旧多能性幹細胞分野　(2012～2014)
Department of Stem Cell Biology

膵臓β細胞は血液中のグルコース 糖の濃度を正常に維持するために必要なインスリンを産生分泌している。インスリン産生が不十分、あるいはインスリンの作用が悪くなると糖尿病を発症する。重篤な糖尿病では、インスリンが作れなくなり、血糖コントロールが困難な状況に陥ってしまい、移植医療が必要になるが、ドナー不足が大きな問題点 となっており、その解決には、多能性幹細胞を用いた再生医療が期待されている。

哺乳類の発生において、膵臓は胚性内胚葉、膵前駆細胞、内分泌前駆細胞を経てインスリンを産生する β 細胞へ分化する。本分野では、特に膵臓と肝臓と腸などの消化器官に焦点を絞り下記の研究を進めている：

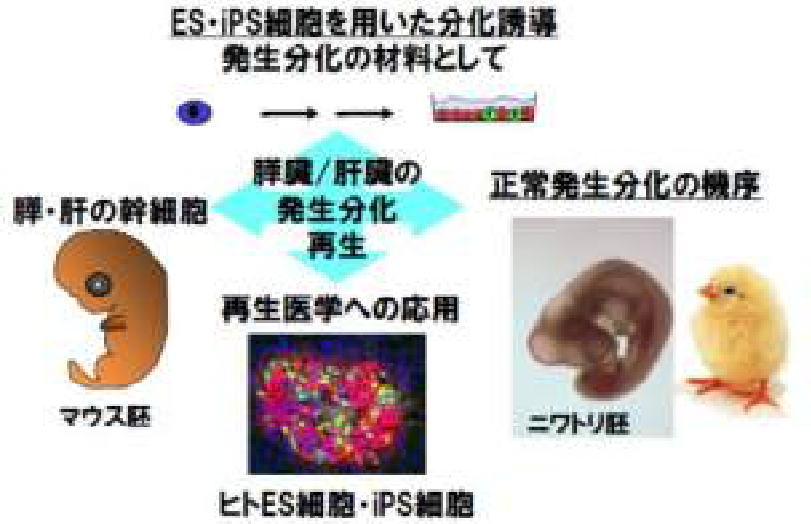
1）幹細胞（ES 細胞および iPS 細胞）から消化器官（膵臓･肝臓・腸）の分化誘導方法の開発

2）消化器官の正常発生分化の機序の解明

3）ヒト ES 細胞・iPS 細胞を用いて、消化器官の幹細胞を単離し、性情について解析する。

The main goal of the lab is to understand the development of pancreas. We use mouse, chick and ES cell models to search for molecules that function in early events of pancreatic development. For this purpose, we currently devote on the following projects

1. Identification of molecular mechanism that regulate pancreatic and hepatic development.
2. Establishment of *in vitro* differentiation procedures to pancreas.
3. Studies on the biology of pancreas stem cells.



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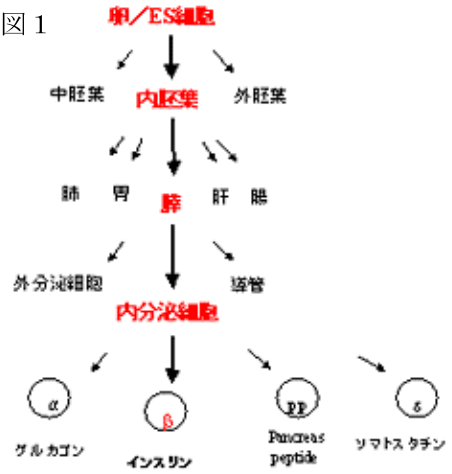
研究概略　Projects

図１には、１つの卵（または ES 細胞）からスタートし、膵臓 β 細胞までに至る発生分化の細胞系譜の模式図を示す。内胚葉の成立過程、領域化する前後の遺伝子発現変化、膵前駆細胞の起源の追跡、膵の発生分化に関わる誘導シグナルの本体などについて正常胚発生・ES 細胞を用いた研究の両方からのアプローチを行っている。

図１



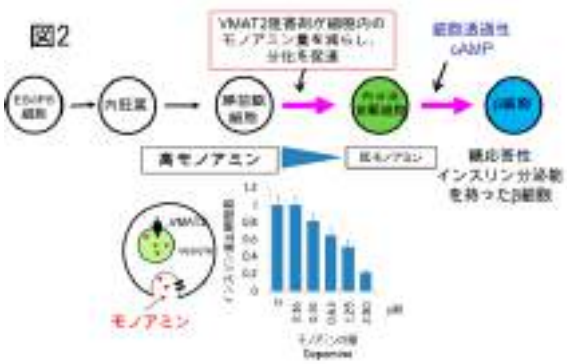
ES・/iPS 細胞を用いた研究では、正常膵臓発生分化の各段階を *In vitro* での培養系を用いて再現する条件を検討している。ES 細胞からインスリンを分泌する膵臓 β細胞に特異的分化誘導することを目指す。そして、試験管内で初期発生のプロセスを ES 細胞が辿ることで、膵臓への分化誘導を試験管内で再現できると考えられる。これまでの研究成果は以下が挙げられる。

１．細胞内の未知なシグナルを活性化させる低分子化合物の探索

従来の ES・iPS 細胞の培養手法は、膵臓の自然な発生過程で使われている液性因子を試験管内で連続的な添加によって Pdx1 陽性の膵前駆細胞まで高い効率で分化誘導することを可能になった（Shiraki ら、2008）。しかし、分化機構が分からない場合では、この手法ではうまく行かない。細胞内の未知なシグナルを活性化させる低分子化合物を見つけることで、膵臓 β 細胞への分化促進することができ、さらにその分子メカニズムを明らかにすることで、分化に関わるシグナル分子を突き止めること、さらに化合物を利用して分化細胞を得ることができる。

私たちは、マウス ES 細胞から膵前駆細胞を誘導した後、1,120 の低分子化合物の中から 小胞型モノアミントランスポーターの １つ VMAT2 の阻害剤が膵前駆細胞から内分泌前駆細胞への分化を促進する効果があることを見出した。モノアミンは β 細胞の分化を阻害する作用があることから、 VMAT2 は発生過程で細胞内のモノアミン量の調節を介して、β 細胞数を制御する役割を担っていると考えられる(図２)。実際、膵臓の発生過程においてもモノアミンにより分化が調節されていることを明らかにした。さらに cAMP により β 細胞の成熟化が促進され、グルコース濃度に応じたインスリン分泌能をもった β 細胞が得られることを見出した。この ２つの低分子化合物（VMAT2 阻害剤および cAMP）を ES 細胞に作用させることにより、成体膵島に近いインスリン含量と高グルコース濃度に応じたインスリン分泌能をもった膵 β 細胞を作製でき、糖尿病モデルマウスの高血糖を正常化できた。（Sakano et al.,Nature Chem Biol 2014）

図2



２．ES 細胞から肝臓細胞への分化誘導法の開発

肝臓は創薬研究において中心的役割を果たす臓器であるが、新薬候補物質の体内での代謝および薬物毒性試験などにおいて、動物肝細胞とヒト肝細胞では物質代謝の大きな違いがあることから、動物実験だけでは毒性と有効性の検定が不可能である。またヒト肝細胞の供給も極めて限定されており、多数の提供者から集められた細胞組織では試験データのばらつきが大きく有

意義な結果を得ることは困難である。そこで、ヒト ES 細胞から肝細胞への分化誘導技術が確立できれば、安定したヒト肝細胞の供給が可能となり、創薬研究分野における極めて重要な技術革新となる。

マウスおよびヒトにおける ES 細胞および iPS 細胞の肝細胞分化誘導を効率化するための最適な細胞足場環境を探索し、その分化効率化を促す分子メカニズムを同定することを目的として、完全合成基材であるナノファイバーに注目した。この培養基材は未分化の ES 細胞の増殖を促進し、初代培養細胞の機能維持に優れていると報告されている。そこで再生医療および研究利用のために、未同定物質を含むウシ胎仔血清を用いない合成培地で分化誘導を確立し、一般的に用いられている細胞外マトリックス成分であるゼラチン・コラーゲンタイプ I ・ファイブロネクチン・マトリゲルと比較したところ、内胚葉分化誘導効率、肝細胞分化マーカーの上昇、肝機能が有意に高いことがわかった。

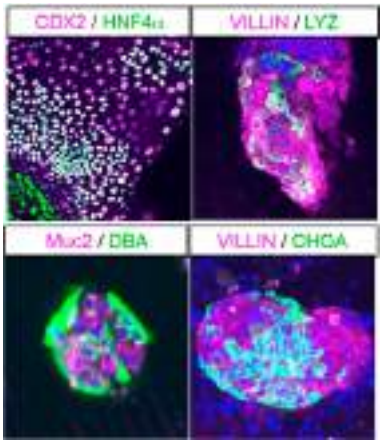
また、このナノファイバーにおける分化誘導効率化に寄与する分子機構の同定を行うために、ES 細胞がドーム状になるという細胞形態変化がナノファイバー上で見られることに注目し、細胞骨格を制御する Rho ファミリーの Rac1 活性を測定したところ未分化状態ならびに分化途中においても活性化していた。ナノファイバーの肝分化誘導効果は Rac1 の活性化が寄与しており、Rac1 の活性化は分化過程のいずれの時期においても重要であることが示唆された。（Yamazoe et al, J Cell Science 2013）

３．ES 細胞から腸細胞への分化誘導法の開発

多能性幹細胞を用いた小腸上皮細胞への分化誘導研究は 発生研究や薬物動態研究において重要であり、また重度の炎症性腸疾患などの治療法として小腸上皮細胞の移植を必要とするため再生医療においても重要である。

多能性幹細胞である ES 細胞から内胚葉の細胞に分化後、様々な液性因子を添加した結果、Wnt シグナルを活性化し、Notch シグナルを抑制することで小腸上皮の細胞への分化が促進され、全体の 88% 程度の細胞が小腸上皮の細胞となった。この ES 細胞由来の小腸上皮細胞には、吸収腸細胞など成体小腸上皮を構成する様々な細胞も存在していた。本研究では、Wnt シグナルと Notch シグナルがマウス、ヒト ES 細胞から内胚葉系譜の小腸上皮の細胞への分化に関与することを初めて明らかにした。今後はこの細胞を用いた小腸の発生メカニズムや炎症性腸疾患の 病因解明などの基礎研究と創薬や再生医療などの応用研究が期待できる。（Ogaki et al, Stem Cell 2013）

（図３）ES 細胞から分化した 4 種類の腸の機能的細胞

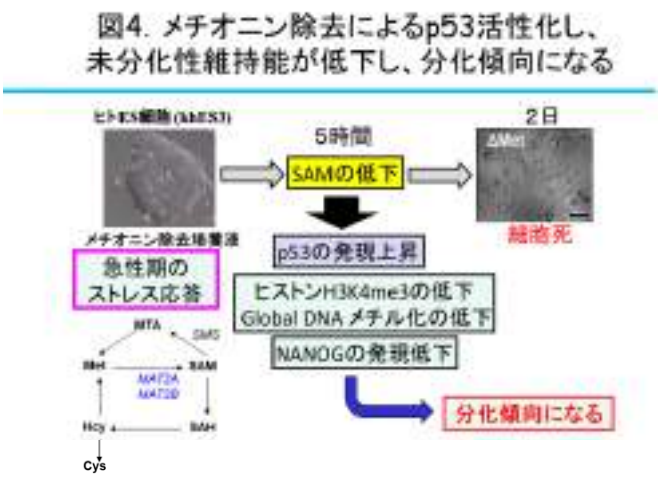


４．メチオニン代謝による未分化性維持の制御

ES/iPS 細胞といった多能性幹細胞では，分化細胞とは異なる代謝プログラムを保持していることがわかっており、その代謝プログラムが幹細胞の未分化維持や自己複製能などに関与することも明らかになってきている。アミノ酸代謝と幹細胞との関係については 2009 年に、マウス ES/iPS 細胞の生存には アミノ酸の一種であるスレオニンが必須であることが報告されていたが、ヒト多能性幹細胞におけるアミノ酸代謝の役割は不明であった。

私たちは、メチオニン代謝がヒト ES/iPS 細胞の未分化維持および分化を制御していることを明らかにし、メチオニンを除去した培養液を利用した分化促進および未分化細胞の選択的除去に世界で初めて成功した。ヒト多能性幹細胞であるヒト ES/iPS 細胞を用いた検討により、生存にはメチオニンが必須であり、その代謝物である S アデノシルメチオニン(SAM)を介してヒト

ES/iPS 細胞の未分化維持および分化を制御することを見出した。さらに、未分化細胞は分化した内胚葉 細胞と比較して、生存により多くのメチオニンが必要であることも見出した。未分化なヒトES/iPS細胞をメチオニン除去培養液で培養すると、細胞内の SAM 濃度が顕著に低下し、それに伴い p53 の発現上昇、ヒストン H3 の 4 番目のリジン残基のトリメチル化(H3K4me3)の低下、未分化マーカーである Nanog の発現低下が起こった。続いて、ヒト ES/iPS 細胞がもつ代謝特性の分化誘導へ応用を試みた結果、未分化過程においてメチオニン除去後に内胚葉・中胚葉・外胚葉へそれぞれ分化誘導すると顕著な分化促進効果を確認した。内胚葉への分化誘導過程においてメチオニン除去培養液で培養することにより、残存する未分化細胞特異的に細胞死を誘導することができ、その後の肝臓分化を効率的に行うことに成功した。これまで不明であったヒトES/iPS細胞におけるメチオニン代謝の役割を明らかにすることができた。未分化細胞の高いメチオニン 代謝特性を利用し、メチオニン除去培養液を利用する未分化状態からの分化促進と内胚葉分化過程での肝臓分化の効率化という新たな分化誘導方法を構築した。(図 4 :模式



図を示す) （Shiraki et al, Cell Metab 2014）

1. Establishment of pancreatic differentiation of human iPS/ES cells *in vitro*, and use PSC differentiation system for high content screening

ES cells have been extensively studied and shown to be able to form cells of all germ layers including endoderm. The generation of specific lineages of the definitive endoderm from embryonic stem (ES) cells is an important issue in developmental biology as well as in regenerative medicine.

Cell replacement therapy for diabetes mellitus requires cost-effective generation of high-quality, insulin-producing, pancreatic β cells from pluripotent stem cells. Development of this technique has been hampered by a lack of knowledge of the molecular mechanisms underlying b-cell differentiation. The present study identified reserpine and tetrabenazine (TBZ), both vesicular monoamine transporter 2 (VMAT2) inhibitors, as promoters of late-stage differentiation of Pdx1-positive pancreatic progenitor cells into Neurog3 (referred to henceforth as Ngn3)-positive endocrine precursors. VMAT2-controlled monoamines, such as dopamine, histamine and serotonin, negatively regulated b-cell differentiation. Reserpine or TBZ acted additively with dibutyryl adenosine 3',5'-cyclic AMP, a cell-permeable cAMP analog, to potentiate differentiation of embryonic stem (ES) cells into β cells that exhibited glucose-stimulated insulin secretion. When ES cell–derived β cells were transplanted into AKITA diabetic mice, the cells reversed hyperglycemia. Our protocol provides a basis for the understanding of b-cell differentiation and its application to a cost-effective production of functional β cells for cell therapy. （Sakano et al, Nature Chem Biol 2014）

2. A synthetic nanofibrillar matrix promotes in vitro hepatic differentiation of embryonic stem cells and induced pluripotent stem cells.

Embryonic stem (ES) cells recapitulate normal developmental processes and serve as an attractive source for routine access to a large number of cells for research and therapies. We previously reported that ES cells cultured on M15 cells, or a synthesized basement membrane (sBM) substratum, efficiently differentiated into an endodermal fate and subsequently adopted fates of various digestive organs, such as the pancreas and liver. Here, we established a novel hepatic differentiation procedure using the synthetic nanofiber (sNF) as a cell culture scaffold. We first compared endoderm induction and hepatic differentiation between murine ES cells grown on sNF and several other substrata. The functional assays for hepatocytes reveal that the ES cells grown on sNF were directed into hepatic differentiation. To clarify the mechanisms for the promotion of ES cell differentiation in the sNF system, we focused on the function of Rac1, which

is a Rho family member protein known to regulate the actin cytoskeleton. We observed the activation of Rac1 in undifferentiated and differentiated ES cells cultured on sNF plates, but not in those cultured on normal plastic plates. We also show that inhibition of Rac1 blocked the potentiating effects of sNF on endoderm and hepatic differentiation throughout the whole differentiation stages. Our results suggest that morphological changes result in cellular differentiation controlled by Rac1 activation, and that motility is not only the consequence, but is also able to trigger differentiation. We believe that sNF is a promising material that might contribute to tissue engineering and drug delivery. （Yamazoe et al, J Cell Science 2013）

3. Wnt and Notch Signals Guide Embryonic Stem Cell Differentiation into the Intestinal Lineages

The studies of differentiation of mouse or human embryonic stem cells (hESCs) into specific cell types of the intestinal cells would provide insights to the understanding of intestinal development and ultimately yield cells for the use in future regenerative medicine. Using an in vitro differentiation procedure of pluripotent stem cells into definitive endoderm (DE), inductive signal pathways' guiding differentiation into intestinal cells was investigated. We found that activation of Wnt /β-catenin and inhibition of Notch signaling pathways, by simultaneous application of 6-bromoindirubin-30-oxime (BIO), a glycogen synthase kinase-3b inhibitor, and N- [(3,5-Difluorophenyl)acetyl]-L-alanyl-2-phenylglycine-1, 1-dimethylethyl ester (DAPT), a known c-secretase inhibitor, efficiently induced intestinal differentiation of ESCs cultured on feeder cell. BIO and DAPT patterned the DE at graded concentrations. Upon prolonged culture on feeder cells, all four intestinal differentiated cell types, the absorptive enterocytes and three types of secretory cells (goblet cells, enteroendocrine cells, and Paneth cells), were efficiently differentiated from mouse and hESC-derived intestinal epithelium cells. Further investigation revealed that in the mouse ESCs, fibroblast growth factor (FGF) and bone morphogenetic protein (BMP) signaling act synergistically with BIO and DAPT to potentiate differentiation into the intestinal epithelium. However, in hESCs, FGF signaling inhibited, and BMP signaling did not affect differentiation into the intestinal epithelium. We concluded that Wnt and Notch signaling function to pattern the anterior-posterior axis of the DE and control intestinal differentiation. (Ogaki et al, Stem Cells, 2013)

4. **Methionine Metabolism Regulates Maintenance and Differentiation of Human Pluripotent Stem Cells**

Mouse embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs) are in a high-flux metabolic state, with a high dependence on threonine catabolism. However, little is known regarding amino acid metabolism in human ESCs/iPSCs. We show that human ESCs/iPSCs require high amounts of methionine and express high levels of enzymes involved in Met metabolism. Methionine deprivation results in a rapid decrease in intracellular S-adenosyl-methionine (SAM), triggering the activation of p53-p38 signaling, reducing NANOG expression, and poising human iPSC/ESCs for differentiation, follow by potentiated differentiation into all three germ layers. However, when exposed to prolonged Met deprivation, the cells undergo apoptosis. We also show that human ESCs/iPSCs have regulatory systems to maintain constant intracellular Met and SAM levels. Our findings show that SAM is a key regulator for the maintenance of undifferentiated pluripotent stem cells and regulating their differentiation.

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80. Shoen Kume, “Signals guiding differentiation of pluripotent stem cells into pancreatic beta cells” 第 55 回日本糖尿病学会「再生医療による糖尿病治療の可能性」横浜パシフィコ　2012 年 5 月 17-19 日（南條輝志男、稲垣暢也先生　オーガナイザー）

81. 粂昭苑　「多能性幹細胞の分化をサポートする細胞外環境」日本組織培養学会 JTCA 第 85 回大会シンポジウム「細胞接着と細胞機能制御の最先端」オーガナイザー（オーガナイザー　絵野沢伸、粂　昭苑）京都 2012 年 5 月 18 日

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アウトリーチ活動　Outreach activity

1. 粂昭苑　熊本県立第二高等学校 SSH 特別講演会（全校生徒　1400 名）「幹細胞と再生医学」平成２６年 11 月 20 日

2. 粂昭苑「女性研究者の育成～熊大の取り組み～」『保育と行動経済学−学内保育園を通じて』大阪 平成 26 年 11 月 15 日

3. 粂昭苑「多能性幹細胞を使った夢の再生医療～糖尿病の再生医療～」『高校生のための熊大ワクワク連続講義』（熊本）平成 24 年 9 月 1 日

4. 粂昭苑『科学技術人材育成費補助金「女性研究者養成システム改革加速」』シンポジウム「熊本大学における男女共同参画事業」2012 年 3 月 26 日（熊本）

5. 粂　昭苑「男女共同参画とは？～熊本大学の取り組みと今後の展望～」岩手医科大学男女共同参画シンポジウム　2012 年 2 月 16 日　(盛岡市)

Journal of Molecular Cell Biology Advance Access published July 3, 2014

doi:10.1093/jmcb/mju029 *Journal of Molecular Cell Biology* (2014), 0(0), 1–15 | 1

Article

Generation of insulin-producing β-like cells from human iPS cells in a defined and completely xeno-free culture system

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Human induced pluripotent stem (hiPS) cells are considered a potential source for the generation of insulin-producing pancreatic β-cells because of their differentiation capacity. In this study, we have developed a five-step xeno-free culture system to efficiently differentiate hiPS cells into insulin-producing cells *in vitro*. We found that a high NOGGIN concentration is crucial for specifically inducing the differentiation first into pancreatic and duodenal homeobox-1 (PDX1)-positive pancreatic progenitors and then into neurogenin 3 (NGN3)-expressing pancreatic endocrine progenitors, while suppressing the differentiation into hepatic or intestinal cells. We also found that a combination of 3-isobutyl-1-methylxanthine (IBMX), exendin-4, and nicotinamide was important for the differentiation into insulin single-positive cells that expressed various pancreatic β-cell markers. Most notably, the differentiated cells contained endogenous C-peptide pools that were released in response to various insulin secretagogues and high levels of glucose. Therefore, our results demonstrate the feasibility of generating hiPS-derived pancreatic β-cells under xeno-free conditions and highlight their potential to treat patients with type 1 diabetes.

Keywords: diabetes, pancreas, cell therapy, hiPS cells, xeno-free differentiation, β-cells

Introduction

Diabetes is a life-long disease characterized by chronic hyperglycemia. Type 1 diabetes is caused by autoimmune destruction of insulin-producing β-cells in the pancreas and its treatment is solely dependent on insulin administration. Islet transplantation from cadaveric donors is a promising therapy for type 1 diabetes; however, due to difficulties such as the scarcity of cadaveric donors compared with the large number of diabetic patients, the low yield of transplantable islets from cadaveric pancreas, and the necessity of chronic immunosuppression (Shapiro et al., 2006; Shapiro, 2011), alternative cell sources for the generation of insulin expressing β-cells are needed.

Human pluripotent stem cells, e.g. human embryonic stem (hES) cells and human induced pluripotent stem (hiPS) cells, possess the capacity for unlimited replication and the potential to differentiate into all major somatic cell lineages (Thomson et al., 1998; Takahashi et al., 2007). Therefore, they have great potential for use in cell therapy and drug discovery. Many studies reported the generation of pancreatic endocrine cells (ECs) *in vitro* from hES/iPS cells in feeder-cell culture systems (D'Amour et al., 2006; Kroon et al., 2008; Chen et al., 2009; Kunisada et al., 2012) or feeder-free culture systems (Jiang et al., 2007a, b; Zhang et al., 2009; Rezania et al., 2012). Studies on the differentiation of hES or iPS cells into endodermal or pancreatic cell lineages have shown that activin A, fibroblast growth factor (FGF), stimulation with retinoic acid (RA), and inhibition of hedgehog, bone morphogenetic protein (BMP), and transforming growth factor (TGF)-β signaling promote the differentiation into endodermal or pancreatic lineages (D'Amour et al., 2006; Kroon et al., 2008; Chen et al., 2009; Mfopou et al., 2010; Kunisada et al., 2012; Rezania et al., 2012). Stepwise differentiation protocols were designed to mimic pancreatic differentiation and to successfully generate insulin

Received February 5, 2014. Revised April 28, 2014. Accepted May 14, 2014.

CellPress

Cell Metabolism
Article

Methionine Metabolism Regulates Maintenance and Differentiation of Human Pluripotent Stem Cells

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http://dx.doi.org/10.1016/j.cmet.2014.03.017

SUMMARY

Mouse embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs) are in a high-flux metabolic state, with a high dependence on threonine catabolism. However, little is known regarding amino acid metabolism in human ESCs/iPSCs. We show that human ESCs/iPSCs require high amounts of methionine (Met) and express high levels of enzymes involved in Met metabolism. Met deprivation results in a rapid decrease in intracellular S-adenosylmethionine (SAM), triggering the activation of p53-p38 signaling, reducing NANOG expression, and poising human iPSC/ESCs for differentiation, follow by potentiated differentiation into all three germ layers. However, when exposed to prolonged Met deprivation, the cells undergo apoptosis. We also show that human ESCs/iPSCs have regulatory systems to maintain constant intracellular Met and SAM levels. Our findings show that SAM is a key regulator for maintaining undifferentiated pluripotent stem cells and regulating their differentiation.

INTRODUCTION

Embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs) have an unlimited ability to replicate; they are pluripotent and can give rise to all cell types. ESCs/iPSCs possess a unique transcriptional circuit that sustains the pluripotent state. These cells are in a specific epigenetic state that is ready for rapid cell-fate decisions. Furthermore, various forms of histone methylation allow dynamic regulation of ESC/iPSC pluripotency and plasticity. ESCs/iPSCs also possess a characteristically high rate of proliferation as well as an abbreviated G1 phase. These unique molecular properties distinguish ESCs and iPSCs from somatic cells (Boheler, 2009).

These unusual features signify that ESCs/iPSCs exist in a specialized metabolic state. ESCs and iPSCs rely specifically on glycolysis (Armstrong et al., 2010; Facucho-Oliveira and St John, 2009), whereas somatic cells utilize mitochondrial oxidative phosphorylation for energy production. This metabolic requirement appears to play a causative role rather than being a consequence of pluripotency acquisition (Folmes et al., 2011). Recent reports have shown that metabolism is tightly linked to cellular signaling, and these two processes reciprocally regulate each other and modulate cell activities such as cell survival, proliferation, and stem cell function (Takubo et al., 2013; Wellen and Thompson, 2012).

Mouse ESCs are in a high-flux metabolic state, with a high dependence on threonine (Thr) catabolism (Alexander et al., 2011; Wang et al., 2009). It was recently reported that Thr metabolism regulates intracellular S-adenosylmethionine (SAM) and histone methylation such that depletion of Thr from the culture medium or knockdown of threonine dehydrogenase (*Tdh*) in mouse ESCs decreases SAM accumulation and trimethylation of histone H3 lysine 4 (H3K4me3), leading to slowed growth and increased differentiation (Shyh-Chang et al., 2013). However, in human cells, *Tdh* is expressed as a nonfunctional pseudogene. Furthermore, little is known regarding amino acid metabolism and its role in human ESCs/iPSCs. These reports highlight the importance of examining the metabolic state of human ESCs/iPSCs, which may improve our understanding of the signaling pathways regulating cell survival, pluripotency maintenance, and differentiation.

Methionine (Met) is an essential amino acid (Finkelstein, 1990). An important metabolite of Met is SAM, which is produced through an intermediate reaction catalyzed by methionine

nature chemical biology

ARTICLE

PUBLISHED ONLINE: 15 DECEMBER 2013 | DOI: 10.1038/NCHEMBIO.1410

VMAT2 identified as a regulator of late-stage β-cell differentiation

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Cell replacement therapy for diabetes mellitus requires cost-effective generation of high-quality, insulin-producing, pancreatic β cells from pluripotent stem cells. Development of this technique has been hampered by a lack of knowledge of the molecular mechanisms underlying β-cell differentiation. The present study identified reserpine and tetrabenazine (TBZ), both vesicular monoamine transporter 2 (VMAT2) inhibitors, as promoters of late-stage differentiation of *Pdx1*-positive pancreatic progenitor cells into *Neurog3* (referred to henceforth as *Ngn3*)-positive endocrine precursors. VMAT2-controlled monoamines, such as dopamine, histamine and serotonin, negatively regulated β-cell differentiation. Reserpine or TBZ acted additively with dibutyryl adenosine 3′,5′-cyclic AMP, a cell-permeable cAMP analog, to potentiate differentiation of embryonic stem (ES) cells into β cells that exhibited glucose-stimulated insulin secretion. When ES cell–derived β cells were transplanted into AKITA diabetic mice, the cells reversed hyperglycemia. Our protocol provides a basis for the understanding of β-cell differentiation and its application to a cost-effective production of functional β cells for cell therapy.

Pancreatic cells arise from definitive endoderm and Pdx1-positive (Pdx1+) pancreatic progenitor cells[1], which proliferate and give rise to all three pancreatic lineages: acini, ducts and endocrine islets[2]. Endocrine precursors are characterized by the transient expression of the basic helix-loop-helix transcription factor neurogenin 3 (Ngn3, also known as Neurog3)[2]. Previous studies showed that Ngn3 specifically establishes the endocrine lineages and that loss of Ngn3 precludes endocrine cell development[2,3]. Production of islet cells occurs through the concerted activation of a combination of transcription factors[4]. However, the coordination of cell fate decisions remains poorly understood.

The prevalence of diabetes mellitus in many populations is high, and development of cell replacement therapy through generation of β cells from ES cells is a research priority. Recent studies have shown that mouse or human ES cells can be induced to recapitulate embryonic development of the pancreas[5]. Studies on ES cell differentiation into endodermal or pancreatic cell lineages have shown that stimulation with activin, FGF or retinoic acid, in addition to inhibition of hedgehog signaling by KAAD-cyclopamine, promotes the differentiation into endoderm or pancreatic fates[6,7]. New signal pathways that promote ES cell differentiation into endodermal[8] or pancreatic[9] lineages have been discovered through large-scale screening of cell-permeable, bioactive small molecules. However, it is still difficult to derive mature β cells that secrete insulin in a glucose-dependent manner. A better understanding is needed of the underlying molecular mechanisms that control the late stages of β-cell development, in which Pdx1+ pancreatic progenitor cells develop into Ngn3+ endocrine progenitor cells and insulin-positive (Ins+) β cells and then further differentiate into mature β cells capable of glucose-stimulated insulin secretion (GSIS).

Here, we identified reserpine and TBZ as potent promoters of pancreatic progenitor cell differentiation into functional β cells. This study highlights the use of chemical compound libraries for the identification of new developmental pathways that control progenitor cell differentiation into mature β cells.

RESULTS

Reserpine and TBZ increase Ins+ cells

The present study used large-scale screening of chemical compounds with an ES cell line, SK7, that expresses GFP under the *Pdx1* promoter[10,11]. The *Pdx1*-GFP ES cell line is useful because the expression of *Pdx1* is biphasic (**Fig. 1a**), which enabled the detection of early-stage Pdx1+ pancreatic progenitors and late-stage Pdx1+ Ins+ β cells . We optimized the culture to promote modest basal differentiation with high reproducibility, so that the markers *Sox17*, *Pdx1*, *Ngn3* and *Ins1* were sequentially expressed (**Fig. 1a**).

To screen for compounds that potentiate the differentiation of ES cell–derived Pdx1+ pancreatic progenitor cells into insulin-expressing cells, we tested a library of 1,120 biologically active compounds arrayed as single compounds in DMSO on cultures, starting on day 11 after confirming the appearance of *Pdx1*-GFP+ cells and conducting the assay on day 17 (**Fig. 1a**). Candidate compounds that increased both *Ins1* expression and the number of Ins+ cells relative to vehicle (1% DMSO) were selected as primary hits. The coefficient of variation of this screen was 0.36 ± 0.0447 (± s.d.), which was difficult to minimize further owing to the long assay

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The Review of DIABETIC STUDIES

REVIEW

Profiling of Embryonic Stem Cell Differentiation

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Manuscript submitted MARCH 22, 2013; resubmitted MAY 6, 2013; accepted JULY 3, 2013

Abstract

Embryonic stem (ES) cells have been shown to recapitulate normal developmental stages. They are therefore a highly useful tool in the study of developmental biology. Profiling of ES cell-derived cells has yielded important information about the characteristics of differentiated cells, and allowed the identification of novel marker genes and pathways of differentiation. In this review, we focus on recent results from profiling studies of mouse embryos, human islets, and human ES cell-derived differentiated cells from several research groups. Global gene expression data from mouse embryos have been used to identify novel genes or pathways involved in the developmental process, and to search for transcription factors that regulate direct reprogramming. We introduce gene expression databases of human pancreas cells (Beta Cell Gene Atlas, EuroDia database), and summarize profiling studies of islet- or human ES cell-derived pancreatic cells, with a focus on gene expression, microRNAs, epigenetics, and protein expression. Then, we describe our gene expression profile analyses and our search for novel endoderm, or pancreatic, progenitor marker genes. We differentiated mouse ES cells into mesendoderm, definitive endoderm (DE), mesoderm, ectoderm, and Pdx1-expressing pancreatic lineages, and performed DNA microarray analyses. Genes specifically expressed in DE, and/or in Pdx1-expressing cells, were extracted and their expression patterns in normal embryonic development were studied by *in situ* hybridization. Out of 54 genes examined, 27 were expressed in the DE of E8.5 mouse embryos, and 15 genes were expressed in distinct domains in the pancreatic buds of E14.5 mouse embryos. *Akr1c19*, *Aebp2*, *Pbxip1*, and *Creb3l1* were all novel, and none has been described as being expressed, either in the DE, or in the pancreas. By introducing the profiling results of ES cell-derived cells, the benefits of using ES cells to study early embryonic development will be discussed.

Keywords: diabetes · embryonic stem cell · differentiation · beta-cell · Pdx1 · Ngn3 · Sox · gene profiling · microRNA

1. Introduction

Endoderm gives rise to respiratory and digestive organs, such as pancreas, liver, lung, stomach, and intestine. Multipotent endoderm has the potential to be used in tissue repair. However, despite the importance of definitive endoderm (DE)-derived tissues, not much is known about how they emerge from the primary gut tube. Fate mapping studies suggest that the DE fate begins to segregate at embryonic day 6-6.5 (E6-E6.5), and that the progenitors fated to become specific tissues of the gut tube appear shortly after the completion of gastrulation [1, 2]. The expression of the region-specific transcription factors has provided clues as to how the endoderm is patterned into different organ domains. *Pancreatic and duodenal homeobox gene 1* (*Pdx1*) expression is the first clear sign of pancreatic differentiation, and is detected at E8.5 in the dorsal endoderm of the gut. *Pdx1* is expressed before the buds become evident, and is required for the progression of pancreatic and rostral duodenal development [3]. Genetic lineage tracing studies have shown that Pdx1-expressing cells give rise to all three cell lineages in the pancreas: endocrine, exocrine, and duct cells [4].

Recent advances in the analysis and identification of early endodermal or pancreatic genes has been remarkable [5-9]. Several reports have dem-

Sexually dimorphic expression of *Mafb* regulates masculinization of the embryonic urethral formation

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Masculinization of external genitalia is an essential process in the formation of the male reproductive system. Prominent characteristics of this masculinization are the organ size and the sexual differentiation of the urethra. Although androgen is a pivotal inducer of the masculinization, the regulatory mechanism under the control of androgen is still unknown. Here, we address this longstanding question about how androgen induces masculinization of the embryonic external genitalia through the identification of the v-maf avian musculoaponeurotic fibrosarcoma oncogene homolog B (Mafb) gene. *Mafb* is expressed prominently in the mesenchyme of male genital tubercle (GT), the anlage of external genitalia. MAFB expression is rarely detected in the mesenchyme of female GTs. However, exposure to exogenous androgen induces its mesenchymal expression in female GTs. Furthermore, MAFB expression is prominently down-regulated in male GTs of *androgen receptor* (*Ar*) KO mice, indicating that AR signaling is necessary for its expression. It is revealed that *Mafb* KO male GTs exhibit defective embryonic urethral formation, giving insight into the common human congenital anomaly hypospadias. However, the size of *Mafb* KO male GTs is similar with that of wild-type males. Moreover, androgen treatment fails to induce urethral masculinization of the GTs in *Mafb* KO mice. The current results provide evidence that *Mafb* is an androgen-inducible, sexually dimorphic regulator of embryonic urethral masculinization.

Mafb | masculinization | urethra | hypospadias | androgen receptor

External genitalia exhibit marked sexual dimorphism in mammals. The organ size of external genitalia is longer in male mice than female mice. Androgen-dependent control of organ size is widely accepted as a masculinization parameter. In addition to androgen-dependent size control, mesenchymal differentiation is an essential process of masculinization to form sexually dimorphic structures such as corporal tissues, the penile bone, and the urethra. Sexual differentiation of corporal tissues and penile bones occurs after birth (1, 2). In contrast, urethral formation shows sexual dimorphism during embryogenesis (Fig. 1) (3). Androgen actions are central to masculinization of external genitalia (4, 5). However, the mechanisms mediating androgen-dependent masculinization processes are largely unknown. Failure of exposure to androgen causes lack of masculinization of male genital tubercles (GTs) (3, 6). Therefore, proper timing of embryonic exposure to androgen is essential for inducing GT masculinization. Androgen actions are mediated through a specific receptor, the androgen receptor (AR). Recent genetic studies on a conditional allele of *Ar* suggest that mesenchymal androgen actions are essential for GT masculinization (3).

Embryonic masculinization processes of male GTs are divided into androgen-dependent organ size control and androgen-dependent mesenchymal differentiation to form the urethra. Researchers have focused on the identification of masculinization genes under the control of androgen actions for many years. However, such genes have not been identified. Recently, growth factor signals have been suggested to regulate the masculinization of the size of GTs (3, 7). However, the modulation of such growth factor signals does not induce the urethral masculinization (3, 7). These observations indicate that distinct regulators may induce embryonic urethral masculinization under androgen actions. Thus, it is still unclear how such unique morphogenetic processes are mediated by androgen-driven regulators.

The external genitalia are the most common sites of congenital abnormalities in humans (8–10). Of those, hypospadias is the frequent abnormality in which the urethral opening is ectopically located on the ventral (lower) side of the penis (8, 10–12). The male urethra connects the bladder to the penis and transports urine and semen outside of the body. In males, the urethra is incorporated into the glans to form the tubular urethra (Fig. 1). The urethral plate is an early, transitory developmental structure that develops into the tubular urethra in males. Fusion of the urethral plate is an essential process for tubular urethral formation (13). Various environmental substances are speculated to modulate developmentally essential hormonal pathways. Maternal exposure to estrogenic compounds may increase the risk of hypospadias because these compounds may interfere with the effects of fetal androgen (14–16). These studies indicate that hypospadias may be caused by disruption of the androgen exposure required for embryonic urethral masculinization. The

Significance

Androgen is essential for the masculinization of external genitalia such as the organ size and the male-type urethra in mammals. However, the genes downstream of androgen, which are responsible for these masculinization processes, have not been identified. Here, we show v-maf avian musculoaponeurotic fibrosarcoma oncogene homolog B (*Mafb*) as an essential masculinization gene for embryonic urethral formation. *Mafb* expression is prominent in developing male external genitalia, driving masculinization of embryonic urethral formation in an androgen-dependent manner. External genitalia of *Mafb* KO males exhibit urethral defects, giving insight into human hypospadias. The current findings indicate that *Mafb* is a crucial mediator of urethral masculinization and is a possible new candidate gene for hypospadias derived from embryonic abnormalities.

Author contributions: K.S. and G.Y. designed research; K.S., T.N., H.S., D.D.R., L.A.I., C.Y., and S.M. performed research; M.H., N.N., R.N., S.K., and S.T. contributed new reagents/analytic tools; K.S. and G.Y. analyzed data; and K.S. and G.Y. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

Freely available online through the PNAS open access option.

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This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1413273111/-/DCSupplemental.

DEVELOPMENTAL BIOLOGY

European Journal of Pharmacology 737 (2014) 194–201

Contents lists available at ScienceDirect

European Journal of Pharmacology

journal homepage: www.elsevier.com/locate/ejphar

Endocrine pharmacology

Potentiation of insulin secretion and improvement of glucose intolerance by combining a novel G protein-coupled receptor 40 agonist DS-1558 with glucagon-like peptide-1 receptor agonists.

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ARTICLE INFO

Article history:
Received 31 January 2014
Received in revised form
14 May 2014
Accepted 14 May 2014
Available online 22 May 2014

Keywords:
Diabetes
GPR40
Insulin
GLP-1

ABSTRACT

G protein-coupled receptor 40 (GPR40) is a Gq-coupled receptor for free fatty acids predominantly expressed in pancreatic *β*-cells. In recent years, GPR40 agonists have been investigated for use as novel therapeutic agents in the treatment of type 2 diabetes. We discovered a novel small molecule GPR40 agonist, (3*S*)-3-ethoxy-3-(4-{[(1*R*)-4-(trifluoromethyl)-2,3-dihydro-1*H*-inden-1-yl]oxy}phenyl)propanoic acid (DS-1558). The GPR40-mediated effects of DS-1558 on glucose-stimulated insulin secretion were evaluated in isolated islets from GPR40 knock-out and wild-type (littermate) mice. The GPR40-mediated effects on glucose tolerance and insulin secretion were also confirmed by an oral glucose tolerance test in these mice. Furthermore, oral administration of DS-1558 (0.03, 0.1 and 0.3 mg/kg) significantly and dose-dependently improved hyperglycemia and increased insulin secretion during the oral glucose tolerance test in Zucker fatty rats, the model of insulin resistance and glucose intolerance. Next, we examined the combination effects of DS-1558 with glucagon like peptide-1 (GLP-1). DS-1558 not only increased the glucose-stimulated insulin secretion by GLP-1 but also potentiated the maximum insulinogenic effects of GLP-1 after an intravenous glucose injection in normal Sprague Dawley rats. Furthermore, the glucose lowering effects of exendin-4, a GLP-1 receptor agonist, were markedly potentiated by the DS-1558 (3 mg/kg) add-on in diabetic *db/db* mice during an intraperitoneal glucose tolerance test. In conclusion, our results indicate that add-on GPR40 agonists to GLP-1 related agents might be a potential treatment compared to single administration of these compounds. Therefore the combinations of these agents are a novel therapeutic option for type 2 diabetes.

1. Introduction

G protein-coupled receptor 40 (GPR40) agonist has been investigated for use as a novel therapeutic agent in the treatment of type 2 diabetes with effects on glucose-stimulated insulin secretion (GSIS) in recent years. GPR40 is a Gq-coupled G protein-coupled receptor (GPCR) for saturated and unsaturated medium and long chain free fatty acids predominantly expressed in pancreatic *β*-cells (Itoh et al., 2003; Tomita et al., 2006). Activation of the Gq-coupled receptor results in the accumulation of second messengers, such as inositol trisphosphate (IP_3) and diacylglycerol, followed by a Ca^{2+} influx from endoplasmic reticulum (ER) and the stimulation of GSIS. Several small molecule GPR40 agonists have been reported, recently (Briscoe et al., 2006; Christiansen et al., 2012; Doshi et al., 2009; Lin et al., 2011; Luo et al., 2012; Negoro et al., 2012;; Schmidt et al., 2011; Tan et al., 2008). It is also reported that [(3S)-6-{(2′,

Abbreviations: ATP, adenosine triphosphate; AUC, area under the curve; BSA, bovine serum albumin; cAMP, cyclic adenosine monophosphate; CHO, Chinese hamster ovary; DHA, docosahexaenoic acid; DMSO, dimethyl sulfoxide; ELISA, enzyme-linked immunosorbent assay; ER, endoplasmic reticulum; GLP-1, glucagon like peptide-1; GPCR, G protein-coupled receptor; GPR40, G protein-coupled receptor 40; GSIS, glucose-stimulated insulin secretion; IP_3, inositol trisphosphate; ipGTT, intraperitoneal glucose tolerance test; KO, knock-out; oGTT, oral glucose tolerance test; PKA, protein kinase A; PKC, protein kinase C; SD, Sprague Dawley; S.E.M., standard error

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http://dx.doi.org/10.1016/j.ejphar.2014.05.014
0014-2999/

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Generation of Functional Insulin-Producing Cells From Mouse Embryonic Stem Cells Through 804G Cell-Derived Extracellular Matrix and Protein Transduction of Transcription Factors

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Key Words. Diabetes • Embryonic stem cells • Pancreatic differentiation • Transcription factors • Induced pluripotent stem cells

ABSTRACT

Embryonic stem (ES) and induced pluripotent stem (iPS) cells have potential applications to regenerative medicine for diabetes; however, a useful and safe way to generate pancreatic β cells has not been developed. In this study, we tried to establish an effective method of differentiation through the protein transduction of three transcription factors (Pdx1, NeuroD, and MafA) important to pancreatic β cell development. The method poses no risk of unexpected genetic modifications in target cells. Transduction of the three proteins induced the differentiation of mouse ES and mouse iPS cells into insulin-producing cells. Furthermore, a laminin-5-rich extracellular matrix efficiently induced differentiation under feeder-free conditions. Cell differentiation was confirmed with the expression of the insulin 1 gene in addition to marker genes in pancreatic β cells, the differentiated cells secreted glucose-responsive C-peptide, and their transplantation restored normoglycemia in diabetic mice. Moreover, Pdx1 protein transduction had facilitative effects on differentiation into pancreatic endocrine progenitors from human iPS cells. These results suggest the direct delivery of recombinant proteins and treatment with laminin-5-rich extracellular matrix to be useful for the generation of insulin-producing cells. STEM CELLS TRANSLATIONAL MEDICINE *2014;3:114–127*

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Received April 12, 2013; accepted for publication August 2, 2013; first published online in SCTM *EXPRESS* November 29, 2013.

1066-5099/2013/$20.00/0

http://dx.doi.org/10.5966/sctm.2013-0075

INTRODUCTION

Diabetes mellitus is a devastating disease, and the World Health Organization expects the number of diabetic patients to increase to 300 million by the year 2025. Although insulin therapy has proved useful for the treatment of diabetes, it is hoped that β-cell-replacement therapy will be even more effective; however, there is a limited supply of donor β cells. The generation of new β cells from expandable stem cell sources is necessary for regenerative medicine. Human embryonic stem (ES) cells hold promise as a source of new β cells. Furthermore, the discovery that adult stem cells can be reprogrammed to revert back to induced pluripotent stem (iPS) cells has raised the possibility of generating patient-specific cell types [1].

Several groups have reported ways of generating pancreatic cell lineages from ES and iPS cells [2–4]. These methods induce definitive endoderm differentiation in the first stage and then pancreatic specialization and maturation in subsequent stages, using combinations of growth factors and small molecules; however, the differentiated cells are immature or not fully functional in culture. Although delivering a specific combination of genes for transcription factors using adenoviral vectors can reprogram differentiated pancreatic exocrine cells in adult mice to become cells that closely resemble β cells [5], this method may be of limited use for patient treatment because of the risk of unexpected genetic modifications by exogenous DNA.

The cellular delivery of various biological compounds such as bioactive proteins has been improved recently by conjugating the compounds to short peptides known as cell-penetrating peptides (CPPs) or protein transduction domains (PTDs) [6–8]. The initial discovery of CPPs or PTDs originated from the unexpected observation that certain full-length proteins or protein domains can translocate across the plasma membrane. This was first shown for the HIV Tat transactivator [9, 10] and for the homeodomain of the *Drosophila melanogaster* transcription factor Antennapedia [11] and has since expanded to include "nonnatural" peptides that share this property. CPPs and PTDs are widely used in research, and, impressively, multiple clinical trials are testing the PTD-mediated delivery of macromolecular drug conjugates in patients with a variety of

Stem Cell Research (2014) 12, 574–583

Available online at www.sciencedirect.com

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

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Received 13 March 2013; received in revised form 13 December 2013; accepted 15 January 2014
Available online 27 January 2014

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.

☆☆ Financial support: This work was supported by Grants-in-Aid for Scientific Research (A) 20253742 and (B) 20253742 from the Ministry of Education, Science, Sports, and Culture of Japan (to YA), and by a grant from the National Institute of Biomedical Innovation (to NS), by a grant from CREST (to NS), and in part by a global COE grant (Cell Fate Regulation Research and Education Unit) from the Ministry of Education, Culture, Sports, Science and Technology (MEXT) Japan (to SK). SK is a member of the Global COE Program (Cell Fate Regulation Research and Education Unit), MEXT, Japan. The funders had no role in the study design, data collection and analysis, decision to publish, or preparation of the manuscript. This study was also supported in part by the Japan Science and Technology Agency's Precursory Research for Embryonic Science and Technology and S-Innovation Programs (to NF), and by grants from the Ministry of Health, Labor, and Welfare of Japan (to TE and NF).

★ Conflict of interest: Nothing to report.

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1873-5061/$ - see front matter
http://dx.doi.org/10.1016/j.scr.2014.01.004

THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 289, NO. 14, pp. 9623–9638, April 4, 2014

High Oxygen Condition Facilitates the Differentiation of Mouse and Human Pluripotent Stem Cells into Pancreatic Progenitors and Insulin-producing Cells*[S]

Received for publication, October 2, 2013, and in revised form, February 18, 2014 Published, JBC Papers in Press, February 19, 2014, DOI 10.1074/jbc.M113.524363

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Background: Oxygen plays a key role in organ development, including pancreatic β-cells.
Results: High oxygen conditions increase Ngn3-positive and insulin-positive cells from both mouse and human pluripotent stem cells.
Conclusion: Culturing under high oxygen conditions has a facilitative effect on pancreatic differentiation.
Significance: This new technique provides an efficient method to utilize patient-specific iPS cells for the treatment of diabetes.

Pluripotent stem cells have potential applications in regenerative medicine for diabetes. Differentiation of stem cells into insulin-producing cells has been achieved using various protocols. However, both the efficiency of the method and potency of differentiated cells are insufficient. Oxygen tension, the partial pressure of oxygen, has been shown to regulate the embryonic development of several organs, including pancreatic β-cells. In this study, we tried to establish an effective method for the differentiation of induced pluripotent stem cells (iPSCs) into insulin-producing cells by culturing under high oxygen (O_2) conditions. Treatment with a high O_2 condition in the early stage of differentiation increased insulin-positive cells at the terminus of differentiation. We found that a high O_2 condition repressed Notch-dependent gene *Hes1* expression and increased *Ngn3* expression at the stage of pancreatic progenitors. This effect was caused by inhibition of hypoxia-inducible factor-1α protein level. Moreover, a high O_2 condition activated Wnt signaling. Optimal stage-specific treatment with a high O_2 condition resulted in a significant increase in insulin production in both mouse embryonic stem cells and human iPSCs and yielded populations containing up to 10% C-peptide-positive cells in human iPSCs. These results suggest that culturing in a high O_2 condition at a specific stage is useful for the efficient generation of insulin-producing cells.

Cell replacement therapy has become possible by utilizing artificially generated cells or organs from embryonic stem cells (ESCs),[3] induced pluripotent stem cells (iPSCs), and adult stem cells. These stem cells have marked potential to develop into many different cell types in the body during early life and growth. Over the last decade, with the advent of new techniques and technologies in modern molecular biology, understanding of the underlying mechanism responsible for organ differentiation has developed rapidly. This knowledge has given rise to various new methods of manipulating stem cells in order to generate deficient organs in various diseases. To date, various differentiation methods have been developed for each cell type, including neurons, cardiomyocytes, and pancreatic endocrine cells. Many of these methods are based on mimicking the *in vivo* development. The development of efficient and safe methods is desired for clinical applications and studying the cause of disease.

Pluripotent stem cells are capable of spontaneous differentiation into insulin-producing cells. This is mainly carried out by preferential differentiation of stem cells into insulin-producing cells by changing the composition of the culture medium and causing the expression of dominant transcription factor genes, which are mainly involved in pancreatic development. Several groups have reported methods of generating pancreatic cell lineages from ESCs and iPSCs (1–8). These methods induce definitive endoderm differentiation in the first stage and then pancreatic specialization and maturation in the following stages, using combinations of growth factors, small molecules, and extracellular matrix. Lumelsky *et al.* (6) first demonstrated the successful differentiation of mouse ESCs (mESCs) to insulin-

* This work was supported by a grant-in-aid for scientific research from the Ministry of Education, Culture, Sports, Science and Technology of Japan, by the Japan Society for the Promotion of Science through its "Funding Program for Next Generation World-leading Researchers," by the Uehara Memorial Foundation, and by the Takeda Science Foundation.
[S] This article contains supplemental Figs. 1 and 2.
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[3] The abbreviations used are: ESC, embryonic stem cell; mESC, mouse embryonic stem cell; iPSC, induced pluripotent stem cell; hiPSC, human induced pluripotent stem cell; HIF, hypoxia-inducible factor; MEF, mouse embryo fibroblast; NEAA, nonessential amino acid(s); β-ME, β-mercaptoethanol; bFGF, basic fibroblast growth factor; qPCR, quantitative PCR.

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Beneficial Effect of Insulin Treatment on Islet Transplantation Outcomes in Akita Mice

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Abstract

Islet transplantation is a promising potential therapy for patients with type 1 diabetes. The outcome of islet transplantation depends on the transplantation of a sufficient amount of β-cell mass. However, the initial loss of islets after transplantation is problematic. We hypothesized the hyperglycemic status of the recipient may negatively affect graft survival. Therefore, in the present study, we evaluated the effect of insulin treatment on islet transplantation involving a suboptimal amount of islets in Akita mice, which is a diabetes model mouse with an *Insulin 2* gene missense mutation. Fifty islets were transplanted under the left kidney capsule of the recipient mouse with or without insulin treatment. For insulin treatment, sustained-release insulin implants were implanted subcutaneously into recipient mice 2 weeks before transplantation and maintained for 4 weeks. Islet transplantation without insulin treatment did not reverse hyperglycemia. In contrast, the group that received transplants in combination with insulin treatment exhibited improved fasting blood glucose levels until 18 weeks after transplantation, even after insulin treatment was discontinued. The group that underwent islet transplantation in combination with insulin treatment had better glucose tolerance than the group that did not undergo insulin treatment. Insulin treatment improved graft survival from the acute phase (i.e., 1 day after transplantation) to the chronic phase (i.e., 18 weeks after transplantation). Islet apoptosis increased with increasing glucose concentration in the medium or blood in both the *in vitro* culture and *in vivo* transplantation experiments. Expression profile analysis of grafts indicated that genes related to immune response, chemotaxis, and inflammatory response were specifically upregulated when islets were transplanted into mice with hyperglycemia compared to those with normoglycemia. Thus, the results demonstrate that insulin treatment protects islets from the initial rapid loss that is usually observed after transplantation and positively affects the outcome of islet transplantation in Akita mice.

Citation: Kikawa K, Sakano D, Shiraki N, Tsuyama T, Kume K, et al. (2014) Beneficial Effect of Insulin Treatment on Islet Transplantation Outcomes in Akita Mice. PLoS ONE 9(4): e95451. doi:10.1371/journal.pone.0095451

Editor: Marcia B. Aguila, State University of Rio de Janeiro, Biomedical Center, Institute of Biology, Brazil

Received September 30, 2013; **Accepted** March 27, 2014; **Published** April 17, 2014

Funding: This work was supported by the "Funding Program for Next Generation World-Leading Researchers" (awarded to S.K., no. LS099), the Japanese Society for the Promotion of Science, the Realization of Regenerative Medicine (awarded to S.K.), the Program for Leading Graduate Schools "HIGO" (awarded to S.K.), Grants-in-Aid from the Ministry of Education, Culture, Sports, Science and Technology, Japan (Grant no. 21390280, awarded to S.K. and #22790653 to D.S.), MEXT, Japan. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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

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Introduction

Diabetes is currently a global health problem. The World Health Organization (WHO) reports that 347 million people have diabetes worldwide. Diabetes is caused by the autoimmune destruction of pancreatic β cells (i.e., type 1 diabetes) or the combination of insulin resistance of all body organs and insulin secretion deficiency (i.e., type 2 diabetes).

Islet transplantation is a promising therapy for severely insulin-dependent diabetes patients in whom the endogenous insulin secretion is insufficient. As sustained insulin independence was reported in type 1 diabetes patients in the Edmonton protocol in 2000 [1], the incidence of islet transplantation has rapidly increased. However, islet transplantation has not yet become a standard therapy for diabetes because of donor shortages and the necessity of lifelong immunosuppressant drug use. Another important issue is the initial loss of many islets immediately after transplantation as a result of graft inflammation, immunorejection, apoptosis, or necrosis [2–4].

Efforts have been made to improve graft survival [5]. Suppression of immunorejection is the most important factor for a successful transplantation. A new immunosuppression trial has reported the combination of co-stimulation blockage via the CD80–CD86 pathways and thymoglobulin T-cell depletion [6]. In addition, some strategies are being developed to suppress inflammation. For instance, heparin and insulin infusions have been shown to significantly prevent instant blood-mediated inflammatory response (IBMIR) [7], the combination of anti-tumor necrosis factor (TNF)-α and interleukin (IL)-1 receptor blockage [8], and the inhibition of interferon (IFN)-γ [9] or caspase [2,10], all of which improve the efficacy of islet engraftment. Moreover, the use of glucagon-like peptide-1 (GLP-1) analog improves human islet survival in culture [11]. Various types of scaffolds such as extracellular matrix protein-coated scaffolds [12] and microporous polymer scaffolds, which allow

A synthetic nanofibrillar matrix promotes *in vitro* hepatic differentiation of embryonic stem cells and induced pluripotent stem cells

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Accepted 9 September 2013
Journal of Cell Science 126, 5391–5399

doi: 10.1242/jcs.129767

Summary

Embryonic stem (ES) cells recapitulate normal developmental processes and serve as an attractive source for routine access to a large number of cells for research and therapies. We previously reported that ES cells cultured on M15 cells, or a synthesized basement membrane (sBM) substratum, efficiently differentiated into an endodermal fate and subsequently adopted fates of various digestive organs, such as the pancreas and liver. Here, we established a novel hepatic differentiation procedure using the synthetic nanofiber (sNF) as a cell culture scaffold. We first compared endoderm induction and hepatic differentiation between murine ES cells grown on sNF and several other substrata. The functional assays for hepatocytes reveal that the ES cells grown on sNF were directed into hepatic differentiation. To clarify the mechanisms for the promotion of ES cell differentiation in the sNF system, we focused on the function of Rac1, which is a Rho family member protein known to regulate the actin cytoskeleton. We observed the activation of Rac1 in undifferentiated and differentiated ES cells cultured on sNF plates, but not in those cultured on normal plastic plates. We also show that inhibition of Rac1 blocked the potentiating effects of sNF on endoderm and hepatic differentiation throughout the whole differentiation stages. Taken together, our results suggest that morphological changes result in cellular differentiation controlled by Rac1 activation, and that motility is not only the consequence, but is also able to trigger differentiation. In conclusion, we believe that sNF is a promising material that might contribute to tissue engineering and drug delivery.

Key words: Hepatic differentiation, *In vitro* differentiation, Embryonic stem cells, Induced pluripotent stem cells

Introduction

The liver is an important organ that performs many complex functions, including the metabolism of carbohydrates, proteins and lipids, as well as storage of essential nutrients and biotransformation of drugs. Drug biotransformation involves detoxification and bioactivation, where the metabolite becomes more toxic. Therefore, drug biotransformation plays an important role in the early stages of drug discovery processes. Primary hepatocyte cultures are often used for pharmacological assays, but they are short-lived and cannot be maintained in long-term culture. In addition, there are considerable donor-dependent variations. By contrast, embryonic stem (ES) cells or induced pluripotent stem (iPS) cells can proliferate infinitely and maintain their pluripotent ability to differentiate into various cell types. There is evidence that ES or iPS cells recapitulate normal developmental processes, and can serve as an alternative resource for hepatological researches, drug development and clinical uses. Through our present knowledge of developmental biology, efficient induction of hepatic lineage cells has been established. For example, based on the evidence that TGFβ–activin–Smad2 signaling is involved in definitive endoderm formation in the mouse (Tremblay et al., 2000), the activation of Activin–Nodal signaling was used for endoderm induction (D'Amour et al., 2005; Kubo et al., 2004). Fibroblast growth factor (FGF) and bone morphogenetic protein (BMP) were added for the specification of liver lineages (Jung, 1999; Mfopou et al., 2010; Shiraki et al., 2008a); this helped to mimic the mesodermal signals from the septum transversum mesenchyme in normal development (Katsumoto et al., 2010; Shin et al., 2007; Rossi et al., 2001). Because hepatocyte growth factors are known to be important effectors in the specification of cell fate and organogenesis of the liver (Schmidt et al., 1995; Sonnenberg et al., 1993), hepatocyte growth factor (HGF), dexamethasone and oncostatin M have been used for induction of hepatocyte maturation (Basma et al., 2009; Kamiya et al., 1999; Si-Tayeb et al., 2010). Compared with the factors described above that direct hepatic differentiation, the role of extracellular matrices (ECMs) and scaffolds remains unclear.

Methods in Molecular Biology
DOI 10.1007/7651_2014_138

Hepatic Differentiation from Murine and Human iPS Cells Using Nanofiber Scaffolds

Taiji Yamazoe, Nobuaki Shiraki, and Shoen Kume

Abstract

The induced pluripotent stem (iPS) cells of murine and human are capable to differentiate into any cell type of the body through recapitulating normal development, similarly as the embryonic stem (ES) cells. Lines of evidence support that both ES cells and iPS cells are induced to differentiate in vitro by sequential treatment of humoral cues such as growth factors and chemicals, combined with the use of certain microenvironments including extracellular matrices and scaffolds.

Here, we describe the procedure to potentiate hepatic lineage cells differentiation from murine and human iPS cells, using growth factor cocktails and nanofiber scaffolds. Nanofiber scaffolds have a three-dimensional surface mimicking the fine structures of the basement membrane in vivo, allow the iPS cells to differentiate into the definitive endoderm and mature hepatocyte-like cells more efficiently than the two-dimensional conventional culture plates.

Keywords: Hepatic differentiation, Microenvironment, Extracellular matrices, Nanofiber scaffolds

1 Introduction

The iPS cells and ES cells have the ability to differentiate into any cell type of our body through mimicking normal developmental processes (1, 2). Therefore, these stem cells can serve as an attractive cell source for a large number of cells needed in biomedical research and regenerative therapies.

There are two majorly considerable conditions to culture ES and iPS cells, one is humoral cues in the culture medium and the other is the components of extracellular matrices and scaffolds.

Based on lines of evidence in developmental biology, hepatic differentiation from stem cells has been established (3, 4). This utilized not only the growth factors that are indispensable for liver organogenesis in vivo but also small chemicals that are theoretically expected to evoke intracellular signaling pathways. Activin A, for instance, is a ligand of the TGF-β superfamily and is used to induce endoderm differentiation, and hepatocyte growth factor is used to differentiate cells to adopt differentiation into the hepatic lineages (3, 5).

Kawabata et al. BMC Psychiatry 2013, 13:281
http://www.biomedcentral.com/1471-244X/13/281

BMC Psychiatry

RESEARCH ARTICLE Open Access

Temporal organization of rest defined by actigraphy data in healthy and childhood chronic fatigue syndrome children

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Abstract

Background: Accumulating evidence has shown a universality in the temporal organization of activity and rest among animals ranging from mammals to insects. Previous reports in both humans and mice showed that rest bout durations followed long-tailed (i.e., power-law) distributions, whereas activity bouts followed exponential distributions. We confirmed similar results in the fruit fly, *Drosophila melanogaster*. Conversely, another report showed that the awakening bout durations, which were defined by polysomnography in bed, followed power-law distributions, while sleeping periods, which may correspond to rest, followed exponential distributions. This apparent discrepancy has been left to be resolved.

Methods: Actigraphy data from healthy and disordered children were analyzed separately for two periods: time out of bed (UP period) and time in bed (DOWN period).

Results: When data over a period of 24 h were analyzed as a whole, rest bouts showed a power law distribution as previously reported. However, when UP and DOWN period data were analyzed separately, neither showed power law properties. Using a newly developed strict method, only 30% of individuals satisfied the power law criteria, even when the 24 h data were analyzed. The human results were in contrast to the *Drosophila* results, which revealed clear power-law distributions for both day time and night time rest through the use of a strict method. In addition, we analyzed the actigraphy data from patients with childhood type chronic fatigue syndrome (CCFS), and found that they showed differences from healthy controls when their UP and DOWN data were analyzed separately.

Conclusions: These results suggested that the DOWN sleep, the bout distribution of which showed exponential properties, contributes to the production of long-tail distributions in human rest periods. We propose that separate analysis of UP and DOWN period data is important for understanding the temporal organization of activity.

Keywords: Sleep, Temporal organization, Activity, Rest, Actigraphy

Background

Most natural phenomena appear to occur stochastically and this random distribution of phenomena is thought to produce Poisson distributions. However, it has been shown recently that many phenomena, such as human behavior, form non-Poisson distributions. For example, interevent intervals of social behaviors such as e-mail communications and trade transactions follow power-law distributions [1]. Nakamura et al. showed that rest bout durations followed long-tailed (i.e., power-law) distributions, whereas activity bouts followed exponential distributions in both humans and mice [2,3]. A similar temporal organization of rest and activity bouts has also been observed in invertebrates. In the insect *Drosophila melanogaster*, waiting intervals between behavioral episodes such as walking, feeding, and flight maneuvers follow the power-law distribution [4-8]. Using a video-recording method, we recently confirmed that the duration of the rest bout for the flies followed a power-law distribution

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PLOS ONE

Secreted Cerberus1 as a Marker for Quantification of Definitive Endoderm Differentiation of the Pluripotent Stem Cells

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Abstract

To date, CXCR4 and E-cadherin double-positive cells detected by flow cytometry have been used to identify the differentiation of embryonic stem (ES) cells or induced pluripotent stem (iPS) cells into definitive endoderm (DE) lineages. Quantification of DE differentiation from ES/iPS cells by using flow cytometry is a multi-step procedure including dissociation of the cells, antibody reaction, and flow cytometry analysis. To establish a quick assay method for quantification of ES/iPS cell differentiation into the DE without dissociating the cells, we examined whether secreted Cerberus1 (Cer1) protein could be used as a marker. Cer1 is a secreted protein expressed first in the anterior visceral endoderm and then in the DE. The amount of Cer1 secreted correlated with the proportion of CXCR4+/E-Cadherin+ cells that differentiated from mouse ES cells. In addition, we found that human iPS cell-derived DE also expressed the secreted CER1 and that the expression level correlated with the proportion of SOX17+/FOXA2+ cells present. Taken together, these results show that Cer1 (or CER1) serves as a good marker for quantification of DE differentiation of mouse and human ES/iPS cells.

Citation: Iwashita H, Shiraki N, Sakano D, Ikegami T, Shiga M, et al. (2013) Secreted Cerberus1 as a Marker for Quantification of Definitive Endoderm Differentiation of the Pluripotent Stem Cells. PLoS ONE 8(5): e64291. doi:10.1371/journal.pone.0064291

Editor: Jennifer Nichols, Wellcome Trust Centre for Stem Cell Research, United Kingdom

Received December 28, 2012; **Accepted** April 11, 2013; **Published** May 22, 2013

Funding: This work was supported by a Grant-in-Aid (#21390280 to SK and NEXT program from JST to SK), National Institute of Biomedical Innovation (to NS), and, in part, by a Global COE grant (Cell Fate Regulation Research and Education Unit). SK received funding from the Dojindo Laboratories. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: Three of the authors are employees and are affiliated to the commercial funders of this research (Dojindo Laboratories). The ELISA kit described in this manuscript will be sold as a product by Dojindo Laboratories. There are no consultancy, patents associated with this manuscript. However, these do not alter the authors' adherence to all the PLOS ONE policies on sharing data and materials.

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Introduction

Embryonic stem (ES) cells are derived from a pluripotent inner cell mass, which can be cultured indefinitely in an undifferentiated state and can be differentiated into most cell types in an organism. Therefore, ES cells have been proposed as a source of surrogate cells for use in regenerative medicine.

The definitive endoderm (DE) gives rise to the gastrointestinal organs, such as stomach, pancreas, liver, and intestine. The gastrointestinal organs are of great importance in their therapeutic aspects. Studies of ES cells have demonstrated that ES cell differentiation recapitulates early signaling events of differentiation into the 3 germ layers. Recent progress has identified several germ layer-specific markers of the early DE. Sox17 (Sry-box–containing gene 17), which encodes an endodermal HMG (high mobility group)-box transcription factor, is a DE-specific marker [1]. CXCR4 (C-X-C chemokine receptor type 4), which is expressed in the mesoderm, is also expressed in the DE and is widely used in combination with E-cadherin for the prospective isolation of embryonic or ES cell-derived DE cells [2]. Our group previously identified DAF1 (decay accelerating factor)/CD55 as a novel DE marker [3]. Yasunaga et al., reported the use of the Sox17 promoter to drive the expression of the surface antigen-GFP (green fluorescent protein) fusion protein, which genetically marked the DE with GFP.

Cerberus1 (Cer1; also known as Cerberus like 1 [Cerl1] or Cerberus related gene [Cerr1]) is a secreted protein, which belongs to the cysteine knot superfamily and includes TGF (transforming growth factor) βs and BMPs (bone morphogenetic proteins). Cer1 is first expressed in the anterior visceral endoderm at E6.5 and at E7.0 in the distal visceral endoderm and the definitive endoderm, which emanates from the anterior portion of the primitive streak. Cer1 is expressed in the anterior DE at E7.5 and is expressed in the foregut at the headfold stage. Later, Cer1 is expressed in a limited region in the somatic mesoderm, the pre-somitic mesoderm, and the presumptive foregut endoderm. Cer1 belongs to the Cer/Dan gene family, which contains the secreted antagonists of Nodal, Wnt, or BMP signaling pathways, and plays an important role in regulating these signals [4] [5][6] [7][8] [9].

We previously established a procedure to induce ES cells to sequentially differentiate into the mesendoderm, DE, and, finally, regional specific definitive endodermal tissues *in vitro* in a manner that mimics early embryonic inductive events *in vivo* by culturing ES cells on a monolayer of M15 cells [10] [11]. This M15 monolayer culture procedure turned out to be useful not only in

Stem Cell Research (2012) 10, 179–194

Available online at www.sciencedirect.com

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Albumin gene targeting in human embryonic stem cells and induced pluripotent stem cells with helper-dependent adenoviral vector to monitor hepatic differentiation

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Received 31 July 2012; received in revised form 10 October 2012; accepted 9 November 2012

Abstract Although progresses in developing differentiation procedures have been achieved, it remains challenging to generate hES/iPS cell-derived mature hepatocytes. We performed knock-in of a monomeric Kusabira orange (mKO1) cassette in the *albumin* (*ALB*) gene, in human embryonic stem (hES) cells and induced pluripotent stem (hiPS) cells, with the use of the helper-dependent adenovirus vector (HDAdV). Upon induction into the hepatic lineages, these knock-in hES/iPS cells differentiated into cells that displayed several known hepatic functions. The mKO1 knock-in (*ALB*/mKo1) hES/hiPS cells were used to visualize hepatic differentiation in vitro. mKO1 reporter expression recapitulated endogenous *ALB* transcriptional activity. *ALB*/mKo1 [Hi] population isolated by flow cytometry was confirmed to be enriched with *ALB* mRNA. Expression profile analyses revealed that characteristic hepatocyte genes and genes related to drug metabolism and many aspects of liver function were highly enriched in the *ALB*/mKo1 [Hi] population. Our data demonstrate that *ALB*/mKo1 knock-in hES/iPS cells are valuable resources for monitoring in vitro hepatic differentiation, isolation and analyses of hES and hiPS cells-derived hepatic cells that actively transcribing *ALB*. These knock-in hES/iPS cell lines could provide further insights into the mechanism of hepatic differentiation and molecular signatures of the hepatic cells derived from hES/iPS cells.

Abbreviations: ALB, Albumin; mKO1, monomeric Kusabira orange; hES, human embryonic stem; hiPS, human induced pluripotent stem; HDAdV, Helper-dependent adenovirus; AFP, Alpha-fetoprotein; GO, Gene ontology

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1873-5061/$ - see front matter © 2012 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.scr.2012.11.003

Methods in Molecular Biology
DOI 10.1007/7651_2014_146

Hepatic Differentiation from Human Ips Cells Using M15 Cells

Kahoko Umeda, Nobuaki Shiraki, and Shoen Kume

Abstract

Here, we describe a procedure of human iPS cells differentiation into the definitive endoderm, further into albumin-expressing and albumin-secreting hepatocyte, using M15, a mesonephros- derived cell line. Approximately 90 % of human iPS cells differentiated into SOX17-positive definitive endoderm then approximately 50 % of cells became albumin-positive cells, and secreted ALB protein. This M15 feeder system for endoderm and hepatic differentiation is a simple and efficient method, and useful for elucidating molecular mechanisms for hepatic fate decision, and could represent an attractive approach for a surrogate cell source for pharmaceutical studies.

Keywords: Hepatic differentiation, Endoderm differentiation, Feeder cells, M15 cells

1 Introduction

Human iPS cells are potential sources of hepatocytes for applications in regenerative medicine and drug development (1). We previously reported a procedure in which ES cells are sequentially induced into the regional specific gut endoderm lineages, such as the pancreas, liver, and intestine, by use of M15, a mesoderm derived cell line (2–4).

M15 is used as a source for signals for in vitro ES differentiation. M15 directs human ES cells to differentiate into the definitive endodermal lineages with the addition of activin and LY294002, a potent PI3 kinase inhibitor, further into the hepatic lineages with the addition of dexamethasone (Dex) and Hepatocyte growth factor (HGF) (2). Approximately 80 % of the human ES cells differentiated into alpha feto protein (AFP)-positive hepatic precursor cells on day 20. On day 40, approximately 9 % of the total cells became Albumin (ALB)-positive hepatocytes and secreted a substantial level of ALB protein (2). Here, we describe an optimized protocol which is more efficient and results in generating a higher portion (85.9 %) of SOX17-positive definitive endoderm by altering the endoderm

STEM CELLS

EMBRYONIC STEM CELLS/INDUCED PLURIPOTENT STEM CELLS

Wnt and Notch Signals Guide Embryonic Stem Cell Differentiation into the Intestinal Lineages

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Key Words. Embryonic stem cells • Endoderm • Intestine differentiation • In vitro differentiation

ABSTRACT

The studies of differentiation of mouse or human embryonic stem cells (hESCs) into specific cell types of the intestinal cells would provide insights to the understanding of intestinal development and ultimately yield cells for the use in future regenerative medicine. Here, using an in vitro differentiation procedure of pluripotent stem cells into definitive endoderm (DE), inductive signal pathways' guiding differentiation into intestinal cells was investigated. We found that activation of Wnt/β-catenin and inhibition of Notch signaling pathways, by simultaneous application of 6-bromoindirubin-3′-oxime (BIO), a glycogen synthase kinase-3β inhibitor, and N-[(3,5-Difluorophenyl)acetyl]-L-alanyl-2-phenylglycine-1,1-dimethylethyl ester (DAPT), a known γ-secretase inhibitor, efficiently induced intestinal differentiation of ESCs cultured on feeder cell. BIO and DAPT patterned the DE at graded concentrations. Upon prolonged culture on feeder cells, all four intestinal differentiated cell types, the absorptive enterocytes and three types of secretory cells (goblet cells, enteroendocrine cells, and Paneth cells), were efficiently differentiated from mouse and hESC-derived intestinal epithelium cells. Further investigation revealed that in the mouse ESCs, fibroblast growth factor (FGF) and bone morphogenetic protein (BMP) signaling act synergistically with BIO and DAPT to potentiate differentiation into the intestinal epithelium. However, in hESCs, FGF signaling inhibited, and BMP signaling did not affect differentiation into the intestinal epithelium. We concluded that Wnt and Notch signaling function to pattern the anterior-posterior axis of the DE and control intestinal differentiation. STEM CELLS *2013;31:1086–1096*

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

INTRODUCTION

The intestinal epithelium is a robust system for the study of cell proliferation and differentiation. There are four differentiated cell types of nonproliferative epithelial cells: enterocytes, goblet cells, enteroendocrine cells, and Paneth cells. Intestinal stem cells (ISCs) and progenitor cells reside in crypts, proliferate vigorously, and function as the source of differentiated epithelial cells. In contrast to adult tissue stem cells, embryonic development and differentiation from embryonic stem cells (ESCs) have not been as well investigated in the intestine. The intestinal epithelium is derived from the definitive endoderm (DE) and is covered by a single layer of epithelial cells, where the cells turnover rapidly. Intestinal endoderm is marked by the expression of the homeobox gene of the *caudal* family, *Cdx2*, which start to be expressed from embryonic day 8.5 (E8.5). *Cdx2* is shown to play a central role in the regionalization of the primitive gut tube. Conditional ablation of *Cdx2* in the DE resulted in the formation of abnormal intestinal epithelia, which was transformed into an anterior identity of the esophageal epithelium [1]. *Cdx2* is also shown to be directly regulated by canonical Wnt/β-catenin signaling molecules Tcf4 and β-catenin [2].

Recently, it was reported that at high concentration of fibroblast growth factor 2 (FGF2), the differentiation of human ESCs (hESCs) into small intestinal progenitors increased at the expense of Pdx1+ pancreatic progenitors [3], and that the combination of Wnt3a and FGF4 was reported to be required for hindgut specification, and that FGF4 alone was sufficient for promoting hindgut morphogenesis [4]. During normal development, bone morphogenetic protein (BMP) signals are reported to be absent in the Hensen's node, and to be high in the posterior region in the chick [5]. However, it is not clear how BMP antagonizing signals exerts its anteriorizing effect. Loss of BMP2b signaling in zebrafish was reported to expand the anterior gut endoderm and reduce the posterior gut endoderm, while loss of chordin signaling resulted in opposite effects [6]. Conversely, posterior endoderm seems to be specified through FGF signal emitted from the posterior ectomesoderm [7, 8]. In the chick, we reported that segregation of the intestinal fate occurs very early, at two to five somite stages (ss), before pancreatic fate decision takes place

Author contributions. S.O.: acquisition of data and analysis and interpretation of data; N.S.: study concept and design and acquisition, analysis, and interpretation of data; K.K.: critical revision of the manuscript and administrative and study supervision; S.K.: study concept and design, drafting and revision of the manuscript, approval of the final version of the manuscript, and obtained funding.

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IVYSPRING INTERNATIONAL PUBLISHER

Theranostics

2013; 3(1):11-17. doi: 10.7150/thno.4806

Review

The Role of CXCL12-CXCR4 Signaling Pathway in Pancreatic Development

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Received: 2012.07.02; Accepted: 2012.09.13; Published: 2013.01.12

Abstract

Chemokine (C-X-C motif) receptor 4 (CXCR4) is the receptor for chemokine (C-X-C motif) ligand 12 (CXCL12, also known as stromal derived factor-1, Sdf1). CXCR4, a protein consisting 352 amino acids, is known to transduce various signals such as cell differentiation, cell survival, cell proliferation, cell chemotaxis and apoptosis [1, 2]. The expression of CXCR4 is observed in embryonic stem cells, blood cells, haematopoietic stem cells, endothelial cells, angioblasts and smooth muscle cells [3-9]. The CXCL12-CXCR4 signaling pathway has very important roles in the embryonic development. Mutant mice for *CXCL12* or *CXCR4* genes showed lethality due to defects in neurogenesis, angiogenesis, cardiogenesis, myelopoiesis, lymphopoiesis and germ cell development [10-13]. Recently, we reported that CXCL12-CXCR4 signaling pathway has a crucial role in regional specification of the gut endoderm during early development [14]. Here, we would like to focus on the role of CXCL12-CXCR4 signaling pathway in pancreatic development and summarize recent findings of its role in the induction of the pancreatic progenitor cells.

Key words: CXCL12, CXCR4, signaling pathway

An overview of early pancreas development

The earliest pancreatic marker gene, *pancreatic and duodenal homeobox 1* (*Pdx1*) starts to be expressed in the pre-pancreatic endoderm at the 9-somite stage (ss), corresponding to embryonic day 8.5 (E8.5) in the mouse and at 8 ss in the chick [14-17]. *Pdx1* is also expressed in the stomach and duodenal endoderm. The pancreas is derived from the dorsal and ventral endoderm, which give rise from two distinct origins. In the mouse, the pancreatic bud emerges from E9.5. Fate map studies revealed that while the dorsal pancreas progenitor cells originated at the 3-6 somite levels at 7-9 ss in the mouse embryo, the ventral pancreatic progenitors are closely associated with hepatic progenitors and that they originates at the 1 ss at the lateral endoderm posterior to the anterior intestinal portal (AIP) lip [18]. Later on, at 3-4 ss, they were observed at the outer side at the AIP lip.

In the chick, the dorsal pancreas progenitors were first observed near the hensen's node at late gastrula stage (stage 5), which then migrate to the 3-6 somite levels at 6 ss, then to the 4-7 somite levels at 8 ss [19]. The ventral pancreas progenitor cells were first observed lateral to the 2 somite level at 10 ss, which then migrate to a site lateral to the 4 somite level at 17 ss [20] (Figure 1).

All mature pancreatic cell types are derived from the common pancreatic progenitors, which expresses *Pdx1* and *pancreas specific transcription factor 1a* (*Ptf1a*) and *SRY-box containing gene 9* (*Sox9*) [21-23]. Gu

Biochemical and Biophysical Research Communications 430 (2013) 1103–1108

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Recovery from diabetes in neonatal mice after a low-dose streptozotocin treatment

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ARTICLE INFO

Article history:
Received 2 December 2012
Available online 19 December 2012

Keywords:
Insulin
Pancreas
Beta-cell
Regeneration
Streptozotocin
Diabetes

ABSTRACT

Administration of streptozotocin (STZ) induces destruction of β-cells and is widely used as an experimental animal model of type I diabetes. In neonatal rat, after low-doses of STZ-mediated destruction of β-cells, β-cells regeneration occurs and reversal of hyperglycemia was observed. However, in neonatal mice, β-cell regeneration seems to occur much slowly compared to that observed in the rat. Here, we described the time dependent quantitative changes in β-cell mass during a spontaneous slow recovery of diabetes induced in a low-dose STZ mice model. We then investigated the underlying mechanisms and analyzed the cell source for the recovery of β-cells. We showed here that postnatal day 7 (P7) female mice treated with 50 mg/kg STZ underwent the destruction of a large proportion of β-cells and developed hyperglycemia. The blood glucose increased gradually and reached a peak level at 500 mg/dl on day 35–50. This was followed by a spontaneous regeneration of β-cells. A reversal of non-fasting blood glucose to the control value was observed within 150 days. However, the mice still showed impaired glucose tolerance on day 150 and day 220, although a significant improvement was observed on day 150. Quantification of the β-cell mass revealed that the β-cell mass increased significantly between day 100 and day 150. On day 150 and day 220, the β-cell mass was approximately 23% and 48.5% of the control, respectively. Of the insulin-positive cells, 10% turned out to be PCNA-positive proliferating cells. Our results demonstrated that, β-cell duplication is one of the cell sources for β-cell regeneration.

1. Introduction

Blood glucose homeostasis is controlled by the insulin producing endocrine β-cells of the pancreas. In mice, rats, and humans, the β-cell mass remains linear with body weight or Body Mass Index (BMI), thus implicating that there is a mechanism to regulate the β-cell mass [1,2]. Neogenesis of β-cells postnatally has been reported to originate from several sources, through activation and differentiation of endogenous progenitor cells in injured adult pancreas [3–5]. However, lineage-tracing studies using genetically marked β-cells in mice showed that in the adult mice, all new β-cells are generated by replication of pre-existing β-cells, either after birth or following 70% pancreatectomy [6]. Additionally, transdifferentiation from acinar cells are reported to occur in culture [7]. Recently, a direct conversion from α-cells was reported to occur after an extensive loss of β-cells reported to represent an alternative cell source for β-cell regeneration [8,9].

Administration of graded doses of streptozotocin (STZ; *N*-nitroso derivative of glucosamine) induces a dose-dependent hyperglycemia and is widely used as an experimental animal models of type I diabetes [10]. In the neonatal rat, STZ injection at subdiabetogenic doses (70–100 mg/kg body weight) on the day of birth caused damages to β-cells, which is followed by a rapid remission by spontaneous β-cell regeneration, so that approximately one week after STZ administration, the β-cell mass was about half of the original one [11,12]. Under this neonatal STZ rat regeneration model, the induction of endocrine cells expressing both insulin and glucagon with proliferative ability, or α-cell hyperplasia was observed [13].

In the adult mice, STZ injection at 100 mg/kg body weight induced slow and progressive damages to β-cells, which caused slow increases in blood glucose, and no remission was observed within 12 weeks [14]. In neonatal mice, it was reported that a low-dose of STZ injection at 50 mg/kg induced diabetes, and after 29 weeks a remission from diabetes was observed [15]. These reports indicate that the sensitivity to STZ differs and the mice β-cell regeneration takes more time compared to the rat. In the neonatal mice STZ model, detailed time dependent dynamics of the regeneration of β-cells was not documented.

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0006-291X/$ - see front matter © 2012 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.bbrc.2012.12.030

nature neuroscience

Identification of a dopamine pathway that regulates sleep and arousal in *Drosophila*

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Sleep is required to maintain physiological functions, including memory, and is regulated by monoamines across species. Enhancement of dopamine signals by a mutation in the dopamine transporter (*DAT*) decreases sleep, but the underlying dopamine circuit responsible for this remains unknown. We found that the D1 dopamine receptor (DA1) in the dorsal fan-shaped body (dFSB) mediates the arousal effect of dopamine in *Drosophila*. The short sleep phenotype of the *DAT* mutant was completely rescued by an additional mutation in the *DA1* (also known as DopR) gene, but expression of wild-type *DA1* in the dFSB restored the short sleep phenotype. We found anatomical and physiological connections between dopamine neurons and the dFSB neuron. Finally, we used mosaic analysis with a repressive marker and found that a single dopamine neuron projecting to the FSB activated arousal. These results suggest that a local dopamine pathway regulates sleep.

Sleep has been observed in many organisms. The cellular basis underlying sleep regulation, however, remains poorly understood. Studies of cellular mechanisms for sleep regulation in mammals are hindered by their complex brain structure. *Drosophila melanogaster*, however, has a relatively simple brain, and is therefore suitable for studying the cellular basis that underlies a specific behavior[1,2]. The phylogenetic conservation of sleep regulating molecular mechanisms between mammals and fruit flies has been established previously[3–8].

Sleep regulation in *Drosophila* involves dopamine[6,7]. Dopamine is synthesized from L-3,4-dihydroxyphenylalanine (DOPA) by DOPA decarboxylase (Ddc), and DOPA is synthesized from tyrosine by tyrosine hydroxylase. Administration of 3-iodo-tyrosine, a tyrosine hydroxylase inhibitor, increases sleep duration, whereas administration of psychostimulants that enhance dopamine signaling, such as cocaine or amphetamine, decreases sleep. DAT^{fmn} mutant flies show a reduction in sleep time as a result of an increase in postsynaptic dopamine signaling[7,9]. Increased dopamine signaling also alters the temporal characteristics of the activities, metabolic rate and temperature sensitivities of these flies[10,11]. Similarly, in mammals, deletion of the dopamine transporter gene or transient activation of dopamine neurons results in a decrease in sleep time[12,13]. However, it is not known whether dopamine transmission between a specific subset of dopamine neurons and defined receiving cells regulates arousal. Alternatively, broad background dopamine levels could be responsible for arousal regulation. Although dopamine was recently shown to regulate arousal, in part, through a pigment dispersing factor (PDF) neuron that regulates circadian rhythm, the primary target for dopamine in sleep regulation remains unknown[13,14]. There are two major subfamilies of dopamine receptors in *Drosophila*: two D1-like receptors, DA1 and DopR2, and a D2-like receptor, D2R, have been identified[15]. Although it has been reported that DA1 mediates wakefulness through caffeine and methamphetamine[16], startle-induced arousal[14], and ethanol-stimulated locomotion[17], its contribution to physiological sleep regulation and the cellular loci in which it functions remain unclear.

Dopamine also mediates other physiological functions, such as memory and attention[18,19]. Specific subsets of dopamine neurons that project to the mushroom bodies mediate aversive and appetitive reinforcement in olfactory memory in *Drosophila*[20–22]. As the mushroom body has been shown to regulate sleep[23,24], an important question arises as to whether the same dopamine neurons can regulate both sleep/arousal and learning. Sleep also interacts with various brain functions, including efficient memory formation[25–27]. Whether the functional interaction between sleep and memory is implemented in the same dopamine neuron(s) remains an open question.

We set out to map the dopamine pathway that is required for sleep and arousal regulation. To this end, we employed a combinatorial approach. We identified FSB as a target for dopamine using cell type–specific genetic manipulation of *DA1*, and presynaptic neurons to the FSB using the GFP reconstitution across synaptic partners (GRASP) method. We measured the physiological response of the FSB neurons to dopamine application and mapped the wake-promoting dopamine neuron to a single posterior protocerebral 3 (PPM3) neuron using mosaic analysis with a repressible cell marker (MARCM).

RESULTS

Dopamine mediates arousal via DA1 in dFSB neurons

To identify the target of dopamine in sleep regulation, we employed a genetic strategy that used the dopamine transporter DAT^{fmn} mutant. Given that presynaptic DAT clears the released dopamine from the synaptic cleft, the short sleep phenotype of DAT^{fmn} flies can be attributed to an increase in postsynaptic dopamine signaling in all dopamine pathways. Flies containing the hypomorphic *DA1* allele *dumb*[2] show a substantial sleep increase when under constant darkness conditions[14].

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Received 27 August; accepted 17 September; published online 14 October 2012; doi:10.1038/nn.3238

PLoS one

Dopamine Modulates Metabolic Rate and Temperature Sensitivity in *Drosophila melanogaster*

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Abstract

Homeothermal animals, such as mammals, maintain their body temperature by heat generation and heat dissipation, while poikilothermal animals, such as insects, accomplish it by relocating to an environment of their favored temperature. Catecholamines are known to regulate thermogenesis and metabolic rate in mammals, but their roles in other animals are poorly understood. The fruit fly, *Drosophila melanogaster*, has been used as a model system for the genetic studies of temperature preference behavior. Here, we demonstrate that metabolic rate and temperature sensitivity of some temperature sensitive behaviors are regulated by dopamine in *Drosophila*. Temperature-sensitive molecules like dTrpA1 and *shi*^{ts} induce temperature-dependent behavioral changes, and the temperature at which the changes are induced were lowered in the dopamine transporter-defective mutant, *fumin*. The mutant also displays a preference for lower temperatures. This thermophobic phenotype was rescued by the genetic recovery of the dopamine transporter in dopamine neurons. Flies fed with a dopamine biosynthesis inhibitor (3-iodo-L-tyrosine), which diminishes dopamine signaling, exhibited preference for a higher temperature. Furthermore, we found that the metabolic rate is up-regulated in the *fumin* mutant. Taken together, dopamine has functions in the temperature sensitivity of behavioral changes and metabolic rate regulation in *Drosophila*, as well as its previously reported functions in arousal/sleep regulation.

Citation: Ueno T, Tomita J, Kume S, Kume K (2012) Dopamine Modulates Metabolic Rate and Temperature Sensitivity in *Drosophila melanogaster*. PLoS ONE 7(2): e31513. doi:10.1371/journal.pone.0031513

Editor: Michael N. Nitabach, Yale School of Medicine, United States of America

Received June 9, 2011; **Accepted** January 11, 2012; **Published** February 7, 2012

Funding: This work was supported by a grant-in-aid (17024049) from the Ministry of Education, Culture, Sports, Science and Technology (MEXT), Japan, and a grant-in-aid (15300108, 17300105, 22300132 to KK) from the Japanese Society for the Promotion of Science (JSPS); a 21st Century Center of Excellence (COE) grant; and a Global COE grant from MEXT. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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

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Introduction

Almost all animals physiologically adjust their energy consumption rate in response to a wide range of environmental changes, including ambient temperature. In order to maintain their proper body temperature, they need more energy at lower temperatures, with the exception of hibernating animals. Mobile animals actively select and relocate to a place of their preferred temperature so that they can maintain their body temperature with less energy expenditure [1]. Thus, in order to understand the mechanisms of energy balance, studies of behavior as well as physiological activity at the organ and cellular levels are required.

Studies on thermotaxis and temperature preference behavior have been performed in model organisms, including the fruit fly *Drosophila melanogaster* (reviewed in Dillon et al. [2]) and *Caenorhabditis elegans* [3,4,5]. However, the molecular mechanisms mediating metabolic regulation and temperature preference behavior remain elusive. In *Drosophila*, several genes related to temperature sensing have been characterized, including *dTRPA1*, *Painless*, *Hsp70*, *Pyrexia*, and *brivido* [6,7,8,9,10]. The biogenic amine histamine was reported to modulate temperature preference behavior in *Drosophila* [11].

The role of catecholamines on energy balance has been studied in mammals. Mice lacking both noradrenaline and adrenaline showed impaired thermogenesis and metabolic regulation [12]. Catecholamine reuptake inhibitors, such as bupropion, promoted thermogenesis and resulted in weight loss [13].

We have employed *Drosophila melanogaster* as a model animal for genetic studies of behaviors including sleep and arousal. We previously reported that a dopamine transporter (DAT)-defective mutant, *fumin (fmn)*, showed a short sleep phenotype, identifying dopamine's function in sleep and arousal regulation in *Drosophila* – a function conserved also in mammals [14,15]. During further analysis of dopaminergic sleep regulation using genetic behavioral assays based on temperature-dependent molecules, we found that the temperature sensitivity of the *fmn* mutant is different from that of the wild type flies. That means we found that the temperatures at which *fmn* mutants showed behavioral changes were significantly lower. In this study, we show that the dopaminergic system participates in regulating metabolic rate and temperature sensitivities of some temperature-dependent behavioral changes, as well as modulating temperature preference in *Drosophila*. Elevated dopamine signaling in the *fmn* mutants resulted in an increased metabolic rate and a thermophobic phenotype – a preference for lower temperatures compared to wild type flies. These results highlight similarities between insects and mammals in the molecular basis of energy homeostasis.

PLoS one

Dopamine Modulates the Rest Period Length without Perturbation of Its Power Law Distribution in *Drosophila melanogaster*

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Abstract

We analyzed the effects of dopamine signaling on the temporal organization of rest and activity in *Drosophila melanogaster*. Locomotor behaviors were recorded using a video-monitoring system, and the amounts of movements were quantified by using an image processing program. We, first, confirmed that rest bout durations followed long-tailed (i.e., power-law) distributions, whereas activity bout durations did not with a strict method described by Clauset *et al.* We also studied the effects of circadian rhythm and ambient temperature on rest bouts and activity bouts. The fraction of activity significantly increased during subjective day and at high temperature, but the power-law exponent of the rest bout distribution was not affected. The reduction in rest was realized by reduction in long rest bouts. The distribution of activity bouts did not change drastically under the above mentioned conditions. We then assessed the effects of dopamine. The distribution of rest bouts became less long-tailed and the time spent in activity significantly increased after the augmentation of dopamine signaling. Administration of a dopamine biosynthesis inhibitor yielded the opposite effects. However, the distribution of activity bouts did not contribute to the changes. These results suggest that the modulation of locomotor behavior by dopamine is predominantly controlled by changing the duration of rest bouts, rather than the duration of activity bouts.

Citation: Ueno T, Masuda N, Kume S, Kume K (2012) Dopamine Modulates the Rest Period Length without Perturbation of Its Power Law Distribution in *Drosophila melanogaster*. PLoS ONE 7(2): e32007. doi:10.1371/journal.pone.0032007

Editor: Mark A. Frye, University of California Los Angeles, United States of America

Received November 24, 2011; **Accepted** January 17, 2012; **Published** February 16, 2012

Funding: This work was supported by the Grant-in-Aid (17024049) from the Ministry of Education, Culture, Sports, Science and Technology (MEXT), Japan, and the Grants-in-Aid (17300105, 22300132) from the Japanese Society for the Promotion of Science (JSPS) to KK; a 21st Century Center of Excellence (COE) grant; and a Global COE grant from MEXT. NM acknowledges support through the Grants-in-Aid for Scientific Research on Innovative Areas "Systems Molecular Ethology"(20115009) and the Grant-in-Aid for Scientific Research (20760258) from MEXT, Japan. TU is a global COE junior research associate and JSPS fellow. SK is a member of the Global COE Program (Cell Fate Regulation Research and Education Unit), MEXT, Japan. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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

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Introduction

Human behavior is highly diverse and profoundly complex. Our decisions are influenced by both our internal drive and our perception of environments. However, recent studies have shown that many human behaviors have shared common characteristics in the temporal organization. Although current models of human dynamics assume that human actions are randomly distributed in time and thus well approximated by Poisson processes, there is increasing evidence that the timing of many human activities follow non-Poisson statistics, characterized by bursts of rapidly occurring events separated by long periods of inactivity. Interevent intervals of social behaviors such as e-mail communications and trade transactions follow power-law distributions [1]. How rest and activity episodes are interwoven in behaving animals was also studied recently [2,3], indicating that rest bouts follow the power-law distribution in both human and mice, whereas activity bouts follow the exponential distribution. Furthermore, Nakamura et al, reported that patients with major depressive disorder exhibited decrease of scaling exponent in power-law distribution of resting period, suggesting novel quantitative strategy for neuropsychiatry [3]. Although power-law distributions are abundant in various natural phenomena and thus do not indicate the universality of animal behaviors by themselves, the biological mechanism underlying the power law distributions attracts attentions and remains an open question.

Similar temporal organization of rest and activity bouts has also been observed in invertebrates. In an insect *Drosophila melanogaster*, waiting intervals between behavioral episodes such as walking, feeding, and flight maneuvers follow the power-law distribution [4,5,6,7,8]. A wide range of similarities have been reported between insects and mammals with respect to the genes that regulate behaviors such as clock genes regulating circadian rhythm and sleep-wake cycle related genes. We believe that understanding the temporal organization of behavior in *Drosophila melanogaster*, the most promising model organism for studying the molecular basis of behavior, would shed light on the biological mechanism underlying common behavioral characteristics. For the analysis of temporal organization, precise fitting method to power-law distribution is indispensable. The previous report drew attention to an issue that conventional methods such as simple linear fitting of data plotted on log-log axes tend to place overweight on large

MECHANISMS OF DEVELOPMENT 128 (2012) 597–609

Available at www.sciencedirect.com
SciVerse ScienceDirect
journal homepage: www.elsevier.com/locate/modo

Fate maps of ventral and dorsal pancreatic progenitor cells in early somite stage mouse embryos

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ARTICLE INFO

Article history:
Received 13 May 2011
Received in revised form
12 December 2011
Accepted 13 December 2011
Available online 30 December 2011

Keywords:
Pdx1
Endoderm
Fate map
Pancreas
Gut
Lineage tracing

ABSTRACT

The origins of liver progenitor cells have been extensively studied, but evidence on the origin of pancreatic precursor cells is currently limited. *Pancreatic and duodenal homeobox gene 1* (*Pdx1*) is one of the earliest known markers for the pancreas. A transgenic mouse line expressing green fluorescent protein (GFP) under the control of the *Pdx1* promoter showed that *Pdx1*/GFP expression was first observed in the mid-region of the anterior intestinal portal (AIP) lip at embryonic day (E) 8.5 at the 5–6 somite stage (ss). The liver progenitors were confirmed to originate from separate domains at the lateral endoderm and the inner part of the medial AIP as previously reported (Tremblay and Zaret, 2005), which turned out to lie caudally to the *Pdx1*/GFP-expressing domain. To confirm if the early *Pdx1*/GFP-positive cells give rise to the pancreatic bud, we labeled the cells on the lip of the AIP using the carbocyanine dye CM-DiI and traced their fates in 1–4 ss, 5–6 ss and 7–9 ss E8.5 embryos using an *ex utero* whole embryo culture method. At 1 ss, the ventral pancreas progenitors were observed in the lateral endoderm, not yet being segregated from the liver or gut progenitors. Cells that contributed solely to the ventral pancreas first appeared at the AIP lip from 5 ss. At 5–6 ss, cells from the medial of the AIP lip contributed to the ventral pancreas. The pancreas fate region become narrower as development progresses. At 7–9 ss, the cells contributing to the ventral pancreas resided in a narrow region of the AIP lip. From 5 ss, the right flanking region contributes to the posterior gut, and the left flanking region contributes to the anterior gut. Dorsal pancreatic progenitors originate from the dorsal endoderm at the 3–6 somite level at 7–9 ss, though they have not yet diverged from the dorsal gut progenitors at this stage.

1. Introduction

The pancreas is derived from the definitive endodermal epithelium of the embryonic foregut. It develops from two separate buds, the dorsal and the ventral buds, at embryonic day (E) 9.5, which then fuse to form the pancreas (Edlund, 2002). *Pancreatic and duodenal homeobox gene 1* (*Pdx1*) is a regional endodermal marker, the expression of which marks the

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0925-4773/$ - see front matter © 2011 Elsevier Ireland Ltd. All rights reserved.
doi:10.1016/j.mod.2011.12.004

Biochemical and Biophysical Research Communications 417 (2012) 812–816

Contents lists available at SciVerse ScienceDirect
Biochemical and Biophysical Research Communications
journal homepage: www.elsevier.com/locate/ybbrc

High calorie diet augments age-associated sleep impairment in *Drosophila*

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ARTICLE INFO

Article history:
Received 8 December 2011
Available online 16 December 2011

Keywords:
Sleep
Locomotor activity
Aging
Diet
Dopamine
Drosophila

ABSTRACT

The fruit fly, *Drosophila melanogaster* is an established model used for aging and longevity studies and more recently for sleep studies. Mammals and *Drosophila* share various physiological, pathological, pharmacological and genetic similarities in these processes. In particular, sleep is essential for survival in both species and both have age-associated sleep quality alterations. Here we report that a high calorie diet, which accelerates the aging process and reduces lifespan across species, also accelerates age-associated sleep changes in *Drosophila*. These changes are more evident in the dopamine transporter mutant, *fumin*, that displays a short sleep phenotype due to enhanced dopaminergic signaling. With normal food, *fumin* mutants sleep for only one third of the time that the control flies do, but still show equivalent longevity. However, when on a mildly high calorie diet, their sleep length shows a marked decrease and they have a reduced longevity. These data indicate that the age-associated change in sleep in *Drosophila* is a physiologically regulated aging process that is tightly linked to calorie intake and that the dopamine level plays an important role. In addition, this provides another evidence that sleep is essential for the longevity of *Drosophila*.

1. Introduction

The fruit fly, *Drosophila melanogaster* has been a good model for the study of aging and longevity for a century [1–3]. First, their lifespan is only several weeks and therefore relatively short. Second, the maturation process during which newly enclosed flies become fully fertile adults is less than 24 h and constitutes only a very limited part of their lifespan. Third, most of their adult tissues, except for reproductive and gastrointestinal systems, are postmitotic, and thus are similar to mammals. In addition, a large number of powerful genetic tools are available for *Drosophila* and the maintenance of a large genetically homogeneous population is easy. Many single alleles have been reported either to shorten or prolong their longevity including methuselah [4], superoxide dismutase [5,6], glutathione S-transferase-like gene [7], *Indy* (sodium dicarboxylate cotransporter-A) [8] and CHICO (insulin receptor substrate protein) [9]. Various conditions influencing longevity have also been reported, including temperature [10], oxidative stress [11], food compositions [12] and population density/social interactions [13,14]. Among them, lifespan extension by dietary intake reduction is most important [15], since it has also been confirmed in many other species including mammals.

Recently, *Drosophila* have also been regarded as a prospective model for studying sleep [16,17]. Their resting states are similar in many ways to mammalian sleep. *Drosophila* have both circadian and homeostatic regulation and an increased arousal threshold. Their sleep characteristics change and sleep quality deteriorates with age [18,19]. In addition, studies have indicated that sleep is required for a long lifespan in *Drosophila*. Sleep deprivation has been reported to result in lethality in cyc^{01} clock gene mutant flies [20]. In addition, a voltage-gated potassium channel mutant, *Shaker*, which decreases the amount of sleep, has reduced longevity [21,22].

We isolated a *Drosophila* mutant with a very short sleep phenotype and named it *fumin* (*fmn*, insomnia in Japanese). We identified the causative insertional mutation in the dopamine transporter (DAT) gene [23,24]. The *fmn* flies sleep for only 20–30% of the time of wild type flies. In addition, they show a learning deficiency [25] and enhanced male-male courtship [26], which is also regarded as the consequence of abnormal dopamine signaling. Unexpectedly, however, the average lifespan of *fumin* mutants was equivalent to that of the control [23].

In this study, we show that the longevity of short sleep mutant *fmn* flies was significantly reduced under high calorie diet conditions. These results indicate a novel relationship between high calorie intake and age-associated changes in sleep in *Drosophila*. We

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0006-291X/$ - see front matter © 2011 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2011.12.041

Biochemical and Biophysical Research Communications xxx (2012) xxx–xxx

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Pan-neuronal knockdown of the *c-Jun N-terminal Kinase* (*JNK*) results in a reduction in sleep and longevity in *Drosophila*

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ARTICLE INFO

Article history:
Received 8 December 2011
Available online xxxx

Keywords:
JNK
Sleep
Aging
Diet
Drosophila

ABSTRACT

Sleep is a unique behavioral state that is conserved between species, and sleep regulation is closely associated to metabolism and aging. The fruit fly, *Drosophila melanogaster* has been used to study the molecular mechanism underlying these physiological processes. Here we show that the *c-Jun N-terminal Kinase* (*JNK*) gene, known as basket (*bsk*) in *Drosophila*, functions in neurons to regulate both sleep and longevity in *Drosophila*. Pan-neuronal knockdown of *JNK* mRNA expression by RNA interference resulted in a decrease in both sleep and longevity. A heterozygous knockout of *JNK* showed similar effects, indicating the molecular specificity. The *JNK* knockdown showed a normal arousal threshold and sleep rebound, suggesting that the basic sleep mechanism was not affected. JNK is known to be involved in the insulin pathway, which regulates metabolism and longevity. A *JNK* knockdown in insulin-producing neurons in the pars intercerebralis had slight effects on sleep. However, knocking down *JNK* in the mushroom body had a significant effect on sleep. These data suggest a unique sleep regulating pathway for *JNK*.

1. Introduction

Sleep is a behavioral state characterized by an extended immobile period with a decreased sensitivity to outside stimuli and is considered to be conserved across a variety of species [1]. The discovery of sleep and sleep mutants in the fruit fly, *Drosophila melanogaster* has accelerated the study of sleep [2,3]. *Drosophila* has been used as a model organism for studying lifespan, and there is a growing evidence to suggest that the relationship between sleep and lifespan is important. Sleep deprivation of the cyc^{01} mutant resulted in a reduced lifespan [4] and short sleep mutants *Shaker*, *sleepless* and *Hyperkinetic* have a short lifespan [5–7]. Interestingly, another short sleep mutant *fumin* (*fmn*), which is a mutation of the dopamine transporter gene, showed a similar lifespan to the wild type when under conventional diet conditions [8,9]. However, *fmn* had a reduced lifespan when under high calorie diet conditions. High calorie diet also caused a reduction in sleep, and sleep time was reduced from approximately 30% to 10% of the day in 20 day-old *fmn* flies [10]. These data suggest a close relationship between metabolic signaling, lifespan and sleep regulation.

During the search for the genes responsible for the difference between control and *fmn* flies on high calorie diet, we found that many genes show differential expression in the head (data not shown). To clarify the functions, we knocked down a selection of genes using RNA interference (RNAi) lines obtained from the Vienna *Drosophila* RNAi Center (VDRC). We then examined the sleep phenotypes and identified novel sleep related genes. Here we describe a pan-neuronal knockdown of the ortholog of mammalian *c-Jun N-terminal Kinase* (*JNK*), named basket (*bsk*) in *Drosophila*, which resulted in a reduction in both sleep and longevity.

2. Materials and methods

2.1. Fly stocks, food conditions and genetic crosses

All stocks were raised at 25 °C in 60% humidity with a 12 h light/dark cycle on a conventional cornmeal, yeast, glucose agar medium (see below for the composition). All the experiments using live flies were performed at 25 °C. Two independent RNAi lines for *JNK* under the control of the upstream activating sequence (UAS) [Transformant ID: 34138 (II) and 104569 (II)] and the w^{1118} line, which is the genetic background of the RNAi lines, were from VDRC. Gal4 drivers [*elav-Gal4* (X), OK107 (IV), eas^{ala9}(X)] were from the Bloomington *Drosophila* Stock Center, Indiana University, Indiana, USA. Dilp2-Gal4 (II) was a kind gift from Dr. Linda Partridge. The *JNK* mutant, bsk^{147e}, has been described previously [11] and was a kind gift from Dr. Julian Ng [12].

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0006-291X/$ - see front matter © 2011 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2011.12.040

Please cite this article in press as: K. Takahama et al., Pan-neuronal knockdown of the *c-Jun N-terminal Kinase* (*JNK*) results in a reduction in sleep and longevity in *Drosophila*, Biochem. Biophys. Res. Commun. (2012), doi:10.1016/j.bbrc.2011.12.040