幹細胞誘導分野
Department of Cell Modulation

多能性幹細胞は試験管内で多能性を維持したままで増幅でき、かつ、様々な細胞へと分化することができる優れた細胞である。私たちは、この多能性幹細胞の研究を行っている。研究目的は、以下の 3 つ specific aim を達成して、その得られた知見を臨床の研究へ応用し、革新的な新規治療戦略のコンセプトの提供や薬剤開発を行うことである。

(1) 組織幹細胞の 1 つである間葉系幹細胞の多能性幹細胞からの分化誘導系を確立し、間葉系幹細胞の発生起源、分化経路、分化と増殖の分子生物学的メカニズムを明らかにすること。

(2) 疾患由来の人工多能性幹細胞(iPS 細胞)を樹立し、それを解析することで疾病の原因・病態を解明すること。

(3) リプログラミング機構の解明

ES and iPS cells are pluripotent stem cell lines which undergo unlimited growth with pluripotency and can give rise to various cell lineages including three germ layers. However, in vitro cell differentiation culture is still very complex as various cell types are simultaneously generated in the culture. This is one of disadvantage of this system. Our lab is studying the differentiation and manipulation of pluripotent stem cells, in particular, is focusing on three specific aims;

(1) To elucidate the differentiation pathways and molecular mechanisms of tissue stem cells including mesenchymal stem cells (MSCs)

(2) To establish disease-derived iPS cells from intractable diseases and to develop new therapeutic concepts based on the results of iPS cell studies

(3) To elucidate mechanisms underlying reprograming in iPS cell generation

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研究概略　Projects

(1) 間葉系幹細胞への発生過程研究

間葉系幹細胞は骨、軟骨、脂肪細胞に分化する能力をもつ幹細胞である。現在、骨髄由来の間葉系幹細胞が臨床への応用が進められている。私たちは、マウス ES 細胞を用いて Sox1 陽性の神経上皮系細胞から PDGFRα陽性間葉系幹細胞を誘導することに成功し、さらにマウス発生過程においても間葉系幹細胞が神経上皮から発生するという発生学的に新しいコンセプトを見出した（Fig. 1)。しかしながら神経上皮由来の間葉系幹細胞は成体での骨髄由来の間葉系幹細胞には一切分化していないことも明らかとなり、新たに別の発生起源があることが示唆された。

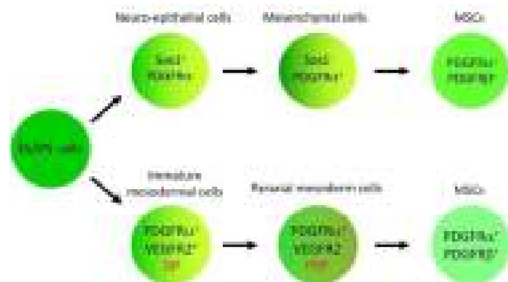

Fig. 1 Differentiation pathway of Mesenchymal Stem Cells

そこでさらに間葉系幹細胞の起源をマウス個体内で詳細に明らかにするために。間葉系幹細胞特異的に Cre 組換え酵素を発現する遺伝子改変マウスを作製した(Fig. 2)。この Cre は変異型エストロゲン受容体を付加してあるので、その活性をタモキシフェンの投与によりコントロールできる。作製したマウスを ROSA26-LacZ レポーターマウスを掛け合わせ、胎仔の各発達段階でタモキシフェンを投与して LacZ 染色を行うことで、発生過程での間葉系幹細胞の追跡が可能となる。また、種々の flox マウスと掛け合わせることで間葉系幹細胞特異的なコンディショナルノックアウトマウスも作製できる。

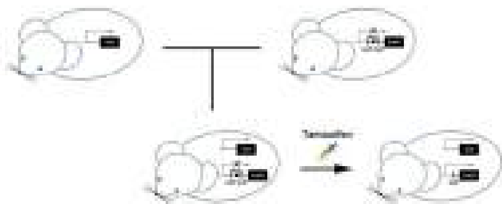

Fig. 2 Strategy for studying MSC origin

このシステムを使い、すべての間葉系幹細胞が PDGFRα陽性の細胞から出現すること、そして成体の骨髄と脂肪組織に存在する間葉系幹細胞は中胚葉由来で発生中期に wave をもって出現してくることを明らかとした。さらに骨折、皮膚潰瘍などの組織損傷モデルにおいてその修復に PDGFRα陽性の間葉系幹細胞が重要な役割を持つことを明らかとした。

(2) 多能性幹細胞からの分化誘導法の開発とその応用

間葉系幹細胞は骨、軟骨などの間葉系組織の修復に関与することから、再生医療への応用が期待されている。しかしながら、患者からの採取には個体差があり常に治療に必要量の細胞が採取できるとはかぎらない。一方、ES/iPS 細胞から間葉系幹細胞を容易に誘導できれば、これらの問題は解決される。そこで、ヒト iPS 細胞から間葉系幹細胞をこれまでの知見に基づいて誘導を試みた。

ヒト iPS 細胞を試験管内で適切な条件下にて誘導すると誘導 6 日目に中胚葉細胞の表面マーカーである PDGFRα (platelet derived growth factor receptor-alpha) 、 VEGFR2 (Vascular endothelial growth factor receptor 2)の２つの分子が発現する。これらのマーカーの発現パターンは、PDGFRα陽性、VEGFR2 陽性分画(double positive cells :DP)、 PDGFRα陽性、VEGFR2 陰性の分画（PDGFRα single positive cells: PSP)、PDGFRα陰性、VEGFR2 陽性分画（VEGFR2 single positive cells: VSP）、PDGFRα陰性、VEGFR2 陰性の分画（Double negative cells: DN）の 4 つに分けられる。これらのマーカー陽性の中胚葉系細胞から間葉系幹細胞を誘導することに成功した。さらにマウス ES 細胞と同様の方法にてヒト iPS 細胞から神経上皮由来の間葉系幹細胞の誘導にも成功した。ヒト iPS 細胞から誘導された由来の異なる２つの間葉系幹細胞はマウスを使った褥瘡モデルと関節障害モデルにおいて、有意に治療効果を発揮した。この成果は将来 iPS 細胞由来の間葉系幹細胞が臨床へ応用できることを示唆している。

(3) 疾患由来 iPS 細胞の樹立、バンク化とそれを使った難治性疾患の研究

医学研究では、疾患由来の生体試料（細胞や血液等）はその疾患の診断法や治療法を開発する上で必須のものである。しかしながら、疾患によっては、病気の標的細胞の採取が困難であったり、症例数が限られる難治性疾患のように生体試料そのものが非常に少ないといった問題が存在する。このことが治療法をはじめとした開発研究の大きな障害となっている。

一方、iPS 細胞は、体細胞に初期化因子を発現させ作製することができる。皮膚生検サンプルから作製できるので、多くの疾患から作製可能である。iPS 細胞は、疾病の標的細胞を誘導し、発症機序の解明や治療法の開発へ利用できると期待されている。また、試験管内で増幅でき、長期保存も可能である。したがって採取困難な標的細胞を有する疾患や希少性が高い難治性疾患からの研究にすぐれた効果を発揮すると考えられる。私たちは、難治性疾患由来 iPS 細胞の樹立と解析、さらに iPS 細胞バンク整備の研究を行っている。血液細胞から容易に iPS 細胞を樹立するために企業とともに新しいセンダイウイルスベクターを開発した。この方法では、iPS 細胞作製に用いる初期化因子が染色体に組み込まれないために、疾患研究により有用な iPS 細胞を作製できる。また２％程度の高効率で血液細胞から iPS 細胞を作成できる。血液細胞を使用できることからこれまで作成が難しかった小児の患者からも容易に作成可能となった。

疾患由来 iPS 細胞を使っての疾患研究もライソゾーム病などの先天性の難治性疾患を中心に進めている。ニーマンピック病 C 型（NPC）は、ライソゾーム内に遊離型コレステロール、糖脂質が蓄積するライソゾーム病である。幼児期に発症し、多くは神経障害にて 20 歳ぐらいまでに死亡する。認可薬ミグルスタットは効果が限定的であり、コレステロール蓄積に対しては無効である。他に有効な治療薬はない。欧米での治験中薬剤、2-Hydroxypropyl-β-Cyclodextrin (HPBCD)は、国内では重篤な肺障害が起こり、以後、全身投与は中止されている。したがって、有効性と安全性に優れる薬剤の開発が NPC 治療の課題である。

私たちは、患者 iPS 細胞から誘導した肝細胞と神経細胞では、疾患と同様に遊離型コレステロールの著明な蓄積が見られることを明らかとし、この系を使って新たに 2-Hydroxypropyl-γ-Cyclodextrin (HPGCD)が蓄積を減少させることを見出した(Fig. 3)。さらに、NPC モデルマウスへの投与実験でも、神経障害の発症を抑制し、生存期間を延長させた。急性毒性試験では HPGCD は先行開発品 HPBCD に比べて遥かに安全性が高かった。したがって HPBCD に対して開発優位な位置にあると考えられる。

疾患由来の iPS 細胞を多くの研究者に提供するために細胞バンク化についても他の研究施設と共同で進めており、現在までに 200 例以上の難治性疾患から iPS 細胞を作成して貯蔵しバンク化している。

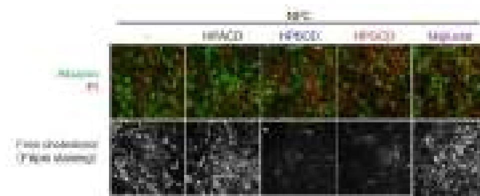

Fig. 3 Effect of cyclodextrins on NPC cells

(4) リプログラミング機構の解明

細胞のリプログラミング機構についても研究を行っている。Calcineurin/NFAT シグナルは、リプログラミングにおいて２つの相反する働きを行うことを明らかとした。

リプログラミング開始から 7 日目までの初期段階では、細胞分裂を維持と間葉-上皮転換（MET）を促すことでリプログラミングを正に制御している。一方、7 日目以降の後期段階では、NFATc2 と呼ばれる転写因子の働きにより未分化維持転写因子である Sox2 と KLF2 の発現を、H3K9me3 と H3K27me3 を制御することで、抑制し、リプログラミングを負に制御している。またこれらのシグナルの上流には G タンパク質にシグナルを伝える Gαq が存在することも同時に明らかにした。

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(1) Origin of MSC

Mesenchymal stem cells (MSCs) are defined by their ability both to undergo sustained proliferation

in vitro and to give rise to multiple mesenchymal cell lineages including bone, cartilage, and fat cells. MSC is going to be applied for clinical medicine. We wish to define the differentiation pathways of MSCs. Using ES cell culture, we show that $Sox1^+$ neuroepithelial cells generate MSCs at the highest efficiency. In early embryos, we can induce MSCs from $Sox1^+$ cells but not from $PDGFR\alpha^+$ mesoderm. However, as the development proceeds, neuroepithelium-derived MSCs gradually decrease and most MSCs in postnatal bone marrow are derived from other origins, which are also enriched in the $PDGFR\alpha^+$ population. Thus, at least, MSCs are generated from two sources, neuronal and non-neuronal, with those derived from neuroepithelium constituting the earliest wave.

To elucidate the differentiation process of MSC, we generated knock-in mice which specifically express Cre recombinase in MSCs. Since this Cre is fused with estrogen receptor, its activity can be controlled by tamoxifen injection. The generated mice were mated with ROSA26 LacZ repoter mice to investigate the MSC fate during mouse development. Additionally, when these mice are mated with various floxed mice, that system can provide us to examine the function of genes specific for MSCs. We first investigated the developmental process of MSC in mouse embryos using PDGFRα as a marker gene. The tracing of MSC origins and destinations indicated that embryonic MSC emerge in waves and that almost all adult BM-MSC and WAT-MSC are originated from mesoderm and embryonic PDGFRα-positive cells. We demonstrated that adult PDGFRα-positive cells play a role in improving the disrupted tissues in the some pathological conditions.

(2) Differentiation of MSC from ES/iPS cells

As they can be easily isolated from bone marrows, MSCs are expected to be applied for clinical medicine. However, their availability of self-renewal and multipotency are limited and the procedures harvesting MSCs from bone marrow or adipose tissues are sometimes invasive. Therefore, it is eager to establish the novel methods or another sources to generate the MSCs extensively and safely. Induced pluripotent stem cells (iPSCs) can serve as an alternative cell source for these MSCs. We attempted to establish MSC induction from human iPSC cells. In vitro iPS cell differentiation culture is still very complex as various cell types are simultaneously generated in the culture. This is one of disadvantages of this system. In order to overcome this problem, we have attempted to establish molecular markers for defining the cell lineages generated in cultures. Our study established the definitive methods for differentiation of hiPSCs into MSCs via mesoderm and neuroepithelium. We adopted two steps for the differentiation to MSCs from iPSCs. At first, we generated PDGFR-α+/KDR- cells, which are MSC progenitor cells, purified by using flow cytometry after mesodermal and neuroepithelial differentiation. Next, the sorted PDGFR-α+/KDR- cells were differentiated into MSCs. Both MSCs exhibited self-renewal and multipotency abilities and therapeutic potentials for skin wounds, pressure ulcers and osteoarthritis mouse models. In addition, the treatment with iPS derived MSC were effective on the pressure ulcer and osteoarthritis models. These results suggest that iPS cell-derived MSC therapy is effective on human disease in the future.

(3) Study for iPS cells derived from intractable diseases

Samples collected from the patients with intractable diseases are useful and indispensable for both studying the molecular mechanism of diseases and developing new therapeutic agents. However, as the number of samples is limited, it is necessary for us to build a system in which researchers can fairly receive samples on their request. To attain this aim, we are studying for the cell bank of the induced-pluripotent stem cells (iPS cells) from the intractable diseases. Since the iPS cells established are able to be easily expanded and stored in deep freezer for a long term, we can widely supply the iPS cells to the researchers on their request.

In order to generate exogenous factors-free iPS cells, we newly generated temperature-sensitive Sendai-virus vector carrying the reprogramming factors including Oct3/4, Sox2, KLF4 and c-Myc. The method is powerful and useful for establishing iPS cells from blood cells because the frequency is higher than previously conventional ones and the genes of four factors are not at all integrated into chromosome of iPS cells. We can easily generate the iPS cells from children.

We concentrate into the study of lysosomal diseases using the patient-derived iPS cells. Niemann-Pick disease type C (NPC) is a lysosomal storage disease characterized by abnormal accumulation of free cholesterol and glycolipids. It develops in early childhood, mostly dying by about 20 years of age in neurological disorders. The approved drug, Miglestat, has limited effect and is ineffective for cholesterol accumulation. There are no other effective drugs. 2-

Hydroxypropyl-β-Cyclodextrin (HPBCD), a clinical trial in the US, experienced severe pulmonary injury in Japan, and systemic administration has since been discontinued. We established induced pluripotent stem cell (iPSC) lines from NPC patients. Hepatocyte-like cells (HLCs) and neural progenitors derived from the iPSC lines accumulated cholesterol and displayed impaired autophagy and ATP production. A molecular signature related to lipid metabolism was also impaired in the NPC-iPSC-derived HLCs. These findings indicate that iPSC-derived cells can phenocopy human NPC. We also newly found that 2-hydroxypropyl-γ-cyclodextrin (HPGCD) could reduce the cholesterol accumulation and restore the functional and molecular abnormalities in the NPC patient-derived cells, and do so more effectively than HPBCD treatment. In addition, NPC model mice showed an improved liver status and prolonged survival with HPGCDs. Thus, iPSC lines derived from patient cells are powerful tools to study cellular models of NPC, and HPGCD is a potential new drug candidate for future treatment of this disease.

Until now, we generated more than 2,000 iPS cell lines from 200 cases of intractable diseases. Distribution of iPS cell from the cell bank enhances the opportunities in which the researchers face and study the intractable diseases, thereby accelerates to develop the new therapies that can cure them.

(4) Study for mechanisms underlying reprograming in iPS cell generation

We are also studying the reprogramming mechanism in the iPS cell generation. Calcineurin/NFAT signal plays two conflicting roles in the reprogramming. At the initial stage from the start of reprogramming to the seventh day, the reprogramming process is positively controlled by the maintaining cell division and promoting mesenchymal-epithelial conversion (MET). On the other hand, at the late stage after day 7, expressions of Sox2 and KLF2, which are undifferentiated state-maintaining transcription factors, are suppressed by NFATc2 transcriptional factor with controlling H3K9me3 and H3K27me3, resulting in the negative regulation of reprogramming. We also found Gαq, which transduces a signal to the G protein, as an upstream molecule of Calcineurin/NFAT signal in the reprograming.

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4. 熊本県難病相談・支援センター記念医療会の講師　10月6日、2013年、熊本市

5. 熊本大学附属中学校「学びの交流会」での出張授業　9月26日、2013年

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マスメディアによる研究成果の報道・発信

1. NHKの午後7時の全国ニュースでiPS細胞を使った研究が紹介された。10月21日、2012年

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STEM CELLS AND REGENERATION

RESEARCH ARTICLE

Tracing the destiny of mesenchymal stem cells from embryo to adult bone marrow and white adipose tissue via Pdgfrα expression

Hiroyuki Miwa*,‡ and Takumi Era‡

ABSTRACT

Mesenchymal stem cells (MSCs) are somatic stem cells that can be derived from adult bone marrow (BM) and white adipose tissue (WAT), and that display multipotency and self-renewal capacity. Although MSCs are essential for tissue formation and have already been used in clinical therapy, the origins and markers of these cells remain unknown. In this study, we first investigated the developmental process of MSCs in mouse embryos using the gene encoding platelet-derived growth factor receptor α (*Pdgfra*) as a marker. We then traced cells expressing *Pdgfra* and other genes (brachyury, *Sox1* and *Pmx1*) in various mutant mouse embryos until the adult stage. This tracing of MSC origins and destinies indicates that embryonic MSCs emerge in waves and that almost all adult BM MSCs and WAT MSCs originate from mesoderm and embryonic Pdgfrα-positive cells. Furthermore, we demonstrate that adult Pdgfrα-positive cells are involved in some pathological conditions.

KEY WORDS: Mesenchymal stem cells, Pdgfrα, Brachyury, Sox1, Pmx1 (Prrx1)

INTRODUCTION

Mesenchymal stem cells (MSCs) display self-renewal capacity, proliferation sustainability *in vitro* and the potential to differentiate into various mesenchymal cell lineages, including adipocytes, chondrocytes, and osteocytes (Prockop, 1997; Pittenger et al., 1999). MSCs are also crucial for several tissue formation processes and injury repair, including bone fracture (Grcevic et al., 2012; Mizoguchi et al., 2014; Miwa and Era, 2015). MSCs have been proposed for use in stem cell therapy because they can be derived from adult bone marrow (BM) and white adipose tissue (WAT); however, the safe and successful application of MSCs for clinical use requires clarification of their origin and the development of reliable markers for fate mapping.

Previously, we have exploited two methods for inducing mesenchymal cell lineages from embryonic stem cells (ESCs) that have also proved useful for dissecting the differentiation process *in vitro* (Wobus et al., 2002; Kawaguchi et al., 2005; Sakurai et al., 2006). The first method, which was culturing ESCs on collagen IV-coated dishes under serum-containing medium, supported the generation of mesoderm cells that can give rise to chondrocytes, osteocytes and myocytes. The other method involved treating ESCs with retinoic acid (Dani et al., 1997), thus mimicking the natural MSC differentiation pathway through neuroepithelium. This second method was demonstrated *in vivo* using a Sox1-Cre knock-in mouse line and indicated that Sox1-positive neuroepithelial cells supply the earliest wave of MSC differentiation that occurs during embryogenesis and is subsequently replaced by MSCs from other origins during postnatal development (Takashima et al., 2007). Nevertheless, our observations in these previous studies did not rule out the possibility that mesoderm could give rise to MSCs under other appropriate conditions. The current study investigates this possibility further *in vivo* using our previously generated Pdgfrα-GFPCreER^{T2} knock-in mouse line together with several other mutant mouse lines. The results define Pdgfrα as a key marker of MSC *in vivo* and show that almost all adult BM-MSC and WAT-MSC originate from mesoderm and embryonic Pdgfrα-positive cells.

RESULTS

Emergence and localization of MSC

To first investigate when MSCs emerge during mouse embryonic development, we sorted total cells from whole embryos at respective developmental stages by flow cytometry using Pdgfrα as a MSC marker, and then performed colony-forming unit-fibroblast (CFU-F) assays on the sorted cells (Fig. 1A, Fig. S1A). Colonies of CFU-F initially emerged at embryonic day 9.5 (E9.5) and were significantly increased in number between E11.5 and E14.5 (Fig. 1A). We observed no colonies in Pdgfrα-negative fractions (Fig. 1A), and E7.5 cells could not be cultured using our methods (data not shown). Correlating with the increased CFU-F colony numbers during embryo development, the percentages of Pdgfrα-positive fractions in total cells also rose from E11.5 to E13.5 (Fig. S1A). The CFU-F colony formation tendency in C57BL/6 mice was similar to that of another mouse strain, ICR (Fig. S1B,C). Furthermore, we divided whole embryos into four parts, head, trunk, limb and tail, and then sorted Pdgfrα-positive cells from each. Trunks showed a similar sorting pattern to whole embryos, whereas CFU-F colonies in heads and limbs only emerged at E13.5 (Fig. 1B). The percentages of Pdgfrα-positive fractions in all segmented parts of embryos peaked at E13.5, similar to the results for whole embryos (Fig. S1A,D). Although we examined expressions of several marker genes in Pdgfrα-positive and -negative fractions by RT-PCR, *Sca1* (*Atxn1*) and *Cd51* (*Itgav*), which have been reported as MSC markers (Morikawa et al., 2009; Pinho et al., 2013), were expressed in both fractions (Fig. S1E). These experiments suggested that embryonic MSCs emerge in waves and that Pdgfrα is a key marker of MSCs (Fig. 1A, Figs S1C and S2A); however, we could not confirm that all of CFU-F colonies were MSCs with the attendant features of self-renewal capacity and multipotency. We therefore picked up some of the CFU-F colonies derived from various stages and tissues, and established cell lines for induction into adipocytes, chondrocytes and osteocytes (Fig. S2B). CFU-F colonies from E9.5 samples showed no multipotency, whereas some was shown for

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Ribosome Incorporation into Somatic Cells Promotes Lineage Transdifferentiation towards Multipotency

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Recently, we reported that bacterial incorporation induces cellular transdifferentiation of human fibroblasts. However, the bacterium-intrinsic cellular- transdifferentiation factor remained unknown. Here, we found that cellular transdifferentiation is caused by ribosomes. Ribosomes, isolated from both prokaryotic and eukaryotic cells, induce the formation of embryoid body-like cell clusters. Numerous ribosomes are incorporated into both the cytoplasm and nucleus through trypsin-activated endocytosis, which leads to cell-cluster formation. Although ribosome-induced cell clusters (RICs) express several stemness markers and differentiate into derivatives of all three germ layers in heterogeneous cell populations, RICs fail to proliferate, alter the methylation states of pluripotent genes, or contribute to teratoma or chimera formation. However, RICs express markers of epithelial–mesenchymal transition without altering the cell cycle, despite their proliferation obstruction. These findings demonstrate that incorporation of ribosomes into host cells induces cell transdifferentiation and alters cellular plasticity.

Terminally differentiated somatic cells are considered to be stable. However, pluripotency can be achieved by transplanting the nuclei of frog somatic cells into eggs[1]. Furthermore, induced pluripotent stem (iPS) cells can be generated by forced expression of specific transcription factors[2]. A recent study showed that pluripotent stem cells can also be generated from mouse somatic cells by using a cocktail of small-molecule compounds[3].

Humans contact microbiota immediately after birth and interact broadly with microbiota throughout life, such as during disease[4], nutrient absorption[5], and immune system development[6]. The microbial community in the human intestine has been widely analyzed, and lactic acid bacteria are common bacteria among the intestinal

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A Skeletal Muscle Model of Infantile-onset Pompe Disease with Patient-specific iPS Cells

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Pompe disease is caused by an inborn defect of lysosomal acid α-glucosidase (GAA) and is characterized by lysosomal glycogen accumulation primarily in the skeletal muscle and heart. Patients with the severe type of the disease, infantile-onset Pompe disease (IOPD), show generalized muscle weakness and heart failure in early infancy. They cannot survive over two years. Enzyme replacement therapy with recombinant human GAA (rhGAA) improves the survival rate, but its effect on skeletal muscle is insufficient compared to other organs. Moreover, the patho-mechanism of skeletal muscle damage in IOPD is still unclear. Here we generated induced pluripotent stem cells (iPSCs) from patients with IOPD and differentiated them into myocytes. Differentiated myocytes showed lysosomal glycogen accumulation, which was dose-dependently rescued by rhGAA. We further demonstrated that mammalian/mechanistic target of rapamycin complex 1 (mTORC1) activity was impaired in IOPD iPSC-derived myocytes. Comprehensive metabolomic and transcriptomic analyses suggested the disturbance of mTORC1-related signaling, including deteriorated energy status and suppressed mitochondrial oxidative function. In summary, we successfully established an *in vitro* skeletal muscle model of IOPD using patient-specific iPSCs. Disturbed mTORC1 signaling may contribute to the pathogenesis of skeletal muscle damage in IOPD, and may be a potential therapeutic target for Pompe disease.

Pompe disease (OMIM 232300, glycogen storage disease type II or acid maltase deficiency) is one of the lysosomal storage disorders, caused by an inborn defect of lysosomal acid α-glucosidase (GAA). GAA is the only enzyme that can degrade glycogen into glucose in the lysosomes. Thus, the lack of GAA causes abnormal accumulation of glycogen within the lysosomes, primarily in the skeletal muscle and heart[1]. Patients with Pompe disease show an extremely wide spectrum in the severity of their symptoms depending on the residual amount of GAA activity, and are generally classified into two categories according to time of onset[2], infantile-onset Pompe disease (IOPD) and late-onset (LOPD). Patients with IOPD develop generalized muscle weakness and heart failure in early infancy, and almost all the patients cannot survive over two years[3,4]. On the other hand, patients with LOPD, having partial defects of GAA, slowly develop progressive skeletal muscle weakness, often resulting in ventilator dependence and shortened lifespans[5]. The only treatment currently available is enzyme replacement therapy (ERT) with recombinant human GAA (rhGAA), which dramatically improves the survival rate in patients with IOPD[6,7]. However, the limitations of ERT have become increasingly evident. ERT is very effective on cardiac symptoms, but its effect on skeletal muscle symptoms is limited, and many patients eventually become dependent on artificial ventilation. In addition, emerging anti-rhGAA antibodies that attenuate therapeutic response to ERT is another serious problem for lifelong treatment[8,9]. Thus, the development of a novel therapeutic approach or adjunctive therapy to the current ERT is urgently needed.

The pathogenesis of skeletal muscle damage in Pompe disease has not been fully elucidated. Formerly, lysosomal rupture due to glycogen accumulation and release of its lytic enzymes into the cytoplasm were considered as the explanation of muscle damage[10,11]. Recent studies of GAA knockout mice or muscle biopsies from patients with LOPD demonstrated that secondary autophagic dysfunction plays an important role in progressive muscle

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Impact of the Niemann−Pick c1 Gene Mutation on the Total Cellular Glycomics of CHO Cells

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Supporting Information

ABSTRACT: Niemann−Pick disease type C (NPC) is an autosomal recessive lipid storage disorder, and the majority of cases are caused by mutations in the *NPC1* gene. In this study, we clarified how a single gene mutation in the *NPC1* gene impacts the cellular glycome by analyzing the total glycomic expression profile of Chinese hamster ovary cell mutants defective in the *Npc1* gene (*Npc1* KO CHO cells). A number of glycomic alterations were identified, including increased expression of lactosylceramide, GM1, GM2, GD1, various neolacto-series glycosphingolipids, and sialyl-T (O-glycan), which was found to be the major sialylated protein-bound glycan, as well as various N-glycans, which were commonly both fucosylated and sialylated. We also observed significant increases in the total amounts of free oligosaccharides (fOSs), especially in the unique complex- and hybrid-type fOSs. Treatment of *Npc1* KO CHO cells with 2-hydroxypropyl-β-cyclodextrin (HPBCD), which can reduce cholesterol and glycosphingolipid (GSL) storage, did not affect the glycomic alterations observed in the GSL-, N-, and O-glycans of *Npc1* KO CHO cells. However, HPBCD treatment corrected the glycomic alterations observed in fOSs to levels observed in wild-type cells.

KEYWORDS: *Niemann−Pick disease, glycomics, N-glycan, O-glycan, glycosaminoglycan, glycosphingolipids, free oligosaccharide, cyclodextrin*

■ INTRODUCTION

Niemann−Pick disease type C (NPC) is an inborn error of metabolism caused by mutations in the lipid transporter genes, *NPC1* and *NPC2*.[1,2] In cooperation with NPC2, NPC1 is involved in the transport of cholesterol between lysosomes and the endoplasmic reticulum (ER). Mutations in the *NPC1* and *NPC2* genes disrupt this transport mechanism, resulting in the accumulation of free cholesterol and glycolipids in lysosomes. NPC patients suffer from hepatological and neurological dysfunctions and eventually die due to respiratory and hepatic failure.[3] Miglustat is a lipogenesis inhibitor indicated for NPC; however, its effects are so limited that patients eagerly await new advances in drug development for NPC therapy.[4] A Phase IIb/III clinical trial of 2-hydroxypropyl-β-cyclodextrin (HPBCD), which can reduce cholesterol and glycosphingolipid (GSL) storage in neurons, is currently being conducted in the United States and Europe. Furthermore, we recently found that 2-hydroxypropyl-γ-cyclodextrin (HPGCD) is more effective than HPBCD in reducing the cholesterol accumulation and restoring the functional and molecular abnormalities in cells derived from NPC patients. Because of the availability of disease-modifying treatments[5] and the development of new therapies, there is an urgent need for reliable and robust biomarkers.

Because most cellular processes are generally regulated by different feedback loops and alterations and glycomic profiles are likely to be amplified compared with upstream genetic variations, a glycomic study of NPC may provide a good opportunity to identify novel biomarkers to monitor disease progression or serve as outcome measures in future clinical trials. Indeed, several pioneering studies reported various glycomic alterations in NPC involving various glycosphingolipids (glucosylceramides (GlcCer), lactosylceramide (LacCer),

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Synthesis of multi-lactose-appended β-cyclodextrin and its cholesterol-lowering effects in Niemann–Pick type C disease-like HepG2 cells

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Full Research Paper

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Keywords:
asialoglycoprotein receptor; cholesterol; cyclodextrin; lactose; Niemann–Pick type C disease

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Abstract
Niemann–Pick type C (NPC) disease, characterized by intracellular accumulation of unesterified cholesterol and other lipids owing to defects in two proteins NPC1 and NPC2, causes neurodegeneration and other fatal neurovisceral symptoms. Currently, treatment of NPC involves the use of 2-hydroxypropyl-β-cyclodextrin (HP-β-CD). HP-β-CD is effective in the treatment of hepatosplenomegaly in NPC disease, albeit at a very high dose. One of the methods to reduce the required dose of HP-β-CD for treatment of NPC is to actively targeting hepatocytes with β-cyclodextrin (β-CD). The aim of the present study was to synthesize a novel multi-lactose-appended β-CD (multi-Lac-β-CD) and to evaluate its cholesterol-lowering effect in U18666A-treated HepG2 (NPC-like HepG2) cells. Further, the study aimed at delivering β-CD to hepatocytes via cholesterol-accumulated HepG2 cells, and indicated that the newly synthesized multi-Lac-β-CD had an average degree of substitution of lactose (DSL) of 5.6. This newly synthesized multi-Lac-β-CD was found to significantly decrease the concentration of intracellular cholesterol with negligible cytotoxicity as compared to HP-β-CD. An increased internalization of TRITC-multi-Lac-β-CD (DSL 5.6) as compared to TRITC-HP-β-CD was observed in NPC-like HepG2 cells. Further, the dissociation constant of peanut lectin with multi-Lac-β-CD (DSL5.6) was found to be extremely low (2.5×10^{-8} M). These results indicate that multi-Lac-β-CD (DSL5.6) diminished intracellular cholesterol levels in NPC-like HepG2 cells via asialoglycoprotein receptor (ASGPR)-mediated endocytosis.

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Identifying the Biphasic Role of Calcineurin/NFAT Signaling Enables Replacement of Sox2 in Somatic Cell Reprogramming

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Key Words. Calcineurin • Nuclear factor of activated T cells • Reprogramming • Induced pluripotent stem cells • SRY (sex determining region Y)-box 2

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ABSTRACT
Induction of pluripotency with defined factors (octamer-binding transcription factor 4 [Oct4], SRY (sex determining region Y)-box 2 [Sox2], Kruppel-like factor 4 [Klf4], c-Myc) raises hopes for successful clinical trials. Despite considerable efforts, the molecular mechanism of reprogramming remains poorly understood. The aim of the present study was to identify the role of calcineurin/nuclear factor of activated T cells (NFAT) in reprogramming. Our results demonstrated a biphasic role for calcineurin/NFAT signaling during reprogramming. In the early phase of reprogramming, calcineurin activity is required to maintain proper cell cycle division and for mesenchymal–epithelial transition. In the late phase, calcineurin exerts a negative effect that is mediated by NFATc2. NFATc2 interacts with Hdac3, Ezh2, and Suv39h1 to increase H3K9me3 and H3K27me3 over the Sox2 enhancer and Klf2 promoter, respectively, resulting in the downregulation of their expression. Moreover, Gαq was identified as a positive upstream regulator for calcineurin. The Gαq/calcineurin/NFATc2 axis negatively regulates the late step of reprogramming. By inhibiting NFATc2 or calcineurin, induced pluripotent stem cells could be established without exogenous Sox2. Thus, the present study revealed another regulatory level of reprogramming, and proposes a biological axis that could be useful for cancer therapy. STEM CELLS *2017;35:1162–1175*

SIGNIFICANCE STATEMENT
Despite enormous contributions to reveal detailed molecular and epigenetic modification during reprogramming, the mechanism is still poorly understood. Here, we uncover another level of reprogramming complexity by identifying the biphasic role of calcineurin in reprogramming which enables to replace Sox2 in reprogramming. We also identified Gnaq as upstream regulator for calcineurin. The newly identified regulatory axis represented by Gnaq/Calcineurin/NFATc2 over Klf2 promoter and Sox2 enhancer provides a new physiological model that can be vital for therapeutic applications.

INTRODUCTION
Ectopic expression of four reprogramming factors, namely octamer-binding transcription factor 4 (Oct4), SRY (sex determining region Y)-box 2 (Sox2), Kruppel-like factor 4 (Klf4), and c-Myc, enables somatic cells to be successfully reprogrammed to the pluripotent state, resulting in induced pluripotent stem cells (iPSCs) [1, 2]. iPSCs provide a powerful tool for studying early embryonic events, drug screening, and remodeling patient-specific diseases. Because of their high potential to differentiate into various cell types, iPSCs hold considerable promise for successful trials of cell transplantation [3].

Ever since iPSCs were established, tremendous efforts have been made to decipher the detailed molecular and epigenetic modifications linked to the reprogramming process. Based on kinetics and molecular aspects, models have been proposed that divide the reprogramming process into either two or three phases. The two-phase model was explained by an early stochastic phase of gene activation of variable latency followed by a late deterministic phase of gene activation of constant latency [4]. Conversely, the three-phase model starts with an initiation phase during which the cell undergoes mesenchymal–epithelial transition (MET), followed by a maturation phase, mediated by upregulation of pluripotency markers, and then finally a stabilization phase, which is characterized by removal of epigenetic memory, telomere elongation, and expression of self-renewal and pluripotency markers in reprogrammed cells that is independent of transgenes [5, 6].

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AMYOTROPHIC LATERAL SCLEROSIS

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The Src/c-Abl pathway is a potential therapeutic target in amyotrophic lateral sclerosis

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Amyotrophic lateral sclerosis (ALS), a fatal disease causing progressive loss of motor neurons, still has no effective treatment. We developed a phenotypic screen to repurpose existing drugs using ALS motor neuron survival as readout. Motor neurons were generated from induced pluripotent stem cells (iPSCs) derived from an ALS patient with a mutation in superoxide dismutase 1 (*SOD1*). Results of the screen showed that more than half of the hits targeted the Src/c-Abl signaling pathway. Src/c-Abl inhibitors increased survival of ALS iPSC-derived motor neurons in vitro. Knockdown of Src or c-Abl with small interfering RNAs (siRNAs) also rescued ALS motor neuron degeneration. One of the hits, bosutinib, boosted autophagy, reduced the amount of misfolded mutant SOD1 protein, and attenuated altered expression of mitochondrial genes. Bosutinib also increased survival in vitro of ALS iPSC-derived motor neurons from patients with sporadic ALS or other forms of familial ALS caused by mutations in TAR DNA binding protein (*TDP-43*) or repeat expansions in *C9orf72*. Furthermore, bosutinib treatment modestly extended survival of a mouse model of ALS with an *SOD1* mutation, suggesting that Src/c-Abl may be a potentially useful target for developing new drugs to treat ALS.

INTRODUCTION

Amyotrophic lateral sclerosis (ALS) is a neurodegenerative disease that causes progressive loss of motor neurons (*1*, *2*). The disease progression is fast, and there is no effective treatment. Most cases are classified as sporadic ALS, whereas about 10% are familial. About 25% of the familial ALS (FALS) cases are associated with mutations in the gene encoding Cu/Zn superoxide dismutase (SOD1) (*3*). Although mutant SOD1 transgenic mice recapitulate ALS phenotypes (*4*) and have been used

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for preclinical studies of ALS drug development, only a limited number of compounds have been tested. Thus, we developed a phenotypic screening assay for testing a number of compounds with a readout of ALS motor neuron survival. In previous studies of ALS, many kinds of causative genes have been discovered and multiple hypotheses about molecular pathogenesis have been proposed. However, it is clear that motor neuron death is an undisputed common phenotype among the heterogeneous forms of ALS (*1*, *2*). Many drug screening platforms for ALS have been developed based on induced pluripotent stem cell (iPSC) technology (*5–11*). Here, we introduced transcription factors using the *piggyBac* vector system (*12*) to generate iPSCs from an ALS patient carrying an *SOD1* mutation and then derived motor neurons from them. Using this phenotypic assay, we screened existing drugs (*13*) and identified Src/c-Abl inhibitors that ameliorated ALS motor neuron degeneration.

Src and c-Abl are ubiquitous nonreceptor tyrosine kinases that were identified as the mammalian homologs of the oncogene products of Rous sarcoma virus and Abelson murine leukemia virus, respectively. Activation of Src, which is associated with cell proliferation, angiogenesis, apoptosis, and invasion, has been observed in tumors and is a target for cancer therapy (*14*). The Bcr-Abl fusion protein, an oncogenic form of the c-Abl fusion kinase, is known to cause chronic myelogenous leukemia (CML) and Philadelphia chromosome–positive adult acute lymphoblastic leukemia, and c-Abl inhibitors have been developed as anti-CML drugs (*15*). Src/c-Abl is associated with various cellular functions (*16*, *17*), and several studies have shown the involvement of Src family proteins and c-Abl in neurodegenerative diseases (*18–24*).

RESULTS

To screen compounds, we used survival of ALS patient iPSC-derived motor neurons as readout. This required large-scale generation of

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Inhibition of Apoptosis Overcomes Stage-Related Compatibility Barriers to Chimera Formation in Mouse Embryos

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SUMMARY

Cell types more advanced in development than embryonic stem cells, such as EpiSCs, fail to contribute to chimeras when injected into pre-implantation-stage blastocysts, apparently because the injected cells undergo apoptosis. Here we show that transient promotion of cell survival through expression of the anti-apoptotic gene *BCL2* enables EpiSCs and Sox17⁺ endoderm progenitors to integrate into blastocysts and contribute to chimeric embryos. Upon injection into blastocyst, *BCL2*-expressing EpiSCs contributed to all bodily tissues in chimeric animals while Sox17⁺ endoderm progenitors specifically contributed in a region-specific fashion to endodermal tissues. In addition, *BCL2* expression enabled rat EpiSCs to contribute to mouse embryonic chimeras, thereby forming interspecies chimeras that could survive to adulthood. Our system therefore provides a method to overcome cellular compatibility issues that typically restrict chimera formation. Application of this type of approach could broaden the use of embryonic chimeras, including region-specific chimeras, for basic developmental biology research and regenerative medicine.

Naive and primed pluripotent stem cells, originating from mouse early pre-implantation blastocysts (~E3.5–E4.5 of mouse development) and later post-implantation egg cylinders (~E5.5–E7.5), respectively, have long been thought to harbor distinct developmental potentials based on the outcome of chimera assays (Silva and Smith, 2008; Nichols and Smith, 2009). Naive mouse embryonic stem cells (mESCs) can integrate into early blastocysts and subsequently contribute to all bodily tissues in chimeric adult animals. By contrast, primed pluripotent stem cells, i.e. post-implantation mouse epiblast cells (Gardner et al., 1985) or cultured epiblast stem cells (EpiSCs) (Brons et al., 2007; Tesar et al., 2007), generally fail to engraft into early blastocysts, although they can sparsely integrate into post-implantation egg cylinders and differentiate into small numbers (dozens) of cells (Huang et al., 2012; Kojima et al., 2014; Wu et al., 2015) and *E-cadherin* expression can enhance blastocyst integration to some extent (Ohtsuka et al., 2012). Based on these findings, the developmental potential of primed mouse EpiSCs has been questioned (Silva and Smith, 2008), and there has been a general recognition that injected cells optimally contribute to chimeras only if introduced back into the developmental stage from which they were originally derived (Mascetti and Pedersen, 2016). However, why the developmental fates of naive and primed mouse pluripotent cells separated by only 24 hr of development (from E4.5 versus E5.5) differ so significantly after blastocyst injection remains unclear (Loh et al., 2015).

Previously we have shown that, after injection into pre-implantation embryos, most EpiSCs rapidly disappeared within 24 hr (Masaki et al., 2015). Based on that observation, we reasoned that EpiSCs might be prone to apoptosis upon introduction into a heterologous (mismatched) developmental environment. Here we show that this is the case, and that prevention of apoptosis enables primed mouse and rat EpiSCs, and even lineage-committed Sox17⁺ endoderm progenitors,

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Regular Article

Cholesterol-Lowering Effect of Octaarginine-Appended β-Cyclodextrin in *Npc1*-Trap-CHO Cells

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Niemann–Pick disease type C (NPC) is an autosomal recessive lysosomal storage disorder, which is an inherited disease characterized by the accumulation of unesterified cholesterol in endolysosomes. Recently, 2-hydroxypropyl-β-cyclodextrin (HP-β-CyD) has been used for the treatment of NPC, and ameliorated a hepatosplenomegaly in the patients. However, to obtain the treatment efficacy, a high dose of HP-β-CyD was necessary. Therefore, the decrease in dose by using active intracellular delivery system of β-CyD to NPC cells is expected. In this study, to efficiently deliver β-CyD to NPC-like cells, we newly synthesized octaarginine (R8)-appended β-CyD with a spacer of γ-aminobutyric acid (R8-β-CyD) and evaluated its cytotoxicity, intracellular distribution, endocytosis pathway and cholesterol-lowering effect in *Npc1*-trap-Chinese hamster ovary (CHO) cells, cholesterol-accumulated cells through the impairment of NPC1 function. R8-β-CyD did not show cytotoxicity in the cells. In addition, Alexa568-labeled R8-β-CyD was actively internalized into *Npc1*-trap-CHO cells, possibly through micropinocytosis. Notably, R8-β-CyD significantly decreased intracellular cholesterol content compared with HP-β-CyD. These results suggest that R8-β-CyD may be a promising therapeutic agent for ameliorating cholesterol accumulation in NPC.

Key words cyclodextrin; octaarginine; Niemann–Pick disease type C; cholesterol; cell-penetrating peptide

Niemann–Pick disease type C (NPC) is an atypical lysosomal storage disorder, which is an inherited disease characterized by the accumulation of unesterified cholesterol in endolysosomes. NPC is elicited by the mutations in either *Npc1* or *Npc2* gene, and elicits hepatosplenomegaly, neurodegeneration and failure to thrive childhood.[1–3] NPC1 protein in endolysosomes is dominantly associated with cholesterol trafficking in cells.[3,4] Therefore, in NPC patients with loss of function of NPC1 protein, an excessive accumulation of unesterified cholesterol in endolysosomes and a shortage of esterified cholesterol in other cellular compartments are observed. Therefore, decreasing the cholesterol level in endolysosomes was found to be crucial approach for the treatment of NPC.

To evaluate the cholesterol-decreasing ability of drug candidates against NPC, *in vitro* cell culture systems by utilizing NPC-like cells are necessary. Recently, Higaki *et al.* established the *Npc1*-deficient Chinese hamster ovary (CHO) cell mutants (*Npc1*-trap-CHO cells) by gene trap mutagenesis.[5] *Npc1*-trap-CHO cells exhibit the phenotype characteristics of *Npc1*-deficient cells; the accumulation of free cholesterol in endocytic vesicles and the upregulation of cholesterol synthesis through mevalonate pathway.[5,6] Therefore, *Npc1*-trap-CHO cells can be applicable for the model of NPC cells.

Cyclodextrins (CyDs) are cyclic oligosaccharides consisting of 6–8 glucose units and have been utilized for improvement of certain properties of drugs such as solubility, stability and bioavailability, *etc.*, through the formation of inclusion complexes.[7,8] Recently, of various CyD derivatives, 2-hydroxypropyl-β-cyclodextrin (HP-β-CyD) has utilized for the treatment of NPC,[4,9–11] based on the several reports demonstrating the gain of longevity of *Npc1*-deficient mice by a cholesterol decreasing ability of HP-β-CyD.[9–11] Unfortunately, HP-β-CyD was found to produce an increasing in hearing threshold.[12] Most recently, it is demonstrated that HP-γ-CyD, which is more biocompatible than HP-β-CyD, can also lower the accumulation of cholesterol in NPC-like cells.[13] However, further dosing as well as dose escalations are needed to ascertain sufficient efficacy of HP-β-CyD or HP-γ-CyD.

To deliver the various bioactive molecules into cells, arginine-rich cell-penetrating peptides (CPPs) such as the oligoarginine peptides and human immunodeficiency virus (HIV)-1 Tat peptide are promising carriers. So far, various cellular uptake mechanisms of arginine-rich CPPs were proposed, *i.e.*, physiological cellular uptake (*i.e.*, endocytosis) and direct permeation through the plasma membranes,[14,15] and recently macropinocytosis (accompanied by actin reorganization, plasma membrane ruffling, and the stimulated engulfment of large volumes of extracellular fluid) has been shown to be an important pathway.[16–20] Therefore, CPP-mediated intracellular delivery is likely to be promising approach to deliver CyDs to NPC disease cells. In this study, to efficiently deliver β-CyD to *Npc1*-trap-CHO cells, we newly fabricated octaarginine (R8)-appended β-CyD with a spacer of γ-aminobutyric acids (R8-β-CyD) and evaluated its cytotoxicity, cholesterol-lowering effect and intracellular distribution in *Npc1*-trap-CHO

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RESEARCH ARTICLE

Cellular Functions and Gene and Protein Expression Profiles in Endothelial Cells Derived from Moyamoya Disease-Specific iPS Cells

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Abstract

Background and purpose

Moyamoya disease (MMD) is a slow, progressive steno-occlusive disease, arising in the terminal portions of the cerebral internal carotid artery. However, the functions and characteristics of the endothelial cells (ECs) in MMD are unknown. We analyzed these features using induced pluripotent stem cell (iPSC)-derived ECs.

Methods

iPSC lines were established from the peripheral blood of three patients with MMD carrying the variant *RNF213 R4810K*, and three healthy persons used as controls. After the endothelial differentiation of iPSCs, $CD31^{+}CD144^{+}$ cells were purified as ECs using a cell sorter. We analyzed their proliferation, angiogenesis, and responses to some angiogenic factors, namely VEGF, bFGF, TGF-β, and BMP4. The ECs were also analyzed using DNA microarray and proteomics to perform comprehensive gene and protein expression analysis.

Results

Angiogenesis was significantly impaired in MMD regardless of the presence of any angiogenic factor. On the contrary, endothelial proliferation was not significant between control- and MMD-derived cells. Regarding DNA microarray, pathway analysis illustrated that extracellular matrix (ECM) receptor-related genes, including integrin β3, were significantly downregulated in MMD. Proteomic analysis revealed that cytoskeleton-related proteins were downregulated and splicing regulation-related proteins were upregulated in MMD.

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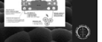
Establishment and gene expression analysis of disease-derived induced pluripotent stem cells of scleroderma



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ABSTRACT

Background: We recently generated induced pluripotent stem cells (iPSCs) from cultured dermal fibroblasts of systemic sclerosis (SSc-iPSC) to study the disease mechanisms.
Objective: In the present study, we have performed gene expression analysis using cultured SSc dermal fibroblasts, SSc-iPSC, and fibroblasts re-differentiated from SSc-iPSC (SSc-iPSC-FB).
Methods: mRNA and protein levels of collagen and integrins were analyzed using PCR array, PCR, immunoblotting, and immunofluorescence.
Results: We compared expression pattern of TGF-β-related genes between normal iPSC (NS-iPSC) and SSc-iPSC by PCR array, and found constitutive and significant down-regulation of S100A8, Smad6, and TGF-β2 in SSc-iPSC.
The expression of these genes was not altered in cultured SSc fibroblasts or SSc-iPSC-FB compared to NS fibroblasts or NS-iPSC-FB, respectively. On the other hand, the expression of collagen, integrin α and β was up-regulated in SSc fibroblasts, while SSc-iPSC-FB showed normalized levels of collagen and integrin β.
Conclusions: So far, there have been no reports investigating disease-derived iPSCs of SSc. Our results suggest that S100A8, Smad6, and TGF-β2 may be the key molecules of this disease. On the other hand, the normalization of collagen and integrins by iPSC reprogramming suggests that epigenetic modifications of genes may play a role in the mechanism of collagen accumulation seen in SSc fibroblasts, and that gene reprogramming may become novel therapeutic approach.
As the limitation of this study, we established only one iPSC line from each patient, which may not be enough to discuss disease-specific phenotypes. Larger studies including increased number of iPSC lines are needed in the future.
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1. Introduction

Tissue fibrosis of skin and internal organs, autoimmune abnormalities and vascular involvements are the characteristics of systemic sclerosis (SSc). Although the pathogenesis is still unknown, the pathogenic triad may affect each other, and may form the complex symptoms of this disease. As the clue, cultured dermal fibroblasts derived from affected lesion of SSc skin are similar to normal dermal fibroblasts stimulated with transforming growth factor (TGF)-β1: TGF-β1 is the most potent inducers of extracellular matrix (ECM) accumulation [1], and SSc dermal fibroblasts produce excessive amounts of various collagens (especially type I and type III collagens), which causes the tissue fibrosis [2–4]. Accordingly, the ECM accumulation in SSc may be a result of stimulation by TGF-β1 signaling.

On the other hand, we previously demonstrated that TGF-β1 levels in the media of cultured SSc fibroblasts are not increased compared to those in normal fibroblasts [5], indicating that TGF-β1 signal is activated without increase of TGF-β1 in SSc fibroblasts. Our previous studies suggested that integrins play central roles in

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RESEARCH Open Access

Modeling Alexander disease with patient iPSCs reveals cellular and molecular pathology of astrocytes

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Abstract

Alexander disease is a fatal neurological illness characterized by white-matter degeneration and formation of Rosenthal fibers, which contain glial fibrillary acidic protein as astrocytic inclusion. Alexander disease is mainly caused by a gene mutation encoding glial fibrillary acidic protein, although the underlying pathomechanism remains unclear. We established induced pluripotent stem cells from Alexander disease patients, and differentiated induced pluripotent stem cells into astrocytes. Alexander disease patient astrocytes exhibited Rosenthal fiber-like structures, a key Alexander disease pathology, and increased inflammatory cytokine release compared to healthy control. These results suggested that Alexander disease astrocytes contribute to leukodystrophy and a variety of symptoms as an inflammatory source in the Alexander disease patient brain. Astrocytes, differentiated from induced pluripotent stem cells of Alexander disease, could be a cellular model for future translational medicine.

Keywords: Alexander disease (AxD), Glial fibrillary acidic protein (GFAP), Induced pluripotent stem cells (iPSCs), Disease modeling, Astrocytes, Rosenthal fibers, Heat-shock protein, Alpha-crystallin, Cytokine, Inflammatory response, Inherited astrocytopathy

Introduction

Alexander disease (AxD) was first described by W. S. Alexander [1]. The clinical phenotypes of AxD are macrocephaly, frontal leukodystrophy and a variety of developmental delays with epileptic seizures, dysphagia, or bulbar/pseudobulbar signs. However, the severity of these clinical features differs among patients, being mostly dependent on the age of onset [2].

The common neuropathological feature of AxD is the presence of Rosenthal fibers, a unique cytoplasmic inclusion within astrocytes. Rosenthal fibers contain glial fibrillary acidic protein (GFAP), major astrocytic intermediate filament protein and molecular chaperones, including alpha-B-crystallin and other heat shock proteins [3, 4]. After extensive neuropathological investigations, missense mutations in GFAP have been identified as a genetic basis for AxD [5]. The discovery of the GFAP mutations opened the way to the development of model systems using tissue culture cells and transgenic mice for the study of AxD. Transgenic models recapitulated GFAP aggregations. However, it remained unclear how AxD mutations lead to protein aggregation in patient astrocytes as well as how mutant GFAP-expressing astrocytes contribute to neuronal degeneration [6].

In 2007, the discovery of a combination of transcription factors that could reprogram somatic cells into cells exhibiting pluripotency, called induced pluripotent stem cells (iPSCs), has provided researchers with a revolutionary tool to study human biology and diseases [7]. iPSCs can be derived from many somatic cell types, including easily accessible dermal fibroblasts and peripheral blood mononuclear cells [8, 9]. Similar to human embryonic stem cells (hESCs), iPSCs can self-renew and expand indefinitely in culture [7]. More importantly, they share the capacity to generate any cell types in the body, a property that is particularly useful for the study of neurological diseases. The pluripotency of iPSCs enables the production of astrocytes for disease modeling [10–12]. This remarkable feature of

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ARTICLE

Disease modeling and lentiviral gene transfer in patient-specific induced pluripotent stem cells from late-onset Pompe disease patient

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Pompe disease is an autosomal recessive inherited metabolic disease caused by deficiency of acid α-glucosidase (GAA). Glycogen accumulation is seen in the affected organ such as skeletal muscle, heart, and liver. Hypertrophic cardiomyopathy is frequently seen in the infantile onset Pompe disease. On the other hand, cardiovascular complication of the late-onset Pompe disease is considered as less frequent and severe than that of infantile onset. There are few investigations which show cardiovascular complication of late onset Pompe disease due to the shortage of appropriate disease model. We have generated late-onset Pompe disease-specific induced pluripotent stem cell (iPSC) and differentiated them into cardiomyocytes. Differentiated cardiomyocyte shows glycogen accumulation and lysosomal enlargement. Lentiviral GAA rescue improves GAA enzyme activity and glycogen accumulation in iPSC. The efficacy of gene therapy is maintained following the cardiomyocyte differentiation. Lentiviral GAA transfer ameliorates the disease-specific change in cardiomyocyte. It is suggested that Pompe disease iPSC-derived cardiomyocyte is replicating disease-specific changes in the context of disease modeling, drug screening, and cell therapy.

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INTRODUCTION

Pompe disease (PD) is an autosomal recessive, lysosomal storage disease caused by deficiency of acid-α-glucosidase (GAA), which is located in lysosome and degrades glycogen.[1] Based on clinical manifestation, PD is divided into infantile form, which is remarkable for neonatal onset progressive weakness of skeletal muscle and cardiac hypertrophy, and late-onset form, which is known for later and slower progressive weakness of skeletal muscle.[2]

Enzyme replacement therapy (Myozyme) was initiated in 2006; however clinical response was known to be variable.[3,4] *Ex vivo* lentiviral gene therapy is promising alternative treatment modality. Several human trials of lentiviral gene therapy have been conducted and efficacy is reported in some monogenic diseases.[5,6] Other treatment strategy, such as chemical chaperon, which enhances the residual enzyme activity by stabilizing mutated enzyme, is still under development.[7] There are few available treatment modalities at this point and novel therapeutic strategy is warranted.

In 2011, Huang *et al.*[8] reported the generation of iPS cells from infantile form PD patient and cardiomyocyte differentiation. Transient expression of GAA was required to generate PD-specific iPS cells. Induced cardiomyocyte contains higher amount of glycogen and manifests disarrayed cardiomyocyte fiber.

Although, it has been considered that cardiovascular complication of PD is limited to infantile onset PD. Several researches showed that cardiac complication was sometimes seen in late-onset Pompe disease (LOPD), even though it is less severe and frequent.[9,10] However, shortage of bio-resource made it difficult to investigate the disease mechanism.

iPS cells are excellent tools for screening drugs. However, some concerns exist if disease phenotypes are maintained after differentiation to specific cells, such as cardiomyocyte. In addition, differentiated pluripotent stem cell replacement might be useful in terms of autologous pluripotent stem cell therapy if combined with gene correction method such as viral transfer and homologous recombination by TALEN or CRISPR/Cas9.[11]

iPS cells from LOPD patient have been successfully generated and even after cardiomyocyte differentiation disease hallmarks were observed in induced cardiomyocyte. We additionally conducted gene transfer to PD iPS cells and rescued the phenotype both biochemically and pathologically.

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Aloe vera Extract Suppresses Proliferation of Neuroblastoma Cells *In Vitro*

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Abstract. *Background/Aim: Neuroblastoma is a pediatric solid tumor refractory to eradication by chemotherapy. To determine whether Aloe vera (AV), a pontial anticancer reagent, could be useful in neuroblastoma therapy, we investigated the anti-proliferative effects of an AV protein extract. Materials and Methods: Human neuroblastoma cell lines (IMR-32, TGW, CHP-126 and NBL-S) were cultured with AV protein extract and proliferation status was assessed by cell counting, Ki-67 staining and gene expression. Results: Among tested lines, the number of viable, AV-treated IMR-32 cells significantly decreased 1.98-fold by day 2 and 1.33-fold by day 5 of culture relative to untreated controls (p<0.05). Treatment also decreased the number of Ki-67(+) IMR-32 cells by 13% by day 5 (p<0.05) and, unlike untreated controls, CCND2 mRNA expression levels became undetectable by day 1. Conclusion: AV-protein extract suppresses human IMR-32 neuroblastoma cell proliferation, possibly by suppressing CCND2 transcript levels in vitro.*

Neuroblastoma, a pediatric solid tumor derived from precursors of sympathetic nervous system, is the third most common cancer in children behind leukemia and brain tumors (1). Average annual incidence of neuroblastoma was 22.3 and 29.8 cases per 100,000 births in the US (1973-1992) and Japan (1980-1998) (2, 3), respectively. Presently, neuroblastoma is treated by a combination of surgery, radiation therapy and intensive chemotherapy. However, 60-70% of neuroblastoma patients who have poor prognosis, such as chromosome duplication and older age of onset, are resistant to chemotherapy (4) and more than 60% of chemotherapy-treated patients show disease recurrence (5). In addition, chemotherapy-related death accounts for 2-12% of patients (5). Therefore, development of more effective treatments for this condition are critical.

Plants are a natural source of bioactive molecules. *Aloe vera* (AV) is a succulent plant species used in traditional herbal medicine to treat burns. Recently, it was reported that AV also possesses anticancer activity (6-8). AV components, such as the emodin or aloe-emodin, reportedly suppress proliferation of human breast cancer cells (9), gastric cancer cells (10), human hepatoma cells (11) and glioma cells (12) *in vitro* and *in vivo* (13) (Table I). However, little is known about the anti-proliferative effects of AV in the case of neuroblastoma. To address this issue, we investigated the effect of an AV protein extract on human neuroblastoma cell proliferation. We report that this treatment suppressed proliferation of one human neuroblastoma cell line *in vitro*.

Materials and Methods

Preparation of an Aloe vera protein extract. The AV protein extract used in this study was provided by Natural Rendez-Vous Co., Ltd. (Ho Chi Minh City, Vietnam). The protein fraction of the extract was concentrated using an Amicon® Ultra Centrifugal Filter (molecular weight cut-off=3,000 Da, Millipore, Billerica, MA, USA). Protein quantity was measured using Quick Start™ Bradford Dye reagent (BIO-RAD, Hercules, CA, USA).

Cell culture and counting. Four human neuroblastoma lines were used in this study, including IMR-32 (14), TGW (15), CHP-126 (16) and NBL-S (17), provided from the Institute of Molecular

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Key Words: Neuroblastoma, *Aloe vera*, proliferation.

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TECHNOLOGY REPORT

Generation and Characterization of PDGFRα-GFPCreER^{T2} Knock-in Mouse Line

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Summary: Platelet-derived growth factor (PDGF) and its receptor play an important role in embryogenesis. PDGF receptor α (PDGFRα) is expressed specifically in the embryonic day 7.5 (E7.5) mesoderm and in the E9.5 neural crest among other tissues. PDGFRα-expressing cells and their descendants are involved in the formation of various tissues. To trace PDGFRα-expressing cells in vivo, we generated a knock-in mouse line that expressed a fusion protein of green fluorescent protein (GFP), Cre recombinase (Cre), and mutated estrogen receptor ligand-binding domain (ER^{T2}) under the control of the PDGFRα promoter. In these mice, Cre activity in PDGFRα-expressing cells could be induced by tamoxifen treatment. Taken together, our results suggest that the knock-in mouse line generated here could be useful for studying PDGFRα-expressing cells and their descendants in vivo at various stages of development. genesis 53:329–336, 2015. © 2015 Wiley Periodicals, Inc.

Key words: PDGFRα; CreER^{T2}; knock-in; mesoderm; neural crest; mesenchyme

INTRODUCTION

The mesoderm and its descendant cells are important in the formation of various tissues and are involved in many injury repair and disease processes (Hamasaki *et al*., 2012; Kitagawa *et al*., 2012). Platelet-derived growth factor (PDGF) signaling is associated with cellular proliferation, survival, migration, and differentiation. The PDGF receptor α (PDGFRα) is one of two receptors for PDGF and binds to all isoforms of PDGFs, including PDGF-AA, PDGF-AB, PDGF-BB, PDGF-CC, and PDGF-DD (Andrae *et al*., 2008). PDGFRα is a marker of mesoderm cells at gastrulation, which is a germ layer that ultimately differentiates into bone, cartilage, skeletal muscle, and the dermis (Orr-Urtreger and Lonai, 1992; Orr-Urtreger *et al*., 1992; Schatteman *et al*., 1992; Takakura *et al*., 1997). PDGFRα is also widely expressed in the mesenchyme during organogenesis and is critical for the development of the palate, hair follicles, and lung tissue (Karlsson *et al*., 1999; Sun *et al*., 2000; Xu *et al*., 2005). Gene disruption of PDGFRα in mice results in abnormal patterning of somites, which are derived from the mesoderm, and pulmonary hypoplasia (Bleyl *et al*., 2007; Soriano, 1997). These findings indicate that PDGFRα signaling and PDGFRα-expressing cells are required for both morphogenesis and organogenesis in mouse development. Other studies have shown that PDGFRα was expressed by mesenchymal cells in the intestine and testis and that the numbers of these cells decreased markedly in PDGFRα-null embryos (Karlsson *et al*., 2000; Brennan *et al*., 2003). In adult human tissues, widespread expression of PDGFRα is also observed during abnormal fibrotic and sclerosing processes associated with inflammation (Heldin and Westermark, 1999). Taken together, these findings support the notion that PDGFRα-expressing cells are important for the normal development of mesenchymal tissues, and abnormal proliferation of such cells triggers pathogenetic events. Furthermore, PDGFRα is also expressed by neural crest cells, which

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Research Paper

Effects of cyclodextrins on GM1-gangliosides in fibroblasts from GM1-gangliosidosis patients

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Keywords
cyclodextrins; fibroblasts; GM1-gangliosidosis; lysosomes

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Abstract

Objectives GM1-gangliosidosis is an inherited disorder characterized by the accumulation of GM1-gangliosides in many tissues and organs, particularly in the brain. Currently, there is no treatment available for patients with ganglioside storage diseases. Therefore, we investigated the effects of cyclodextrins (CyDs) on the GM1-ganglioside level in EA1 cells, fibroblasts from patients with GM1-gangliosidosis.
Methods The concentrations of cholesterol and phospholipids in supernatants were determined by Cholesterol E-test Wako and Phospholipid C-test Wako, respectively. The effects of CyDs on GM1-ganglioside levels in EA1 cells using fluorescence-labelled cholera toxin B-subunit, which can bind to GM1-gangliosides specifically, were investigated by flow cytometry and confocal laser scanning microscopy.
Key findings The treatment with methylated CyDs, hydroxypropylated CyDs and branched CyDs decreased GM1-ganglioside levels in EA1 cells at 1 mM for 24 h. Unexpectedly, there was no significant change in the efflux of cholesterol or phospholipids from the cells after treatment with CyDs under the same experimental conditions, indicating that the efflux of membrane components is not associated with down-regulation of GM1-ganglioside levels in EA1 cells upon CyDs treatment.
Conclusions CyDs may have the potential as drugs for GM1-gangliosidosis, although the mechanism should be thereafter clarified.

Introduction

GM1-gangliosidosis is a rare lysosomal storage disorder characterized clinically by a wide range of variable neurovisceral, ophthalmological and dysmorphic features.[1] Without enough functional β-galactosidase which in humans is encoded by the *GLB1* gene, GM1-gangliosides cannot be degraded in lysosomes, and eventually GM1-gangliosides accumulate to toxic levels in many tissues and organs, particularly in the brain.[1] Several approaches for the treatment of GM1-gangliosidosis were developed, such as enzyme replacement therapy,[2] gene therapy,[3] chemical chaperone therapy.[4] However, at present, there is no treatment available for patients with ganglioside storage diseases.[5] Therefore, development of novel drugs for GM1-gangliosidosis is needed.

Cyclodextrins (CyDs) are cyclic oligosaccharides forming inclusion complexes with a wide range of hydrophobic molecules, and are used widely in pharmaceutical region.[6] Uekama and his colleagues previously reported that CyDs extracted cell membrane components such as cholesterol and phospholipids from lipid rafts, which contain high concentrations of cholesterol and glycosphingolipids including GM1-gangliosides.[7,8] Meanwhile, the administration of 2-hydroxypropyl-β-CyD (HP-β-CyD) to mice lacking Niemann-Pick disease type C (NPC) protein was reported

BEILSTEIN JOURNAL OF ORGANIC CHEMISTRY

Cholesterol lowering effects of mono-lactose-appended β-cyclodextrin in Niemann–Pick type C disease-like HepG2 cells

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Full Research Paper

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asialoglycoprotein receptor; cholesterol; cyclodextrin; lactose; Niemann–Pick disease type C

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Abstract

The Niemann–Pick type C disease (NPC) is one of inherited lysosomal storage disorders, emerges the accumulation of unesterified cholesterol in endolysosomes. Currently, 2-hydroxypropyl-β-cyclodextrin (HP-β-CyD) has been applied for the treatment of NPC. HP-β-CyD improved hepatosplenomegaly in NPC patients, however, a high dose of HP-β-CyD was necessary. Therefore, the decrease in dose by actively targeted-β-CyD to hepatocytes is expected. In the present study, to deliver β-CyD selectively to hepatocytes, we newly fabricated mono-lactose-appended β-CyD (Lac-β-CyD) and evaluated its cholesterol lowering effects in NPC-like HepG2 cells, cholesterol accumulated HepG2 cells induced by treatment with U18666A. Lac-β-CyD (degree of substitution of lactose (DSL) 1) significantly decreased the intracellular cholesterol content in a concentration-dependent manner. TRITC-Lac-β-CyD was associated with NPC-like HepG2 cells higher than TRITC-β-CyD. In addition, TRITC-Lac-β-CyD was partially localized with endolysosomes after endocytosis. Thus, Lac-β-CyD entered NPC-like HepG2 cells via asialoglycoprotein receptor (ASGPR)-mediated endocytosis and decreased the accumulation of intracellular cholesterol in NPC-like HepG2 cells. These results suggest that Lac-β-CyD may have the potential as a drug for the treatment of hepatosplenomegaly in NPC disease.

STEM CELLS

EMBRYONIC STEM CELLS/INDUCED PLURIPOTENT STEM CELLS

HPGCD Outperforms HPBCD as a Potential Treatment for Niemann-Pick Disease Type C During Disease Modeling with iPS Cells

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Key Words. Induced pluripotent stem cells • Transgene-free • Niemann-Pick disease type C • Experimental models

ABSTRACT

Niemann-Pick disease type C (NPC) is a lysosomal storage disease characterized by abnormal accumulation of free cholesterol and glycolipids. Here, we established induced pluripotent stem cell (iPSC) lines from NPC patients. Hepatocyte-like cells (HLCs) and neural progenitors derived from the iPSC lines accumulated cholesterol and displayed impaired autophagy and ATP production. A molecular signature related to lipid metabolism was also impaired in the NPC-iPSC-derived HLCs. These findings indicate that iPSC-derived cells can phenocopy human NPC. We also newly found that 2-hydroxypropyl-γ-cyclodextrin (HPGCD) could reduce the cholesterol accumulation and restore the functional and molecular abnormalities in the NPC patient-derived cells, and do so more effectively than 2-hydroxypropyl-β-cyclodextrin treatment. In addition, NPC model mice showed an improved liver status and prolonged survival with HPGCDs. Thus, iPSC lines derived from patient cells are powerful tools to study cellular models of NPC, and HPGCD is a potential new drug candidate for future treatment of this disease. STEM CELLS *2015;33:1075–1088*

INTRODUCTION

Induced pluripotent stem cells (iPSCs), which are artificially produced from human somatic cells, can be further induced to undergo sustained, unlimited growth, and exhibit multipotency (i.e., the ability to give rise to various cell types in vitro) [1, 2]. Because of these features, iPSCs are a potential source for cell therapy applications in clinical medicine. The process of iPSC generation, known as reprogramming, is triggered by the expression of four transcription factors, Oct3/4, Sox2, Klf4, and c-Myc, which are the same core factors underlying pluripotency in other pluripotent stem cells such as embryonic stem cells (ESCs) [3–5]. In particular, many procedures have now been reported to easily generate iPSCs from human fibroblasts and peripheral blood cells [6, 7].

Numerous iPSC lines derived from the somatic cells of patients harboring pathogenic mutations have been established and shown to phenocopy the disease [8–13]. These studies clearly demonstrated that disease-derived iPSC lines represent a powerful tool not only for cell therapy, but also for biomedical research and drug development [14, 15]. In particular, biomaterial samples obtained from patients with intractable diseases are indispensable for studying the underlying molecular mechanisms and developing new therapeutic agents. However, because the number and size of samples available from such patients are usually limited, disease-derived iPSCs are expected to be useful mainly as a replacement or supplemental source of biomaterials for developing new therapies.

Niemann-Pick disease type C (NPC) is a hereditary intractable disease associated with mutations in the lipid transporter genes, *NPC1* and *NPC2* [16, 17]. NPC1 helps to transport cholesterol between lysosomes and endoplasmic reticulum (ER) in cooperation with NPC2. Mutations in the *NPC1* and *NPC2* genes disrupt this transporting system, resulting in the accumulation of free cholesterol and glycolipids in lysosomes [18]. NPC patients suffer from liver and neurological dysfunctions and eventually die due to respiratory and hepatic failure [18]. Miglustat is a lipogenesis inhibitor indicated for NPC; however, its effect is so limited that patients eagerly await new advances in drug development for NPC therapy [19].

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Ectopic Cerebellar Cell Migration Causes Maldevelopment of Purkinje Cells and Abnormal Motor Behaviour in *Cxcr4* Null Mice

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Abstract

SDF-1/CXCR4 signalling plays an important role in neuronal cell migration and brain development. However, the impact of CXCR4 deficiency in the postnatal mouse brain is still poorly understood. Here, we demonstrate the importance of CXCR4 on cerebellar development and motor behaviour by conditional inactivation of *Cxcr4* in the central nervous system. We found CXCR4 plays a key role in cerebellar development. Its loss leads to defects in Purkinje cell dentritogenesis and axonal projection *in vivo* but not in cell culture. Transcriptome analysis revealed the most significantly affected pathways in the *Cxcr4* deficient developing cerebellum are involved in extra cellular matrix receptor interactions and focal adhesion. Consistent with functional impairment of the cerebellum, *Cxcr4* knockout mice have poor coordination and balance performance in skilled motor tests. Together, these results suggest ectopic the migration of granule cells impairs development of Purkinje cells, causes gross cerebellar anatomical disruption and leads to behavioural motor defects in *Cxcr4* null mice.

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Competing Interests: The authors have declared that no competing interests exist.

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Introduction

CXC chemokine receptor 4 (CXCR4) is a seven-transmembrane G-protein-coupled receptor. It acts as a receptor for CXC chemokine stromal cell derived factor-1 (SDF-1, also called CXCL12). It is widely expressed in a variety of tissue types but is predominantly expressed by immune cells and in the brain. While the immune function of CXCR4 has been much studied, little is known about its role in the brain.

During embryonic mouse brain development, *Cxcr4* is expressed in ventricular zones. These are sites of stem cell proliferation. In late embryonic stages, *Cxcr4* is expressed in the hippocampus and cerebellum [1]. Embryonic data (E18.5 and P0) from *Cxcr4* knockout (KO) mice show that the cerebellum develops abnormally with an irregular external granule cell layer (EGL) and ectopically located Purkinje cells [2,3]. These studies imply that defects in SDF-1/CXCR4 signaling result in premature migration from the EGL during embryonic cerebellar development. Indeed, SDF-1 has been shown to function as a chemoattractant and is secreted from the meninges. It attracts embryonic but not postnatal cerebellar EGL cells [4]. In SDF-1 KO mice at E15.5, premature granule cells have been detected migrating into the cerebellar anlage [5].

Cxcr4 is highly expressed from E18.5 to P4 in the cerebellum. Subsequently, expression becomes very low or non-detectable at P14 (according to the Allen Brain Atlas [6]). Currently, the effect of CXCR4 deficiency in postnatal cerebellar development is poorly understood. This is because *Cxcr4* KO mice are embryonic lethal as a result of defects in cardiogenesis and hematopoiesis [3]. To date there has been no study into postnatal cerebellar development in CXCR4 KOs since the work of Zou *et al.* in 1998. Consequently, in order to study postnatal development and its impact on function we conditionally inactivated *Cxcr4* in the central nervous system (CNS). We here report the functional characterization of conditional inactivation of *Cxcr4* in postnatal cerebellar development.

Materials and Methods

Ethics Statement

All experiments were carried out in strict accordance with the recommendations in the Guide for Laboratory Animals Facilities and Care as promulgated by the Council of Agriculture. Executive

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Data Availability: The authors confirm that all data underlying the findings are fully available without restriction. All DNA array data can be found at the Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/). The accession number is GSE 62572. Additional materials can be obtained by request from the corresponding author.

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Competing Interests: HB is an employee of DNAVEC Corporation. M. Hasegawa is a founder and adviser of DNAVEC Corporation. NF was an employee of DNAVEC Corporation until January 2013 but not now. The commercial product developed by DNAVEC Corporation is similar to the vectors described in this paper but the component is different. The patent of the Sendai virus vectors to generate iPS cells that was applied by and of DNAVEC Corporation is pending (WO/2010/008054). NF and HB have waived the right of the patent. These do not alter the authors'

RESEARCH ARTICLE

New Type of Sendai Virus Vector Provides Transgene-Free iPS Cells Derived from Chimpanzee Blood

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Abstract

Induced pluripotent stem cells (iPSCs) are potentially valuable cell sources for disease models and future therapeutic applications; however, inefficient generation and the presence of integrated transgenes remain as problems limiting their current use. Here, we developed a new Sendai virus vector, TS12KOS, which has improved efficiency, does not integrate into the cellular DNA, and can be easily eliminated. TS12KOS carries *KLF4*, *OCT3/4*, and *SOX2* in a single vector and can easily generate iPSCs from human blood cells. Using TS12KOS, we established iPSC lines from chimpanzee blood, and used DNA array analysis to show that the global gene-expression pattern of chimpanzee iPSCs is similar to those of human embryonic stem cell and iPSC lines. These results demonstrated that our new vector is useful for generating iPSCs from the blood cells of both human and chimpanzee. In addition, the chimpanzee iPSCs are expected to facilitate unique studies into human physiology and disease.

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Generation of familial amyloidotic polyneuropathy-specific induced pluripotent stem cells☆,☆☆,★

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Abbreviations: ALP, alkaline phosphatase; ATTR, amyloidogenic transthyretin; FAP, familial amyloidotic polyneuropathy; LT, liver transplantation; SELDI-TOF MS, surface-enhanced laser desorption/ionization time-of-flight mass spectrometry; SeV, Sendai virus; TTR, transthyretin.

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Original Article

Dual origin of melanocytes defined by Sox1 expression and their region-specific distribution in mammalian skin

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Melanocytes are pigment-producing cells generated from neural crest cells (NCCs) that delaminate from the dorsal neural tube. The widely accepted premise that NCCs migrating along the dorsolateral pathway are the main source of melanocytes in the skin was recently challenged by the finding that Schwann cell precursors are the major cellular source of melanocytes in the skin. Still, in a wide variety of vertebrate embryos, melanocytes are exclusively derived from NCCs. In this study, we show that a NCC population that is not derived from *Sox1*$^{+}$ dorsal neuroepithelial cells but are derived from *Sox1*$^{-}$ cells differentiate into a significant population of melanocytes in the skin of mice. Later, these *Sox1*$^{-}$ cells clearly segregate from cells that originated from *Sox1*$^{+}$ dorsal neuroepithelial cell-derived NCCs. The possible derivation of *Sox1*$^{-}$ cells from epidermal cells also strengthens their non-neuroepithelial origin.

Key words: Cre recombinase, hair follicle, K14, melanocyte lineage tracing, neural crest, Sox1.

Introduction

Melanocytes themselves and/or the pigment they synthesize and sequester in melanosomes and deliver to keratinocytes provide photoprotection and thermoregulation for the skin. Melanocytes are originally derived from neural crest cells (NCCs) (Liem *et al.* 1995; Nakagawa & Takeichi 1998; Le Douarin & Kalcheim 1999; Wilson *et al.* 2004; Morales *et al.* 2005; Hall 2009) and undergo extensive migration to reach their various destinations in hair follicles, in the basal layer and the dermis of the skin, and also to various internal parts of the body, such as the uvea, Harderian gland, inner ear and heart (Erickson 1993; Nordlund *et al.* 2006; Uehara *et al.* 2009). NCCs delaminate from the dorsal neural epithelium of the neural tube by undergoing an epithelium to mesenchyme transition. NCCs then migrate through stereotypical pathways and differentiate into a variety of cell types in vertebrate embryos, including neuronal, glial, endocrine, skeletal and pigment cells. NCCs migrating along the dorsolateral pathway between the dermamyotome and the skin were thought to become melanocytes; however, recent work has uncovered that a significant population of follicular melanocytes in mice is produced by the re-differentiation of Schwann cell precursors that migrated from the dorsal root ganglia toward the dermal surface, which in turn had originated from NCCs that had taken the ventral migration pathway (Adameyko *et al.* 2009).

In mice, tissue or cell-type specific genetic tracing are frequently used to address the cellular and molecular identity of NCC-derived cells including melanocytes. When we used transgenic mice expressing Cre recombinase under control of the *Sox1* promoter crossed to the *Rosa26-YFP* reporter strain that contains a floxed stop cassette preventing yellow fluorescent protein (YFP) expression until the Cre-induced deletion (*Sox1-Cre/+; Rosa26R-YFP/+* mice: Srinivas *et al.* 2001; Takashima *et al.* 2007), we noticed that melanocytes were derived differently from *Sox1-Cre*$^{+}$ and from *Sox1-Cre*$^{-}$ populations in *Sox1-Cre/YFP* embryos. We show here that a significant population

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Efficient and Reproducible Myogenic Differentiation from Human iPS Cells: Prospects for Modeling Miyoshi Myopathy *In Vitro*

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Abstract

The establishment of human induced pluripotent stem cells (hiPSCs) has enabled the production of *in vitro*, patient-specific cell models of human disease. *In vitro* recreation of disease pathology from patient-derived hiPSCs depends on efficient differentiation protocols producing relevant adult cell types. However, myogenic differentiation of hiPSCs has faced obstacles, namely, low efficiency and/or poor reproducibility. Here, we report the rapid, efficient, and reproducible differentiation of hiPSCs into mature myocytes. We demonstrated that inducible expression of *myogenic differentiation1* (*MYOD1*) in immature hiPSCs for at least 5 days drives cells along the myogenic lineage, with efficiencies reaching 70–90%. Myogenic differentiation driven by *MYOD1* occurred even in immature, almost completely undifferentiated hiPSCs, without mesodermal transition. Myocytes induced in this manner reach maturity within 2 weeks of differentiation as assessed by marker gene expression and functional properties, including *in vitro* and *in vivo* cell fusion and twitching in response to electrical stimulation. Miyoshi Myopathy (MM) is a congenital distal myopathy caused by defective muscle membrane repair due to mutations in DYSFERLIN. Using our induced differentiation technique, we successfully recreated the pathological condition of MM *in vitro*, demonstrating defective membrane repair in hiPSC-derived myotubes from an MM patient and phenotypic rescue by expression of full-length *DYSFERLIN (DYSF)*. These findings not only facilitate the pathological investigation of MM, but could potentially be applied in modeling of other human muscular diseases by using patient-derived hiPSCs.

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Competing Interests: The authors have declared that no competing interests exist.

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Introduction

The establishment of human induced pluripotent stem cells (hiPSCs) [1,2] has paved the way for the generation of patient-specific stem cell resources. Directed differentiation of pluripotent stem cells into a variety of cell types provides a powerful tool for *in vitro* disease modeling [3]. Although the number and genetic diversity of patient-derived hiPSC lines continues to increase, the difficulty of differentiating hiPSC into mature cell types remains a major obstacle in understanding disease.

Effective differentiation into affected cell types is a critical step in the production of *in vitro* disease models from hiPSCs. In the case of myopathies, significant efforts have been made to generate skeletal muscle cells from human pluripotent stem cells [4,5,6]. However, previously reported differentiation protocols suffer from complex time-consuming procedures, low differentiation efficiencies, and/or low reproducibility. Reproducibility is perhaps the greatest hurdle facing robust differentiation protocols from human pluripotent stem cells, especially considering the high levels of clonal variation previously reported [7].

Directed myogenic differentiation of adult somatic cells mediated by the master transcriptional factor, MYOD1 [8,9], was initially established in 1987 [8]. Following this first demonstration, various types of cells have been shown to give rise to myocytes in response to forced expression of *MYOD1* [9,10,11], including hiPSC-derived fibroblasts treated with *MYOD1* mRNA [12]. Considering the inherent potential of hiPSCs, differentiation into fibroblasts prior to myogenic induction is a redundant step. Recently, Tedesco et al. showed that hiPSC-derived mesoangio-

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Genetic ablation of *Rest* leads to in vitro-specific derepression of neuronal genes during neurogenesis

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SUMMARY

Rest (RE1-silencing transcription factor, also called Nrsf) is involved in the maintenance of the undifferentiated state of neuronal stem/progenitor cells in vitro by preventing precocious expression of neuronal genes. However, the function of *Rest* during neurogenesis in vivo remains to be elucidated because of the early embryonic lethal phenotype of conventional *Rest* knockout mice. In the present study, we have generated *Rest* conditional knockout mice, which allow the effect of genetic ablation of *Rest* during embryonic neurogenesis to be examined in vivo. We show that *Rest* plays a role in suppressing the expression of neuronal genes in cultured neuronal cells in vitro, as well as in non-neuronal cells outside of the central nervous system, but that it is dispensable for embryonic neurogenesis in vivo. Our findings highlight the significance of extrinsic signals for the proper intrinsic regulation of neuronal gene expression levels in the specification of cell fate during embryonic neurogenesis in vivo.

KEY WORDS: Rest (Nrsf), Mouse model, Neurogenesis

INTRODUCTION

The establishment and maintenance of neuronal identity underlie the core of neuronal development. The transcriptional repressor RE1-silencing transcription factor [Rest; also known as neuron-restrictive silencer factor (Nrsf)], was initially discovered as a negative regulator of neuron-specific genes in non-neuronal cells (Chong et al., 1995; Schoenherr and Anderson, 1995). Rest is expressed throughout early development, where it represses the expression of neuronal genes and is involved in the transcriptional silencing of neuronal promoters in conjunction with CoRest (Rcor1/2) (Ballas et al., 2001), which recruits additional silencing machinery, including the methyl DNA-binding protein MeCP2, histone deacetylase (HDAC) and the histone H3K9 methyltransferase G9a (Ehmt2) (Andres et al., 1999; Lunyak et al., 2002; Roopra et al., 2004; Shi et al., 2003; You et al., 2001). Rest targets include a number of genes encoding ion channels, neurotrophins, synaptic vesicle proteins and neurotransmitter receptors (Bruce et al., 2004; Johnson et al., 2006; Otto et al., 2007). Indeed, a targeted mutation of *Rest* in mice caused derepression of neuron-specific tubulin in a subset of non-neuronal tissues, leading to embryonic lethality (Chen et al., 1998).

Mosaic inhibition of *Rest* in chicken embryos using a dominant-negative form of *Rest* also caused derepression of neuronal tubulin, as well as several other neuronal target genes, not only in non-neuronal tissues but also neuronal progenitors (Chen et al., 1998). These results suggest that *Rest* is required to repress the expression of neuronal genes in undifferentiated neuronal tissue. Expression of Rest is highest in embryonic stem cells (ESCs) and is downregulated as ESCs differentiate into neuronal stem cells (NSCs), and it is completely silenced in mature adult neuronal cells (Ballas et al., 2005). Given the fact that Rest represses the expression of a large number of neuronal genes, it is reasonable to expect that it plays a central role in the inhibition of the precocious expression of neuronal genes in NSCs, and that its downregulation upon receipt of neuronal differentiation cues permits the robust expression of differentiation-related neuronal genes, resulting in terminal differentiation (Ballas et al., 2005).

In addition to the involvement of Rest in neurogenesis, recent studies have demonstrated that Rest modulates glial lineage elaboration (Abrajano et al., 2009; Kohyama et al., 2010), suggesting that it also mediates the coupling of neurogenesis and gliogenesis, which might contribute to the neuronal-glial interactions that are associated with synaptic and neuronal network plasticity and homeostasis in the brain. Despite the expectation of a fundamental role of Rest in brain development, the function of Rest in NSCs and neuronal progenitors in the brain in vivo remains to be elucidated. *Rest* null mice survive to embryonic day (E) 9 without obvious morphological defects, by which time all three germ layers and the neural tube have formed, clearly demonstrating that neuronal progenitors can develop in vivo in the absence of *Rest* (Chen et al., 1998). However, *Rest* null mice die by E11.5 accompanied by gross morphological changes starting ~E9.5. This early embryonic lethality has precluded further analysis of the role of *Rest* in the maintenance and differentiation of NSCs and neural progenitor cells (NPCs) in vivo.

In addition to the possible role of *Rest* in neuronal/glial development, recent studies have indicated that the breakdown of these processes accompanies and promotes neurodegenerative disorders. The disruption of the interaction of Rest with its target genes was reported in epileptic seizures (Bassuk et al., 2008), Huntington's disease (Zuccato et al., 2007) and Down's syndrome (Canzonetta et al., 2008; Lepagnol-Bestel et al., 2009). In these disorders, Rest dysfunction is suggested to be a cause of aberrant changes in neuronal gene expression. Considering that abnormal expression of Rest has been seen in a variety of neurological and neurodegenerative diseases, it is important to uncover the

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Phf14, a Novel Regulator of Mesenchyme Growth via Platelet-derived Growth Factor (PDGF) Receptor-α*[S]

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Background: *Phf14* is identified as a novel gene that regulates proliferation of mesenchymal cells.
Results: Phf14-null mice show interstitial pulmonary hyperplasia and mesenchymal fibroblasts exhibit increased proliferation rates accompanied by enhanced expression of PDGFRα.
Conclusion: Phf14 acts as a negative regulator of PDGFRα expression in mesenchymal cells.
Significance: Phf14 has potential as a target for the treatment of lung fibrosis.

The regulation of mesenchymal cell growth by signaling molecules plays an important role in maintaining tissue functions. Aberrant mesenchymal cell proliferation caused by disruption of this regulatory process leads to pathogenetic events such as fibrosis. In the current study we have identified a novel nuclear factor, Phf14, which controls the proliferation of mesenchymal cells by regulating PDGFRα expression. Phf14-null mice died just after birth due to respiratory failure. Histological analyses of the lungs of these mice showed interstitial hyperplasia with an increased number of PDGFRα^+ mesenchymal cells. PDGFRα expression was elevated in Phf14-null mesenchymal fibroblasts, resulting in increased proliferation. We demonstrated that Phf14 acts as a transcription factor that directly represses PDGFRα expression. Based on these results, we used an antibody against PDGFRα to successfully treat mouse lung fibrosis. This study shows that Phf14 acts as a negative regulator of PDGFRα expression in mesenchymal cells undergoing normal and abnormal proliferation, and is a potential target for new treatments of lung fibrosis.

The development and maintenance of adult and embryonic tissues requires fine control by cell surface receptors and extracellular ligands. Disruption of receptor-mediated signaling underlying these control processes is associated with developmental dysplasia, tissue degeneration, and tumorigenesis. Mesenchymal cells, which provide structural support for various types of tissues, can proliferate in response to extracellular stimuli, and aberrant growth of these cells causes pathogenetic fibrosis in adult tissues (1). PDGF and its receptor, PDGFRα, play a crucial role in the proliferation of mesenchymal cells (2). PDGFRα is widely expressed in mesenchyme during organogenesis and is critical to development of the palate (3), hair follicles (4), and lung tissues (5). It is also a marker of paraxial mesoderm cells at gastrulation, which is a germ layer committed to differentiate into bone, cartilage, and skeletal muscle (6). Gene disruption of *Pdgfrα* in mice results in abnormal patterning of somites, which are derived from paraxial mesoderm (7), and pulmonary hypoplasia (8). These findings indicate that PDGFRα signaling is required for both morphogenesis and organogenesis in mouse development. A knock-out mouse study showed that mesenchymal cell numbers in the intestine and testis markedly decreased in PDGFRα-null embryos (9, 10). In adult human tissues, widespread expression of PDGFRα is also observed in abnormal fibrotic and sclerosing processes associated with inflammation, during which expression of PDGFRα is induced by extracellular factors such as transforming growth factor β, interleukin-1α, basic fibroblast growth factor (bFGF), tumor necrosis factor α, and lipopolysaccharide (11). Taken together, these findings support the notion that PDGF/PDGFRα signaling is important for the normal development of mesenchymal tissues, but it also underlies the pathogenetic events triggered by abnormal mesenchyme proliferation. However, the mechanisms controlling PDGFRα expression in normal and abnormal growth of mesenchyme remain unclear. This study aimed to elucidate the role of PDGFRα expression and the regulatory mechanism governing PDGFRα expression in normal development and related diseases. We used an *in vitro* ES cell culture system using PDGFRα as a marker to identify progenitors of paraxial mesoderm (12–14). The advantage of this system is that gene expression in differentiating ES cells mimics that observed in embryonic development (15–17), enabling us to monitor the intermediate stages of embryonic development and differentiation *in vitro*

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[S] This article contains supplemental Tables S1–S3, Figs. S1–S10, and Movie S1.
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Pathogenic Mutation of ALK2 Inhibits Induced Pluripotent Stem Cell Reprogramming and Maintenance: Mechanisms of Reprogramming and Strategy for Drug Identification

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Key Words. Induced pluripotent stem cells • Reprogramming • Pluripotency • Experimental models

ABSTRACT

Fibrodysplasia ossificans progressiva (FOP) is a rare congenital disorder characterized by progressive ossification of soft tissues. FOP is caused by mutations in activin receptor-like kinase 2 (ALK2) that cause its constitutive activation and result in dysregulation of BMP signaling. Here, we show that generation of induced pluripotent stem cells (iPSCs) from FOP-derived skin fibroblasts is repressed because of incomplete reprogramming and inhibition of iPSC maintenance. This repression was mostly overcome by specific suppression of ALK2 expression and treatment with an ALK2 inhibitor, indicating that the inhibition of iPSC generation and maintenance observed in FOP-derived skin fibroblasts results from constitutive activation of ALK2. Using this system, we identified an ALK2 inhibitor as a potential candidate for future drug development. This study highlights the potential of the inhibited production and maintenance of iPSCs seen in diseases as a useful phenotype not only for studying the molecular mechanisms underlying iPS reprogramming but also for identifying drug candidates for future therapies. STEM CELLS *2012;30:2437–2449*

Disclosure of potential conflicts of interest is found at the end of this article.

INTRODUCTION

Fibrodysplasia ossificans progressiva (FOP) is a congenital disorder of progressive and widespread postnatal ossification of soft tissues and muscles [1–3]. Severe debilitation, reduced life expectancy due to joint fusion, and restrictive ventilatory impairment with thoracic involvement are major symptoms of this disease. Patients with FOP have gradual worsening of pulmonary function and eventually die by 40 due to respiratory failure if they do not receive the appropriate support. There is no effective therapy for preventing the ectopic ossification associated with FOP. Recent studies have revealed that this disorder is caused by mutations in Activin A receptor type I, the gene encoding the bone morphogenetic protein (BMP) type I receptor activin receptor-like kinase 2 (ALK2) [4–9]. The most common mutation is R206H, which is thought to alter ALK2 and confer constitutive activity to the mutant receptor. Mesenchymal cells derived from primary teeth of FOP patients showed elevated basal expression of RUNX2 and alkaline phosphatase (AP), which are involved in bone formation [4]. These data suggest that the dysregulation of BMP signaling seen in FOP patients results in ectopic expression of osteogenesis-related genes and aberrant ossification. Several other mutations in ALK2, such as G356D, underlie phenotypic variations of FOP and these also alter ALK2 and confer constitutive activity to the mutant receptor [10]. The weaker kinase activity of ALK2 (G356D) compared to that of ALK2 (R206H) suggests that clinical variation is due to differences in the bioactivity of ALK2 mutants [11].

Induced pluripotent stem cells (iPSCs) derived from patients with incurable diseases represent a powerful tool not

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