UNIVERSITI SAINS MALAYSIA
GERAN PENYELIDIKAN UNIVERSITI PENYELIDIKAN
LAPORAN AKHIR

ROLE OF TRANSFORMING GROWTH FACTOR-BETA1 (TGFB-1) AND THE CELL SIGNALLING PATHWAY IN THE DIFFERENTIATION OF SHED INTO EPITHELIAL-LIKE CELLS

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FINAL REPORT
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ESEARCH TITLE: **Role of Transforming Growth Factor Beta1 (TGFβ-1) and the cell signaling pathway in the differentiation of SHED into epithelial-like cells.**

HASE & YEAR: **3 & 2015**

TART DATE: **1 June 2012**
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ROJECT LEADER: **Dr Azlina Ahmad**
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JECT ACHIEVEMENT *(Prestasi Projek)*

ACHIEVEMENT PERCENTAGE			
Project progress according to milestones achieved up to this period	0 - 50%	51 - 75%	76 - 100%
Percentage (please state #%)			90%

RESEARCH OUTPUT		
Number of articles/ manuscripts/ books *(Please attach the First Page of Publication)*	Indexed Journal	*Non-Indexed Journal*
		1
Conference Proceeding *(Please attach the First Page of Publication)*	International	*National*
	1	2
Intellectual Property *(Please specify)*		

HUMAN CAPITAL DEVELOPMENT

Human Capital	Number				Others (please specify)
	On-going		Graduated		
Citizen	Malaysian	Non Malaysian	Malaysian	Non Malaysian	
No. PHD STUDENT					
Student Fullname: IC / Passport No: Student ID:					
No. MASTER STUDENT	1				
Student Fullname: IC / Passport No: Student ID:					
No. UNDERGRADUATE STUDENT					
Student Fullname: IC / Passport No: Student ID:					
Total	1				

EXPENDITURE *(Perbelanjaan) as Borang K1(RMC)*

C

Budget Approved *(Peruntukan diluluskan)*
Amount Spent *(Jumlah Perbelanjaan)*

Balance *(Baki)*
Percentage of Amount Spent
(Peratusan Belanja)

ADDITIONAL RESEARCH ACTIVITIES THAT CONTRIBUTE TOWARDS DEVELOPING SOFT AND HARD SKILLS
(Aktiviti Penyelidikan Sampingan yang menyumbang kepada pembangunan kemahiran insaniah)

D

International

Activity	Date (Month, Year)	Organizer
(e.g : Course/ Seminar/ Symposium/ Conference/ Workshop/ Site Visit)	1. International Conference On Medical & Health Sciences (ICMHS) 22-24 May 2013	1. College of Pathologists, Health Campus, USM.

National

Activity	Date (Month, Year)	Organizer
(e.g : Course/ Seminar/ Symposium/ Conference/ Workshop/ Site Visit)	1. Health and Life Sciences Postgraduate Conference 2014 10-11 June 2014 2. 10th Malaysia Genetics Congress 2013 (MGC10) 3-5 December 2013	1. Institute for Research in Molecular Medicine (INFORMM) 2. Persatuan Genetik Malaysia

E **PROBLEMS / CONSTRAINTS IF ANY** *(Masalah/ Kekangan sekiranya ada)*

1. Protein expression analysis takes longer times than expected.

ECOMMENDATION (*Cadangan Penambahbaikan*)

ıture direction: Since this study showed that KGM media was not successfully to completely differentiated SHED into ɔithelial-like cells, we suggest that maybe addition of keratinocyte growth factor protein in the KGM media will help in e differentiation. However, we could not do this experiment because the growth factor is expensive.

ESEARCH ABSTRACT – Not More Than 200 Words (*Abstrak Penyelidikan – Tidak Melebihi 200 patah perkataan*)

ɔithelial cells plays crucial role in human body and in oral cavity, epithelial formation is important during the healing rocess after injuries. Previous study had shown that primary cells from human exfoliated deciduous teeth (SHED) can e differentiated into epithelial-like cells when cultured in Keratinocyte Growth Medium (KGM). This study aim to etermine the effects of TGF-β1 and ALK-5 inhibitor in the differentiation of SHED into epithelial-like cells cultured in ɔecific differentiation medium (KGM). MTT and alamarBlue assay was done to investigate and determine the best ɔncentration to be used in the study. Protein expression using flowcytometer was conducted to identify the presence of tem cell markers, CD105, and epithelial marker, E-Cadherin. The cell was positive for stem cell protein marker, but we ill optimizing the protein expression level of E-Cadherin. Then, gene expression of mesenchymal, epithelial, and TGF- signaling marker was carried out on SHED cultured with KGM treated with 1.25 ng/ml of TGF-β1 or 0.625 µM of ALK-5 hibitor. The treated SHED was harvested at day 1, 3, 7, 14, and 21 and subjected to RNA extraction. Then, study was receded with two-step Reverse Transcriptase Polymerase Chain Reaction (RT-PCR). The presence of mesenchymal narker, *Nanog* on all samples except on SHED cultured in KGM + 0.625 µM on day 21. Based on epithelial marker, *eratin5* showed there is no expression on all treated samples. For TGF-β signaling marker which were *ALK-5* and *mad4*, *Smad4* showed expression on day 1 until 7 for all treatments and only SHED in KGM on day 14 showed a mild xpression. *ALK5* expressed in most of samples analysed but only SHED in KGM with TGF-β1 showed no expression. s conclusion, this study showed that TGF-β1 and ALK-5 inhibitor might give a little effect to the differentiation of SHED nto epithelial-like cells but not enough to induce the differentiation process.

)ate :
Tarikh

Project Leader's Signature
Tandatangan Ketua Projek

H	**COMMENTS, IF ANY/ ENDORSEMENT BY RESEARCH MANAGEMENT CENTER (RMC)** *(Komen, sekiranya ada/ Pengesahan oleh Pusat Pengurusan Penyelidikan)*

.....

Pembentangan,

.....

.....

Name:
Nama:

PROF. DR LEE KEAT TEONG
Pengarah
Pejabat Pengurusan & Kreativiti Penyelidikan
Universiti Sains Malaysia

Signature:
Tandatangan:

Date:
Tarikh:

Title: Role of Transforming Growth Factor Beta1 (TGFβ-1) and the cell signaling pathway in the differentiation of SHED into epithelial-like cells.

ABSRACT

Epithelial cells plays crucial role in human body and in oral cavity, epithelial formation is important during the healing process after injuries. Previous study had shown that primary cells from human exfoliated deciduous teeth (SHED) can be differentiated into epithelial-like cells when cultured in Keratinocyte Growth Medium (KGM). This study aim to determine the effects of TGF-β1 and ALK-5 inhibitor in the differentiation of SHED into epithelial-like cells cultured in specific differentiation medium (KGM). MTT and alamarBlue assay was done to investigate and determine the best concentration to be used in the study. Then, gene expression of mesenchymal, epithelial, and TGF-β signaling marker was carried out on SHED cultured with KGM treated with 1.25 ng/ml of TGF-β1 or 0.625 μM of ALK-5 inhibitor. The treated SHED was harvested at day 1,3,7,14, and 21 and subjected to RNA extraction. Then, study was proceeded with two-step Reverse Transcriptase Polymerase Chain Reaction (RT-PCR). The presence of mesenchymal marker, *Nanog* on all samples except on SHED cultured in KGM + 0.625 μM on day 21. Based on epithelial marker, *Keratin5* showed there is no expression on all samples treatment except control which is keratinocyte cells. For TGF-β signaling marker which were *ALK-5* and *Smad4*, *Smad4* showed expression on day 1 until 7 for all treatmets and only SHED in KGM on day 14 showed a mild expression. *ALK5* expressed in most of samples analysed but only SHED in KGM with TGF-β1 showed no expression. As conclusion, this study showed that TGF-β1 and ALK-5 inhibitor might give a little effects to the differentiation of SHED into epithelial-like cells but not enough to induce the differentiation process.

1. INTRODUCTION

Epithelial formation is crucial in human part of the body especially during the process of healing from injuries due to accidents and burns. Previous study has shown that stem cells from human exfoliated deciduous teeth (SHED) can be induced to differentiate into epithelial-like cells with induction media (KGM) (Nam and Lee, 2009). Stem cells have been widely known and intensely studied by many researchers. Based on their potential and capabilities, many researchers have been working to determine the stem cells possibilities to regenerate damaged human cells due to illness, developmental defects and accidents. Stem cells are defined as unspecialized cells which generated daughter cells that either have the same properties or become more restricted cells with more specialized functions and have a limited replication potential. Among various adult stem cells, mesenchymal stem cells (MSCs) have been found to be the widest distribution in human body and among diverse organs. MSCs are defined as multipotent cells, where they have limited capabilities of specialization and are derived from other non-marrow tissues such as umbilical cord blood, adipose tissue, adult muscle, corneal stroma or dental pulp of deciduous baby teeth. In view with the advancement in tissue engineering together with stem cell regeneration's potential, researchers started to explore stem cells from different sources and one of the promising stem cells is stem cells from human exfoliated deciduous teeth (SHED) which was first discovered by Miura and his colleague in 2003 (Miura *et al.*, 2003).

There are many growth factors or cytokines which are involve during cell proliferation and differentiation. One of interested growth factor is Transforming Growth Factor-Beta (TGF-β). This growth factor has been recognized as secreted protein which is involved in cell proliferation, differentiation and other functions in most of the cells. This TGF-β molecule consists of three isoforms which are TGF-βI, TGF-βII, and TGF-βIII. The most prominent isoforms among three isoforms is TGF-βI molecule. In TGF-β signalling, there are TGF-β Type I receptor (TβRI) and Type II receptor (TβRII). Once TGF-βI molecule recognized it binding site at TβRII and become activated, the TβRII will recruit and phosphorylates a type I TGF-β receptor (TβRI) which is also known as activin receptor-like kinase (ALK) to form heterocomplexes. Out of the seven known type I (ALK) receptors, Miyazawa *et al.*, (2002) reported that ALK-5 is the most specific receptor for TGF-β, while it closely

related ALKs, which are ALK-4 and ALK-7 (similar in their kinase domain with ALK-5) interact with other members of TGF-β superfamily. Hence, this study focused on effects of TGF-β1 and ALK-5 inhibitor on differentiation of SHED cultured in Keratinocyte Growth Medium (KGM) into epithelial like cells.

2. JUSTIFICATION OF STUDY

Epithelial cells have been found to be lining in skin, oral mucosa, and blood vessel. Formation of epithelial cells have become crucial to regenerate new cells thus replacing damaged human cells due to illness, developmental defects and accidents. SHED has been widely known as one of stem cell sources for therapeutic application. The ability of SHED to differentiate to other cell lineage such as epithelial cell, when cultured in specific medium highlights its potential for application in future tissue regeneration. Interestingly, the novel epithelial stem cell-like cells from SHED have been identified recently (Nam and Lee, 2009). Thus, suggesting the ability of dental pulp stem cell to be differentiated to epithelial like cells. TGF-β1 is a growth factor that is mostly produced by epithelial cells. TGF-β1 is also a growth factor that is involved during the process of epithelial-mesenchymal interaction during organogenesis. Meanwhile, ALK-5 inhibitor played a role as an inhibitor to the type I TGF-β receptor (ALK-5) which involved in TGF-β signalling pathway. Although many studies have been done looking into the function of TGF-β signalling pathway on cell proliferation; the role of TGF-β1 molecule especially in controlling the process of epithelial cell differentiation from stem cell is still limited. Hence, the effects of the TGF-β1, ALK-5 inhibitor, and the molecules involved in cell signalling pathway in the differentiation process of SHED into epithelial-like cells will be identified and highlighted. This study may provide a better insight and understanding on the mechanism and cellular signalling works for stem cell and tissue regeneration process.

3. OBJECTIVES

3.1 General objective

To study the effect of ALK-5 inhibitor and TGF-β1 molecule in the differentiation of SHED to epithelial-like cells cultured in specific differentiation medium.

To study the effect of TGFβ-1 and its inhibitor (ALK-5) in the induction of SHED to epithelial-like cells

3.2 Specific objectives

i. To determine the effects of TGFβ-1 and ALK-5 inhibitor on the population doubling time of epithelial-like cells derived from SHED

ii. To investigate the gene expression levels of specific genetic markers for epithelial-like cells derived from SHED treated with TGFβ-1 and ALK-5 inhibitor

iii. To determine the protein expression levels of epithelial-like cells derived from SHED treated with TGFβ-1 and ALK-5 inhibitor (Not completed)

iv. To determine the gene expression levels of specific molecules associated in the cell signaling pathway of TGFβ-1 involved in the differentiation of SHED into epithelial-like cells

*Objective (iii) is not able to be achieved due to some technical problems involved during the process of cell culture whereby higher number of cells was required for protein extraction. Additionally, the protein analysis which involves immunofluorescence and Western Blotting also took longer time than our expectation.

4. MATERIALS AND METHODS

Stem cell culture

SHED primary cell-line was obtained commercially from ALLCELLS, USA. SHED was cultured and maintained in serum-free keratinocyte growth medium (KGM; Lonza) with supplements provided. The medium was changed and cell was sub-cultured at 70% confluence. At each passage, cells was counted and photographed. Morphology of the cells treated differently was analysed accordingly (SHED + TGFβ-1), (SHED + ALK-5 inhibitor) and control (SHED alone) and observed using phase-contrast microscope.

Preparation of TGFB1

The recombinant human TGF-β1 powder (10μg) (cat no. PHG9214, Life Technologies, USA) was prepared by reconstituted in an acidic buffer such as 40 mM acetic acid (cat no.100063, Merck, Germany) to a concentration of 1 mg/mL where this solution can be stored at 2 °C to 8 °C for up to one week. For extended storage, the solution was further diluted in a buffer containing a carrier protein, such as 0.1% Bovine Serum Albumin (BSA) (cat no. AM2616, Ambion, Life Technologies, USA). The solution then was stored at -20 °C.

Preparation of ALK5 inhibitor (SB431542)

The SB431542 powder (MW: 384.4) (CAS no. 301836-41-9, Sigma Aldrich, Germany) was prepared by diluted with Dimethyl sulfoxide (DMSO) (S4317, Merck, USA) as a stock concentration. The mixture was incubated overnight at 37 °C, 5% CO_2 so that the powder will dissolved. Then, solution was aliquot for working solution and stored at -20 °C.

To achieve objective (i)

4.1 MTT assay

$1x10^5$ SHED was cultured in KGM and seeded in 96 well-plate. A serial dilution of TGF-βI and ALK-5 inhibitor (SB431542) was done and the cells were treated with 100μl of each treatment separately. The cells were incubated for 72 hours in 37°C, 5% CO_2 incubator. MTT assay was conducted to determine concentrations which

produced highest cell viability and was compared to control (without treatment). After 72 hours, 100µl DMSO was added and further incubated for 4 hours. The plate then was read at ELISA reader with 570nm wavelength and 0nm as reference wavelength.

4.2 AlamarBlue assay

$1x10^4$ SHED was cultured in α-MEM since KGM does not help the proliferation of the cells. The cells were seeded in 96 well-plate. From MTT assay, 3 concentrations were selected to be further investigated. For TGF-βI, 0.3125, 0.625, and 1.25 ng/ml were choose and for ALK-5 inhibitor, 0.156, 0.3125, and 0.625µM were selected. The assay was investigated for day 1,3,7, and 10. The cells were treated with each treatment and the cells in media only as control. Prior 4 hours before the incubation period of each day, 10µl of alamarBlue was added to the cells treatment and was analysed using ELISA reader at 570nm wavelength and 600nm as reference wavelength.

Multiplication cell rate and population doubling time was calculated based on the formula:

$$\text{Multiplication rate } (r) = 3.32 (\log NH - \log N1) / (t2 - t1)$$

Where,

NH = total cell harvested

N1 = total cell seeded

$t2$ = time when cells harvested

$t1$ = time when cells seeded

Population doubling time (PDT) = $1/r$

= 1/ multiplication rate = x hour per doubling

To achieve objectives (ii) and (iv)

4.3 Gene expression analysis

RNA was extracted from cell pellet using RNA extraction kit. Then, RNA was converted into cDNA using MMLV Reverse Transcriptase Kit. This method involved two steps Polymerase Chain Reaction (PCR) which required cDNA conversion to amplify PCR product specific to gene of interest. The gene expression was analysed for day 1, 3, 7, 14, and 21. Keratin5 gene was selected as the epithelial markers while *Alk-5* and *Smad4* was selected as the molecule involved in TGF-β cell signalling pathway. B-actin was used as internal amplification control. The sequences and cycling condition of the primers used in this study as below:

Sequences of the primers used in RT-PCR

Gene	Sequences (5' to 3')	Amplicon size (bp)	Reference
β-actin	F: TGGCACCACACCTTCTACAATGAGC	437	Karaöz *et al.*, 2010
	R: GCACAGCTTCTCCTTAATGTCACGC		
	R: CCCCCTGTCCCCCATTCCTA		
Nanog	F: CCCAAAGGCAAACAACCCACTTCT	107	Park *et al.*, 2012
	R: AGCTGGGTGGAAGAGAACACAGTT		
	R: GCAGGTAGCACACCTCCTG		
	R: GACGCAGAATCAGAATTAGGAAAGCAAG		
	R: AGCTCATGGTTGGGGCAC		
Keratin 5	F: GCTGAGCTCTGTTCTCTCCAGCAC	966	Liovic *et al.*, 2001
	R: CATCAGTGCATCAACCTTGGCCTC		
ALK-5	F: GGGGCGACGGCGTTACAGTGTTTCTGCCAC	345	Finnson *et al.*, 2008
	R: TGAGATGCAGACGAAGCACACTGGTCCAGC		
	R: ATCGCCAGGAATTGTTGCTGTAT		
	R: CTCCTTGGAAGGTGCTGAAG		
Smad4	F: CCATTTCCAATCATCCTGCT	221	Banas *et al.*, 2007
	R: ACCTTTGCCTATGTGCAACC		

Cycling conditions of the RT-PCR

Gene	Step	Temperature (°C)	Time (sec)	Number of cycles
β-actin	Activation	95	60	1
	Denaturation Annealing Extension	95 59.5 72	15 17 10	30
	Holding	4	∞	-
Nanog	Activation	95	60	1
	Denaturation Annealing Extension	95 58.1 72	15 15 10	35
	Holding	4	∞	-
Keratin 5	Activation	95	60	1
	Denaturation Annealing Extension	95 63.8 72	15 15 10	35
	Holding	4	∞	-
ALK-5	Activation	95	60	1
	Denaturation Annealing Extension	95 66 72	15 17 10	35
	Holding	4	∞	-
Smad4	Activation	95	60	1
	Denaturation Annealing Extension	95 52.2 72	15 15 10	35
	Holding	4	∞	-

4.4 To achieve objectives (iii)

For two-steps flowcytometry, primary antibodies and fluorescence secondary were involved. Mouse anti-human CD105 was used as mesenchymal stem cells marker meanwhile mouse anti-human E-cadherin was used as epithelial marker. SHED was cultured in αMEM and HEK001 as a positive control; SHED cultured in KGM only, SHED cultured in KGM with presence of 1.25 ng/mL of TGF-β1, and SHED cultured in KGM with addition of 0.625 μM of ALK-5 inhibitor for Day 7, Day 14, and Day 21 were used in this study. First, the cells were harvested by trypsinization process. Then, the cells were re-suspended with 1 ml of media to determine the number of cells. For SHED cultured in KGM, the cells need to be re-suspended with PBS and centrifuged at 1200 rpm for 5 minutes. This step was repeated two times. This step was important because serum contained in previous culture media medium (αMEM) will stopped differentiation process. Then, 1 ml of KGM media was added, re-suspended and counted to determine the number of cells. The cells suspension which contained $5x10^5$ cells was aliquot into 1.5 ml eppendorf tube and centrifuged at 1200 rpm for 5 minutes. Then, supernatant was discarded and 100 μl of cold PBS was added to cell pellet and the cell pellet was re-suspended. After that, primary antibodies were added to the cell suspension. In this study, CD105 (dilution 1:200) was E-cadherin (dilution 1:100) were used as primary antibodies. The eppendorf tube was covered with aluminium foil to protect from light and was incubated for 3 hours at 4°C on belly dancer to maximize the interaction between antibodies and cells. The cell suspension was washed with PBS by centrifugation at 1200 rpm for 5 minutes. This step was repeated 3 times. After that, supernatant was discarded and 100 μl of cold PBS was added to the cell pellet to re-suspend the cells. Next, secondary antibody (Goat-anti mouse FITC) was added and incubated for 3 hours at 4°C on belly dancer. After 3 hours incubation, the cell suspension was centrifuged at 1200 rpm for 5 minutes and the supernatant was discarded. This step was repeated 3 times. 100 μl of cold PBS was added to the cell pellet so that the cells were re-suspended and ready for flowcytometry analysis.

5. RESULTS

5.1 MTT assay

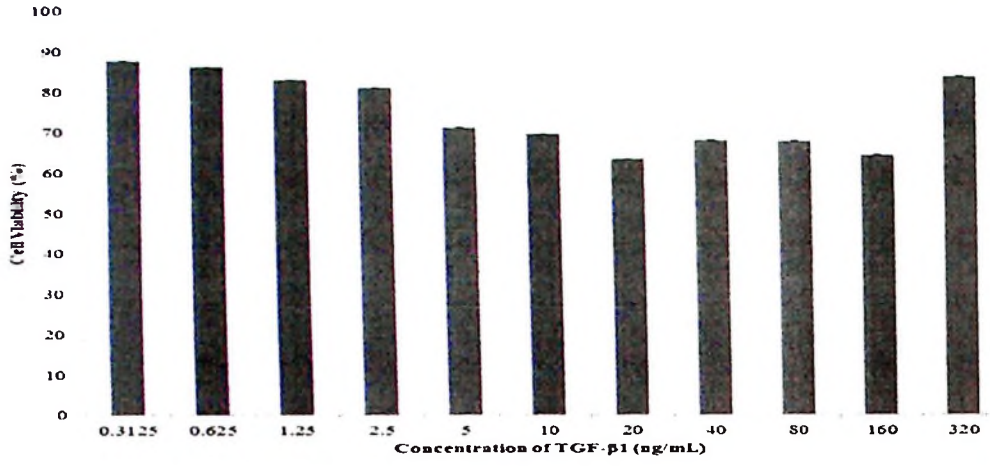

Figure 1(a): Dose-viability graph of TGF-β1 on SHED cells by MTT assay.

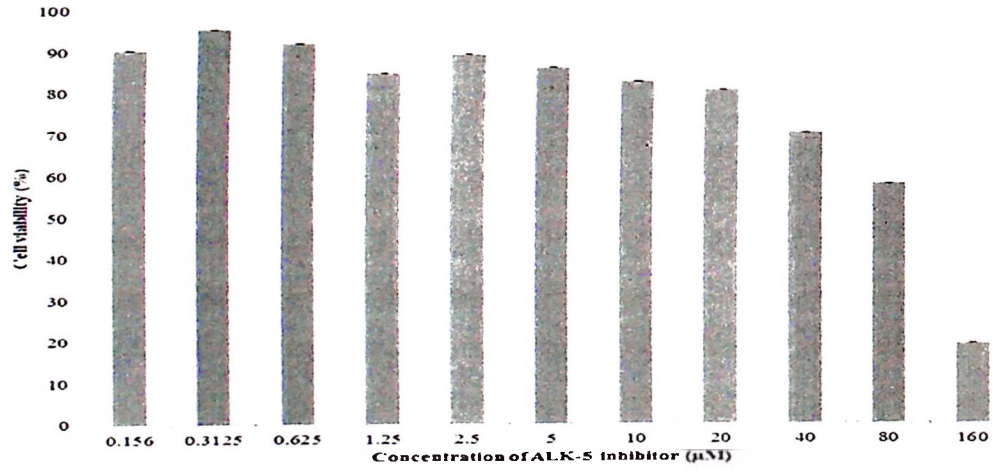

Figure 1(b): Dose-viability graph of ALK-5 inhibitor concentration on SHED cells.

Based on Figure 1(a) and (b), the percentage of cell viability for most cell treatments with addition of TGF-β1 or ALK-5 inhibitor was more than 50% except SHED treated with 160 μM of ALK-5 inhibitor.

For SHED treated with TGF-β1, cell viability of the cells started to decrease with the increasing TGF-β1 concentrations until 20 ng/ml of TGF-β1. At 40 until 80 ng/ml of TGF-β1, viability of cells became increasing, decreasing at concentration 160 ng/ml, and increasing again at 320 ng/ml of TGF-β1.

Meanwhile, for SHED treated with ALK-5 inhibitor, viability of cells started to increase from 0.156 μM until 0.3125 μM of ALK-5 inhibitor. Then, cell viability became decreased until 1.25 μM, increased at 2.5 μM, and became decreasing from concentration of 5 μM until 160 μM of ALK-5 inhibitor.

Prior to serial dilution of TGF-β1 and ALK-5 inhibitor concentration, 0.3125, 0.625, and 1.25 ng/ml of TGF-β1, and 0.156, 0.3125, and 0.625 μM of ALK-5 inhibitor has been choose as best concentrations to be further investigated since they produced most higher percentage of cell viability compare to other concentration although the percentage was not exceed percentage of control, where we assume as 100%.

5.2 AlamarBlue assa

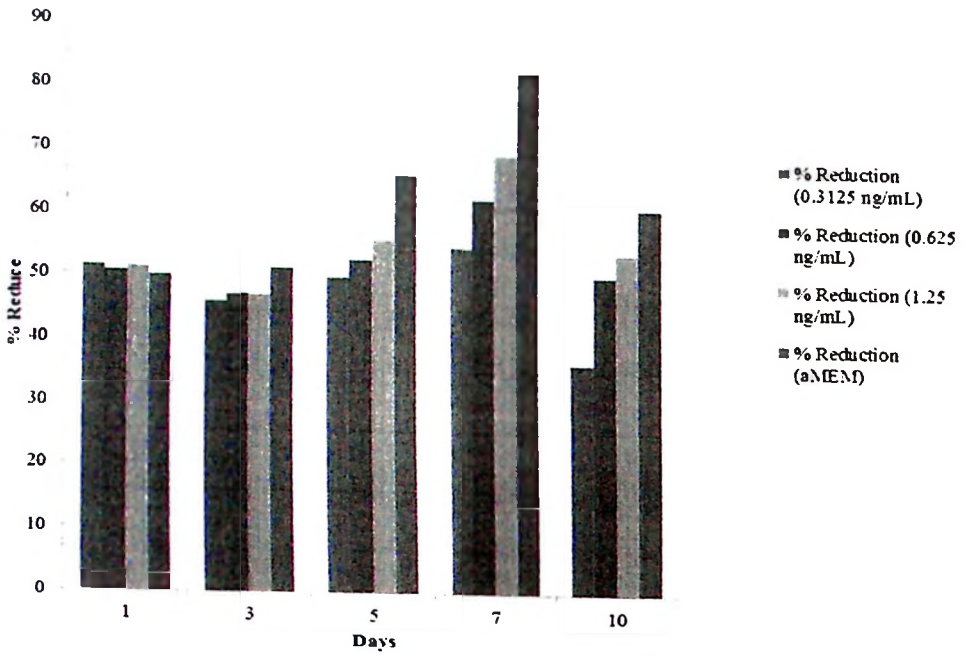

Figure 2 (a): Percentage of alamarBlue reduction's graph on SHED with TGF-β1 treatment.

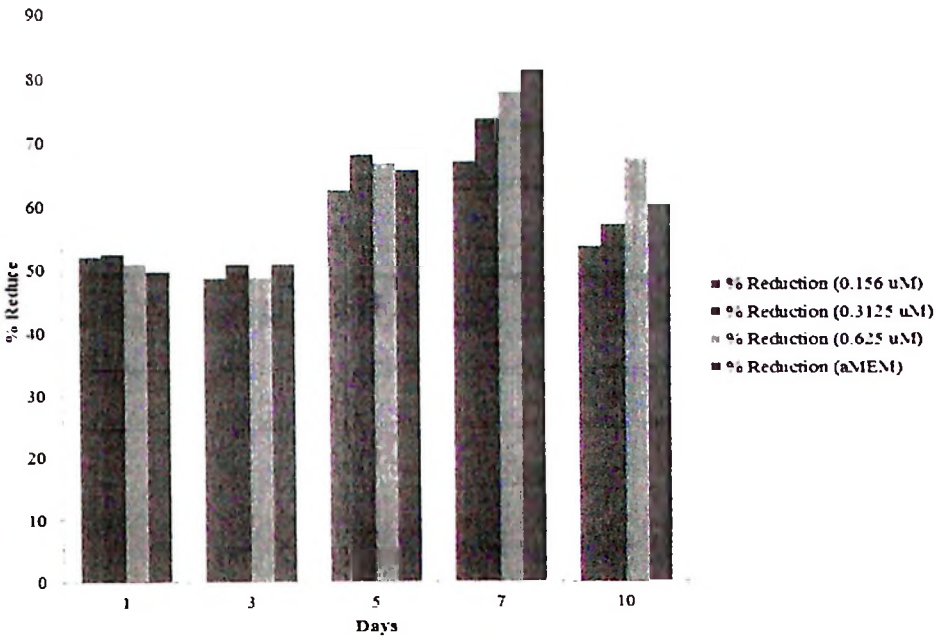

Figure 2 (b): Percentage of alamarBlue reduction's graph on SHED with ALK-5 inhibitor treatments.

Refer to Figure 2 (a) and (b), alamarBlue assay's reduction represent cell proliferation of cells where oxidation by cells reduced the blue colour of alamarBlue into pinkish colour. Both figure showed the same pattern where from day 1 to 3, the percentage of cell reduction was decreased, then increased started day 5 to 7, and decreased again at day 10. Day 7 has the highest percentage of cell reduction compared to day 1, 3, 5, and 10. For TGF-β1 treatment, 1.25 ng/ml showed the highest reduction percentage compare to 0.3125 and 0.625 ng/ml of TGF-β1. Meanwhile for ALK-5 inhibitor, 0.625 μM have the highest reduction percentage as compared to 0.156 and 0.3125 μM of ALK-5 inhibitor.

To calculate population doubling time (PDT), the standard curve was first done to get a linear equation. Standard curve was done where serial dilution number of cells was done for overnight and evaluate by reduction of alamarblue. In this study, the linear equation as below:

$$y= 18.982\ln(x)-80.102$$

Based on our result, the PDT for each cell treatment as below:

- in α-MEM only = 41.1h per doubling
- 0.3125 ng/mL TGFβ-1 = 149.9h per doubling
- 0.625 ng/mL TGFβ-1 = 84.7h per doubling
- 1.25 ng/mL TGFβ-1 = 57.7h per doubling
- 0.156 μM ALK-5 inhibitor = 68.5h per doubling
- 0.3125 μM ALK-5 inhibitor = 54.7h per doubling
- 0.625 μM ALK-5 inhibitor = 42.9 per doubling

5.3 Morphology of cells

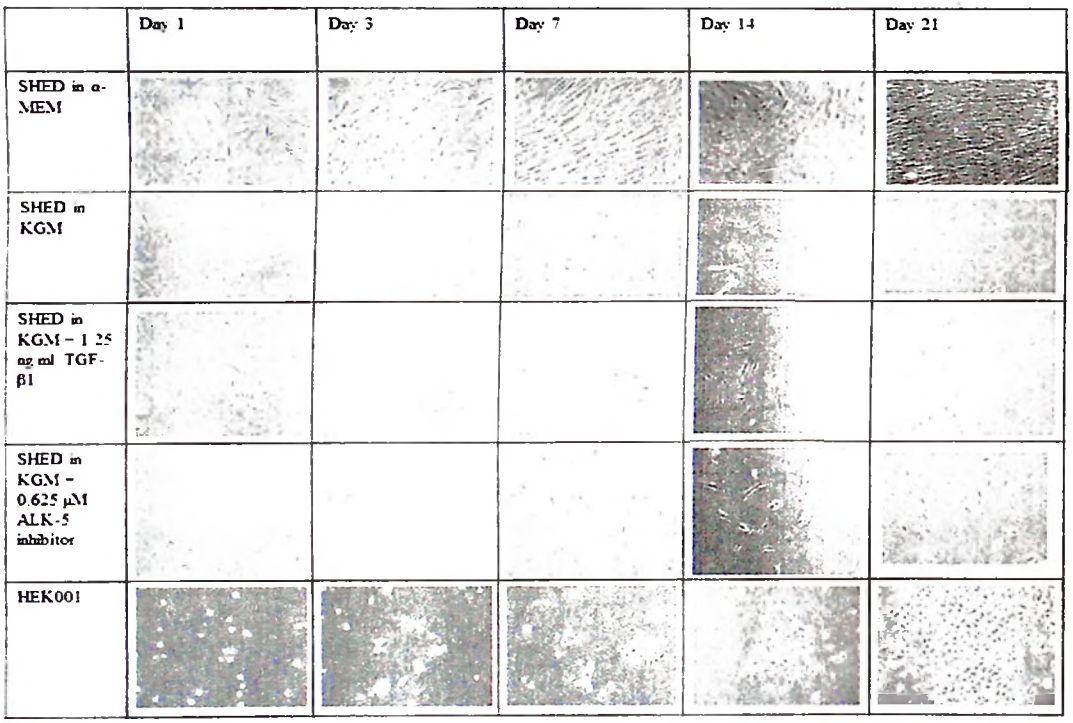

Figure 3: Morphology of cells from day 1 until 21 using phase inverted-phase contrast microscope.

Based on Figure 3, SHED in KGM, SHED in KGM with the addition of 1.25 ng/ml TGF-β1 or 0.625 μM ALK-5 inhibitor showed SHED started to have morphological changes on day 7. After 3 weeks of induction, there are still morphological changes where the cells started to become shortened and appeared to be cuboidal-like shape compared to positive control; keratinocyte cells.

5.4 Gene expression analysis

5.4.1 Extraction of RNA-Quality of RNA

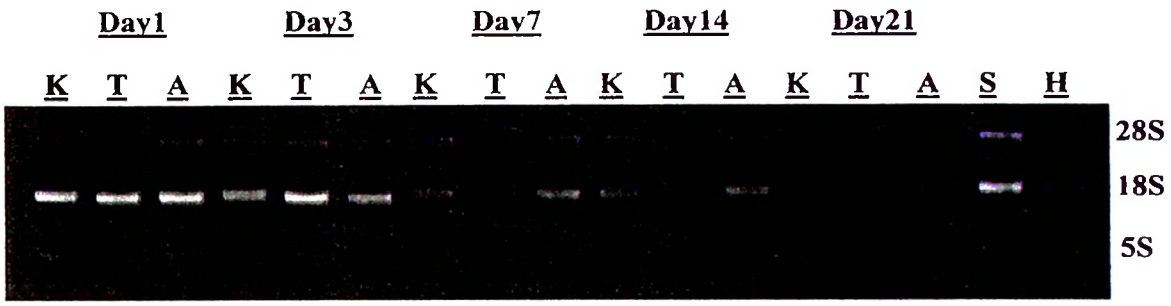

Figure 4: Agarose gel electrophoresis of RNA extracted from SHED cultured in KGM and SHED cultured in KGM with the presence of 1.25 ng/ml TGF-β1 or 0.625 μM ALK-5 inhibitor. The samples showed clear 18S and 28S bands which indicated that integrity of RNA was good. Only certain samples showed 5S band which indicated a very good integrity of RNA. K, SHED cultured in KGM only. T, SHED cultured in KGM with addition of 1.25 ng/ml TGF-β1. A, SHED cultured in KGM with addition of 0.625 μM ALK-5 inhibitor. S, SHED cultured in α-MEM. H, keratinocyte cultured in keratinoctye media.

RNA extraction produced high quality and good RNA integrity. Clear 18S and 28S bands with some samples showing 5S band were observed in all samples analyzed.

5.4.2 Gene expression of stem cells and epithelial markers

Housekeeping gene: *β-actin*

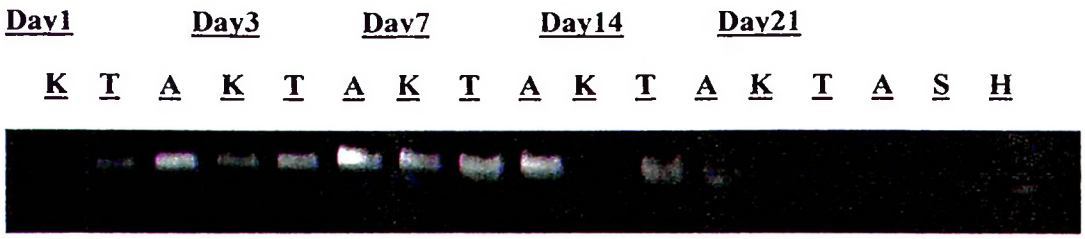

Figure 5: Agarose gel electrophoresis of *β-actin* expression from SHED cultured in KGM, and SHED cultured in KGM with the presence of 1.25 ng/ml TGF-β1 or 0.625 μM ALK-5 inhibitor. K, SHED cultured in KGM only. T, SHED cultured in KGM with addition of 1.25 ng/ml TGF-β1. A, SHED cultured in KGM with addition of 0.625 μM ALK-5 inhibitor. S, SHED cultured in α-MEM AND H, HEK001 (control).

In our study, *β-actin*, the housekeeping gene, was expressed equally throughout the study period. This gene is an endogenous control gene which regulates gene expression. Constant and equal expression of this gene indicates that the RNA concentrations used in PCR were having same concentration. It is crucial to calibrate the *β-actin* expression as different concentration of RNA which can give rise to different level of gene expression.

Stem cell marker: *Nanog*

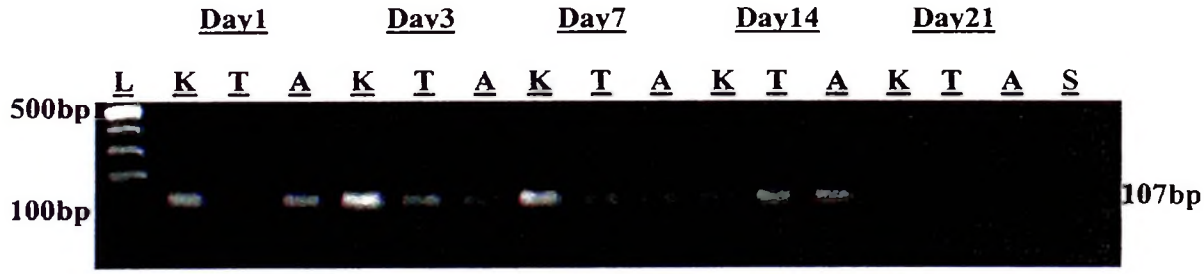

Figure 6: Agarose gel electrophoresis of *Nanog* expression (107bp) from SHED cultured in KGM, and SHED cultured in KGM with the presence of 1.25 ng/ml TGF-β1 or 0.625 μM ALK-5 inhibitor. K, SHED cultured in KGM only. T, SHED cultured in KGM with addition of 1.25 ng/ml TGF-β1. A, SHED cultured in KGM with addition of 0.625 μM ALK-5 inhibitor. S, SHED cultured in α-MEM (control).

All samples were subjected to RT-PCR for *Nanog* (Figure 6). *Nanog* was expressed in all samples except sample of SHED in KGM treated 0.625 μM ALK-5 inhibitor on day 21. For SHED cultured in KGM only, the expression on *Nanog* from day 1 until day 14 was increased compared to day 21. On day 14, *Nanog* expression started to decrease until day 21. For SHED cultured in KGM with addition of 1.25 ng/ml TGF-β1 (T), *Nanog* expression was slightly increased until day 14 and decreased on day 21. SHED cultured in KGM with the presence of 0.625 μM ALK-5 inhibitor (A) showed expression of *Nanog* slightly decreased from day 1 until day 3, and at day 7, there was no change on gene expression. At day 14, the expression was slightly increased but was not detected on day 21.

Epithelial marker: *Keratin5*

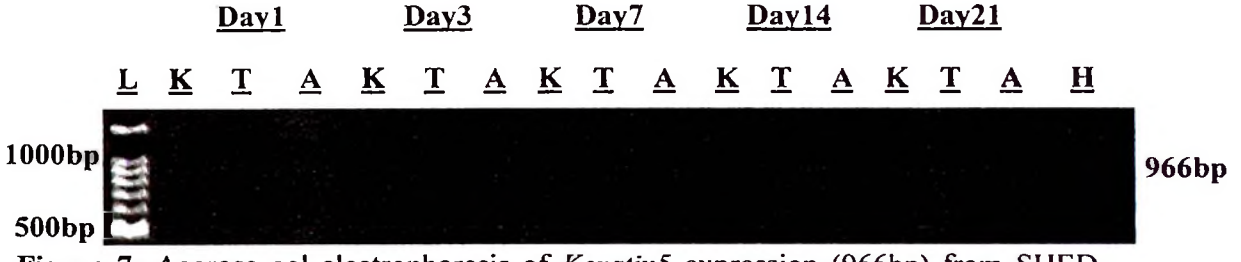

Figure 7: Agarose gel electrophoresis of *Keratin5* expression (966bp) from SHED cultured in KGM, and SHED cultured in KGM with the presence of 1.25 ng/ml TGF-β1 or 0.625 μM ALK-5 inhibitor. K, SHED cultured in KGM only. T, SHED cultured in KGM with addition of 1.25 ng/ml TGF-β1. A, SHED cultured in KGM with addition of 0.625 μM ALK-5 inhibitor. H, keratinocyte cultured in keratinoctye media (control).

Keratin5 expression was not detected in all samples analysed except for keratinocytes (positive control; Figure 7).

5.4.3 Gene expression of molecules involved in TGF-β signalling markers

ALK5

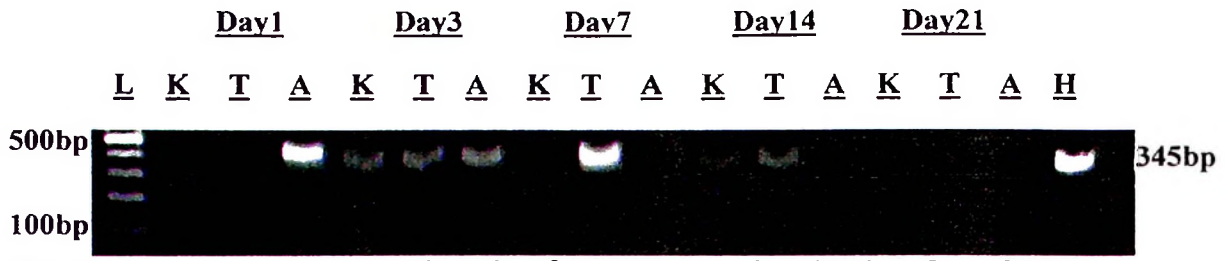

Figure 8: Agarose gel electrophoresis of *ALK5* expression (345bp) from SHED cultured in KGM, and SHED cultured in KGM with the presence of 1.25 ng/ml TGF-β1 or 0.625 μM ALK-5 inhibitor. K, SHED cultured in KGM only. T, SHED cultured in KGM with addition of 1.25 ng/ml TGF-β1. A, SHED cultured in KGM with addition of 0.625 μM ALK-5 inhibitor. H, keratinocyte cultured in keratinoctye media (control).

Figure 8 showed that most of samples analysed expressed *ALK5* except on day 1 of treatment; no expression for *ALK5* was observed in SHED treated with TGF-β1 (T). *ALK5* was expressed in SHED cultured in KGM only (K) from day 1 to 21, however the expression was fluctuated. On day 1, expression of *ALK5* decreased and then increased on day 3. On day 7, the expression was decreased and increased again at day 14 and decreased at day 21. Meanwhile, *ALK5* was expressed on day 3- day 14 although it was reduced from day 14 to day 21 in SHED cultured in KGM + TGF-β1(T). *ALK5* was down regulated in SHED cultured in KGM and treated with ALK-5 inhibitor (A) from day 1 to day 21. *ALK5* expression was strongly expressed in keratinocyte cell.

Smad4

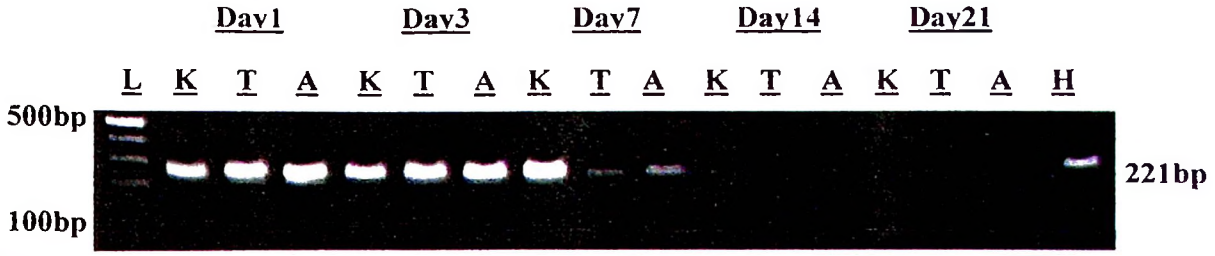

Figure 9: Agarose gel electrophoresis of *Smad4* expression (221bp) from SHED cultured in KGM, and SHED cultured in KGM with the presence of 1.25 ng/ml TGF-β1 or 0.625 μM ALK-5 inhibitor. K, SHED cultured in KGM only. T, SHED cultured in KGM treated with 1.25 ng/ml TGF-β1. A, SHED cultured in KGM treated with 0.625 μM ALK-5 inhibitor. H, keratinocyte cultured in keratinoctye media (control).

Smad4 was expressed from day 1 until day 7 in all samples analysed. However, *Smad4* was down regulated at day 14 in both SHED cultured in KGM only (K) and SHED cultured in KGM and treated with the TGF-β1 (T). Additionally, *Smad4* was not expressed in SHED cultured in KGM and treated with ALK-5 inhibitor (A). At day 21, *Smad4* expression was not detected in all samples analysed (Figure 9). *Smad4* expression was strongly expressed in keratinocyte cell.