CULTURE OF OCT-4 POSITIVE CELLS FROM PREPUTIAL EPIDERMIS

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(Abstrak) The medical waste of preputial skin is easily obtained in Indonesia. The cells isolated from six samples are expected to have pluripotency and able to differentiate to other lineage for medical treatment. This research uses the epidermal layer of preputial skin to obtain keratinocytes, these cells are taken using dispase and trypsin solution overnight respectively. Then, the keratinocytes are subsequently cultured using DMEM complete high glucose to increase the number of cells. The cultured cells are then taken for immunocytochemistry (ICC) of Oct-4 since it is the marker of pluripotency. The other half of cultured samples are continued for over confluency analysis for fourteen days to observe spontaneous differentiation. This research has successfully used an alternative and cost effective culture medium for keratinocytes. It consist of DMEM high glucose, PRP 10%, heparin 1%, FBS 10%, penstrep 1%, and fungizone 1%. The result of ICC is partially positive with keratinocytes nuclear being stained dark brown in five hpf from each sample. However, spontaneous differentiation analysis using alcian blue shows negative result of chondrogenic formation from keratinocytes.

Keywords: prepuce epidermis, pluripotency, Oct-4, immunocytochemistry, isolation, culture, spontaneous differentiation, alcian blue staining.

1. INTRODUCTION

This experiment is based on the epidermis of human. The essence of this experiment is for the study of stem cell factor in the prepuce as a medical waste. The positive stem cells factor from this tissue would give rise to an opportunity of stem cell treatment using this tissue lineage. This layer of skin is isolated and cultured to give rise to colonies of keratinocytes. This culture is then checked for the availability of Oct-4 positive cells and the ability to differentiate spontaneously to the other lineage such as chondrogenic lineage. Oct4 itself is the marker of pluripotency which is high in stem cells and/or pluripotent cells.

The source of epidermis in this research is preputial skin post-circumcision. The prepuce is from the external genitalia of male. Prepuce is usually operated due to some cultural believe and having some medical background as to avoid infection in the Langerhans cells of the prepuce. 1

Circumcision has been done since the history of humanity such as medieval times. Even though circumcision has been controversial in some parts of the world because of its questionable efficacy in dealing with HIV infection risk factor, it has been widely used to treat infection in the prepuce which is called as prostatitis, infection in the gland area named as balanitis, and also conjoining infection which is called as balanoprostatitis. Some other indication of circumcision are phimosis and paraphimosis which are alteration at the urinary tract. In all malignancy case, prepuce must be circumcised as well.2

Since another background of circumcision are beliefs of the religion and culture. This surgical procedure is elaborated, widely in Indonesia, without any scientific backgound in the society. Every year substantial amount of prepuce are produced as medical waste. So these prepuce use in this research are out of medical waste which are taken recently after the surgical procedure.2

Stem cells is a type of cell that can be found in some specific places of the human body and this cell could have many advance usage. Since it can keep itself being renewed so there could be many identical copy. Furthermore, these cells can also live for a long time and avoid the mechanism of apoptosis. It is essential to notice that the ability of stem cells to
differentiate into many different kinds of cells would be highly beneficial in the future.¹

There are two kinds of stem cells which are used in most research based on their differential capacity, embryonic stem cells and somatic or adult stem cells. In this research preputial epidermis is expected to acquire stem cell characteristic through observation of oct4 by immunohistochemistry. Since stem cells of a tissue have the ability to differentiate, this research expect epidermal derived stem cells to differentiate to the other lineage of cells.⁴

The difference between adult stem cells and embryonic stem cells are in the number and types of differential capacity they could produce. Embryonic stem cells are considered as pluripotent stem cells since they can produce all cell types in human being, whilst adult stem cells could only produce specific cell types according to their obtained site. In case stem cells exist in the epidermis of prepuce, it is highly suspected to acquire adult stem cells characteristic instead of embryonic stem cells. This is because prepuce is not and embryonic tissue which could differentiate to various tissues. Example of embryonic tissue is umbilical cord.⁴

To observe the presence of adult stem cell in the sample, transcription factors analysis can be done, such as the presence of Oct4 and Nanog. Both of these transcription factors are identical to the stem cell self-renewing capability.⁵

Since stem cells are type of cells that are not specialized in any function and form yet, it can produce or give rise to a specific type of cell that can work for a specific function, this procedure is called differentiation. Differentiation is a gradual process which is controlled by signal from inside of the cell (internal signals) which are coded by the DNA of the cell, and from the microenvironment signaling (external signals) which are mostly produce by the nearby cells in vivo. Both of these signaling would join and trigger the epigenetic marks.⁴

Scientists have made some basic protocols for directed differentiation, such as by altering the culture medium, surface of the culture dish, insertion of specific genes. In normal condition, the adult stem cells change itself to the tissue where they reside before removal and culture. Adult stem cells differentiation are specific to the organ or tissue where they come from in vivo. In normal condition, the adult stem cells from the basal layer of the epidermis and at the base of the follicles tend to generate keratinocytes for the surface of the skin and the follicular stem cells produce hair follicle.⁴

The differentiation of adult stem cells involve numerous signaling such as peroxisome proliferator activated receptor γ (PPARγ) and Runt-related transcription factor 2 (Runx2) are the most important signaling for adipogenesis and osteogenesis. While for the differentiation of adult stem cells into chondrocytes uses signaling such as type II TGF-β receptor (TGFβRII) in low glucose concentration.⁵⁻⁷ However spontaneous differentiation does take place to either one lineage of cells mentioned above.

This research is conducted to give an answer whether the cultured stem cells taken from the epidermis of the preputial skin could differentiate spontaneously or not to other cells lineage, which is chondrocyte lineage. This research does not include any kind of kits for differentiation.

The desire to observe the existence of stem cells factor and differentiate spontaneously are due to their prospective usage as regenerative or reparative medicine in the near future for cell-based therapies. These cells might be used to replaces dead cells in any specific disease in the future because of their renewal property, dividing, and specializing stem cells characteristic.⁵

Some diseases that are having increase in opportunity to be treated in the future by the stem cell differentiation are such as diabetes, trauma of the spinal cord, muscular dystrophy, heart disease, hearing and vision loss.⁴

Problem Identification is to increase the usage of epidermis derived from the prepuce, it is important to determine the appropriate cost effective culture medium; Oct-4 positive cells which can determine the pluripotent capacity of keratinocytes, and the possibility of spontaneous differentiation of keratinocytes.

Hypothesis is keratinocytes can be cultured directly from preputial skin using a complete high glucose DMEM, fungizone 1.5%, penstrept 1.5%, PRP 10%, heparin 1%, and FBS 10%. These cells which are obtained from the prepuetial epidermal layer might contain OCT4 and have the potency to differentiate spontaneously to other cells lineage such as chondrocytes.

2. LITERATURE REVIEW

A. Culture of Keratinocytes

It is very interesting to note that keratinocytes have started to be cultured since 1975 by Rheinwald and Green, followed by Boyce and Ham in 1983. They found out that keratinocytes can be cultured under defined niche without any serum. This condition includes low concentration of calcium. Some aspects of sample characteristic that must be put into consideration to experiment using epidermal skin is that; first, it is best to obtain sample from young donor; second, the sample is best taken from regions without any mechanical interference, no hair, and scar tissue.¹⁴

There are two different method of epidermal skin culture. The first one is feeder layer-dependent and serum-containing culture medium. Both of these methods are legible to prevent epidermal cells from apoptosis. This includes inhibition of adenoviral infection. However, feeder layer-dependent condition is believed to limit keratinocytes growth to certain extent. While serum-containing medium is known to lessen keratinocytes attachment.¹⁴

B. Spontaneous differentiation to Chondrogenic Cells

It is believed that epidermis differentiation process is coded genetically. One important gene is STAT3, which alteration of this gene would result in changes of terminal differentiation as well as hyperplasia.¹⁵

Spontaneous differentiation is a process of differentiation which does not require specific induction of cells genetically. An example of spontaneous differentiation is using 10% fetal bovine serum (FBS) on plastic culture medium. This experiment found out that spontaneous differentiation is not applicable for bone marrow stem cells. It was successfully suitable for fibroblastic-like cells.¹⁶

The experiment conducted by Naruseet al.¹⁶ proved that fetal mice’s differentiated blood cells could differentiate spontaneously to many lineage, especially chondrocytes. This was examined by PCR, immunohistology, and von Kossa staining which will not be described further. It was found that collagen type II and X which were found in the differentiated cells resembles the chondrocytes in the rat ribs cartilage. Other evidences of differentiation which were found in his experiment of spontaneous differentiation were the presence of parathyroid hormone receptors, alkaline phosphatase, osteocalcin, and other markers that prove the existence of adipocytes such as...
methyllisobutyl xanthine, insulin, dexamethasone, and indomethacin. Based on this experiment, it is found that the capability of cells to differentiate spontaneously to other lineage depends on TGF-β and other three-dimensional culture environment.  

*In vitro* experiment of MSCs proved that differentiation to chondrocytes require inhibition of epithelial growth factor (EGF). In this experiment, collagen type-II expression were examined to observe the availability of chondrocytes.  

### 3. METHODS

This research was conducted using cross-sectional observation design. It was because this experiment was laboratory oriented without any intervention. From transport, isolation, cultural, and differentiation of the keratinocytes obtained from the specimen highly needed laboratory tools.

This research took 7 months from 10th September 2015 until 31st May 2016 in UPT-RSCM and Histology department FKUI. This included optimization of the method and laboratory orientation. The actual research was conducted in between 1st and 20th May 2016.

Samples were taken from the mass circumcision using cauter and normal surgery, each consist of three samples. Directly after the procedure, samples were cleaned by povidone-iodine and alcohol. The samples were transported by PBS 15ml in falcon 50 ml with added penstep and fungizone 2% respectively. After the samples had been obtained, they were put inside a cool box over the weekend.

The process of isolation was started by forming dispase. It consisted of 13.5 mg dispase II powder and DMEM high glucose 27 ml. This solution was important to separate epidermis from the dermis layer.  

All samples were first washed by PBS 1.5 ml to clean the hematomas. The following process was washing by betadine and alcohol for three times respectively. After the samples were ready and cleaned, the samples were incubated using dispase in -3-degreesecelsius for overnight.

In the second day, the samples had to be warmed using water bath for an hour in 15 ml falcon. The samples were then washed by sterile PBS. The epidermis was separated using surgical set and was stored by PBS 5 ml mixed with triple select 15µl in falcon 15 ml for one night.

The six samples were started to be cultured by making complete medium for epidermal layer. This complete medium consisted of DMEM high glucose, fungzone 1.5%, penstep 1.5%, PRP 10%, heparin 1%, FBS 10%. Finally, the processing of this medium had to be sterilized using filter and syringe.

In the twelve day, the samples were moved to Eppendorf after trypsinization and centrifugation from each of the 3 well of 6 well plate culture. This cells were fixated using methanol 200µl per Eppendorf for further processing of immunocytochemistry.

The test of OCT4 using immunocytochemistry was resumed after being fixated using methanol 200µl per well. After the cells had been fixated by methanol in the Eppendorf, the solution was mixed using pipet and put on a coated slide for 100µl after marking the region using PAP pen. This slides were named and put inside the oven for 10 minutes. After that, the cells had been fixated on the coated slide. Next, slides were washed using aquadest for one minute. They were blocked using H2O2 3% for 10 minutes inside the moist chamber. The next step, the slides were washed by water in the jar. This was done for 3x each for 1 minute. To block the other antigens, the each of the sample was given blocking serum for 100µl (1.5% donkey serum + 98.5% PBS) for 30 minutes. Primary antibody oct4 1:50 was applied for an hour in the moist chamber. Samples were washed using PBS again for 3x each for 5 minutes. Now the samples were ready to be given secondary antibody (60 µl biotinylated secondary antibody + 90 µl normal blocking serum + 6 µl PBS to be applied for thirty slides) for 30 minutes in the moist chamber. The slides were washed again using PBS for 3x, each was for 5 minutes. Next, the samples were given avidin-biotin HRP (100 µl avidin + 100 µl biotin diluted in the PBS 5 ml) for 30 minutes. The slides were washed again using PBS for 3x, each approximately for 5 minutes. After that, the slides were washed using DAB solution (6 drops of 50x peroxidase substrate + 6 drops of 50x DAB + 30 drops 10x substrate buffer + 9.6 ml aquadest) to make 10 ml DAB solution, this solution had to be made just before being used) for 1 minutes. The slides were then counterstained using Hematoxylin for 5 seconds (or less). The slides were irrigated using water. The following step, the slides were dehydrated using alcohol 80%, alcohol 95%, and alcohol 100% for 5 minutes respectively. The last step of the immunocytochemistry was purification of the slides using Xyol for a minute before being covered using cover slide, which was joined by glue. Now the slides were ready to be observed. The 3 samples were then compared with the positive control obtained from the testicle tissue.

The trans-differentiation experiment was done using 2 wells of the 24 well plate from 3 prepuce samples. This wells were then administered 300µl DMEM complete high glucose, which was changed every 2 days to prevent dryness and supply nutrient for the cells. The cells for the over-conflury test was finally determined in the fourteenth day, whether there was or no changes to other lineage such as to the chondrocytes. In this experiment, the cell density were examined merely by microscope overall observation.

To observe the over confluent experiment, alcian blue staining was used. This first reagent that had to be formed was 3% acetic acid (3ml glacial acetic acid + 97ml distilled water). Then, 100 ml acetic acid 3 % had to be made which was mixed with alcian blue 1 g, the pH had to be adjusted to 2.5 by acetic acid. The following reagent was 0.1% nuclear fast red solution which were made of aluminum sulfate 5 g and 100 ml distilled water, this solution was finally added nuclear fast red and boiled. After the solution had cooled down, the staining process could be proceeded.

First, the slide had to be hydrated. The next step was administration of 3% acetic acid solution and alcian blue for 30 minutes respectively. Then, it was irrigated using water for 2 minutes. The following step was rinsing by aquadest. The slide was then counter-stained by nuclear fast red solution for 5
minutes. The preceding step was irrigation by water for 1 minute. The slide was dehydrated by alcohol 95% and 100% for 5 minutes respectively. The last step was purification the slide appearance using Xylol for 10 minutes. Now the slide could be observed under the microscope for the existence of chondrocytes. Positive result appeared as blue in the matrix extracellular.32

The data obtained from this research was analyzed by microscope and displayed in the form of pictures which were compared with the known histological tissue of OCT4 staining in testicular tissue and appearance of chondrocytes for spontaneous differentiation.

The Oct-4 positive cells were interpreted using Optilab camera (Optilab, Indonesia), Olympus Microscope (Olympus, Japan). The analysis of Oct-4 cells were based on the 5 high power fields (HPF) with positive signals. The negative cells in the same field were not counted. The total 5 high power fields (HPFs) average were compared from the three samples A, B, and C.

While the analysis of alcian blue staining and keratinocytes culture were accomplished using Zeiss inverted Microscope and Zeiss camera (Zeiss, Germany).

A software of the computer named as “Image Raster” from the Optilab cooperation (Optilab, Indonesia) was used to analyze the images from the microscope. This include cell counting and arrows to describe the images.

A. Transport Medium

The transport medium was PBS 15 ml as well as 2% fungizone and 2% penstrep in 50 ml falcon tube for each sample. Before the sample prepuce was situated in the falcon, the sample was cleaned 3x using povidone iodine and 3x alcohol. This procedure was important to maintain sample sterility. The falcon used for the transport was sterile. In this experiment, the prepuce was located in the transport medium for nearly 48 hours.

B. Isolation Technique

The isolation of prepuce sample was done using dispase enzyme. It composed of dispase II powder for 13.5 mg added with DMEM high glucose 27 ml. Each of the prepuce was expose to this solution overnight in -4 degree Celcius. The next day after washing by PBS and being warmed using water-bath for an hour, samples could be easily separated from the dermis layer. The epidermal layers were incubated overnight again by PBS 5ml and 15µl triple select 1x. The cells were eventually ready to be planted in the well plates.

C. Culture Medium of Keratinocytes

The culture medium of keratinocytes in this research was made out of DMEM high glucose, fungizone 1.5%, penstrep 1.5%, PRP 10%, heparin 1%, and FBS 10%. This culture medium was proven in this research to be viable for the culture of keratinocytes derived from the epidermis (figure 4.3). Heparin was used to prevent clothing of PRP. This is because PRP which is prothrombin which could form clothing.

In this experiment, the cells were not counted using hemocytometry trypan blue. This is because this research is not aiming to acquire certain amount of cells, nevertheless the aim is to conduct a study of the appropriate medium to culture and give rise of keratinocytes in general, without expecting certain number of cells.

Figure 1. Keratinocytes Culture from Day 1-9: A. first-day culture with explant (arrowheads), mag 4x; B. first day culture, mag. 40x; C. second day culture, mag 40x; D. third day culture after trypsinization (explant removal and medium change), mag 40x; E. fourth day culture, mag 40x; F. fifth day culture, mag 40x; G. apoptotic cell culture, mag 40x; H. end of the culture experiment, mag 40x.

Oct4 Characterization in Keratinocytes Derived from Prepuce Skin

Figure 2. Oct4 Positive Immunocytochemistry of The Keratinocytes, magnification 40x: A. Sample A-1; B. Sample A-4; C. Sample B-5.
In this research, the cultured keratinocytes were proven to express oct4 since there were positive signals as depicted above (figure 1). The signals were shown obviously inside the nucleus of the keratinocytes. This sample could be compared with the control positive using testicular tissue. The testicular tissue which is rich in spermatogonia as germ cells express abundant of oct4. The cells were viable under the microscope after immunohistochemistry. The signals of oct4 were found using immunocytochemistry 1:50 primary antibody oct4. The average positive cells were 0.2-0.4 cells of all samples (table 1).

### Table 1. Oct4 Samples Analysis using Five Random HPF Counting

<table>
<thead>
<tr>
<th>Samples</th>
<th>Random High-Power Field (HPF)</th>
<th>Average</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>1 0 0 1 0</td>
<td>0.4</td>
</tr>
<tr>
<td>B</td>
<td>0 0 0 1 0</td>
<td>0.2</td>
</tr>
<tr>
<td>C</td>
<td>0 0 0 1 0</td>
<td>0.2</td>
</tr>
</tbody>
</table>

The counting result of Oct4 positive cells were depicted in the table above (table 1). The average positive cells of sample A, B, and C were 0.4, 0.2, 0.2 respectively. In total, there were four positive Oct4 cells.

### D. Spontaneous Differentiation of Keratinocytes to Chondrocytes

After fourteen days of culturing keratinocytes in a small well of 2 wells of 24 well plate, the cells were then observe of the possibilities of spontaneous differentiation to the other lineage, especially chondrocytes. The small well space was expected to increase the cells over-confluency status for this experiment. To observe this change, Alcian blue staining using counter-stain Kernechtrot solution (nuclear fast red and aluminum ammonium sulfate) were used. The result of this examination was negative. There were no signs of chondrocytes stained blue in the extracellular matrix of the clone.

![Figure 3. Controls of Oct4 ICC Experiment, magnification 40x: A. Represent the positive oct4 in circle, B. negative control (without primary Oct4 antibody) no signal of Oct4.](image)

![Figure 4. Fourteen Days Over Confluency Culture Stained Using Alcian Blue and Counter-stain Kernechtrot, magnification 40x: arrow heads are areas with combination of alcian blue and fast red staining in the extracellular matrix of the cells, this figure may represent some chondrocytes. Nevertheless, the number of this kind of appearance was extremely rare.](image)

### 5. DISCUSSION

#### A. Transport Medium of the Sample

The transport medium in this research is using PBS added with fungizone 2% and penstrep 2%. This proves that PBS can retain epidermis cells for the culture and further use. Even though the samples are processed after more than 48 hours in this experiment, the epidermis derived keratinocytes are still viable and cultured successfully. However, the other layers of the prepuce do not sprout new cells such as fibroblast-like cells during the culture of prepuce for more than ten days. Ideally, the samples must be transported and processed within an hour after obtaining the tissue using PBS. Due to the failure of other layers to sprout, this research cultivates a hypothesis that the layers beneath epidermis might need a more nutritious transport medium and different type of culture medium.

#### B. Method of Obtaining Epidermal Layer

After prepuce is processed using dispase enzymatic solution to separate the epidermis layer from the other layer next day. The next day samples are administered triple select 1x, this enzymatic solution is important to separate each and every cells from each other and the lamina propria of epidermal layer, it is incubated over the night in room temperature. The epidermis layer is not crushed using syringe tip as in Huang. 33

In this research, the cells are simply cut and planted in a medium. Crushing the epidermis layer is not possibly done when using the tip of syringe’s rubber.

Based on the experiment and paper by Somuncu Ö, the samples from younger prepuce can be obtain easier after the treatment using dispase. This could be seen during the process of separating the epidermis layer from the other layers using surgical tools.34 It is highly recommended to use younger samples for better cell growth.

#### C. Culture Medium

Overall, the culture process of this research is successful. Furthermore, it is important to use the medium described earlier for a short-term culture, since the culture of this research is successful to produce large amount of cells in just nine days. The medium must be administered for more than 200µl in over the weekend culture. This is to prevent dryness of the medium.

The culture process of keratinocytes are using DMEM complete high glucose. DMEM high glucose which consist of 4500mg/l glucose is considered potent medium to grow...
keratinocytes culture as proven in this research.

The standard clinically proven keratinocytes culture medium is in vitro lifespan of human epidermal keratinocytes (EpiLife®). This brand is a sterile culture medium, which is usable for a wide period of culture. Unlike the culture medium used in this research, the advantage of EpiLife® culture medium is free of serum. It is noticeable that further research to improve the culture medium of this research is important to be clinically proven. This includes changing FBS to other substance animal free.39

D. Removal of the Remaining Explant

This process requires centrifugation to remove the old medium with the new medium, which at the same time to maintain the cultured cells’ existence and removal of the explant. However, based on this research and paper of J Pawitan, the optimal centrifugation speed to remove the debris from the solution is 800 G-force, which is equal to around 2500 RPM. Instead of 1200x RPM for 10 minutes which is done in this research. It is because the faster RPM is proven to clear the supernatant very well. However, 2500x RPM is hypothesize not to damage the cultured cells.

E. Immunocytochemistry of the Cultured Keratinocytes

The objective of this research, which is to find the OCT4 signals in the prepuse is successfully achieved from the first trial using primary OCT4 antibody of the concentration 1:50. The primer’s control which is testicular tissue is also showing a positive result, it is because of the existence pluripotent germ cells in the testicular tissue which is rich in OCT4.

It is noticeable that the cells found in immunocytochemically stained slides are in various stages. For example, there are small sized keratinocytes, mature keratinocytes with large cytoplasm, and keratinocytes that are losing the nuclear structure, and the last observable finding is the keratin cells, which do not acquire any nuclear organelle.

F. Spontaneous Differentiation of Epidermal Layer

This experiment is tested using ALCian Blue as it would stain chondrocytes in the matrix extracellular in blue. The Alcian Blue is counter-stained using Kernechtrot solution (it is a nuclear fast red stain, contains Aluminum Sulfate). However, because of the lacking of aluminum sulfate reagent in the laboratory, this research optimize the usage of substitution of aluminum ammonium sulfate and aluminum potassium sulfate. Based on both experimentation of control positive tissue from mouse, aluminum ammonium sulfate is proven to be potent reagent of counter-stain combined with Kernechtrot. This is because it does not produce debris such as in aluminum potassium sulfate as substitution.

Corresponding to the negative result, it is important to note that there are no chondrocyte progenitor cells in the beginning of culture process. This is important because the result of over confluent test shows mature and apoptotic keratinocytes. However, if the keratinocytes are cultured using specific kit of differentiation to chondrogenic lineage, there is some possibilities of trans-differentiation.37

6. CONCLUSION AND RECOMMENDATION

A. Conclusion

This research has successfully cultured and checked pluripotency maker of Oct4 positive cells from the epidermal layer. However, the spontaneous differentiation indicates negative chondrocyte cells by alcian blue staining. The culture medium used in this research is DMEM high glucose, fungizone 1.5%, penstrep 1.5%, PRP 10%, heparin 1%, FBS 10%. The average Oct 4 positive cells are 0.2-0.4.

B. Recommendation

- It is much better to transport the sample in less than a day. A perfect sample would be processed an hour after it is obtained
- It is not recommended to crush the epidermal layer using the rubber part of syringe since it is not effective
- Culture medium should be more than 200µl over the weekend
- ICC is recommended to use at least 1:50 primary antibody
- Alcian blue staining is recommended to be counterstained by nuclear fast red mixed with either aluminum sulfate or aluminum ammonium sulfate.

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