由来繊維芽細胞に特異的な作用であると考えられる。なお、ヒト間葉系幹細胞はヒトiPS 細胞の生着性・造腫瘍性をむしろ抑制する可能性も考えられる。

E．結論

本研究では造腫瘍性リスクの質的把握に関し、特に、原材料としての各種ヒト多能性幹細胞の造腫瘍性に与える、ヒト多能性幹細胞の特性指標の同定を目指している。本分担研究においては、初年度である本年度は、NOG マウスへの移植試験プロトコールの検討および至適化を試みた。複数の細胞株を比較する際、投与時の細胞の初期条件を揃えることが不可欠と考え、単一細胞にまで分散しても安定的に生着し、造腫瘍性の評価が可能となる試験系として、ヒト多能性幹細胞とヒト新生児由来繊維芽細胞および ROCK 阻害剤の同時投与の系を確立した。今後は本試験系を用い、様々な細胞株について造腫瘍性および形成された腫瘍の特性の評価を行い、悪性度等とその他の細胞特性指標との関係を検討する予定である。

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F．研究危険情報

なし

G．研究発表

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H．知的財産権の出願・登録状況

H-1. 取得特許

なし

H-2. 実用新案登録

なし

H-3. その他

なし

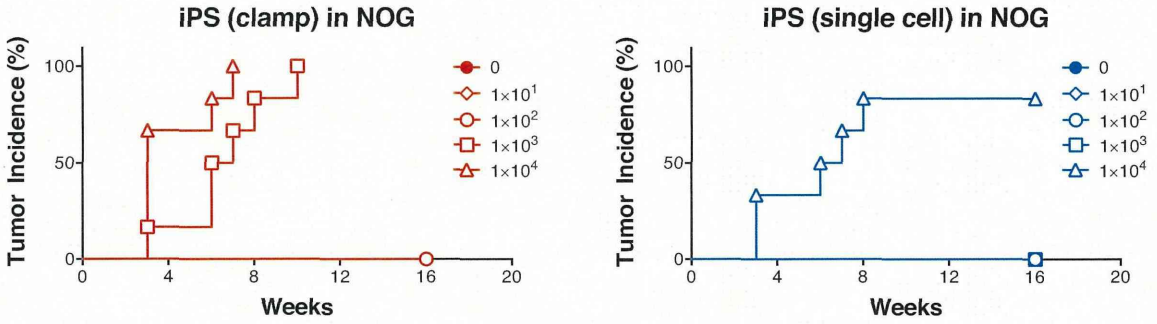

Figure 1 NOG マウスへの iPS 細胞の生着性

iPS 細胞をクランプまたは単一細胞の状態で NOG マウスに移植した。移植後 3 週目から移植細胞由来と推定される明らかな結節形成が観察され始めた。iPS クランプ移植群では移植後約 8 週目までには結節形成の用量依存性が明らかになった。一方、iPS 単一細胞移植群では、クランプ移植群に比べ生着性がかなり低く、最高用量である 10^4 個移植群でしか結節形成が認められなかった。

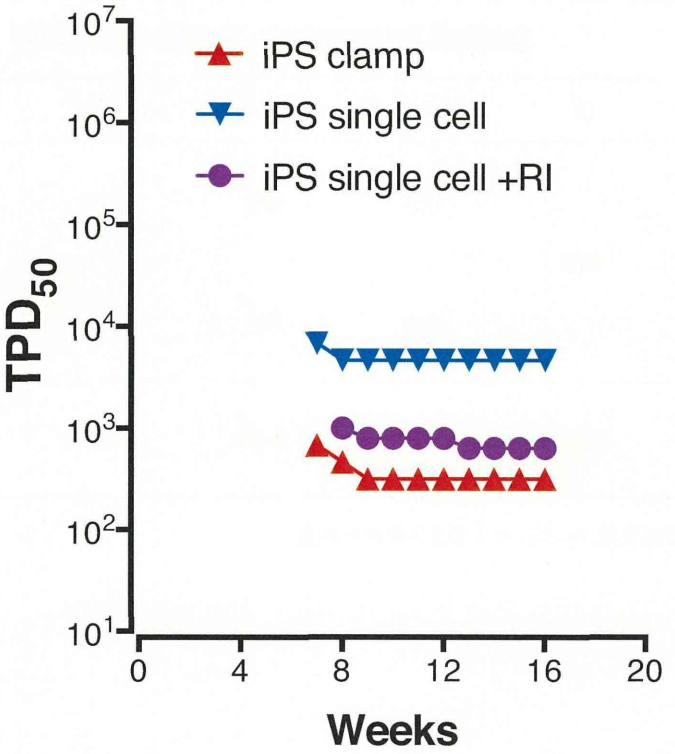

Figure 2 NOG マウスへの iPS 細胞移植における TPD_{50} 値の推移

iPS クランプ移植群（iPS clamp）では 8 週目の時点以降で TPD_{50} 値はほぼ安定し、iPS 単一細胞移植群（iPS single cell）ではわずかに遅れ 9 週目以降で TPD_{50} 値が安定した。ROCK 阻害剤を添加した iPS 単一細胞移植群（iPS single cell+RI）では、13 週目以降で TPD_{50} 値が安定した。

Table 1　NOG マウスにおける iPS 細胞生着能（16 週目の結果）

系統	移植群	腫瘍形成率（腫瘍を呈した頭数／供試頭数）					TPD_{50}
		0[a]	1x10	$1x10^2$	$1x10^3$	$1x10^4$	
NOG	iPS/NHDF（クランプ）	0/6	0/6	0/6	6/6	6/6	$3.1x10^2$
NOG	iPS/NHDF（単一細胞）		0/6	0/6	0/6	5/6	$4.6x10^3$
NOG	iPS/NHDF+RI（単一細胞）	0/10	0/10	2/10	7/10	8/10	$6.3x10^2$

[a] : iPS 細胞混入数, RI: ROCK 阻害剤, NHDF: ヒト新生児繊維芽細胞

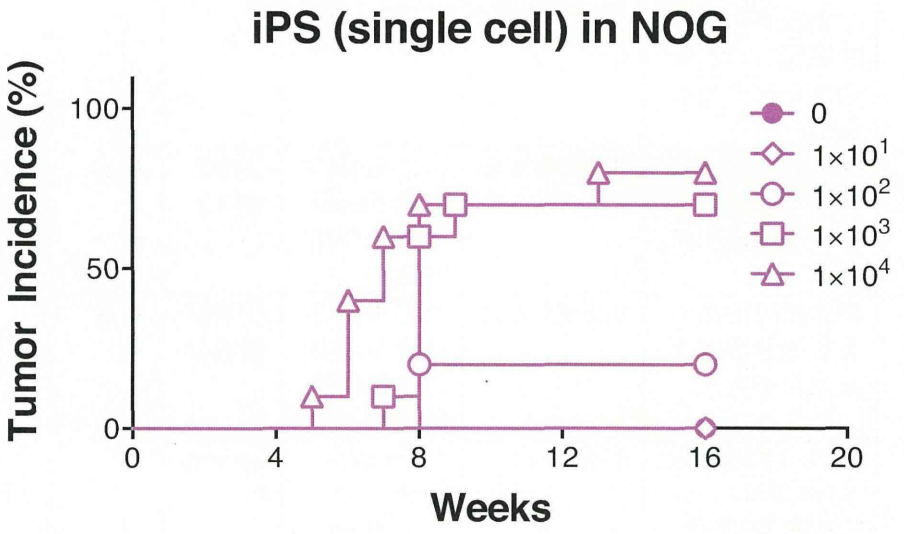

Figure 3 iPS 単一細胞を ROCK 阻害剤を添加して NOG マウスに移植した際の結節形成

iPS 単一細胞移植後、5 週目以降になって結節形成が観察され始めた。10^2、10^3 個と少ない数の移植群でも結節形成が認められており、ROCK 阻害剤の添加によって細胞の生着性が大きく向上していることが確認された。

研究成果の刊行に関する一覧表

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佐藤陽治	ヒト iPS 細胞由来移植細胞の製造管理のための *in vitro* 造腫瘍性評価系の開発	*Cytometry Research*			印刷中

指針草案作成

草案作成者	文書名	発出元	文書番号	発出日
早川堯夫，青井貴之，梅澤明弘，小澤敬也，佐藤陽治，澤芳樹，松山晃文，大和雅之，山中伸弥	ヒト（自己）体性幹細胞加工医薬品等の品質及び安全性の確保について	厚生労働省	薬食発0907第2号	平成24年9月7日
早川堯夫，青井貴之，梅澤明弘，小澤敬也，佐藤陽治，澤芳樹，松山晃文，大和雅之，山中伸弥	ヒト（同種）体性幹細胞加工医薬品等の品質及び安全性の確保について	厚生労働省	薬食発0907第3号	平成24年9月7日
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早川堯夫，青井貴之，梅澤明弘，小澤敬也，佐藤陽治，澤芳樹，松山晃文，大和雅之，山中伸弥	ヒト（同種）iPS（様）細胞加工医薬品等の品質及び安全性の確保について	厚生労働省	薬食発0907第5号	平成24年9月7日
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西田幸一，飯田知弘，梅澤明弘，小沢洋子，瓶井資弘，平形明人，万代道子，大和雅之，森永千佳子，佐藤陽治	次世代医療機器評価指標策定事業　再生医療審査WG報告書　「自己iPS細胞由来網膜色素上皮細胞に関する評価指標（案）」	厚生労働省	パブリックコメント案件番号495120405	平成25年3月19日（案の公示日）

政策提言

厚生労働省医薬食品局「「再生医療等製品原料基準」のあり方に関する検討」での提言
経済産業省　「グローバル認証基盤整備事業再生医療等基準検討委員会」での提言

研究成果の刊行物・別刷

Role of Notch signaling in the maintenance of human mesenchymal stem cells under hypoxic conditions

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Running title: MESENCHYMAL STEM CELLS UNDER HYPOXIA

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Abstract

Human adipose tissue-derived multilineage progenitor cells (hADMPCs) are attractive for cell therapy and tissue engineering because of their multipotency and ease of isolation without serial ethical issues. However, their limited *in vitro* lifespan in culture systems hinders their therapeutic application. Some somatic stem cells including hADMPCs are known to be localized in hypoxic regions; thus, hypoxia may be beneficial for *ex vivo* culture of these stem cells. These cells exhibit a high level of glycolytic metabolism in presence of high oxygen levels and further increase their glycolysis rate under hypoxia. However, the physiological role of glycolytic activation and its regulatory mechanisms are still incompletely understood. Here we show that Notch signaling is required for glycolysis regulation under hypoxic conditions. Our results demonstrate that 5% O_2 dramatically increased the glycolysis rate, improved the proliferation efficiency, prevented senescence, and maintained the multipotency of hADMPCs. Intriguingly, these effects were not mediated by hypoxia-inducible factor (HIF), but rather by the Notch signaling pathway. 5% O_2 significantly increased the level of activated Notch1 and expression of its downstream gene, *HES1*. Furthermore, 5% O_2 markedly increased glucose consumption and lactate production of hADMPCs, which decreased back to normoxic levels upon treatment with a γ-secretase inhibitor. We also found that HES1 was involved in induction of GLUT3, TPI, and PGK1 in addition to reduction of TIGAR and SCO2 expression. These results clearly suggest that Notch signaling regulates glycolysis under hypoxic conditions and thus likely affects the cell lifespan via glycolysis.

Introduction

Human adipose tissue-derived mesenchymal stem cells (MSCs), also referred to as human adipose tissue–derived multilineage progenitor cells (hADMPCs), are multipotent stem cells that can differentiate into various types of cells, including hepatocytes [1], cardiomyoblasts [2], pancreatic cells [3], and neuronal cells [4-6]. They can be easily and safely obtained from lipoaspirate without posing serious ethical issues and can also be expanded *ex vivo* under appropriate culture conditions. Moreover, MSCs, including hADMPCs, have the ability to migrate to injured areas and secrete a wide variety of cytokines and growth factors necessary for tissue regeneration [7-11]. In addition, because of their hypoimmunogenicity and immunomodulatory effects, hADMPCs are good candidates as gene delivery vehicles for therapeutic purposes [12]. Thus, hADMPCs are attractive seeding cells for cell therapy and tissue engineering. However, similar to other somatic stem cells or primary cells, hADMPCs have limited growth potential and ultimately stop proliferation as a result of cellular senescence [13], which hinders their therapeutic application.

Conversely, embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs) are immortal under standard culture conditions. Recently, several groups have reported that these cells greatly rely on glycolysis for energy production even under high-oxygen conditions [14-16]. This phenomenon is known as the Warburg effect and was originally described for cancer cells by Otto Warburg in the 1920s [17]. Although mitochondrial respiration is more efficient than glycolysis in generating ATP (net yield of 30 ATPs vs. 2 ATPs), glycolysis is able to produce ATP considerably faster than mitochondrial respiration as long as glucose supplies are adequate. Thus,

a metabolic shift from mitochondrial respiration to glycolysis would provide a growth advantage for actively proliferating cells. Moreover, Kondoh *et al.* demonstrated that enhanced glycolysis is also involved in cellular immortalization through reduction of intrinsic ROS production [14,18,19]. Because accumulation of intrinsic ROS levels could be a major reason for replicative senescence [20], enhancing glycolysis in cultured cells might improve the quality of the cells by suppressing premature senescence. One candidate method for induction of glycolysis is application of low-oxygen conditions to activate the transcription factor, hypoxia-inducible factor (HIF). HIF-1 is known to increase the expression of most glycolytic enzymes and the glucose transporters GLUT1 and GLUT3 [20]. Thus, several studies have reported that hypoxia is beneficial for the maintenance of hESCs in a pluripotent state [21,22]. Moreover, low oxygen tension has been reported to enhance the generation of induced pluripotent stem cells both from mouse and human primary fibroblasts [23].

Recently, hypoxic culture conditions have also been reported to confer a growth advantage, prevent premature senescence, and maintain undifferentiated states in somatic stem cells, e.g., hematopoietic stem cells (HSCs) [24], neural stem cells [25], and bone marrow-derived MSCs [26]. These stem cells reside in their local microenvironments called the “stem cell niche”, where the oxygen tension is relatively low (in the range of 1%–9%). Thus, hypoxic culture may be beneficial to these stem cells with regard to in vitro proliferation, cell survival, and differentiation. Takubo et al. reported that HSCs activated Pdk through HIF1α in hypoxic culture conditions, resulting in maintenance of glycolytic flow and suppression of the influx of glycolytic metabolites into mitochondria, and this glycolytic metabolic state was shown to be indispensable for the maintenance of HSCs [27]. Several studies have reported that MSCs exhibit a high level of glycolytic metabolism in the presence of high oxygen

levels and further increase their rate of glycolysis upon culture under hypoxia [28,29]. However, a relationship between beneficial effects of hypoxic conditions and metabolic status in addition to involvement of HIFs in the metabolic changes has not been investigated in these reports.

In this study, we aimed to investigate the effect of 5% oxygen on hADMPCs. Our results demonstrate that culture under 5% oxygen increased the glycolysis rate, improved the proliferation efficiency, prevented the cellular senescence, and maintained the undifferentiated status of hADMPCs. Intriguingly, these effects were not mediated by HIF, but rather by Notch signaling, an important signaling pathway required for the development of many cell types and maintenance of stem cells [30,31]. 5% oxygen activated Notch signaling, resulting the upregulation of *SLC2A3*, *TPI*, and *PGK1* in addition to the downregulation of *TIGAR* and *SCO2*, which may contribute to the increase in the glycolysis rate. These observations thus provide new regulatory mechanisms for stemness maintenance obtained under 5% oxygen conditions.

Material and Methods

Adipose tissue samples

Subcutaneous adipose tissue samples (10–50 g each) were resected during plastic surgery from 5 female and 2 male patients (age 20–60 years) as discarded tissue. The study protocol was approved by the Review Board for Human Research of Kobe University Graduate School of Medicine Foundation for Biomedical Research and

Innovation, Osaka City University Graduate School of Medicine, and Kinki University Pharmaceutical Research and Technology Institute (reference number: 12-043). Each subject provided signed informed consent.

Cell culture

hADMPCs were isolated as previously reported [32-35] and maintained in a medium containing 60% DMEM-low glucose, 40% MCDB-201 medium (Sigma Aldrich, St. Louis, MO, USA), 1× insulin-transferrin-selenium (Life Technologies, NY, USA), 1 nM dexamethasone (Sigma Aldrich), 100 mM ascorbic acid 2-phosphate (Wako, Osaka, Japan), 10 ng/mL epidermal growth factor (PeproTech, NJ, USA), and 5% fetal bovine serum. The cells were plated to a density of 5×10^3 cells/cm^2 on fibronectin-coated dishes, and the medium was replaced every 2 days. For hypoxic culture, cells were cultured in a gas mixture composed of 90% N_2, 5% CO_2, and 5% O_2. For maintenance of the hypoxic gas mixture, a ProOx C21 carbon dioxide and oxygen controller and a C-Chamber (Biospherix, NY, USA) were used.

Senescence-associated β-galactosidase staining

Cells were fixed with 2% paraformaldehyde/0.2% glutaradehyde for 5 min at room temperature and then washed 2 times with phosphate-buffered saline (PBS). The cells were then incubated overnight at 37 °C with fresh senescence-associated β-galactosidase (SA-β-Gal) chromogenic substrate solution (1 mg/mL Bluo-gal (Life

Technologies), 40 mM citric acid (pH 6.0), 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, 150 mM NaCl, and 2 mM $MgCl_2$).

Measurement of reactive oxygen species production

Cells were harvested and incubated with 10 μM 5-(and-6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate, acetyl ester (CM-H_2DCFDA). The amount of intracellular ROS production was proportional to the green fluorescence, as analyzed using a Guava EasyCyte 8HT flow cytometer (Millipore, MA, USA) using an argon laser at 488 nm and a 525/30 nm band pass filter, and dead cells were excluded using the Live/Dead Fixable Far Red Dead Cell Stain Kit (Life Technologies).

EdU proliferation assay

For assessment of cell proliferation, hADMPCs were seeded on a fibronectin-coated 6-well plate at a density of 5 × 10^3 cells/cm^2 and cultured for 3 days. Cell proliferation was detected by incorporating of 5-ethynyl-2'-deoxyuridine (EdU) and using the Click-iT EdU Alexa Fluor 488 Flow Cytometry Assay Kit (Life Technologies). Briefly, according to the manufacturer's protocol, cells were incubated with 10 μM EdU for 2 h before fixation, permeabilized, and stained with EdU. EdU-positive cells were then analyzed using the 488 nm laser of a Guava EasyCyte 8HT flow cytometer (Millipore).