mouse IgG + IgM (H + L) (Jackson ImmunoResearch Laboratories, Inc.) and mouse anti-mouse Thy1.2 IgM antibodies (MCA02R; AbD Serotec, Oxford, UK). The panning plate was washed with PBS, and adherent RGCs were released by treatment with 0.125% trypsin for 10 min at 37°C. The RGC suspension was mixed with 30% fetal bovine serum and centrifuged at 200 *g* for 10 min. RGCs were suspended in medium containing 1 mM glutamine, 5 μg/ml insulin, 60 μg/ml N-acetylcysteine, 62 ng/ml progesterone, 16 μg/ml putrescine, 40 ng/ml sodium selenite, 0.1 mg/ml BSA, 40 ng/ml triiodothyronine, 0.1 mg/ml transferrin, 1 mM sodium pyruvate, 2% B27 supplement (Invitrogen), 10 μM forskolin (Sigma), 50 ng/ml brain-derived neurotrophic factor (BDNF; PeproTech, Rocky Hill, NJ), 50 ng/ml ciliary neurotrophic factor (CNTF; PeproTech), and 50 ng/ml basic fibroblast growth factor (bFGF; PeproTech) in Neurobasal medium (Invitrogen). Ninety-six–well culture plates were coated with poly-D-lysine (Sigma) and laminin (Sigma) and mouse RGCs were plated at a density of 4,000 cells/well (or 4,000 cells/culture insert for μ-dishes (ibidi)) and cultured for at least 10 days before the experiments.

Induction and detection of apoptosis induced by glutamate

RGCs were washed twice (15-min incubation, ×2) with Hank's Balanced Salt Solution (HBSS; Invitrogen) containing 2.4 mM $CaCl_2$ and 20 mM HEPES without magnesium. Subsequently, RGCs were incubated for 2 h at 37°C with or without 300 μM glutamate and 10 μM glycine (a co-activator of NMDARs) in HBSS containing 2.4 mM $CaCl_2$ and 20 mM HEPES without magnesium. After HBSS or glutamate treatment, RGCs were cultured for 22 h at 37°C in the same medium to culture the RGCs, but without forskolin, BDNF, CNTF, and bFGF. To detect apoptosis using Hoechst 33342 (Dojindo), RGCs were washed once with PBS and incubated with 1 μg/ml Hoechst 33342 for 15 min at room temperature. Fluorescent images were randomly taken (four images/well) using an Olympus IX71 fluorescence microscope. For each treatment, at least eight images were taken from two wells of a 96-well plate. Fragmented or shrunken nuclei stained with Hoechst dye were deemed apoptotic, and neurons with round and smooth nuclei were counted as healthy. More than 200 neurons for each treatment were counted by a researcher blinded to the identity of the samples.

Measurement of intracellular calcium

Mouse RGCs were incubated for 30 min at 37°C in culture medium with 3 μM Fluo-8 acetoxymethyl ester (AAT Bioquest). Cells were washed twice (15-min incubation, ×2) with HBSS containing 2.4 mM $CaCl_2$ and 20 mM HEPES without magnesium, then stimulated with 300 μM glutamate and 10 μM glycine. Fluorescence images were acquired every 500 msec using an ORCA-R2 digital CCD camera (Hamamatsu Photonics) and analyzed using the MetaFluor fluorescence-ratio imaging software (Molecular Devices).

Surface-biotinylation assay

Neuro 2A cells were plated at a density of 2×10^5/well in 6-well plates and cultured in 95% air/5% CO_2 at 37°C. The cells were transiently co-transfected with the cDNAs encoding NR1 and NR2D with (Dock3+) or without (Dock3-) Dock3. Forty-eight hours after transfection, cells were incubated in PBS containing 1.5 mg/ml Sulfo-NHS-SS-biotin (Pierce) for 20 min at 4°C. Surface biotinylation was stopped by removing that solution and incubating the cells in 10 mM ice-cold glycine in PBS for 20 min. Cells were rinsed twice in PBS and then lysed in 200 μl PBS with Complete Protease Inhibitor Cocktail, 0.1% SDS, and 1% Triton X-100. A fraction (15%, 30 μl) of the cell lysate was removed to measure total protein concentration and for total input; the remaining 85% (170 μl) of the cell lysate was incubated with 70 μl of 50% avidin-agarose (Sigma) overnight at 4°C. After washing three times with lysis buffer, bound proteins were resuspended in 30 μl of 2× sample buffer and boiled. Samples were analyzed by SDS-PAGE followed by Western blotting using anti-NR2D guinea-pig polyclonal antibody (1:1000). The data were quantified by measuring the ratios between intensities of the biotinylated and total NR2D bands using the Image Lab software (Bio-Rad). Surface/total ratios from the Dock3- control were assigned a value of 1. Ratios of the Dock3+ groups were expressed relative to the controls and averaged.

Statistical analysis

All data are expressed as mean ± S.E. Statistical analyses were conducted using Student's *t*-test for comparison between two samples, or one-way ANOVA followed by Bonferroni's test for multiple comparisons, using the SPSS 17.0 software package. P values < 0.05 were considered statistically significant.

Abbreviations

CNS: Central nervous system; DHR-1: Dock homology region 1; DHR-2: Dock homology region 2; Dock3: Dedicator of cytokinesis 3; GCL: Ganglion cell layer; GLAST: Glutamate aspartate transporter; HEK: Human embryonic kidney; INL: Inner nuclear layer; MOCA: Modifier of cell adhesion protein; NMDAR: N-methyl-D-aspartate receptor; NTG: Normal tension glaucoma; PBP: Presenilin binding protein; PBS: Phosphate-buffered saline; PCP: Phenycyclidine; PMSF: Phenylmethylsulfonyl fluoride; PS: Presenilin; PVDF: Polyvinylidene difluoride; RGC: Retinal ganglion cell; TCL: Total cell lysate; WT: Wild-type.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

KT, TA and NB conceived and designed the experiments. NB carried out all experiments except the experiments performed on primary cultured RGCs and analyzed the data. HH carried out the experiments performed on primary cultured RGCs and analyzed the data. KN, TH and MM contributed

reagents and materials. KT and NB wrote the paper. All authors have read and approved the manuscript for publication.

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特集2

中枢神経のトランスポーター・チャネル：新たな創薬標的として

精神神経疾患とグルタミン酸神経伝達：基礎医学的観点から

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SUMMARY

グルタミン酸は，中枢神経系において主要な興奮性神経伝達物質であり，記憶・学習などの脳高次機能に重要な役割を果たしている．しかし，その機能的な重要性の反面，興奮毒性という概念で表されるように，過剰なグルタミン酸は神経細胞障害作用を持ち，さまざまな精神神経疾患に関与すると考えられている．著者らは，グルタミン酸の細胞外濃度を制御するグリア型グルタミン酸トランスポーターの機能を阻害したマウスを作製し，そのマウスに神経細胞変性や社会行動の障害・強迫性行動・統合失調症様の行動異常が起こることを発見した．グリア型グルタミン酸トランスポーターを活性化する化合物は，新しい抗精神神経疾患薬として有用であると期待される．

KEY WORDS
グルタミン酸
トランスポーター
統合失調症
うつ病
神経変性疾患

はじめに

グルタミン酸は哺乳類の中枢神経系において記憶・学習などの高次機能を調節する主要な興奮性神経伝達物質として知られている[1]．一方で，細胞外グルタミン酸の上昇は，グルタミン酸受容体の過剰な活性化によりグルタミン酸興奮毒性とよばれる神経細胞障害作用を持ち，多くの精神疾患に関与している（**図1,2**）[2]．細胞外グルタミン酸濃度は，グルタミン酸トランスポーターにより厳密に制御されている．これまで，5種類のグルタミン酸トランスポーターサブタイプ，EAAT1（GLAST），EAAT2（GLT1），EAAT3（EAAC1），EAAT4，EAAT5が単離され，その分子的実態が明らかにされている．GLAST，GLT1は主にアストロサイトに，EAAC1とEAAT4は神経細胞に，EAAT5は網膜に発現している．シナプス間隙におけるグルタミン酸の除去は，主にアストロサイトに存在する2種類のグルタミン酸トランスポーターGLAST,GLT1の活性により制御されている．本稿では，GLAST,GLT1の機能障害に着目し，その機能障害がどのような精神神経疾患の発症に関与するかを概説する．

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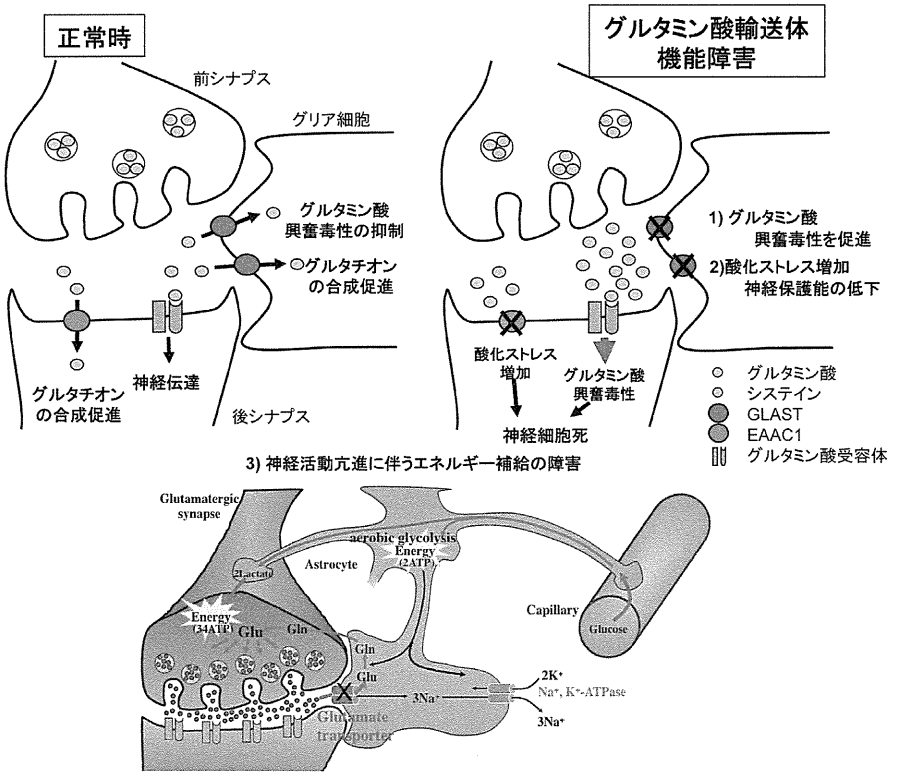

図1　グルタミン酸トランスポーターの機能障害が神経機能におよぼす影響

（p.5 カラー図参照）

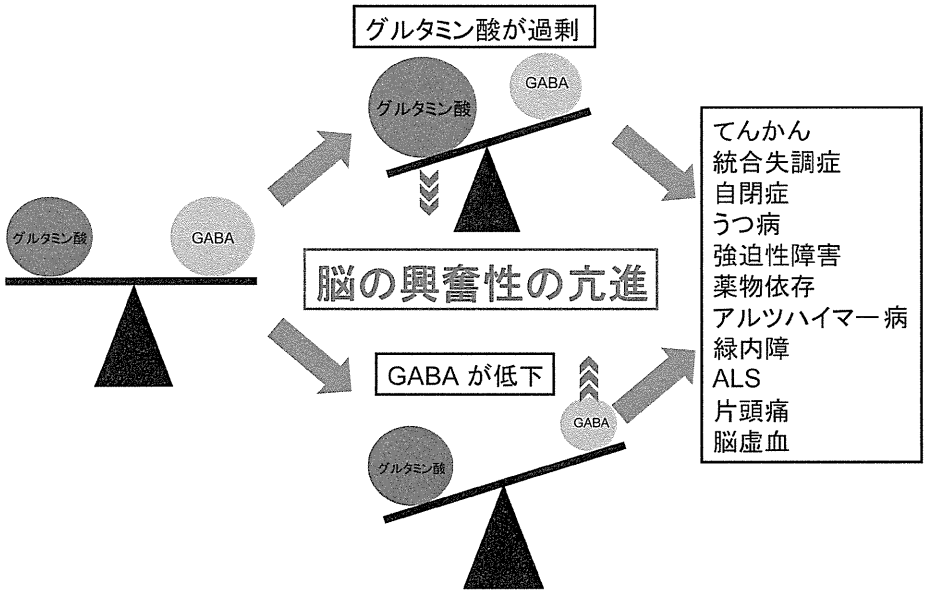

図2　グルタミン酸トランスポーターの機能異常による興奮と抑制のアンバランスがさまざまな精神神経疾患を引き起こす

I. グルタミン酸トランスポーターと精神疾患

1. 統合失調症における GLAST・GLT1 の関与

統合失調症は，幻覚・妄想などの陽性症状と，無為自閉，感情鈍麻，意欲の減退などの陰性症状，ワーキングメモリーなどの障害による認知障害を示し，世界中でおよそ 100 人に 1 人が発症する精神疾患である．NMDA 受容体欠損マウスや NMDA 受容体阻害剤を投与された動物が統合失調症様の症状を示すことから，グルタミン酸神経伝達の低下が統合失調症の有力な病態であると考えられている．しかし，最近の臨床試験結果から，グルタミン酸の放出を抑制する代謝型グルタミン酸受容体 mGluR2/3 のアゴニストが統合失調症の治療薬として有望であることが報告された[3]．この報告は，統合失調症では細胞外グルタミン酸濃度が上昇し，脳全体として興奮性優位となっている可能性を示唆している．さらに，統合失調症患者の遺伝子解析から，GLAST 遺伝子座の欠失や GLT1 のミスセンス変異がある症例が報告された[4,5]．そこで，まず GLAST 欠損マウスの行動解析を行った．GLAST 欠損マウスは，新規環境下に置かれると，野生型に比べ行動量が増加し（**図 3**），その増加はハロペリドールや mGluR2/3 のアゴニスト（LY379268）などの統合失調症治療薬で改善される[6]．さらに，グルタミン酸受容体阻害剤である MK-801 により誘発される行動量の増加が，GLAST 欠損マウスでは悪化する（**図 3**）[6]．これらの行動異常は，統合失調症の陽性症状に相当する．また，GLAST 欠損マウスは，新奇マウスに対する sniffing などの行動時間が減少し（**図 3**），巣作り行動も障害されており，社会行動に障害がみられた[7]．これらの行動異常は，統合失調症の陰性症状に相当する．さらに，GLAST 欠損マウスは pairwise discrimination task が障害され，統合失調症の認知障害に類似した症状を示す[7]．これらの結果は，GLAST 欠損マウスが統合失調症のモデル動物であることを示しており，「アストロサイトに発現するグルタミン酸トランスポーター GLAST の機能異常によるグルタミン酸機能亢進」が統合失調症の発症に重要な役割を果たすと考えられる．GLT1 に関しては，統合

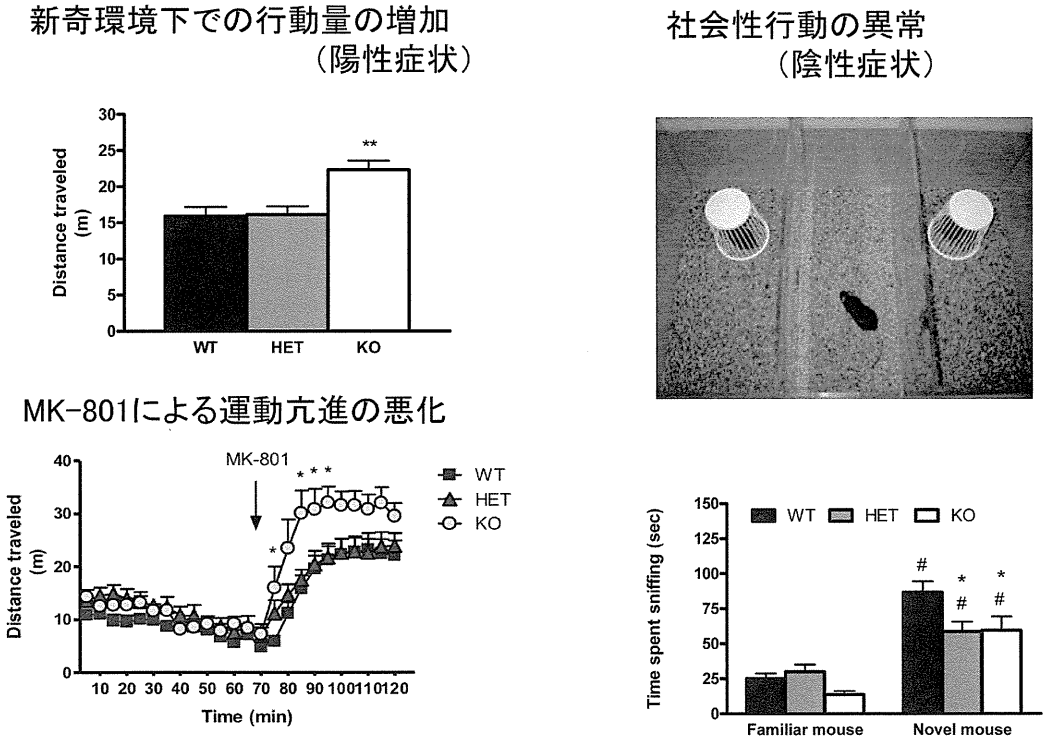

図 3　グルタミン酸輸送体 GLAST 欠損マウスは統合失調症に似た行動異常を示す

失調症の患者さんの約0.1%にArg106Hisのミスセンス変異がみられることが，最近報告された[5]．著者らは，このミスセンス変異によりGLT1のグルタミン酸取り込み活性が低下することを見出している．現在，GLT1の発現を低下させたマウスを用いて，統合失調症様行動異常が観察されるかどうか解析している．

統合失調症の主要な仮説の一つとして，「神経発達障害仮説」がある．統合失調症患者では，海馬・扁桃体の細胞構築の異常や萎縮，側脳室・第Ⅲ脳室の拡大が認められる．そこで著者は，グルタミン酸トランスポーターの機能障害が統合失調症に似た神経発達障害を引き起こすのではないかと考えた．統合失調症患者で遺伝子異常が報告されているGLASTおよびGLT1の両者を欠損させたダブル欠損マウス（DKマウス）を作製し，脳形成期におけるグルタミン酸機能亢進状態を再現した[8]．DKマウスは胎生17日以降に死亡し，大脳皮質・海馬の層形成障害，扁桃体の核形成障害，側脳室・第Ⅲ脳室の拡大など統合失調症に似た神経発達障害を示した．これらの脳発達障害は，グルタミン酸受容体NR1の欠損により正常化された[9]．さらに，DKマウスの脳では，視床に神経細胞死がみられた．統合失調症患者では，視床の体積減少，課題遂行中の血流低下，代謝異常が報告されている．以上の結果は，胎児期のグルタミン酸トランスポーターGLAST・GLT1の機能障害は，統合失調症に似た神経発達障害を起こすことを示している．また，統合失調症のリスク要因として報告されている胎児期・周産期の栄養障害・脳虚血・ウイルス感染は，グルタミン酸トランスポーターの機能を阻害することが知られている．これらのことは，アストロサイトに発現するグルタミン酸トランスポーターの機能障害は，統合失調症の発症に関与することを示している．

最近，統合失調症の発症危険状態を発症へと移行させる要因として，海馬における細胞外グルタミン酸濃度の上昇が報告された[10]．著者らは，グルタミン酸トランスポーターを一過性に欠損させるマウスを作製しており，そのマウスの解析は統合失調症の発症危険状態を発症へと移行させる機序を解明するのに貢献すると期待される．統合失調症の発症危険状態を発症へと移行させる機序の解明は，早期介入による統合失調症の発症予防法の開発に役立つと考えられる．

2. 強迫性障害・自閉症におけるGLT1の関与

強迫性障害は，強迫観念・強迫行為を特徴とする疾患である．多くは思春期過ぎから発症し，人口の2～3%が罹患歴を持つ．従来，セロトニン神経伝達の異常が強迫性障害に関与すると考えられてきた．しかし，セロトニン神経伝達を上昇させる抗うつ薬などの治療では，一部の患者さんにしか効果がなく，セロトニン神経伝達の障害だけでは強迫性障害の病態を説明できない．最近，グルタミン酸神経伝達の亢進が強迫性障害の発症にも重要な役割を果たすことが報告されている．主なものに，(1) 強迫性障害患者の脳内ではグルタミン酸量が増加し，これによる神経伝達が亢進している (2) グルタミン酸神経伝達に関わる遺伝子の一塩基多型頻度が強迫性障害患者では増加している (3) グルタミン酸神経伝達を抑制する薬剤に強迫性障害の治療効果がある，といったことがあげられる[11]．

自閉症は，社会性行動の喪失や言語発達の遅延を特徴とする脳高次機能の発達障害である．グルタミン酸神経伝達系の亢進は自閉症の重要なリスクであり，脳の形成にきわめて重要な役割を持つ．自閉症様の行動を示す脆弱X症候群や結節性硬化症の患者ではグルタミン酸神経伝達の亢進が報告されている．自閉症のゲノムワイドな連鎖解析により，11番染色体の11p12-13が自閉症に関連があることが明らかになった[12]．この領域はGLT1の遺伝子座である．

最近，著者らは誘導型Cre & loxPシステムを使って生後3週齢にGLT1遺伝子の欠損を誘導すると，マウスの思春期に相当する7週齢の時点で，GLT1蛋白質の量は，対照群と比べて約30%にまで減少した．このマウスは，過度な毛繕い行動を示すようになり，顔面には激しい裂傷が生じた．このマウスの痛覚や皮膚に異常がみられないことから，過度な毛繕い行動は中枢神経系の異常に起因すると考えられる．この行動異常は，強迫性障害と自閉症に共通する主要な症状である「繰り返し行動」に相当する．このモデルでは，不安の亢進や社会性行動の異常がみられず，純粋な「繰り返し行動」のみを再現しており，疾患モデルではなく症状モデルと考えられる．現在，このモデルを用いて，「繰り返し行動」の神経基盤や新規治療薬の開発を行っている．

II. グルタミン酸トランスポーターと神経疾患

1. アルツハイマー病における GLT1 の関与

アルツハイマー病は，神経変性による起こる認知症で，高齢化により患者数は増加し，超高齢化社会を迎える日本にとって根本的な治療法の確立が望まれている．アルツハイマー病の病態に過剰なグルタミン酸受容体の活性化が関与することは，グルタミン酸受容体阻害剤であるメマンチンが治療薬として用いられていることからも明らかである．グルタミン酸トランスポーターの障害がアルツハイマー病の発症に関与することを示す証拠として，(1) アルツハイマー病患者の脳では GLAST，GLT1，EAAC1 の発現量が減少している[13]，(2) アルツハイマー病モデルマウスの GLT1 の発現量を低下させると空間学習の障害が促進される[14]，(3) アルツハイマー病における神経変性の原因物質と考えられているβアミロイド蛋白により GLT1 の機能が障害される，などがある．GLT1 の活性化化合物が，新規アルツハイマー病の治療薬として有効かどうか，モデル動物による評価が期待される．

2. 正常眼圧緑内障における GLAST の関与

緑内障は，40 歳以上では約 5%が潜在的に罹患していると考えられており，日本人の中途失明原因の第 1 位である．さらに，高齢化により患者数は増加し，その治療は活力ある高齢化社会を作るためには必要不可欠である．わが国の緑内障の約 70%は正常眼圧緑内障であり，その病態は不明である．著者らは，グルタミン酸トランスポーター GLAST 欠損マウスが，正常眼圧緑内障と同じ症状を示すことを明らかにした（眼圧が正常であるにも関わらず，網膜神経節細胞が加齢に伴い選択的に変性し，視神経乳頭陥凹が拡大する）（**図 4**）[15]．さらに，緑内障患者の約 1%が，GLAST の機能障害を伴うミスセンス変異を持つことをみつけた．この結果は，GLAST の遺伝子異常によるグルタミン酸輸送活性の低下が，緑内障の発症に関与していることを示している．また，GLAST の発現を増加させる化合物をみつけ，その化合物が GLAST ヘテロマウスの緑内障様症状を改善することを見出した．

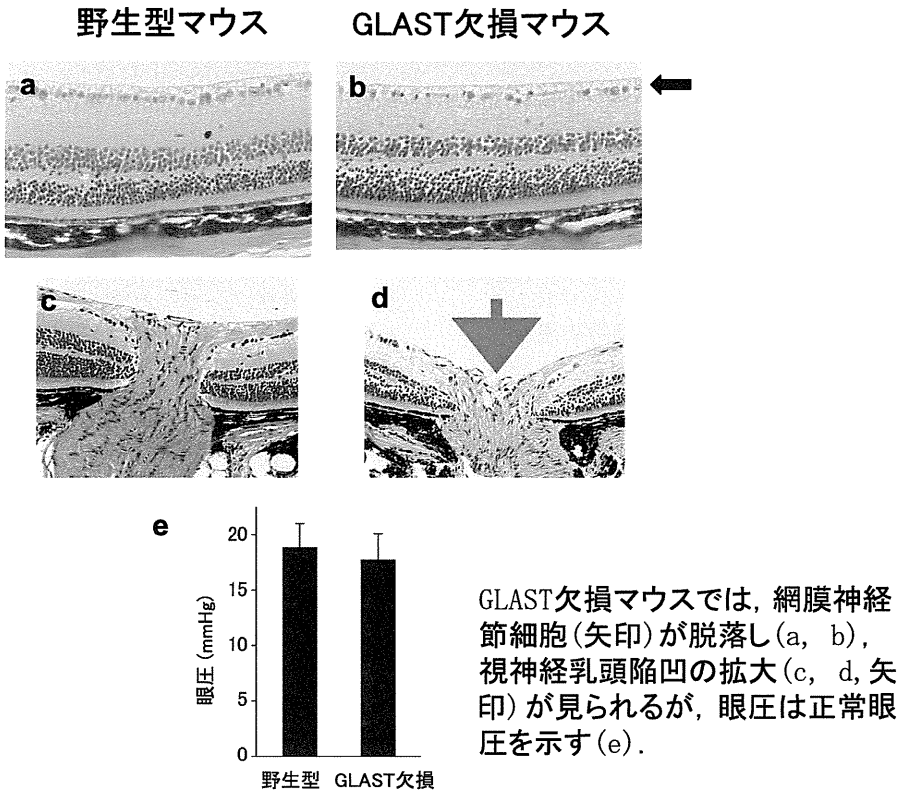

図 4　GLAST 欠損マウスはヒト正常眼圧緑内障と同様の異常を示す

（p.5 カラー図参照）

おわりに

著者らは，グルタミン酸の細胞外濃度を制御するグルタミン酸トランスポーターGLAST，GLT1の機能を阻害したマウスを作製し，そのマウスに社会性行動の異常・繰り返し行動・統合失調症の陰性症状や陽性症状に相当する行動異常が起こることを発見した．また，これらのマウスに，細胞種特異的な神経細胞死が起こることを見出した．さらに，統合失調症・うつ病・強迫性障害・筋萎縮性側索硬化症・ハンチントン病・アルツハイマー病・緑内障などさまざまな精神神経疾患において，グリア型グルタミン酸トランスポーターの異常が報告されている．

グリア型グルタミン酸トランスポーターの機能障害は，どのようにして神経細胞死および神経細胞の形成・機能障害をもたらすのか？現在までわかっている機序としては，以下の三つが考えうる（**図1**）．1）細胞外グルタミン酸濃度の上昇によるシナプス外グルタミン酸受容体および隣接シナプスのグルタミン酸受容体の活性化，2）グリア内グルタミン酸の枯渇がもたらすグルタチオン合成の減少による酸化ストレスの亢進，3）神経活動が亢進している部位への選択的エネルギー補給システムの障害[16]．

著者らは，さまざまな精神神経疾患の中に，グルタミン酸トランスポーターの異常が原因で発症する患者が一定の割合存在し，「グルタミン酸トランスポーター機能異常症候群」として分類できると考えている．グルタミン酸トランスポーター機能異常症候群の患者には，グルタミン酸トランスポーターの取り込み活性を促進する薬物が，共通した治療薬として有効であると期待される[17]．

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Review Article

Translating human genetics into mouse: The impact of ultra-rapid *in vivo* genome editing

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Gene-targeted mutant animals, such as knockout or knockin mice, have dramatically improved our understanding of the functions of genes *in vivo* and the genetic diversity that characterizes health and disease. However, the generation of targeted mice relies on gene targeting in embryonic stem (ES) cells, which is a time-consuming, laborious, and expensive process. The recent groundbreaking development of several genome editing technologies has enabled the targeted alteration of almost any sequence in any cell or organism. These technologies have now been applied to mouse zygotes (*in vivo* genome editing), thereby providing new avenues for simple, convenient, and ultra-rapid production of knockout or knockin mice without the need for ES cells. Here, we review recent achievements in the production of gene-targeted mice by *in vivo* genome editing.

Key words: clustered, regularly interspaced, short palindromic repeats- associated endonuclease, genome editing, mouse, transcription activator-like effector nucleases, zinc-finger nucleases.

Introduction

The mouse has become the most commonly used animal model system in the biological and medical sciences because its genome can be specifically and precisely modified as desired (Capecchi 2005). The invaluable advantage of the mouse is the ability to perform homologous recombination in embryonic stem (ES) cells, an essential step in gene targeting and a technology that was unavailable in the majority of other mammalian species. Since the first success of gene targeting in mouse, thousands of mice, mainly knockout mice created by insertion of a selection marker or reporter into a target gene locus, have been created, unveiling the *in vivo* functions of the genes. As an extension of these efforts, large-scale international consortia were organized to provide knockout mice for all protein-coding genes and systematically analyze the resulting phenotypes (Sung *et al.* 2012; Menke 2013). The International Knockout Mouse Consortium (IKMC) released targeted ES cell lines, including knockout, conditional, and gene-trapped alleles, for more than 18 000 genes, in addition to mice targeted for over 2600 loci (Skarnes *et al.* 2011). Further, the Sanger Institute Mouse Genetics Project (MGP), a founding member of the International Mouse Phenotyping Consortium (IMPC), recently released pilot data from a large-scale systematic phenotype analysis of 489 knockout mouse strains, derived from more than 900 IKMC knockout ES cell lines (White *et al.* 2013). Surprisingly, unbiased screening by the MGP identified many previously unknown phenotypes in both new knockout mice and strains that were the subject of earlier reports. The IMPC will expand this phenotypic screening to cover 5000 knockout mouse lines over the next 4 years and to all 20 000 protein-coding genes in the future. These genome-wide and large-scale systematic knockout mouse resources are now publicly available; thus, researchers can focus their efforts on the detailed functional analysis of genes of interest, rather than on the construction of mouse lines.

Recent advances in genomic microarray and next generation sequencing technologies have revealed the landscape of human genetic diversity, which comprises tens of millions of common and rare variants associated with health and disease (Raychaudhuri 2011; 1000 Genomes Project Consortium *et al.* 2012). Although large-scale genome-wide association studies

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(GWAS), based on high-density genomic microarray technology, have identified hundreds of frequent variants associated with common and complex human diseases and traits, the majority of these are responsible for only a small amount of the disease risk (Manolio *et al.* 2009). In contrast, with recent advances in unbiased whole-genome and whole-exome sequencing approaches, inherited and rare *de novo* single nucleotide variants (SNVs) are now also believed to be important in common and complex diseases (Cirulli & Goldstein 2010; Veltman & Brunner 2012). The best examples are large-scale exome sequencing studies on hundreds of patient-parent trios or quartets for autism spectrum disorders (ASDs), which are neurodevelopmental conditions characterized by impairments in social interaction and stereotyped behaviors with a strong genetic component (Iossifov *et al.* 2012; Neale *et al.* 2012; O'Roak *et al.* 2012; Sanders *et al.* 2012). These studies consistently report higher rates of *de novo*, especially nonsense, splice site, or frameshift SNVs in patients with ASD than in their unaffected siblings (Veltman & Brunner 2012). Further, they also unveiled extreme genetic heterogeneity in ASD, as evidenced by the uncovering of *de novo* SNVs in hundreds of different genes in different individuals. This indicates the need for further efforts to investigate the biological consequences of these rare *de novo* SNVs (Veltman & Brunner 2012).

One possible approach to address the biological function of these SNVs, as well as determining whether they are causal for the human phenotype of interest, is the use of genetic mouse models incorporating the identified variants. Precisely modified knockin mouse models carrying such human SNVs provide a unique and direct approach for the investigation of the functional consequence of variants *in vivo*. Pioneer work in this field by Südhof and colleagues reported that a knockin mouse carrying a neuroligin-3 R451C SNV found in a subset of ASD patients exhibited abnormal behaviors that resembled those of human patients, in addition to abnormal synaptic transmission (Tabuchi *et al.* 2007). Importantly, in contrast to R451C knockin mouse, neuroligin-3 knockout mouse did not exhibit such abnormalities, suggesting that the R451C SNV represents a gain-of-function mutation (Tabuchi *et al.* 2007). Similarly, in Rett syndrome, an ASD caused by mutations in methyl-CpG-binding protein 2 (MeCP2), many SNVs throughout the *MECP2* gene were identified and several knockin mouse lines carrying each SNV provided important insights into the biological and phenotypic significance of each variant, as well as identifying downstream targets of MeCP2 (Tao *et al.* 2009; Jentarra *et al.* 2010; Cohen *et al.* 2011; Goffin *et al.* 2011; Ebert *et al.* 2013; Lyst *et al.* 2013). Taken together these studies demonstrate that mouse models for human SNVs, rather than simple knockout mice, are essential and extremely valuable tools for the biological and phenotypic interpretation of human variants and the development of novel treatments.

Although the demand for precisely modified knockin mouse models carrying human SNVs is growing, a recent review by Menke reported that only 600 such mice could be found in the Mouse Genome Informatics database (Menke 2013). This is partially due to the difficulty of generating such mice by conventional gene targeting technology using homologous recombination in ES cells; a time-consuming, laborious, and expensive process (Capecchi 2005).

In vivo genome editing in mice

The recent emergence and drastic evolution of genome editing technologies is revolutionizing gene targeting in the mouse (Sung *et al.* 2012; Menke 2013). The methods are based on molecular tools, including zinc-finger nucleases (ZFNs), transcription activator-like effector (TALE) nucleases (TALENs), and clustered, regularly interspaced, short palindromic repeats (CRISPR) and the CRISPR-associated endonuclease (Cas), known as the CRISPR/Cas system. These methods provide exciting and groundbreaking opportunities, enabling direct and rapid gene targeting in fertilized mouse eggs, with no need for ES cells. The basic characteristics and various applications of these genome editing technologies will be discussed by others in this special issue of DGD. Here, we focus on gene targeting in the mouse by *in vivo* genome editing and review current achievements, issues to be solved, and future applications.

The principle of *in vivo* editing involves targeting of the genome by direct microinjection of plasmid DNA or mRNA encoding editing tools (ZFNs, TALENs, or CRISPR/Cas) into the cytoplasm or pronuclei of one-cell embryos to generate a DNA double-strand break (DSB) at a specific target locus (Fig. 1, Urnov *et al.* 2010; Joung & Sander 2013). The DSB is subsequently repaired by two major cellular endogenous DNA damage repair pathways; the error-prone, non-homologous end-joining (NHEJ) route, which results in small deletions or sequence insertions into the DSB site, and the homology-directed repair (HDR) pathway, which relies on a donor DNA template with homology to the DSB site to achieve precise homologous recombination. NHEJ occurs rapidly and preferentially, often leading to frameshift mutations and loss-of-function of the targeted genes, resulting in a knockout mouse when the protein-coding sequence is targeted (Fig. 1,

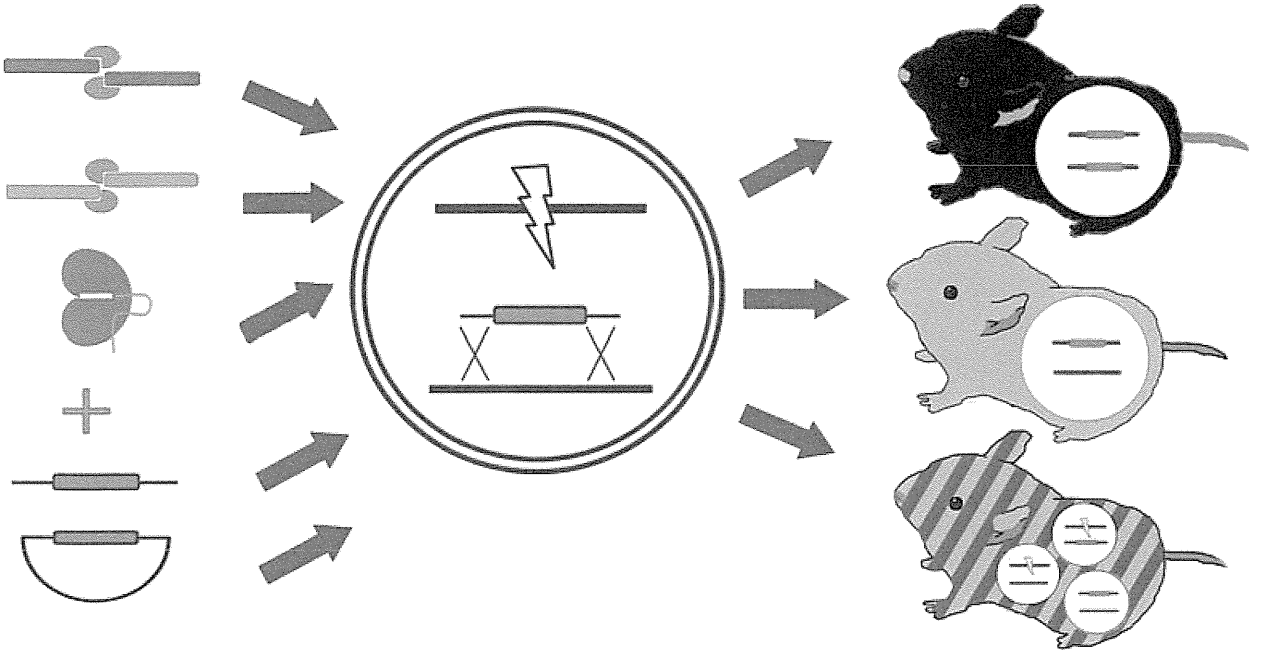

Fig. 1. Schematic diagram of *in vivo* genome editing in mouse. Transcription activator-like effector nucleases (TALENs) (blue), zinc-finger nucleases (ZFNs) (pink), or clustered, regularly interspaced, short palindromic repeats-associated endonuclease (CRISPR/Cas) (green) are microinjected into one-cell fertilized eggs (circle in the middle) derived from wildtype mice with or without ssOligo or targeting vector for knockin mouse production. Then, double-strand break (DSB) and subsequent homology-directed repair (HDR) (red) and/or non-homologous end-joining (NHEJ) (yellow) are induced within one-cell fertilized eggs, resulting in genetically-mosaic (striped), monoallelically-targeted heterozygous (gray-colored), or biallelically-targeted homozygous (black-colored) mutant mice at F0.

Carbery *et al.* 2010; Sung *et al.* 2013; Shen *et al.* 2013; Wang *et al.* 2013a). HDR infrequently occurs and leads to precise and specific genome modifications, such as SNV substitutions, insertions, deletions, or gene replacement, when a targeting vector or synthetic single-strand oligonucleotide (ssOligo) is co-microinjected into mouse embryos, resulting in a knockin mouse (Fig. 1, Meyer *et al.* 2010; Cui *et al.* 2011; Meyer *et al.* 2012; Wefers *et al.* 2013; Wang *et al.* 2013a; Yang *et al.* 2013). A series of groundbreaking successes indicate that *in vivo* genome editing in the mouse is robust and has great potential as an alternative to the conventional gene targeting approach.

Knockout mice

After the first success of NHEJ-mediated gene knockout by *in vivo* genome editing in mammals was achieved in rat with ZFNs targeting three different genes (Geurts *et al.* 2009), it was rapidly applied to mouse (Table 1). Cui and colleagues from Sigma-Aldrich, the exclusive supplier of ZFNs, reported the first knockout mice by *in vivo* genome editing with ZFNs (Carbery *et al.* 2010). They targeted three endogenous genes in both FVB/N and C57BL/6J strains with targeting efficiencies from 20 to 75% of live newborns and no off-target effects at 20 potential sites. The founder mice were heterozygous or genetically mosaic, carried more than one mutant allele, and successfully produced F1 mutant progeny. Importantly, homozygous mice were generated from F1 mutants within 4 months (Fig. 2).

Similar work was performed for the targeting of the *ROSA26* locus, a safe harbor often used for gene targeting (Hermann *et al.* 2012). Although the efficiency in this study was <10%, one of the founders had a biallelic modification, resulting in an F0 biallelic knockout mouse that drastically reduced the time taken to produce homozygous knockouts (Fig. 2).

Although the simple modular DNA recognition code of TALENs and the existence of publicly available resources has resulted in the rapid expansion of TALENs as versatile genome editing tools, the first knockout mice by TALEN-mediated *in vivo* genome editing were reported in 2013 (Sung *et al.* 2013). Two endogenous genes were targeted with efficiencies from 49 to 77% in live newborns with no off-target effects. All alleles present in F0 founders were successfully transmitted to F1 progeny. Importantly, targeting and biallelic modification efficiencies were increased when microinjection was performed with a high dose of TALEN mRNA; when 50 ng/μL of TALEN

Table 1. Summary of non-homologous end-joining (NHEJ) in mice by *in vivo* genome editing

Nucleases	Targets	Genes	NHEJ (%)	Off-target	F1	Refs
ZFNs	3	*Mdr1a, Jag1, Notch3*	19.5–77.3	None	Yes	Carbery *et al.* (2010)
ZFNs	1	*ROSA26*	22.4[†]	n.d.	n.d.	Meyer *et al.* (2010)
ZFNs	1	*Mdr1a*	7.5–75.0[‡]	n.d.	n.d.	Cui *et al.* (2011)
ZFNs	1	*ROSA26*	4.6–8.7[†]	n.d.	n.d.	Hermann *et al.* (2012)
TALENs	2	*Pibf1, Sepw1*	48.7–76.9[†]	None	Yes	Sung *et al.* (2013)
TALENs	1	*Rab38*	2.0–6.0	n.d.	Yes	Wefers *et al.* (2013)
TALENs	1	*Zic2*	10.0–46.7	n.d.	Yes	Davies *et al.* (2013)
TALENs	10	*Lepr, Pak1ip1, Gpr55, Rprm, Fbxo6, Smurf1, Tmem74, Wdr20a, Dcaf13, Fam73a*	12.5–66.7[†]	None[§]	Yes	Qiu *et al.* (2013)
TALENs	1	*Mlkl*	18.2[†]	n.d.	Yes	Wu *et al.* (2013)
TALENs	2	*C9orf72, Fus*	7.5–41.2	None	Yes	Panda *et al.* (2013)
CRISPR/Cas	2	*Pouf5-EGFP, CAG-EGFP*	14.3–20.0	n.d.	n.d.	Shen *et al.* (2013)
CRISPR/Cas	3	*Tet1, Tet2, Tet3*	66.7–100.0[†,¶]	None	n.d.	Wang *et al.* (2013a)
CRISPR/Cas	3	*Th, Rheb, Uhrf2*	75.0–91.7[††]	None	Yes	Li *et al.* (2013)

[†]Including biallelic modification, [‡]mouse data only, [§]for *Smurf1*, [¶]57.8–78.5% for biallelic modification for Tet1 and Tet2, [††]RNA and mouse data only. n.d., not determined. F1 represents germline transmission. Percentages of NHEJ were calculated using the number of NHEJ positive pups as the numerator and the number of the total pups as the denominator. Fetus data were included in some studies.

mRNA targeting the *Pibf1* gene was injected, six of eight F0 founders had biallelic modifications. Similar results were obtained for the *Sepw1* gene, and the absence of Sepw1 protein was confirmed in the biallelically targeted F0 mutants. This work suggests that highly active TALENs are critical for efficient targeting and biallelic modification. Homozygous knockout mice can be generated within 1 month by TALEN-mediated *in vivo* genome editing (Fig. 2).

Since the first report, a flood of knockout mice generated by TALEN-mediated *in vivo* genome editing has been reported. Davies *et al.* (2013) targeted the *Zic2* gene by TALENs in three different mouse strains, including CD1, C3H, and C57BL/6J. Targeting efficiencies producing live newborns or blastocysts varied, with 10%, 23%, and 46% for C57BL/6J, C3H, and CD1, respectively.

Li and colleagues generated a series of knockout mice for 10 genes, revealing the utility, convenience, and robustness of TALEN-mediated *in vivo* genome editing (Qiu *et al.* 2013). Targeting efficiencies varied from 13 to 67%, with an average of 40%, of live newborns. By using one TALEN for the *Lepr* gene, which encodes the Leptin receptor, they showed that there was no difference in the targeting efficiency between two different mouse strains (C57BL/6N and FVB/N).

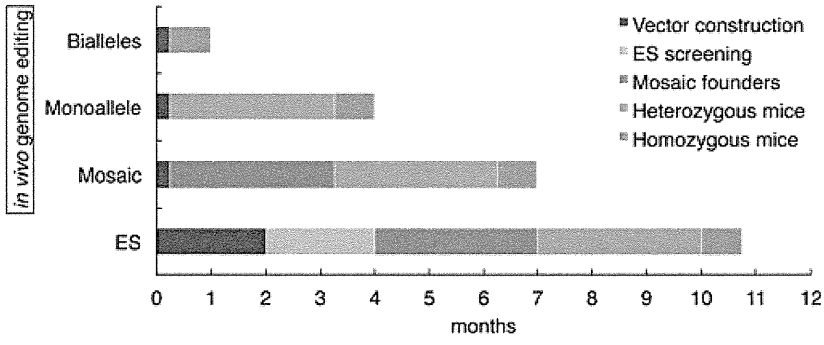

Fig. 2. Time course for homozygous mutant mouse production. ES: embryonic stem (ES)-cell based traditional gene targeting method. Mosaic: genetically-mosaic founders are obtained at F0 by *in vivo* genome editing. Monoallele: monoallelically-targeted heterozygous founders are obtained at F0 by *in vivo* genome editing. Bialleles: biallelically-targeted homozygous founders are obtained at F0 by *in vivo* genome editing. Green: the term to obtain F0 adult mosaic founders from microinjection of genome editing tools, or F0 adult chimeric founders from ES cell microinjection. Orange: the term to obtain adult heterozygous mice from mating of F0 founders with wildtype mice or from microinjection of genome editing tools. Magenta: the term to obtain homozygous newborns from mating of heterozygous mice or from microinjection of genome editing tools. Best cases are shown.

Among the F0 founders, one had biallelic modifications with different frame-shift deletions and exhibited an obese phenotype resembling that of Lepr mutant db/db mice. Further, there were no off-target effects, even at sites with only one mismatch to each TALEN. All the F0 founders tested transmitted the mutant alleles to F1 mice with high efficiency. These results suggest that this method of genome editing is highly accurate and efficient.

Further, Han and colleagues generated knockout mice for *Mlkl* gene (which encodes the mixed lineage kinase domain-like protein, essential for tumor necrosis factor induced necrosis) by TALEN-mediated *in vivo* genome editing (Wu *et al.* 2013). They injected TALEN mRNA into nearly 3000 embryos and obtained 71 mutants from 390 newborns (18% efficiency); of these, four were homozygous mutants.

The latest player, CRISPR/Cas system, is having a drastic impact on the field, due to its simplicity, incredibly high efficiency, and multiplexing capability. Huang and colleagues first generated a knockout mouse in which a green fluorescent protein (GFP) transgene was disrupted by CRISPR/Cas-mediated *in vivo* genome editing (Shen *et al.* 2013). They targeted GFP in two different mouse strains carrying transgenes that encoded GFP, and obtained GFP-deficient mice with targeting efficiencies from 14 to 20% of live newborns.

Next, Jaenisch and colleagues published a revolutionary paper describing the production of mice knocked out for multiple genes with extremely high efficiency (Wang *et al.* 2013a). In this study, they targeted three functionally redundant genes (*Tet1, Tet2, and Tet3*) encoding Ten-eleven translocation (Tet) enzymes that convert 5-methylcytosine to 5-hydroxymethylcytosine. They first investigated the optimal conditions for CRISPR/Cas-mediated *in vivo* genome editing by injecting various amounts (20–200 ng/μL) of Cas9 mRNA, with *Tet1, Tet2* or *Tet3* guide RNAs, into fertilized eggs, and found Cas9 dose-dependent increments in targeting efficiency, with low toxicity in newborn mice. Surprisingly, the vast majority (60–100%) of newborns carried biallelic modifications in each target gene. Further, they simultaneously targeted both *Tet1* and *Tet2*, and obtained double mutants in which all four alleles of these genes were targeted in 80% of newborns without off-target effects. CRISPR/Cas-mediated *in vivo* genome editing can be completed within a month, since construction of CRISPR/Cas vector only takes a few days. This represents an incredible shortcut for the generation of single or double knockout mice, which often takes several years using conventional gene targeting methods (Fig. 2).

Liu and colleagues confirmed the high efficiency of CRISPR/Cas-mediated *in vivo* genome editing (Li *et al.* 2013). They generated three knockout mice targeting *Th, Rheb,* and *Uhrf2*, with targeting efficiencies from 75 to 92% and no off-target effects. They also targeted two adjacent sites, spanning 86 bp, in the *Uhrf2* locus. Importantly, the mutations were successfully transmitted to the next generation, suggesting that the CRISPR/Cas system is the third tool after ZFNs and TALENs to allow heritable *in vivo* genome editing in mice.

Knockin mice

Although the success of *in vivo* genome editing has enabled the rapid generation of knockout mice, developing this technique for the production of knockin mouse models would fully exploit its capabilities (Table 2). In 2010, Kühn and colleagues reported

Table 2. Summary of homology-directed repair (HDR) in mice by *in vivo* genome editing

Nucleases	Targets	Genes	Inserts	Donors	HDR (%)	Off-target	F1	Refs
ZFNs	1	*ROSA26*	*LacZ, Venus*	Plasmid	1.7–4.5	n.d.	n.d.	Meyer *et al.* (2010)
ZFNs	1	*Mdr1a*	*NotI, GFP*	Plasmid	5.0–25.0†	n.d.	n.d.	Cui *et al.* (2011)
ZFNs	1	*ROSA26*	*GFP*	Plasmid	2.0	n.d.	Yes	Hermann *et al.* (2012)
ZFNs	1	*Rab38*	*SNV*	Plasmid, ssOligo	1.7–3.5	n.d.	Yes	Meyer *et al.* (2012)
TALENs	1	*Rab38*	*SNV*	Plasmid, ssOligo	0.9–2.0	n.d.	Yes	Wefers *et al.* (2013)
TALENs	1	*Fus*	*SNV*	ssOligo	4.0–8.4	None	Yes	Panda *et al.* (2013)
CRISPR/Cas	2	*Tet1, Tet2*	*EcoRI*	ssOligo	70.0–80.0‡,§	None	n.d.	Wang *et al.* (2013a)
CRISPR/Cas	4	*Sox2, Nanog, Oct4, MeCP2*	*V5, mCherry, GFP-Neo, LoxP*	Plasmid, ssOligo	8.1–61.3¶,††	Present	n.d.	Yang *et al.* (2013)

†Mouse data only, ‡including biallelic modification, §60% for double HDR of both *Tet1* and *Tet2*, ¶61.3% for loxP site integration, ††16% for two loxP sites in one allele. n.d., not determined. F1 represents germline transmission. Percentages of HDR were calculated using the number of HDR positive pups as the numerator and the number of the total pups as the denominator. Fetus data were included in some studies.

pioneering work in the production of the first knockin mice using ZFN-mediated *in vivo* genome editing and targeting vectors (Meyer *et al.* 2010). They co-injected mRNAs encoding ZFNs for the *ROSA26* locus and a targeting vector, containing a 4.2 kb *LacZ* reporter cassette, with homology arms flanking the target site of the locus, into one-cell mouse embryos. Fifty-eight embryos were analyzed and one was found to be a precisely modified knockin mouse and was functionally confirmed by X-gal staining. Next, the same ZFN mRNAs were co-injected with a targeting vector containing a 1.1 kb *Venus* reporter cassette, instead of *LacZ*. Among the 22 embryos obtained, they identified one as a precisely modified knockin mouse. The targeting efficiencies of the two experiments were 1.7 and 4.5%, respectively.

Soon afterwards, Cui *et al.* (2011) also reported production of knockin mice and rats with relatively high efficiency. First they tested the targeted integration of a small 8 bp NotI site fragment flanked on each side by 800 bp of homology arms. Co-injection of a targeting vector with ZFNs for two genomic loci (*Mdr1a* and *PXR*) in both rats and mice resulted in knockin mutants with efficiencies of 6.7–25% of embryos. Next, they tested targeted integration of a long DNA fragment using the same donor vector, in which the NotI site was replaced with a 1.5 kb GFP cassette. They injected the GFP vector in the same way as the NotI sequence, and obtained knockin mice and rats for the two genomic loci with efficiencies from 2.4 to 8.3% of embryos or newborns. Further, they confirmed efficient germline transmission in both Mdr1a- and PXR-GFP knockin rats, with 50% of the F1 progeny corresponding to heterozygous mutants. Similar work was performed to target the *ROSA26* locus to integrate a GFP fragment, and the efficiency reported was 2% (Hermann *et al.* 2012).

Although the donor vectors used in *in vivo* genome editing contain relatively short homology arms for the targeted integration of SNVs that require only a few nucleotide substitutions, their construction is still a disproportionately laborious and time-consuming task. The use of synthetic single stranded DNA oligonucleotides (ssOligos) as donors for HDR can bypass this process. Davis and colleagues reported ZFN-mediated targeted integration of point mutations with ssOligos in several human cell lines with efficiencies that were up to twice those achieved using conventional targeting vectors (Chen *et al.* 2011). Kühn and colleagues applied ssOligo donor to produce knockin mice carrying SNVs (Meyer *et al.* 2012). They first generated a knockin mouse carrying a G19V missense and several silent SNVs in the *Rab38* gene, which encodes a small GTPase whose mutation results in a brown coat color, by co-injecting ZFNs with a conventional targeting vector. The targeting efficiency was 3.5% (three of 87 newborns), which is comparable to the efficiency they reported in their pioneering work (Meyer *et al.* 2010). All three founders exhibited efficient germline transmission, resulting in a brown coat color in F2 homozygous mutants (Meyer *et al.* 2012). Next, they co-injected the same ZFNs with a 144 nucleotide (nt) ssOligo containing seven substitutions into one-cell mouse embryos. They obtained one partially targeted mutant from 60 newborns with an efficiency of 1.7%, and the mutation was successfully transmitted to the F1 progeny. This work clearly reveals the enormous potential that ssOligos have for the replacement of conventional gene-targeting vectors in *in vivo* genome editing, which should greatly facilitate the rapid production of knockin mice.

In early 2013, Kühn and colleagues also generated *Rab38* G19V knockin mice by TALENs using an ssOligo (Wefers *et al.* 2013). They first constructed TALENs targeting the same region of the *Rab38* gene previously targeted by ZFNs, and found that the activity of the TALEN system was approximately twice that of ZFNs. Next, they co-injected TALENs and a ssOligo into one-cell mouse embryos and obtained one founder mouse carrying a partially targeted G19V allele from 117 newborns (an efficiency of 0.9%). The G19V allele was successfully transmitted to the F1 progeny. They also co-injected TALENs with a conventional targeting vector, rather than the ssOligo, and obtained one knockin founder carrying the G19V allele from 50 newborns (efficiency 2%). The G19V allele was also successfully transmitted to F1 progeny. As the construction of TALENs is much simpler than that of ZFNs, rapid production of knockin mice can be achieved using a combination of TALENs and ssOligos. However, the relatively low knockin efficiency of TALENs is a bottleneck that limits the dissemination of the method. To expand the applicability of TALEN-mediated *in vivo* genome editing, we developed highly active TALENs in collaboration with Dr Yamamoto's group at Hiroshima University. We focused on glutamate transporters, which are essential molecules that keep extracellular glutamate concentrations below neurotoxic levels (Tanaka *et al.* 1997; Watase *et al.* 1998; Matsugami *et al.* 2006; Aida *et al.* 2012). We previously reported *GLAST*, a glial glutamate transporter, knockout mouse as the first model for normal tension glaucoma (Harada *et al.* 1998, 2007; Bai *et al.* 2013a,b; Namekata *et al.* 2013). We also recently discovered deleterious missense mutations in *EAAT1*, a human orthologue of *GLAST*, in patients with glaucoma (Yanagisawa *et al.* unpubl. data, 2013). To

generate knockin mice carrying these SNVs in the *GLAST* gene, we co-injected highly active TALENs targeting *GLAST* into one-cell mouse embryos with ssOligos carrying each SNV. We obtained several germline-competent knockin founders with targeting efficiency of approximately 20% (Aida *et al.* unpubl. data, 2013). This study yielded the highest reported efficiency, which was almost 25-fold higher than the efficiency in a previous report by Wefers *et al.* As a single microinjection is sufficient to obtain several knockin founders, our TALEN technology provides a fast and efficient approach for the production of genetic mouse models that reproduce the disease-associated SNVs of complex diseases. Recently, Kühn and colleagues reported improved knockin efficiencies that were up to 8%, using TALEN mRNAs transcribed from plasmids containing a poly A tail (Panda *et al.* 2013).

Later, Jaenisch and colleagues reported the groundbreaking production of mice carrying multiple knockin alleles in different genes using CRISPR/Cas-mediated *in vivo* genome editing with extremely high efficiency (Wang *et al.* 2013a). They co-injected fertilized eggs with Cas9 mRNA, *Tet1* and *Tet2* guide RNAs, and 126 nt ssOligos to substitute a SacI site in *Tet1* and an EcoRV site in *Tet2* with EcoRI sites. Surprisingly, the vast majority (70–80%) of newborns carried EcoRI sites at *Tet1* or *Tet2* loci and some were homozygous for the EcoRI sites. Further, 60% of the newborns had EcoRI sites at both *Tet1* and *Tet2* loci.

Soon after this major accomplishment, Jaenisch and colleagues also produced knockin mice carrying longer DNA insertions by CRISPR/Cas-mediated *in vivo* genome editing (Yang *et al.* 2013). They first targeted the last codon of the *Sox2* gene with an ssOligo containing 42 nt short V5 epitope tag, and obtained targeted embryos and newborns with 34% efficiency. Next, they targeted the last codon of the *Nanog* gene with larger plasmid vector containing p2A-mCherry reporter cassette, and obtained targeted embryos and newborns with 8% efficiency. Further, they targeted the 3′ end of the *Oct4* gene with a plasmid vector containing 3 kb sequence of IRES-EGFP-loxP-Neo-loxP reporter cassette, and obtained targeted newborns with an efficiency of 30%. Finally, they also successfully generated knockin mice carrying a conditional allele of *Mecp2*, by simultaneously targeting with two loxP-containing ssOligos, and obtained targeted embryos and newborns carrying two loxP sites in one allele with an efficiency of 16%. Thus, knockin mice carrying, not only a SNV, but also longer DNA fragments, can now be created within a month using *in vivo* genome editing with high efficiency (Fig. 2). Taken together, almost everything achieved by ES cell-based gene targeting can now be performed by the *in vivo* genome editing technologies. Further, the new techniques allow previously impossible achievements, such as ultra-rapid production, biallelic targeting in F0 mice and multiplexing, leading genome editing to be the method of first choice for gene targeting.

Off-target effects

The off-target effect, which involves non-specific recognition and digestion at non-targeted regions by ZFNs, TALENs, and the CRISPR/Cas system, has been extensively discussed in the field of genome editing. When compared to ZFNs, TALENs produce only minimal off-target effects (less than a tenth), even at highly similar non-specific target sites with only two mismatches in the TALEN recognition sequence in human cells (Mussolino *et al.* 2011). Consistent with *in vitro* data, three papers describing TALEN-mediated *in vivo* genome editing in mice reported no off-target effects at a total of 15 potential off-target sites, containing only one mismatch, for four TALEN pairs (Panda *et al.* 2013; Qiu *et al.* 2013; Sung *et al.* 2013). Thus, in addition to basic research, TALENs may be applicable to therapeutics, a field that demands high-specificity.

Although the CRISPR/Cas system is an easy, quick, and highly efficient genome editing tool, the small size of the sequence (20 nt) required for DNA-RNA hybridization may make off-target effects more frequent with the CRISPR/Cas system than with TALENs or ZFNs. Recent large-scale systematic reports revealed an unexpectedly high frequency off-target effects using the CRISPR/Cas system in several human cell lines (Fu *et al.* 2013; Hsu *et al.* 2013; Pattanayak *et al.* 2013). According to these reports, the CRISPR/Cas system can cleave off-target sites containing even up to five mismatches (Fu *et al.* 2013). Jaenisch and colleagues investigated potential off-target sites using their knockout and knockin mice, as well as newly established mouse ES cells, to examine the specificity of the CRISPR/Cas system *in vivo* (Wang *et al.* 2013a; Yang *et al.* 2013). Through analyses of 54 potential off-target sites for seven guide RNAs, they found several non-specific digestions at three sites containing one or two mismatches. These results indicate that off-target effects in the CRISPR/Cas system do exist *in vivo*, but may be lower than predicted from *in vitro* studies using human cell lines.

As the majority of recent studies have focused on selected candidates for potential off-target effects, unbiased and genome-wide characterization of off-target sites through whole-genome sequencing will be required to guide the more sophisticated and specific design of RNAs.

To reduce non-specific off-target effects in the CRISPR/Cas system, Cas9 nickase, a mutant form of Cas9 that cleaves single stranded DNA, may provide an alternative for the induction of HDR (Cong *et al.* 2013; Mali *et al.* 2013). Zhang and colleagues recently reported that off-target effects could be reduced by using nickase and a pair of guide RNAs, without affecting on-target cleavage activity (Ran *et al.* 2013). They also revealed that this double-nicking strategy could efficiently cleave on-target sites in mouse zygotes. In combination with future developments using mutant Cas9 variants or other more specific Cas9 orthologues, these methods could reduce off-target effects in the CRISPR/Cas system.

The impact of *in vivo* genome editing

In Figure 2, we summarize the time course for the production of gene-targeted mice by conventional ES cell-based methods and *in vivo* genome editing by ZFNs, TALENs, and the CRISPR/Cas system. At best, it takes approximately 1 year to obtain a homozygous mutant by the conventional ES cell-based method. Also, it is common to spend a year or more obtaining germline competent chimeric founders. However, *in vivo* genome editing is revolutionizing these complex processes and enables ultra-rapid production of gene-targeted mice. In many cases, a genetically mosaic F0 founder and F2 homozygous knockout or knockin mouse can be obtained within a month and approximately 7 months, respectively. Further, in the best cases, as reported by several groups (Meyer *et al.* 2010; Hermann *et al.* 2012; Qiu *et al.* 2013; Sung *et al.* 2013; Wang *et al.* 2013a; Wu *et al.* 2013), biallelically targeted homozygous knockout or knockin mice can be obtained within a month. Thus, the genome editing revolution provides practical and exciting opportunities for the research community to freely and rapidly generate gene-targeted mice.

We now have the means to functionally investigate the consequences of millions of rare SNVs *in vivo* using "humanized" mice carrying equivalent variants. The cutting-edge work of Gleeson and colleagues demonstrated an interdisciplinary, sequencing era approach that integrates human genetics and mouse models (Novarino *et al.* 2012). They performed exome sequencing in consanguineous families with ASD, epilepsy, and intellectual disability and identified homozygous gene-disrupting SNVs in the *BCKDK* gene, which inactivates an enzyme complex essential for the catabolism of branched-chain amino acids (BCAAs). Because the SNVs resulted in disruption of the BCKDK gene, instead of generating knockin mice carrying the SNVs, they were able to investigate BCKDK knockout mice that showed reduced BCAAs in various tissues and neurological abnormalities, similar to other mouse models for ASD. In addition to BCAAs, they discovered imbalanced amino acid levels in the mutant brain that may contribute to the defects in neurotransmitter synthesis and subsequent neurological abnormalities. Finally, they tried to treat the mutant mice and patients using dietary supplementation with BCAAs and successfully reversed neurological abnormalities in the mutant mice and normalized plasma BCAA levels in patients (Novarino *et al.* 2012). Because the vast majority of rare SNVs are missense or synonymous, instead of gene-disrupting nonsense, splice site, or frameshift variants (Veltman & Brunner 2012), the production of knockin mouse models carrying such variants will be essential. Thus, *in vivo* genome editing drastically accelerates functional investigation of rare SNVs.

In vivo genome editing also accelerates functional research of common SNVs in intronic or intergenic regions indicated by the ENCODE (Encyclopedia of DNA Elements) project or GWAS studies (ENCODE Project Consortium *et al.* 2012; Maurano *et al.* 2012), in addition to those in protein-coding sequences. Cutting-edge work by Taipale and colleagues demonstrated the utility of gene-targeted mouse models in investigating the function of a GWAS-identified SNV (Sur *et al.* 2012). They focused on a conserved 500 kb region upstream of the *MYC* oncogene, where multiple cancer-associated SNVs have been mapped. They generated mutant mice lacking the region containing the SNV strongly associated with cancer, and found that the mutant mice were resistant to tumorigenesis (Sur *et al.* 2012). Further, Sabeti and colleagues used precisely modified knockin mice carrying a V370A SNV in the ectodysplasin receptor, which resulted in the identification of GWAS as one of the strongest candidates for recent positive selection in human evolution (Kamberov *et al.* 2013). They found that the knockin mice not only recapitulated the human phenotype, but also had previously unknown traits which were, surprisingly, also confirmed in human. As most common SNVs identified by GWAS can only explain relatively small contributions to disease risk, and the functional interpretation of non-coding SNVs is difficult, the generation of knockin mice by the time-consuming, laborious, and expensive process of ES cell-based conventional gene targeting is now considered disproportionate. The advent of *in vivo* genome editing technology has transformed this situation, enabling the mouse as a useful animal model system for the functional analysis of common, non-coding SNVs.

Further, genome editing technologies allow previously impossible gene targeting in mice. First, the methods allow gene targeting at the locus where traditional homologous recombination cannot be applied, such as the Y chromosome. Because the Y chromosome has unique structure containing many palindromes, conventional gene targeting in ES cells has failed. Jaenisch and colleagues targeted *Sry* and *Uty* genes on Y chromosome in mouse ES cells by using TALENs, and successfully obtained knockout mice lacking *Sry* or *Uty* (Wang *et al.* 2013b). Thus, high sequence specificity of TALENs provides a novel approach for genetic manipulation of the Y chromosome. Second, the methods allow double gene targeting at the neighboring loci. When two genes are located next to each other on the same chromosome, it is almost impossible to obtain double knockout mice by crossing two single knockout mice. Thus, the researchers have generated double-targeted ES cells by sequential targeting, a process much more time-consuming, laborious, and expensive than single gene targeting (Kitajima *et al.* 2000). As Jaenisch and colleagues demonstrated (Wang *et al.* 2013a), now, multiple genes can be targeted simultaneously by *in vivo* genome editing, thus providing opportunities to investigate cooperative roles of functionally redundant, clustered genes. Third, the methods allow gene targeting in diverse genetic backgrounds of mouse strains. In traditional gene targeting, ES cells derived from 129 mouse strain are most often used due to high efficiency of gene targeting. However, it is preferred to perform subsequent analyses of targeted mice on C57BL/6 genetic background. Thus, time-consuming backcrossing which takes at least 1 year is essential. As several groups demonstrated (Davies *et al.* 2013; Qiu *et al.* 2013), *in vivo* genome editing can be applicable to any mouse strain and provide opportunities to analyze the targeted mice immediately without backcrossing.

Overall, *in vivo* genome editing technology drastically accelerates the translation of human genetics into the mouse, in addition to other higher species such as primates (Sasaki *et al.* 2009), and should revolutionize our understanding of the functional consequences of human genomic diversity in health and disease.

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