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Neuronal conversion of dermal fibroblasts as a disease model

Hastalık modeli olarak dermal fibroblasttan dönüştürülmüş nöron

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SUMMARY

Objective: Disease modelling applications are gaining importance especially for the need of patient-specific studies. Because some cell types cannot be obtained from patients, somatic cell differentiation is needed for disease modelling. There are many methods for somatic cell reprogramming. Induced Pluripotent Stem Cells (iPSCs) and direct conversion are the main techniques. However, due to the disadvantages of iPSCs, such as expense, high mutation rate and differentiation defects, many researches choose direct conversion for reprogramming.
In this study, we aimed to reprogram dermal fibroblasts into neurons with direct conversion in order them to be potentially used for patient-specific disease modelling applications.
Method: Commercially purchased dermal fibroblasts were seeded on coverslips and then induced with a medium containing small chemicals for 24 hours. Cells were visualized by scanning electron microscopy and transcriptome analysis was done by next generation RNA sequencing.
Results: Neuronal cell morphology was observed after 24 hour induction. According to the transcriptomic data, neuronal genes were upregulated.
Conclusions: Dermal fibroblasts were successfully induced into neurons by direct conversion with using small chemicals.
Keywords: Dermal fibroblast, disease modelling, direct conversion, neuron, transcriptomics.

ÖZET

Amaç: Kişiselleştirilmiş tıp çalışmaları için hastalık modeli oluşturmak önem kazanmıştır. Hastalardan bazı hücre türlerinin alınamıyor olması, hastalık modeli için somatik hücre farklılaştırılmasını gerektirmiştir. Hücrelerin yeniden programlanması için pek çok yöntem bulunmaktadır. İndüklenmiş Pluripotent Kök Hücre (iPKH) ve doğrudan dönüştürme en temel iki yöntemdir. Ancak, iPKH'nin pahalılık, yüksek mutasyon oranı ve farklılaşma hasarları gibi dezavantajlarından dolayı, pek çok araştırmacı doğrudan dönüştürmeyi seçmektedir.
Bu çalışmada, dermal fibroblastların doğrudan dönüştürme ile nöronlara farklılaştırılarak kişiye özel hastalık modeli uygulamalarında kullanılabileceği amaçlanmıştır.
Yöntem: Ticari olarak satın alınmış dermal fibroblastlar lamel üzerine ekilerek küçük kimyasallar içeren besiyeri ile 24 saat süresince indüklenmiştir. Hücreler taramalı elektron mikroskobu ile görüntülenmiş ve yeni nesil RNA dizileme ile transkriptom analizi gerçekleştirilmiştir.
Bulgular: 24 saatlik indükleme sonucunda nöral hücre morfolojisi görülmüştür. Transkriptom sonuçlarında nöral genlerin artmış olduğu tespit edilmiştir.
Sonuç: Dermal fibroblastlar küçük moleküller kullanılarak doğrudan dönüştürme ile başarılı olarak indüklenmiştir.

Anahtar sözcükler: Dermal fibroblast, hastalık modeli, doğrudan dönüştürme, nöron, transkriptomik.

INTRODUCTION

For the last decade, disease modelling applications has emerged due to the need for patient-specific studies. Cell lines or animal models may not always reflect the disease phenotype exactly. Therefore, modelling the disease pathogenesis with patient-derived samples are beneficial for future drug research. However, all cell types cannot be obtained from healthy donors or patients, especially needed by the interventional methods. At this stage, differentiation studies become very critical [1]. In detail, differentiating any somatic cell of the patient into a desired cell type plays a key role for disease modelling. There are many techniques to differentiate somatic cells into other types, two of the most popular ones are induced pluripotent stem cells (iPSCs) and direct conversion/reprogramming. iPSCs are firstly achieved by introducing 'Yamanaka factors' (Oct3/4, Sox2, Klf4 and c-Myc) into somatic cells and taking them into pluripotent stage [2]. Direct conversion or reprogramming (DC) is converting a somatic cell into another cell type by using exogenic molecules (transcription factors, small chemicals etc.), without forcing them to go to a pluripotent stage [3]. When compared to DC, iPSCs require more time and expense, cells could gain de novo mutations in iPSCs production process [4], age-related gene expression may be affected due to the hypermethylation [5] and forms teratomas. In addition, iPSCs process can generate differentiation defective iPSCs which cannot differentiate into neurons [6]. However, DC is more easy and cheaper, conserves age-related gene expression, has nearly 100% conversion efficiency and does not form any cancerous or differentiation defective cells [5]. Therefore, differentiating cells with DC is more beneficial for cell differentiation studies. In this study, we differentiate dermal fibroblasts into neurons by using small chemicals and investigate the global gene expression differences between these two cell types.

MATERIAL AND METHODS

Cell Culture

Primary human dermal fibroblasts (DFs) (Cat. No: PCS-201-012™) were purchased from American Type Culture Collection (ATCC), which were obtained from a 28 years old male African American donor's abdominal skin (Lot: 63792061). Passage 3 cells were used for further experiments. Cells were incubated at 37^0C and 5% CO_2, culture medium (DMEM-LG supplemented with 10% FBS, 1% Penicillin-streptomycin and 1% L-glutamine) was changed twice a week.

Neuronal Induction

Six thousand DFs were seeded per cm^2, on coverslips in a multi well plate. Before induction, cells were incubated in a medium (DMEM-LG supplemented with 10% FBS, 1% Penicillin-streptomycin, 1% L-glutamine and 1% MEM Non-essential aminoacids) for 24 hours. Neuronal induction medium was added after washing with PBS (phosphate buffered saline). Induction medium was prepared according to a recent study [7], details were given below:

DMEM F12 : Neurobasal Medium (1:1) (DN) was mixed; bFGF (100ug/ml), B27 (50X in DN) N2 (100X in DN), Penicillin/streptomycin (100X), and the small chemicals [VPA (50mM), CHIR99021 (10mM), Repsox (10mM), Forskolin (50mM), SP600125 (50mM), GÖ6983 (10mM) and Y-27632 (10mM)] were added.

Scanning Electron Microscopy (SEM)

Culture medium was removed, cells were washed with PBS and were fixated with 2.5% glutaraldehyde for 15 minutes. For drying, fixated samples were treated with 50%, 75%, 90%, 95% and 100% ethanol sequentially, for 5 minutes each. Then, 75 µl Hexamethylenedisilazane (HMDS) was dropped. Dried samples were coated with gold/palladium (1:1) with sputter, carbon and e-beam coater (Leica EM ACE600). Then visualized under SEM (Tescan, GAIA3).

RNA Isolation and RNA-Sequencing

After 24 hour induction, culture medium was removed and washed with PBS. Total RNA from cultured cells was extracted using TRIzol (Sigma). SENSE mRNA kit for Ion Torrent (Lexogen) was used for strand-specific library preparation. Briefly, 1000 ng total RNA was enriched for poly(A) mRNAs using oligo-dT magnetic beads. For random hybridization of starter/stopper heterodimers, RNA/magnetic beads mixes were incubated on 37^0C for 2 hours. Then, first and second strand cDNA synthesis was

performed. After library amplification libraries were quantified with Qubit 2.0 Fluorometer (Thermo Fisher Scientific). Clonal amplification of the libraries (emulsion PCR) was performed on Ion OneTouch™ 2 system using the Ion PI Hi-Q OT2 200 Kit and Ion sphere particles (ISP) were enriched using the One Touch™ ES module, according to the manufacturer's instructions. Finally, ISPs were loaded on Ion PI chips v.3 and sequenced by using Ion Proton Sequencer (Thermo Fisher Scientific). For data analysis, sequencing reads were mapped by using TMAP (Torrent Mapping Alignment Program) which runs on the Ion Torrent Server. Then, pathway analysis was done using STRING web tool (https://string-db.org/).

RESULTS

When dermal fibroblasts are cultured at induction medium they undergo neural differentiation (Figure 1). Morphological characteristics of neurons were appeared in cell culture in the first 12 hours. We observed unipolar-, bipolar- and multipolar-like neural cells. The neural network was observed at 18 hours. At the 48 hours of induction, most of the cells undergone differentiation.

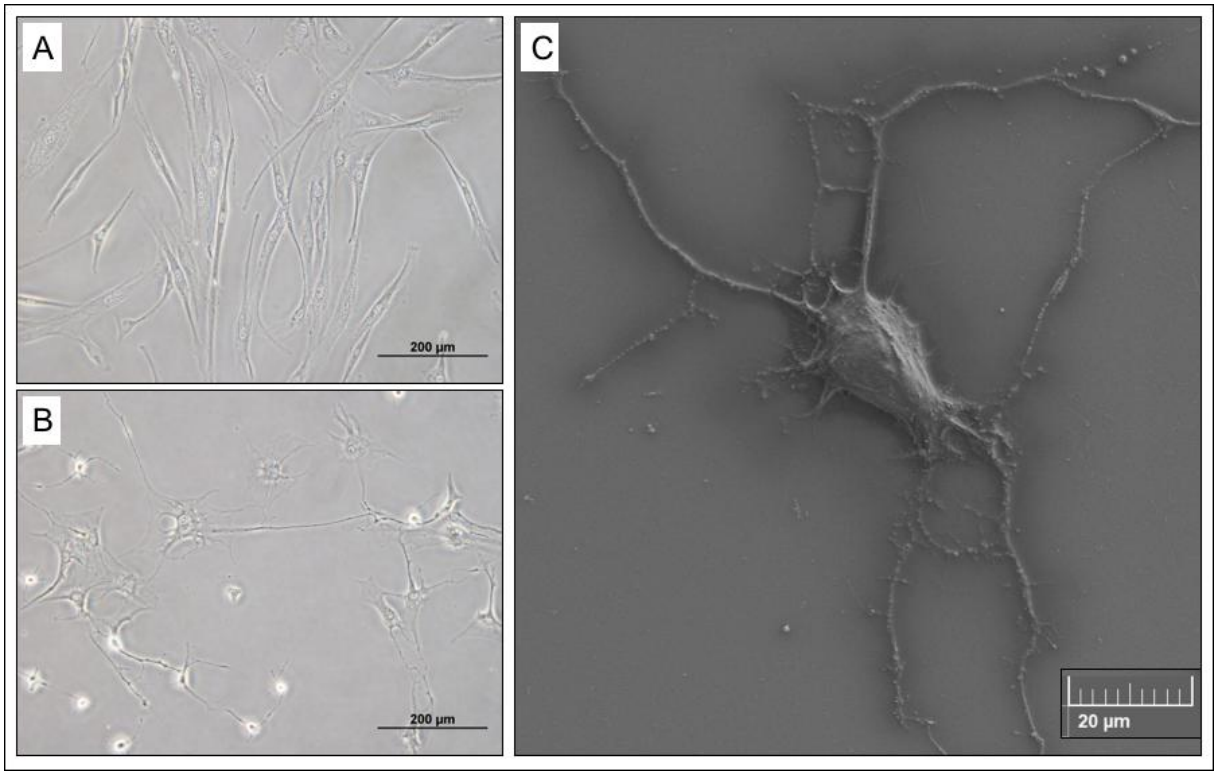

Figure: Microscopic images of the cells. A. light microscopy of dermal fibroblast, B. light microscopy of 24 hour induced neurons, C. Scanning Electron Microscopy (SEM) of 24 hour induced neurons.

In order to analyze the gene expression profile of these cells we performed total mRNA sequencing. We focused on the 24th hour of induction so that we could investigate the early changes of global gene expression between dermal fibroblasts and induced cells. Comparative analysis showed that 1291 genes were up-regulated (minimum 4-fold) after neuronal induction. Addition to this, 785 genes were relatively increased in induced cells compared to dermal fibroblasts. We performed pathway analysis using STRING with these totally nearly 2000 genes (Gene list is available upon request). We obtained GO annotation of induction-related genes in two domains. We showed that that several pathways were closely related to neuronal cells according to biological process (Table 1).

Table 1: Pathway analysis according to biological process.

Pathway ID	Pathway Description	Observed Gene Count	False Discovery Rate
GO.0071840	cellular component organization or biogenesis	464	1.83e-06
GO.0032502	developmental process	441	4.33e-05
GO.0044237	cellular metabolic process	713	0.000277
GO.0048856	anatomical structure development	388	0.000277
GO.0048869	cellular developmental process	314	0.000657
GO.0030182	neuron differentiation	117	0.000663
GO.0048666	neuron development	100	0.000852
GO.0030154	cell differentiation	299	0.00123
GO.0050790	regulation of catalytic activity	224	0.00125
GO.0050803	regulation of synapse structure or activity	37	0.00242
GO.0050804	modulation of synaptic transmission	41	0.00242
GO.0048701	embryonic cranial skeleton morphogenesis	12	0.0179
GO.0016070	RNA metabolic process	321	0.019
GO.0007411	axon guidance	52	0.0196
GO.0045595	regulation of cell differentiation	149	0.0199
GO.0061564	axon development	63	0.0202
GO.0007409	axonogenesis	61	0.0212
GO.0000904	cell morphogenesis involved in differentiation	77	0.0229
GO.0006836	neurotransmitter transport	25	0.0229

Addition to this, we observed neuron-related genes controlling several cellular components (Table 2). The most increased top 30 genes were given in Table 3. Most of them are primarily related to a neuronal function.

Table 2: Pathway analysis according to cellular components.

Pathway ID	Pathway Description	Observed Gene Count	False Discovery Rate
GO.0030054	cell junction	138	2.44e-06
GO.0043233	organelle lumen	395	0.000171
GO.0012505	endomembrane system	339	0.000619
GO.0044432	endoplasmic reticulum part	123	0.00123
GO.0005634	nucleus	597	0.00295
GO.0097458	neuron part	122	0.00299
GO.0044421	extracellular region part	349	0.00363
GO.0005783	endoplasmic reticulum	163	0.00496
GO.0005578	proteinaceous extracellular matrix	47	0.00712
GO.0030055	cell-substrate junction	51	0.00894
GO.0005925	focal adhesion	50	0.00939
GO.0031982	vesicle	331	0.0148
GO.0005912	adherens junction	55	0.0155
GO.0005739	mitochondrion	164	0.0171
GO.0098793	presynapse	24	0.0171
GO.0070161	anchoring junction	56	0.0194
GO.0043005	neuron projection	94	0.0207
GO.0036477	somatodendritic compartment	71	0.0236
GO.0009986	cell surface	79	0.0308
GO.0005730	nucleolus	93	0.0353
GO.0045202	synapse	72	0.0371
GO.0043025	neuronal cell body	50	0.0475

Table 3: The most increased 30 genes after 24 hour neuronal induction.

Gene Name	Fold Change (Increase)	Function/Location	Reference
AC005682.6	83,59		
GPHN	78,95	Component of the postsynaptic protein network	10
RP11-115D19.1	77,78	-	-
GCK	52,24	-	-
THRB-AS1	49,92	-	-
BCAN	47,87	Neural proteoglycan	11
EGFLAM	44,37	Localized at the ribbon synapses	12
EIF2B2	42,95	Guanine nucleotide exchange factor activity of EIF2B	13
RP11-104H15.8	38,53	-	-
CDC7	38,31	-	-
LINC00880	36,65	-	-
PDE2A	35,99	Phosphodiesterase	14
FER1L6-AS2	35,72	-	-
FARP1	35,63	Dendritic filopodial dynamics in immature neurons	15
RP11-501J20.5	33,86	-	-
CTD-2514K5.4	33,67	-	-
KCNV2	33,67	Regulatory subunit of voltage-gated potassium channels	16
RP11-21L19.1	33,61	-	-
TMEM151B	32,51	-	-
C9orf69	31,35	-	-
STK24	31,35	Regulates axon outgrowth	17
CTC-441N14.2	30,19		
MAPK8IP1	27,86	Regulation of autophagosomes trafficking	18
ALKBH4	26,85	-	-
RP1-102K2.6	26,85	-	-
RP1-102K2.8	26,73	-	-
PHACTR1	26,72	Expressed in the central nervous system	19
C9orf9	26,70	-	-
RP11-845M18.6	26,70	-	-

DISCUSSION

The use of patient-specific cells for modelling is critical to investigate molecular mechanisms of human diseases. Using reprogramming technologies it is also possible to create in vitro models for neurological diseases [1, 3, 5, 7]. Large scale omics data (such as genomics, transcriptomics, and proteomics) has a great power in identifying disease-mechanisms, drug targets and biomarkers in human diseases [8]. Transcriptomics has been one of the most popular approach to obtain global gene expression data [9]. In this study, we try to create neuron-like cells from dermal fibroblasts using direct conversion and investigate molecular differences in transcriptomic level. Because direct conversion has many advantages against other reprogramming methods, we choose this method for reprogramming of dermal fibroblasts. With this purpose, we used a small chemical cocktail for neuronal induction medium and followed them up to 72 hours. Because the most striking change (neuronal cell morphology) was observed at 24th hour, we performed RNA-Seq at that time point. We focused on the increased/up-regulated genes on directly converted neuronal cells. The most increased genes list contains proteins specifically related to neuronal functions. Similar to this, pathway analysis showed strong neuronal signatures in increased/up-regulated genes (nearly 2000 transcripts). Together both gene expression signatures and cellular characteristics showed that neuronal conversion was successful in the first 24 hours of induction.

Future studies should focused on functional capabilities of these directly converted neurons and investigate transcriptomic profiles in different time points in order to enlighten the whole differentiation process in vitro. By doing this, it will be possible to create different cell types of neuronal lineage. Additional studies with omic platforms (such as proteomics and metabolomics) might contribute to investigate the differentiation process in detail. In the age of precision medicine, it is important to identify disease-causing mechanisms. Patient specific in vitro modelling of neurological diseases is possible with direct conversion of dermal fibroblasts. This approach may be beneficial for drug discovery studies for rare disorders in the near future.

CONCLUSION

In conclusion, our study confirmed that human dermal fibroblast can be directly converted into neurons by using small chemicals in vitro. Cellular morphology and gene expression signatures verified this differentiation even at 24th hour of induction.

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