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精子幹細胞を用いた創薬モデルラット作成技術の開発

平成２３年度　総括研究報告書

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別紙３

厚生労働科学研究費補助金（創薬基盤推進研究事業）
（総括）研究報告書

精子幹細胞を用いた創薬モデルラットの作成技術の開発

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研究要旨

本研究ではES細胞でなく、精子幹細胞に基づく新しいノックアウト技術による創薬モデルラット作成技術の開発を目的としている。精子幹細胞は精子形成の源となる細胞であり、一生を通じて精巣内で自己複製増殖により維持され、かつ分化して多数の精子を産生する。我々の研究グループは２００３年にマウス精子幹細胞の長期培養系を確立し、さらに２００６年に培養細胞（Germline Stem; GS細胞と命名）に遺伝子導入した後、精巣内移植により精子へと分化させることで、ノックアウトマウスの作成に成功した。本研究ではこの技術をラットに展開し、ラットの精子幹細胞を遺伝子トラップとジーンターゲッティングにより遺伝子改変し、ホモ変異個体を作成する技術の確立を目指す。

平成２２年度までの研究ではラットGS細胞が長期培養後も精子幹細胞としての機能的活性を維持していることを明らかにした。また血清成分がラットGS細胞の維持に悪影響を及ぼすことから無血清培地を開発し、マウスGS細胞において６ヶ月以上の長期培養に成功した。平成２３年度ではラットGS細胞の無血清培地による培養を検討し、無血清培地でも血清添加培地と同様に増殖する条件を見いだした。しかし増殖が亢進する条件は得られなかった。

平成２２年度までに遺伝子トラップベクターを導入したGS細胞においてどのような遺伝子が破壊されているかを5’-RACE法により解析した。結果、St8sia1, Hnrpc, Itgα6の遺伝子欠損精子幹細胞が得られていることが判明した。このうち、Itgα6欠損クローンをヌードマウス精巣に移植した後、顕微授精を行って産仔を作成した。PCR genotypingによりヘテロラットを同定し、ヘテロ同士の交配によりノックアウトラットの作製を試みた。生存産仔が得られなかったため妊娠中期で胎児の形態を解析したところ、発生が途中で停止し胎生致死となっていることが確認された

平成２２年度までの研究ではラット由来のノックアウトベクター（Lesch-Nyhan病の原因遺伝子であるHprt、癌抑制遺伝子p53、接着因子occludinの破壊ベクター）の相同組み換えクローンを同定した。これらのクローンをヌードマウスおよびラットのレシピエント精巣に移植を行ったところ、コロニー形成およびドナー細胞由来の減数分裂像が認められた。これにより得られた円形精子細胞を卵子に直接注入し産仔作製を試みた。卵子に円形精子細胞を直接注入して受精卵を作製し、偽妊娠ラット子宮に移植した。しかしながら産仔を得ることができなかった。そこで精子幹細胞クローン#502, #2054の核型解析を行ったところ、正常な核型（42 chromosomes）を有する細胞はそれぞれ12.5,% 7.5%であった。このため培養中に染色体異常が蓄積した結果産仔形成能が下がっていると考えられ、変異を起こしにくい培養法の開発や移植による産仔作成法の改善などが課題として明らかになった。

A．研究目的

遺伝子ノックアウト動物の作成は現在マウスしか一般的方法が確立していない。それは他種のEmbryonic Stem(ES)細胞から子孫を作成できないためである。しかし創薬や神経疾患、糖尿病などで必要とされる生理学的解析に、マウスでは限界がある。小型実験動物のラットは、ヒトに近い薬剤反応を示す他、マウスより手術操作が容易で、高次な神経機能を持つという利点がある。そのため次世代の疾患モデルとして期待でき、遺伝子ノックアウト技術開発の必要性が唱えられてきた。

本研究ではES細胞でなく、精子幹細胞に基づく新しいノックアウト技術による創薬モデルラット作成技術の開発を目的としている。研究代表者らはこれまで精子幹細胞を中心とした生殖工学技術の開発を行い、2003年にマウス精子幹細胞の長期培養系（Germline stem, GS細胞）を世界に先駆けて確立した（Kanatsu-Shinohara M., et al., Biol. Reprod. 69,612-6,2003）。これを用いて相同組換えにより遺伝子ノックアウトマウス作成にも成功した（Kanatsu-Shinohara M., et.al., Proc. Natl. Acad. Sci. USA 103, 8018-23,2006）。本研究全体では、遺伝子トラップ法の改善および相同組換えにより遺伝子ノックアウトラットの作成を試みる。平成23年度ではマウスGS細胞で開発したSerum & Feeder-free培養をラットGS細胞に応用し、増殖が亢進されるか検討した。遺伝子トラップについては、トラップにより破壊された遺伝子の同定とホモ個体の作成を試み、表現型を解析した。相同組換えでは平成２２年度に得られた組換えクローンから産仔の作成を試みた。

B．研究方法

(1) 新規無血清培地の開発によるラットGS細胞のSerum&Feeder-free culture

これまでの研究で、ラットGS細胞はマウスGS細胞と異なり、１％以上の高濃度の血清で維持できない事が分かっている。また、フィーダーとの共培養ではラミニンによるフィーダーフリー培養と比べ増殖速度が著しく低下することから、フィーダー細胞や血清がＧＳ細胞の維持に悪影響を及ぼす可能性が高い。血清成分には未知の物質が含まれており、用いる血清の種類によりGS細胞の増殖・維持に及ぼす作用も一定でないことから、GS細胞をフィーダー細胞や血清を用いずに培養する方法の確立が必要である。これまでGS細胞のSerum&Feeder-free培養はできなかったが、平成22年度の研究で新規無血清培地を開発し、マウスGS細胞のSerum&Feeder-free条件による長期培養に成功した。平成23年度ではこのSerum&Feeder-free条件による培養をラットGS細胞に応用することを試みた。

(2) 遺伝子トラップ遺伝子改変ラットの作成

平成２２年度までにMolonyウイルス由来のプロモータートラップベクター（ROSA-b-geo）をラットGS細胞に導入し、G418を用いた薬剤選択にてクローンを樹立した。それらのGS細胞をヌードマウスの精巣に移植を行った後、顕微授精にて産仔の作成を試み、産まれた産仔の発育と胎盤重量の測定・インプリンティング遺伝子のメチル化への影響などを調べた。平成23年度では得られたトラップクローンで欠損した遺伝子を5'-RACE法により同定した。さらに得られた遺伝子改変ヘテロラットをホモ化し、表現型を解析した。

(3) 遺伝子ターゲッティングによるノックアウトラットの作成

平成22年度までにOccludin遺伝子およびHprt遺伝子（Lesch-Nyhan病の原因遺伝子でX染色体上にある）のノックアウトベクターを作成し、エレクトロポレーションにてSDラットから樹立したGS細胞に導入した。neomycin耐性能を獲得したクローンをG418添加により選択し、最終的に安定的に遺伝子導入されたクローンを樹立した。さらにこれらのクローンをPCR法とサザンブロッティングにより調べ、相同組み換え体のスクリーニングを行った。平成23年度ではそれらのGS細胞をヌードマウスの精巣に移植を行った後、顕微授精にて産仔の作成を試みた。また相同組換えクローンの染色体異常の有無を調べるため、細胞をコルセミドで処理、カルノア液で固定した後Hoechst33452で染色して染色体数を数えた。

（倫理面への配慮）

実験動物の取り扱いについては、京都大学大学院医学研究科の実験動物規定に従い、その内容につき審査で適当とされたものにのみ取り組んだ。また実験方法・飼育方法を含め、米国の国立衛生研究所(NIH)が詳細なガイドラインを提示しているが、本研究はこのガイドラインに準拠して行った。

本研究にはヒト組織を利用した研究は含まれていない。

C．研究結果

(1) 新規無血清培地の開発によるラットGS細胞のSerum&Feeder-free culture

我々が２００３年に報告した初めのマウスGS細胞培養系は１％の血清とGDNF,bFGF, EGF を含む培地で MEF をフィーダー細胞として用いていた。その後 laminin を使ったフィーダーフリー培養を報告しているが、血清の存在下でのみフィーダーフリーが可能だが、無血清培地ではフィーダーを用いなければ長期維持ができなかった。本研究で、無血清条件下において GS 細胞への接着性を亢進する因子をスクリーニングした結果、血清成分の約４５％を占める Fetuin という糖タンパク質に効果があることが分かった。Fetuin を加えた培地にさらに脂質を添加することによりGS 細胞の増殖が亢進することが分かり、Serum&Feeder Free 培養を確立した。このSerum&Feeder Free 培養による GS 細胞では精原細胞マーカーの integrin や EPCAM、CD9 などの発現が血清のある場合と同様に保たれていた。核型やゲノムインプリンティングも、正常なパターンを維持しており、移植により精巣でコロニーを形成し、完全な精子への分化が認められた。産仔および子孫の作成にも成功していた。平成 23 年ではこの培地をラット GS 細胞に応用することを試みた。通常の血清添加培地と無血清培地、無血清培地にFetuin および脂質を添加した培地、無血清培地に Fetuin、脂質、さらに FGF9 を添加した培地を用いて、laminin 上のフィーダーフリー培養で増殖を比較した（図１）。その結果、無血清培地に Fetuin および脂質を添加した培地で細胞がよく増殖していることが分かった。さらに FGF9 を添加した培地では血清添加培地と同程度の増殖がみられた。このためラットGS細胞も Serum&Feeder Free 培養が可能であることが示された。しかしながら血清添加培地と比較して増殖が亢進しなかったため、遺伝子改変に用いることは断念した。

(2) 遺伝子トラップ遺伝子改変ラットの作成

これまでに BN ラットとクローズドコロニーの SD ラットなど、多数の系統から樹立した GS 細胞を用いて、Molony ウイルス由来のプロモータートラップベクター（ROSA-b-geo）を導入した。導入に先立ち、エレクトロポレーションにより neomycin と puromycin を発現するベクターを SD ラット GS 細胞に導入し、薬剤選択効率を比較した。薬剤耐性遺伝子は neomycin にくらべ puromycin の方が 3 倍程度クローン化効率が良いことが分かった。全体で 44 個のクローンを樹立し、一部についてはサザンブロッティングにて integration の頻度を調べ、ほぼ 1～2 個程度の integration であることを確認した。それらのクローンでどのような遺伝子が破壊されているかを 5’-RACE 法により解析した。結果、St8sia1, Hnrpc, Itg α 6 の遺伝子破壊精子幹細胞が得られていることが判明した。

遺伝子破壊ラットの作製のため、Itg α 6 遺伝子破壊精子幹細胞をヌードマウスの精細管内に移植、3 ヶ月経過の後顕微授精により産仔を得た。PCR genotyping によりヘテロラットを同定し、ヘテロ同士の交配によりノックアウトラットの作製を試みた。生存産仔が得られなかったため妊娠中期で胎児の形態を解析したところ、発生が途中で停止し胎生致死となっていることが確認された（図２）。

(3) 遺伝子ターゲッティングによるノックアウトラットの作成

これまでに EGFP を発現するトランスジェニックラット TgN (act-EGFP)Osb4 から樹立した GS 細胞に、エレクトロポレーションにて Occludin 遺伝子のノックアウトベクターを導入し、G418 を用いて neomycin 耐性能を獲得したクローンを選択し、安定的に遺伝子導入された 146 個のクローンを樹立した。これらのクローンから PCR・サザンブロットにて相同組換え体をスクリーニングし、二個の相同組み換え体を同定した。これらの GS 細胞をヌードマウスの精細管内に移植し、3-5 ヶ月後精巣を摘出し UV 照射にて観察を行ったところ、EGFP を発現するドナー細胞由来のコロニー形成が認められた。さらに synaptonemal complex protein 3 (SYCP3) 抗原に対する抗体にて免疫組織染色を行ったところ、synaptonemal complex の形成が認められることから、減数分裂の進行が確認された。これにより得られた円形精子細胞（図３）を卵子に直接注入し産仔作製を試みた。合計 1586 個の卵子に円形精子細胞を直接注入し、1207 個の受精卵を作製、内 1081 個を偽妊娠ラット子宮に移植したが、産仔を得ることができなかった（表１）。そこで精

子幹細胞クローン#502, #2054の核型解析を行ったところ、正常な核型（42 chromosomes）を有する細胞はそれぞれ12.5,% 7.5%であった（図４）。遺伝子トラップによる遺伝子破壊精子幹細胞の中で産仔を得られたクローン#886では20%が正常核型であった。

D．考察

ラットＧＳ細胞はマウスＧＳ細胞にくらべ増殖が遅く、培養条件や薬剤選択・クローン化の効率が極めて低かった。そのためまずこれらの条件を詳細に検討した。これまでラットではsingle integration cloneの薬剤選択が困難であったが、平成21年度の研究では血清濃度や低酸素下での培養、ラット系統の検討、薬剤選択法の改善などにより、安定的に樹立することができるようになった。しかしながら増殖速度はマウスに比べると遅く、クローンの樹立までに４～５ヶ月かかることから、更なる改善が必要であった。平成２２年度にマウスGS細胞にて新規に開発した無血清培地にて長期の幹細胞活性の維持が可能であることが分かった。これをもとに検討した結果、FetuinとLipid ConcentrateとFGF-9を添加した無血清培地がラットGS細胞の増殖を維持可能であることが分かった。しかし増殖の亢進は見られなかった。ラットGS細胞は依然マウスに比べると増殖速度が劣り、クローン樹立効率が著しく低いことから、さらなる改善が必要である。その原因の一つとして、ラットGS細胞のライン間のばらつきの大きさが考えられる事や、ラットの系統が影響する可能性が示唆される。

本研究では遺伝子トラップ法と相同組み換え法の２種類の方法での遺伝子破壊を試みた。まず遺伝子トラップ法についてトラップクローンが得られ、それをマウス精巣へ移植して円形精子細胞を顕微授精することにより、遺伝子改変ラット個体を作製することができた。さらに、トラップクローンについて欠損している遺伝子を同定することに成功した。この遺伝子改変ラットの表現型解析と発育エピジェネティックな解析を行った結果、Itgα6遺伝子破壊ラットの解析を行い、ホモの場合耐性致死であることを突き止めた。相同組み換え法については、特定の遺伝子をねらったターゲティング法によりOcln遺伝子を欠損したラットGS細胞を作成し、ラット精巣に移植、産仔作製に使用可能な円形精子細胞形成させることに成功した。この顕微授精により産仔の作成を試みたが、十分と考えられる数の顕微授精を行っても産仔は得られなかった。相同組換えクローンでは高頻度に染色体数の異常を示す異数体が見られた。このことにより相同組換えクローンを作成する際の培養中の染色体不安定性が原因で、得られたクローンの産仔形成能が下がっていると考えられる。

E．結論

平成23年度では培養方法の改善として、マウスGS細胞で開発したSerum & Feeder-free培養のラットGS細胞への応用を目指した。条件検討の結果、ラット GS 細胞も Serum & Feeder-free 条件で維持できることが明らかになった。しかしながら血清添加培地と比べて増殖の有意に亢進することはなかった。そのため遺伝子トラップや相同組換えなどへの応用は検討しなかった。

トラップクローンから得られた遺伝子改変ラット個体の解析を行った。まずトラップにより破壊された遺伝子を同定した。さらに遺伝子欠損GS細胞からヘテロ個体を作成、この交配によりホモ欠損ラットを作成して表現型を解析することに成功した。

相同組み換え法により遺伝子改変 GS 細胞が得られ、精巣への移植により円形精子細胞を得ることができた。この顕微授精により産仔の作成を試みたが、十分と考えられる数の顕微授精を行っても産仔は得られなかった。そこで相同組換えクローンの染色体数を解析すると、高頻度に染色体数の異常を示す異数体が見られた。このことにより相同組換えクローンを作成する際の培養中に染色体異常が蓄積し、得られたクローンの産仔形成能が下がっていることが示唆される。このため染色体不安定性を低く保つ培養条件の検討や効率の良い産仔形成方法の開発など、今後改善すべき課題が明らかになった。

F．健康危険情報

得られた成果の中で健康危険情報に該当するものはなかった。

G．研究発表

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2. 学会発表

なし

H．知的財産権の出願・登録状況

1. 特許取得

なし。

2. 実用新案登録

なし。

別紙４

研究成果の刊行に関する一覧表

雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
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Kanatsu-Shinohara M, Takashima S, Ishii K, Shinohara T.	Dynamic changes in EPCAM expression during spermatogonial stem cell differentiation in the mouse testis.	***PLoS One.***	6(8)	e23663	2011
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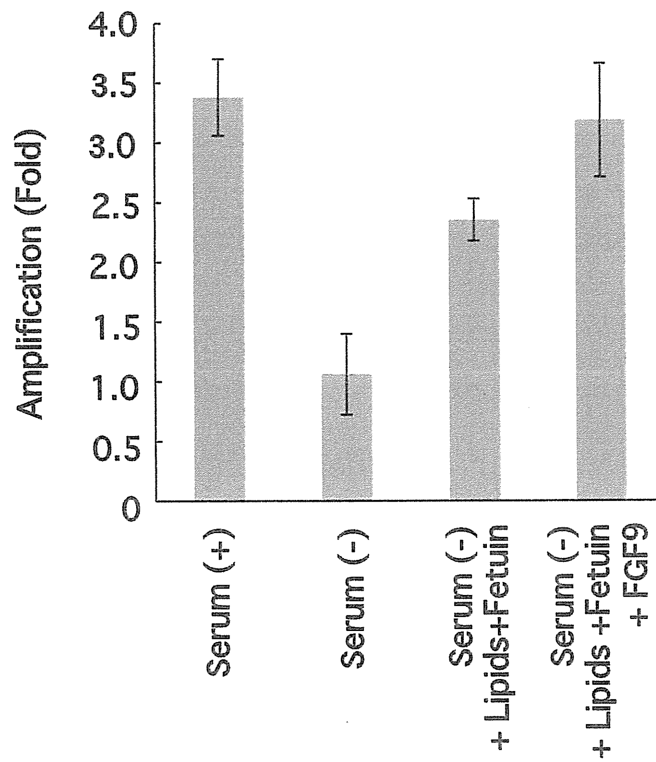

図1： ラットGS細胞のSerum & Feeder Free培養
ラットGS細胞はラミニン上において、Lipids, Fetuin, FGF9を加えたSerum Free培地でSerum添加培地と同様に増殖する。

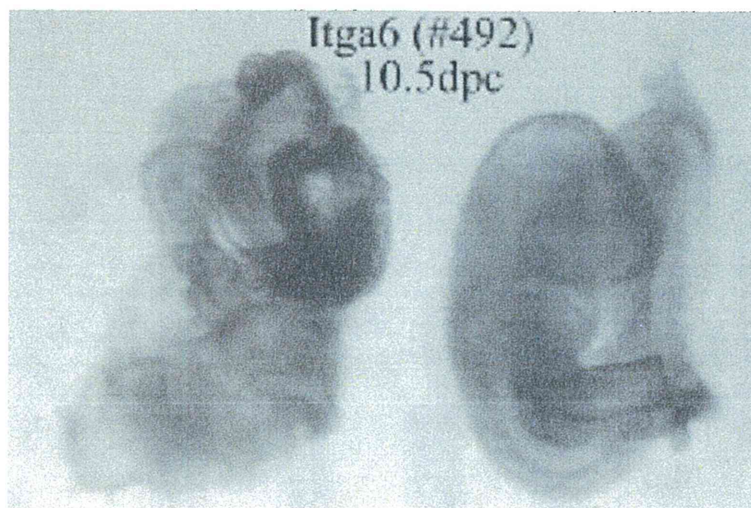

図2：遺伝子トラップ法によるItga6 KO Rat
右の野生型胎仔は正常に発生しているが、左のItga6 ホモ個体は胎性致死となっている。

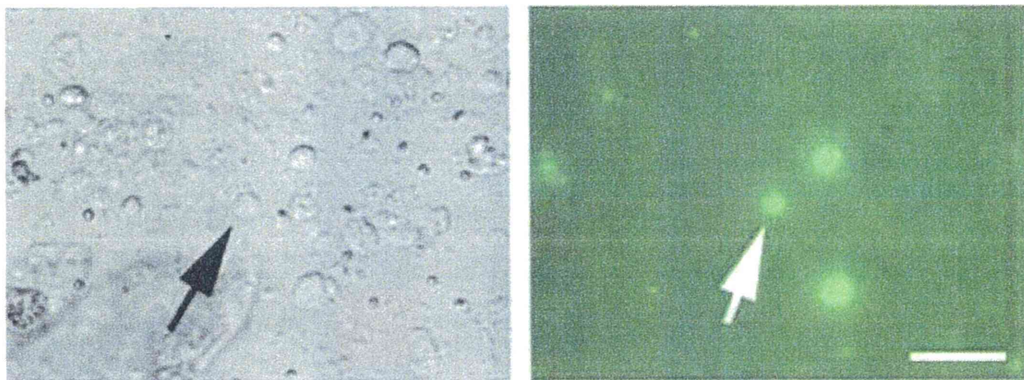

図3： 相同組換えクローン由来の円形精子細胞
相同組換えを起こしたラットGS細胞クローン#2054をマウス精巣に移植して生じた円形精子細胞。GFPによる緑色蛍光がドナー由来の細胞。Bar=50μm

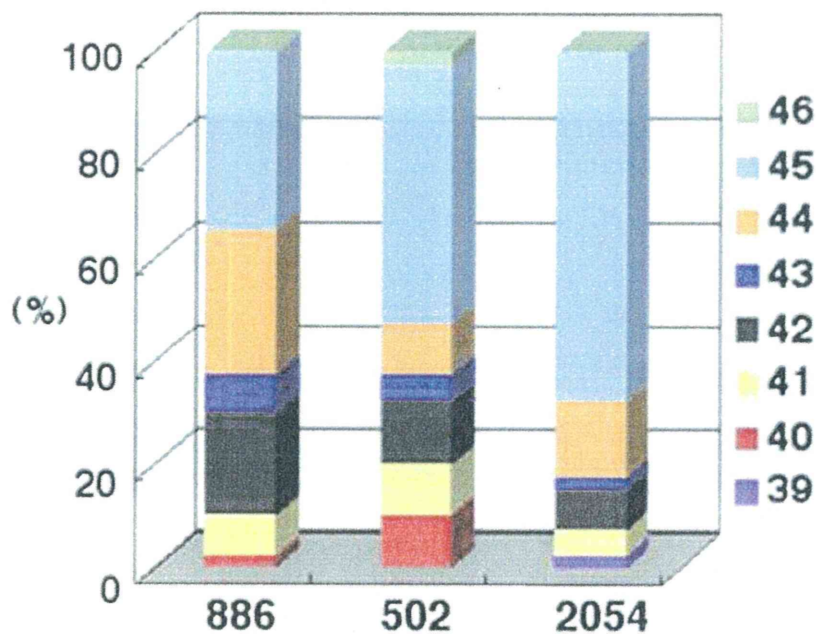

図4： 遺伝子トラップおよび相同組換えクローンの染色体数
遺伝子トラップクローン#886および相同組換えを起こしたラットGS細胞クローン#502, 2054の染色体数を調べた。ラット細胞の正常な染色体数は42本である。#886では20%、#502, #2054では12.5%, 7.5%の細胞が正常な染色体数であった。

表１：Occludin 遺伝子破壊ラット精子幹細胞由来円形精子細胞の顕微授精結果

Clone	Host oocyte strain	No. of oocytes (%)			No. (%)	
		Injected	Survived	Transferred	Implantations	Pups born
502	SD	900	688 (76)	572	22 (4)	0 (0)
2054	SD	276	211 (76)	210	6 (3)	0 (0)
2054	Wistar	221	170 (77)	162	9 (6)	0 (0)
2054	Wistar Hannover	171	138 (81)	137	6 (4)	0 (0)
Total		1568	1207 (77)	1081	43 (4)	0 (0)

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Homologous Recombination in Rat Germline Stem Cells[1]

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ABSTRACT

Spermatogonial stem cells (SSCs) are the only stem cells in the body with germline potential, which makes them an attractive target for germline modification. We previously showed the feasibility of homologous recombination in mouse SSCs and produced knockout (KO) mice by exploiting germline stem (GS) cells, i.e., cultured spermatogonia with SSC activity. In this study, we report the successful homologous recombination in rat GS cells, which can be readily established by their ability to form germ cell colonies on culture plates whose surfaces are hydrophilic and neutrally charged and thus limit somatic cell binding. We established a drug selection protocol for GS cells under hypoxic conditions. The frequency of the homologous recombination of the *Ocln* gene was 4.2% (2 out of 48 clones). However, these GS cell lines failed to produce offspring following xenogeneic transplantation into mouse testes and microinsemination, suggesting that long-term culture and drug selection have a negative effect on GS cells. Nevertheless, our results demonstrate the feasibility of gene targeting in rat GS cells and pave the way toward the generation of KO rats.

developmental biology, gametogenesis, sertoli cells, spermatogenesis, testis

INTRODUCTION

Techniques that produce knockout (KO) animals have been established by using embryonic stem (ES) cells. The application of ES cell-based technology to a wide range of animals, however, is limited due to a lack of germline-competent ES cells in many animal species [1]. As laboratory mice do not serve as adequate models, particularly for physiology and complex disease and pharmacological studies, attempts have been made to extend KO technologies to a wider range of animal species [2]. For example, recent studies showed the advantage of using three inhibitors for glycogen synthase kinase 3 beta, mitogen-activated protein kinase kinase, and fibroblast growth factor (FGF) receptor in the derivation of germline-competent rat ES cells [3–5]. Spermatogonial stem cells (SSCs) provide the foundation of spermatogenesis throughout the life cycle of male animals. While there are only a few of these cells in the testes (0.02%–0.03% of total germ cells) [6, 7], they produce a vast amount of sperm through self-renewal and differentiation. In 1994, the spermatogonial transplantation technique was developed in which SSCs recolonize the niche and proliferate to differentiate into sperm when injected into the seminiferous tubule of testes [8]. Offspring can be produced from donor-derived SSCs [9]. Although it has been shown that SSCs rarely acquire pluripotency [10], SSCs are normally unipotent and are specialized for sperm production. However, because SSCs can proliferate indefinitely by self-renewal and can transmit genetic information to the next generation via sperm, the spermatogonial transplantation technique opened up a new avenue for germline modification.

In 2003, we established a long-term culture of mouse SSCs that were designated germline stem (GS) cells [11]. In the presence of the glial cell line-derived neurotrophic factor (GDNF), a self-renewal factor of SSCs [12], germ cell colonies of unique shape were derived from dissociated testes cells in vitro. Mouse GS cells continued to proliferate without losing their normal karyotype and genomic imprinting patterns. Moreover, GS cells possess a very stable germline potential and produce normal, fertile offspring even after 2 yr of culture [13]. In contrast, ES cells often lose germline potential during long-term culture and accumulate changes in karyotype and DNA methylation patterns [14, 15]. GS cells can be cultured under serum- and feeder-free conditions and can be genetically manipulated [16, 17]. We produced transgenic and KO mice through genetic transduction and drug selection [18]. The frequency of homologous recombination was 1.7% in mouse GS cells, which was comparable to that achieved in ES cells. Thus, SSCs have the potential to serve as an alternative to ES cells in germline modification.

The rat has been used as an animal model for medical research for over 150 yr and has advantages over mice in several areas, particularly for physiological and pharmacological studies [2]. Methods to manipulate rat SSCs have now

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been developed, and recent studies have shown that xenogeneic transplantation of rat SSCs in immunodeficient nude mice not only allowed spermatogenesis but also resulted in rat offspring production following microinsemination [19]. Transgenic rats were also produced by transducing SSCs in vitro and transplanting them into mouse or rat testes [20, 21]. Furthermore, long-term culture of rat SSCs has been established and used for generating KO rats by transposon mutagenesis [22–24]. However, unlike SSCs in vivo, rat GS cells proliferate more slowly than those of mice, and the overall efficiencies of the in vitro culture and transplantation technique are still limited. In this study, we report a simple method for deriving rat GS cells and also demonstrate homologous recombination in rat GS cells.

MATERIALS AND METHODS

Animals and GS Cell Culture

GS cells were established using two transgenic rat lines, *TgN(act-EGFP)Osb4*, Sprague-Dawley (SD) background (a gift from Dr. M. Okabe, Osaka University, Japan) or *Tg(CAG-Venus)* rats (Wistar background). The spermatogonia, spermatocytes, and round spermatids of these transgenic rats express the enhanced green fluorescent protein (EGFP) or green fluorescent protein Venus, respectively. In some experiments, *TgN(act-EGFP)Osb4* rats were mated with wild-type (wt) rats of Wistar, Donryu, Brown Norway (BN), or Lewis background to derive F1 offspring (Japan SLC, Hamamatsu, Shizuoka, Japan).

To derive GS cells, testis cells were dissociated by using a two-step enzymatic digestion using collagenase type IV and trypsin (both from Sigma, St. Louis, MO), as previously described [11]. Dissociated cells were plated on a low-cell-binding dish (Nalge Nunc International KK, Tokyo, Japan) at a density of 10^6 cells/9.6 cm^2. After being incubated for 7 to 10 days, the floating cells were removed by aspiration, and the plates were washed with fresh medium to recover adherent cells. The cells were then transferred to plates coated with laminin (20 μg/ml; BD Biosciences, Bedford, MA) at a concentration of 3×10^3 to 1×10^4 cells/cm^2. After testicular somatic cells were eliminated, the cells were maintained on laminin or mouse embryonic fibroblasts (MEFs) and were passaged every 5–7 days by incubation with Accutase (Sigma) for 10 min.

The culture medium consisted of StemPro-34 SFM (Invitrogen, Carlsbad, CA), 25 μg/ml insulin (Nacalai Tesque Inc., Kyoto, Japan), 100 μg/ml transferrin, 60 μM putrescine, 30 nM sodium selenite, 6 mg/ml D-(+)-glucose, 30 μg/ml pyruvic acid, 1 μl/ml DL-lactic acid, 2 mM L-glutamine, 5×10^{-5} M 2-mercaptoethanol, 10^{-4} M ascorbic acid, 10 μg/ml D-biotin, 30 ng/ml β-estradiol, 60 ng/ml progesterone, 3 μg/ml heparin (all from Sigma), MEM vitamin solution, MEM nonessential amino acids solution (both from Invitrogen), 20 ng/ml mouse epidermal growth factor (BD Biosciences), 10 ng/ml human FGF2, 10 ng/ml mouse FGF9, 15 ng/ml recombinant rat GDNF (all from Peprotech Inc., Rocky Hill, NJ), and 10^3 units/ml ESGRO (murine leukemia inhibitory factor; Millipore, Billerica, MA). After cells were transferred from the low-cell-binding plate, the medium was supplemented with 0.06% fetal bovine serum (Hyclone Laboratories, Inc., Logan, UT) and vitamin A-deficient B27 (Invitrogen). At approximately 1 mo after the culture was initiated, ESGRO was removed, and the serum concentration was increased to 0.2%. For culturing on MEFs, bovine serum albumin (MP Biomedicals Inc., Irvine, CA) was added at a concentration of 5 mg/ml, and the concentration levels of FGF2, FGF9, and GDNF were increased to 50, 30, and 45 ng/ml, respectively.

Flow Cytometry

The primary antibodies used were biotin-conjugated anti-rat/mouse CD90.1 (HIS51; eBioscience, San Diego, CA), purified mouse anti-rat CD9 (RPM.7), biotin-conjugated hamster anti-rat CD29 (Ha2/5; both from BD Biosciences), and mouse anti-rat GFRA1 (81401; R&D Systems, Minneapolis, MN). Allophycocyanin-conjugated goat anti-mouse immunoglobulin G (BD Biosciences), and allophycocyanin-conjugated streptavidin (eBioscience) were used to detect the primary antibodies. Stained cells were analyzed by using a FACSCalibur unit (BD Biosciences).

Transfection of GS Cells

GS cells, which were cultured for 2–5 mo (5–20 passages) after the initiation of culture, were used for transfection. For retroviral transfection, GS cells were infected with ROSAbetageo retrovirus (a gift from Dr. P. Soriano, Fred Hutchinson Cancer Center) [25]. Virus particles were produced by transient transfection of Plat-E cells with ROSAbetageo and pCMV-vesicular stomatitis virus glycoprotein plasmids as described previously [18, 26]. The titer of the virus supernatant was determined by using a Retro-X quantitative RT-PCR (qRT-PCR) titration kit (Clontech, Mountain View, CA). The infection of GS cells with the retrovirus was performed as described previously [27]. In brief, 1×10^5 GS cells were suspended in 0.8 ml of virus supernatant (0.8 ml in a 12-well plate) in the presence of 10 μg/ml polybrene (Sigma) and plated on MEFs. The plate was then centrifuged at 3000 × *g* for 1 h at 32°C. The plate was incubated overnight, and the cells were passaged on the next day after infection. The final titer of the retrovirus concentrate was adjusted to 2.9×10^6 colony-forming units (cfu) per milliliter. In experiments carried out under normoxic conditions, GS cells were transduced at 9.1×10^7 cfu/ml.

Plasmid DNAs were electroporated into GS cells using cell line Nucleofector kit T (Lonza, Münster, Germany) according to the manufacturer's instructions. Briefly, 4×10^6 GS cells were suspended in 100 μl of Nucleofector solution T mixed with 5 μg of DNA and subjected to electroporation using program A-23. For homologous recombination, a gene targeting vector was constructed with the use of pNT1.1 containing the *Neo* and thymidine kinase genes [28]. A 2.8-kb *Not*I-*Xho*I fragment and a 6.5-kb *Kpn*I-*Xba*I fragment of the *Ocln* gene were inserted as short and long arms, respectively. In some experiments, the vector was linearized by *Not*I digestion.

After electroporation, cells were split into 3 wells of a 12-well culture plate that had been plated with G418-resistant MEFs. Genetic selection was carried out with 40 μg/ml G418 (Invitrogen) [17]. After incubation with G418 for approximately 2–3 wk, the contents of each well were passaged at a 1:1 ratio in a well of a fresh 12-well culture plate. As the growth of GS cells was density dependent [17], 1×10^5 nontransfected wt GS cells were added two to three times to each well at passage. It generally took 3–4 mo to establish G418-resistant clones. A negative selection marker (*tk*) was not used in these experiments. To enhance the efficiency of genetic selection, the cells were cultured in 5% O_2 in some experiments. Colonies resistant to G418 were screened by PCR, using the specific primers listed in Supplemental Table S1 (available online at www.biolreprod.org). The selected cells were cryopreserved in a cryopreservation solution (Cellbanker; Dia-Iatron, Tokyo, Japan), using previously described procedures [29].

Southern Blot Analyses

Positive clones were confirmed by Southern blot analysis using *Eco*RV digestion and hybridization with a 344-bp probe derived from intron 1, which was produced by PCR amplification using the specific primers listed in Supplemental Table S1.

Transplantation Procedure

GS cells were microinjected into the seminiferous tubules of KSN nude mice (Japan SLC, Inc., Shizuoka, Japan) that were treated with 44 mg/kg busulfan at 4 wk of age. Busulfan-treated mice received transplants of syngeneic bone marrow cells 2–4 days after treatment to avoid bone marrow suppression. These animals were used for germ cell transplantation at least 4 wk after busulfan treatment. A single-cell suspension containing approximately 4×10^4 to 8×10^4 cells (for counting colonies) or 10^6 cells (for microinsemination) was introduced into the seminiferous tubules via the efferent duct. Each injection filled 75%–85% of the total tubules. The Institutional Animal Care and Use Committee of Kyoto University approved all of the animal experimentation protocols.

Analysis of the Recipient Testes

To quantify germ cell colonies, we recovered recipient testes 3 mo after transplantation and exposed them to ultraviolet (UV) light. The donor cell-derived fluorescence was observed under a stereomicroscope. The donor cell clusters were defined as colonies when they occupied the entire basal surface of the tubule and were at least 0.1 mm in length.

For histological analysis of the testes, testis samples were collected and fixed in 2% paraformaldehyde. The sample was then embedded in Tissue-Tek OCT compound (Sakura Finetechnical, Tokyo, Japan) for cryosectioning. Meiosis was detected by immunofluorescence, using rabbit antisynaptonemal complex protein 3 (SYCP3) antibodies, which were prepared by using a synthetic oligopeptide (a gift from Dr. S. Chuma, Kyoto University, Japan) [30]. Alexa 568-conjugated goat anti-rabbit immunoglobulin was used as a secondary antibody (Invitrogen). Sections were counterstained with Hoechst 33342 dye (Sigma). Images of the sections were obtained using a confocal microscope (Fluoview FV1000D; Olympus, Tokyo, Japan).

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Combined Bisulfite Restriction Analysis (COBRA)

Genomic DNA was treated with sodium bisulfite, which deaminates unmethylated cytosines to uracils but does not affect 5-methylated cytosines. With this template, differentially methylated regions (DMRs) of the indicated genes were amplified by PCR with the specific primers listed in Supplemental Table S1. The PCR products were digested with the indicated restriction enzymes, which recognize sequences containing cytosine-phosphate-guanine in the original unconverted DNA. The intensity of the digested bands was quantified using Image Gauge software (Fuji Photo Film, Tokyo, Japan).

Microinsemination

Spermatogenic cells were collected from the recipient testes by repeated pipetting of the seminiferous tubule segments that showed EGFP or Venus fluorescence. Microinsemination was performed as described previously, using round spermatids [19]. The constructed embryos were cultured for 24 h and transferred into the oviducts of pseudopregnant Wistar rats. Offspring were recovered by cesarean section at 21 days after transfer.

Karyotype Analysis

Cultured cells were harvested, treated with 75 mM KCl for 15 min, and fixed with a methanol-acetic acid (3:1) solution. Metaphase spreads were prepared using standard procedures, and the slides were stained with Hoechst 33258 dye (Sigma).

Statistical Analysis

The results are presented as means ± SEM. Data were analyzed using a Student *t*-test for independent samples with equal variance.

RESULTS

Derivation of Rat GS Cells by Selection with a Low-Cell-Binding Plate

To establish rat GS cells, testis cells from 12- to 18-day-old *TgN(act-EGFP)Osb4* rats were dissociated with enzymatic digestion. Given that the culture medium for mouse GS cells induces overgrowth of somatic cells and inhibits germ cell growth, we modified the culture medium by reducing the serum concentration and supplementing it with FGF9, which has beneficial effects on primordial germ cells and spermatogonia [31, 32]. We also employed a low-cell-binding plate to reduce the attachment of somatic cells. Under these conditions, spermatogonia preferentially attached to the plate, whereas most somatic cells floated in the medium (Fig. 1A). After 6–8 days of culture, spermatogonia clumps were dissociated with gentle pipetting and transferred to a laminin-coated culture plate. The cells then started to form chain-type colonies (Fig. 1B) and could be easily distinguished from somatic cells, which showed a larger, fibroblast-like appearance. In contrast, germ cells plated after gelatin selection could not produce colonies due to somatic cell overgrowth (Fig. 1C). Whereas somatic cells stopped proliferating during repeated passages, germ cells continued to proliferate on laminin-coated plates. Approximately 1 mo after culture was initiated, these colonies consisted entirely of germ cells (Fig. 1D, left). Flow cytometric analysis confirmed that the cultured cells expressed several SSC markers, including ITGB1, CD9, and THY1 (formerly CD90) (Fig. 1E). The cells also expressed GFRA1, but its expression level was variable, indicating that they are a heterogeneous population. Established GS cells proliferated constantly for a long term, and the total cell number increased 2.6×10^{55}-fold over 822 days (between 100 and 922 days) (Fig. 1F).

Stem cell activities of cultured cells were assessed by spermatogonial transplantation. Because rat SSCs reinitiate spermatogenesis and produce offspring following transplantation into xenogeneic mouse testes [19], we used immunodeficient nude mice as recipients. Two independent cultures were maintained and injected into the seminiferous tubules of busulfan-treated nude mice. Three months after transplantation, the testes were examined for the presence of EGFP-expressing germ cell colonies under UV fluorescence. While culture 1 showed 2.2×10^{55}-fold increase in the number of SSC cells over 822 days of culture (between 100 and 922 days), SSCs in culture 2 showed 6.1×10^{11}-fold expansion after 222 days (between 116 and 338 days; Table 1). Colonization efficiencies did not change significantly during the experimental period. The cells maintained in vitro for 116 or 443 days produced offspring resulting from microinsemination using round spermatids in the recipient testes.

Genetic Selection of Rat GS Cells

Next, we examined conditions for genetic selection. GS cells established from *TgN(act-EGFP)Osb4* or *Tg(CAG-Venus)* rats were transduced with the retrovirus vector ROSAbetageo, which expresses beta-galactosidase and becomes G418-resistant under the promoter of trapped genes [25]. Transduced cells were cultured on laminin, and G418 was added to the culture at 10 days after infection. Although LacZ staining confirmed the transduction of GS cells, we could not establish GS cell transfectants with G418 selection under those culture conditions.

Then we selected G418-resistant cells using the MEF feeder. Although GS cells form flat chain- or monolayer-type colonies on laminin, they form three-dimensional clump-type colonies on the MEF feeder (Fig. 1D, right). However, proliferation of rat GS cells was less efficient on MEFs than on laminin (Fig. 1G). Under the MEF-based culture, we obtained a few G418-resistant colonies. Given that the

TABLE 1. SSC expansion in long-term culture.

Culture	Days to transplant[a] (passage)	Colonies/10^5 GS cells (no. of recipient testis)[b]	Increase in total cell no. (fold)[c]	Increase in SSC no. (fold)[d]
1	100 (8)	251.4 ± 13.1 (4)	–	–
	172 (16)	70.6 ± 15.7 (4)	1.7×10^3	466.2
	351 (37)	493.3 ± 149.6 (6)	3.4×10^{13}	6.7×10^{13}
	443 (48)[e]	310.0 ± 63.4 (6)	5.2×10^{18}	6.4×10^{18}
	922 (114)	210.0 ± 58.6 (3)	2.6×10^{55}	2.2×10^{55}
2	116 (10)[e]	137.3 ± 20.3 (4)	–	–
	338 (35)	332.5 ± 33.8 (8)	2.5×10^{11}	6.1×10^{11}

[a] The number of days from initiation of culture to transplantation.
[b] Values are means ± SEM.
[c,d] The increase in the total cell or SSC number from the initial transplantation indicated.
[e] These cultures produced offspring by microinsemination.

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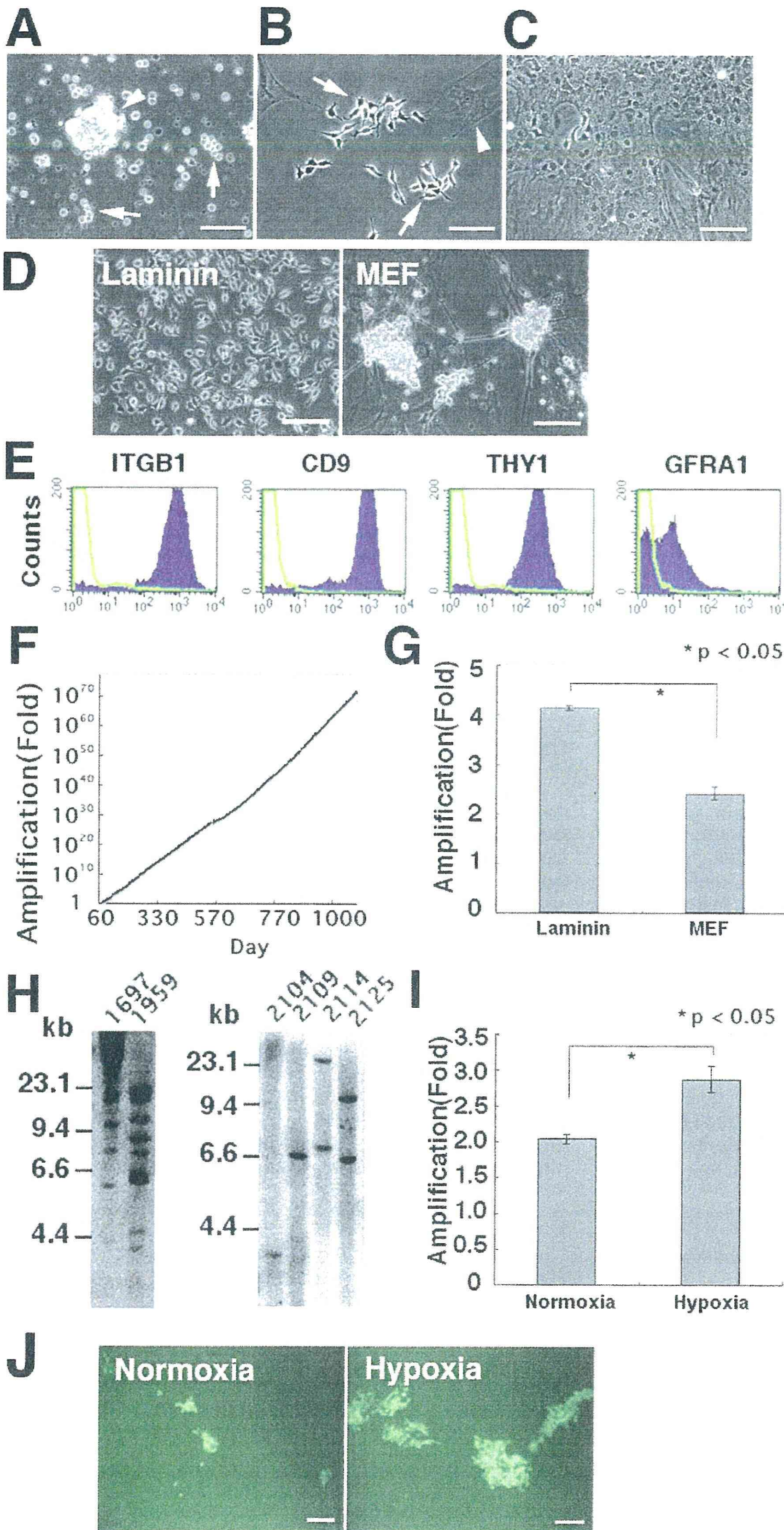

FIG. 1. Long-term culture and drug selection of rat GS cells. **A**) Germ cell colonies that developed on a low-cell-binding plate 7 days after culture initiation are shown. While germ cells were loosely attached to the plate and proliferated (arrows), somatic cells aggregated to form floating clumps (arrowhead). **B**) Proliferation of germ cells is shown 4 days after transfer to a laminin-coated plate. While few somatic cells (arrowhead) remained in culture, germ cells were relatively enriched (arrows). **C**) Rat testis cells cultured on tissue culture dish are shown 6 days after gelatin selection. Germ cell proliferation was suppressed by the overgrowth of testicular somatic cells. **D**) Established rat GS cells cultured on a laminin-coated plate (left) and MEFs (right) are shown. **E**) Flow cytometry of GS cells was performed. Green lines indicate control staining. **F**) Growth curve of GS cells on laminin is shown. **G**) Proliferation of GS cells grown on laminin and MEFs are shown during 5 days of culture (n = 4). The difference was significant. **H**) Southern blot analyses of G418-resistant GS cell clones are shown. A full-length *Neo* fragment was used as a probe. Genomic DNA was digested with *Eco*RI, which does not cut the retrovirus vector. Clones with multiple integrations were observed when the cells were selected under normoxic conditions (left), whereas selection under hypoxic conditions allowed clones to establish a single/low number of integrations (right). **I**) Enhanced proliferation of rat GS cells on hypoxic MEFs (n = 3) is shown. GS cells, which had been cultured for 3 mo from the time of culture initiation, were plated at 3×10^5 cells/9.4 cm^2. Increase in GS cell numbers after 5 days of culture is shown. **J**) Large colony formation after 11 days of G418 selection on hypoxic MEFs (right) is shown. Identical GFP-expressing *Neo*-resistant GS cells (8000 cells), which had been cultured for 7 mo, were mixed with 4×10^4 cells/9.4 cm^2 on MEFs and incubated in the presence of G418 under normoxic and hypoxic conditions, respectively. Significantly smaller colonies were formed under normoxic conditions (left). Bar = 100 µm (**A**–**D**) and 50 µm (**J**).

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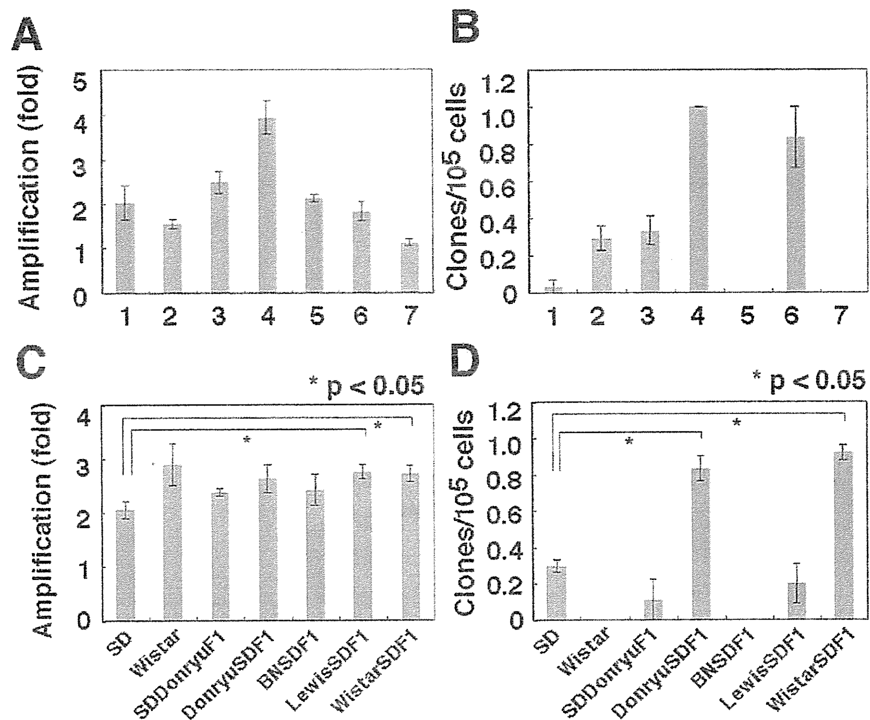

FIG. 2. Variations in the efficiencies of drug selection among GS cell clones are shown. **A**, **B**) Efficiencies of proliferation (**A**) and clone establishment (**B**) are shown using seven GS cell lines (1–7) in an SD background. To evaluate proliferation, GS cells were harvested at 2.8×10^5 cells/9.6 cm^2 on a laminin-coated dish, and the number of cells after 6 days of culture was counted (n = 4). To evaluate drug selection efficiency, GS cells were infected with ROSAbetageo virus, and the number of clones established by infecting 1×10^5 cells/3.8 cm^2 GS cells after selection with G418 in at least six experiments is shown. **C**, **D**) Efficiencies of proliferation (**C**) and clone establishment (**D**) are shown using GS cell lines of variable genetic backgrounds. To evaluate proliferation, GS cells were harvested at 2.8×10^5 cells/9.6 cm^2 on a laminin-coated dish, and the number of cells after 6 days of culture were counted. Three independent GS cell lines of each strain were examined (n = 10). Asterisks indicate strains that are significantly different from SD ($P < 0.05$). To determine drug selection efficiency, the number of clones established by infecting 1×10^5 cells/3.8 cm^2 GS cells after selection with G418 in at least nine experiments is shown. At least two lines were used in each experiment.

efficiency of G418-resistant colony development was significantly lower, we then used a virus supernatant, which was concentrated by centrifugation, and established 45 clones from $\sim 1.6 \times 10^7$ cells in 13 experiments. However, Southern blot analysis using a *Neo* probe showed multiple transgene integrations in all clones (Fig. 1H, left). This result suggested that only clones expressing the G418-resistant gene at high levels were selected by this method.

Because limited proliferation of GS cells on MEFs reduced drug selection efficiency, we tried to improve the GS cell culture conditions by reducing oxygen levels [22]. The proliferation of rat GS cells was 1.4-fold enhanced within 5 days of culturing on hypoxic MEFs (Fig. 1I). Hypoxia also improved drug selection efficiency. While the G418-resistant cells formed colonies of moderate size after 11 days of selection under normoxia (Fig. 1J, left), we observed significantly larger colonies under hypoxia during the same period (Fig. 1J, right). In contrast, hypoxia did not influence viability of GS cells; viability levels of wt GS cells after 11 days of selection, as assessed by trypan blue exclusion, were 23.7% ± 0.9% (n = 6) and 28.6% ± 3.2% (n = 6) for normoxia and hypoxia, respectively, and the difference was not significant. Under this condition, 50 clones with ~1–2 integrations were reproducibly established from $\sim 4.2 \times 10^7$ GS cells in 70 experiments by transducing 30 independent GS cell lines (Fig. 1H, right).

We then examined the efficiencies of growth and drug selection by using several GS cell lines (Fig. 2, A and B). Although seven GS clones in an SD background appeared morphologically indistinguishable, no clones were established from the two lines. The growth rate of seven GS cell lines also varied, but the growth rate did not correlate necessarily with the genetic selection efficiency. To examine whether the selection efficiency was influenced by the genetic background of GS cells, we established GS cell lines from various strains and compared the efficiencies of growth and genetic selection (Fig. 2, C and D). Although these cells proliferated at a comparable speed, we observed significant variations in selection efficiencies. While GS cells of SD background produced ~0.3 clones/10^5 cells, no clones were established from GS cells from a Wistar background. GS cells from both DonryuSDF1 and WistarSDF1 hybrids exhibited significantly higher efficiency,

TABLE 2. Offspring production from ROSAbetageo-infected GS cells by microinsemination.

Source of round spermatid	Host oocyte strain	No. of oocytes (%) Injected	Survived	Transferred	No. (%) Implantations	Pups born
ROSAbetageo-infected GS cells						
886 (SD)	SD	1482	1184 (80)	1089	75 (7)	18 (2)
1122 (Wistar)	SD	154	115 (75)	100	61 (61)	33 (33)
1176 (Wistar)	SD	587	401 (68)	334	154 (46)	64 (19)
Total		2223	1700 (76)	1523	290 (19)	115 (8)
Control						
Nonmanipulated GS cells (SD)	SD	144	94 (65)	74	20 (27)	4 (5)
Fresh round spermatid (SD)	SD	150	130 (87)	129	49 (38)	21 (16)
Fresh round spermatid (Wistar)	SD	306	220 (72)	205	83 (40)	34 (17)

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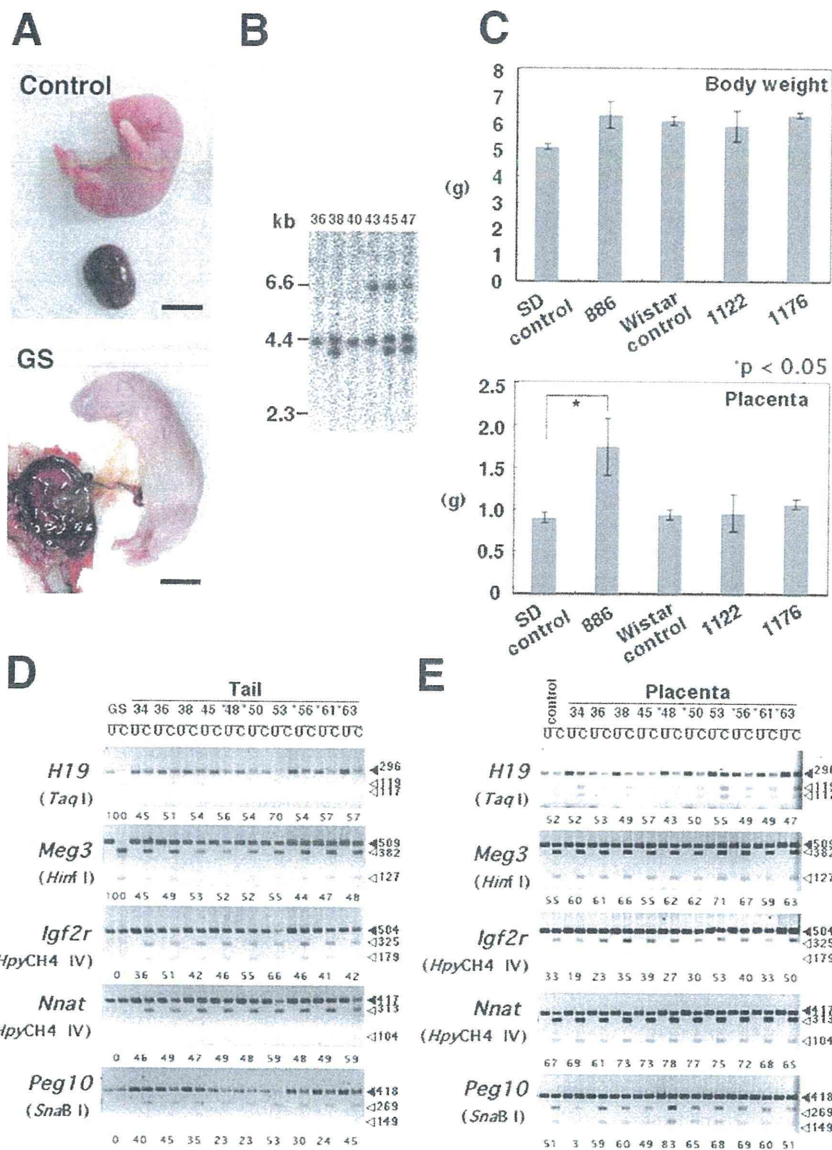

FIG. 3. Offspring produced from GS cell clones are shown. **A**) Offspring derived from the wt (SD) (top) or GS cell (clone 886)-derived round spermatids (bottom) by microinsemination are shown. Note the enlarged placenta of the offspring derived from GS cells. **B**) Southern blot analysis of offspring DNAs derived from GS cell (clone 886)-derived round spermatids is shown. Genomic DNAs from six offspring were digested with *Eco*RI, which does not cut the retrovirus vector. A full-length *Neo* fragment was used as a probe. **C**) Weights of offspring (top) and placenta (bottom) are shown. Offspring from GS cells (886) had significantly enlarged placentas (n = 12 for clone 886 and clone 1122; n = 21 for the control SD round spermatid; n = 33 for the control Wistar round spermatid, and n = 34 for clone 1176). **D**, **E**) COBRA of the tail (**D**) and placenta (**E**) DNA in 10 different offspring. Asterisks indicate placentas that weighed >2.0 g. Open and closed arrowheads indicate the size of methylated and unmethylated DNA, respectively. Placenta derived from microinsemination using wt sperm of SD rat was used as a control. The enzymes used to cleave each locus are indicated in parentheses. U, uncut; C, cut. Bar = 1 cm (**A**).

but those from other F1 hybrids (SDDonryuF1, BNSDF1, and LewisSDF1) showed limited efficiency. These results suggest that growth speed is not correlated with the genetic selection efficiency.

Offspring Production from Genetically Selected Rat GS Cells

To investigate whether genetically manipulated GS cells maintain the potential to produce normal offspring after drug selection, we injected several clones into seminiferous tubules of busulfan-treated nude mice. At 3–6 mo after transplantation, EGFP-expressing colonies were dissected and used for microinsemination. Round spermatids from clones 886, 1122, and 1176 were recovered from the recipient testes and microinjected into SD oocytes. In total, 2223 oocytes were injected, and 115 offspring were recovered by cesarean section (Fig. 3A; Table 2). Southern blot analysis with *Neo* probe confirmed germline transmission of transgene (Fig. 3B).

Although the offspring produced from the GS cells appeared normal, we noted large placentas for several of them. While clones 1122 and 1176 exhibited a normal range of placenta weight (0.95 ± 0.22 g, n = 12 for clone 1122; 1.02 ± 0.06 g, n = 34 for clone 1176), the average weight of the placentas derived from clone 886 was 1.74 ± 0.33 g (n = 12), which was significantly heavier than the control, using fresh wt round spermatids (0.93 ± 0.06 g, n = 33 for Wistar; 0.90 ± 0.06 g, n = 21 for SD) (Fig. 3, A and C; and see Supplemental Table S2). We also examined the DNA methylation patterns in DMRs of several imprinted genes. Genomic DNAs from GS cells, tails, and placentas of the offspring derived from clone 886 were analyzed by using COBRA. Methylation at the loci of the paternally imprinted *H19* and *Meg3* genes and maternally imprinted *Igf2r*, *Nnat* (formerly *Peg5*), and *Peg10* genes was analyzed. GS cells showed typical androgenetic imprinting patterns, with hypermethylation in the *H19* and *Meg3* DMRs and hypomethylation in the *Igf2r*, *Nnat*, and *Peg10* genes, whereas DMR methylation in the tail DNA showed somatic

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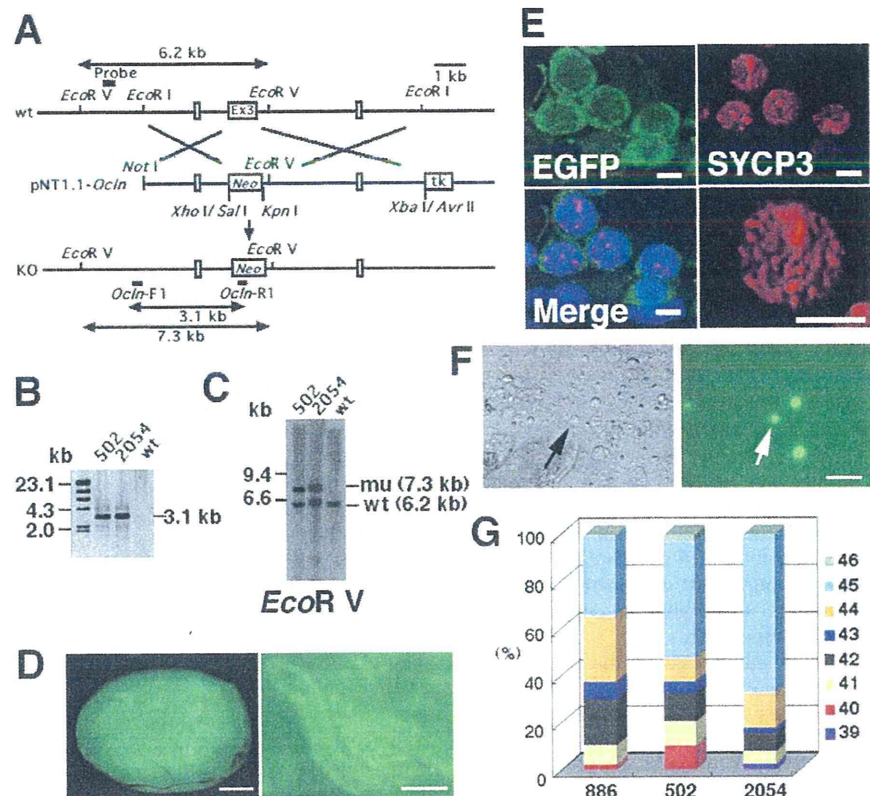

FIG. 4. Homologous recombination in GS cells is shown. **A**) Targeting vector of the *Ocln* gene. Exon 3 (Ex3) was replaced with the *Neo* gene. PCR primers and a probe for Southern blotting are shown. **B**, **C**) PCR (**B**) and Southern blot (**C**) analyses of two clones demonstrating homologous recombination are shown. Genomic DNAs of GS cell clones were digested with *Eco*RV. **D**) Colonization of a GS cell clone (2054) in seminiferous tubules of nude mice 3 mo after transplantation. Low (left) and high (right) magnification. **E**) Histological appearance of a recipient mouse testis stained with anti-SYCP3 antibodies. The donor-derived germ cells (green) displayed the formation of synaptonemal complexes with SYCP3 fluorescence (red), indicating meiosis of the donor rat cells in the mouse testis. The section was counterstained with Hoechst 33342 dye (blue). **F**) A round spermatid derived from donor SSCs is shown. Green fluorescence indicates the donor cell origin. **G**) Karyotype analysis is shown. Chromosome numbers of clone 886, 502, and 2054 are shown. mu, mutant. Bar = 1 mm (**D**, left), 200 μm (**D**, right), 5 μm (**E**), and 50 μm (**F**).

cell imprinting patterns. Although we noted hypermethylation of the *Peg10* gene in the placenta in one of the offspring, we did not find apparent abnormalities in other offspring with large placentas (Fig. 3, D and E). The large-placenta phenotype did not persist in the germline because two of the clone 886-derived male offspring produced normal F2 healthy offspring by microinsemination, and the sizes of their placentas were normal.

Homologous Recombination Using Rat GS Cells

Finally, to examine whether gene targeting technology is applicable to rat GS cells, we constructed a gene targeting vector for *Ocln*. GS cells established from *TgN(act-EGFP)Osb4* rats were electroporated with the targeting vector (Fig. 4A) and treated with G418 on a MEF feeder at 7–10 days after electroporation. Immediately after electroporation, 51.4% ± 3.6% (n = 3) of the input cells were recovered, and 86.9% ± 1.8% (n = 3) of them were viable. In total, by transducing 20 independent GS cell lines, 33 clones were established from $\sim 6.4 \times 10^8$ GS cells with circular DNAs in 159 experiments; whereas 15 clones were established from $\sim 5.8 \times 10^8$ GS cells with linearized DNAs in 144 experiments. Genomic DNAs were isolated from cultured cells and screened for clones of homologous recombination by PCR. Homologous recombination occurred in two clones transduced with circular DNAs (Fig. 4B), which was confirmed by Southern blotting (Fig. 4C). These two clones were established from two independent GS cell lines. To produce offspring, these cells were transplanted into busulfan-treated nude mice. Three months after transplantation, EGFP-expressing donor-derived colonies were detected in the recipient testes under UV light (Fig. 4D). Immunohistological examination showed that EGFP-expressing donor-derived germ cells underwent meiosis in the mouse testes, as indicated by the formation of synaptonemal complexes, which were detected by SYCP3 expression (Fig. 4E).

We collected EGFP-expressing round spermatids and performed microinsemination (Fig. 4F). A total of 1568 eggs were constructed and transferred into the uteri of pseudopregnant mothers. Although 43 embryos were implanted in the uteri, no offspring were produced from these animals (Table 3). To investigate whether genetically selected cells have normal

TABLE 3. Offspring production from gene-targeted GS cells by microinsemination.

Clone	Host oocyte strain	No. of oocytes (%)			No. (%)	
		Injected	Survived	Transferred	Implantations	Pups born
502	SD	900	688 (76)	572	22 (4)	0 (0)
2054	SD	276	211 (76)	210	6 (3)	0 (0)
2054	Wistar	221	170 (77)	162	9 (6)	0 (0)
2054	Wistar Hannover	171	138 (81)	137	6 (4)	0 (0)
Total		1568	1207 (77)	1081	43 (4)	0 (0)

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