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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

平成２１年度　総括研究報告書

研究代表者　篠原　美都

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目　　次

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

研究代表者　篠原　美都　京都大学・医学研究科・助教

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。ラットの精子幹細胞に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21年度の研究では精子幹細胞の培養の基本的なプロトコールの改善と、クローンの樹立を中心に行った。ラット精子幹細胞培養条件の改善については、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、安定的に遺伝子導入クローンを樹立することができるようになった。またこれまで無血清・フィーダーフリー培養は成功していなかったが、マウス精子幹細胞細胞にて成功した。またこれらの条件検討を行いながらトラップクローンの樹立とターゲッティングベクターの作成・遺伝子導入を行った。これによりターゲッティングについてはHprt遺伝子につき44個、 Occludin遺伝子にて146個のクローンを樹立できた。

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替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21年度はGS細胞への遺伝子導入・薬剤選択法の改善と、それによるクローン樹立を目指した。

B．研究方法

GS細胞における遺伝子導入とクローンの樹立は、マウスと比べてラットでは薬剤選択の効率が依然低く、特にウイルスベクターではクローン樹立の効率が良いがプラスミドを用いた場合には低いこと、その他にも精巣内移植の効率に改善の余地があるなど、実用的なプロトコールの確立のために多くの改善点があった。特にランダムな遺伝子挿入でなく遺伝子相同組み換えによるターゲッティングのためには効率の改善が必須である。平成21年度の研究では培養条件の改善を行うとともに、改善したプロトコールによって遺伝子トラップ・ターゲッティングクローンの樹立を目指し、以下の点について検討した。

(1) 培養条件の改善：

生後10-15日齢のラット精巣細胞をGlial cell-line derived neurotrophic factor (GDNF)やbasic Fibroblast growth factor (bFGF)を含む培養液にて培養することによりＧＳ細胞を樹立した。マウスの場合に用いられている培養液の組成ではラットGS細胞は難しいので、血清成分や、各種添加物、サイトカインの濃度などを改変することでラットGS細胞の増殖に適した

組成を検討した。

GS 細胞の c DNA を定法により採取し、マイクロアレイデータを参考に精子幹細胞の増殖を促進する分子の候補を選定した。これらの候補となるサイトカインを培養中に添加して新規増殖因子をスクリーニングした。GS 細胞に培養中添加し、約 6 日~8 日後に細胞の増殖率から効果を判定した。

また、培養液の改変により無血清・フィーダーフリー培養を試みた。マウス GS 細胞を通常の MEF(mouse embryonic fibroblast)でなくラミニン上にて培養し、血清成分を代替できる因子をスクリーニングした。確立した培養条件にて継代培養を行い、4 ヶ月の増殖維持を試みた。また培養細胞を精巣内移植し、幹細胞活性を調べた。精巣への移植は内因性の精子形成の欠損している W マウスをレシピエントとして用いた。約 3 ヶ月後に精巣におけるコロニー形成を GS 細胞に発現する EGFP の発光により検知した。フローサイトメトリーを用いて EpCAM, Integrin,など精原細胞マーカーの発現を調べた他、c-Kit, SSEA-1 などの発現を調べた。同時に cDNA を作成し、RT-PCR 法により精原細胞マーカーの発現を調べた。

(2) ラット系統や年齢による差異の検討：

様々な系統のラットにおいて、生後 10-18 日齢の精巣から GS 細胞の樹立を試み、その樹立・増殖効率を比較した。SD の他、Wistar, Lewis, Long-Evans, Donryu などの系統や、これらの系統間の F1 種を作成し、それぞれ GS 細胞を樹立して、樹立効率や増殖速度・クローン樹立効率を比較した。

(3) 薬剤耐性遺伝子：

neomycin と puromycin の耐性遺伝子を pgk プロモーターの下流に挿入したベクターを作成し、Amaxa 社の Nucleofector Kit T を用いて SD ラット GS 細胞にエレクトロポレーションにより導入した。導入後、約 5 日後から neomycin と puromycin を添加した。細胞の密度が低下すると GS 細胞の増殖が低下するため、継代の際適宜野生型の細胞を混ぜ、数回薬剤選択を繰り返した。最終的にクローンの樹立頻度から薬剤選択効率を比較した。

(4) ラット精子幹細胞の精巣内移植技術の改善：

ラットの精巣内移植は、レシピエントの Busulfan による前処理の効率が悪い。平成 21 年度の研究ではラットとヌードマウスをホストとして移植の効率を検討した。生後および妊娠中の SD ラットに Busulfan を腹腔内注射にて投与し、約 10 日-12 日後に精巣内に EGFP を発現する GS 細胞を移植した。約 3 ヶ月後に精巣を摘出し、UV 照射によりコロニー形成率を調べた。また一部のレシピエントについて野生型 SD ラット雌と自然交配を行った。

一方、マウスに関しては従来用いてきた Balb/nu で効率が不安定なため、他の系統（KSN/nu）を用い、比較検討した。

(5) 遺伝子トラップ遺伝子改変ラットの作成

Molony ウイルス由来のプロモータートラップベクター（ROSA-β-geo）を PlatE 細胞に Lipofection（Roche; Fugene6）にて導入し、上清を回収した。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 また、遺伝子導入に用いる GS 細胞数、薬剤選択の濃度やタイムコースなどを調整し、single integration のクローンを樹立する条件を確立した。

(6) 遺伝子ターゲッティングベクターの作成

Neomycin と Tk による二段階選択が可能なターゲッティングベクターを設計した。プロモーターには pgk を用いた。Occludin 遺伝子(接着因子･マウス GS 細胞でジーンターゲッティングに成功した)、Hprt 遺伝子（Lesch-Nyhan 病の原因遺伝子で X 染色体上にある)、p53 遺伝子（癌抑制遺伝子）について SD ラット由来のゲノム DNA を用いて作成した。Occludin 遺伝子については promoterless ノックアウトベクターも作成した。

(7) 遺伝子相同組み換えクローンの樹立

エレクトロポレーションにて SD ラットから樹立した GS 細胞に上記のコンストラクトを導入した。neomycin 耐性能を獲得したクローンを G418 を用いて選択した。GS 細胞の増殖効率は細胞の密度により影響を受けるため、薬剤選択の際には遺伝子導入しない GS 細胞を適宜混合して増殖・薬剤選択を繰り返し、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。promoterless ノックアウトベクターについても stable clone の樹立効率を調べた。

（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

平成21年度の研究ではラットＧＳ細胞培養条件の改善を試みた。またこれまで無血清・フィーダーフリー培養は成功していなかったが、マウスＧＳ細胞にて成功した。また、ラット系統、薬剤選択法の改変、などによりクローン樹立効率を改善した。上記の改善点を取り入れ、遺伝子トラップクローンを樹立した他、ジーンターゲッティングベクターを導入したクローンを樹立した。

(1) 培養条件の改善：

Glial cell-line derived neurotrophic factor (GDNF)や basic Fibroblast growth factor (bFGF)を含む培養液にて生後 10-15 日齢のラット精巣細胞を培養することによりＧＳ細胞を樹立した。　血清を0.2%まで下げること、また培地に B27 を添加することにより樹立効率・増殖速度の改善が見られた。

GS 細胞のマイクロアレイデータなどを参考にサイトカインを培養中に添加して新規増殖因子をスクリーニングした。Wnt family 分子や BMP family 分子、TGFb family 分子などを含め多数調べたが、有意に効果のあるものはなかった。

平成21年度の研究では培養液の改変により無血清・フィーダーフリー培養を試みた。ラット培養は血清の濃度が高いと増殖が維持できないこと、Mouse embryonic fibroblast などのフィーダーを用いるとフィーダーフリー培養（ラミニンを基質とする）より増殖効率が下がること、などから無血清・フィーダーフリー培養の開発が必要である。マウスではフィーダーを用いた無血清培養、もしくは血清を用いたフィーダーフリー培養には成功しているが、無血清・フィーダーフリー培養には成功していない。DBA/2 マウス由来の GS 細胞を用いてラミニンコートしたプレート上で培養を行うと、血清の存在下ではラミニンへの接着と増殖が持続するが、無血清では接着と増殖が損なわれる。研究代表者らは血清の活性を代替する因子をスクリーニングした結果、Fetuin により GS 細胞の接着が保たれることが分かった。無血清・フィーダーフリー培養にて４ヶ月以上増殖維持ができ、また精子幹細胞に特有の遺伝子発現パターンを保持していた（α6/β1-integrin 陽性、EpCAM 陽性、SSEA-1 陰性、など）。この細胞を現在精巣内に移植し、顕微授精にて子孫を作成を試みている。ラットでは特に血清とフィーダーの存在により GS 細胞の増殖に悪影響があることがこれまでの研究から示唆されることから、平成２２年度の研究では類似の培養方法をラットにて検討する。

(2) ラット系統や年齢による差異の検討：

GS 細胞を樹立するラットの年齢を検討した。生後 10-18 日齢の精巣からは GS 細胞の樹立ができるが、20 日齢以上の精巣からは樹立できなかった。

マウスにおいては GS 細胞の樹立効率や増殖速度が系統により大きく異なることが分かっている。平成 21 年度の研究では SD の他、Wistar, Lewis, Long-Evans, Donryu などの系統や、これらの系統間の F1 種を作成し、それぞれ GS 細胞を樹立して、樹立効率や増殖速度・クローン樹立効率を比較した。どの系統でも樹立に成功し、増殖速度も大きく変わらなかった。ところが、クローン樹立効率については Lewis, Long-Evans は悪く、SD や Wistar と SD の F1 や、Donryu と SD の F1 の成績が良かった。

(3) 薬剤耐性遺伝子：

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。

(4) ラット精子幹細胞の精巣内移植技術の改善：

ラットの精巣内移植は、レシピエントの Busulfan による前処理の効率が悪い。内因性の

精子形成を除去するためにレシピエントに投与する Busulfan の効果が不安定であり、過度に除去すると、移植された細胞からの精子形成も抑制してしまうため、投与条件を検討する必要があった。

平成21年度の研究ではラットをレシピエントとした移植効率の改善のため、生後でなく胎生期のラットに対し Busulfan を投与した。妊娠 SD ラットに Busulfan を投与し、生後 1-2 週齢にて精巣内移植を行った。移植後２ヶ月から野生型のメスと自然交配を行ったところ、一部のレシピエントから子供が得られた。しかしながら妊性を獲得するレシピエントの割合が低く、不安定な点において改善の必要がある。

一方、ヌードマウスをホストとした異種移植ではラットよりも安定したコロニー形成が見られた。Balb/nu より KSN/nu の方が成績がよいなどのホストの系統差もあることが分かった。

(5) 遺伝子トラップ遺伝子改変ラットの作成

これまでの方法では複数遺伝子が挿入されたクローンのみが選択的に樹立されるという問題があった。そこで 21 年度の研究では以下の点で改善を試みた。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-β-geo）を導入した。

改善した培養条件を取り入れた他、遺伝子導入に用いる GS 細胞数、薬剤選択の濃度やタイムコースなどを調整し、single integration のクローンを樹立する条件を確立した。

Wistar や SD、その F1 など複数の系統の GS 細胞にてクローンの樹立を行った。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ー2 個程度の integration であることを確認した。

(6) 遺伝子ターゲッティングベクターの作成

Occludin遺伝子（接着因子･マウスGS細胞でジーンターゲッティングに成功した）、Hprt遺伝子（Lesch-Nyhan病の原因遺伝子でX染色体上にある）、p53 遺伝子（癌抑制遺伝子）についてSDラット由来のノックアウトベクターを作成した。Occludin 遺伝子については promoterless ノックアウトベクターも作成した。Neomycin と Tk による二段階選択が可能なベクターを設計した。プロモーターには pgk を用いた。

(7) 遺伝子相同組み換えクローンの樹立

エレクトロポレーションにて SD ラットから樹立した GS 細胞に上記のコンストラクトを導入した。neomycin 耐性能を獲得したクローンを G418 を用いて選択した。tk によるネガティブセレクションが理論上可能であるが、ラット GS 細胞では gancyclovir への反応性が良くないことが分かったため、G418 のみにて選択を行った。GS 細胞の増殖効率は細胞の密度により影響を受けるため、薬剤選択の際には遺伝子導入しない GS 細胞を適宜混合して増殖・薬剤選択を繰り返し、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。Hprt 遺伝子につき 44 個、Occludin 遺伝子にて 146 個のクローンを樹立した。promoterless ノックアウトベクターは同じ Occludin 遺伝子でも、stable clone の樹立効率が著しく悪かったため、pgk promoter のもののみを使うこととした。

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・クローン化の効率の改善が必要である。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、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以前マウスに比べると遅く、クローンの樹立までに４ー５ヶ月かかることから、更なる改善が必要である。

E．結論

平成21年度はこれらの条件検討を行いながらトラップクローンの樹立とターゲッティングベクターの作成・遺伝子導入を行った。平成22年度の研究ではこららのクローンを精巣内に移植して個体化することを重点的に進めるとともに条件検討を継続して行い、より実用的なプロトコールの確立を目指す。

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

1. 論文発表

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Yoshimoto M., Heike T., Chang H., Kanatsu-Shinohara M., Baba S., Varnau J.T., Shinohara T., Yoder M,C., Nakahata T. Bone marrow engraftment but limited expansion of hematopoietic cells from multipotent germline stem cells derived from neonatal mouse testis. Exp.Hematol. 37(12):1400-10 (2009)

2. 学会発表

平成 21 年 9 月 1-3 日
特定領域「細胞増殖制御」班会議
篠原美都「精子幹細胞の細胞周期の調節機構の解明」
ホテルアンビエント安曇野

平成 21 年 9 月 15-16 日
特定領域「細胞外環境」班会議
篠原美都「精子幹細胞の癌化における細胞外環境の関わり」
京都国際会議場

平成 21 年 10 月 13-14 日
新学術領域研究 「配偶子制御」第 2 回領域会議
篠原美都「精子幹細胞の自己複製制御機構」
総合研究大学

平成 21 年 12 月 18-19 日
特定領域 「統合脳」班会議
篠原美都「精子幹細胞を用いた神経疾患モデルラット作成技術の開発」
東京・学士会館

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
Kanatsu-Shinohara M., Takashima S., Shinohara T.	Transmission distortion by loss of p21 or p27 cyclin-dependent kinase inhibitors following competitive spermatogonial transplantation.	Proc. Natl. Acad. Sci. USA	107(14)	6210-5	2010
Morimoto H., Kanatsu-Shionohara M., Takashima S., Chuma S., Nakatsuji N., Shinohara T.	Phenotypic plasticity of mouse spermatogonial stem cells.	PLoS One.	4(11)	e7909	2009
Kanatsu-Shinohara M., Ogonuki N., Miki H., Inoue K., Morimoto H., Takashima S., Ogura A., Shinohara T.	Genetic influences in mouse spermatogonial stem cell self-renewal.	J.Reprod.Dev.	56(1)	145-153	2010
Yoshimoto M., Heike T., Chang H., Kanatsu-Shinohara M., Baba S., Varnau J.T., Shinohara T., Yoder M,C., Nakahata T.	Bone marrow engraftment but limited expansion of hematopoietic cells from multipotent germline stem cells derived from neonatal mouse testis.	Exp.Hematol.	37(12)	1400-10	2009

Transmission distortion by loss of p21 or p27 cyclin-dependent kinase inhibitors following competitive spermatogonial transplantation

Mito Kanatsu-Shinohara[a,1], Seiji Takashima[a], and Takashi Shinohara[a,b]

[a]Department of Molecular Genetics, Graduate School of Medicine, Kyoto University, Kyoto 606-8501 Japan; and [b]Japan Science and Technology Agency, Japan Science and Technology Agency, Kyoto 606-8501 Japan

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Spermatogonial stem cells (SSCs) undergo self-renewal division to support spermatogenesis. Although several positive regulators of SSC self-renewal have been identified, little is known about the mechanisms that negatively regulate SSCs. Here we developed a novel transplantation assay for SSCs and demonstrate that p21 and p27 cyclin-dependent kinase inhibitors play critical roles in SSC self-renewal and differentiation. Overexpression of p21 or p27 abrogated proliferation of cultured SSCs in vitro, and their expression levels were downregulated by exogenous self-renewal signals. In contrast, no apparent defects were found in p21 or p27-deficient SSCs by spermatogonial transplantation. However, competitive spermatogonial transplantation with WT SSCs revealed that the loss of either gene causes distortion of germline transmission: p21-deficiency facilitated mutant offspring production, whereas germline transmission was limited by p27-deficiency. Serial transplantation also showed that the loss of p27, but not p21, decreases secondary colony formation, suggesting that appropriate amounts of p27 are necessary for sustaining SSC self-renewal. Thus, p21 and p27 cyclin-dependent kinase inhibitors play critical roles in germline transmission by regulating the balance between SSC self-renewal and differentiation, and competitive spermatogonial transplantation technique will be useful for analyzing subtle defects in spermatogenesis that are not evident by traditional spermatogonial transplantation.

germline transmission | spermatogenesis | stem cell | cyclin-dependent kinase inhibitor | self-renewal

Spermatogenesis is a dynamic and complex process based on the self-renewal division of spermatogonial stem cells (SSCs). Very few SSCs are found in the testis (0.02–0.03% in a testis cell suspension), but SSCs proliferate throughout life to support spermatogenesis (1, 2). The balance between SSC self-renewal and pool size necessitates strict control, because excessive self-renewal or differentiation can cause male infertility. Although stem cells are relatively cytokine resistant in many tissues, glial cell line-derived neurotrophic factor (GDNF) promotes self-renewal division of SSCs (3). Changes in GDNF levels greatly impact on spermatogenesis in vivo: Excessive GDNF expression induces the accumulation of undifferentiated spermatogonia, whereas decreases in GDNF cause hypospermatogenesis and the gradual loss of SSCs (3). Thus, the regulation of SSC self-renewal is governed by a subtle balance between negative regulatory pathways that maintain mitotic quiescence and positive growth-promoting signals involving GDNF. Although we recently discovered that the Ras-cyclin D2 pathway acts downstream of GDNF to promote SSC self-renewal (4), little is known about how the balance between self-renewal and differentiation is maintained in vivo.

Cyclin-dependent kinase inhibitors (CDKIs) are good candidates for the negative regulation of SSC proliferation. Two families of CDKIs promote cell cycle withdrawal by blocking the activity of cyclin/CDK complexes: the Cip/Kip family, including p21, p27, and p57, and the INK4 family, including p15, p16, p18, and p19 (5). Whereas INK4 proteins bind to CDK4 or CDK6 and inhibit their activity, Cip/Kip proteins show a broader range of activities and interact with all cyclin/CDK complexes. Cip/Kip proteins are distinct from the INK4 family in that they also stimulate the formation of cyclin D/CDK4/6 complexes to promote proliferation (6). Importantly, Cip/Kip proteins have additional functions beyond regulating cell divisions and regulate differentiation. For example, enforced expression of p21 or p27 induces the differentiation of neuroblastoma cells and myelomonocytic leukemia cells (6). Although KO mice have been produced for Cip/Kip family genes, no apparent SSC phenotype has been reported, and the effects of these genes remain unclear (7, 8).

Because stem cells comprise only a small population and are defined retrospectively through the analysis of daughter cells, studies to analyze SSCs have been hampered by difficulties in distinguishing SSCs from committed spermatogonia. In 1994, however, a spermatogonial transplantation technique was developed (9). With this technique, SSCs can be detected by their ability to generate germ cell colonies after microinjection into seminiferous tubules of infertile recipient animals. Eventually, recipient animals can produce donor-derived offspring by mating with WT females. Because differentiated progenitor cells do not have self-renewal capacity, only SSCs can produce these results. Although this technique has been used extensively to study SSCs, the degree of SSC self-renewal and differentiation is difficult to evaluate by morphological analyses of germ cell colonies. Moreover, measurements of SSC numbers do not necessarily correlate with long-term functional capacities. In fact, declining fertility is reported after serial SSC transplantation, which raised question about whether transplanted SSCs are fully competitive as WT SSCs before transplantation (10).

In the present study, we report a unique SSC transplantation assay, in which stem cell function of a mutant donor is assayed by mixing with WT SSCs. Although spermatogonial transplantation failed to reveal abnormalities in p21 and p27 KO SSCs, p21 and p27 deficiencies have contrasting effects on germline transmission efficiency when they were mixed with WT SSCs. Moreover, serial transplantation showed decreased secondary colony production from p27 KO SSCs. This strategy of SSC analysis will be useful for comparing long-term SSC function and is capable of detecting subtle defects that escape detection by conventional transplantation technique.

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[1]To whom correspondence should be addressed. E-mail: mshinoha@virus.kyoto-u.ac.jp.

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Results

Effects of p21 and p27 CDKIs on Cultured SSCs. To identify molecules that negatively regulate SSC proliferation, we used germline stem (GS) cells, cultured SSCs (11). The addition of GDNF and EGF and/or basic fibroblast growth factor (bFGF) induces SSC proliferation in vitro. Approximately 1–2% of the GS cells have SSC activity (4). GS cells from WT mice were infected with lentivirus vectors expressing candidate genes and Venus fluorescent marker gene under the control of an elongation factor2 promoter. Using this strategy, we found that overepression of p21 or p27 CDKIs compromises GS cell proliferation. When the transfected cells were analyzed 3 d after infection, significantly smaller numbers of Venus$^+$ cells were found after CDKI overexpression in all six separate experiments (Fig. S1 *A* and *B*). In particular, very few germ cell clumps showed fluorescence after p21 overexpression, suggesting that the transfected cells proliferated more slowly than WT cells or died shortly after transfection. Some clumps, however, remained after p27 overexpression at 21 d, but significantly fewer clumps were observed as compared to controls (Fig. S1*C*). In contrast, cells transduced with an empty vector showed strong fluorescence and the cells could be passaged every 5–6 d. Flow cytometric analysis at 10 d postinfection also confirmed the reduction in Venus$^+$ cells after CDKI overexpression. Cell cycle analyses also showed the growth suppression due to p21 overexpression (Fig. S1*D*).

We then examined whether p21 or p27 expression is influenced by exogenous cytokines. Real-time PCR analysis showed a significant reduction in p21 and p27 expression due to cytokine stimulation (Fig. S1*E*). The combination of EGF and bFGF showed a comparable effect as GDNF, and the addition of all cytokines suppressed the p21 and p27 levels to 25.8% and 19.0%, respectively. These in vitro results indicated that p21 and p27 CDKI levels are regulated by exogenous cytokines and also suggested that both genes may negatively regulate SSC proliferation in vivo.

SSC Activity of p21 and p27 KO Testis Cells After Spermatogonial Transplantation. To examine the effects of p21 and p27 genes in vivo, we analyzed testes of p21 and p27 KO mice. Both mutant mice survived to birth and matured into adults. Whereas p21 KO mice appeared normal, p27 KO mice exhibited multiple organ hyperplasia and females were infertile due to poor ovarian follicle development (7, 8). In contrast, both p21 and p27 KO males produced spermatozoa and were fertile, suggesting normal spermatogenesis. However, whereas testes from p21 KO mice were comparable to those of WT mice, testes from p27 KO mice were significantly larger (Fig. 1*A*). Although flow cytometry showed normal size of p21 and p27 KO spermatogonia, cell cycle analyses revealed that EpCAM$^+$ spermatogonia from both mutants proliferated more actively than those of WT mice, and significantly more cells were found in the G2/M phase (Fig. 1*B*). However, whereas real-time PCR analyses showed no compensatory upregulation of p21 or p27 in either type of KO mice, expression of E-cadherin or promyelocytic leukemia zinc finger (PLZF), markers for undifferentiated spermatogonia (12–14), was significantly reduced (Fig. 1*C*), suggesting that undifferentiated spermatogonia in these mice comprise relatively smaller population.

To study the function of SSCs, we used spermatogonial transplantation (Fig. 1*D*). Donor testis cells were marked by mating the KO animals with a transgenic mouse line C57BL6/Tg14 (act-EGFP-OsbY01) (green mice) that ubiquitously expresses enhanced green fluorescent protein (EGFP). Preliminary transplantation of mutant cells into nonablated WT testis did not result in colonization, thus suggesting that mutant SSCs do not have an enhanced ability to compete for the niche that is occupied by WT SSCs. We then transplanted the mutant cells into empty tubules to quantify SSC number. In three separate experiments, the same number of mutant and WT testis cells were transplanted into adult WBB6F1-W/W^v(W) mice that were deficient for spermatogenesis due to c-kit gene defects (15). Two months after transplantation, the recipient mice were sacrificed and their testes were analyzed for colonization under UV light.

Both p21 and p27 KO donor cells produced germ cell colonies that were similar to those of WT mice. We found no apparent abnormalities in the length and the morphology of the colonies, which suggested normal proliferation and differentiation of transplanted SSCs (Fig. 1*D*). The p21 KO cells and WT cells produced 4.7 ± 0.8 and 4.2 ± 0.5 colonies of donor-derived spermatogenesis/10^5 cells injected, respectively, and this difference was not statistically significant (Fig. 1*E*). In contrast, the p27 KO cells produced significantly smaller numbers of colonies as compared to WT cells, and the number of colonies generated was 2.0 ± 0.5 and $3.8 \pm 0.5/10^5$ cells injected for p27 KO cells and WT cells, respectively. Because each colony was derived from a single transplanted SSC (16, 17), these results indicated that the SSC concentration in p27 KO mice was significantly lower than that of WT mice. Although the total number of SSCs per testis (cell recovery × concentration of SSCs in the injected cell suspension determined by transplantation) was increased for the WT background (1482 vs. 1160 for WT and p27 KO cells), the difference was not significant. These results show that both p21 and p27 KO testis contain similar numbers of SSCs.

Transmission Distortion by Competitive Spermatogonial Transplantation. Although these transplantation experiments showed no apparent SSC abnormalities, we speculated that subtle imbalances in SSC self-renewal and differentiation might not be evident in a morphological analysis of germ cell colonies using a simple transplantation assay. To overcome this problem, we cotransplanted WT and mutant cells into the same recipient, and analyzed cell differentiation potential by confirming the genotype of the offspring (Fig. 2*A*). We collected single cell suspensions from p21 or p27 KO pup testes that were enriched for SSCs due to the absence of differentiated cells (18). Testis cells from each mutant mouse were mixed at a 1:1 ratio with those from control WT mice of the same age. The mixed testis cells were then transplanted into 5–10 day old W recipient pups to produce offspring from the transplanted SSCs. Previously, we showed that immature recipients are superior to adult recipients in restoring fertility due to the enhanced SSC colonization (18). Recipient males were housed with two or three WT B6 females, at least 4 weeks after transplantation. Two separate experiments were performed for p21 KO mice, whereas three experiments were carried out for p27 KO mice. About 3×10^5 cells were transplanted into each recipient testis.

Within 3 months after transplantation, 3/8 and 3/9 recipient males that received p21 or p27 KO SSCs, respectively, became fertile (Table S1). Histological analyses of both types of recipients confirmed normal spermatogenesis (Fig. 2*B*). When the offspring genotypes were confirmed by genomic PCR using tail DNA, transmission rate distortion of the donor haplotype was noted in both experiments (Fig. 2 *C* and *D*). For p21 experiments, 212 offspring were produced from the three recipients, and the animals were maintained as long as 304 d after transplantation. PCR analysis revealed that 206/212 offspring contained the neo gene. All of these offspring were heterozygous for the p21 gene, because the recipient males were mated with WT females. The percentage of these heterozygous males was $97.2 \pm 1.8\%$ from each recipient. In contrast, for p27 experiments, a total of 98 offspring were produced during 205 d from the three recipients that received p27 KO testis cell transplantation. WT offspring, however, were predominantly produced from the recipients, and only 12/98 offspring were heterozygous for the p27 gene. Although the overall percentage of heterozygous offspring was 12.2%, one of the recipient males produced as many as 10 heterozygous offspring. Moreover, these offspring were born

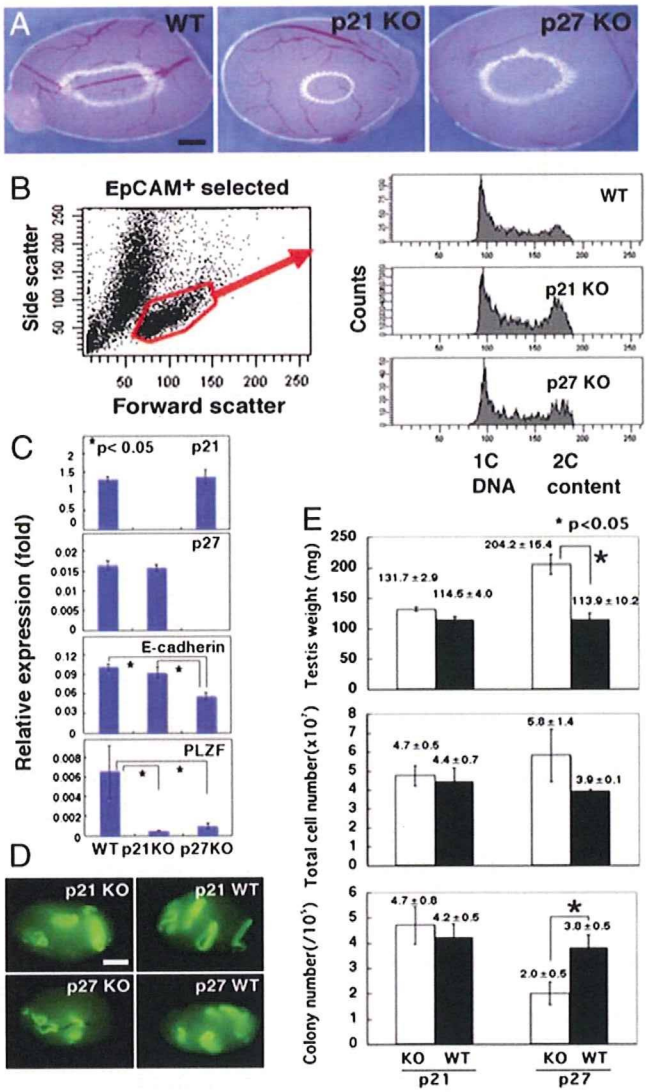

Fig. 1. Phenotypic and functional analyses of p21 or p27 KO testis cells. (*A*) Appearance of mutant testes. Note the larger size of the p27 KO testis. (*B*) Cell cycle analysis of EpCAM$^+$ spermatogonia. Note the enhanced proliferation of p21 and p27 KO cells. WT mice were used as a control. (*C*) Real-time PCR analyses of p21, p27, E-cadherin, and PLZF expression. Expression of E-cadherin or PLZF was significantly downregulated in the mutant mice. (*D*) Macroscopic appearance of the recipient testes. Green fluorescence indicates donor cell colonization. (*E*) Testis weight (*Upper*, $n = 3$), total cell number after enzymatic digestion (*Middle*, $n = 3$), and colony number (*Lower*, $n = 15$–17). Asterisks denote significant differences compared to the control ($P < 0.01$). Bar = 1 mm (*A* and *D*).

within 98 d after transplantation, and no heterozygous offspring were produced from this male up to 195 d.

Impact of p21 and p27 Deficiencies in Self-Renewal Divisions of SSCs. To directly examine the impact of p21- and p27-deficiency on SSC self-renewal, we performed serial transplantation (Fig. 3*A*). In normal testes, SSCs are kept under constant pressure to differentiate and produce sperm. The most generally accepted hypothesis is that SSCs undergo only two types of cell division: They produce either two stem cells (self-renewal division) or two progenitor cells (differentiating division) (1). Each division occurs at about the same frequency. After transplantation, however, SSCs are thought to undergo self-renewal divisions more frequently than differentiation divisions, and thus increase their numbers (19). Because the number of colonies in the recipient testis indicates the number of SSCs that initially colonized the testis, the number of SSCs that were produced by subsequent divisions may be determined by transplantation into another testis. We collected testis cells from WT and mutant donors that contained the EGFP transgene. After dissecting out colonies in each recipient testis at 10 weeks posttransplantation, the tubules were dissociated into single cells and suspended in 15–21 μl of injection medium. The number of cells recovered from the three types of recipients ranged from $0.4 - 3.0 \times 10^6$ cells, with an average of 1.6×10^6 cells. Differences among donors were not significant. Approximately 4 μl of the cell suspension was microinjected into three secondary recipient testes.

Analysis of the secondary recipient testes at 2 months after transplantation revealed that significantly fewer SSCs were produced from the p27 KO donor testis cells (Fig. 3 *B* and *C*). Although 13/14 transplantations showed colony number increases (total regenerated colony number—primary colony number used for transplantation) in experiments using WT and p21 KO mice, only 5/14 transplantations showed increase in experiments using p27 KO mice, indicating that the SSCs in p27 KO mice produced smaller numbers of secondary colonies. Assuming that 10% of the SSCs can colonize (19), and that each colony is produced by one SSC (16, 17), the multiplication of colony numbers (total regenerated colony number × 10/primary colony number used for serial transplantation) were

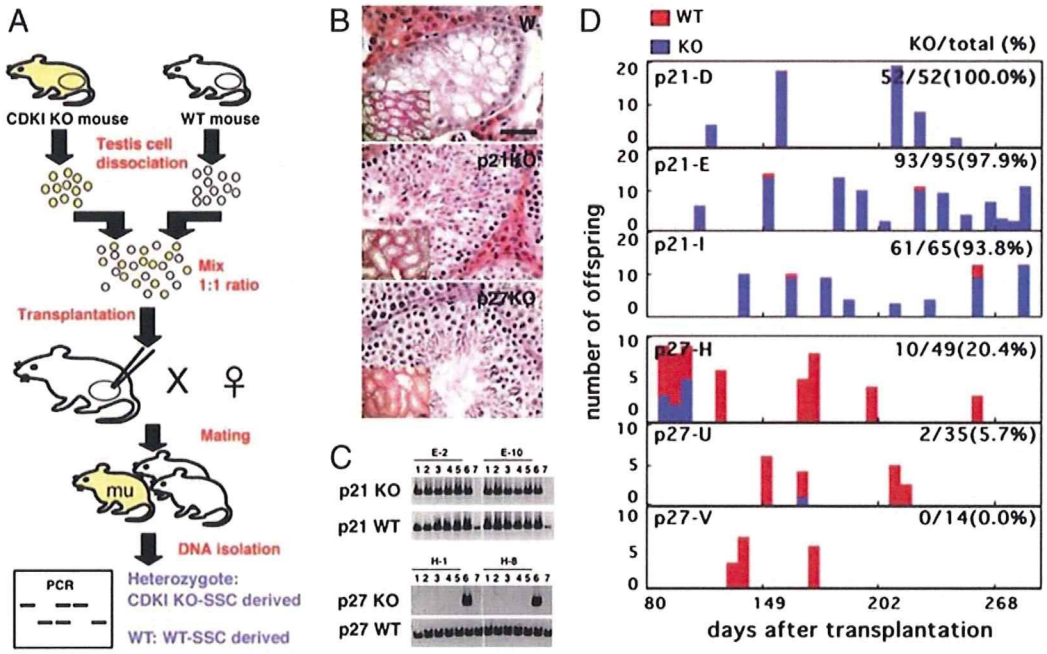

Fig. 2. Competitive spermatogonial transplantation. (*A*) Experimental procedure. Two populations of testis cells, one from a WT mouse and the other from a KO mouse, were mixed at a 1:1 ratio and transplanted into W mice to produce offspring. Tail DNA of the F1 offspring were analyzed by PCR for genotyping. (*B*) Normal appearing spermatogenesis in the recipient testes. (*C*) Genotyping by PCR. (*Upper*) Offspring from recipient E, which were transplanted with a mixture of p21 KO and WT testis cells. Analyses of the second and tenth littermates are shown (*Lanes 1–5*). Lane 6: p21 heterozygous; lane 7: WT control tails. All F1-derived offspring showed p21 mutant bands. (*Lower*) Offspring from recipient H, which were transplanted with a mixture of p27 KO and WT testis cells. Analyses of the second and tenth littermates are shown (*Lanes 1–5*). Lane 6: p21 heterozygous ; lane 7: WT control tails. None of the offspring showed p27 mutant bands. (*D*) Temporal analysis of offspring. Bar = 50 μm, (*B*); 100 μm (*B*, *Inset*).

7.2 ± 1.6 ($n = 14$) and 29.3 ± 4.0 ($n = 14$) for p27 KO cells and WT cells, respectively (Table S2). The difference between p27 KO and WT cells was significant. In contrast, p21 KO cells produced comparable numbers of secondary colonies with WT cells, and the average number of colonies per primary colony was 26.6 ± 5.9 ($n = 14$). No significant difference was observed in the self-

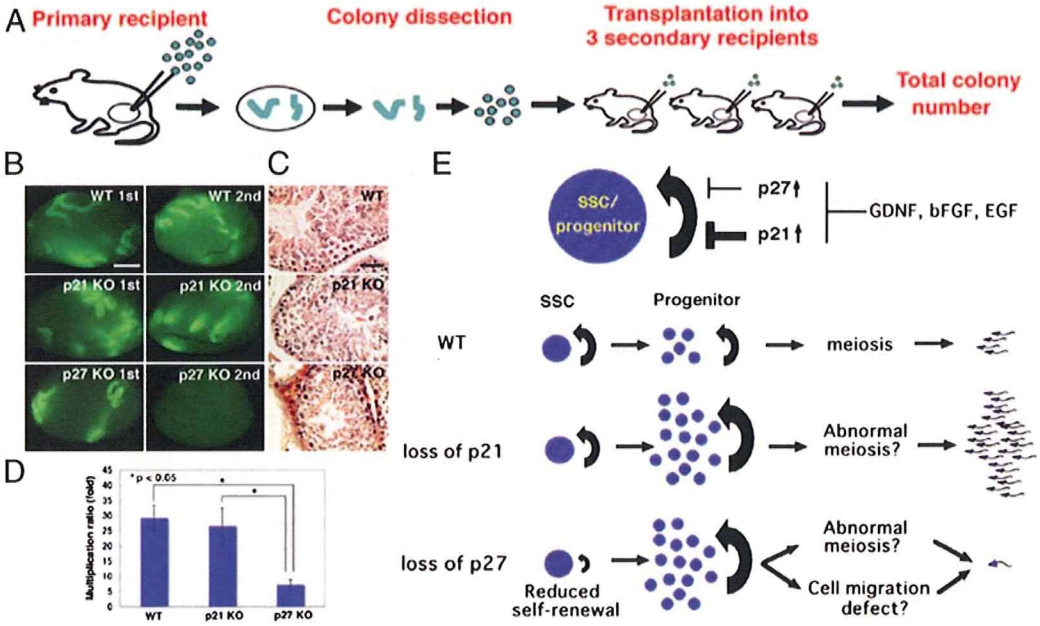

Fig. 3. Serial transplantation. (*A*) Experimental procedure. Colonies in the primary recipients were dissected out at 10 weeks after transplantation, and a portion of them was transplanted into the three testes of the secondary recipients. (*B*, *C*) Macroscopic (*B*) and histological (*C*) appearance of the recipient testes. Green fluorescence indicates donor cell colonization. Note the decrease in secondary colony numbers from p27 KO testis cells. (*D*) The degree of secondary colony formation, indicated by the ratio of SSC number between the two time points. Asterisks denote significant differences compared to the control ($P < 0.01$). (*E*) Summary of results and models for transmission distortion of p21 and p27 SSCs. (*Upper*) Upregulated p21 or p27 inhibits SSC/progenitor cell proliferation. Exogenous cytokines downregulate p21 and p27 expression. (*Lower*) Loss of p21 does not influence SSC self-renewal, but likely enhances progenitor cell proliferation. In contrast, loss of p27 decreased SSC self-renewal but may enhance progenitor cell proliferation. However, these progenitor cells have defects in meiosis and/or cell migration, both of which may have caused decreased spermatogenesis efficiency. Bar = 1 mm (*B*); 100 μm (*C*).

renewal capacity of SSCs between p21 KO and WT cells. The doubling times of the SSCs during the 10-week period were 14.4, 14.8, and 24.6 d for WT, p21 KO, and p27 KO SSCs, respectively (Fig. 3*D*).

Discussion

Spermatogonial transplantation technique provided the first functional assay for SSCs. It has been used to detect SSCs and determine whether abnormal spermatogenesis is caused by germ cell defects or their respective microenvironments. This technique is also useful for assessing SSC numbers quantitatively. For example, when a mixture of two testis cell populations were transplanted at a 1:1 ratio, recipient testes contained a near 1:1 ratio of colonies with each of the two genotypes (16). However, spermatogenesis is not completely normal after transplantation, and multiple abnormalities that are associated with transplantation, such as increased apoptosis or missing layers of germ cells (20), raise questions about the efficiency and quality of spermatogenesis after transplantation. In this study, we developed a competitive spermatogonial transplantation technique to provide a selective pressure to identify SSCs with high capacity for competitive long-term repopulation. In its concept and design, the technique is somewhat similar to competitive repopulation technique in hematopoietic stem cells (HSCs) (21). Forced competition against WT SSCs allowed direct functional comparisons between the two donors in a quantitative manner under consistent microenvironmental stimuli. Using this method, growth factors, nutrient conditions, and systemic environments such as hormonal levels are provided equally for both donors in the same recipient animal. This was particularly important in this study, because abnormalities are reported for both Sertoli and Leydig cells in p27 KO mice (22, 23). Furthermore, although periodic sperm sampling from the same individual is difficult in mice, this technique allows for the monitoring donor cell dynamics over a long period. Thus, competitive spermatogonial transplantation will be useful for functional analyses of spermatogenesis.

Using this technique, we found transmission distortion of mutant SSCs with contrasting results: p21-deficiency facilitated the production of mutant offspring, whereas it was severely limited by p27-deficiency. In contrast, p21 and p27 overexpression inhibited GS cell proliferation. These results suggested that p21 and p27 levels are important in maintaining normal proliferation of SSCs and/or progenitor cells. In fact, studies of other self-renewing tissues also suggested the involvement of CDKIs in regulating the stem cell quiescence and pool size. In general, p21 is thought to act on quiescent stem cells, whereas p27 is a progenitor-specific inhibitor for repopulation efficiency (24). During hematopoiesis, for example, whereas p21 governs cell cycle entry of HSCs, p27 does not affect HSC number, cell cycling, or self-renewal but has an impact on the cell cycle of progenitors (24). Likewise, p21-deficiency influences the number and proliferation of neural stem cells (NSCs) (25). Although mutant animals initially have increased numbers of NSCs due to excessive proliferation, the levels decrease as they age due to exhaustion.

Recent studies using male germ cells also suggest that p21 plays an important role in SSCs: Atm-deficient undifferentiated spermatogonia upregulates p21, which is responsible for cell cycle arrest upon DNA damage (26). Suppression of p21 can partially restore SSC activity in Atm KO mice, but its normal function remains unclear. In this study, we showed that p21 levels are regulated by exogenous cytokines and that ectopic overexpression of p21 leads to growth inhibition. In contrast, p21-deficiency does not alter SSC number or self-renewal activities, which suggested that transmission distortion was caused by defects in more differentiated cells. On the other hand, reduced PLZF expression suggested smaller size of undifferentiated spermatogonia population, and these conflicting observations make it difficult to explain why germline transmission occurred predominantly from p21 KO cells. Nevertheless, given the increased mitotic activity of whole spermatogonia population, we speculate that more differentiated type of spermatogonia, such as type A_{1-4} or B spermatogonia, are proliferating more actively and caused transmission distortion by increasing the population size or differentiating faster. Alternatively, it may result from an advantage of p21 KO germ cells to progress through meiosis; p21 is most strongly expressed in pachytene spermatocytes and spermatids in normal testis.

On the other hand, p27-deficiency had a direct effect on SSC. We initially assumed that p27-deficiency would not influence germline transmission, because Sertoli cells have been hypothesized as being responsible for the large testis phenotype of p27 KO mice: p27 has been detected only in Sertoli cells and germ cells were thought to be normal (27). However, offspring were rarely produced from p27 KO cells after competitive transplantation, which suggested defects in germ cells. Although the possibility of Sertoli cell colonization cannot be totally excluded, another study also showed the important role of p27 in germ cells: mice deficient in Skp2, which mediates ubiquitin-dependent degradation of p27, exhibited a progressive loss in spermatogenic cells (28). Furthermore, disruption of p27 in these mice restored fertility, suggesting that testicular hypoplasia of Skp2 mutant mice is attributable to the antiproliferative effects of p27 accumulation. Therefore, although p27 has not been detected at protein levels in the germline, these results suggest that germ cells also contribute to the large testis phenotype of p27 KO mice, and that it probably has an important influence on the fate decision of SSCs.

Although p27 KO mice show a comparable number of SSCs per testis, our serial transplantation experiments showed reduced secondary colony formation from p27 KO SSCs. It is possible that loss of p27 might have accelerated senescence/differentiation. However, because p27 KO mice remain fertile for long-term and produce significantly more sperm than WT mice (23), we rather speculate that decreases in p27 levels in SSCs may enhance the production of progenitor cells by increasing the relative frequency of differentiating divisions. On the other hand, we also showed using GS cells that p27 overexpression compromises proliferation and that increase in self-renewal factors decreases p27 mRNA levels. Besides transcriptional regulation, p27 is also regulated by protein degradation (29). We recently found that self-renewal signals promote the export of p27 from the GS cell nucleus (4). Therefore, appropriate levels of p27, as well as its cellular location, are required for undergoing self-renewal divisions, and this appears to be regulated in a sophisticated manner by changes in the local self-renewal factor levels in the seminiferous tubules. Disturbance in this regulation may cause abnormalities in SSC self-renewal.

Besides regulating mitosis, p27 is involved in meiosis. Testes from p27 KO mice have a significant number of abnormal leptotene spermatocytes that cannot enter the meiotic prophase (27). Strikingly, some of these spermatocytes attempted to carry out mitotic divisions instead of entering into prophase. Furthermore, p27 KO cells may have impaired migratory activity: p27 binds to RhoA and inhibits its activation by interfering with the interaction between RhoA and its activators (30). Impaired migratory activity can interfere with several steps of spermatogenesis, such as the migration of preleptotene spermatocytes through the blood–testis barrier. Collectively, these factors may have decreased the efficiency of spermatogenesis and caused transmission distortion despite the expanded pool of progenitor cells (Fig. 3*E*).

Our study revealed a critical role for p21 and p27 CDKIs in regulating germline transmission from SSCs. Although the small population size of SSCs makes it difficult to study their dynamics, competitive spermatogonial transplantation techniques have proved to be more sensitive in detecting subtle abnormalities

in spermatogenesis in a quantitative manner. Moreover, serial transplantation techniques were instrumental in analyzing effects on SSC self-renewal. This strategy of analyzing the dynamics of SSCs in vivo can be extended to different mouse mutants, and further analysis of CDKIs will help to determine how self-renewal and differentiation are differentially regulated in SSCs.

Materials and Methods

Animals and Transplantation. The p21 KO mice were purchased from the Jackson laboratory, and p27 KO mice were kindly provided by K. Nakayama (Kyushu University). Both strains of mice were maintained on a B6 background. In some experiments, we also used a Green mouse that expressed the EGFP gene ubiquitously to mark the donor cells (M. Okabe, Osaka University). For spermatogonial transplantation to quantify SSC number, testis cells were collected from 4–6 week old mice. Donor cells were collected by two-step enzymatic digestion using collagenase and trypsin (both from Sigma). Dissociated cells were introduced into the seminiferous tubules of pup (5–10 d old) or adult (4–6 weeks old) W mice (Japan SLC) via an efferent duct. To produce offspring, testis cells were obtained from 5–10 d old mice. The Institutional Animal Care and Use Committee of Kyoto University approved all of the animal experimentation protocols.

Cell Culture and Transfection. GS cells were cultured on mouse embryonic fibroblasts (MEFs) as described previously using StemPro-34 SFM (Invitrogen) (11). For real-time PCR, the cells were cultured on laminin (20 μg/mL; BD Biosciences) to avoid contamination of MEF-derived RNA. For lentivirus transduction, mouse cDNAs encoding p21 or p27 (both from Addgene) were cloned into CSII-EF-IRES-Venus, and virus particles were produced by transient transfection of 293T cells, as described previously (4). All infections were conducted on MEFs by centrifuging at 3000 × *g* for 1 h at 32 °C in the presence of 10 μg/mL polybrene (Sigma). GS cells were infected overnight at a multiplicity of infection of 35 for overnight (4×10^4 cells/cm^2 in 6-well plate).

PCR. Total RNA was isolated using Trizol reagent, and first strand cDNA was synthesized using Superscript™ II (both from Invitrogen). Real-time PCR was performed using the StepOnePlus™ Real-Time PCR system and *Power* SYBR Green PCR Master Mix (Applied Biosystems) with the PCR primers listed in Table S3. All transcript levels were normalized to those of Hprt1. The PCR conditions were 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 s, and 60 °C for 1 min. Experiments were performed on at least two independent samples, and each PCR was run in triplicate. Genotypes of p21 and p27 KO mice were determined using the PCR primers listed in Table S4.

Analysis of the Recipient Testes. In experiments using Green mice, SSC colonization was determined by observation of fluorescence under UV light. Colonies were defined as germ cell clusters longer than 0.1 mm occupying the entire circumference of the tubule. For the morphological examination, testes were processed for paraffin sectioning and counterstained with hematoxylin and eosin.

Cell Cycle Analysis. Testis cells were selected by rat antimouse EpCAM antibody (G8.8; BD Bioscience) using procedures as previously described (26). EpCAM$^+$ cells were then incubated with Hoechst 33342 (Sigma) at 12.5 μg/mL for 45 min at 37 °C and suspended in phosphate-buffered saline/1% fetal calf serum containing 1 μg/mL propidium iodide (PI; Sigma). Cells were analyzed on a FACSAria2 equipped with a 375-nm UV laser (BD Biosciences). Dead cells and nonspermatogonial cells were gated out by high PI staining and forward scatter.

Statistical Analysis. Results are presented as the mean ± SEM. Data were analyzed using a Student's *t* test. Significant differences in the CDKI expression and serial transplantation were determined by a Tukey's honestly significant differences multiple comparisons test.

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Phenotypic Plasticity of Mouse Spermatogonial Stem Cells

Hiroko Morimoto[1๑], Mito Kanatsu-Shinohara[1๑], Seiji Takashima[1], Shinichiro Chuma[2], Norio Nakatsuji[2], Masanori Takehashi[1¤], Takashi Shinohara[1,3]*

1 Department of Molecular Genetics, Graduate School of Medicine, Kyoto University, Kyoto, Japan, **2** Department of Development and Differentiation, Institute for Frontier Medical Sciences, Kyoto University, Kyoto, Japan, **3** Japan Science and Technology Agency, CREST, Kyoto, Japan

Abstract

Background: Spermatogonial stem cells (SSCs) continuously undergo self-renewal division to support spermatogenesis. SSCs are thought to have a fixed phenotype, and development of a germ cell transplantation technique facilitated their characterization and prospective isolation in a deterministic manner; however, our in vitro SSC culture experiments indicated heterogeneity of cultured cells and suggested that they might not follow deterministic fate commitment in vitro.

Methodology and Principal Findings: In this study, we report phenotypic plasticity of SSCs. Although c-kit tyrosine kinase receptor (Kit) is not expressed in SSCs in vivo, it was upregulated when SSCs were cultured on laminin in vitro. Both Kit^- and Kit^+ cells in culture showed comparable levels of SSC activity after germ cell transplantation. Unlike differentiating spermatogonia that depend on Kit for survival and proliferation, Kit expressed on SSCs did not play any role in SSC self-renewal. Moreover, Kit expression on SSCs changed dynamically once proliferation began after germ cell transplantation in vivo.

Conclusions/Significance: These results indicate that SSCs can change their phenotype according to their microenvironment and stochastically express Kit. Our results also suggest that activated and non-activated SSCs show distinct phenotypes.

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* E-mail: tshinoha@virus.kyoto-u.ac.jp

¤ Current address: Laboratory of Pathophysiology and Pharmacotherapeutics, Osaka Ohtani University, Tondabayashi, Osaka, Japan

๑ These authors contributed equally to this work.

Introduction

Spermatogonial stem cells (SSCs) provide the foundation for spermatogenesis throughout the life of male animals [1,2]. These cells produce differentiating cells and also maintain an undifferentiated state by undergoing self-renewal division. Despite their unique biology, the regulatory mechanism of SSC self-renewal has remained unclear. During the last decade, however, attempts have been made to characterize the surface phenotype of SSCs. Studies have established that SSCs express α6- and β1-integrin, GFRα1, CD9, Thy-1, and EpCAM but are negative for c-kit (Kit) or SSEA-1 [3]. Expression of these markers was analyzed using a germ cell transplantation technique transplanting cells freshly prepared from testes, because SSC activity, by definition, is examined only retrospectively after examining the characteristic of daughter cells [4]. These surface markers proved to be useful to purify SSCs in a deterministic manner by combining multiple parameters using cell sorter [5].

Recent studies revealed important functions of surface molecules on SSCs. For example, β1-integrins on SSCs play pivotal roles in migration into a germline niche after transplantation [6]. Another study also showed that GFRα1, which comprises a receptor for glial cell line-derived neurotrophic factor (GDNF), regulates SSC self-renewal. GDNF from Sertoli cells maintains SSCs in an undifferentiated state by binding to the GFRα1-c-ret receptor complex [7]. GFRα1 is expressed in a small population of undifferentiated spermatogonia, and changes in GDNF or GFRα1 levels can influence the fate of SSCs. For example, when GDNF is overexpressed in testes, spermatogenesis is impaired and clumps of undifferentiated spermatogonia accumulate in seminiferous tubules [7]. By contrast, a decrease in GDNF or GFRα1 level induces SSC differentiation and male infertility [7,8]. In addition to GDNF, Sertoli cells secrete another cytokine, Steel factor (Sl). Sl binds to Kit on germ cells, and a lack of Sl-Kit interaction also results in impaired spermatogenesis [9]. However, Kit is not expressed in SSCs, but it promotes proliferation and suppresses apoptosis of differentiating spermatogonia [5,9–11]. Nevertheless, the number of SSCs in Steel/Steel dickie (Sl^d) mutant mice, which lack membrane-bound Sl, is reduced to ~5% of wild-type (WT) mice. SSCs in these mice do not regenerate to the basal number,

suggesting that Sl-Kit interaction influences SSC number in Sl^d mice [12]. Thus, how environmental stimuli influence SSCs in the decision between self-renewal and differentiation via surface molecules remains unclear.

In 2003, a long-term culture system for SSCs was reported [13]. Cultured SSCs, designated as germline stem (GS) cells, continued to proliferate for more than 2 years while maintaining stable genetic and epigenetic properties [14]. Development of this culture systems provided possibilities to study SSCs in vitro. However, the percentage of SSCs in GS cell culture was unexpectedly low, and only 0.04–1.26% could colonize and reconstitute seminiferous tubules of infertile animals [15]. Moreover, a variable proportion of the cells express Kit, suggesting that a majority of GS cells are differentiating spermatogonia. In contrast, transfection experiments suggested that a significant proportion of GS cells can act as SSCs. When GS cell clones were established by electroporation with a neo-resistant gene, ~20% of picked GS cell colonies colonized seminiferous tubules and produced transgenic offspring [16]. These conflicting experiments suggest that SSC frequency is much higher than previous estimates by direct transplantation and also suggested that SSCs in vitro may exhibit properties that are distinct from those sustaining spermatogenesis in vivo.

In the present study, to clarify the phenotype of SSCs in vitro, we fractionated GS cells according to Kit expression, and examined the SSC activity using a germ cell transplantation technique. We found that GS cells show a constant level of SSC activity regardless of Kit expression. Kit was also strongly expressed in SSCs in vivo when they actively increase their number to colonize seminiferous tubules.

Results

Heterogeneity of GS Cells

We previously reported that a significant proportion of GS cells express Kit [13]. We therefore assumed that SSCs would be enriched by removing Kit^+ cells from the culture, because Kit is expressed in differentiating spermatogonia. However, Kit expression in mouse embryonic fibroblast (MEF)-based GS cell culture varied depending on the timing of analysis, and we could not get consistent results. On the other hand, GS cells proliferate for long periods on laminin-coated dishes [15]. GS cells on laminin differ from those on MEFs in colony morphology. Although they form three-dimensional clump-like colonies similar to GS cells on MEFs, they can also form two-dimensional flat colonies (Figure 1A). When these cells were analyzed by flow cytometry, they were different from those on MEF in Kit expression levels (Figure 1B). Whereas the percentage of Kit-expressing cells increased up to ~90% in the flat colony, clump-type colonies showed little or no Kit expression. In both conditions, >95% of the cultured cells remained viable.

Of the several factors examined (laminin concentration, incubation time, and temperature), we found that the development of two kinds of colonies was most strongly influenced by plating density (Figure 1C). When cells were plated at 1×10^5 cells/3.8 cm^2, 60–90% of the cultured cells showed Kit expression. However, very little expression was observed when cells were plated at $<3.3\times10^4$/3.8 cm^2. Seeding density also had an impact on GS cell proliferation, and GS cells in clump-like colonies did not proliferate as much as did those in fibroblast-like colonies (Figure 1D). Consistent with this observation, Akt, which promotes GS cell proliferation [3,17,18], was strongly phosphorylated when GS cells were plated at the higher cell density (Figure 1E).

Using two different cell densities (1×10^5 and 3.3×10^4 cells/cm^2), we analyzed the expression of other cell surface markers by flow cytometry (Figure 1B). Although the two types of cells showed a significant difference in Kit expression level, GFRα1, a marker for A single (A_s) and A paired (A_{pr}) spermatogonia, and E-cadherin, a marker for undifferentiated spermatogonia [3], were expressed at comparable levels regardless of the type of colonies. We did not find significant changes in other spermatogonia or SSC markers, including α6- and β1-integrins.

Because a difference in cell shape implicated changes in cytoskeletal tension [19], we checked whether actin cytoskeleton was involved in Kit expression by adding actin-disrupting cytochalasin D. Cytochalasin D not only changed the shape of GS cells but it also decreased Kit expression (Figure 1F and G). Because small G proteins are central regulators of cell contractility, we also checked the effect of small G proteins by producing GS cells that stably express Rac, RhoA, and cdc42 dominant-negative mutants. Although no apparent morphological differences were noted among transfectants, dominant-negative RhoA mutants clearly decreased Kit expression (Figure 1F and G). These results suggested that cytoskeletal tension plays an important role in regulation of Kit expression.

Analysis of Kit Function in GS Cell Self-Renewal and Homing into Niche

Although strong Kit expression in feeder-free culture conditions suggested that the undifferentiated state of SSCs is not maintained effectively, GS cells on laminin could be maintained for 6 months without losing SSC potential [15], which raised the possibility that Kit expression was correlated with SSC activity. To examine whether Kit is necessary for GS cell proliferation on laminin, we used a Kit inhibitor (ISCK03) to study the role of Kit in GS cells on laminin. Although the inhibitor prevented proliferation of control Kit-dependent F-36P leukemic cells in a dose-dependent manner [20], it did not show any effects on GS cells (Figure 2A and B). Addition of ACK2, a Kit neutralizing antibody, also did not influence GS cell proliferation (data not shown). These results agreed with the previous observation that Kit is dispensable for proliferation of undifferentiated spermatogonia [9–11]. On the other hand, we also examined whether Kit expression can promote GS cell proliferation. Different concentrations of soluble Sl (5 to 150 ng/ml) were added to the laminin culture, but the number of cells that recovered after a 5 day-period did not show a significant increase compared with control, and they maintained their fibroblastic morphology (data not shown).

Although these results suggested that Kit is dispensable in GS cell proliferation, it was still possible that soluble Sl did not provide a strong signal through Kit; it is known that membrane-bound Sl can activate Kit more strongly [21]. Indeed, Sl^d mutant mice, which lack the membrane-bound form of Sl, are deficient for spermatogenesis despite the expression of soluble Sl [12]. To overcome this problem, we stably transfected WT Sl and dominant active Kit cDNA (Val559 to Gly mutation; Kit-G559) into GS cells derived from an enhanced green fluorescent protein (EGFP)-expressing transgenic mouse [22]. While Kit-G559-transfected cells ($GS^{Kit\text{-}G559}$) did not change morphology, Sl-transfected cells (GS^{Sl}) produced elongated colonies and did not show flat appearances despite being plated at high cell density (Figure 2C). Although Western blot showed phosphorylation of Kit in WT and the transfected GS cells, the transgenes could not replace any of the cytokines used in GS cell culture (Figure 2D).

We further examined the effect of the transgenes in SSC colonization by germ cell transplantation [4]. GS^{Sl}, $GS^{Kit\text{-}G559}$ and GS^{WT} cells were transplanted into WBB6F1-W/W^v (W) mice, which lack endogenous differentiating germ cells [11]. Two months after transplantation, numbers of colonies in recipient

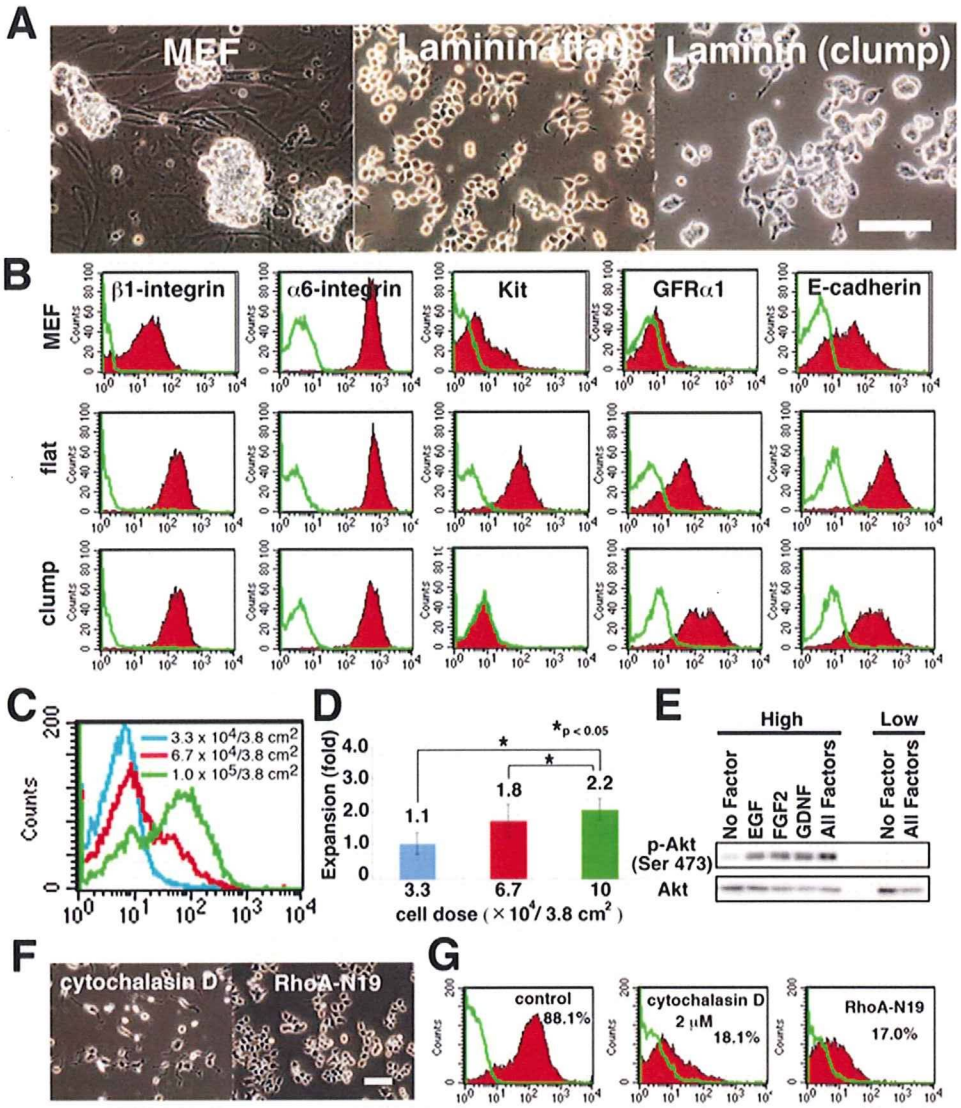

Figure 1. GS cells express Kit. (A) Morphological appearance. (B) FACS analysis of surface markers. Green line indicates the control. (C, D) Effect of cell density on Kit expression (C) and GS cell expansion (D). Cells were plated at the indicated density on laminin (n = 6). Values indicate the degree of expansion from the initially plated cells. (E) Western blot analysis of GS cells plated at 5×10^5 or 3×10^4 cells/9.5 cm^2. (F, G) Appearance (F) and Kit expression (G) of GS cells after cytochalasin D treatment or transfection of RhoA-N19 cDNA. Bar = 100 μm (A, F).
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testes were counted under UV light (Figure 2E). Although both transgenes did not influence SSC homing (Figure 2F and G), we noticed abnormalities in subsequent colony development. Interestingly, while GS^{WT} and $GS^{Kit\text{-}G559}$ could differentiate normally, GS^{Sl} cells could not initiate vertical differentiation in the recipient testes (Figure 2E, inset), suggesting that regulation of Kit activation is critical for completing spermatogenesis. Thus, activation of Kit did not influence GS cell proliferation or SSC homing into the germline niche but has an impact on subsequent differentiation.

SSC Activity of GS Cells with Kit Expression

To directly test whether Kit-expressing GS cells on laminin can colonize seminiferous tubules, we used magnetic activated cell sorting (MACS) (Figure 3A). EGFP-expressing fibroblastic GS cells were selected by anti-Kit antibody, and were used for selection. After selection, 5.2±1.8% (n = 3) of the cultured cells could be recovered, and cells were then microinjected into seminiferous tubules of W mice. Two months after transplantation, analysis revealed that Kit-expressing cells have SSC activity. Whereas control unfractionated cells produced 17.2±2.4 colonies/10^4 injected cells, Kit-expressing cells showed 13.3±2.3 colonies/10^4 injected cells (n = 18). The value was not statistically significant (Figure 3B).

To extend this observation, we next used fluorescence activated cell sorting (FACS) to fractionate GS cells on laminin according to Kit expression levels (Figure 3A and C). We initially characterized sorted cells by real-time PCR for spermatogonia marker expression. Real-time PCR analysis confirmed a difference in Kit expression levels, and showed stronger expression of several SSC markers, including Pou5f1, Zbtb16, and GFRα1, in Kit^+ cells (Figure 3D). Because GFRα1 is specifically expressed in A_s, and A_{pr} undifferentiated spermatogonia in vivo and, therefore, the $GFR\alpha1^+$ population did not express Kit [3], we also checked expression patterns of GFRα1 at the protein level by flow cytometry. FACS analysis of GS cells showed that GFRα1 expression is found in both Kit^+ and Kit^- cells (Figure 3E).

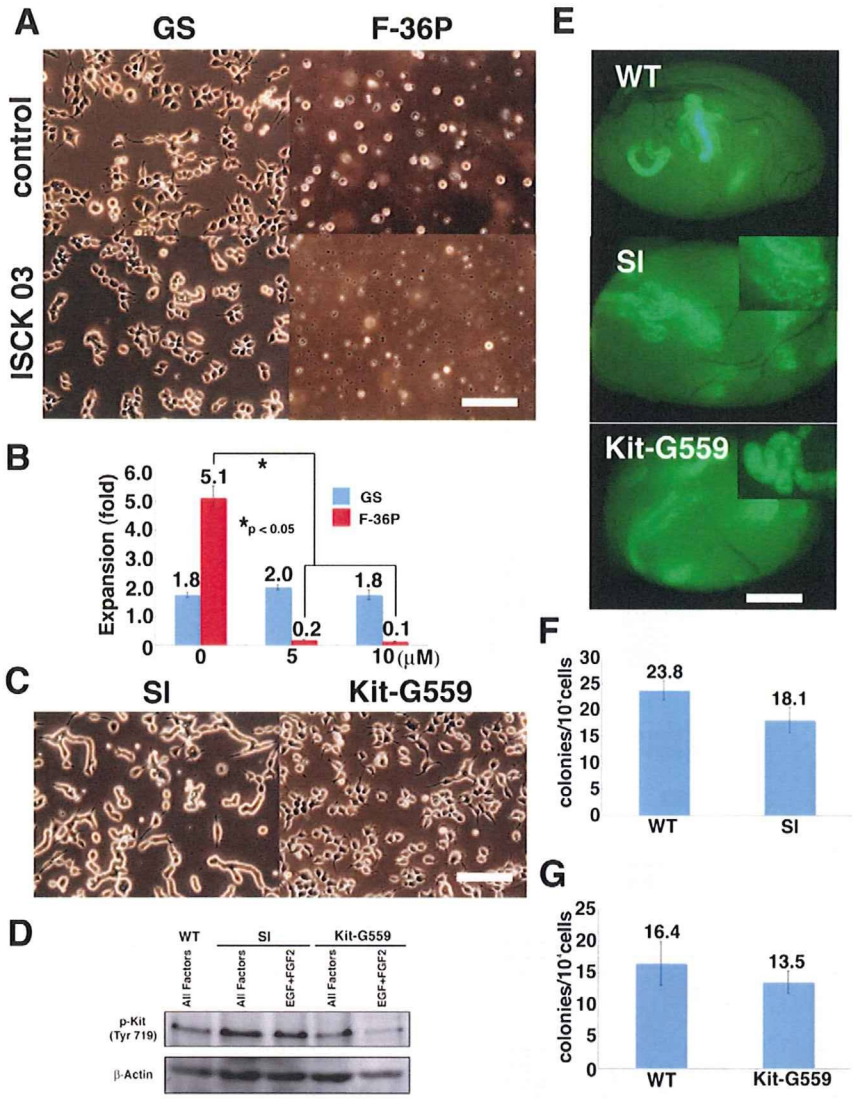

Figure 2. Dispensable role of Kit in GS cells. (A, B) Effect of Kit inhibitor (ISCK03) in colony morphology (A) and proliferation (B) of GS cells. Whereas the inhibitor could suppress the growth of the F-36P lymphocyte cell line effectively, no significant effect was found for GS cells. Cells were plated at $1\times10^5/3.8$ cm^2 and cultured with indicated cytokines for 5 (GS) or 3 (F36P) days. (C) Appearance of transfected GS cells. Note the elongated colonies of GS^{SI}. (D) Western blot analysis of transfected cells. GS^{SI} showed an enhanced phosphorylation of Kit. (E) Macroscopic appearance of recipient testes that received transfected GS cells. Whereas GS$^{Kit\text{-}G559}$ cells differentiated normally, GS^{SI} cells proliferated on the basement membrane and no vertical differentiation was observed (inset). (F, G) Homing efficiency of transfected cells. Approximately 8×10^3 cells were transplanted into each testis. No significant changes were induced by Kit-G559 (F) or SI (G) transfection. Bar = 100 μm (A, C); 1 mm (E).
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To compare proliferative potential, we cultured the sorted cells in vitro. Before initiating culture, cells from both fractions were microinjected into W mice directly to evaluate initial SSC content (Figure 3F). The remainder of the sorted cells was plated in culture for in vitro expansion. In these experiments, cells were plated on MEFs, because they promoted the survival of sorted cells more efficiently than did laminin possibly because of damage after sorting. In three sets of experiments, total cell numbers from both fractions expanded 8 to 55-fold during these 2 weeks of culture, regardless of Kit expression levels. After 2 weeks of culture, cells were transplanted into W mice to measure the increase in SSC numbers.

Analyses of recipient animals confirmed the results of MACS experiment; fresh Kit$^+$ produced 18.4 ± 1.2 colonies/10^4 injected cells ($n=14$), whereas Kit$^-$ cells yielded 16.5 ± 1.6 colonies/10^4 injected cells ($n=17$, Figure 3G). Differences between the two fractions were not statistically significant. Moreover, the concentration of SSCs in GS cell culture was also comparable after in vitro culture. Cultured Kit$^+$ and Kit$^-$ cells produced 20.0 ± 1.3 and 22.5 ± 2.2 colonies/10^4 injected cells ($n=14$), respectively. The overall increase in SSC number (SSC concentration × cell increase) in Kit$^+$ and Kit$^-$ cells was 17.5 and 16.0-fold, respectively, and the difference was not statistically significant (Figure 3H). Histological analysis confirmed normal spermatogenesis (Figure 3I). These results indicated that Kit$^+$GS cells not only had SSC activity but also underwent self-renewal division at a level comparable to Kit$^-$ GS cells.

Changes in SSC Phenotype In Vivo

Finally, we examined whether SSCs undergo phenotypic changes in vivo. We hypothesized that active proliferation of

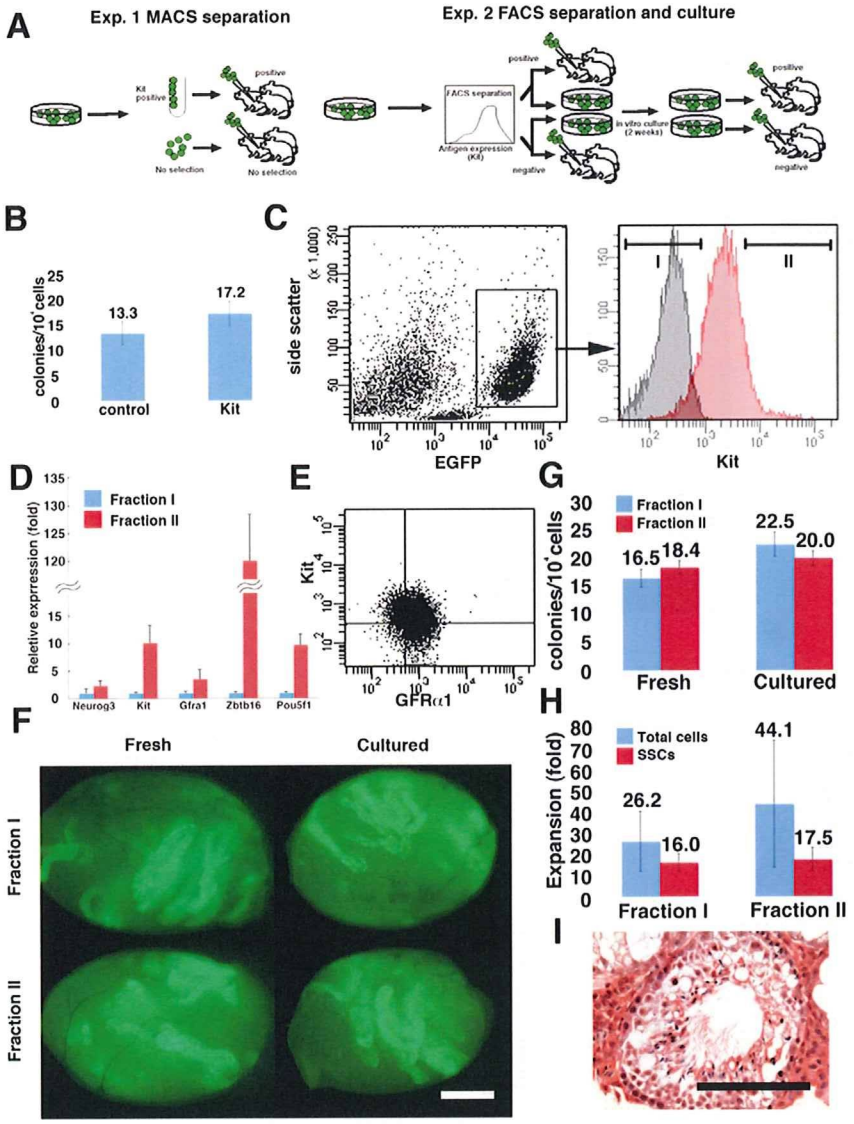

Figure 3. Fractionation of GS cells by Kit. (A) Experimental strategy. In the first experiment, Kit-expressing cells were selected by MACS. In the second experiment, GS cells were separated according to Kit expression levels by FACS. A portion of sorted cells was directly injected in each testis, and the rest of the cells were cultured for 2 weeks before transplantation. (B) SSC activity of MACS-separated cells. No significant difference was found. (C) Fractionation of GS cells by FACS. Distributions of stained (red) or control (black) are shown. (D) Real-time PCR analyses of sorted cells (n = 3–8). (E) Double immunostaining of GS cells by Kit and GFRa1. (F) Appearance of testes that received fresh and cultured cells. (G) SSC activity of fresh and cultured cells. No significant difference was found. (H) Increase in cell and SSC number after 2 weeks of culture. No significant difference was found. (I) Spermatogenesis in the recipient testis. Bar = 1 mm (F), 100 μm (I).
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SSCs might induce such changes and examined phenotypes of SSCs after germ cell transplantation. It is considered that SSCs expand in seminiferous tubules by increasing the probability of self-renewal division during the early phase of transplantation [23]. Three months after transplantation, however, transplanted cells establish a spermatogenic wave and produce spermatozoa.

We microinjected EGFP-expressing GS cells into the seminiferous tubules of W mice (primary recipients). The recipient animals were sacrificed at early (2 to 4 weeks) and late (3 to 4 months) time points after transplantation, and single cells were obtained by enzymatic digestion (Figure 4A). Expression of Kit or GFRα1 in donor cells could be specifically analyzed by gating cells with an EGFP donor marker (Figure 4B), which was downregulated during meiosis [11]. Whereas $EGFP^+$ cells showed a low side-scatter value in recipients at the early time point, they exhibited higher side-scatter value at late time point, indicating the progression of spermatogenesis [5]. Interestingly, development of this Kit^+ population in recipients did not depend on membrane-bound Sl, because ~20% of Kit^+ cells were found when GS cells were transplanted into Sl^d testes (Figure 4C–E). On the other hand, Sl^d testes were enriched with $GFR\alpha1^+$ cells, suggesting that germ cells in Sl^d testes were relatively undifferentiated. No significant difference in β1-integrin expression was observed.

We fractionated the $EGFP^+$ donor cells in the primary W recipient mice according to Kit or GFRα1 levels by cell sorting, and cells were retransplanted into seminiferous tubules of W mice (secondary recipients) to evaluate SSC activity. The number of colonies was smaller than were those from GS cells, suggesting that SSCs undergo more predominant differentiating divisions in vivo. Nevertheless, SSC activity was found in both Kit^- and Kit^-

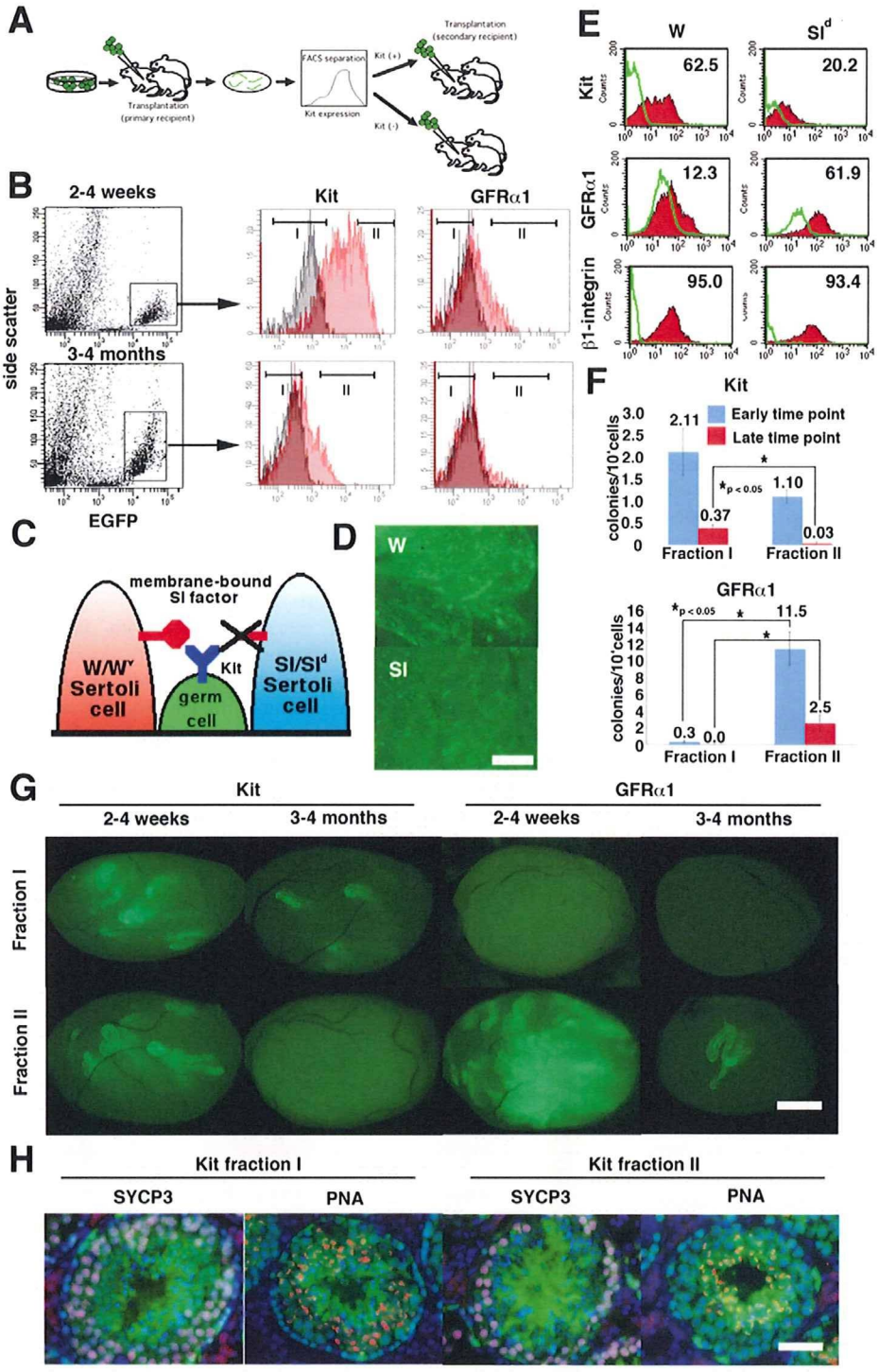

Figure 4. Changes in Kit expression in vivo. (A) Experimental strategy. After transplantation of GS cells, EGFP-expressing donor cells were fractionated according to Kit or GFRα1 levels. Sorted cells were transplanted into W mice. (B) Fractionation of donor spermatogenic cells. EGFP$^+$ cells were gated and fractionated into two groups according to Kit or GFRα1 levels. Distributions of stained (red) or control (black) are shown. (C) Sl-Kit interaction in W and Sl^d mice. Germ cells in W mice have a defect in Kit and cannot respond to Sl, whereas Sertoli cells in Sl^d mice do not express membrane-bound Sl and cannot support differentiation. (D) Appearance of W and Sl^d recipient testes 2 weeks after transplantation. Differentiation was limited in Sl^d testis. (E) FACS analysis of W and Sl^d recipient testis after transplantation. EGFP$^+$ cells were gated for analysis. (F) SSC activity of sorted cells. Both Kit$^-$ and GFRα1$^+$ cells showed significant enrichment of SSCs at both time points. (G) Appearance of recipient testes that received sorted cells. (H) Immunohistological section of the recipient testes that received Kit$^+$ or Kit$^-$ cells. The donor cells were collected from the primary recipient testes 2 weeks after transplantation, and the recipient testes were stained 2 months after cell sorting. The sections were stained with Rhodamine-PNA (red) for acrosomes and with anti-SYCP3 antibody (blue) for meiotic cells. Bar = 20 μm (D), 100 μm (G), 50 μm (H).
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