Isolation and Characterization of Fibroblast from Normal and Thalassemia Foreskin

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Abstract

Thalassemia is a genetic blood disorder impacting hemoglobin production, varies in severity, and requires lifelong treatments. The advancement in somatic cell reprogramming through induced pluripotent stem cells (iPSCs) represents a personalized medicine approach that holds promise as a treatment for individuals with thalassemia. One source that could be reprogrammed into iPSCs can be generated from foreskin fibroblast cells. This study aimed to isolate and characterize human fibroblast cells from normal (NFF) and thalassemia (TFF) foreskin surface markers (CD44, CD90, CD105, CD73), and negative lineage (CD45, CD34, CD11b, CD19, HLA-DR). Fibroblast cells were isolated out of normal and thalassemia foreskin using the explant method. Characterization of NFF and TFF isolates was carried out using flow cytometry to disclose the expression of CD90, CD44, CD73, CD105, and negative lineage. Isolation of fibroblast cells from normal and thalassemia foreskin was successfully carried out using the explant method with NFF and TFF characterized as expressing CD90, CD44, CD105, CD73, and negative lineages (CD45, CD11b, CD34, CD19, and HLA-DR). This result could lead to a continuation of reprogramming into iPSCs.

Keywords: Characterization, Fibroblast, Flow cytometry, Isolation, Thalassemia

Introduction

Thalassemia is a genetic disease that affects the hemoglobin (Hb) production process due to mutations in the genes for the formation of α and β globin chains [1]. There are various types of thalassemia, ranging from clinical manifestations without symptoms to severe symptoms. Thalassemia is categorized into major, intermedia, and minor types, depending on its classification. Thalassemia major patients require lifelong blood transfusions with indications of suffering from severe anemia [2]. Thalassemia intermedia is identified by mild to moderate anemia, often necessitating treatment such as occasional blood transfusions. On the other hand, individuals with thalassemia minor typically exhibit good physical health and generally do not need blood transfusions [3]. Epidemiological data shows that 300.000 - 500.000 newborn babies

suffer from Hb abnormalities, and 50.000 to 100.000 children succumb to thalassemia [4,5]. The incidence of thalassemia patients is believed to be more pronounced in Asia, the Middle East, and the Mediterranean regions compared to North America or Europe [6]. In Indonesia, 5.8 % of the 12,038 people examined were detected as carriers of thalassemia, and every year, 2,500 babies are born with thalassemia [7].

Most thalassemia patients require lifelong clinical treatment using routine blood transfusions and chelation therapy [4,8]. A concern in thalassemia patients who require chronic blood transfusions is iron overload. This leads to significant complications, including cardiomyopathy, diabetes, osteoporosis, endocrinopathy, hearing disorders, and growth failure [9]. Deferiprone, deferoxamine, and deferasirox are 3 widely used and commercially available chelation therapy agents. Overall, chelation therapy aims to maintain safe levels of iron in the body. However, the 3 currently available chelation agents have different safety profiles, benefits, and patient responses [10].

The innovative aspect of personalized medicine lies in somatic cell reprogramming's process using induced pluripotent stem cells (iPSCs). It has the potential to address thalassemia by targeting specific genes in the treatment of patients. Wang *et al*. [11] carried out a study indicating that the editing of the β-globin gene in induced pluripotent stem cells (iPSCs) attained from skin fibroblasts of thalassemia patients with a homozygous 41/42 deletion in the β-globin gene can be rectified through homologous recombination, showcasing potentiated clinical applicability. The advancement of somatic cell reprogramming produced by induced pluripotent stem cells (iPSCs) is a personalized medication that could be used to treat thalassemia patients through gene editing of mutated DNA. iPSCs were successfully created for the first time using adult fibroblast cells, and until now, fibroblast cells have been widely used for iPSCs [22]. Fibroblast cells can be isolated from various anatomical sites, one of which is the foreskin [23].

Human foreskin tissue removed after circumcision can be a valuable source for isolating fibroblast cells that can be used in several fields of research and medicine [12]. Fibroblasts are referred to as cells that create and maintain anatomically diverse extracellular matrix (ECM)-rich connective tissue to support various important organ functions, such as resistance to blunt and sharp injuries to the skin or stretching of entire organs and elastic rolling in intact respiratory lungs. Fibroblasts provide important information and positioning to surrounding cells through microarchitectural, biochemical, and biomechanical cues in the ECM and regulate the secretion of soluble mediators [13].

Fibroblasts share numerous markers with Mesenchymal stem cells (MSCs), such as surface markers (CD44, CD90, CD105, and CD73) and negative lineage (CD45, CD34, CD11b, CD19, and HLA-DR). They can be prompted to transform into adipocytes, chondrocytes, and osteoblasts. Additionally, like MSCs, fibroblasts exhibit immunomodulatory properties [14]. In normal tissues, MSCs actively participate in maintaining tissue homeostasis. They follow a shared pathway and display common traits, such as a spindle-shaped structure, presence in connective tissue, and the capacity to undergo differentiation into various cell types [15]. With similar characteristics, fibroblast cells can be characterized with the same markers as MSCs. This study aimed to isolate and characterize normal foreskin fibroblast cells, examining thalassemia surface markers (CD44, CD105, CD90, and CD73), as well as negative lineage (CD45, CD34, CD19, CD11b, and HLA-DR).

Materials and methods

Analysis of globin gene mutations in thalassemia patients

Around 5 mL of blood samples were gathered from 3 individuals with thalassemia, utilizing tubes containing the anticoagulant EDTA (BD vacutainer 5 mL). Globin gene mutation analysis was conducted on blood samples [16]. Subsequently, DNA extraction was carried out for 1 h and followed the procedure based on the manufacturer's protocol (DNA Prep Kit, Hybribio Medicine Technology, Ltd, Guangzhou, China). Meanwhile, PCR amplification and flow-through hybridization methods followed the manufacturer's protocol as well (HBGA-THAL-b31, Hybribio Medicine Technology, Ltd, Guangzhou, China). DNA amplification was then conducted using PCR Mix1 and Mix2. PCR Mix1 and Mix2 were thawed at room temperature. Centrifugation of DNA Taq Polymerase and PCR Mix was performed for 60 s at 8000 rpm. Amplification reagents was vortexed and set in PCR tube. As much as 5 µL of extracted DNA was added to each PCR Mix tube. Centrifugation of DNA and amplification reagents was done briefly. The PCR tube was moved to the PCR thermal cycler instrument afterwards. PCR amplification was executed for approximately 3.5 h. The temperature set for the PCR program was initiated at 95°C for 15 min, followed by 97 °C for 50 s, 60 °C for 60 s, 72 °C for 90 s, and 72 °C for 10 min. The cycles of 35 was carried out. Denaturation program was set at 95 °C for 5 min. PCR reagents were placed at −20°C when not in use.

Flow-through hybridization was conducted subsequently. The biotinylated PCR amplified samples were denatured for 5 min in 95 °C and was chilled on ice immediately. HybriMax was set to 42 °C. Pre-warmed Hybridization solution in 42 °C was added as much as 0.8 mL. Incubation was performed for 3 min. The solvent was then pumped away. Pre-warmed Hybridization solution was added to designated sample wells. The amplified DNA samples were placed into wells and subjected to incubation at 42 °C for 30 min. Pre-warmed WB1 was employed to wash the membrane. The HybriMax was then set to 25 °C. At this temperature, the materials used were blocking solutions and enzyme conjugates. Before the temperature was changed to 36 °C, the membrane was washed using Solution A for 4 times. The materials added to 36 °C HybriMax were NBT/BCIP solution and hybridization solution. Washing with the hybridization solution was done 3 times. Lastly, the membrane was rinsed with distilled water. The separator and fixing cover were removed afterwards. DNA amplification, flow-through hybridization, and result interpretation referred to the Thalassemia GenoArray Diagnostic Kit (Hybribio, HBGA-THAL-b31). Interpretation was carried out by color visualization for 1 h.

Isolation of normal and thalassemia foreskin fibroblasts

Foreskin was obtained by the circumcision method in children aged 3 - 15 years with ethic number NO: 016/KEP/II/2023. Circumcision was performed on 1 normal subject and 3 thalassemia patients. Previously, the 4 donors had undergone examinations including routine hematology in normal subjects and globin gene mutation analysis in thalassemia patients. The foreskin was put into a 15 mL tube (SPL, 50015) containing Dulbecco's Phosphate Buffered Saline 1x (Biowest, X0520-500), 1 % antibiotics - antimycotics (Biowest, L0010-100), and 1 % Penicillin-Streptomycin-Amphotericin B Solution (100×) (Elabscience, PB180121) in an ice bag. The foreskin was positioned in a petri dish (100 mm) and washed with 1× PBS containing 1 % Antibiotic-Antimycotic (ABAM) (Biowest, L0010-100) through 5 wash cycles until the foreskin was free of blood. The epidermis and dermis layers of the foreskin were separated, and the dermis part of the foreskin was chopped (1 - 2 mm). The tissue is placed in a 6-well plate, and let stand for 1 h (until the tissue adheres) [17]. Added to each well 1 mL of complete medium containing MEM-α (Biowest, L0475-500), 20 % Fetal Bovine Serum (FBS) (Biowest, S181B-500), 1 % Nanomycopulitine Concentrate 20× (Biowest, L-X16-100), 1 % ABAM, 1 % Amphotericin B (Biowest, L0009-100), 1 % MEM Non Essential Amino Acids 100× (Biowest, X0557-100), 1 % L-Glutamine 100× (Biowest, X0550-100), 1 % MEM Vitamins 100× (Biowest, X0556-100), 1 % MEM Non Essential Amino Acids 100× (Biowest, X0557-100), and 0.1 % Gentamicin (Gibco, 2517932). Explants were incubated for 1 week in a 5 % CO_2 incubator at a temperature of 37 °C (Thermo Scientific, 8000DH) [18]. Observation of cell growth was carried out with an inverted microscope (Olympus, CKX41-F32FL), then cells in 6-well plates were harvested and transferred into a T75 tissue culture flask ± 2 weeks after isolation [19,17].

Normal and thalassemia foreskin fibroblast cell culture

The foreskin fibroblast cell culture method modified from Jagadeeshaprasad *et al*. [20]. Harvest of normal and thalassemia foreskin fibroblast cells was done by removing the culture medium. The cells were rinsed twice with 1 mL of 1× ABAM. 1 mL trypsin EDTA 0.25 % (Biowest, L0932) was applied and incubated for 5 min in a 5 % CO_2 incubator at 37°C. Cells detachment was confirmed by observing the cells under an inverted microscope. To halt trypsin activity, 1 mL of MEM α basal medium was introduced. Subsequently, the cell suspension was moved to a 15 mL centrifuge tube and subjected to centrifugation at 4000 rpm for 5 min. The supernatant was removed, the pellet was mixed with 500 µL of fetal bovine serum (FBS), and the resulting cell suspension was transferred into a 75-flask containing complete MEM α medium with 10 % FBS. Cell growth was observed and the medium was refreshed every 48 h. Cells were propagated at 80 % and sub cultured until ready for characterization.

Characterization of normal and thalassemia foreskin fibroblast cells

Fibroblast cells that had reached passage 4 with 80 % confluency were harvested for counting and markers analysis using flow cytometry (Macsquant, Analyzer 10, Miltenyi Biotec, Germany). The cells were stained with distinct antibodies (CD90 FIT C, CD44 PE, CD105 PerCP-Cy5, CD73 APC, and negative lineage CD45 PE, CD34 PE, CD11b PE, CD19 PE, and HLA-DR PE) according to the manufacturer's protocol BD StemflowTM hMSC Analysis Kit (BD 562245). Surface marker measurement were conducted in triplicate [21].

Results and discussion

Globin gene mutation analysis in blood samples of thalassemia patients

The results of globin gene mutation analysis in 3 blood samples from thalassemia patients is discernible in **Table 1**. The results of the analysis show that 3 donors had positive thalassemia with different types of globin gene mutations. The globin gene mutations for each patient were IVS-I-5 G->C, Del 4,2 kb, and Codon 26 (GAG to AAG) or HbE, respectively.

Table 1 Identification of α-globin and β-globin gene mutations in blood samples from thalassemia patients

Case	Genotypes	Mutation	HGVS Name
Patient 1	Homozygous IVS1nt5 (G > C)	IVS-I-5 G- > C	HBB: c.92+5G > C
Patient 2	Heterozygote of 4.2kb-alpha thalassemia 2	Del 4,2 kb	4.2 kb deletion involving the alpha2 gene alpha-Thal-2
Patient 3	Compound heterozygote of IVS1nt1 and HbE	Codon 26 (GAG to AAG) or HbE	HBB: c.79G > A

Normal and thalassemia foreskin fibroblast cells culture

Result of fibroblast cells morphology of NFF and TFF cells can be seen in **Figure 1**. NFF and TFF cells began to appear on day 7 after planting explants in 6 well plates. After the 14th day, the cells had reached 50 % confluence, then the explant was carefully removed, and the cells were re-cultured. In **Figure 1**, it can be seen that there is no difference in the morphology of NFF and TFF cells. NFF and TFF cells are spindle-shaped, like fibroblast cells. On day 21 the cells had reached 80 % confluence.
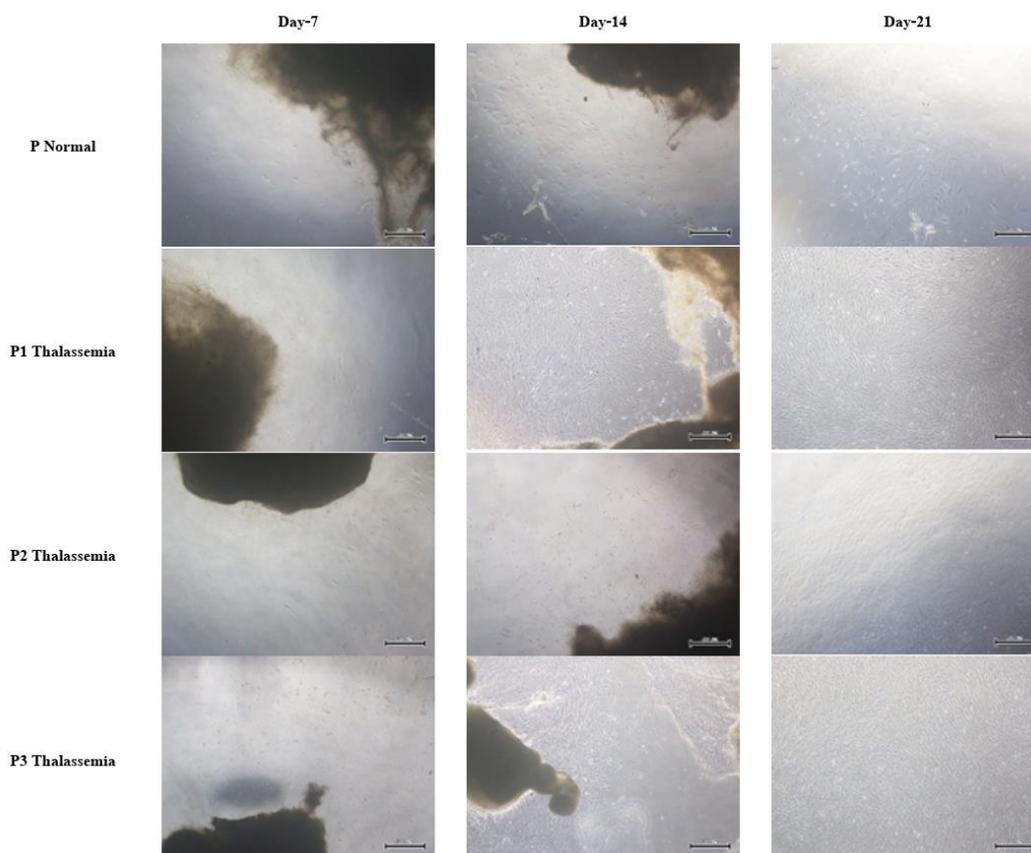

Figure 1 Normal and thalassemia foreskin fibroblast cell culture. *P Normal: Patient Normal; P1 Thalassemia: Patient 1 Thalassemia; P2 Thalassemia: Patient 2 Thalassemia; P3 Thalassemia: Patient 3 Thalassemia.

The results showed that fibroblast cells were successfully isolated and cultured from the foreskin of normal subjects and thalassemia patients within 21 days. These findings corroborate previous studies that have extracted fibroblasts from various anatomical sites including the neck, breast, arms, abdomen, and thigh dermal tissues, demonstrating consistent spindle-shaped fibroblast morphology across these diverse locations [24].
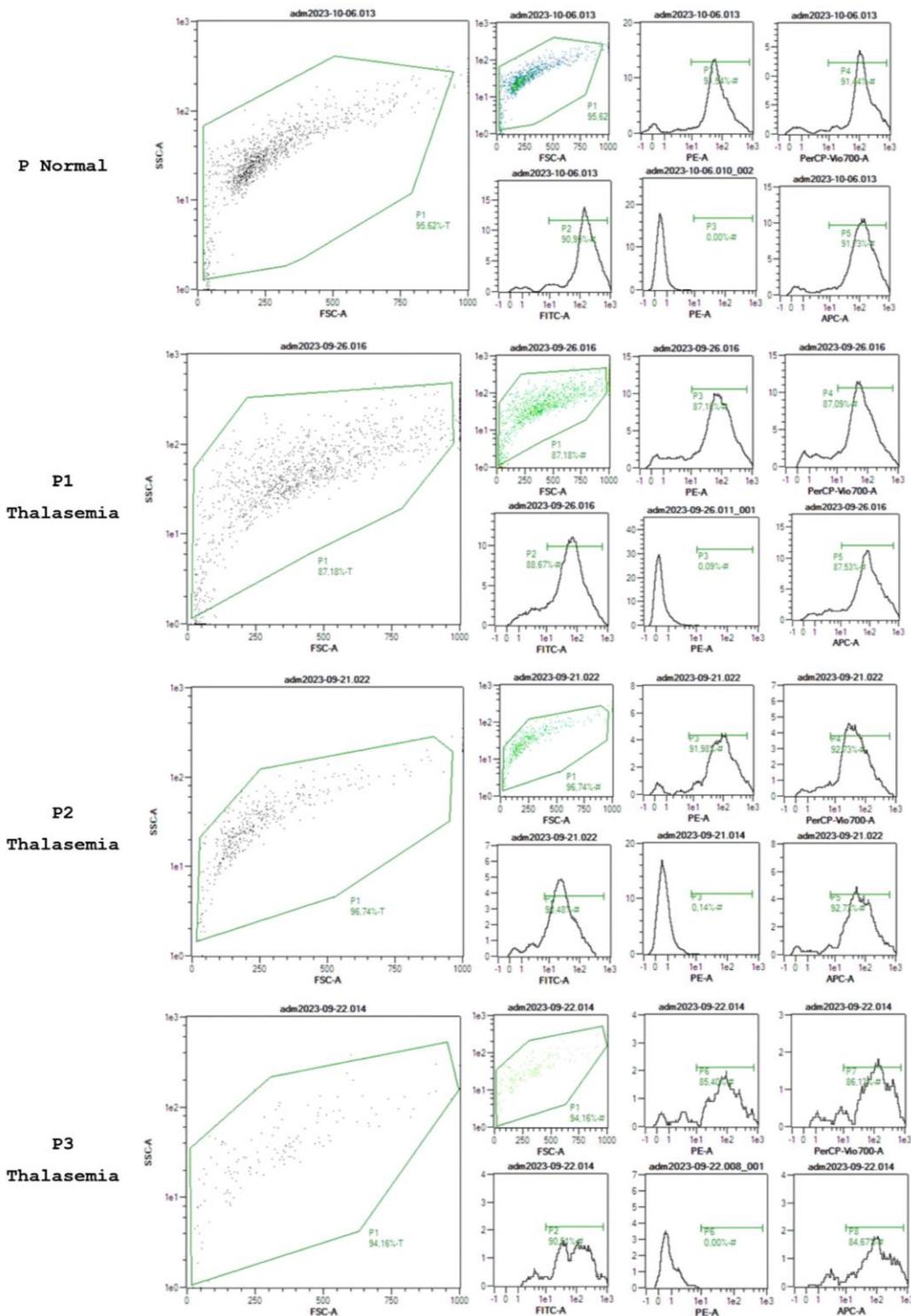

Figure 2 Dot blot representative of positive and negative lineage surface markers of NFF and TFF. *P Normal: Patient Normal; P1 Thalassemia: Patient 1 Thalassemia; P2 Thalassemia: Patient 2 Thalassemia; P3 Thalassemia: Patient 3 Thalassemia.

Characterization of normal and thalassemia foreskin fibroblast cells

The isolated markers, including CD44, CD90, CD105, CD73, and the non-appearance of negative lineage markers (CD45, CD34, CD11b, CD19, and HLA-DR), closely resemble those of MSCs. **Tables 2** and **3** display the proportions of positive and negative lineage surface markers for NFF and TFF. Dotblot representation of NFF and TFF's surface markers can be seen in **Figure 2**. The fibroblasts on every patient at 3 repetitions show the MSCs characteristic. Normal subject exhibited fibroblasts positive markers percentages, including CD44, CD90, CD105, and CD73, with an average of > 90 % Meanwhile, lineages negative for CD45, CD34, CD11b, CD19, and HLA-DR exhibited an average percentage of 0.18 %. In Thalassemia patients, fibroblasts demonstrate a positive expression of over 84 % for DC90, CD73, CD44, and CD105, while exhibiting a negative expression of less than 0.19 % for negative lineages such as CD34, CD19, CD45, CD11b, and HLA-DR.

Numerous studies indicate that fibroblast cells exhibit similar characteristics to those of mesenchymal stem cells (MSCs). Non-hematopoietic stromal cells MSCs have the capacity to regenerate and modulate the immune system. They are recognized as a source of reparative cells [25]. Fibroblast cells exhibit similar markers to MSCs, including the existence of the glycoproteins CD90, CD44, CD105, and CD73 [14]. CD90 is a cell surface glycoprotein that is involved in regulating diverse cellular functions, including the processes of proliferation and differentiation [26]. CD44 is a receptor for hyaluronan, which plays a role in migration and adhesion. CD105 functions as a transmembrane glycoprotein involved in regulating cell growth and angiogenesis, whereas CD73 is an enzyme that participates in the control of immune responses and inflammation. [27]. These markers are commonly used to identify and characterize MSCs [28,29]. The results showed that fibroblast cells isolated from normal subject and thalassemia patients were positive for CD44, CD105, CD90, and CD73 (**Tables 2** and **3**). The results were consistent with prior studies, which denoted that fibroblast cells were positive for CD90, CD44, CD105, and [14,30].

In fibroblast characterization, CD34, CD19, CD45, CD11b, and HLA-DR were also used as negative lineage markers. That protein is found in blood cells or the immune system and is not found in MSC cells [31]. The results showed that the isolated fibroblasts had a value of 0 % in lineage-negative, which means the cells were pure fibroblast cells and were not mixed with blood cells (**Tables 2** and **3**). Thus, the isolated cells that have been characterized as fibroblast cells could be reprogrammed into IPSCs.

Table 2 Percentage of positive and negative lineage surface markers of NFF.

Sample	Repetition	CD44 (%)	CD90 (%)	CD105 (%)	CD73 (%)	Neg. Lin (%)
P Normal	1	91.54	90.95	91.44	91.73	0
	2	92.7	92.89	93.56	92.22	0.35
	3	93.29	93.61	91.77	91.34	0.18
	Average	92.51 ± 0.89	92.48 ± 1.38	92.26 ± 1.14	91.76 ± 0.44	0.18 ± 0.18

Table 3 Percentage of positive and negative lineage surface markers of TFF.

Sample	Repetition	CD44 (%)	CD90 (%)	CD105 (%)	CD73 (%)	Neg. Lin (%)
P1 Thalassemia	1	87.18	88.67	87.09	87.53	0.09
	2	85.53	88.02	86.34	84.72	0
	3	85.72	87.31	86.04	85.46	0
	Average	86.14 ± 0.90	88.00 ± 0.68	86.49 ± 0.54	85.90 ± 1.46	0.03 ± 0.05
P2 Thalassemia	1	91.98	90.48	92.73	92.73	0.14
	2	91.03	87.62	93.61	94.02	0

Sample	Repetition	CD44 (%)	CD90 (%)	CD105 (%)	CD73 (%)	Neg. Lin (%)
	3	94.34	93.06	95.32	96.25	0
	Average	92.45 ± 1.70	90.39 ± 2.72	93.89 ± 1.32	94.33 ± 1.78	0.05 ± 0.08
P3 Thalassemia	1	85.4	90.51	86.13	84.67	0
	2	80.9	86.63	81.6	80.38	0
	3	87.39	93.69	86.49	88.29	0.58
	Average	84.56 ± 3.32	90.28 ± 3.54	84.74 ± 2.73	84.45 ± 3.96	0.19 ± 0.33

Conclusions

Fibroblast cells from the foreskin of normal and thalassemia patients were successfully isolated using the explant method. Fibroblast cells from the foreskin of normal and thalassemia patients were detected expressing CD44, CD105, CD90, CD73, and negative lineages for CD45, CD34, CD11b, CD19, and HLA-DR.

Acknowledgments

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