collected every 20 minutes. Cells were plated on irradiated NIH 3T3 cells and cultured in limbal medium (Kolli *et. al*, 2008). The native corneal cells were used as positive control. Bone marrow was obtained from interlocking nail fixation procedure with written consent. Bone marrow was loaded over a Ficoll-Paque solution and centrifuged at 3000rpm. Cells obtained were resuspended in Dulbecco's Modified Eagle Medium (DMEM) and Ham's F12 and limbal medium. Bone marrow culture was expanded until P2. Total RNA was extracted at P2 using TRI reagent and subjected to quantitative Real Time Polymerase Chain Reaction (q-RTPCR) to detect β-integrin and CEBPδ expression

RESULTS

Genes / Samples	B-integrin	CEBPδ	p value
Cornea (Control)	0.410 ± 0.034	0.019 ± 0.003	-
Uninduced BMSC	$3.553 \times 10^{-6} \pm 9.99 \times 10^{-7}$	$1.55 \times 10^{-5} \pm 1.259 \times 10^{-6}$	$p<0.05$ (Uninduced vs Induced group in β-integrin)

Induced BMSC	0.275 ± 0.049	$2.64 \times 10^{-3} \pm 6.77 \times 10^{-4}$	$p<0.05$ (Uninduced vs Induced group in CEBPδ)

Table1: Gene expression of corneal epithelial cells (control), uninduced bone marrow and induced bone marrow using q-RTPCR.

Bone marrow mesenchymal stem cells grew rapidly and became fibroblast-like with rich cytoplasm. Table 1 shows the expression of β-integrin and CEBPδ marker genes in differentiated BMSC. Corneal cells expressed higher β-integrin and CEBPδ as compared to induced and uninduced bone marrow. Induced bone marrow showed up-regulation of both corneal markers as compared to uninduced bone marrow.

DISCUSSION

The differentiated bone marrow using limbal medium showed up-regulation of corneal markers resembling native corneal epithelium. β-integrin has been localized to basal cells of healthy adult limbal epithelium, precisely the site of the limbal stem cells. CEBPδ is positively regulated gene loci, suggesting a direct role of this transcription factor in determining limbal stem cell identity. This study showed the successful differentiation of human BMSC cells to corneal lineages.

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Lentiviral Transgene Silencing in Transduced Haematopoietic Stem Cells

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SUMMARY

Lentivirus (LV) is an attractive tool for gene therapy as it can transduce hematopoietic stem cells (HSCs) efficiently. Unfortunately, few studies have shown that the duration of transgene expression by LV is transient. To substantiate this, we transduced HSCs with LV carrying GFP and the levels of expression were measured at different time points. As expected, GFP expression declined by day 2 post-transduction. This may due to the *de novo* methylation of the transgene and chromatin modifications at the transgene-integrated region. We hypothesized that 5-azacytidine (5-azaC), a DNA demethylating agent, and Trichostatin A (TSA), an inhibitor of histone deacetylase, would be able to improve the level of transgene expression. First, either 5-azaC or TSA was added to HSCs a day after transduction or on the day following GFP silencing. 5-azaC was able to prevent and reverse the silencing effect. However, TSA was unable to reverse the GFP silencing, but it prevented the GFP silencing at high concentration. Next, we investigated the effects of 5-azaC and TSA combination on GFP expression. Both drugs were added a day after transduction or on the day following the GFP silencing. Combination of both drugs prevented GFP silencing when high concentration of TSA was used but there was no effect in reversing the silenced GFP. Here we show that transgene silencing in HSCs can be prevented and reversed by 5-aza-C, while TSA or the combination of both drugs do not demonstrate any silencing reversal by transgene deacetylation inhibition.

Bio-functional Nanoparticles for Transfection of Embryonic Stem Cells

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SUMMARY

Stem cells possessing the inherent capability of transforming into many cell types have shown tremendous potential for cell-based therapies in regenerative medicine for neurological disease or injury, diabetes and myocardial infarct. The in vitro differentiated derivatives of stem cells are thought to be able to repair or replace damaged cells, tissues or organs. Thus, the use of both adult and embryonic stem cells is currently under intensive investigation. However, compared to embryonic stem cells, adult stem cells are likely more difficult to be implemented into useful therapies considering their limited pluripotency. Transgene delivery could be a powerful strategy for specific differentiation of embryonic stem cells since several transcription factors have been demonstrated to regulate stem cell differentiation to specific cell types of heart, pancreas, liver and neurons. Among the existing approaches for transgene delivery, viral systems suffer from their potential life-threatening effects of immunogenicity and carcinogenicity whereas non-viral ones, although safe, possess significant limitation in terms of efficacy. Development of a safe as well as an efficient carrier is, therefore, an urgent requirement for effective implementation of stem cells in regenerative medicine. Recently, we have developed an efficient DNA delivery and expression system based on some fascinating properties of carbonate apatite- ability of preventing crystal growth for generation of nano-scale particles as needed for efficient endocytosis and fast dissolution rate in endosomal acidic compartments to facilitate DNA release from the particles and endosomes. Moreover, through complexation of these nano-crystals with naturally occurring fibronectin and genetically engineered E-cadherin-Fc, we showed a dramatic enhancement both in transgene delivery and expression in mouse embryonic stem cells and embryonic stem cell-like carcinoma (F9) cells which possess both transmembrane fibronectin-specific integrin and E-cadherin. Here, we report on the development of an effective formulation process for such cell-adhesive protein embedded particles that dramatically improved binding DNA and cell-recognizable proteins with growing inorganic crystals and reducing the size of the resulting composite particles for highly efficient transgene delivery and expression in mouse stem cells.

Young Coconut Juice, a Potential Therapeutic Agent for Postmenopausal Women: Novel Findings

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SUMMARY

After menopause, estrogen levels in women decrease dramatically. This decrease in endogenous estrogen is associated with an increased risk of cardiovascular disease and osteoporosis. Decreasing levels of estrogen are also claimed to be associated with an increased risk of dementia and Alzheimers disease (AD). A relationship between estrogen levels and dementia is biologically plausible and, if proven, may have major implications for the prevention or treatment of dementia. Many studies have reported a lower prevalence and incidence of dementia and AD in postmenopausal (PMS) women who used estrogen replacement therapy. This indicates that the feeding of exogenous estrogen may reduce the risk of dementia. However exogenous estrogen could also increase the risk of developing breast and ovarian cancer, as well as causing other side effects. Phytoestrogen derived from plants might be a viable alternative source for hormone replacement therapy (HRT). According to folk medicine, coconut juice (*Cocos nucifera* L., Arecaceae), contains many compounds with therapeutic properties. For centuries, coconut juice especially young coconut juice (YCJ) has been used as a "temporary contraceptive" drink for both Thai and Indonesian women. It was thought that this could be due to the presence of estrogen-like compounds such as phytoestrogen. With this background, YCJ, a known source of phytoestrogen, was investigated for its possible beneficial effects for postmenopausal women by first using ovariectomized (ovx) rats, a standard model for postmenopausal women.

The main investigations have concentrated on 3 aspects of the use of YCJ

1) Can it halt dementia? Investigations include measurements of the levels of β-amyloid 1-42, Tau 1 and GFAP antibodies.
2) Does it accelerate cutaneous wound healing? Its effect on both female and male rats has been investigated.
3) Does it prevent osteoporosis?

Our studies have shown that young coconut juice containing estrogen-like hormones helped to reduce Alzheimer pathologies. Feeding young-coconut juice to ovx rats helped to accelerate wound healing. YCJ did have beneficial effects for the treatment of osteoporosis in both male and female rats. All these studies are novel and it indicates the YCJ can have beneficial effects and prevent or delay the development of many diseases. A "functional drink" containing young-coconut juice and other herbal plant extracts has been developed and is now patented.

Strobilanthus crispus: A Potential Herb as Anticancer for Liver and Breast.

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SUMMARY

Strobilanthus crispus (*S. crispus*) or pecah beling has long been used as medicinal plants against various conditions and cancer. This study was conducted to determine the effect of aqueous extracts of *S. crispus* with four different doses on experimental male albino rats species *Rattus norvegicus* with induced diethylnitrosamine as initiator and 2-acethylaminoflourene as promoter agent. The rats were divided into normal with basal diet, normal supplemented with *S. crispus* aqueous extract group, cancer induced group with DEN/AAF and cancer induced group with DEN/AAF and supplemented with 4 different doses of *S. crispus* (1%, 2.5%, 5% and 7.5%) and glycyrrhizin as comparison. This study was designed according to Solt and Farber (1976) without partial hepatectomy. Transmission electron micrograph showed the ultrastructural features of cells such as nucleus, mitochondria, rough endoplasmic reticulum (RER) were observed in DEN/AAF induced groups. However, the shape of the nucleus and the arrangement of rough endosplasmic reticulum (RER) and mitochondria appeared normal in DEN/AAF induced rat treated *S. crispus* group. As a conclusion this study revealed *S. crispus* aqueous extract has a potential as an inhibition agent during hepatocarcinogenesis without interfering the normal growth of cells. Studies on the cytotoxicity effects, antitumor components and their mechanism of action of *S. crispus* were carried out in the study. The IC_{50}-values of *S. crispus* against liver cancer cell line was 29.3 µg/ml for methanolic extract while the chloroform extract showed IC_{50}= 28 µg/ml. The mechanism of action was studied by apoptosis pathway and expression of *c-myc* oncogene. The TUNEL assay staining revealed cells with intensely yellow fluorescence of PI-FITC. The common features were condensation of chromatin, fragmentation of DNA, and formation of apoptotic bodies. Chemopreventive properties of *S. crispus* extract was investigated via screening against a panel of human cancer cell lines and the normal fibrolast cells to screen for selective cytotoxicity and anti-proliferative activity. *S. crispus* extract was found to display selective cytotoxicity and anti-proliferative against breast cancer cells but not on normal cells. Exposure of *S. crispus* extract (30 µg/mL) resulted in inhibition of cell growth in hormone dependent breast cancer MCF-7. Further analysis indicated the presence of subG1 population in MCF-7 cells treated with *S. crispus* extract (30 µg/mL), a classical feature of apoptotic cells. Detection of apoptotic MCF-7 cells was also apparent in flow cytometry Annexin V/FIT-C staining and via detection of double or single DNA break stands in TUNEL assay. Mitochondrial activated apoptosis induction by *S. crispus* in MCF-7 cells was found to involve activation of caspases and release of Cytochrome C in the cytosol, thus activating initiator and effector CASPASE 3/7. Upregulation of tumor suppressor p53 protein was detected upon *S. crispus* exposure however, apoptosis induction in treated MCF-7 cells was found to be p53 transcriptive independent as pro-apoptotic bax and Bcl-2 protein were not activated upon activation of apoptosis machinery. In this research, the targeted modulation or restoration of the intracellular signaling network by *S. crispus* extract towards breast cancer cells offered a potential strategy in preventing abnormal cell proliferation and promoting cell death of neoplastic cells in an *in vitro* model. In conclusion, *S. crispus* has potential as anticancer agents for liver and breast.

Effect of L-Carnitine on the Antioxidant Status and Neurochemical Hormones in 6-Hydroxydopamine-Induced Neurotoxicity in the Substantia Nigra of Aged Rats

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SUMMARY

Parkinson's disease (PD) is one of the commonest neurodegenerative diseases, and oxidative stress has been evidenced to play a vital role in its causation. In the present study, we evaluated L-carnitine (LC) (β-hydroxy-γ-trimethyamino butyric acid) an iron-chelating antioxidant and enhancer of biogenic amines, can attenuate the neuronal injury in a 6-hydroxydopamine (OHDA)-induced PD in aged rats. Aged male Sprague-Dawley rats (24 months) were divided into 4 groups: a control, LC-treated, OHDA-treated and LC+OHDA-treated groups. Neurotoxicity was induced by a single intrastriatal infusion of 2µl of 6-OHDA (at a rate of 1 µl per min) into the *substantia nigra pars compacta* (SNc) (12µg in 0.01% in ascorbic acid-saline, while the sham-operated groups received 2µl of vehicle) and a pharmacological dose of L-Carnitine (300 mg/kg b.wt, i.p in saline, started 3 days prior to OHDA) was administered for 21 days. At the end of the experimental period animals were killed under anaesthesia and the estimation of lipid peroxidation (LPO), total antitioxidants (TA), protein carbonyl content (PCC), reduced glutathione (GSH), the activities of glutathione-S-transferase (GST), glutathione reductase (GR), glutathione peroxidase (GPx), superoxide dismutase (SOD) and catalase (CAT), quantification of catecholamines (Dopamine (DA) and noradrenaline (NE), melatonin and serotonin, were carried out in the substantia nigra dopaminergic neurons. Lesioning was followed by an increased LPO, PCC and a significant depletion of GSH content in the substantia nigra, which was prevented with L-carnitine pretreatment. The activities of glutathione-dependent enzymes (GDEs), CAT and SOD in substantia nigra, which were reduced significantly by OHDA-lesioning were restored by L-carnitine. A significant decrease in the level of catecholamines, melatonin and serotonin were observed after 6-OHDA injection, and were significantly recovered following L-carnitine treatment. This study indicates that L-carnitine ameliorates xenobiotics induced neurodegeneration and might be helpful in the attenuation and prevention of Parkinsonism.

Optimal Transfection Conditions and the Safety Profiles of Dextran-Spermine/Plasmid DNA as a Potential Gene Transfer Vector to Mouse Airway

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SUMMARY

The emergence of gene therapy offers a new paradigm to the field of molecular medicine. However, current viral and non-viral gene transfer vectors are not efficient and often restricted by dose-limiting toxicity. Thus, generation of a new gene delivery vector, which is efficient and with good safety profile is highly required. In this study, optimal transfection conditions and safety profile a novel biodegradable cationic polymerdextran-spermine (D-SPM) in mouse airways were ascertained. The highest level of gene expression in the lungs of BALB/c mice was detected at D-SPM to plasmid DNA (pDNA) weight ratio (w/w) of 16, with 13.5 µg pDNA. No significant induction of pro-inflammatory cytokines in the broncho alveolar lavage fluids was observed, which implies no overt toxicity occurred in the mouse lungs. In short, these results demonstrate that D-SPM has moderate gene transfer efficiency but with acceptable safety profile in the mouse airways.

INTRODUCTION

The advancement in the gene transfer technology has made possible for the development of gene therapy. However, the main challenge for a successful gene therapy is the development of a safe and effective gene transfer vector.The viral vectors have been extensively used in gene therapy clinical trials, as they are able to deliver gene efficiently to target cells.Yet, the risk of insertional mutagenesis by the provirus poses a serious safety concern [2]. Due to this reason, non-viral vectors are currently emerging as a favorable option to viral vectors. Hosseinkhan i*et al.* has shown that a new biodegradable polycation,dextran-spermine (D-SPM), was efficient attransfecting cells and tissues *in vitro* and *in vivo*.However, no study has been performed to ascertain the effectiveness and the safety of this gene transfer agent exclusively in the lung of mouse via intranasal delivery. Therefore, this study aims:(1) to determine the optimum weight-mixing ratio of D-SPM to pDNA and the loading capacity ofpDNA in the complexthat can give the maximum level of gene expression, and (2)to measure the induction of pro-inflammatory cytokines in the mouse airway.

MATERIALS AND METHODS

Plasmid pCIKLux expressing luciferase reporter gene was kindly provided by the Gene Medicine Research Group, Oxford University, UK. QIAGEN EndoFree® Plasmid Mega kit (Qiagen, Germany) was used to prepare a large-scale quantity of plasmid.Complexes of D-SPM and pDNA were prepared at various D-SPM/pDNA weight-mixing ratios of 13 to 18 (µg D-SPM/ µg pDNA) (w/w). To determine the most optimal amount of pDNA, increasing amount of pDNA(9.5 to 17.5 µg) was added to D-SPM with the w/w kept at a constant ratio. Female BALB/c mice, 6 to 8 weeks of age were used in the experiment, with n=6 for each experimental group. All animal experiments were carried out in accordance to the Guidelines for Animal Experiments of Universiti Putra Malaysia.Mice were euthanized by neck-dislocation. The lung and trachea of the mice were harvested for reporter gene expression analysis. The Luciferase Assay System (Promega, USA) was used for luciferase reporter quantification on a GloMax™ 20/20 luminometer (Promega, USA).The obtained relative light units (RLUs) from luciferase assay were normalized against total protein, which was quantified using a Bio-Rad DC Protein Assay (Bio-Rad Laboratories, USA).The levels IFN-γand TNF-α in the bronchio alveolar lavage fluids (BALF) were quantified using Duoset® ELISA Development System kits (R&D Systems, USA).

RESULTS

The D-SPM/pDNA complex ranging from ratio 13 to 18 showed almost similar levels of gene expression to the untreated group. The highest reporter gene expression was observed at weight-mixing ratio of 16, where 91.23 ± 30.18 RLU/mg protein was obtained (Fig. 1a). For the optimal amount of pDNA, the highest gene expression level was detected from the D-SPM/pDNA complex containing 13.5 µg of pDNA, accounting for 158.1 ± 79.6 RLU/mg protein. This reading was approximately 3-fold higher compared to the untreated group, although it was not statistically significant (Fig. 1b). Mice were weighed prior to the gene delivery and after they were sacrificedat day 1 post-treatment. Although the D-SPM and D-SPM/pDNA treated mice showed a massive reduction of weight (results not shown), the mice appeared healthy with no physical sign of illness. For pro-inflammatory cytokines assay, theadministration of D-SPM/pDNA resulted in no significant difference in the levels of IFN-γ and TNF-α between the D-SPM/pDNA treated group and the control untreated group (Fig. 2).

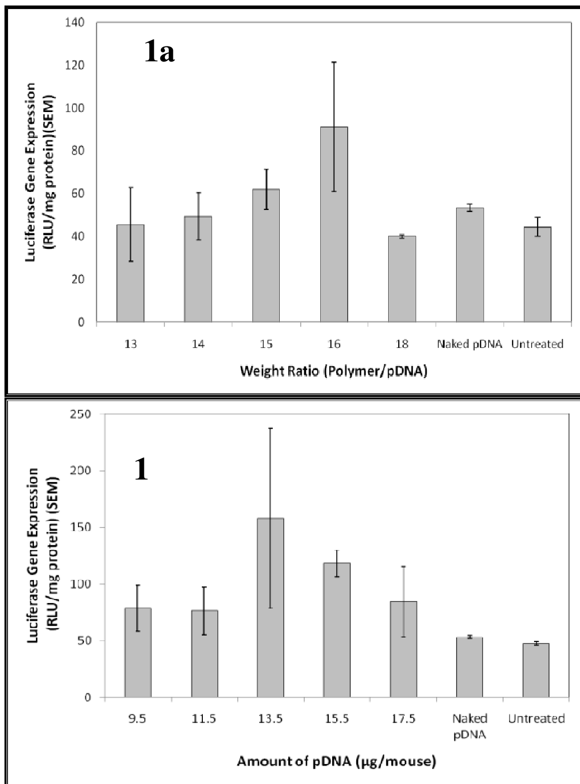

Fig. 1a. Luciferase expression in the trachea and lung of BALB/c mice following the administration of various weight-mixing ratios of D-SPM/pCIKlux complexes day 2 post-treatment.**Fig. 1b.** Luciferase expression in the trachea and lung of BALB/c mice following the administration of D-SPM with different amount of pCIKLux at day 2 post-treatment**.**

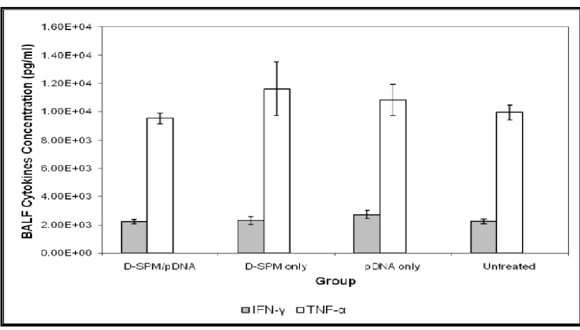

Fig. 2. Pulmonary inflammatory indicators, IFN-γ and TNF-α concentrations in the BALF of D-SPM/pDNA in comparison to naked pDNA and dextran-spermine only (D-SPM).

DISCUSSION

A modest increase of reporter gene expression by D-SPM/pDNA was observed at w/w of 16containing 13.5 µg of pDNA compared to the untreated group. No improvement of reporter gene expression was seen as the w/w or pDNA was increased. A possible reason to this is that increasing the w/w or pDNA may increase the cationic dextran moiety, resulting in the increase of the size of the complex. This eventually impedes its uptake once it reaches certain size due to the steric effect of the random charge distribution surface of the cationic carrier. [1] Although there was a massive reduction of mice weight seen in the D-SPM and D-SPM/pDNA treated groups, the doses were well tolerated and none of the mice died during the course of the experiment. No increase in the levels of TNF-αand IFN-γ was seen, implying no overt toxicity occurred in the lung of mouse following the delivery of the D-SPM/pDNA.These results suggest that although D-SPM/pDNA has moderate gene delivery ability, it was well tolerated and was relatively safe for gene transfer to the lung of mouse.

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Cord Blood Banking: State-of-the-art

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SUMMARY

Cord blood is an unlimited source of stem cells for allogeneic haematopoietic stem cell transplant and regenerative medicine. Cord blood banks (CBB) have been established for related or unrelated umbilical cord blood transplant (UCBT) with more than 400,000 units available and more than 20,000 umbilical cord blood transplants performed in children and in adults. UCB has many theoretical advantages due to the immaturity of newborn cells. UCB hematopoietic progenitors are enriched in primitive stem/***progenitor*** cells able to produce ***in vivo*** long-term repopulating stem cells. Hematopoietic stem cell transplantation (HSCT) can be curative in a large variety of selected malignant and non-malignant diseases. In comparison with other sources of allogeneic HSCT, UCB offers substantial logistic and clinical advantages such as (i) significantly faster availability of banked cryopreserved UCB units, with patients receiving UCB transplantation in a median of 25–36 days earlier than those receiving BM; (ii) extension of the donor pool due to tolerance of 1–2 HLA mismatches out of 6 (higher HLA mismatched is associated with lower probability of engraftment); (iii) lower incidence and severity of GVHD; (iv) lower risk of transmitting infections by latent viruses; (v) lack of donor attrition; (vi) lack of risk to the donor, and finally (vii) higher frequency of rare haplotypes compared to bone marrow registries, since it is easier to target ethnic minorities. Non-Hematopoietic stem cells have been isolated from cord blood and placenta. These cells can be grown and differentiate in various tissues including MSC, bone, cartilage, liver, pancreas, neurones, endothelial cells, muscle and keratinocytes. They have an advantage over other sources of stem cells, embryonic stem (ES) cells or induced pluripotent stem cells (iPS), because the supply is unlimited, they can be used in autologous or allogeneic situations, they need minimal manipulation, and they raise no ethical concerns.

Autologous Bone-Marrow Mononuclear Cell Implantation Increases Long- Term Major Amputation Free Survival in Patients with Critical Limb Ischemia.

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SUMMARY

Objectives: The purpose of this study was to evaluate long-term clinical outcome after autologous bone-marrow mononuclear cell (BM-MNC) implantation in patients with critical limb ischemia (CLI). Background: Surgical bypass and percutaneous transluminal angioplasty and combination with pharmacological therapy are options for revascularization and improvement in limb ischemic symptoms in patients with peripheral arterial disease (PAD). PAD patients with no other treatment option must undergo amputation. Cell therapy involving implantation of bone-marrow or peripheral mononuclear cells as well as endothelial progenitor cells is effective in patients with CLI who have no other treatment option[1-3]. We have also shown that autologous BM-MNC implantation improves not only limb ischemic symptoms and findings of angiography but also endothelial function in patients with CLI.[4] BM-MNC implantation increases collateral vessel formation and improves ischemic symptoms in patients with CLI[2-4]. Methods: We assessed the long-term clinical outcome after BM-MNC implantation in 51 patients with CLI, including 25 atherosclerotic PAD patients and 26 Buerger's disease patients. Forty-six CLI patients who had no BM-MNC implantation served as controls. Results: Median follow-up period for the whole study population was 4.8 years. The 4-year major amputation-free survival (MAFS) among the patients receiving BM-MNC-Implantation was significantly higher compared to the patients without BM-MNC implantation. In subset analysis, it had been found that patients with Buerger's disease receiving BM-MNC implantation had 95% MAFS after 4 years, whereas it was only 6% in control Buerger's patients (P<0.0001). Among the atherosclerotic PAD patients with BM-MNC implantation, MAFS after 4 years were 48% while no control patients (0%) with atherosclerotic PAD could have 4 year MAFS (P<0.0001).

Cumulative incidence of death among the patients with atherosclerotic PAD with BM-MNC-I or without BM-MNC-I had 24% and 33% respectively at 4 years, whereas no patient with Buergers disease (either receiving BM-MNC implantation or not) died during this follow-up period. Interestingly, among the patients with Buerger's disease, ankle brachial pressure index (ABPI) was significantly increased after 1 month and remained high during whole of the study period. Multivariate Cox proportional hazards analysis revealed that BM-MNC implantation was an independent predictor for prevention of major amputation (p<0.0001), while hemodialysis (p=0.024) and diabetes mellitus (p=0.043) were independent predictors of major amputation. Conclusions: These findings suggest that BM-MNC implantation is a safe and effective option for limb salvage for the patients with CLI, and it could be more effective among the patients with Buerger's disease compared to the patients with atherosclerotic PAD.
This study was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science and Culture of Japan (1859081500 and 21590898).

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From Bench to Bedside: "The Challenges and Results of Clinical Trials I & II for Testing Locally-fabricated Bioceramic in Orthopaedic Applications"

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SUMMARY

Hydroxyapatite granules (GranuMas™) manufactured by the Advance Materials Research Centre (AMREC), SIRIM Bhd. SIRIM Bhd has developed a novel method (Patent No. PI 2004 0748) for the fabrication of HA from local raw materials, namely high grade limestone with a 96% Ca(OH)2 purity. The Research Programme is being performed in collaboration with MINT (the lead institution), SIRIM Bhd, UKM, IIUM and USM. A Memorandum of Agreement has been signed by the heads of all 5 institutions in June of 2003 to further consolidate the collaboration and research efforts being undertaken. Objectives: A proposal was prepared for the purpose of applying for approval from the Institutional Research Board (IRB), Kulliyyah of Medicine, IIUM to perform a Phase 2 Clinical Trial as part of the IRPA Prioritised Research Project (No. 03-02-08-0001-PR0026/05-05) under the purview of the Department of Orthopaedics, Traumatology & Rehabilitation. General Objective of Research: To determine the suitability of using a locally produced calcium phosphate based synthetic bone graft substitute, GranuMaS™, for specific fractures in the distal end of the radius, proximal tibia and distal femur that requires bone graft surgery in suitable human patients. Specific Objective of Research: To evaluate the effectiveness and the osteointegration properties of GranuMaS™, a locally produced calcium phosphate based synthetic bone graft substitute, in the treatment of fractures requiring bone graft surgery at the distal end of the radius, proximal tibia and distal femur in suitable human patients by radiological means. PreClinical Evaluation: GranuMas™ have been fully characterised using X-ray diffraction (XRD – Bruker, Germany), Scanning electron microscopy (SEM - Leo, Germany), Energy dispersive x-ray (EDAX – Oxford Instruments, UK), Fourier transform infra-red spectroscopy (FTIR – Nicolet, USA) and Inductively Coupled Plasma (ICP – Perkin Elmer, USA). It was also compared against HA synthesised from 'Captal 60' (Plasma Biotal, UK), commercially pure Ca(OH)2 (Fluka, Switzerland) and also a mixture of pure chemical precursors (Ca(OH)2 (Fluka, Switzerland) + CaCO3 (MERCK, Germany) and has been found to be comparable to or better to them in practically all aspects. GranuMas™ has also been shown to comply with the ASTM F1185 - 88 (1993) specification standards, which covers material requirements for ceramic HA intended for surgical implants. Biocompatibility studies have been performed on the product by UKM and USM (Dental), which include Neutral Red Cytotoxicity Assay using cultured fibroblast (up to 102% viability) and CRL-1427 osteoblast cells (107% viability); MTT (Tetrazolium Salt) assay (up to 99.5% viability); cytotoxicity studies for apoptosis using Acridine Orange / Propidium Iodide (AO/PI) dual staining of V79 cells in DMEM with positive and negative test controls; and COMET assay genotoxicity studies using L-929 (normal mouse epithelial) cells with positive and negative controls (1.7% tail DNA – similar to negative controls). Animal studies that were performed on GranuMas™ are systemic (acute) toxicity tests (intraperitoneal injection of extract with negative control – negative results); dermal sensitisation assay (topical applicaton of extract 3 times per week for 3 weeks followed by challenge dose for 24 hours 12 days after last induction – negative results); and primary skin irritation assay (single application of extract on intact and abraded skin – 0.208/5.0, therefore not a primary irritant. Implantation studies have also been performed on New Zealand White rabbits (IIUM) and Merino sheep (USM Dental). The animals were observed clinically and the samples were harvested from 2 to 12 weeks post-implantation. These samples were processed to produce undecalcified hard tissue sections for analysis under the light microscopy using Haematoxylin & Eosin, Toluidine Blue, Masson Goldner's Trichrome or von Kossa stains. Phase 1 clinical trial on young healthy adults for the obliteration of their root socket following an uncomplicated tooth extraction was performed at USM (Dental). The results showed excellent outcome. The product has no deleterious effect on patients in the Phase 1 clinical trial study. Phase 1

clinical trials are done to see if an experimental medication or treatment is safe. After a treatment is tested in the lab or on animals, it enters a phase 1 clinical trial that is done with humans. A phase 1 clinical trial usually involves only a small number of people to determine if a drug or treatment is safe, and to determine the best dose of a drug. If a treatment appears safe at the end of a phase 1 clinical trial, it may then enter a phase 2 clinical trial, a study done to see if a treatment is safe and effective. Phase 2 clinical trial was done by IIUM team. Young healthy adults (age above 18) who have had recent trauma resulting in a closed fracture that requires bone grafting with no other complications were included in the study. Non union which is uninfected was included in the case study. Most of the patient was involved in road traffic accident. Inclusion and exclusion criteria were used to guide the selection. Clinical and radiological observations were made and recorded. Radiograph was taken at post-operatively, 6, 12 weeks, 6 months and yearly basis. The results of 35 patients that were treated using the GranuMas as bone substitute showed excellent and good results. No collapse of bony part noted during follow up. Union was noted in all of the cases. The HA granules showed osteoconduction functions as for the bone to heal. Callus formation noted in all cases. The Challenges: Every research will face its own challenge. We faced similar task during our clinical studies. The study was started from concept to clinics. Prototype preparation, evaluation of medical devices, biological evaluation, laboratory and local expertise, equipment availability, animal study and its protocol and availability of animals, Clinical trial have its own task to discuss and need to handle it properly. The availability of Good Clinical Practice protocol was very helpful.

The Journey of Stem Cells in Veterinary Practice

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SUMMARY

The purpose of this paper is to discuss the uses of stem cells in the veterinary profession. A stem cell is a remarkable cell, as it has the amazing ability to change into a variety of different cell types in the body such as heart muscle cells, brain cells, and skin cells. Stem cells, which are often referred to as one of the body's "master cells," can grow into any one of the body's more than 200 cell types. Stem cells assist the body in maintaining, renewing and repairing tissue and cells damaged by disease, injury and everyday life. There are two types of stem cells that are currently being used in the veterinary practice: i. embryonic stem cells and ii. adult stem cells such as adipose, bone marrow and some nerve cells. The most commonly used stem cells in the veterinary practice are the adipose stem cells. Adipose stem cells are being used for the treatment of conditions affecting joints and ligaments and spinal degeneration in horses, dogs and in cats. As for the embryonic stem cells, there are many potential uses for these cells in veterinary medicine. These include treatments for injuries especially in cats involved in road traffic accidents, repair of joints, specific heart murmurs, epilepsy in dogs and diabetes. However, one of the potential drawbacks with using the stem cells in animals is the cost and ethical issues. Overall, stem cells are miracles in themselves, with their potential to influence the attitude of the veterinary profession towards untreatable diseases and conditions not likely to wane in the future.

The Journey towards Clinical Trials in Cell and Tissue Therapy

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SUMMARY

There are many potential cell and tissue based products that have arose from the research and development of the Tissue Engineering Centre, UKM Medical Centre. Products that have completed preclinical trials are the bi-layered tissue engineered skin, human articular chondrocytes for the treatment of focal defect, engineered bone using bone marrow stem cells with ceramic scaffold, engineered respiratory epithelium made from the nasal turbinate, engineered cornea made from limbal and bone marrow stem cells and a few more. These products have to undergo proper clinical trials in order to be used in patients as a mode of therapy. Clinical trial is also the pathway to develop marketable products. We see the need for a certified facility that can help this academic research participate in translational research. cGMP-compliant labs are expensive to design, build and operate. It is critical to establish the user-requirement specification early in the process. Institutional support for the long-term maintenance and operation of this cGMP facility is also essential to success. UKM Medical Centre who is hosting the facility shares the same long-range vision and must make sure that resources will be there after GMP compliance is achieved to ensure that the laboratory's operation can be successfully continued. cGMP are a set of scientifically sound methods, practices and principles that are implemented and documented during product development and production to ensure consistent manufacture of safe, pure and potent products. The building of this cGMP facility for Cell and Tissue Therapy on the 12th Floor of Universiti Kebangsaan Malaysia Medical Centre would not have been possible without grants from Malaysian Technology Development Corporation Sdn Bhd (MTDC) and UKM; consultants from Cell Therapies Pty Ltd, Australia and Intran Technologies Sdn Bhd. Last but not least is the contractor company that took the challenge to help us build this lab, HMS Corporation Sdn Bhd.

Inducible Transgene Expression of DKK1 in Mouse Embryonic Stem (Es) Cells and Embryoid Bodies

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SUMMARY

In this study, we aimed to induce conditional overexpression of Dkk1 in undifferentiated ES cells as well as upon differentiation through the formation of embryo bodies (EBs). Combining two techniques, *Cre/loxP*-based genetic recombination and ligand-dependent activation of Cre, we have generated transgenic *CAG-floxed-neopA-Dkk1* embryonic stem (ES) cell line. The expression of Dkk1 transgene was observed in undifferentiated ES cells after treatment with tamoxifen and maintained upon differentiation. This new ES cell line would be useful for us to examine the effects of Dkk1 overexpression during different time points of neural differentiation process in the future.

INTRODUCTION

Embryonic stem (ES) cells have two unique characteristics; self-renewal and pluripotency[1]. They are able to long-term renewal without differentiation *in vitro,* and under appropriate conditions, they can also be directed to differentiate into cells of specific lineages, including neurons. Hence, they are useful as a candidate to study the mechanisms that regulate neural differentiation process of ES cells. Inhibition of Wnt activity at early stage of neural differentiation process has been shown to promote differentiation of ES cells into neurons[2]. Dkk1 protein, which belongs to Wnt antagonist family, acts as Wnt signalling inhibitor. Thus, overexpression of Dkk1 at different time points would enable us to monitor the effects of inhibiting Wnt signaling during the process. In this study, ES cell conditional expression system was used. *Cre/loxP*-based genetic recombination and ligand-dependent activation of Cre which allow for the temporal control of expression and activity of *Dkk1* were utilized[3]. A transgenic ES cell line carrying p*CAG-floxed-neopA-Dkk1* was generated by transfecting the plasmid into Cre-expressing ES cell line, R26CT2S. It is important to observe the expression of the transgene in ES cells as well as upon differentiation. Therefore, we aim to observe the ability of this transgenic ES cell line to tightly control the expression of *Dkk1* in undifferentiated ES cells as well as in differentiated derivatives through the formation of EBs *in vitro*.

MATERIALS AND METHODS

Transgenic *CAG-floxed-neopA-Dkk1* cell line was established by transfecting p*CAG-floxed-neopA-Dkk1* plasmid into R26CT2S cell line using GeneJuice (Novagen). Selection for positive clones was done by G418. Induction of the transgene expression in undifferentiated ES cells and EBs was carried out by exposing the cells to various concentrations of 4'-hydroxytamoxifen (4OHT) for 24 or 48 hours. Immuunocytochemistry (ICC) and quantitative RT-PCR were used to examine the protein and RNA expression of *Dkk1* in response to 4'OHT, respectively.

RESULTS

There were no significant differences between the expression levels of Dkk1 when differentiated control ES cell line (EBs) were treated with 400 nM 4'-OHT indicating that the endogenous Dkk1 is maintained upon treatment (Figure 1). Exposing the cells to 400 nM 4'-OHT for 48 hours was found to be the optimum conditions to induce the overexpression of Dkk1 in undifferentiated ES cells up to as high as 20-fold when compared to untreated cells (Figure 2). The optimum conditions to induce the expression of Dkk1 transgene in EBs were found to be at 800 nM 4'-OHT for 48 hours (Figure 3). The highest expression was observed to be as high as 20-fold compared to the expression in untreated cells. ICC results also demonstrate that the expression of Dkk1 transgene was induced upon treating the undifferentiated ES cells at 400 nM 4'-OHT either with or without the presence of LIF (Figure 4).

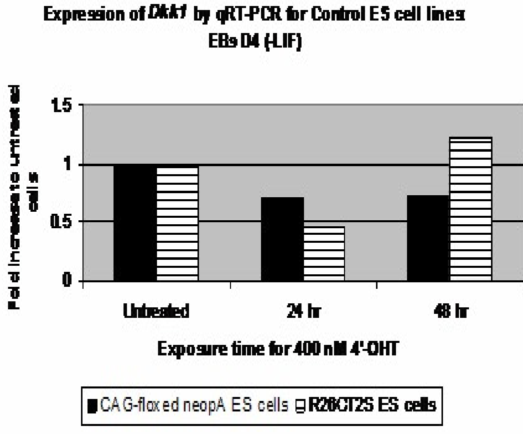

Fig. 1: Tamoxifen (4'-OHT) has no effect on the expression of endogenous Dkk1.

CAG-floxed-*neo*pA-*Dkk1* ES cells (+LIF)

Expression of Dkk1 by qRT-PCR

Fold induction to untreated cells

25
20
15
10
5
0

Untreated 24 hrs 48 hrs

400 nM 4'-OHT

Fig. 2: Exposure to 400 nM 4'-OHT for 48 hours was the optimum condition to induce Dkk1 transgene at high level

Inducible Dkk1 Expression in EBs (-LIF) by qRT-PCR

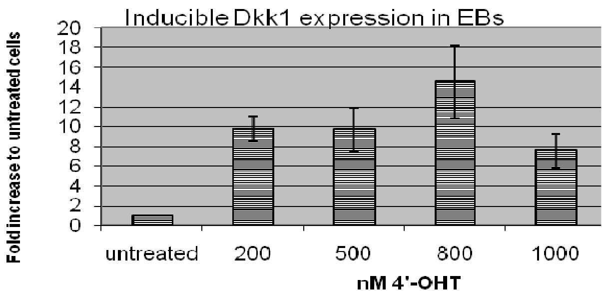

Fig. 3: 800 nM 4'-OHT was found to give the highest level of Dkk1 expression in EBs.

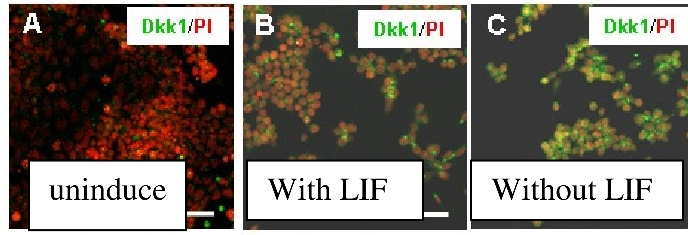

Fig. 3: ICC results showing the expression of Dkk1 transgene upon exposure to 400 µM of 4'-OHT either with or without the presence of LIF. Scale bar is 50µm

DISCUSSION

Our findings demonstrate the stability and functionality of the transgenic construct in inducing the transgene expression upon treatment with non-detrimental doses of tamoxifen. The system is certainly useful in overexpressing Dkk1 at specific time points. Thus, beneficial in monitoring the effects of inhibiting Wnt signaling at different stages during differentiation process of ES cells into neurons. The identification of exactly how Wnt genes work will be very important in regenerating and restoring neurons for neurodegenerative diseases such as Parkinson, Alzheimer's and spinal cord injury.

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Human Mesenchymal Stem Cells Maintain their Phenotypes and Differentiation Capacity to Adipogenic and Osteogenic Lineages after Manipulation with Nucleofection

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SUMMARY

Mesenchymal Stem Cells (MSCs) are an attractive cell target to become a vehicle for gene delivery. Hence, this study aimed to determine the capability of MSCs to maintain its renewability, phenotypes and differentiation capacity following transfection by nucleofection. Our results showed that transfected MSCs maintained its renewability and multiplied in culture. The single cell could form a clone and was found to express CD90 and CD105 strongly. The cells were also able to differentiate into adipogenic and osteogenic lineages. Thus, nucleofection is an efficient non-viral technique for transferring gene into human MSCs.

INTRODUCTION

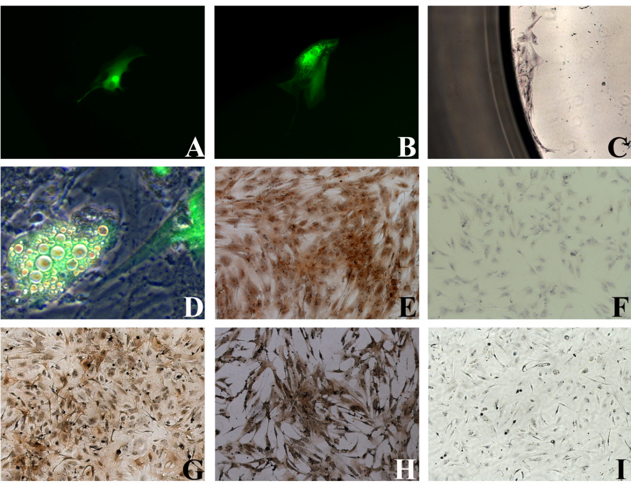
Mesenchymal Stem Cells (MSCs) are attractive cell targets because it can be a good vehicle for gene delivery [1-4]. Properties contributing to their attractiveness include (a) ease of isolation, (b) robust growth potential, (c) amenability to gene transfer, (d) low immunogenicity, and (e) capability to migrate to injury site [5-8]. We have previously isolated MSCs from adult human bone marrow [9-10]. Thus, the objective of this study was to determine the capability of MSCs to maintain its renewability, phenotypes and differentiation capacity following transfection by nucleofection.

MATERIALS AND METHODS

Human bone marrow MSCs were transfected with plasmid encoding green fluorescent protein (plasmid pmaxGFP) by nucleofection using pulsing programme U23. Single transfected cell was then isolated into wells of 96 well-microplate by manual cell dilution. The single positive cell that expressed GFP was cloned and tested for their surface markers (CD90 and CD105) by immunocytochemical staining. The propagated cells were also tested for their capability to differentiate into the adipogenic and osteogenic lineages by incubating them in adipogenic and osteogenic induction medium. The induced cells were observed under phase contrast microscope and the presence of calcium deposition in osteogenic lineages was assessed by Alizarin Red S staining.

RESULTS

Nucleofection with pulsing programme U23 yielded transfection efficiency with a score of 26.1 ± 5.6 %. The single nucleofected cell was able to be isolated and expanded. Thus, MSC after transfection maintained its renewability and multiplied in culture. The single cell could form a clone and was found to express CD90 and CD105 strongly. The cells were also able to differentiate into the adipogenic and osteogenic lineages.



MSC nucleofected with plasmid encoding GFP using programme U23 were isolated by single cell dilution into a 96 well microplate. The single cell adhered and showed green fluorescence 24 hours post-nucleofection (Fig. A). After five days, the cell started to duplicate and the daughter cell had also expressed the green fluorescence (Fig. B). A week later, the cells have formed a small colony (Fig. C). The cells were then culture-expanded and demonstrated positive staining to CD90 and CD105 markers. After two weeks of incubation in adipogenic induction medium, the cells showed lipid accumulation (Fig. D). Under the microscopic view,

the adipogenic cells exhibited both green fluorescence and lipid vacuoles (Fig. D). The cells were also stained positively with Alizarin Red S after two to three weeks incubation in osteogenic induction medium (Fig. E). The control for non-induced cells showed negative staining in Alizarin Red S (Fig. F). The expanded cells also showed positive result to immunocytochemical staining for CD 105 and CD90 (Fig. G & H). A negative control for immunocytochemical staining was shown in Fig. I.

DISCUSSION AND CONCLUSION

Nucleofection is an efficient non-viral technique for transferring gene into human Mesenchymal Stem Cells. Following nucleofection with plasmid encoding reporter gene for green fluorescent protein, the cells could still maintain their proliferative activity, exhibited the surface markers of CD90 and CD105; and was capable of differentiating into adipogenic and osteogenic lineages.

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Differentiation Potential of C-Kit Positive Cells Derived from Full Term Rat Amniotic Fluid to Neural Lineage

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SUMMARY

Rat full-term amniotic fluid is believed to contain stem cells of high potency. We have managed to isolate a population of cells that express the marker for stem cell factor receptor, c-kit and the pluripotent marker, Oct4, indicating the ability of these cells to differentiate into derivatives of the three primary germ layers including neural lineage. Cells with neuronal-like morphology have been observed upon spontaneous differentiation through the formation of multicellular aggregates, embryoid bodies (EBs). The ability for these cells to undergone directed differentiation into specific lineage namely neural lineage was also observed.

INTRODUCTION

Mid-term amniotic fluid-derived stem (AFS) cells have been shown to express Oct4 indicating the ability of the cells to differentiate into derivatives representing the three primary germ layers[1]. However, the generation of mid-term AFS cells involves an invasive technique (amniocentesis). We have managed to isolate c-kit positive cells from full-term amniotic fluid that are expressing Oct4 and TERT genes, indicating the existence of pluripotent stem cells from rat full-term amniotic fluid. In this study we aim to observe the differentiation potential of these c-kit positive cells into neural lineage. Monolayer differentiation [2] was carried out to direct the differentiation of these cells into neurons.

MATERIALS AND METHODS

Immunoselection of c-Kit positive cells were performed using miniMACS kit (Miltenyi Biotec) according to the manufacturer's suggestion. The c-Kit positive cells were then cultured in ES medium in a gelatin-coated flask and subcultured by mild trypsinization. $6x10^4$ cells were seeded into gelatin-coated 4 wells plates (Nunc). Cells were incubated with DMEM/F12/N2 medium (Gibco) overnight. Then, neurobasal medium (Gibco) supplemented with B27 were added into each sample. After 2 hours of incubation at 37□C and 5% carbon dioxide, the cells were harvested for nestin and Oct4 immunofluorescence staining. For β-tubulin immunofluorescence staining, the cells were harvested only after 24 hours of incubation. The cells were fixed with 4% paraformaldehdye (PFA) in PBS for 30 minutes. Then, the cells were permeabilised with 1% triton-X 100 in PBS for 15 minutes prior to blocking the cells using blocking solution (freshly prepared with 0.3% BSA, 1% goat serum, 0.1% Tween-20 in PBS) for 30 minutes. The cells were then incubated in primary antibody for Oct4 (Chemicon), nestin and β-tubulin (Sigma) at 4□C overnight. The cells were rinsed with PBS thrice before they were incubated in the secondary antibody (Alexa Flour) for 2 hours in dark. Nuclear counterstain was performed using Propidium Iodide.

RESULTS

Undifferentiated c-kit positive cells were observed to express Oct4 (Figure 1). The expression was not observed upon monolayer differentiation after 2 hours in N2/B27 medium (Figure 2) Expression of nestin (Figure 3) and β-tubulin (Figure 4) was observed after 2 hours and 24 hours incubation in N2/B27 medium, respectively. These markers were not expressed in undifferentiated cells (data not shown).

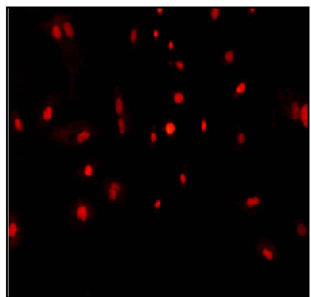
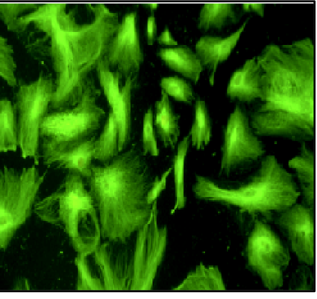
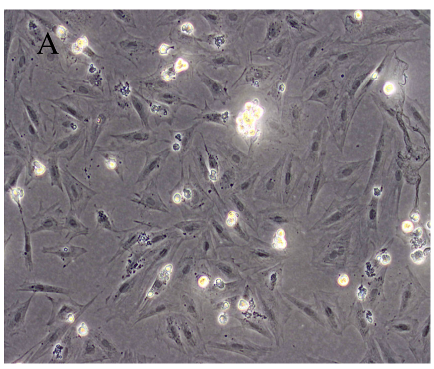
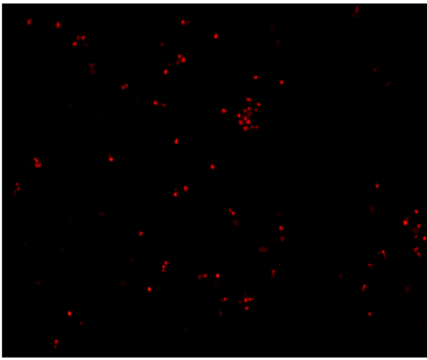



Fig 1: ICC results show the expression of Oct 4 in undifferentiated rat c-Kit positive cells. 20X magnification.

Fig. 2: ICC results show no expression of Oct 4 after 2 hours in N2/B27 medium. 10X magnification.





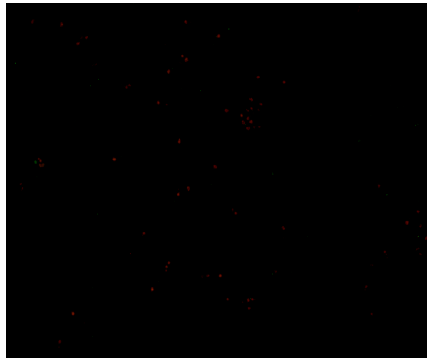
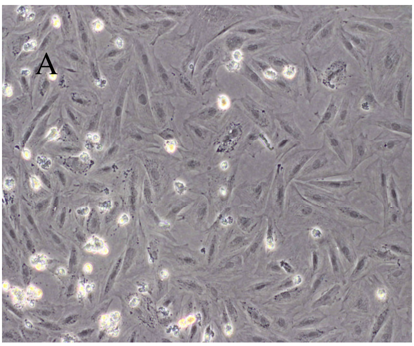
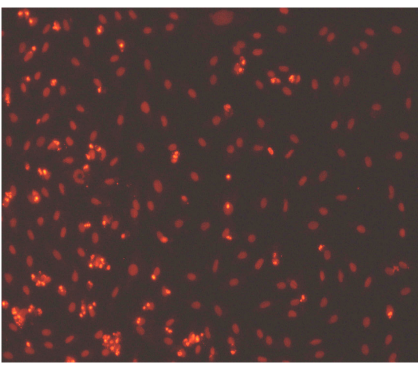


Fig. 3: ICC results clearly show the expression of nestin after 2 hours in N2/B27 medium, indicating that the cells have been differentiated into neural lineage (nestin is a marker for neural precursor cells).





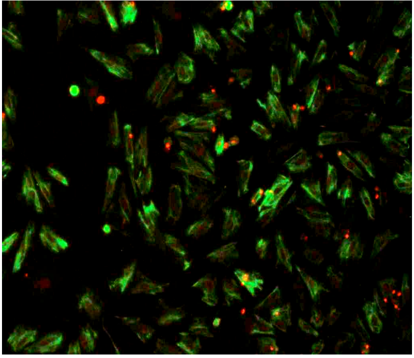
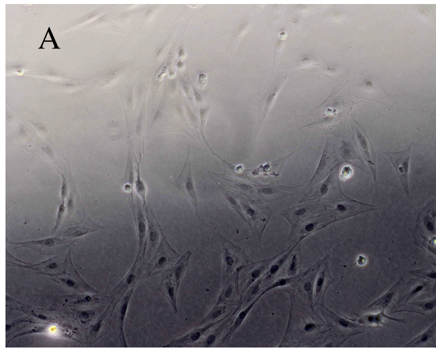
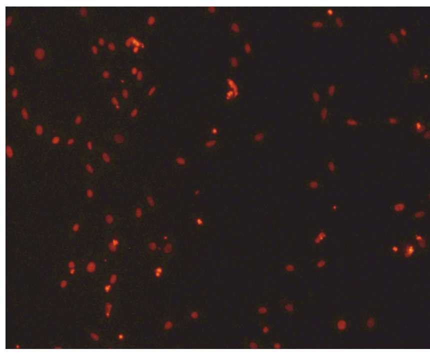
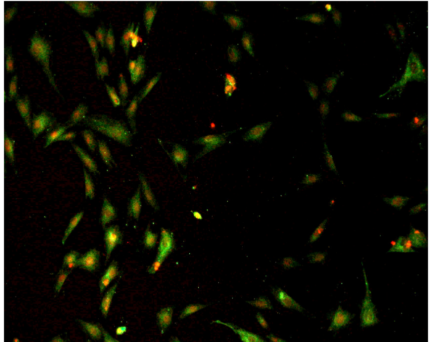


Fig. 4: ICC results demonstrate the expression of β-tubulin after 24 hours in N2/B27 medium indicating the presence of neurons (β-tubulin is a marker for post-mitotic neurons).







DISCUSSION

The expression of Oct-4 in undifferentiated c-kit positive cells highly suggests that the cells may contain stem cells of high potency, pluripotent. The expression of both nestin and β-tubulin after monolayer differentiation is very much suggesting the success in directing these c-kit positive cells to differentiate into neural lineage derivatives. Thus, our findings may demonstrate the presence of stem cell population in full-term amniotic fluid which could be directed to differentiate into specific lineage.

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Suppression of Interleukin-6 in Human Bone Marrow-derived Mesenchymal Stem Cells Post siRNA Transfection

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SUMMARY
Studies have shown that Interleukin-6 (IL-6) produced by mesenchymal stem cells (MSC) promotes growth of multiple myeloma (MM) cells. We report suppression of IL-6 expression in human bone marrow-derived mesenchymal stem cells (BM-MSC) post siRNA transfection. Conditioned medium from transfected BM-MSC with suppressed IL-6 inhibited the growth of U266 MM cells *in vitro* when compared to conditioned medium from control BM-MSC. The results from this study suggest that IL-6 suppression could be potentially useful as a novel therapeutic approach to treat multiple myeloma.

INTRODUCTION
IL-6 is a major growth factor for MM cells. Studies demonstrated that MSC from bone marrow stroma produced high concentration of IL-6 that promoted MM expansion in paracrine manner[1-3]. In this study, we report suppression of IL-6 expression in BM-MSC post siRNA transfection. Additionally, we also studied the growth of U266 multiple myeloma cells when cultured with conditioned medium from transfected BM-MSC with suppressed IL-6.

MATERIALS AND METHODS
BM-MSC (1×10^5) were transfected with 100 pmol IL-6 siRNA (SASI_Hs01_00155911) using 5 µl Lipofectamine 2000 (Invitrogen) with untransfected BM-MSC as control. Total RNA was extracted post-transfection using RNeasy plus micro kit (Qiagen) and reverse-transcribed to generate cDNA. Real-Time PCR was carried out in duplicates using IL-6 and β-actin Taqman probes. IL-6 mRNA expression was determined using the comparative C_T method with β-actin as endogenous control. Supernatant from BM-MSC was also collected and IL-6 protein level was determined using ELISA (Thermo Scientific). U266 cells (1×10^4) were cultured with conditioned medium from control and transfected BM-MSC. A luminescent-based viability assay was carried out 72h after exposure to assess the effect of BM-MSC conditioned medium on U266 growth.

RESULTS
Real-Time PCR data analysis showed a significant 5.9 fold suppression of IL-6 mRNA 72h post transfection when compared to control BM-MSC. IL-6 mRNA continued to be suppressed at 6.3 fold and 2.1 fold at 96h and 120h post transfection [Figure 1]. These results corresponded with the suppression of IL-6 protein in the supernatant of transfected BM-MSC [Figure 2]. At 72h post-transfection, IL-6 protein was suppressed to 66.4 pg/ml when compared to 190.2 pg/ml in control BM-MSC ($P<0.05$). IL-6 proteins at 96h and 120h post transfection were 80.3 pg/ml (vs. 170.5 pg/ml) and 50.8 pg/ml (vs. 185.3 pg/ml) respectively ($P<0.05$). The growth of U266 in control and transfected BM-MSC conditioned medium relative to control U266 (100%) were 269% and 154% respectively. U266 growth was inhibited when cultured in conditioned medium of transfected BM-MSC with suppressed IL-6 expression when compared to conditioned medium from control BM-MSC [Figure 3].

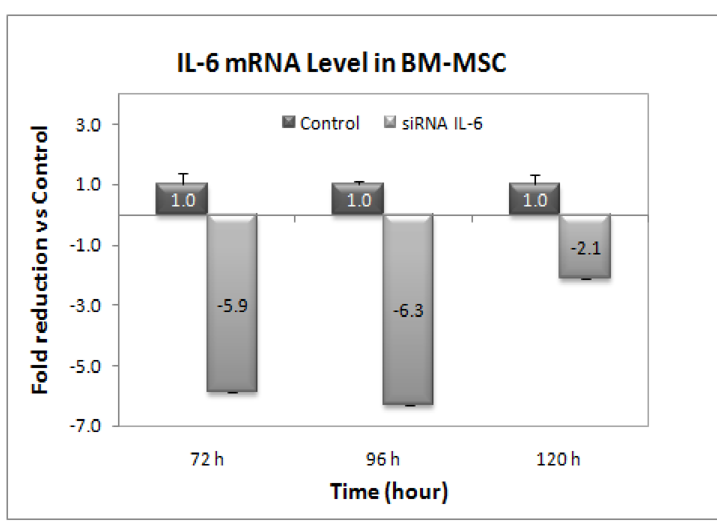

Figure 1

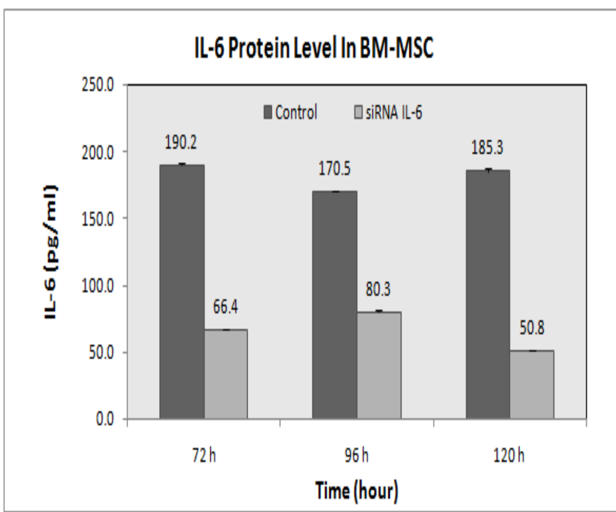

Figure 2

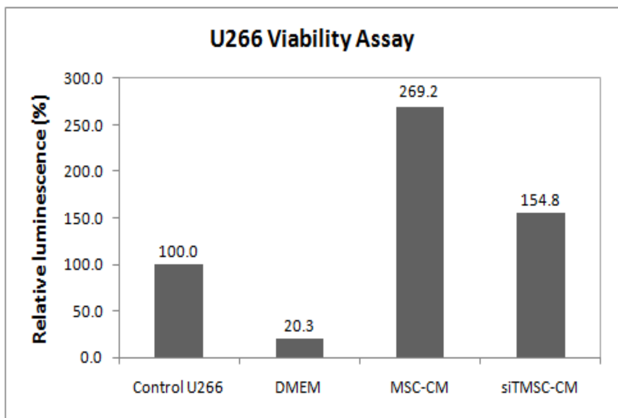

Figure 3

DISCUSSION AND CONCLUSION

IL-6 siRNA transfection suppressed IL-6 expression in BM-MSC 72h post transfection. Additionally, preliminary *in vitro* culture showed growth inhibition of multiple myeloma cells when cultured with the supernatant from transfected MSC. These results suggest that IL-6 suppression could be potentially useful as a novel therapeutic approach to treat multiple myeloma.

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Prospective Pluripotent Stem Cells Derived from Human Full-term Amniotic fluid during Deliveries

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SUMMARY

Isolation and characterisation of amniotic fluid-derived stem cells were conducted. Cells of amniotic fluid from full-term pregnancies were cultured, and the cells expressing the marker for stem cell factor receptor, c-kit, were isolated using magnetic-activated cell sorting (miniMACS) technique. RNA and protein analyses using RT-PCR, fluorescent-activated cell sorting (FACS) and immunocytochemistry (ICC) demonstrated an enriched population of c-kit positive cells upon isolation, and importantly revealed the expression of a marker for pluripotency, Oct4.Formation of embryoid bodies was also observed by spontaneous differentiation. These results indicate the pluripotent character of amniotic fluid-derived stem cells.

INTRODUCTION

The field of therapeutic and regenerative medicine are now focusing on new sources for pluripotent stem cells. The wide spectrum of differentiation capacity of these cells has made them a promising powerful tool in curing diseases. Amniotic fluid (AF) of mid-term pregnancies have been discovered to home a subpopulation of high potency cells termed amniotic-fluid derived stem (AFS) cells. However, several complications have been associated with the procedure (amniocentesis) in obtaining AF at this stage. The aim of this study was to isolate pluripotent stem cells from human full-term amniotic fluid, specifically during delivery.

MATERIALS AND METHODS

Amniotic fluid from full-term pregnancies obtained through artificial rupture membrane (ARM) or Caesarean section deliveries were used as the source to derive the cells upon approval from the ethical committee of Faculty of Medicine and Health Sciences, UPM. Human amniotic fluid sample cells cultured in Amniomax-C100 media (Gibco) were isolated by miniMACS (Miltenyi biotech) for c-Kit positive cells. The direct staining method was used as suggested by the manufacturer. The c-Kit positive and negative cells were cultured in Chang medium. Pluripotency is characterized by looking at the expression of Oct4 in c-kit positive cells by RT-PCR, FACS and ICC, as well as the ability of these cells to differentiate by undergoing spontaneous differentiation through the formation of three dimensional multicellular aggregates, embryoid bodies (EBs). PCR conditions were optimized according to the amplified Oct-4 gene (Bossolasco *et al.,* 2006). FACSCalibur flow cytometer and CellQuest software were used for acquisition and for analysis, respectively. Hanging drop method (Valli *et al.,* 2009) was used for the spontaneous differentiation and EBs formation was viewed under phase-contrast inverted microscope.

RESULTS

Human amniotic fluid cells were successfully cultured. Heterogenous population with morphology of epitheloid, fibroblastic and amniotic fluid-like cells was observed after 25-30 days in culture. Isolation using MiniMACS was also successfully conducted (Figure 1) with a clear enrichment of c-kit positive cells as shown by FACS and ICC (Figure 2). The presence of a large number of c-Kit positive cells expressing Oct-4 was also revealed by RT-PCR, FACS and ICC (Figure 3). Spontaneous differentiation of the c-Kit positive cells clearly displayed the formation of EBs (Figure 4).

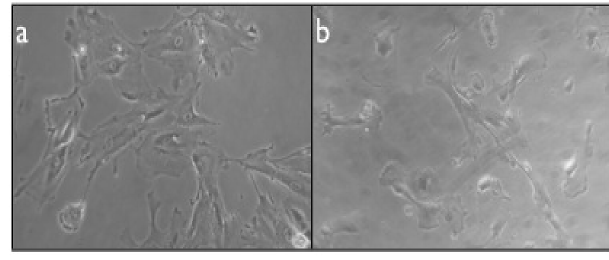

Fig. 1: The morphology of the cells after miniMACS showing the c-kit positive cells (a) and c-kit negative cells (b). The magnification is 10x

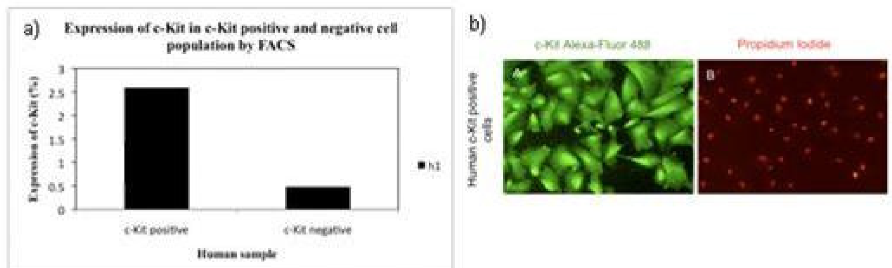

Fig. 2: Enrichment of c-kit positive cells after isolation by miniMACS shown by FACS (a) and ICC (b) analyses. (a) Comparison of c-Kit expression in human c-Kit positive and negative cells population. (b) Immunofluorescence staining shows the expression of stem cell factor, c-Kit, on human c-Kit positive cells (A). Nuclei were counterstained with PI (B).

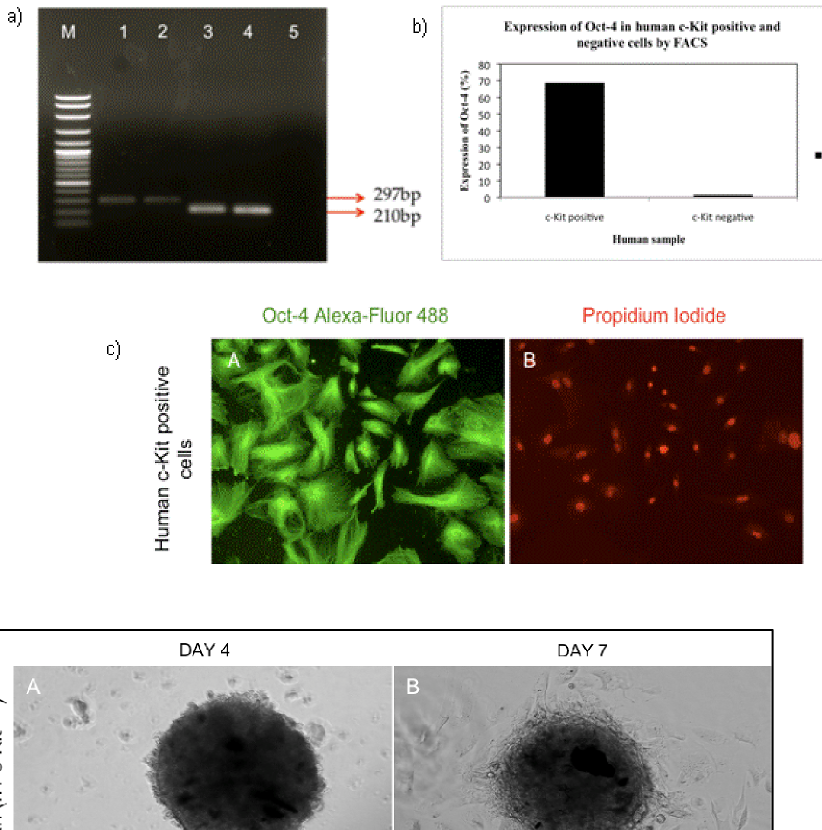

Fig. 3: Expression of pluripotent marker, Oct4 in c-kit positive cells revealed by RT-PCR (a), FACS (b) and ICC (c). (a) Lane 1 shows the expected band size of *Oct-4* expression for positive control; MSC (297bp). Lane 2 indicates the *Oct-4* expression of human c-Kit positive cells (297 bp). Lane 3 and 4 indicate the internal control GAPDH of MSC and human c-Kit positive cells, respectively (210 bp). Lane 5 is the negative control. M indicates the 100bp marker. (b) Comparison of Oct-4 expression between human c-Kit positive and negative cells. (c) Green immunofluorescence staining shows expression of pluripotency marker, Oct-4 on human c-Kit positive cells (A) with the nuclei stained with PI (B).

Fig. 4: Formation of embryoid bodies (EB) from human c-Kit positive cells. An EB of human c-Kit positive cells on day 4 shows a solid round shape (A). On day 7, the cells in the EB start to grow and expand (B). The magnification is 10x. Scalebar: 100µm.

DISCUSSION

The expression of Oct4 as well as the formation of EBs may indicate that the c-kit positive cells may contain stem cells of high potency, pluripotent. Overall, our preliminary findings demonstrate that full-term AF may serve as an excellent source for AFS, where they are usually discarded after delivery, thus making them more accessible than mid-term AF. Therefore this represent a potential future alternative source for stem cell therapy.

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Effects of High Gentamicin Concentration on Viability and *In Vitro* Proliferation of Bone Marrow-derived Human Mesenchymal Stem Cells

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SUMMARY

This study investigated the effects of gentamicin at different concentrations on bone marrow-derived mesenchymal stem cells (MSC) *in vitro*. MSC were cultured in different concentrations of gentamicin before the cells were examined for cell viability and for cell proliferation. Gentamicin did not show any adverse effect on the viability of MSC at the concentrations tested. However, gentamicin at concentration of 100 µg/ml and greater has a substantial inhibitory effect on MSC.

INTRODUCTION

MSC is known to contribute to tissue regeneration of bone, cartilage, tendon, and fat.[1] These cells display a stable phenotype when isolated and can be expanded in culture.[2] Antibiotics are commonly used in cell or tissue culture to prevent bacterial and fungal contamination. However, high concentration of antibiotics might cause a toxic or adverse effect to cell *in vivo*.[3] This, in turn, might affect cell viability, cell proliferation, cell metabolism, and cell functionality.[4-5] The aim of this study was to investigate the effects of gentamicin at different concentrations on MSC *in vitro*.

MATERIALS AND METHODS

MSC were isolated from bone marrow of healthy adult donors after informed consent and were cultured in complete culture medium (Dulbecco's Modified Eagle's medium containing low glucose supplemented with 10% fetal bovine serum and 2 mM L-glutamine) with different concentrations of gentamicin (0 µg/mL, 50 µg/mL, 100 µg/mL, and 200 µg/mL) for 7 days. The cultures were maintained at 37 °C in 5% CO_2 in air. Fresh medium with different gentamicin concentrations were replaced respectively at day 4. After 7 days of incubation with gentamicin, cell viability and cell proliferation were determined with trypan blue exclusion staining and MTT assay respectively. MSC cultured in medium without gentamicin (0 µg/ml) served as a control.

RESULTS

Gentamicin did not showed adverse effect on the viability of MSC at the concentrations tested (Figure 1). However, high concentrations of gentamicin inhibited cell proliferation. The percentage of proliferation (percentage of absorbance relative to 0 µg/ml group) decreased significantly to 78% ± 9% and 60% ± 10% for cells cultured with 100 µg/mL and 200 µg/mL gentamicin respectively, when compared with control group in which MSC were cultured in medium without gentamicin (Figure 2).

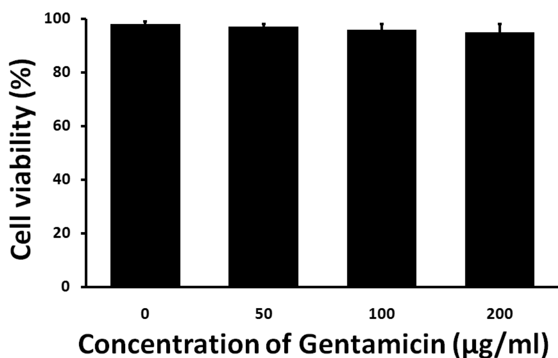

Fig 1. Cell viability of MSC was measured with trypan blue exclusion staining after a 7 days incubated with gentamicin at different concentrations. MSC cultured without gentamicin was the control group.

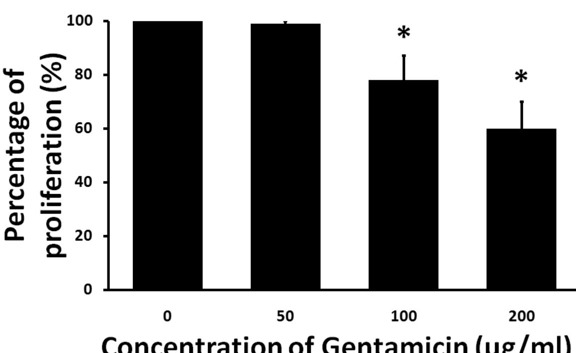

Fig 2. Percentages of cell proliferation MSC (percentage of absorbance relative to 0 µg/ml group) were measured after 7 days incubation with gentamicin at different concentrations. MSC cultured without gentamicin was the control group. Data are expressed as mean ± standard error of mean (SEM) for cells from three donors treated in triplicate for each dose of gentamicin. * Significance compared with 0 µg/ml group. ($p < 0.05$)

DISCUSSION

It is important to use only optimal concentrations of antibiotics in cell culture to avoid contributing any adverse effect to the cells. This study demonstrated that gentamicin at 100 µg/ml concentration and greater has a substantial inhibitory effect on MSC *in vitro*.

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Generation and Characterisation of Mesenchymal Stem Cells Derived from Human Cardiac Tissues

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SUMMARY

In last one decade, overwhelming research work has promised stem cells as an ideal tool for tissue regenerative therapy. In line with this mesenchymal stem cells (MSC) have been actively investigated on their role in repopulating parenchymal and mesenchymal tissues in organ specific diseases. In this study, MSC from the human cardiac tissue were generated and characterised based on immunophenotyping; their gene expression by RT-PCR and multipotential ability to differentiate into adipocytes and osteoblasts.

INTRODUCTION

Myocardial infarction is often become a fatal disease due to inability of local cardiac stem cells at side of injury to regenerate mature functioning mycoardiocytes[1]. This underlying pathology could be reflection of persistent inflammation, insufficient stem cell pool or the demand of injury that supersede the supply of stem cell physiology[2]. Therefore an ex-vivo expansion of cardiomyocytes derived mesenchymal stem cells serve an ideal reagent to treat heart diseases[3]. To realise this notion, myocardial biopsy of stage III heart failure patients were collected and their ability of stem cell generation were assessed.

MATERIALS AND METHODS

MSC were generated from adult human cardiac tissue by enzymatic digestion and cultured using commercially available media together with growth factors. After 10 days, adherent cells were detached, passaged and characterized by immunophenotyping using a panel of MSC cell surface markers, gene expression and their differentiation potential into adipocytes and osteocytes.

RESULTS

MSC generated from human cardiac tissue attained confluency by day 10 (Fig 1A) and immunophenotype profile of MSC at passage 1 (Fig 1B) indicated positive mesenchymal stem cell surface markers. MSC expressed Nanog, Sox2, Rex-1 and OCT4 (Fig 2A), and also NKX2.5 and GATA4 (Fig 2B). Adipogenic and osteogenic induction of MSC cultures resulted in the formation of adipocytes and osteocytes when stained red in Oil-Red-O and orangy red in Alizarin Red respectively (Fig 3).

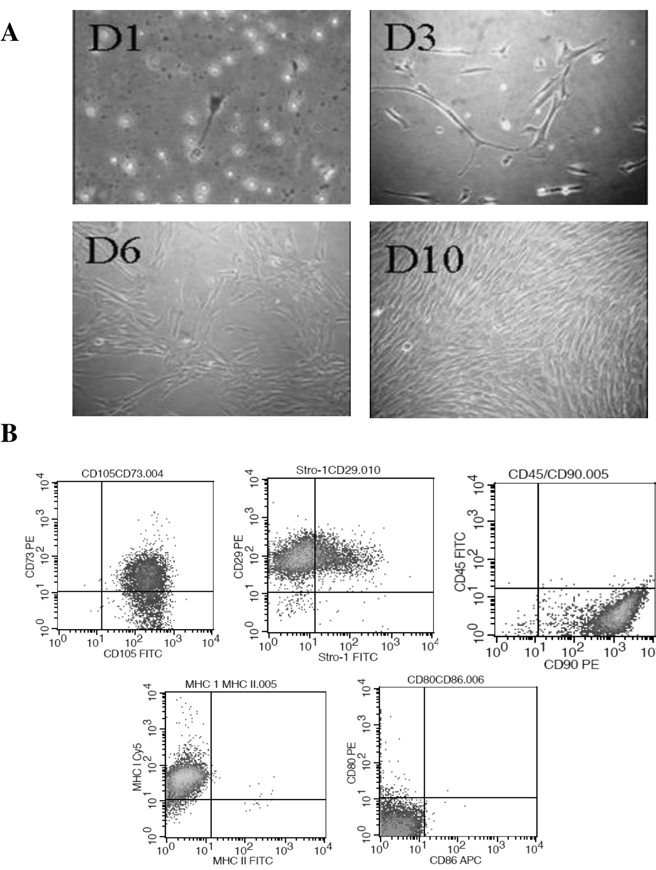
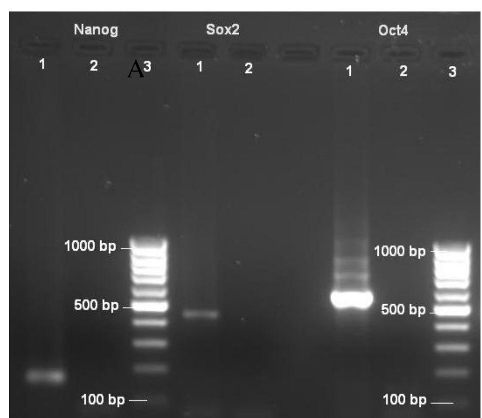
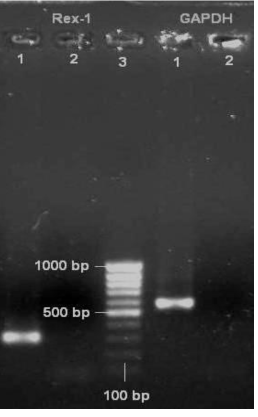


Fig 1: Characterisation of MSC. (A) Phase contrast microscope images of primary culture MSC (100x). (B) Immunophenotyping of MSC for cell surface markers.





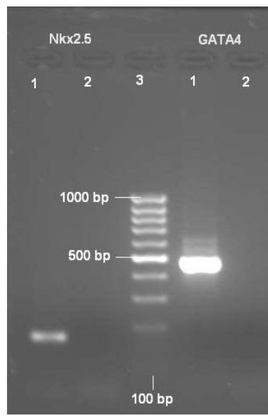
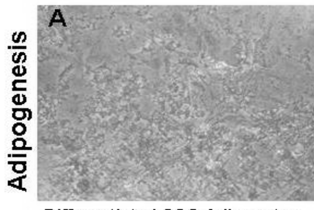
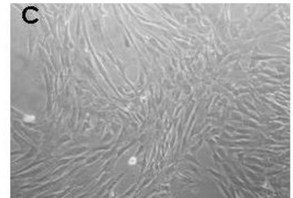


Fig 2: Gene expression by RT-PCR of MSC. Lane 1 MSC samples; lane 2, non DNA template control as negative control; lane 3, 100 bp DNA ladder .





Osteogenesis

B

Differentiated CSC-Osteocytes

Fig 3: Differentiation potential of MSC. MSC after 3 weeks in adipogenic (A) or osteogenic (B) or normal cell culture medium (C,D).

DISCUSSION

Our results show that homogenous population of adherent fibroblastic cell with similar characteristics with MSC can be generated from human myocardiac tissue. Immunophenotyping, gene expression and differentiation assays further confirm the notion that, the culture expanded cells were MSC. Despite their multipotential differentiation ability as indicated by expression of Oct4, Sox2 and Nanog, cardiac derived MSC also express cardiac-specific transcriptional factors NKX2.5 and GATA4. Our data suggest that MSC derived from human cardiac tissues possess a multipotential ability as other stem cells, yet retain their resident tissue differentiation capacity. This finding further explores the potential use of MSC derived from post-operative cardiac tissue for myocardiac regeneration of infracted patients. In conclusion, myocardium could potentially serve as cardiac stem cell source and can be exploited toward treating myocardiac infarct patients.

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Expression of OCT4 in c-kit Positive Cells Derived from Rat Full Term Amniotic Fluid

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SUMMARY
Rat full-term amniotic fluid is believed to contain stem cells of high potency. We have managed to isolate a subpopulation of cells that express the marker for stem cell factor receptor, c-kit. These c-kit positive cells have been found to express the pluripotent marker, *Oct4,* and *TERT*, which codes for the telomerase reverse transcriptase. The expression of these markers may indicate the presence of pluripotent stem cells in rat full term amniotic fluid.

INTRODUCTION
Mid-term amniotic fluid-derived stem (AFS) cells have been shown to have the ability to differentiate into derivatives representing the three primary germ layers, where the growth rate was found to be as fast as embryonic stem (ES) cells, thus pluripotent[3]. Pluripotency is defined based on the expression of the pluripotent marker, *Oct4,* indicating the ability of these cells to differentiate into derivatives of the three primary germ layers[1]. Expression of *TERT,* which codes for the telomerase reverse transcriptase, is also normally used as another defining feature of pluripotent stem cells which is required for the self-renewal capability of the stem cells[2]. The generation of mid-term AFS cells, however, involves an invasive technique (amniocentesis). Using animal model, we are interested to isolate high potency stem cells from full term amniotic fluid. Subpopulation of cells expressing the marker for stem cell factor receptor, c-kit, was isolated using magnetic-activated cell sorting (MACS) technique. In this study, we aimed to further characterize these c-kit positive cells for the expression of *Oct4* and *TERT* genes.

MATERIAL AND METHODS
Four rat full-term amniotic fluid (AF) samples were obtained from time-mated Sprague Dawley rats, sacrificed by cervical dislocation and excess CO_2. The amnion sacs were removed from the uterus by cesarean section and washed with PBS before placed in a petri dish. Animal sampling procedures were approved and performed according to the guidelines established by Institutional Animal Care and Use Committee (IACUC) of UPM. AF were then drawn and pooled from each sac under sterile condition and the cells were cultured in Amniomax medium and

incubated at 37°C in 5%CO_2. Immunoselection of c-Kit positive cells were performed using miniMACS kit (Miltenyi Biotec) according to the manufacturer's suggestion. The c-Kit positive cells were then cultured in ES medium in a gelatin-coated flask and subcultured by mild trypsinization. The same medium was used for c-Kit negative cells. Fluorescent-activated cell sorting (FACS) was performed to characterize the cell surface marker (c-Kit) and intracellular marker (Oct-4) following established protocol. Briefly, the c-Kit positive cells were grown to 80% confluence and detached from the flasks by mild trypsinization prior to incubation with primary antibodies. Secondary antibodies were rabbit IgG-FITC (Abcam) or mouse IgG AlexaFluor-488 (Molecular Probes) diluted in 5ug/ml. Acquisition was performed using FACSCalibur flow cytometer and analysed using CellQuest software. RNA extraction was performed using RNeasy mini plus kit (Qiagen). cDNA was then synthesized according to the manual of Reverse Transcription system (Promega). Then, RT-PCR was carried out by amplifying the cDNA following the conditions described in Bossolasco *et al.,* 2006 for *Oct4*, and Bossolasco *et al.,* 2006 for *TERT*. PCR products were analyzed on 1% agarose gel electrophoresis using gel documentation imaging system (Alpha Innotec)

RESULTS
Heterogenous population of cells was observed five days after the AF cells were cultured. Immunoselection of c-Kit using miniMACS (Miltenyi Biotec) has successfully managed to isolate the c-Kit positive cells from the heterogenous culture based on the cell morphology (Figure 1) and FACS analysis (Figure 2). High expression of Oct4 was also observed in every sample as analysed by FACS (Figure 3). The expression was also detected by RT-PCR (Figure 4). Expression of TERT gene was also detected by RT-PCR (Figure 5).

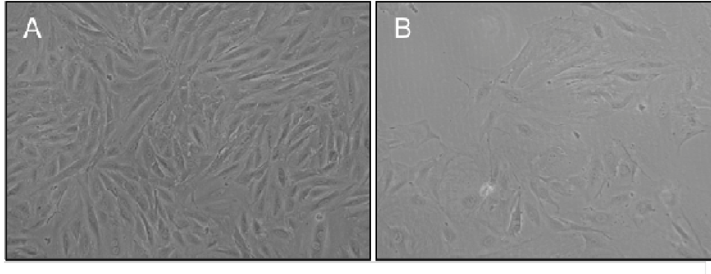

Fig. 1: Morphology of c-Kit positive cells (A) and c-Kit negative cells (B). Magnification is 10x

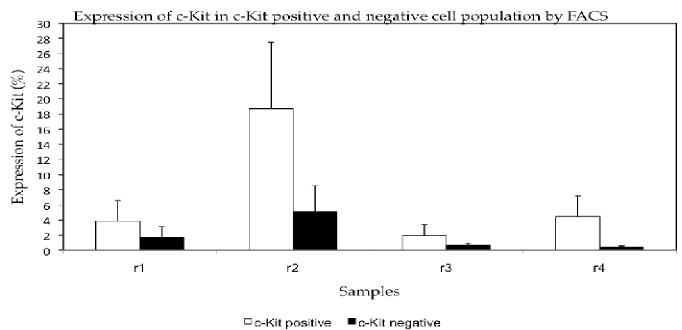

Fig. 2: FACS analysis clearly demonstrates higher expression of c-kit in c-Kit positive cells. Error bar is the mean ± S.E.M from three independent experiments, n=3.

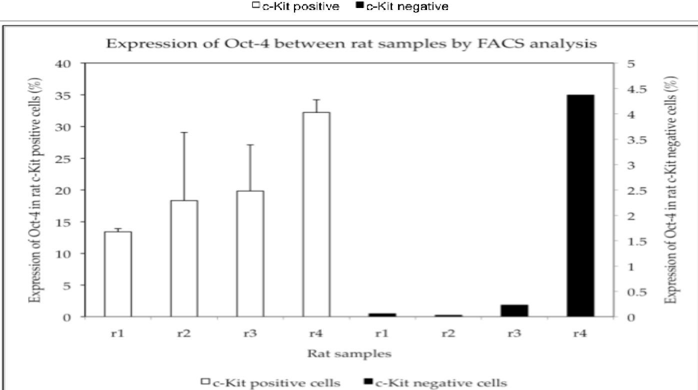

Fig. 3: FACS analysis showing higher expression of Oct 4 in the c-kit positive cells than in the negative cells. Error bar is the mean ± S.E.M from three independent experiments, n=3.

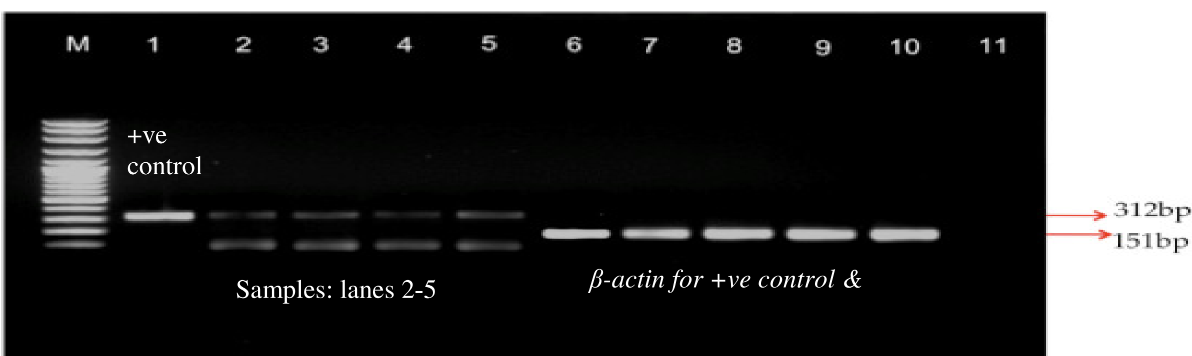

Fig. 4: Expression of pluripotency marker, *Oct- 4,* (lanes 2-5, 312 bp) was detected in all samples by RT-PCR. Lane 11 is the negative control. M is the 100bp marker.

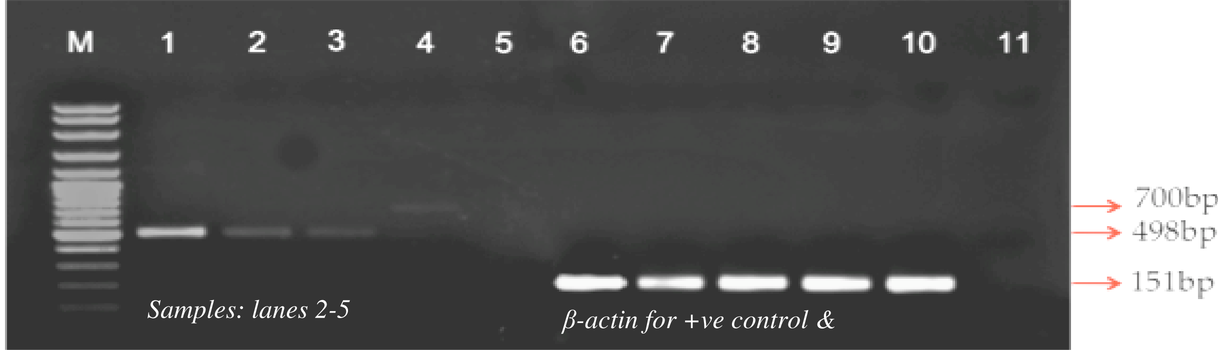

Fig. 5: Expression of *TERT* gene was faintly detected in rat c-Kit positive cells by RT- PCR (lane 2-4). Lane 11 is the negative control. M shows the 100bp marker.

DISCUSSION

It is clearly shown that the isolated c-kit positive cells express the pluripotent marker, Oct4 and TERT genes. These results suggest that the full-term amniotic fluid of mammals may contain the stem cells of high potency, including human. Hence, full term amniotic fluid may serve as a potential source for pluripotent stem cells.

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