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Regenerative Capacity of Post-circumcision Preputial Skin Evaluated by Tissue Expression of Oct-4, Ki-67 and Tenascin C

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Abstract

Background: Induced pluripotent stem cells (iPSCs) using 4 pluripotency transcription factor (c-myc, Klf, Oct-4, Sox) has turned skin fibroblasts into pluripotent stem cells. The aim of this study is to evaluate preputial skin regenerative capacity from three parameters: (i) epidermal cells proliferative capacity, (ii) presence of pluripotent stem cells in preputial skin, (iii) tissue repair prognosis in surgical incision margin.

Method: Design of this study is experimental study in accordance with ethical approval by ethical commission Faculty of Medicine Universitas Indonesia (FMUI)- Cipto Mangunkusumo National General Hospital. Ten post-circumscissed-preputial-skin samples were collected from mass circumscission under signed informed consent. This research is conducted in Jakarta from June 2014 to June 2015. The skin samples were transported in fixative solution to Histology Lab FMUI. After tissue processing, the samples were embedded in paraffin wax, sectioned by microtome and mounted on slides. The slides then underwent hematoxylin-eosin staining and immunohistochemistry specific for Ki-67 (cell proliferation marker), Oct4 (pluripotency marker), and tenascin-C (tissue repair marker). Mean of Ki-67$^+$ cells was observed from five randomly selected high power fields (magnification 400x). Identification of Oct-4$^+$ cells were documented from five randomly selected high power field (magnification 400x). For correlation with gross tissue repair, data was obtained from a follow up interview and physical observation.

Results and discussion: Mean of Ki-67 positive basal keratinocyte in inner mucosal epithelium was relatively higher than outer cutaneous epithelium (5.9 cells per high power field v/s 3.6 cells per high power field respectively). This indicates higher proliferative capacity in inner mucosa epidermal cells. 85.7% of tenascin-c positive samples exhibit normal inflammation resolution thus Tenascin-C may indicate normal tissue repair outcome. 8 out of 10 preputial skin samples had Oct4$^+$ cells, specifically at sites previously reported as skin stem cell's niche i.e. the sebaceous gland, hair root follicle, blood vessel lumen, and hypodermis layer of the skin.

Conclusion: Regenerative capacity of post circumcision preputial skin is indicated by higher proliferation activity in inner mucosa epidermal layer, normal tissue repair outcome as indicated by majority of Tenascin-C expression at wound incision area and presence of Oct-4$^+$ cells in majority of post-circumcision preputial skin.

Keywords: Regenerative tissue; Preputium; Oct4; Ki-67; Tenascin-C

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Introduction

Regenerative medicine is an interdisciplinary field combining principles of engineering and life science to promote regeneration of tissues or organs [1,2]. Regenerative medicine has progressed significantly with induced pluripotent stem cells (iPSCs) technology [3,4]. iPSCs are produced by reprogramming differentiated cells back into a pluripotent state by transducing 4 transcription factors: Oct4, Sox2, c-Myc, and Klf-4 [5,6]. One of the sources of differentiated cells for iPSCs is skin fibroblast. Circumcision is a common practice in Indonesia which yield preputial skin [7,8]. Post-circumcision preputial skin is the potential source for iPSCs as reported by Yu and Thomson [9]. Alternately Aasen et al. [10] reported other cell type from the skin which is keratinocytes as source of iPSC.

Regenerative capacity of preputial skin is investigated by expression of stem-cell marker Oct-4, tissue repair marker Tenascin C, and proliferation marker Ki-67 [11]. Oct4 has been acknowledged as a main marker for pluripotency [12]. One of tissue repair marker expressed in response to tissue injury is tenascin-c [13]. Proliferation marker Ki-67 indicates active mitosis for highly turn-over tissue i.e. preputial skin epidermis [14]. This study aims to describe expression of Oct4, Ki-67, and tenascin-c on different sites of the preputium skin.

Methods

Sample collection

Ten preputial skin samples were collected from 10-16 year old males participating in a mass circumcision held at Panti Asuhan Vincentius held by Tim Bantuan Medis Fakultas Kedokteran Universitas Indonesia in June 2014. Panti Asuhan Vicentius is a foster care facility, in which the children were exposed to similar daily conditions of living and nutrition. Collected samples were immediately kept in a fixative solution (4% formaldehyde) to preserve its quality before further processing in the laboratory.

Histotechnique process

Samples that were preserved in 4% formaldehyde underwent dehydration regimen, which includes putting samples in 70%, 80%, 95%, and absolute ethanol solution. Afterwards, samples underwent clearing by putting them into xylene solution. Samples were then embedded into paraffin wax to be sectioned and mounted on to slides. Samples were processed by different stainings, which include hematoxylin-eosin staining, or immunohistochemistry for tenascin-c, Oct4+, or Ki-67. After the staining process, the samples were covered using coverslip and labeled and further observed under microscope.

Hematoxylin-eosin staining

Samples were de-paraffined in xylol, followed by rehydration in different concentrations of alcohol (absolute, 95%, 70%) and aquadest. Samples were incubated in Harris Hematoxylin, rinsed in water, and counterstained in Eosin. Afterwards the samples were dehydrated again in alcohol (95% and 100%), cleared using xylol, and covered with coverslip.

Immunohistochemistry staining

Samples were de-parafinned in xylol, followed by rehydration in different concentrations of alcohol (absolute, 95%). Samples were rinsed in distilled water, followed by incubation of slides in H_2O_2 3% solution in the moist chamber within room temperature to block endogenous peroxidase. Samples were rinsed in distilled water again, followed by incubation in blocking serum (normal donkey serum 10% v/v) in the moist chamber within room temperature to block non-specific binding. Lastly, primary antibodies aliquots, which have been diluted in normal donkey serum (TNC 1:50, Oct4 1:100, Ki-67 1:50), were added to its designated samples, and incubated overnight in 4°C. Note that negative controls were not added primary antibodies. After the overnight incubation, slides were washed in PBS solutions before incubated using the biotin conjugated secondary antibody inside the moist chamber within room temperature. Slides were rinsed in PBS solutions again, followed by incubation using avidin biotin enzyme reagent within room temperature. Slides were washed in PBS again before incubated with a mixture of Diaminobenzidine (DAB) for TNC and Oct-4 staining and Vina Green for Ki-67 staining, peroxidase substrate, substrate buffer, and distilled water. Afterwards, slides were rinsed in distilled water. Slides stained for tenascin-c were counterstained with hematoxylin. All slides were then dehydrated in different concentrations of alcohol (95%, absolute) and cleared using xylol. Slides were covered using a coverslip.

Microphoto

Microscope images were saved as pictures using the Optilab software. Obtained images were manually marked for immunohistochemistry expressions using the Imageraster software, allowing marking by adding arrowheads, circles, and calculating expression site circumference.

Post circumcision follow up

Exclusively for tenascin-c, the immunohistochemistry data was correlated with data on wound healing progression, which was obtained by a follow up 14 days post-circumcision. To assess the current state of wound healing, subjects were interviewed on the wound healing process and underwent physical observation of the wound-healing site. Results were collected as a filled-in questionnaire and sketches of physical observation findings.

Ethical consideration

This study has received ethics approval by Faculty of Medicine Universitas Indonesia 635/UN2.F1/ETIK/2014.

Results

Subject's characteristics

There were ten samples of prepuce obtained from ten boys aged

10-16 years old during the mass circumcision procedure. All were members of the Panti Asuhan Vincentius (Vincentius Foster Care), thus they are exposed to similar living conditions, nutrition, and hygienic facilities.

Control positive and control negative

Control positive and control negative (Supplementary Figure 1) used were different for each marker: testicular tissue for Oct4, brain tissue sample for Tenascin-c, and breast carcinoma tissue for Ki-67. Positive expression of Oct4^{+} in the testis was shown by the appearance of brown stains in the nuclear regions. While tenascin-c positive brain tissue samples revealed specific binding of tenascin-c within the extracellular matrix marked with brown stain. In the histological section of breast carcinoma, there were green-stained nuclei, which belong to Ki-67 positive cells.

Oct4 is expressed in preputial skin tissues

Oct4+ cells were found in 8 out of 10 subjects. Four specific structures with putative skin stem/progenitor cells were observed for expression of Oct4^{+} cells: sebaceous gland, hair follicle, blood vessel, and hypodermis (Supplementary Figure 2A). However, not all samples appeared to possess these structures (**Table 1**). Hair follicle and sebaceous gland were only present in 1 and 3 samples, respectively. Brown dots appeared in the immunohistochemistry staining of these structures (Supplementary Figure 2B) that indicate the presence of Oct4^{+} cells. Although blood vessel and hypodermis were present in HE stain result of all samples, Oct4^{+} cells in these structures could not be seen in all samples.

Expression of tenascin-c at the wound margin of post circumcised preputial skin

From the tenascin-c positive tissue samples, the author evaluated the length of positively expressed area (Supplementary Figure 3A-E) compared to the length of the incision margin (Supplementary Figure 3F) within the particular HPF. Positive tenascin-c expression area was taken into account when they were found at the wound margin or incision site, and excluded when they were found in blood vessels and dermal connective tissue. Through immunohistochemistry staining, the author was able to evaluate expressions of tenascin-c within the 10 samples (**Table 2**). 7 out of 10 subjects expressed positive expression of tenascin-c ranging from 12%-77%. Meanwhile, the remaining 3 subjects did not exhibit tenascin-c expressions within the 5 randomly selected high power fields (HPF).

Tissue repair and expression of tenascin-c

Based on the interview conducted, 3 out of 10 patients exhibited signs of inflammation thus categorized as “delayed inflammation resolution”. Meanwhile, the other 6 subjects were no longer experiencing signs of inflammation (heat, pain, swelling, and redness) (**Table 3**). Author conducted the physical observation to confirm the previously obtained data from the interview (**Figure 1**). From this examination, 3 subjects who did not confirm the presence of redness during the interview revealed to show redness. One out of the 3 experienced secondary inflammation due to removal of crusts by force. Using 5 random high power fields (HPF) from each sample, we were able to conclude positive expression of tenascin-c in 7 out of 10 patients as seen in **Table 4**.

Ki-67 expression in the inner mucosal and outer cutaneous epithelium of penile preputial skin: We found that the Ki-67 positive cells reside in the basal and parabasal layers of the inner mucosal (Supplementary Figure 3B) and outer cutaneous epithelium (Supplementary Figure 3D) of penile preputial skin. However, we observed that the green-stained nucleus in the parabasal layer was weaker in intensity compared to the Ki-67 green-stained nucleus in the basal layer. The assessed quantification done in this study was the Ki-67 expressing cell found along the basal layer of epithelium, that was the Ki-67 positive basal keratinocyte (**Table 5**) observed in 5 random HPF. The highest quantity of Ki-67 positive basal keratinocyte in inner mucosal epithelium was at a mean of 9.2 positive Ki-67 cells (SD: 2.48), meanwhile the lowest was at 3.4 cells (SD: 1.5), with a total average of 5.9 cells in 7 subjects. The highest quantity of Ki-67 positive basal keratinocyte in outer cutaneous epithelium was at a mean of 4.6 positive basal keratinocyte (SD: 0.49), meanwhile the lowest was at 3.4 positive basal keratinocyte (SD: 0.4). The total average number of Ki-67 positive basal keratinocyte for these five subjects was 3.56 cells.

Table 1 Distribution of Oct4+ cells in The Preputial Skin Tissue of all 10 subjects.

No	Subject	Location of Oct4+ Cells			
		Hair Follicle	Sebaceous Gland	Blood Vessel	Hypodermis
1	AD	N/A	(+)	(+)	(+)
2	AL	N/A	N/A	(+)	(+)
3	CR	N/A	(+)	(+)	(+)
4	DO	(+)	(+)	(-)	(+)
5	FI	N/A	N/A	(+)	(-)
6	PE	N/A	N/A	(-)	(+)
7	RA	N/A	N/A	(-)	(-)
8	SI	N/A	N/A	(-)	(-)
9	ST	N/A	N/A	(-)	(+)
10	TO	N/A	N/A	(+)	(+)

Positive sign (+) : Positive expression of Oct4 in the structures in preputial skin
Negative sign (-) : Negative expression of Oct4 in the structures in the preputial skin
N/A : Structures are not available in the subject’s specimen

Table 2 Immunohistochemistry Result of Tenascin-c at Wound Margin.

No	Subject	Tenascin-c Expression (µm)						Length of Incisional Wound Edge (µm)						Percentage
		HPF 1	HPF 2	HPF 3	HPF 4	HPF 5	Total	HPF 1	HPF 2	HPF 3	HPF 4	HPF 5	Total for TNC positive HPFs	
1	TO	203.2	2307.2	2624.8	1920.4	3652.8	**10708.4**	2429.9	2446.1	3628.8	2401.8	2944.5	**13851.1**	**77%**
2	ST	2324.7	0	0	0	1387.4	**3712.1**	3400.3	1962.1	2061.8	1703.1	1537.8	**4938.1**	**75%**
3	SI	1446	0	0	2166.9	884.7	**4497.6**	1711.9	1937.3	1432.2	1858.2	3085.2	**6655.3**	**68%**
4	PE	316.4	804.6	0	751.8	825	**2697.8**	1877	1846.4	2146.5	1946.1	1858.7	**7528.2**	**36%**
5	DO	718.9	1415.3	461.3	727	382.9	**3705.4**	2713.4	2666.6	2621.4	1435.4	1967	**11403.8**	**32%**
6	AD	0	0	660.2	0	0	**660.2**	1762.8	2152	2145.6	1967.7	2591.7	**2145.6**	**31%**
7	AL	0	0	0	522.2	286.4	**808.6**	1685.4	2213.9	2174.1	4124.8	2467	**6591.8**	**12%**
8	CR	0	0	0	0	0	**0**	1999	2699	1965.5	2117.5	2716.7	**0**	
9	FI	0	0	0	0	0	**0**	2712.6	2261.2	2424.5	2076.3	2466.3	**0**	
10	RA	0	0	0	0	0	**0**	2578.4	2025.8	1871.9	2671.4	1940.8	**0**	

Table 3 Interview Result of Inflammation Characteristic.

No	Name	Pain on Light Pressure	Localized Increase of Temperature	Redness	Swelling	Itching	Wound tissue has dried	Conclusion
1	AD	No	No	No	Mild	Yes	Yes	Normal inflammation resolution
2	AL	No	No	No	No	No	Yes	Normal inflammation resolution
3	CR	No	No	No	**Yes**	No	Yes	**Delayed inflammation resolution**
4	DO	No	No	No	No	No	Yes	Normal inflammation resolution
5	FI	No	No	No	Mild	Yes	Yes	Normal inflammation resolution
6	PE	No	No	No	No	No	Yes	Normal inflammation resolution
7	RA	**Yes**	**Yes**	**Yes**	No	Yes	Yes	**Delayed inflammation resolution**
8	SI	No	No	No	No	No	Yes	Normal inflammation resolution
9	ST	**Yes**	No	No	No	Yes	Yes	**Delayed inflammation resolution**
10	TO	No	No	Mild	No	No	Yes	Normal inflammation resolution

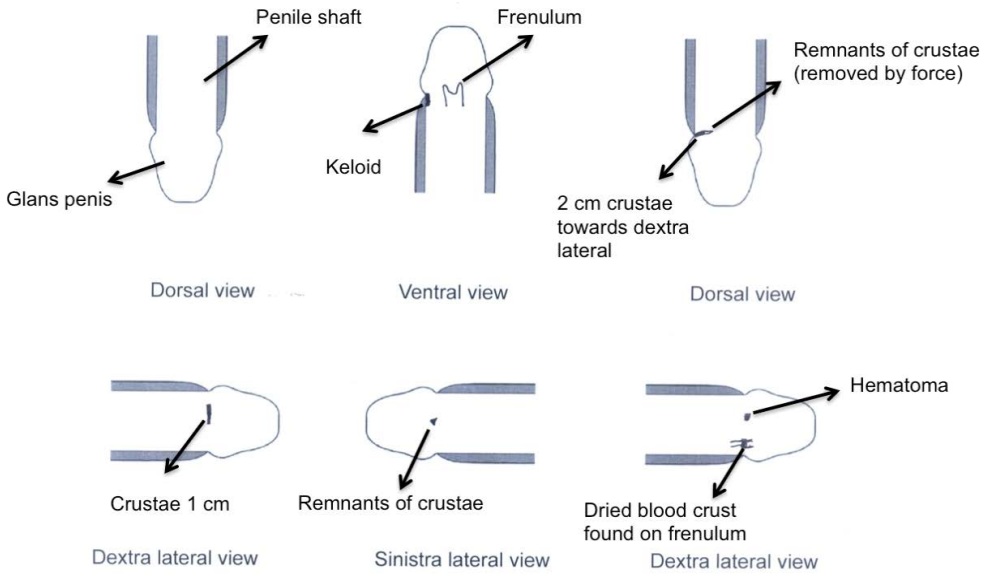

Figure 1 Physical Observation Result Sketch 1 (subject SI), Sketch 2 (subject TO), Sketch 3 (subject CR).

Table 4 Result of Tenascin-c and Inflammation Resolution.

	TNC +	TNC -
Normal	6	1
Delayed	1	2
Total	7	3

Table 5: Number of Ki-67 Positive Basal Keratinocye in Inner Mucosal and Outer Cutaneous Epithelium of Penile Preputial Skin Tissue.

No	Subject	Epithelium	Number of Ki-67 Positive Basal Keratinocyte						
			HPF 1	HPF 2	HPF 3	HPF 4	HPF 5	Mean	SD
1	AL	Inner mucosal	12	10	11	8	5	9.2	2.48
2	ST	Inner mucosal	11	7	8	6	9	8.2	1.72
3	PE	Inner mucosal	7	8	4	5	11	7	2.45
4	DO	Inner mucosal	9	5	5	6	2	5.4	2.25
5	FI	Inner mucosal	5	5	6	1	4	4.2	1.72
6	CR	Inner mucosal	5	6	3	3	3	4	1.27
7	TO	Inner mucosal	5	3	3	1	5	3.4	1.5
							TOTAL	**5.91**	
1	SI	Outer cutaneous	4	4	6	8	3	5	1.79
2	AD	Outer cutaneous	5	4	4	5	5	4.6	0.49
3	ST	Outer cutaneous	4	1	6	7	4	4.4	2.06
4	RA	Outer cutaneous	3	2	4	2	2	2.6	0.8
5	DO	Outer cutaneous	1	2	1	1	1	1.2	0.4
							TOTAL	**3.56**	

Discussion

In this study, 10 boys aged 10-16 years old residing in a foster care participated in this pilot project. 80% of the preputial skin samples in this study showed positive expression of Oct4 as pluripotency marker, which indicates the presence of pluripotency in the preputial skin. This was seen specifically in four locations (sebaceous gland, hair follicle, blood vessel, and hypodermis layer) as mentioned in some study of the skin stem cell [15,16]. The result is supported by a study by Tai, Chang and co-workers [17], stated that Oct4 expression is detected in the breast epithelial, mesenchymal tissue, gastric, kidney, liver, pancreatic and skin epidermal stem cell. Furthermore, Ogliari, Brum and co-workers [16], mentioned that stem cell is present in the microenvironment of the skin.

The presence of $Oct4^+$ cells in hair follicle is in agreement for previous study, conducted by Limbourget et al. [18], Oct4 expressions are found in the skin of postnatal mice. The study used GFP expression in order to get positive expression of the Oct4. The result shown that GFP^+ cells, which indicate pluripotency-related Oct4 expression is high in the hair follicle especially in the first hair cycle. However, the study stated that these $Oct4^+$ populations does not represent pluripotency of the cells in the epidermal environment, yet $Oct4^+$ cells present amongst the multipotent epidermal stem cell may indicates the nuclear, pluripotency-related Oct4 isoform.

The presence of $Oct4^+$ cells in hypodermis seems to be related to the origin of hypodermis in the mesodermal layer. Thus, existence of stem cell in this area is considered as mesenchymal stem cell, which is associated with blood vessels and other structure that involved in the repair mechanism [19]. This explains why $Oct4^+$ cells in hypodermis only present in some of the subject. The content of the hypodermis in the specimen is varies between each subject, which some may have looser connective tissue than the other, indicates the presence of blood vessel. The presence of $Oct4^+$ cells in sebaceous gland corresponds with the existence of sebaceous stem cell [20,21].

Tenascin-C is known to play a role in tissue regeneration [22] and has been identified as one of the extracellular matrix substrates favored by iPSCs [23]. This section focuses on examining the tenascin-c activity within the preputium, particularly at the site of injury and preceding regeneration. It's up-regulation at sites of inflammation, regardless of cause, location, or insult, is best observed at the location of insult, which is at the incision area or wound edges of the dermis [14]. This study provides the initial evidence based research, which confirms tenascin-c expressions found at incision site of preputial skin, as it is positively expressed in 7 out of 10 subjects.

From the interview results, it is revealed that 3 out of 10 subjects exhibit inflammation characteristics. As inflammation is the first phase of wound healing that would typically resolve within 4-8 days post-injury, presence of inflammation characteristics (redness, heat, swelling, and pain) on day 14 would indicate delayed wound healing [24]. These results were confirmed by physical observation conducted by the researcher.

Three subjects revealed redness on observation, despite negatively confirming presence of redness during the interview. One out of the 3 experienced redness as a result of secondary inflammation from removing crusts by force. One subject, who admitted to experiencing redness, was confirmed through observation to experience normal wound healing, however interfered by secondary inflammation from removing crusts by force.

Immunohistochemistry staining reveals that 85.7% (6 out of 7) tenascin-c positive samples exhibit normal inflammation resolution. This supports the association between tenascin-c expression and inflammation resolution. As 66.67% (2 out of 3) tenascin-c negative samples exhibit delayed inflammation resolution, this also favors tenascin-c's role in resolution of inflammation.

Keratinocytes in inner mucosal and outer cutaneous epithelium of the preputium were positive for Ki-67, a cell-marker in proliferation, thus indicating the active process of

proliferation that eventually results in replacement of injured cells to become its appropriate structure [11,13]. In the future, expression of proliferation marker that in turn can propose as an indicator whether the tissue can massively regenerate a certain differentiated cell type. This study pointed out positive expressions of Ki-67 as green-stained nucleus situated in basal and parabasal layers of the epithelium. Unlike previous studies that showed Ki-67 positive cell as brown-stained nucleus, this study used different chromogen substrate, Vina Green, to detect the presence of antigen rather than 3,3-diaminobenzidine (DAB) + peroxidase substrate [25,26]. The reason for this purpose was to avoid misinterpretation of Ki-67 positive cell and the brown-colored melanin pigment found in the basal layer of the epithelium.

In accordance to Biradjar and co-workers [27], basal and parabasal layers in the epithelium showed to be a site for actively proliferating cell. The basal layer serve as a bed for basal keratinocyte, meanwhile the parabasal layer that sits on top of it is the site for migrating cells that undergo active proliferation. We found that the green stained nucleus in the parabasal layer showed weaker intensity compared to those lying on the basal layer. This result was contradictory to a study conducted by Edriss [28] in 2005 aimed to look for the expression of Ki-67 antigen in epidermal keratinocytes located basal, parabasal and intermediate zones acquired from skin biopsy specimen.

This study also showed that the inner mucosal epithelium of penile preputial skin has a higher mean number of Ki-67 positive basal keratinocyte of 9.2 cells. This result suggests higher proliferation rate in the inner portion of preputial skin compared to the outer portion of preputial skin that showed 3.56 cells with Ki-67 positive expression. This may be due to difference in activities of various cytokines, such as epidermal growth factor, keratinocyte growth factor, interleukin-1 and transforming growth factors alpha and beta). From the present work, the researcher could not determine the possible factor that contributes to the variation in epithelial proliferation. Further research would be necessary to address this issue.

In conclusion, Regenerative capacity of post circumcision preputial skin is indicated by higher proliferation activity in inner mucosa epidermal layer, normal tissue repair outcome as indicated by majority of Tenascin-C expression at wound incision area and presence of Oct-4+ cells in majority of post-circumcision preputial skin.

Conflict of Interest

The authors declare that there is no conflict of interest.

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