Published OnlineFirst February 23, 2011; DOI:10.1158/1078-0432.CCR-10-2008

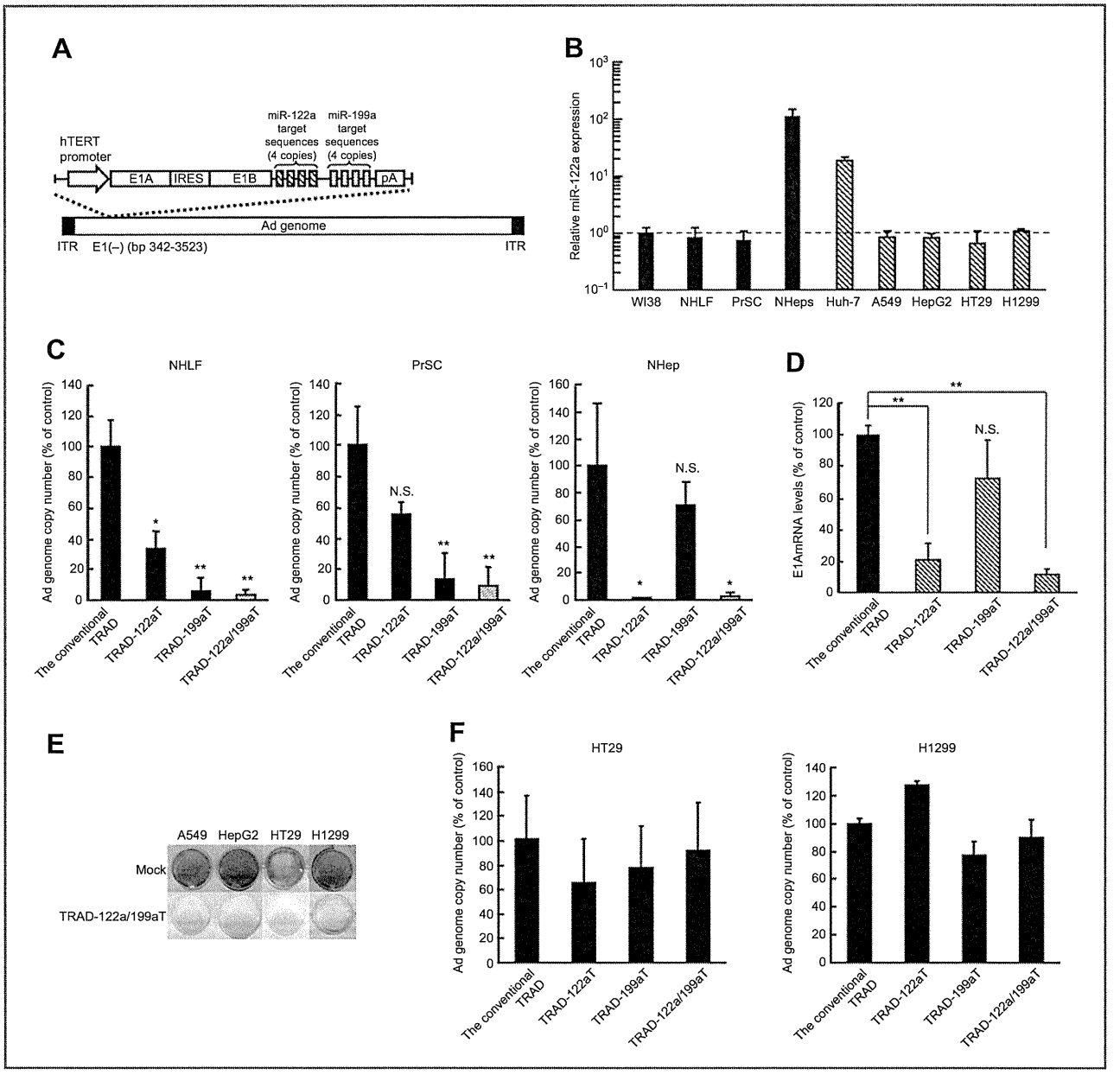

Figure 5. Tumor cell lysis activity and enhanced safety profile of TRAD-122a/199aT. A, a schematic diagram of TRAD-122a/199aT. B, miR-122a expression levels in the normal and tumor cells. C, the viral genome copy numbers of TRAD-122a/199aT in normal human cells. D, the E1A mRNA levels in NHep. E, crystal violet analysis for the cytopathic effects of TRAD-122a/199aT. The results are representative of 2 independent experiments. F, the viral genome copy numbers of TRAD-122a/199aT in tumor cells. The tumor and normal cells were infected with the TRADs at an MOI of 2 (tumor cells) or 10 (normal cells) for 2 hours. The cells were stained with crystal violet 3 days after infection. The viral genome copy numbers were determined 3 (tumor cells) or 5 days (normal cells) after infection. For determination of the E1A mRNA levels, total RNA was isolated from NHep 24 hour after infection with the TRADs at an MOI of 10, and the E1A mRNA levels were determined by real-time RT-PCR. The data was normalized by the data of the conventional TRAD group. All the data are shown as the means ± SD (n = 3–6). N.S.: not significantly different. *, $P < 0.05$; **, $P < 0.005$.

addition to the transcriptional targeting system via tumor-specific promoters.

As described above, TRAD replicates in the injected tumors and is disseminated from the injected tumors into the systemic circulation, leading to infection of distant, uninjected tumors (11, 13, 14). This property of TRAD had led to a concern that TRAD could infect normal cells over the whole body, including the hepatocytes, after dissemination from the injected tumors. It is crucial that such unexpected infection of normal cells by TRAD is prevented. Previous studies have shown that insertion of sequences complementary to liver-specific miR-122a reduced the replication of oncolytic Ads in Huh-7 cells, which are a model cell for hepatocytes (31–33). It is especially crucial

Published OnlineFirst February 23, 2011; DOI:10.1158/1078-0432.CCR-10-2008

to prevent the replication of TRAD in the liver, because Ad vectors have strong hepatotropism. However, TRAD also might infect normal cells other than hepatocytes, indicating that replication of oncolytic Ads in normal cells other than hepatocytes should also be suppressed. To prevent the replication of TRADs in other normal cells, we incorporated the sequences complementary to miR-143, -145, -199a, or let-7a, which are downregulated in the tumors and widely expressed in normal cells. The expression levels of these miRNAs in the tumor cells were lower than those in the normal cells in this study, and insertion of sequences complementary to miR-143, -145, or -199a significantly reduced the E1A mRNA levels and the replication of TRADs in the normal cells.

Overall, among the miRNA complementary sequences, the miR-199a complementary sequences appeared to be the most efficient at suppressing the replication of TRADs across all the normal cells except for hepatocytes; however, insertion of miR-199a target sequences alone failed to significantly reduce the replication of TRADs in the hepatocytes. To simultaneously prevent the replication of TRADs in various types of normal cells, including hepatocytes, we incorporated sequences complementary to miR-122a, which is abundantly expressed in hepatocytes, in addition to miR-199a target sequences. Brown and colleagues reported that a desired transgene expression pattern was achieved, depending on the miRNA expression profile, by incorporation of target sequences for 2 distinct miRNAs (34). TRAD-122aT/199aT exhibited more than 10-fold reduction in the replication in all the normal cells except for SAEC, although insertion of target sequences for miR-122a or miR-199a alone failed to suppress the replication of TRADs in either of the normal cells. Furthermore, TRAD-122aT/199aT and the parental TRAD mediated similar cytopathic efficacies in the tumor cells. These results indicate that replication of TRADs in not only hepatocytes but also other normal cells is simultaneously reduced by insertion of both miR-122a complementary sequences and sequences complementary to miRNAs highly expressed in normal cells, without altering the tumor cell lysis activity.

TRADs containing miR-122a complementary sequences are also considered to be promising for the treatment of liver cancer because miR-122a is significantly downregulated in liver cancer cells (35–37) leading to efficient replication and lytic activity of TRADs containing miR-122a complementary sequences in liver cancer cells. This study has shown that TRAD-122aT/199aT caused efficient cell lysis in a hepatocellular carcinoma cell line, HepG2 cells, while the replication of TRADs containing the miR-122a complementary sequences in normal hepatocytes, which highly express miR-122a, was significantly inhibited.

The expression levels of miRNAs are a crucial factor to suppress the gene expression by miRNAs. Brown and colleagues showed that miRNAs should be expressed at a concentration above the threshold (>100 copies/pg small RNA) to induce miRNA-regulated suppression of transgene expression (34). We were not able to precisely show the expression levels of miRNAs as the ratio of copies/pg small RNA in this study; however, comparing the miRNA levels in this study with those reported by Brown and colleagues (34), we consider that the expression levels of miR-143, -145, and -199a in the normal cells were higher than 100 copies/pg small RNA, leading to efficient suppression of the replication of TRADs.

Several studies have shown that let-7, including let-7a, is significantly downregulated in tumor cells (16, 19, 20). Edge and colleagues reported that insertion of let-7a complementary sequences into the matrix protein expression cassette of the vesicular somatitis virus (VSV) suppressed the replication of VSV in human primary fibroblast MG38 cells; on the other hand, VSV carrying let-7a target sequences efficiently replicated in A549 cells (38). However, our data showed that cancer cell lines other than HepG2 cells expressed similar or higher levels of let-7a than the normal cells. In addition, the expression levels of let-7a were more than 10-fold higher than those of the other miRNAs in the tumor cells. Abundant let-7a expression leads to a reduction in the replication of TRAD-let7aT in tumor cells. Furthermore, the members of the let-7 family, including let-7b and let-7c, have the same seed sequence, suggesting that let-7 family members other than let-7a would also contribute to the significant suppression of replication of TRAD-let7aT. These results suggest that not only expression profiles of miRNAs but also absolute amounts of miRNA expression in the cells are of great importance for miRNA-regulated gene expression.

Our data showed that the E1A mRNA levels were reduced by approximately 30% to 50% for TRAD-143T, -145T, and -199aT, compared with the conventional TRAD 24 hour after infection with the normal cells. These reduction levels in the E1A mRNA were much smaller than those in the Ad genome copy numbers at 5 days after infection; however, these reductions in the E1A mRNA levels would lead to large differences in the Ad genome copy numbers after several virus replication cycles. More than 5-fold reductions in the E1A mRNA were found for TRAD-143T, -145T, and -199aT, compared with the parental TRAD, 5 days after infection with the normal cells (data not shown).

A phase I clinical trial of the parental TRAD was conducted, and serious adverse events were not observed (3). In this study, efficient replication of the conventional TRAD in WI38 cells was found at an MOI of 10; however, the conventional TRAD did not exhibit a high level of replication at an MOI of 2. It might be unlikely that such a high titer (MOI 10) of oncolytic Ad would infect organs distal from the injection points in clinical trials; however, normal cells around the injection points might be infected with a high titer of oncolytic Ad. In addition, even though no apparent replication of TRADs is observed in normal cells after infection of TRADs, the expression of Ad proteins, including E1A and E4 proteins, affects the cellular functions via various mechanisms (39–41). This study indicates that inclusion of an miRNA-regulated *E1* gene expression system in oncolytic Ads enhances the safety of oncolytic Ads and makes it possible to increase the injection doses, leading to superior therapeutic effects.

Published OnlineFirst February 23, 2011; DOI:10.1158/1078-0432.CCR-10-2008

In summary, we developed TRADs in which the *E1* gene expression is controlled by miRNAs more highly expressed in normal cells than tumor cells. The TRADs containing the sequences complementary to miR-143, -145, or -199a exhibited reduced replication in the normal cells without altering the tumor cell lysis activity. Furthermore, incorporation of both miR-199a and miR-122a target sequences significantly suppressed the replication in all human primary cells examined, including hepatocytes. TRAD-miRT has enhanced both the safety profiles and comparable tumor cell lysis activity to the parental TRAD, suggesting that TRAD-miRT offers great potential for the treatment of tumors.

Disclosure of Potential Conflicts of Interest

Toshiyoshi Fujiwara and Hiroyuki Mizuguchi are consultants to Oncolys BioPharma, Inc. No other potential conflicts of interest were disclosed.

Acknowledgments

We thank Takako Ichinose, Koyori Yano (National Institute of Biomedical Innovation, Osaka, Japan), and Sayuri Okamoto (Graduate School of Pharmaceutical Sciences, Osaka University, Osaka, Japan) for their help.

Grant Support

Support was received from a grant-in-aid for Young Scientists (A) F. Sakurai from the Ministry of Education, Culture, Sports, Science, and Technology (MEXT) of Japan (F. Sakurai), and a grant from the Takeda Science Foundation (H. Mizuguchi).

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Received July 28, 2010; revised November 18, 2010; accepted December 14, 2010; published OnlineFirst February 23, 2011.

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—Review—

アデノウイルスベクターを用いたES, iPS細胞への高効率遺伝子導入

田代克久

Optimization of Adenovirus Vectors for Transduction in Embryonic Stem Cells and Induced Pluripotent Stem Cells

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(Received May 28, 2011)

Because embryonic stem (ES) cells and induced pluripotent stem (iPS) cells can differentiate into various types of cells *in vitro*, they are considered as a valuable model to understand the processes involved in the differentiation into functional cells as well as an unlimited source of cells for therapeutic applications. Efficient gene transduction method is one of the powerful tools for the basic researches and for differentiating ES and iPS cells into lineage-committed cells. Recently, we have developed an adenovirus (Ad) vector for efficient transduction into ES and iPS cells. We showed that Ad vectors containing the cytomegalovirus enhancer/β-actin promoter with β-actin intron (CA) promoter or the elongation factor (EF)-1α promoter were the appropriate for the transduction into ES and iPS cells. We also found that enforced expression of a *PPARγ* gene or a *Runx2* gene into mouse ES and iPS cells by an optimized Ad vector markedly augmented the differentiation of adipocytes or osteoblasts, respectively. Thus, a gene transfer technique using an Ad vector could be an advantage for the regulation of stem cell differentiation and could be applied to regenerative medicine based on ES and iPS cells.

Key words——gene transfer; adenovirus vector; embryonic stem cell; induced pluripotent stem cell; differentiation

1. はじめに

幹細胞は自己複製能と多分化能を有する細胞であり，培養条件により *in vitro* で種々の細胞に分化可能であることから，再生医療や創薬研究等への医療応用が期待されている．医療応用が期待されている幹細胞として，受精卵（胚）から樹立された胚性幹（embryonic stem: ES）細胞[1,2]や生体に存在する間葉系幹細胞,[3] そして体細胞に4あるいは3種類の遺伝子（Oct-3/4, Sox2, Klf4, (c-Myc)）を導入することにより作製された人工多能性幹（induced pluripotent stem: iPS）細胞[4,5]などが挙げられる．これらの幹細胞を医療へ応用するには，幹細胞から目的とする機能細胞を効率よく分化誘導する技術の確立が必須であるが，サイトカインや増殖因子等の液性因子のみを用いたこれまでの分化誘導法では，分化効率が十分とは言い難い．そこで，われわれは幹細胞へ分化関連遺伝子を導入することにより，機能細胞を高効率に誘導できるのではないかと考え研究を進めてきた．今回，ES細胞，iPS細胞へ高効率に遺伝子導入可能なアデノウイルス（Ad）ベクターを最適化するとともに，本Adベクターを用いて機能遺伝子を導入した際の分化効率について評価したので，その結果を紹介する.[6-8]

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本総説は，平成22年度日本薬学会近畿支部奨励賞（生物系薬学）の受賞を記念して記述したものである.

2. マウスES, iPS細胞への遺伝子導入

これまでマウスES細胞に対しては，エレクトロポレーション法やレトロウイルスベクターなどが外来遺伝子の導入法として汎用されてきた.[9-11] しかしこれらの方法では，導入遺伝子がランダムに染色体に組み込まれるだけでなく，細胞分化後も遺伝子発現が続き，細胞機能に影響を及ぼす可能性がある．医療への応用を考慮すると，一定の時期にのみ幹細胞に導入遺伝子を発現させて目的細胞へ分化させ，細胞分化完了後は導入遺伝子の発現が消失する

ことが好ましい．すなわち，ES 細胞や iPS 細胞を含む幹細胞の分化誘導には，導入遺伝子を一過性に効率よく発現させるベクターが望まれる．この点，Ad ベクターは，遺伝子発現効率に優れているだけでなく，宿主染色体への遺伝子挿入を伴わないため，効率面及び安全面において“細胞分化の方向付け”を行う目的に適していると考えられる．そこで筆者らは，まず Ad ベクターを用いてマウス ES 細胞，iPS 細胞への遺伝子導入法の確立を行った．まず，プロモーターの異なる 4 種類（RSV, CMV, CA（β アクチンプロモーターと CMV エンハンサーからなるハイブリッドプロモーター），EF-1α）の β-ガラクトシダーゼ（LacZ）発現 Ad ベクターを調製した．マウス ES 細胞，iPS 細胞へ各種 Ad ベクターを 3000 vector particles (VP)/cell の濃度で作用させて LacZ 発現を解析した結果，従来の遺伝子導入実験で汎用されている RSV プロモーターや CMV プロモーターではほとんど LacZ の発現が検出されず，CA 及び EF-1α プロモーターを有する Ad ベクターを用いることにより極めて効率よく遺伝子導入できることが明らかとなった［Fig. 1 (A)］.[7,12]

次に Ad ベクターによる遺伝子導入がマウス iPS 細胞の多分化能に影響を与えるかどうかを検討した．Ad ベクターにより外来遺伝子（mCherry）を導入したマウス iPS 細胞を免疫不全マウスに皮下注射

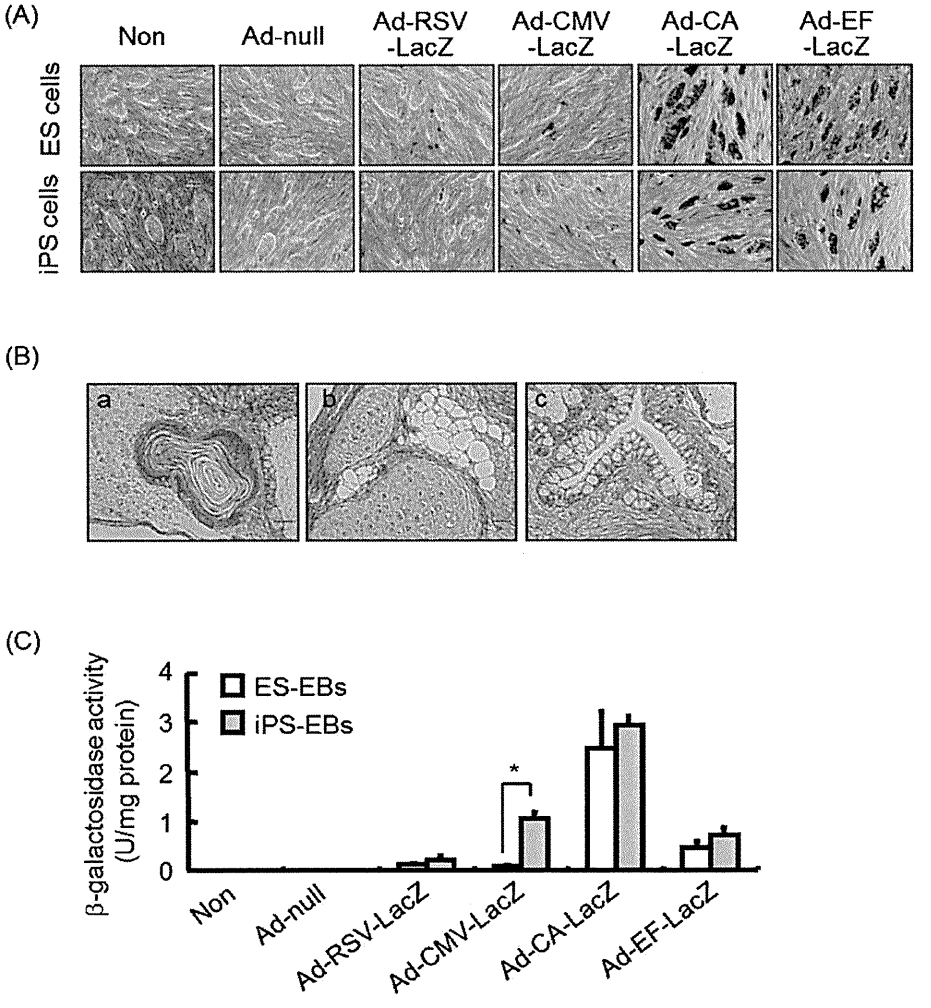

Fig. 1. Efficient Transgene Expression in Mouse ES and iPS Cells by an Ad Vector

(A) Mouse ES cells or iPS cells were transduced with LacZ-expressing Ad vector at 3000 VP/cell for 1.5 h. On the following day, LacZ expression in the cells was detected by X-gal staining. Data shown are from one representative experiment of three performed. (B) Paraffin sections of the teratomas derived from Ad-CA-mCherry-treated iPS cells were prepared, and sections were stained with hematoxylin and eosin. a, ectoderm (epidermis); b, mesoderm (cartilage and adipocyte); c, endoderm (gut epithelium). (C) ES-EBs or iPS-EBs were transduced with each Ad vector at 3000 VP/cell. After 48 h, β-galactosidase luminescence assay was carried out. Results shown were the mean of five independent experiments with indicated standard deviations.

し，形成させた奇形腫を解析した．その結果，Ad ベクターを作用させて形成した奇形腫は，皮膚（外胚葉），軟骨・脂肪（中胚葉）及び消化管様構造（内胚葉）などを含んでいたことから，多能性を保持していることが確認された［Fig. 1(B)］.[7] なお，マウス ES 細胞においても同様の結果が得られた．したがって，Ad ベクターはマウス ES, iPS 細胞の多分化能を妨げることなく，効率よく外来遺伝子を導入可能であることが示された．

マウス ES 細胞から目的の細胞へ分化させる場合，まず，胚様体（Embryoid body; EB）を形成させ，その後液性因子などを加えることにより目的細胞に分化させる手法が一般的である．そこで上述の LacZ 発現 Ad ベクターを用いて，ES-EB, iPS-EB への遺伝子導入効率を評価した．その結果，ES-EB 及び iPS-EB において CA プロモーターを有する Ad ベクターを作用させた場合に最も高い LacZ 発現が観察された［Fig. 1(C)］．以上の結果から，マウス ES, iPS 細胞，そしてこれらの細胞由来の EB への遺伝子導入には CA プロモーターを有する Ad ベクターが適していることが明らかとなった.[6,7]

3. 脂肪細胞，骨芽細胞への分化誘導

Ad ベクターを用いた遺伝子導入法が分化誘導系に応用可能か否か検討するため，機能遺伝子の導入を試みた．分化モデルとして ES, iPS 細胞から脂肪細胞への分化誘導を行うとともに，脂肪細胞分化に必須の PPARγ（peroxisome proliferator-activated receptorγ）遺伝子を ES, iPS 細胞へ導入することに

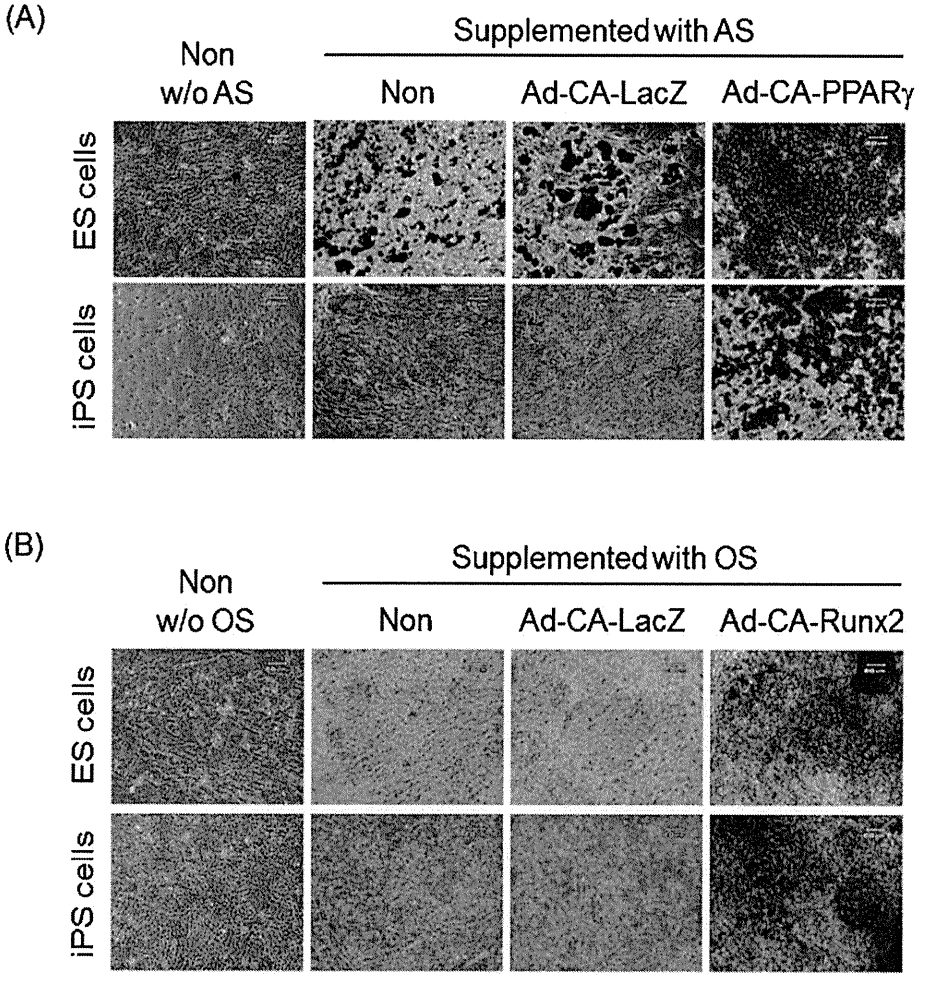

Fig. 2. Increased Adipocyte or Osteoblast Differentiation from Mouse ES and iPS Cells by Ad Vector-mediated *PPARγ* Gene or *Runx2* Gene Transduction

(A) ES-EBs or iPS-EBs were transduced with Ad-CA-LacZ or Ad-CA-PPARγ. After plating onto a gelatin-coated dish on day 7, ES-EBs and iPS-EBs were cultured for 15 d in the presence or absence of adipogenic supplements (AS). After cultivation, lipid accumulation was detected by oil red O staining. Data shown are from one representative experiment of three performed. (B) ES-EBs or iPS-EBs were transduced with Ad-CA-LacZ or Ad-CA-Runx2, and were then cultured for 15 days with or without osteogenic supplements (OS). Matrix mineralization in the cells was detected by von Kossa staining. Data shown are from one representative experiment of three performed. Abbreviation; w/o, without.

より，脂肪細胞への分化効率が向上するかどうかを検討した．PPARγ遺伝子をES, iPS細胞へ導入し，脂肪細胞分化用の液性因子（インスリン等）中で培養することにより脂肪細胞への分化誘導を行った．その結果，液性因子のみを作用させる従来の誘導法では約40%の細胞が脂肪滴を蓄積していたのに対し，PPARγ遺伝子の導入と液性因子を併用した誘導法では，90%以上の細胞が脂肪滴を蓄積していた［Fig. 2(A)］.[6,7] すなわち，マウスES/iPS細胞から脂肪細胞への分化効率はAdベクターを用いたPPARγ遺伝子の導入により飛躍的に改善できることが示された．

次に，骨芽細胞への分化誘導系においても，Adベクターによる機能遺伝子の導入した際の分化効率について検討した．Adベクターを用いてES, iPS細胞へ骨芽細胞分化のマスター遺伝子であるRunx2（Runt-related transcription factor 2）遺伝子を導入し，βグリセロリン酸等の液性因子を含む培地中で培養した．骨芽細胞への分化効率を解析した結果，Runx2遺伝子を導入したES, iPS細胞は，液性因子のみで培養した細胞及びLacZ遺伝子（コントロール）を導入した細胞と比較し，石灰化した細胞が著明に増加していること明らかとなった［Fig. 2(B)］.[7] 以上の結果から，Adベクターを用いたRunx2遺伝子の導入により，骨芽細胞へ効率よく分化誘導可能であることが示された．

4. ヒトES, iPS細胞への遺伝子導入

ヒトES, iPS細胞は，アルカリフォスファターゼやOct-3/4, Nanogの発現など，マウスES, iPS細胞と同様の特徴を有する一方で，SSEA-4, TRA-1-60などの分子の発現や増殖速度，継代方法など，マウスES, iPS細胞との性質の違いも明らかとなっている．そこで，マウスES, iPS細胞で確立した遺伝子導入法がヒトES, iPS細胞においても応用可能か否か，LacZ発現Adベクターを用いて検討した．その結果，CA及びEF-1αプロモーターを有するAdベクターを作用させたヒトES, iPS細胞においてLacZの発現がコロニー全体でみとめられた［Fig. 3(A)］.[8] なお，ヒトiPS細胞のOct-3/4及びNanogの発現はAdベクターによる遺伝子導入後も維

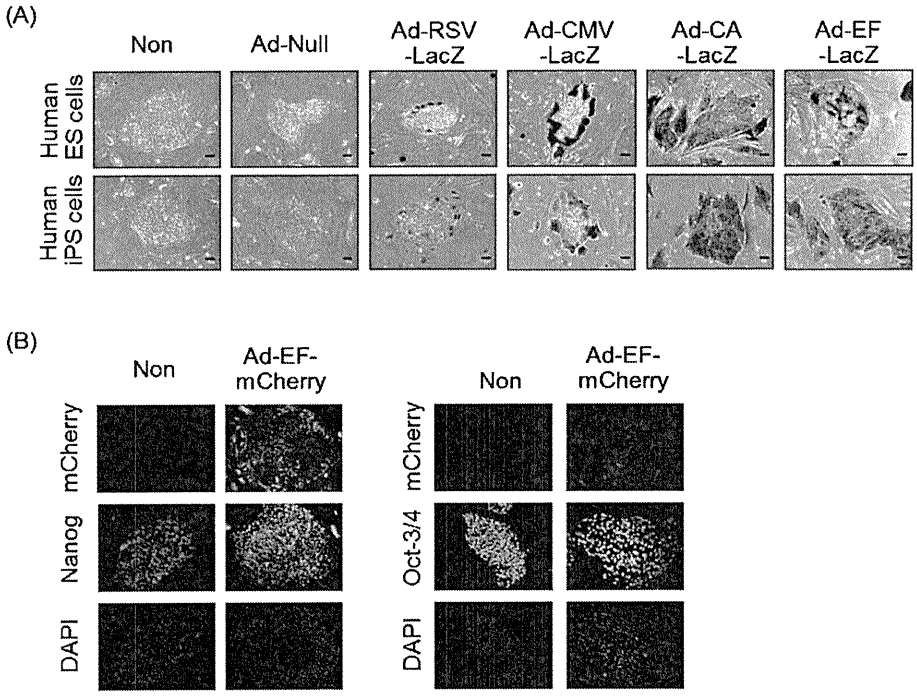

Fig. 3. Ad Vector Could Efficiently Transduce a Foreign Gene in Human ES and iPS Cells without Any Decrease in the Expression of Pluripotent Genes

(A) Human ES cells (KhES-1) and iPS cells (201B7) were passaged into culture plates in the presence of ROCK inhibitor, Y-27632. On the following day, they were transduced with LacZ-expressing Ad vectors containing various types of promoters at 3000 VP/cell for 1.5 h. Forty-eight hours later, X-gal staining was performed. Data shown are from one representative experiment of three performed. (B) Human ES cells and iPS cells were plated into culture plates using Y-27632. On the following day, they were transduced with Ad-EF-mCherry at 3000 VP/cell for 1.5 h. Two days later, the expression of Nanog (left) and Oct-3/4 (right) was detected by immunostaining.

持されていたことから，ヒトiPS細胞は未分化を維持していることが示唆された［Fig. 3(B)］.[8] したがって，マウスES, iPS細胞と同様に，ヒトES, iPS細胞への外来遺伝子の導入もプロモーターの選択が重要であること，そしてCA又はEF-1αプロモーターがヒトES, iPS細胞への遺伝子導入に適していることが明らかとなった.

5. おわりに

今回，筆者らはAdベクターを用いたマウスES, iPS細胞及びヒトES, iPS細胞への高効率遺伝子導入法の確立に成功し，さらに，Adベクターを利用して分化関連遺伝子をマウスES，iPS細胞へ導入することにより特定の細胞へ効率よく分化誘導することに成功した．なお，異なるiPS細胞株についても今回と同様の結果が得られており，最適化されたAdベクターによる遺伝子導入法は種々のES, iPS細胞株に適用可能であることが示唆されている．現在，筆者らのグループではAdベクターを用いた遺伝子導入技術を駆使してES, iPS細胞から他の細胞種への分化誘導も行っており，肝細胞様細胞[13]や血液細胞（未発表）を効率よく誘導することにも成功している．また，筆者らはAdベクターを用いて間葉系幹細胞，ヒト造血幹細胞への高効率遺伝子導入法も確立しており，これらの細胞を用いて医療応用を目指した研究を進めている.[14-16] 一過性発現を示すAdベクターを用いたES, iPS細胞を含む幹細胞への遺伝子導入技術は，幹細胞の分化誘導研究や再生医療研究において重要なツールになるものと考えられ，今後，ますますの応用が期待される.

謝辞　本稿で紹介した研究は，独立行政法人医薬基盤研究所幹細胞制御プロジェクトにて行われたものであり，多大なご協力を頂いた大阪大学大学院薬学研究科水口裕之教授（幹細胞制御プロジェクトチーフプロジェクトリーダー併任），幹細胞制御プロジェクトプロジェクトリーダー川端健二先生及び大阪大学大学院薬学研究科櫻井文教准教授を始めとする関係者の皆様に深く感謝いたします．また，マウスiPS細胞，ヒトiPS細胞をご供与頂きました京都大学山中伸弥教授，ヒトES細胞をご供与頂きました京都大学中辻憲夫教授に心より感謝いたします.

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