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H．知的財産権の出願・登録状況

H-1. 特許取得 なし

H-2. 実用新案登録 なし

H-3.その他

【政策への提言】

1） 「再生医療等の安全性の確保等に関する法律」、「再生医療等の安全性の確保等に関する法律施行令」及び「再生医療等の安全性の確保等に関する法律施行規則」の取扱いについて（平成 26 年 10 月 31 日医政研発 1031 第 1 号厚生労働省医政局研究開発振興課長通知）

2） 生物由来原料基準の一部を改正する件」（平成 26 年厚生労働省告示第 375 号）；

Ⅲ. 研究成果の刊行に関する一覧表

書籍

著者氏名	論文タイトル名	書籍全体の編集者名	書籍名	出版社名	出版地	出版年	ページ
佐藤大作, 佐藤陽治			規制関連『再生医療用語集』（集』			印刷製本中	
草川森士, 佐藤陽治	再生医療製品の造腫瘍性評価		最新医学 増刊号	最新医学社	大阪	2014	745-765
佐藤陽治	再生医療／細胞治療における細胞培養に関する規制	（監修：紀ノ岡正博）	『再生医療の細胞培養技術開発と応用展開』	式会社シーエムシー出版	東京	2014	27-36
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発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
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Takao Hayakawa, Takashi Aoi, Akihiro Umezawa, Keiya Ozawa, Yoji Sato, Yoshiki Sawa, Akifumi Matsuyama, Shinya Yamanaka, and Masayuki Yamato.	Study on Ensuring the Quality and Safety of Pharmaceuticals and Medical Devices Derived from the Processing of Allogenic Human Somatic Stem Cells.	Regenerative Therapy			In press

Takao Hayakawa, Takashi Aoi, Akihiro Umezawa, Keiya Ozawa, Yoji Sato, Yoshiki Sawa, Akifumi Matsuyama, Shinya Yamanaka, and Masayuki Yamato.	Study on Ensuring the Quality and Safety of Pharmaceuticals and Medical Devices Derived from Processing of Autologous Human Induced Pluripotent Stem Cell (-Like Cells).	Regenerative Therapy			In press
Takao Hayakawa, Takashi Aoi, Akihiro Umezawa, Keiya Ozawa, Yoji Sato, Yoshiki Sawa, Akifumi Matsuyama, Shinya Yamanaka, and Masayuki Yamato.	Study on Ensuring the Quality and Safety of Pharmaceuticals and Medical Devices Derived from Processing of Allogenic Human Induced Pluripotent Stem Cell (-Like Cells).	Regenerative Therapy			In press
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Moriyama H, Moriyama M, Isshi H, Ishihara S, Okura H, Ichinose A, Ozawa T, Matsuyama A, Hayakawa T.	Role of notch signaling in the maintenance of humanmesenchymal stem cells under hypoxic conditions.	Stem Cells Dev.	23(18)	2014	2211-24
Moriyama M, Moriyama H, Uda J, Matsuyama A, Osawa M, Hayakawa T.	BNIP3 plays crucial roles in the differentiation and maintenance of epidermal keratinocytes.	J Invest Dermatol.	134(6)	2014	1627-35
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中島啓行，佐藤陽治	薬事法改正と再生医療等安全性確保法を踏まえた再生医療／細胞治療の開発	ファームステージ	10	2014	1-5
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Park SJ, Umemoto T, Saito-Adachi M, Shiratsuchi Y, Yamato M et al.	Computational promoter modeling identifies the modes of transcriptional regulation in hematopoietic stem cells.	PLoS One	9(4)	2014	e93853
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様式第１９

学 会 等 発 表 実 績

委託業務題目「再生医療実用化加速のための幹細胞等由来製品評価に最低限必須・共通の技術要件・基準に関する研究（H26－医薬B －一般－ 018 ）」

機関：近畿大学、神戸大学、東京大学、国立成育医療研究センター、医薬基盤研究所、国立医食品研究所、大阪大学、東京女子医科大学

１．学会等における口頭・ポスター発表

発表した成果（発表題目、口頭・ポスター発表の別）	発表者氏名	発表した場所（学会等名）	発表した時期	国内・外の別
科学的合理性に基づくバイオ医薬品開発のあり方について - 規制面からの視点（口頭）	早川堯夫	BIOJAPAN	2014/10/15	国内
Aspects of Quality Evaluation and Control Corresponding to the Type of Cell-based Products for Regenerative Medicine. （口頭）	Takao Hayakawa	CMC Strategy Forum Japan（国際会議）	2014/12/8	国内
Acceptance of William Hancock Award. （口頭）	Takao Hayakawa	WCBP2015 - 4th William Hancock Award Ceremony	2015/1/27-29	国外
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再生医療製品・遺伝子治療薬等の品質評価の上での科学的妥当性とは(口頭)	早川堯夫	第10回医薬品レギュラトリーサイエンスフォーラム	2013/12/12	国内
ヒトメラノーマ細胞のグライコフォームフォーカストプロテオミクス(口頭)	木下充弘,三ツ井洋介,原沙也香,山田佳太,早川堯夫,掛樋一晃	第32回日本糖質学会年会	2013/8/6	国内
Supressive effects of prenylcoumarins from Mammea siamensis on iNOS synthase expression in RAW264.7 cells(口頭)	T. Morikawa, M. Sueyoshi, S. Chaipech, H. Matsuda, Y. Nomura, M. Yabe, T. Matsumoto, K. Ninomiya, M. Yoshikawa, Y. Pongpiriyadacha, T. Hayakawa, O. Muraoka	14th Tetrahedron Symposium	2013/6/26	国外
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INDISPENSABLE ROLES OF BNIP3, AN INDUCER OF AUTOPHAGY, IN BOTH DIFFERENTIATION AND MAINTENANCE OF EPIDERMAL KERATINOCYTES （口頭）	M. Mariko, M. Hiroyuki, U. Junki, M. Akifumi, O. Masatake, H. Takao	2013 International Investigative Dermatology Meeting,	2013/3/8-11	国外
ROLE OF NOTCH SIGNALING IN THE MAINTENANCE OF HUMAN MESENCHYMAL STEM CELLS UNDER HYPOXIC CONDITIONS （ポスター）	M. Hiroyuki, M. Mariko, U. Ayaka, N. Yusuke, O. Hanayuki, M. Akifumi, H. Takao	11th ISSCR	2013/6/12-15	国外
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ヒト脂肪組織由来多系統前駆細胞(hADMPC)を用いたインスリン産生細胞の作製(ポスター)	曽根千晶, 森山麻里子, 大倉華雪, 松山晃文, 早川堯夫, 森山博由	第4回生命機能研究会	2013/6/12-15	国内
ヒト脂肪組織由来多系統前駆細胞(hADMPC)を用いた効率的なドパミン産生細胞作製(ポスター)	大森重成, 森山麻里子, 大倉華雪, 松山晃文, 早川堯夫, 森山博由	第4回生命機能研究会	2013/6/12-15	国内
Bcl-2ファミリー分子BNIP3が表皮構築に及ぼす影響 （ポスター）	森山麻里子, 宇田純輝, 北川綾弓, 野村昇吾, 早川堯夫, 森山博由	第63回日本薬学会近畿支部総会	2013/10/12	国内
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２．学会誌・雑誌等における論文掲載

掲載した論文（発表題目）	発表者氏名	発表した場所（学会誌・雑誌等名）	発表した時期	国内・外の別
Role of notch signaling in the maintenance of humanmesenchymal stem cells under hypoxic conditions.	Moriyama H, Moriyama M, Isshi H, Ishihara S, Okura H, Ichinose A, Ozawa T, Matsuyama A, Hayakawa T.	Stem Cells Dev.	2014	国外
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CCAAT/enhancer binding protein-mediated regulation of TGFβ receptor 2 expression determines the hepatoblast fate decision	Kazuo Takayama, Kenji Kawabata, Yasuhito Nagamoto, Mitsuru Inamura, Kazuo Ohashi, Hiroko Okuno, Tomoko Yamaguchi, Katsuhisa Tashiro, Fuminori Sakurai1, Takao Hayakawa, Teruo Okano, Miho Kusada Furue and Hiroyuki Mizuguchi.	Development	2014	国外
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The role of maternal-specific H3K9me3 modification in establishing imprinted X-chromosome inactivation and embryogenesis in mice.	Fukuda A, Tomikawa J, Miura T, Hata K, Nakabayashi K, Eggan K, Akutsu H, Umezawa A.	Nat Commun	2014	国外

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A novel in vitro method for detecting undifferentiated human pluripotent stem cells as impurities in cell therapy products using a highly efficient culture system.	Tano K, Yasuda S, Kuroda T, Saito H, Umezawa A, Sato Y.	PLoS One	2014	国外
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N-glycans: phenotypic homology and structural differences between myocardial cells and induced pluripotent stem cell-derived cardiomyocytes.	Kawamura T, Miyagawa S, Fukushima S, Yoshida A, Kashiyama N, Kawamura A, Ito E1, Saito A1, Maeda A, Eguchi H, Toda K, Lee JK, Miyagawa S, Sawa Y.	Plos One	2014	国外
Therapeutic potential of human adipose tissue-derived multi-lineage progenitor cells in liver fibrosis.	Okura H, Soeda M, Morita M, Fujita M, Naba K, Ito C, Ichinose A, Matsuyama A.	Biochem Biophys Res Commun.	2014	国外
Computational promoter modeling identifies the modes of transcriptional regulation in hematopoietic stem cells.	Watanabe Y, Sasaki R, Matsumine H, Yamato M, Okano T.	Plos One	2014	国外

（注１）発表者氏名は、連名による発表の場合には、筆頭者を先頭にして全員を記載すること。

（注２）本様式はexcel形式にて作成し、甲が求める場合は別途電子データを納入すること。

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厚生労働科学研究委託費

医薬品等規制調和・評価研究事業

再生医療実用化加速のための幹細胞等由来製品評価に最低限必須・共通の技術要件・基準に関する研究

平成26年度　委託業務成果報告書

（論文別刷一式）

研究代表者　早川堯夫

平成27(2015)年 3月

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Role of Notch Signaling in the Maintenance of Human Mesenchymal Stem Cells Under Hypoxic Conditions

Hiroyuki Moriyama,[1,*] Mariko Moriyama,[1,*] Haruki Isshi,[1] Shin Ishihara,[1] Hanayuki Okura,[2] Akihiro Ichinose,[3] Toshiyuki Ozawa,[4] Akifumi Matsuyama,[2] and Takao Hayakawa[1]

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 the 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. Five percent 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 on 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 that are necessary for tissue regeneration [7–11]. In addition, due to 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

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intrinsic reactive oxygen species (ROS) production [14,18,19]. Since 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 iPSCs 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; for example, 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 on 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 at investigating 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]. Five percent oxygen activated Notch signaling, resulting in 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.

Materials and Methods

Adipose tissue samples

Subcutaneous adipose tissue samples (10–50 g each) were resected during plastic surgery from five female and two 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 [11,32–34] and maintained in a medium containing 60% DMEM low glucose, 40% MCDB-201 medium (Sigma Aldrich), 1× insulin-transferrin-selenium (Life Technologies), 1 nM dexamethasone (Sigma Aldrich), 100 mM ascorbic acid 2-phosphate (Wako), 10 ng/mL epidermal growth factor (PeproTech), 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) 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 twice 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 ROS 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) 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 six-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).

Flow cytometry analysis

Flow cytometry analysis was performed as previously described [34]. Briefly, hADMPCs were harvested and resuspended in staining buffer (PBS containing 1% BSA, 2 mM EDTA, and 0.01% sodium azide) at a density of