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Original Investigation

Effect of Hepatic Differentiation on Fatty Acid Composition of Induced Pluripotent Stem Cells Derived from Human Dermal Fibroblasts

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Abstract

Introduction: Human induced pluripotent stem cells (hiPSCs) have been recognized as key progenitor cells for liver regeneration, which could potentially be used in the repair of liver injury. Differentiation of stem cells involves specific changes in lipid metabolism to form competent hepatic cells. However, the relationship between the hepatic differentiation of hiPSCs and change in cellular lipids has not been well characterized. The aim of the present study was to determine the fatty acid pattern during hepatic differentiation of hiPSCs derived from dermal fibroblasts.

Methods: Hepatocyte differentiation was induced in three stages using Wnt family member 3A, hepatocyte growth factor, and oncostatin M and was impaired by a chemical extracellular signal-regulated kinase signaling inhibitor. Hepatocyte-specific metabolic markers including gamma-glutamyltransferase and aminotransferases were evaluated using kinetic chromogenic assay kits. During the hepatic differentiation of hiPSCs, changes in individual fatty acids were determined at multiple time points using gas-liquid chromatography.

Results: Significant increases were observed in hepatic enzyme markers during hepatic differentiation. Endodermal induction produced a transient increase in saturated fatty acids (33%, $p<0.01$) and decrease in monounsaturated fatty acids (-15%, $p<0.01$). Total n-6 polyunsaturated fatty acid was elevated in undifferentiated hiPSCs, and a gradual downward trend was observed after endodermal induction (-10%, $p=0.08$) and hepatic lineage commitment (-19%, $p<0.01$). All of these changes in metabolic differentiation markers and individual fatty acids were suppressed by impaired induced hepatic differentiation.

Conclusion: Our findings indicate that the pattern of cellular fatty acids is dynamic and changes with the progress of hepatic differentiation, which includes both transient fluctuations and linear trends.

Keywords: Adult stem cells, cell lineage, endoderm, lipids

Introduction

Human induced pluripotent stem cells (hiPSCs), mostly derived from fibroblasts, have been recognized as key progenitor cells for liver regeneration, which could potentially be used for the repair of liver injury. The process of hepatic differentiation involves the sequential expression of hepatic markers such as α-fetoprotein, albumin, urea, and liver-specific metabolic enzymes, including aminotransferases (1, 2). In addition, hepatic differentiation is marked by major metabolic changes related to lipid metabolism (3-5). Such specific changes in lipid metabolism are important for the formation of competent hepatic cells. The unique metabolomics pattern of hiPSCs has been described in detail in another study; as identified some lipid species were associated with maintaining pluripotency(6). However, the association of hepatic differentiation of hiPSCs with the expression of hepatic enzyme markers and change in cellular lipids has not been well characterized.

Cellular lipid composition is tightly regulated by de novo synthesis, metabolic conversion, and degradation processes. However, the level of multiple lipid species, including fatty acids, may be influenced by the process of hepatic differentiation. Owing to this complexity, an assay for these changes is not readily available. Alteration of the cellular fatty acid pool affects cell membrane functions and signaling pathways that control cell fate determination. Furthermore, fatty acid metabolism is key to guaranteeing adequate energy supply during specific lineage differentiation (7). This indicates that such perturbations strongly impact adipogenic and cardiac and neuronal differentiation (8, 9). Therefore, profiling cellular fatty acids during hepatic differentiation may be a valuable addition to the study of hepatic cells. In a previous study, we have reported a coordinate gene expression and activity index pattern of a fatty acid-metabolizing enzyme during hepatic differentiation of hiPSCs (10). The aim of the present study was to characterize patterns of individual fatty acids during hepatic differentiation of hiPSCs. In the present study, we describe hepatic enzyme markers and fatty acid composition at different stages of in vitro differentiation of hiPSCs that are generated from fibroblasts. Different fatty acid patterns occur in cells undergoing hepatic differentiation. Furthermore, these specific changes in fatty acid pattern were suppressed by impaired induced hepatic maturation.

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Methods

The present study was approved by the local ethics committee. Skin fibroblasts used for hiPS cell generation were obtained from a single donor who provided written informed consent (11).

All culture materials were purchased from Invitrogen/GIBCO (Carlsbad, CA, USA). All other materials were from Sigma, unless stated otherwise. Conditioned media and cell extracts from a human hepatoma cell line (HepG2, National Cell Bank, Pasteur Institute of Iran) served as positive controls (12).

Cell culture and induced differentiation

Expansion medium comprised mouse embryonic fibroblast feeder cells inactivated with mitomycin C, Iscove's Modified Dulbecco's Medium, and 20% knockout serum replacement, supplemented with 100 ng/mL basic fibroblast growth factor, 1 mmol/L L-glutamine, 100 µM nonessential amino acids, 50 U/mL penicillin, and 50 mg/mL streptomycin. The hiPS cell line, R1-hiPSC9, was generated from harvested human skin fibroblasts by means of viral transduction of reprogramming factors (Oct4, Sox2, Klf4, and c-Myc) as described in another study (11). This cell line reportedly expressed the pluripotency-associated marker Oct-4 and was able to spontaneously differentiate into the three embryonic germ layers in vitro (11, 13). The hiPSCs were passaged every 5-7 days.

The method of hepatic differentiation was similar to that used in a previous study (12) and according to that in the study by Chen et al. (14). Briefly, the differentiation protocol included endodermal induction, hepatic lineage commitment, and hepatic maturation stages.

Impaired induction of hepatocyte differentiation

The inhibition of the mitogen-activated protein kinase/extracellular signal-regulated kinase (ERK) signaling pathway is known to negatively affect hepatic differentiation of stem cells (15). In an alternate approach to control the effect of differentiation, the ERK inhibitor, PD98059, at a concentration of 5 µM was added from days 2-10 during the hepatic differentiation of hiPSCs.

Determination of hepatic enzymes

For the quantification of extracellular and intracellular hepatic markers, the culture medium was collected from each well and centrifuged at 800g at 4°C for 10 min, and the supernatant was filtered to remove cell bodies. Conditioned medium was then concentrated approximately 25× using ultrafiltration units with a molecular weight cut-off value of 10 kDa (Amicon Ultra-2, Millipore, MA, USA). Finally, the collected medium, containing secreted hepatic markers, was stored at –80°C until analyzed (16). After the removal of culture medium, the cells in each well were washed with PBS and harvested in a lysis buffer containing 1% SDS, 10 mM EDTA, 50 mM Tris-HCl, and pH 8.1. The lysates were centrifuged, and the supernatant was stored as described above.

Protein concentration of the lysate was determined by the Lowry protein assay using bovine serum albumin as the standard (17). Enzyme activities were measured using commercially available kinetic chromogenic assay kits on a BT 3000 autoanalyzer (Biotecnica Institute, Italy). Determination of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) was based on the oxidation of 2-oxoglutarate, leading to the indirect oxidation of nicotinamide adenine dinucleotide (NADH). Gamma-glutamyltransferase (GGT) assay was based on the monitoring of the enzyme-catalyzed transfer of γ-glutamyl group from the substrate L-γ-glutamyl-p-nitroanilide, liberating the chromogen p-nitroanilide. Measurement of lactate dehydrogenase (LDH) in both the fractions was based on the consumption of NADH in the reduction of pyruvate to L-lactate.

Gas-liquid chromatography of cellular fatty acids

Total lipids were extracted from the whole cell lysate using the conventional Bligh-Dyer method (18). Fatty acid methyl esters were prepared by transesterification of the extracted lipids using methanol with acetyl chloride, acting as an acid catalyst, at 100°C (19). Fatty acid methyl esters formed were extracted using 1.5 mL of n-hexane, dried under N_2, and reconstituted into 0.5 mL n-hexane for analysis.

Data from gas-liquid chromatography (GLC) were obtained using a Buck Scientific model 610 gas chromatograph (SRI Instruments, Torrance, USA) fitted with a highly polar biscyanopropyl polysiloxane capillary column (TR-CN100 60 m×0.25 mm id, 0.2 µm film thickness; Teknokroma, Spain). The gas chromatograph was equipped with a split injector, a flame ionization detector, a hydrogen generator (H_2 NM Plus; LNI Schmidlin, Switzerland), a computer interface, and PeakSimple software version 3.59. The operating conditions for GLC were: injection, 1 µL, split mode (1:90) with 60 s valve time; carrier gas, helium at 20.0 cm/s; and injector and linear temperature, 250°C. The oven temperature of GLC was programmed as follows: 5 min at 190°C then ramped to 210°C at 1°C/min and then isothermal for 15 min.

Statistical Analysis

Data were presented as mean ± standard deviation of three separate experiments performed in duplicate. Statistical differences between the groups were assessed using analysis of variance with post-hoc Tukey's test for multiple comparisons. A $p<0.05$ was considered to be statistically significant. Data were analyzed using statistical functions in Microsoft Excel (Microsoft Corporation, Redmond, WA, USA).

Results

In a previous study, the differentiation protocol used was efficient at directing hiPSCs toward a hepatic-like lineage *in vitro*, which was confirmed by a robust increase in specific markers, including HNF4α, CYP7A1, α-fetoprotein, albumin, and urea (10). An increase in the levels of ALT, AST, and GGT was detected from day 3 to day 7 (Figure 1a). No significant alteration was noticed in the cell-associated lactate during the process of hepatic differentiation (Figure 1b). The highest level of GGT in cells and media was observed 12 days after induced differentiation, followed by a significant decrease on subsequent days. Marked increases in the levels of ALT and AST were noted from day 7 to day 15 at the end of the protocol. These findings in cellular extracts were corroborated by similar secretion patterns of ALT, AST, and GGT (Figure 1c). Fully differentiated hiPSCs exhibited enzyme marker levels comparable to those

exhibited by HepG2 control cells ranging from 83% to 91%. Inhibition of the ERK signaling pathway markedly decreased the levels of α-fetoprotein, albumin, urea (data not shown), ALT, AST, and GGT (Figure 1a, c) in differentiating hiPSCs, indicating impaired hepatic differentiation. There was also no significant change in culture medium lactate during the process of hepatic differentiation (Figure 1d), suggesting that no significant lactate release occurred.

Oleic acid was the major fatty acid in undifferentiated hiPSCs, followed by palmitic acid and linoleic acid. Figure 2 shows the changes in individual and main fatty acid groups throughout hepatic differentiation. Endodermal induction produced a transient increase in saturated fatty acids (SFAs; 33%, $p<0.01$) primarily because of palmitic acid. In contrast, a transient decrease was observed in monounsaturated fatty acids (MUFAs) following endodermal induction (-15%, $p<0.01$). A decrease in MUFA palmitoleic acid continued to the lineage commitment stage. The return of SFAs and MUFAs to base levels after lineage commitment was accompanied by corresponding increase in hepatic enzyme markers. The level of total polyunsaturated fatty acids (PUFAs) increased in hiPSCs and decreased progressively after endodermal induction (-10%, $p=0.08$) and hepatic lineage commitment (-19%, $p<0.01$). The PUFA linoleic acid dramatically decreased to the lowest value measured during differentiation after the hepatic maturation stage (-37% versus undifferentiated cells, $p<0.01$; Figure 2a). Differentiated cells at day 15 exhibited a trend toward an increased level of SFAs (14%, $p<0.05$) and MUFAs (11%, $p=0.07$) relative to undifferentiated hiPSCs (Figure 2b).

Further, hiPSCs treated with PD98059 from day 2 displayed only mild differential pattern of fatty acids in different stages of hepatic

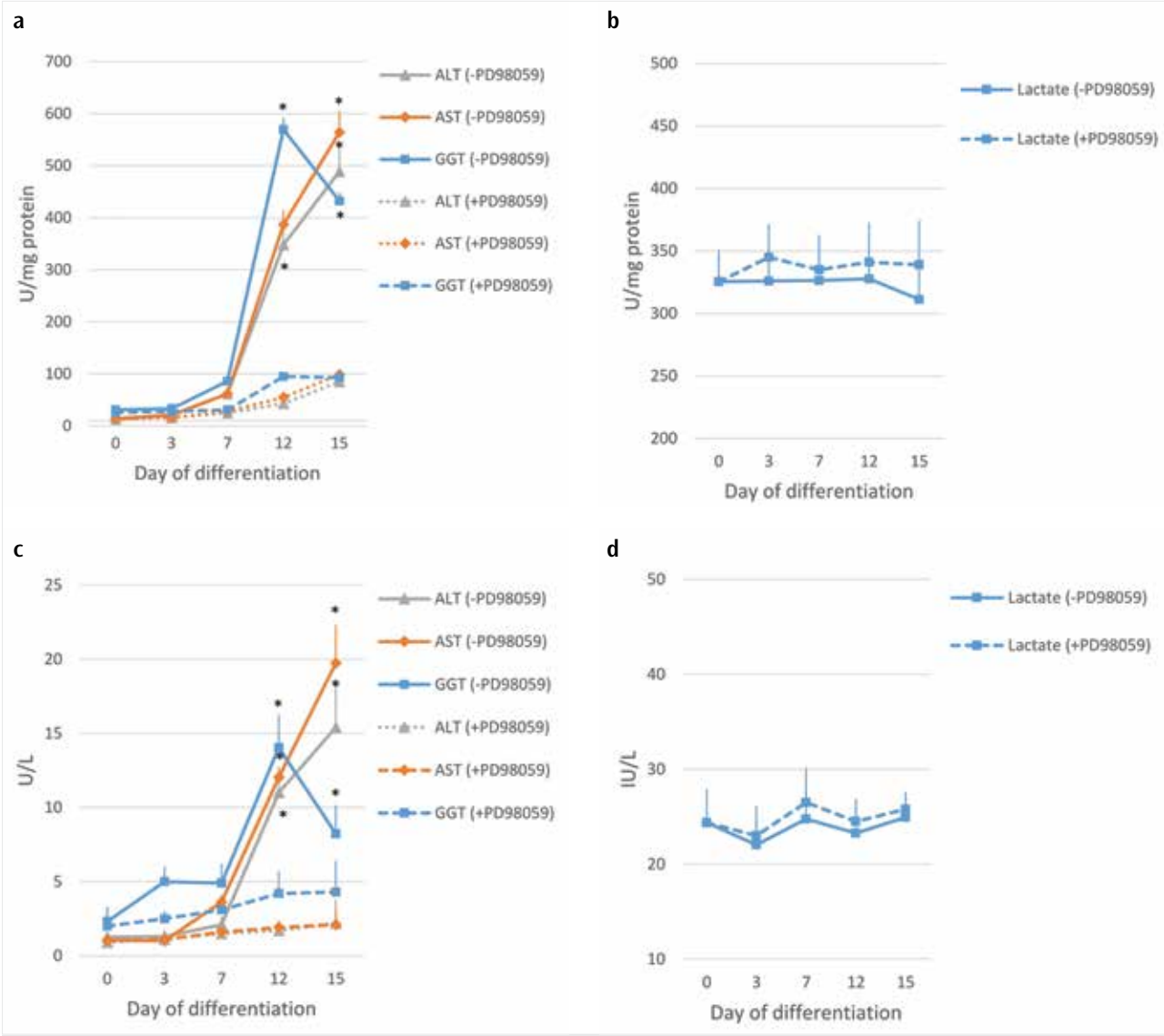

Figure 1. a-d. Changes in enzymatic hepatic markers (a, c) and LDH (b, d) in cellular protein extract and in conditioned culture medium, respectively, of differentiating hiPSCs. Hepatic differentiation is induced by the sequential addition of activin A (100 ng/mL), Wnt-3a (50 ng/mL), HGF (10 ng/mL), and oncostatin M (20 ng/mL) in the presence or absence of PD98059 at 5 µM. Culture supernatant and cell lysates were collected at sequential stages of differentiation and analyzed for ALT, AST, GGT, and LDH spectrophotometrically. Data measures are presented as the mean ± standard error values from three independent experiments. †Values are multiplied by 100.
*$p<0.05$ versus PD98059 treated control. ALT: alanine aminotransferase; AST: aspartate aminotransferase; GGT: gamma-glutamyltransferase; HGF: hepatocyte growth factor; hiPS: human induced pluripotent stem; LDH: lactate dehydrogenase; Wnt-3a: Wnt family member 3a

induction (Figure 2). However, marked changes in SFAs and MUFAs during endodermal induction and hepatic lineage commitment were not observed. Instead, gradual increase in PUFAs was marked during hepatic induction (8%, p=0.07). Thus, impaired hepatic differentiation caused a shift from SFAs and MUFAs toward more polyunsaturated ones in cellular lipids.

Discussion

The use of hiPS-derived hepatocyte-like cells to model human liver physiology requires in-depth knowledge and understanding of the model. The aim of the present study was to analyze the activity pattern of hepatic metabolic enzymes and fatty acid profile of differentiating hiPSCs. Our study took advantage of two features of hiPSCs. First, as iPSCs are sourced from adult somatic cells by means of reprogramming factors, these cells share several characteristics of both embryonic and adult stem cells. Second, hiPSCs-derived hepatocyte-like cells exhibit functional characteristics such as those of mature hepatocytes (20).

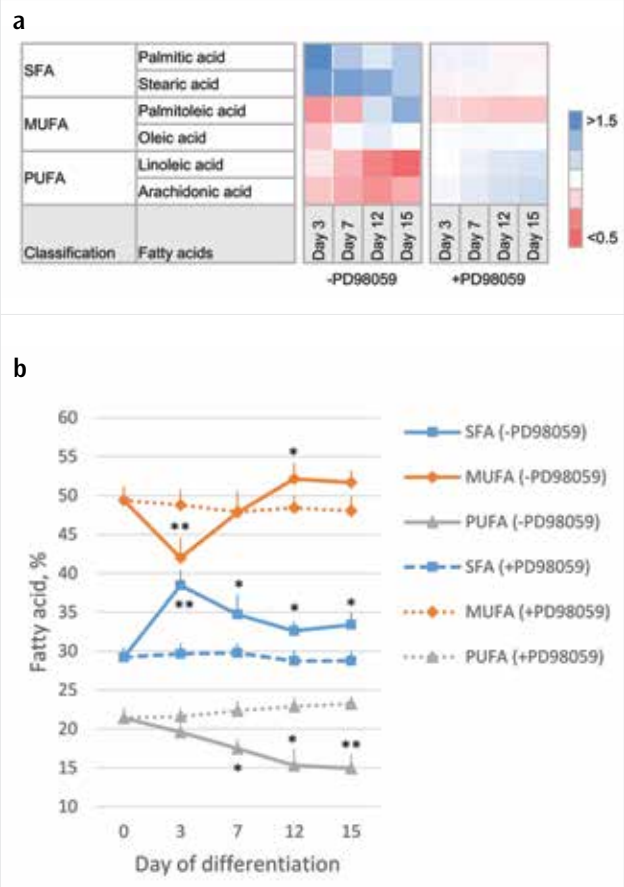

Figure 2. a, b. Changes in fatty acids in cellular lipid extract of differentiating hiPSCs. Relative levels of fatty acids (a) and percentage of SFA, MUFA, and PUFA (b) in differentiating hiPSCs. Hepatic differentiation is induced by the sequential addition of activin A, Wnt-3a, HGF, and oncostatin in the presence or absence of PD98059 at 5 μM. Cell lysates are collected at sequential stages of differentiation and analyzed for fatty acids by gas-liquid chromatography. The level of individual fatty acids is calculated as the percentage of total fatty acids for each condition. Colors correlate to the fold change relative to a reference set of hiPSCs. The changes are presented as the mean ± standard deviation values from three independent experiments.
*p<0.05 versus PD98059 treated control. HGF: hepatocyte growth factor; hiPS: human induced pluripotent stem; MUFA: monounsaturated fatty acid; PUFA: polyunsaturated fatty acid; SFA: saturated fatty acid; Wnt-3a: Wnt family member 3a

Aminotransferases and GGT are essential for amino acid metabolism and can obviously act as key participants in hepatic differentiation through the synthesis of differentiation-related proteins. We observed that induced hepatic differentiation of the hiPSCs used was accompanied by significant increase in liver-specific metabolic factors including aminotransferases and GGT, a phenotype similar to that reported by other studies of human mesenchymal stem cells (1, 2). A previous study showed that the expression of GGT in hepatoblasts is significantly higher than that in hepatocytes (21). Furthermore, the production of soluble GGT is substituted by membrane-bound GGT during maturation from fetal liver to adult liver (22). Consistent with these findings, we found that hepatic maturation was accompanied by significant decrease in GGT at the end of the differentiation process. An elevated release of metabolic enzymes, including aminotransferases, GGT, and LDH, is associated with liver toxicity. Our data showed that the patterns of secretion and intracellular hepatic metabolic enzymes were similar, indicating that no cellular damage was induced during the differentiation of hiPSCs.

Studies employing liquid chromatography-electrospray ionization-tandem mass spectrometry have demonstrated that unsaturated metabolites, including several unsaturated free fatty acids, were abundant in undifferentiated human mesenchymal stem cells and decreased on induced cardiac and neuronal differentiation (9). In support of these findings, the supplementation of embryonic stem cell media with SFAs led to a significant increase in neuronal and cardiac differentiation (9). Esmaeli et al. (4) reported that during hepatic maturation of umbilical cord-derived mesenchymal stem cells, the levels of PUFAs decrease, whereas those of SFAs increase. Oleic acid, a MUFA, has previously been demonstrated to support hepatic differentiation during induced differentiation of hiPSCs and human primary mononuclear cells isolated from umbilical cord blood (10). Overall, these observations suggest that differentiation is associated with alterations in cellular fatty acids. Such observations are supported by the present analysis in which SFAs and MUFAs displayed transient inverse changes and decreasing trends, whereas n-6 PUFA content had a linear increase over hepatic differentiation of hiPSCs.

In general, the processes of differentiation and organ development from stem cells are significantly affected by exogenous fatty acids (8, 23, 24). Madsen et al. (8) have shown that adipocyte differentiation is differently affected by certain fatty acids. Indeed, while n-3 PUFAs effectively stimulated the differentiation of 3T3-L1 cells, n-6 PUFAs prevented adipogenic differentiation and triacylglycerol biosynthesis (8). Moreover, in the latter experimental study, SFA and MUFA did not affect differentiation. It is likely that such effects are related to agonistic and antagonistic effects on transcription factors that control cell fate specification and differentiation, including sterol regulatory element-binding proteins, liver X receptors, and peroxisome proliferator-activated receptors (8).

Finally, we found that the effect of induced hepatic differentiation on the fatty acid profile of hiPSCs changes with ERK inhibi-

tor treatment. ERK signaling pathway participates in hepatocyte differentiation and proliferation through the relay of hepatocyte growth factor signaling toward cell cycle machinery and differentiation pathways (15). Consistent with this finding, we observed that ERK inhibitor treatment during induced differentiation markedly reduced the production of functional hepatic markers. Thus, the lack of marked changes in fatty acids during endodermal induction and hepatic lineage commitment state implies that differentiation-inducing factors in the absence of functional differentiation had no significant effect on cellular fatty acids. Future biochemical and molecular studies of hepatic differentiation should focus on the role of selected enzymatic reactions in the synthesis or modification of fatty acids.

Conclusion

In conclusion, our findings indicate that the pattern of cellular fatty acids is dynamic and changes with the progress of hepatic differentiation, which includes both transient fluctuations and linear trends. Our result is of interest because hiPSCs derived from human dermal fibroblasts are promising candidates for regenerative medicine and drug discovery in addition to being applied to addressing the basis of cellular fatty acid metabolism. This finding can aid efficient differentiation of hiPSCs, particularly for hepatic regeneration.

Ethics Committee Approval: Ethics committee approval was received for this study from the ethics committee of Tabriz University School of Medicine (Approval Date: 05.12.2014; Approval Number: 5/104/1207).

Informed Consent: Written informed consent had been obtained from the patient who donated skin fibroblasts for this study.

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Conflict of Interest: The authors have no conflicts of interest to declare.

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