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Astonishing advances in mouse genetic tools for biomedical research

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Summary

The humble house mouse has long been a workhorse model system in biomedical research. The technology for introducing site-specific genome modifications led to Nobel Prizes for its pioneers and opened a new era of mouse genetics. However, this technology was very time-consuming and technically demanding. As a result, many investigators continued to employ easier genome manipulation methods, though resulting models can suffer from overlooked or underestimated consequences. Another breakthrough, invaluable for the molecular dissection of disease mechanisms, was the invention of high-throughput methods to measure the expression of a plethora of genes in parallel. However, the use of samples containing material from multiple cell types could obfuscate data, and thus interpretations. In this review we highlight some important issues in experimental approaches using mouse models for biomedical research. We then discuss recent technological advances in mouse genetics that are revolutionising human disease research. Mouse genomes are now easily manipulated at precise locations thanks to guided endonucleases, such as transcription activator-like effector nucleases (TALENs) or the CRISPR/Cas9 system, both also having the potential to turn the dream of human gene therapy into reality. Newly developed methods of cell type-specific isolation of transcriptomes from crude tissue homogenates, followed by detection with next generation sequencing (NGS), are vastly improving gene regulation studies. Taken together, these amazing tools simplify the creation of much more accurate mouse models of human disease, and enable the extraction of hitherto unobtainable data.

Key words: *mouse models; genome manipulation; genetic engineering; gene-targeting; transcriptome; translatome; gene expression; neurodegenerative disease; TALEN; CRISPR/Cas9*

Abbreviations

4-TU 4-thiouracil
Ago2 Argonaute 2 protein
BAC bacterial artificial chromosome
Cas9 CRISPR-associated system nuclease 9
CRISPR clustered regularly interspaced short palindromic repeats
DSB double strand break
ESCs embryonic stem cells
Floxed flanked by LoxP sites
HR homologous recombination
LCM laser capture microdissection
NGS next generation sequencing
NHEJ nonhomologous end joining
Prnp prion protein gene
RGENs RNA-guided endonucleases
RISC RNA-induced silencing complex
RIT random integration transgenic
Rpl22 ribosomal protein L22
sgRNA synthetic guide RNA
TALENs transcription activator-like effector nucleases
TALEs transcription activator-like effectors
ZFN zinc finger nuclease

Introduction

Despite their diminutive size, and our lack of body fur and a tail, there are deep, genetically encoded, incontrovertible parallels in human and mouse biology. Like humans, mice have colour vision and close their eyes during sleep, and their four-chambered hearts pump warm blood, which carries gases via haemoglobin proteins. The mouse brain has the same major regions present in humans, including those most severely affected in neurodegenerative diseases. Human and mouse behaviour, metabolism and bodies change with aging [1–3]. Importantly, genomes of mice and humans have a similar linear length and gene content, both are typically methylated on silenced regions and hydroxymethylated on transcribed regions [4, 5], and many features of genome organisation and control are conserved [6–9]. All these similarities, together with their small size and fast life-cycle, have made the mouse a favourite model system for studying human diseases.

The utility of mouse models was dramatically augmented with the advent of recombinant DNA technologies, enabling the modelling of genetic alterations linked to human diseases, and the manipulation of gene networks hypothesised to be involved in disease mechanisms. An important example involves a mouse model of one of the most notorious genetic diseases: sickle cell anaemia. The mice were genetically engineered to express the human sickle cell mutation and developed blood phenotypes that closely resembled those in humans [10]. Remarkably, the mice

were experimentally "cured" by a combined gene therapy / stem cell therapy approach, much like what is envisioned to be commonly used in humans one day [10, 11]. The importance of the mouse's role in medical research has been thoroughly articulated by others [12–14] (see also http://ec.europa.eu/research/health/pdf/summary-report-25082010_en.pdf). Herein we review several cutting edge technologies that are revolutionising the utility of the mouse as a model system for biomedical research and are certain to help bridge the gap between the bench and the bedside.

Strategies for genome manipulation

The first successful introduction of exogenous DNA into the mouse genome was accomplished by injection of viral DNA into mouse blastocyst stage embryos [15]. Several years later, the first examples of genetically engineered mice expressing a specific foreign gene [16] and transmitting it to their progeny [17] were reported. These types of mouse models are referred to as random integration transgenics (RITs), as foreign DNAs (transgenes) integrate into random genomic locations, typically in multiple copies [18]. To inactivate an endogenous gene required a combination of two techniques. First, the endogenous DNA recombination machinery was harnessed to insert a new gene into a specific genomic location of cultured cells, a process known as gene targeting [19, 20]. The next step was to convert gene-targeted mouse cells into fully developed, viable animals. This was accomplished by combining cultured embryonic stem cells (ESCs) with a blastocyst stage mouse embryo, leading to a hybrid (chimeric) mouse consisting of the original (host) blastocyst and the injected ESCs [21]. Importantly, the ESCs can contribute to all tissues of the chimeric embryo. Thus, with a little luck, a chimera is born in which the germ cells were derived from the ESCs and thus engineered genes carried by ESCs can be transmitted to offspring [21–25]. This technology was initially used to engineer interruptions in genes that prevented synthesis of a functional product and the resulting mice were thus called knockouts [26–28]. Soon after, it was possible to place mutant genes intended for expression into specific genomic loci, creating lines now known as knock-ins [29–32]. The practical and experimental differences between RIT and knock-in mice are critical to consider when designing and interpreting experiments (see fig. 1).

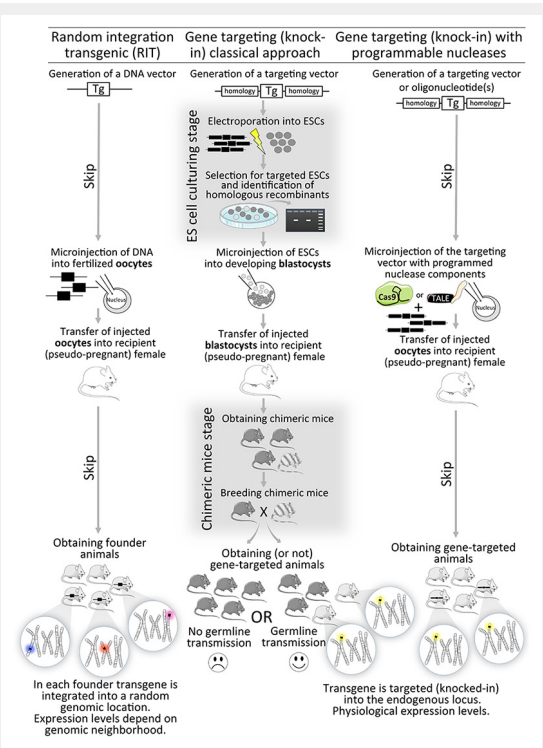

Figure 1

A comparison of strategies to genetically engineer mice. The random integration approach (left workflow) is useful for adding new genes, and is technically straightforward, but suffers from position effects (transgenes on different chromosomes on the bottom). The gene targeting approach (middle workflow) is more laborious, requiring embryonic stem cell (ESC) culturing, and breeding of chimeras, which often fail to transmit the transgene to their progeny. The breakthrough of the gene targeting approach employing guided nucleases (right workflow), is that the final result of gene-targeted mice is obtained without the ESC culturing and chimera breeding hassles.

Location matters – effects of genomic position

When RITs are developed, multiple 'founder' lines are typically created by independent integrations of the transgene into different genomic loci, typically resulting in different expression levels and patterns. Why does this happen? The answer is not completely understood but we can turn to other fields of genetics research for clues. Although mind bogglingly expansive, the mammalian genome is not a sea of randomly distributed elements that simply float around in the cell nucleus. Instead, the genome is contorted into very specific structures. Genes that are distantly localised in linear DNA space can be neighbours in three-dimensional space for coordinated regulation [33–35]. Indeed, neighbourhoods appear to give cells their identities [36, 37]. With the concept of genomic neighbourhoods in mind, it is conceivable that the same transgene behaves differently depending on the genomic context of the integration site. A transgene employing a promoter selective for a specific cell type would likely not be expressed if integrated into a genomic region that is silenced in that cell type. The endogenous genomic elements would likely dominate, and the transgene promoter would fail to recruit a sufficient compilation of activating transcription factors to outcompete the native elements suppressing transcription.

An important example of how this can affect mouse models of disease was published 20 years ago as a description of a new model of a neurodegenerative disease, spinocerebellar ataxia 1 [38]. The authors were as rigorous as possible. Not only did they generate and analyse multiple independent lines carrying the mutant transgene (table 1), but they did the same for a control transgene [38]. By intuition, we assume that the more of a toxic transgene is present, the more harmful its effects. However, the number of copies of the transgene did not reliably correlate with expression level and, even more surprisingly, the expression level was not predictive of the severity of the

phenotype (table 1: BO4 vs BO1, BO2 and BO6). None of the lines expressing the control transgene showed any signs of the disease, indicating it was the mutation, and not simply overexpression of a human protein, that was causing the disease in these mice. This example highlights the importance of examining multiple independent lines expressing a mutant transgene, and a series expressing a control transgene, when working with RITs. The transgene vector employed in these lines is expressed in a very specific cell type, Purkinje cells of the cerebellum (directed by *Pcp2* promoter elements). However, even more broadly expressed transgene vectors are vulnerable to position effects. For example, the thy1 vector, which is widely used for its tendency to express in neurons, is highly prone to position effects [39, 40], and sometimes even functions in glial cells [41]. A more broadly expressed transgene vector MoPrP.Xho1 [42], built from a modified mouse prion protein gene [43], is also prone to expression pattern differences. For example, sometimes it is expressed in the striatum (a brain region affected in Huntington's disease), but sometimes not [42]. Even within brain regions there is variability, as sometimes it is active in Purkinje cells [44] but sometimes it is not [43, 45]. Since the thy1 and MoPrP.Xho1 vectors are widely employed to model brain diseases, recognising that this variability happens and controlling it is extremely important.

The fidelity of expression can be improved by employing larger transgene vectors, such as bacterial artificial chromosome (BAC) or yeast artificial chromosome constructs [46]. They can carry up to 300 kilobase-pairs of DNA, enough to include even distant enhancers or insulators, and significantly increase the probability that a transgene will be expressed in the correct pattern, though some variability still remains [46]. Finally, a tangentially related but nonetheless important issue is that RITs alter the integration site. Besides the presence of new DNA, which might interrupt a gene [47], very large deletions can be created, and the modified loci are often unstable and change through generations [18]. Moreover, the regulation of genes neighbouring the inserted transgene can also be affected [18, 48], certainly an undesirable and difficult-to-detect consequence. Thus, although relatively easy to create, RITs are prone to difficult-to-control confounders related to their genomic location of insertion.

Table 1: Variable expression and ataxia.

Line	Copy no.	Expression (mRNA)*	Ataxia? (weeks)†
BO3	50	0X	No (20)
BO4	3-5	10X	Yes (16)
BO1	30	50X	No (36)
BO2	10	50X	No (36)
BO6	10	50X	Yes (26)
BO5	30	100X	Yes (12)

* fold expression above endogenous levels
† hemizygous mice only
Data obtained from [38]

Targeted genome modifications: the trouble with knock-ins

The knock-in approach has its own set of important disadvantages. The first is that mutations engineered into endogenous genes obtain only natural expression levels. In many cases, phenotypes can be accelerated or enhanced by increased expression, which can be an important strength of RITs despite the caveats raised in the previous section. Indeed, in our own work with knock-in mouse models of neurodegenerative diseases, the onset of phenotypes is typically after midlife, like in humans, which translates to 12 to 18 months in the mouse. Understandably, many researchers do not have the resources or patience to develop and study such slowly progressing models. Nonetheless, they can be viewed as models of early stage disease. These models often have neuropathological changes similar to findings in humans, such as disease-associated protein aggregates, neuronal degeneration, and reactive gliosis. Interestingly, the tissue tropism characteristic of the human disease is often recapitulated with knock-in mice [10, 49–53].

A second disadvantage of knock-in mouse models of disease is the inherent difficulty in creating them (fig. 1). The procedure includes the expensive and tedious process of genetically modifying ESCs, which are then injected into early stage mouse embryos. In comparison, the typical RIT approach requires only that a comparatively simple DNA construct be created and directly injected into mouse oocytes, providing greater flexibility in the DNA construction, and bypassing the ESC work.

Luckily, newly developed technologies make the generation of knock-in and knock-out lines technically quite similar to the random integration approach.

Combining the strengths of knock-ins and RITs

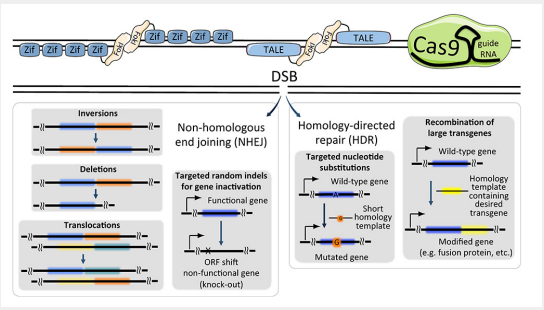

Figure 2

Comparisons of programmable nucleases and possible outcomes. Zinc finger nucleases (top left), transcription activator-like effector nucleases (TALENs; top middle) and CRIPSR/Cas9 (top right) systems introduce a site-directed double strand break (DSB) in DNA. Depending on the repair mechanism, nonhomologous end joining (NHEJ) or homology-directed repair (HDR), the site of interest can be left with random mutations, designed mutations, insertion of large transgenes, or (in case more than one DSB is generated), inversions, deletions and translocations.

Cas9 = CRISPR-associated system nuclease 9; CRISPR = clustered regularly interspaced short palindromic repeats; FokI = FokI nuclease; ORF = open reading frame; TALE = transcription activator-like effector; Zif = zinc finger nuclease

DNA double-strand breaks (DSBs) in the eukaryotic genome trigger two types of repair mechanisms, nonhomologous end joining (NHEJ) [54] and homologous recombination (HR) [55]. NHEJ simply fuses two free ends of DNA, sometimes with a random nucleotide insertion or deletion, resulting in a frameshift in the open reading-frame of a gene, potentially creating a gene knock-out. HR recombines the damaged genomic site with a separate piece of DNA (template) that is homologous to it, in effect switching the two [56]. This can be co-opted for genetic engineering by adding transgenic DNA to serve as the template, allowing one to introduce precise genome changes, such as gene knock-outs or knock-ins (see "strategies for genome manipulation" above). However, the efficiency of HR in a locus strongly depends on its chromatin state [57]. Genes in condensed chromatin are relatively protected from DSBs and thus render miserably low efficiency of targeted mutagenesis, potentially explaining why different loci differ in their susceptibility to gene targeting. For example, the first attempts to knock-out the prion protein gene (*Prnp*) required screening of approximately 10 000 ESC colonies for a single targeted clone [58]. This is likely because the *Prnp* locus remains rather silent in ESCs, and would rarely require and recruit the DSB-HR repair machinery [59]. On the other hand, in certain loci HR occurs with higher efficiency. Two such permissive loci are HPRT or ROSA26 [60, 61]. The ROSA26 locus is an especially popular choice for making expression reporter mouse lines [62], as it provides strong, uniform and ubiquitous transgene expression across multiple tissues [61, 63]. A recently identified locus that is also easy to manipulate (TIGRE) will complement, and may even be better for ubiquitous activity, than the ROSA26 locus [64, 65]. However, modifying or disabling most endogenous genes in mice remained inefficient and variable, making the stimulation of HR sorely needed. This motivated the development of programmable endonucleases to introduce double strand breaks into specific genomic locations and thereby triggering DNA repair mechanisms.

The first programmable nucleases routinely applied to mouse genome manipulation were created by fusing zinc finger DNA-binding domains to FokI endonuclease [66]. These chimeric restriction enzymes (termed zinc finger nucleases, ZFNs) were applied with amazing results in cells and *in vivo* [67–72] and even reached clinical trials [73]. However, this ground-breaking technology suffered from high cost of production and insufficient selectivity [74–76]. More recently, a related technique emerged, transcription activator-like effector nucleases (TALENs). TALENs proved to offer greater flexibility (they can target virtually any DNA sequence of interest, whereas ZFNs require a guanine-rich region, thereby limiting the density of targetable sites), and were easier to make thanks to their modular structure (see also fig. 2 and box 1). Most recently, RNA guided endonucleases (RGENs) burst on to the scene, with high efficiency and design flexibility for genetic modification of a multitude of species (fig. 2) [77–81]. The range of applications of TALENs and RGENs is expanding exponentially and a number of detailed reviews summarising their advantages and potential applications were published recently [72, 82–86].

The ground-breaking advantage of RGENs is the simplicity of construction, as the target specificity is directed by a 20-bp guide RNA molecule template which can be easily cloned into a vector encoding all the remaining components of the system: Cas9 nuclease and synthetic guide RNA (sgRNA) (fig. 2). Many dual expression vectors encoding Cas9 and sgRNA are freely available, and several online tools facilitate the identification of optimal guide sequences (see fig. 2 for details). Ease of design initially came at the expense of specificity, resulting in mutations in unintended locations [87, 88]. However, several recent advances diminish this problem. For example, a Cas9 mutant that cuts one DNA strand instead of two stimulates HR but insertions/deletions are not created in the case of NHEJ repair [89]. A second approach employs a catalytically inactive mutant version of Cas9 protein fused to FokI restriction endonuclease. Since this Cas9::FokI fusion protein requires dimerisation to function, two guide RNAs must bind in close proximity, greatly enhancing specificity [90]. A similar approach utilising TALENs successfully targeted a large construct injected into mouse oocytes [91]. Finally, when genetically engineering mice, most off-target mutations can be eliminated through backcrossing.

Compared with RGENs, ZFNs and TALENs require complex engineering of DNA recognition domains, as it is the protein, not RNA, which confers target specificity. The modular structure of TALENs greatly facilitates their design and construction, and many bioinformatics tools and assembly kits are currently available from open sources [92–95]. However, the design flexibility offered by RGENs is much greater. Consequently, they have rapidly become the method of choice for modifying multiple loci simultaneously. This was employed to generate multiple mouse lines simultaneously using "multiplexed" ESCs [96]. The ease of packaging of the small target specificity component into gene delivery systems should make it a useful tool for modifying genes *in vivo*, globally or in selected tissues. To simplify this process, a mouse line was genetically engineered to express Cas9 nuclease in specific cell types by use of Cre recombinase [97] (Cre is explained in fig. 3). In this inventive tool, a variety of established methods deliver guide RNAs, directing the modification of multiple loci simultaneously in specific cell-types of intact mice or cultured primary cells [97]. An interesting alternative is the recently reported delivery of both Cas9 (in this case a smaller version) and sgRNA packaged into adeno-associated virus particles [98]. The latter technique does not require transgenic mice and thus can be applied directly to mouse models of disease without the added expense of time and money to breed in additional lines. This amazing bid to specifically modify a single gene in a living organism once again highlights the therapeutic potential of RGENs. In human cells, CRISPR/Cas9 was already used with promising results to treat muscular dystrophy [99], human immunodeficiency virus infection [100], and Fanconi anaemia [101].

This therapeutic potential can be inverted to model multigenic diseases, including cancer. In addition to generating multiple mutations simultaneously, guided nucleases can be employed to engineer tumour-specific chromosomal rearrangements [102–104] (fig. 2). These possibilities were

recently described in detail in two excellent reviews [105, 106].

Another important feature is that programmable endonucleases can be employed to create gene-targeted transgenic mice by injection or electroporation into fertilised oocytes [81, 96, 107]. This approach saves precious time and resources that would otherwise be spent on culturing, targeting, screening, and karyotyping ESCs, and the subsequent generation and (often futile) breeding of chimeras (fig. 1). Moreover, the efficiency of *in vivo* gene targeting with RGENs can be enhanced through inhibition of the NHEJ pathway [108]. These improvements could make the generation of gene-targeted mice nearly as simple as the generation of RIT mice, but with the enormous advantage that the integration site of the transgene is precisely controlled.

Finally, modifications of TALEN and Cas9 systems are enabling the manipulation of gene expression [109, 110], sometimes controlled by light [111, 112]. Even the epigenome of specific genomic loci can be manipulated [113, 114]. TALEs- and CRISPR/Cas9-guided transcriptional activators were successfully used to remodel chromatin, enforcing gene activation in silenced regions [115]. Such specific control on gene-expression patterns *in vivo* will have a profound impact on disease research.

Context matters too – effects of genetic background

Engineered alleles (RITs, knock-outs or knock-ins) are often transferred from one genetic background to another by repeated backcrossing, occasionally with unwanted consequences. For example, mouse lines with different knock-out alleles of the prion protein gene were engineered in ESCs derived from 129S7, 129S4 or 129P2 mouse strains (hereafter collectively referred to as 129) and the resulting chimeras were typically bred to a very different genetic background, C57Bl/6 (B6 hereafter) [32, 116–119]. Even after backcrossing to B6 for 15 generations, a small amount of 129 genomic DNA flanking the *Prnp* gene remained [120]. This residual 129-derived sequence caused a phenotype, originally attributed to the mutant *Prnp* gene [121], that was later determined to be independent of the *Prnp* deletion [120]. Unfortunately, this was not an isolated event [122–124]. So how can it be avoided?

If using inbred mice is not possible one solution is to create a knock-in control allele that is built like the mutant allele but lacks the element expected to create the phenotype [50, 52, 122, 125–127]. This approach develops mutants and controls with approximately the same amount of ESC derived genome flanking the engineered locus [122]. However, creating the extra mouse line essentially doubles the work. An alternative solution is to utilise the plethora of available genome mapping and sequence data to identify natural genomic features in that locus that differ between the two strains [28]. This would enable one to backcross the wild-type allele from the donor background into the recipient background guided by a genotyping assay, thus bypassing the extra work required to engineer a knock-in control line, and providing a strategy for RITs if the integration site is known. Finally, with the rapid advances in the RGENs field, routine engineering of the same genetic ele-

Box 1: A brief comparison of key features of ZFNs, TALENs and RGENs.

	ZFNs **Zinc finger nucleases**	**TALENs** **Transcription activator-like effector nucleases**	**RGENs** **RNA guided endonucleases**
Origin	Zinc finger DNA binding protein domains	TALEs from plant pathogenic bacteria xanthomonas.	CRISPR-associated system (CRISPR-Cas) of found in many bacteria and archea species.
Mechanism of DNA recognition	Arrays of 30 amino acid-long Cys2-His2 zinc finger domains separated by linker sequences [162]	Arrays of 33–35 amino-acid repeats (TALEs). Each repeat recognises a single DNA base pair in the major groove [163].	Cas9 nuclease recognises target DNA sequence through a short RNA molecule, which includes a 20-bp fragment complimentary to the target DNA.
Off-target cleavage	High	Low	High for many applications, but being improved [89, 90].
Generation time		1–4 weeks	±3 days
Construction	DNA synthesis or several noncommercial DNA engineering methods [164]	DNA synthesis or several noncommercial DNA engineering methods [92, 94, 95, 165–168].	Only one-step cloning of the guide sequence into gRNA/Cas9 expression vector. For increased efficiency, Cas9 mRNA or protein can be made *in vitro* [96].
Other important features	Lower target density as compared with RGENs and TALENs [74] Higher risk poor DNA recognition or cytotoxicity when using newly designed ZFN [169, 170]	Higher specificity than original CRISPR/Cas9	Fast and relatively inexpensive Preferable for targeting multiple loci Flexible design Cre dependent Cas9-expressing mouse line available from the Jackson Lab: http://jaxmice.jax.org/strain/024857.html Cas9 variant for AAV delivery [98]
DNA recognition determinant	Protein (zinc finger domains)	Protein (TALE arrays)	Synthetic guide RNA (sgRNA)
Nuclease component	FokI	FokI	Cas9
Online tools and other resources	http://www.zincfingers.org http://www.zincfingertools.org	http://www.e-talen.org http://taleffector.genome-engineering.org/	http://www.e-crisp.org http://crispr.genome-engineering.org/ http://www.rgenome.net/

AAV = adeno-associated virus; bp = base-pair; Cas9 = CRISPR-associated system nuclease 9; CRISPR= clustered regularly interspaced short palindromic repeats; gRNA = guide RNA; sgRNA = synthetic guide RNA; TALE = transcription activator-like effector

ment into mice of multiple inbred genetic backgrounds is foreseeable.

Cutting-edge tools to study gene regulation

Diseases typically cause changes to tissues that are marked by changes in gene expression, essentially serving as a quantitative phenotype. For example, in neurodegenerative diseases, astrocytes often convert into a reactive phenotype and therefore changes in related gene products could be detected by *in situ* hybridisation or northern blots [128]. In the 1990s the high-throughput method of DNA microarrays transformed this field, suddenly allowing for hundreds, and eventually thousands, of mRNAs to be tested systematically and simultaneously [129, 130]. Just as dramatically, the field took another exponential leap in the 2000s with the invention of next generation sequencing (NGS) technologies, which basically determine the sequence of tens of millions of small fragments of DNA (obtained by reverse transcription of RNAs) in parallel, which are used to calculate the relative fraction of transcripts of tens of thousands of genes in a sample [131]. These techniques generate very accurate quantitative data, with a large dynamic range, and can provide additional information (for example mRNA splice variants, posttranscriptional edits, etc.). Although contention exists [132, 133], many experiments reveal that gene expression changes observed in humans also occur in the corresponding mouse models [134–136], providing solid validation of the models. New techniques are constantly improving the sequencing side of the NGS approach, for example by reducing the amount of RNA needed while improving the quantitative nature of the technique [137–140]. Until recently a major limitation was rooted in the profound biological problem that tissues consist of an extraordinary mixture of multiple cell types, and cell types with opposing gene expression programmes will be masked if RNAs are purified from a homogenate containing both. Thus, analysis of RNAs from specific cell types would provide a huge leap forward.

It's all about the lure – cell type-specific gene expression studies

One logical approach is to physically isolate specific cell types using a variety of sophisticated techniques. In one, tissues are gently dissociated into cell suspensions, labelled with antibodies targeting cell type-specific epitopes, and then separated by fluorescence activated cell sorting or immunopanning [141, 142]. This works extremely well for tissues that are easy to dissociate, such as blood, allowing a rapid separation procedure to be completed before too many changes to RNA levels occur. It is also perfectly suited for experiments where cells are sorted and subsequently cultured, allowing recovery from any gene expression changes induced by the dissociation and separation. This approach has yielded important insight into the transcriptomes of specific cell types in the mouse brain [141, 142]. Alternatively, cells in tissue slices can be hand-picked with laser capture microdissection (LCM) [143]. The LCM strategy limits unwanted changes in gene expression because the tissues are typically fixed or frozen, and can be combined with spatial information (e.g. proximity to a disease lesion). Though the cell bodies are typically the only parts captured and much mRNA is located far away from there, e.g. mRNAs translated at neuronal synapses [144] much important insight into disease has been acquired with LCM [135, 145].

A new and radically different approach is to isolate RNAs from specific cell types directly from crude tissue homogenates. The various versions of this strategy share the common theme of labelling biomolecules in specific cells and then using the introduced labels to affinity purify (or co-purify) RNAs from homogenised tissue. The isolated tissues can be rapidly frozen, thereby maximally preserving the native (patho)physiological state of gene expression. Many of these tools employ Cre for activation (fig. 3). Cre can activate the expression of a RNA tagging tool encoding transgene in a subset of cells based on, for example, cell identity, developmental stage or activity state (fig. 3) [146–149].

One cell type-specific RNA labelling technique employs a uracil phosphoribosyltransferase (UPRT) enzyme from *Toxoplasma gondii* [150]. In contrast to the endogenous

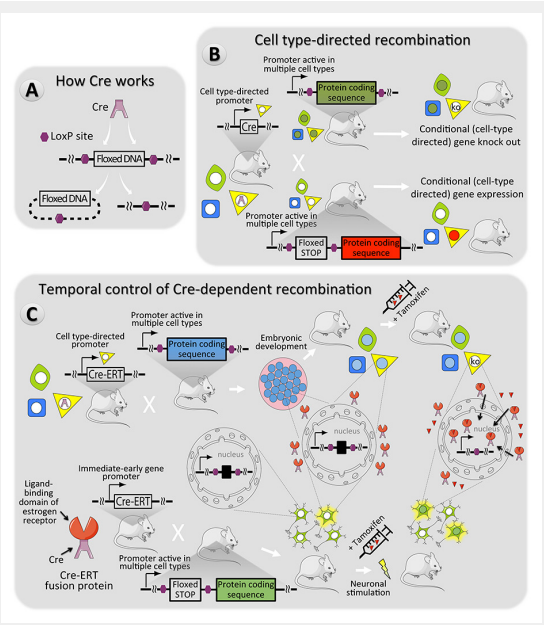

Figure 3

Cre recombinase – a versatile mouse genome manipulation tool. (A) In the systems described here, the activity of the Cre enzyme results in the removal of DNA sequences between two LoxP sites in the mouse genome [160]. (B) Spatially restricted expression of Cre can be employed to inactivate or activate genes in specific cell types [146] (cell type 3 – yellow). (C) Temporal control of Cre activity can be accomplished with fusion proteins consisting of Cre with an oestrogen receptor fragment (ERT) which prevents Cre-mediated recombination, until tamoxifen is present to direct the Cre-ERT fusion into the nucleus [161]. The top pathway features an example where the promoter driving Cre-ERT is cell type-specific. This is convenient in cases where cellular phenotypes change during development or through time (e.g. neural precursor cells can become neurons or glia). The bottom pathway features an example where Cre-ERT expression is induced by stimuli that happen often but when Cre activity is desired for a specific time. In this example an immediate early gene promoter drives expression, which would happen often, but Cre activity would occur only when tamoxifen is present [149].

mouse UPRT, the exogenous enzyme efficiently activates a uracil analogue (4-thiouracil, 4TU), which is then incorporated into RNAs transcribed in the cell type of interest (fig. 4). Such 4TU-containing RNA can be easily biotinylated and efficiently separated from total RNA with routine biotin-streptavidin enrichment tools [151].

A second strategy aims to capture specifically miRNAs, small RNAs that modulate gene expression by guiding the RNA induced silencing complex (RISC) to specific mRNAs and driving their degradation or impairing their translation [152]. This was accomplished by cell type-specific expression of an epitope-tagged protein component of RISC called Argonaute2 (Ago2, fig. 4) [153, 154]. Tissue homogenates are then probed with antibodies against the engineered epitope tags, co-capturing the RISC and associated miRNAs [154].

Two slightly different methods using a concept similar to the Ago2 method were developed to capture, from specific cell types, functional ribosomes and their associated mRNAs, enabling the study of translatomes. In the first method, called RiboTag [155], a modified ribosomal protein is expressed from its endogenous genomic location, but only once activated by Cre (figs 4 and 5). In the second method, called bacTRAP, a modified ribosomal protein is expressed by a BAC transgene (fig. 5) [156, 157]. Besides being a tool to isolate mRNA from specific cell types, mRNAs associated with ribosomes more closely represent the proteome than total mRNAs [158]. Although both methods to study the translatome are broadly similar there are some important differences. One important difference is that for the RiboTag approach, not one but two mouse lines are required: the knocked-in ribosomal gene plus a Cre activator that determines the cell type-specificity. In an effort to study a genetic mouse model of a disease, the addition of two new loci to the mix requires complicated breeding schemes and lots of costly cage space. However, the RiboTag approach has an important advantage over the bacTRAP method. With the bacTRAP method a single promoter determines both cell type-specificity and expression levels. In addition to the issues of random integration (see above), the promoters will have variable strengths in different cells, which will make quantitative comparisons between lines complicated (fig. 5). Moreover, the effects of gene-expression changes on cell type markers might change during disease [36, 159], which would also alter the amount of the tagged ribosome in the bacTRAP method. Both approaches are very clever, but it is critical to identify the most important features and disadvantages of each when designing a project. In all, these various techniques for cell type-specific gene expression studies will vastly improve the information obtained from disease models.

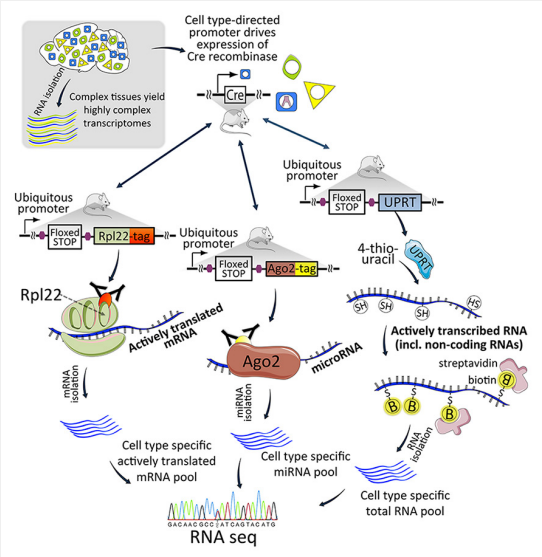

Figure 4

Transgenic techniques for cell type-specific RNA capture. A mouse expressing Cre recombinase is bred with a mouse encoding transgenes for RNA capture components. Each transgene is activated by Cre to drive cell type-specificity. The Rpl22 (ribosomal protein L22) protein captures ribosomes and ribosome-bound mRNAs during translation (the Ribotag method). The Ago2 protein captures the RISC complex and associated miRNAs. The UPRT enzyme converts 4-thiouracil into a nucleotide that is incorporated into newly transcribed RNA.

Ago2 = Argonaute2; miRNA = microRNA; RISC = RNA-induced silencing complex; UPRT = uracil phosphoribosyltransferase

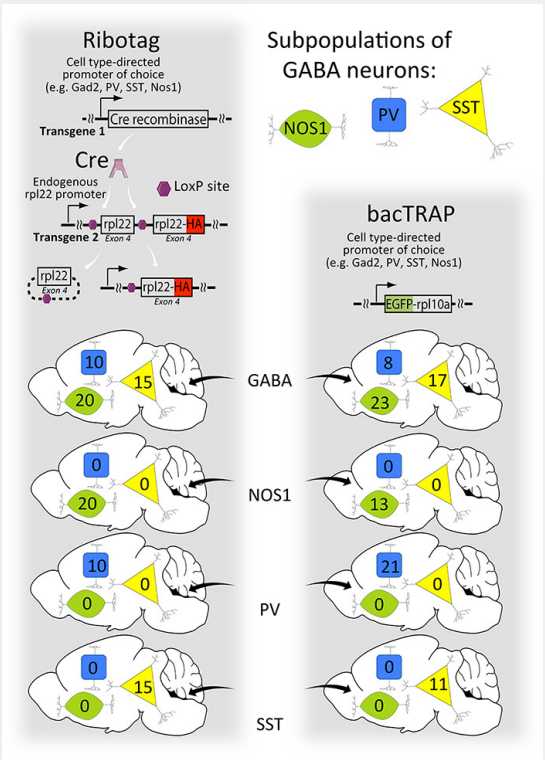

Figure 5

A comparison of Ribotag and bacTRAP methods. In the left pathway, a ubiquitous promoter drives expression of the Ribotag (rpl22-HA) protein only after Cre recombinase activity releases the inhibition of Ribotag expression. The endogenous rpl22 exon 4 functions as a 'floxed STOP' for the Ribotag method, as shown in fig. 4. The cell type specificity is determined by the pattern of Cre expression. Therefore, the same cell targeted by different Cre activators will have the same expression level of the Ribotag protein because the same ubiquitous promoter drives its expression from the same place in the genome. In contrast (right pathway), different promoters drive the expression of bacTRAP resulting in different expression levels in this hypothetical example. An important advantage of bacTRAP is that one mouse line is needed versus two for Ribotag.

Conclusion

In this short review we have presented a handful of examples of new genetic tools that are transforming biomedical research involving mouse models of disease. Some notable examples that we omitted include optogenetics and RNA interference, though they have existed for a while longer and are thus more widely known. Nonetheless, the examples we highlighted make for a very powerful compilation of tools that will harmonise. The gene-expression analysis tools will enhance the discovery of disease mechanisms and targets, and will also function as robust phenotyping tools to measure the efficacy of experimental therapeutic strategies. In turn, the new genome sequence and expression manipulation techniques, especially when applied to specific cell-types, will be a powerful approach to interrogate these pathways. In the 1980s, the development of gene-targeted mice made scientists absolutely giddy with excitement. That giddy feeling is back!

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Figures (large format)

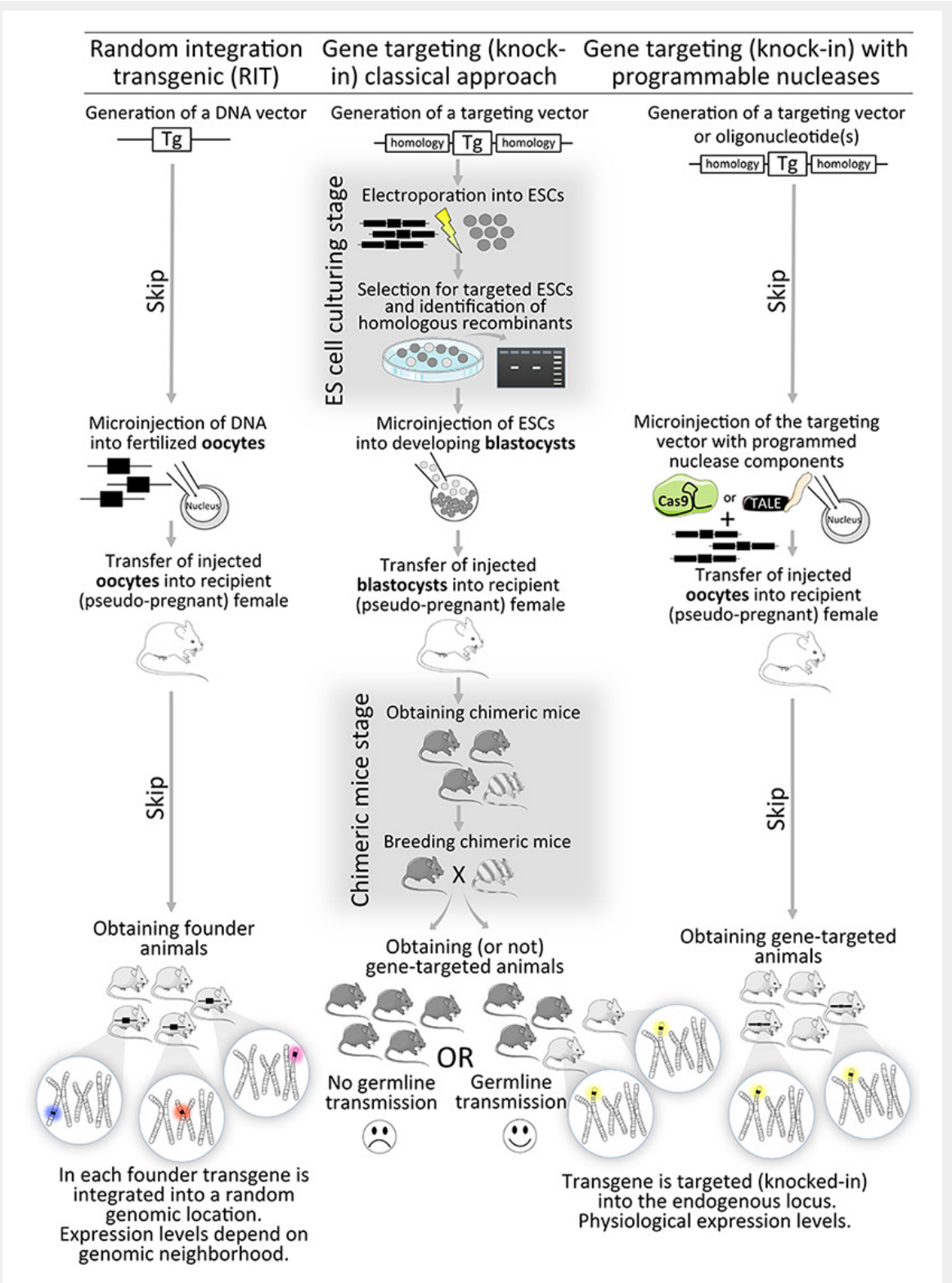

Figure 1

A comparison of strategies to genetically engineer mice. The random integration approach (left workflow) is useful for adding new genes, and is technically straightforward, but suffers from position effects (transgenes on different chromosomes on the bottom). The gene targeting approach (middle workflow) is more laborious, requiring embryonic stem cell (ESC) culturing, and breeding of chimeras, which often fail to transmit the transgene to their progeny. The breakthrough of the gene targeting approach employing guided nucleases (right workflow), is that the final result of gene-targeted mice is obtained without the ESC culturing and chimera breeding hassles.

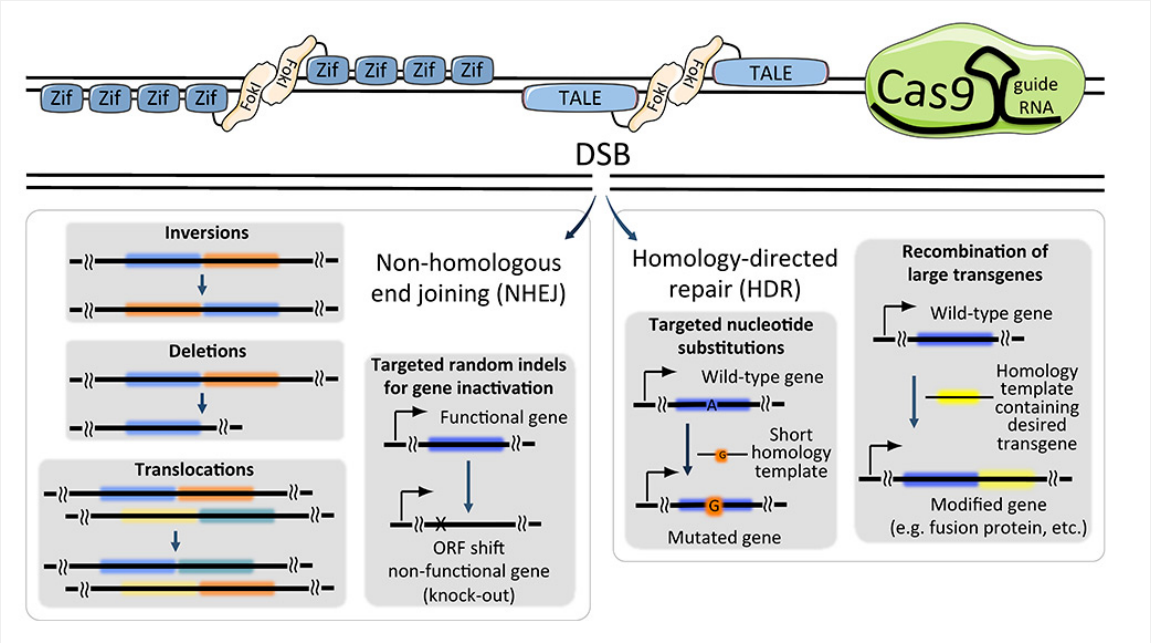

Figure 2

Comparisons of programmable nucleases and possible outcomes. Zinc finger nucleases (top left), transcription activator-like effector nucleases (TALENs; top middle) and CRIPSR/Cas9 (top right) systems introduce a site-directed double strand break (DSB) in DNA. Depending on the repair mechanism, nonhomologous end joining (NHEJ) or homology-directed repair (HDR), the site of interest can be left with random mutations, designed mutations, insertion of large transgenes, or (in case more than one DSB is generated), inversions, deletions and translocations.

Cas9 = CRISPR-associated system nuclease 9; CRISPR = clustered regularly interspaced short palindromic repeats; FokI = FokI nuclease; ORF = open reading frame; TALE = transcription activator-like effector; Zif = zinc finger nuclease

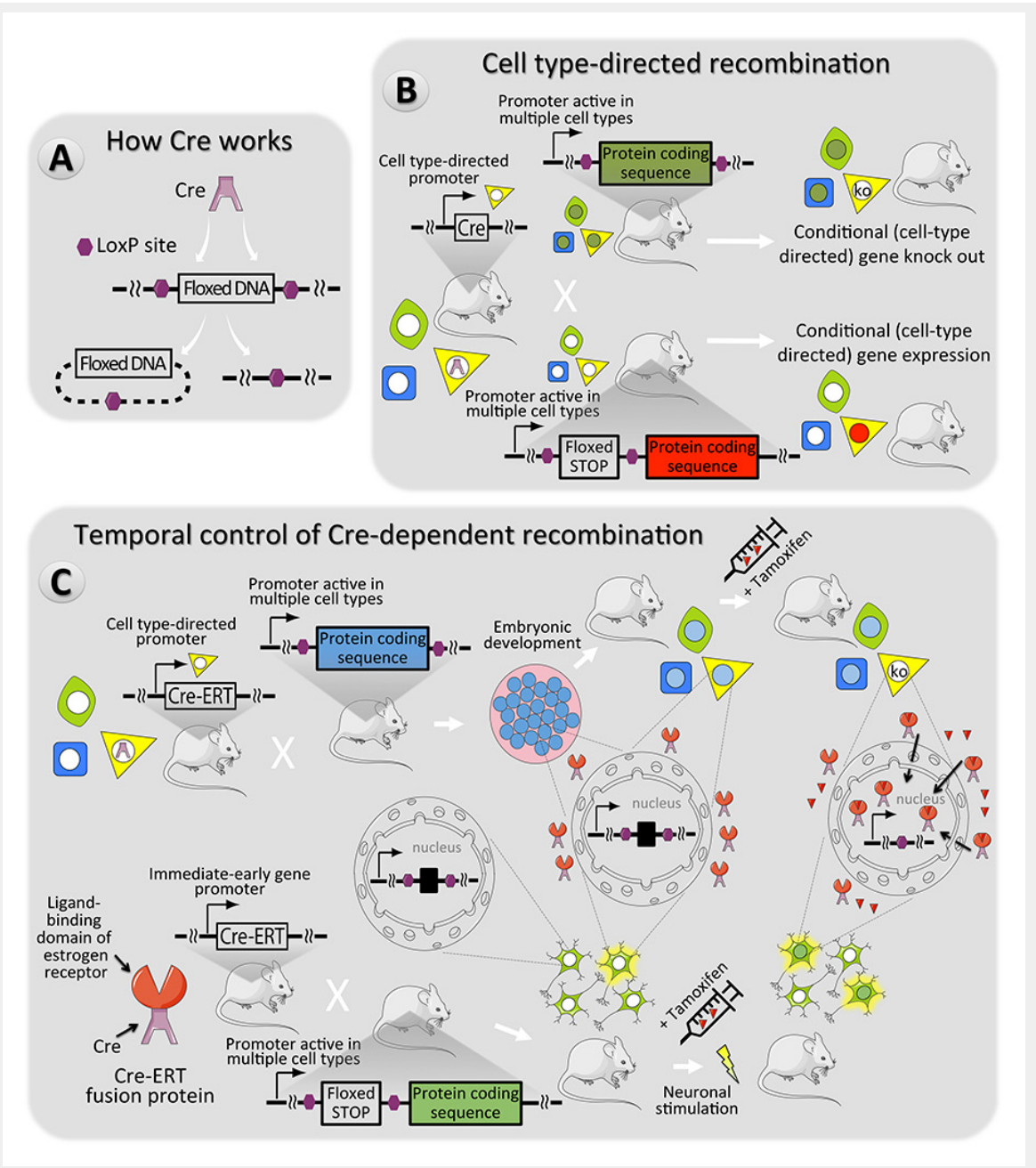

Figure 3

Cre recombinase – a versatile mouse genome manipulation tool. (A) In the systems described here, the activity of the Cre enzyme results in the removal of DNA sequences between two LoxP sites in the mouse genome [160]. (B) Spatially restricted expression of Cre can be employed to inactivate or activate genes in specific cell types [146] (cell type 3 – yellow). (C) Temporal control of Cre activity can be accomplished with fusion proteins consisting of Cre with an oestrogen receptor fragment (ERT) which prevents Cre-mediated recombination, until tamoxifen is present to direct the Cre-ERT fusion into the nucleus [161]. The top pathway features an example where the promoter driving Cre-ERT is cell type-specific. This is convenient in cases where cellular phenotypes change during development or through time (e.g. neural precursor cells can become neurons or glia). The bottom pathway features an example where Cre-ERT expression is induced by stimuli that happen often but when Cre activity is desired for a specific time. In this example an immediate early gene promoter drives expression, which would happen often, but Cre activity would occur only when tamoxifen is present [149].

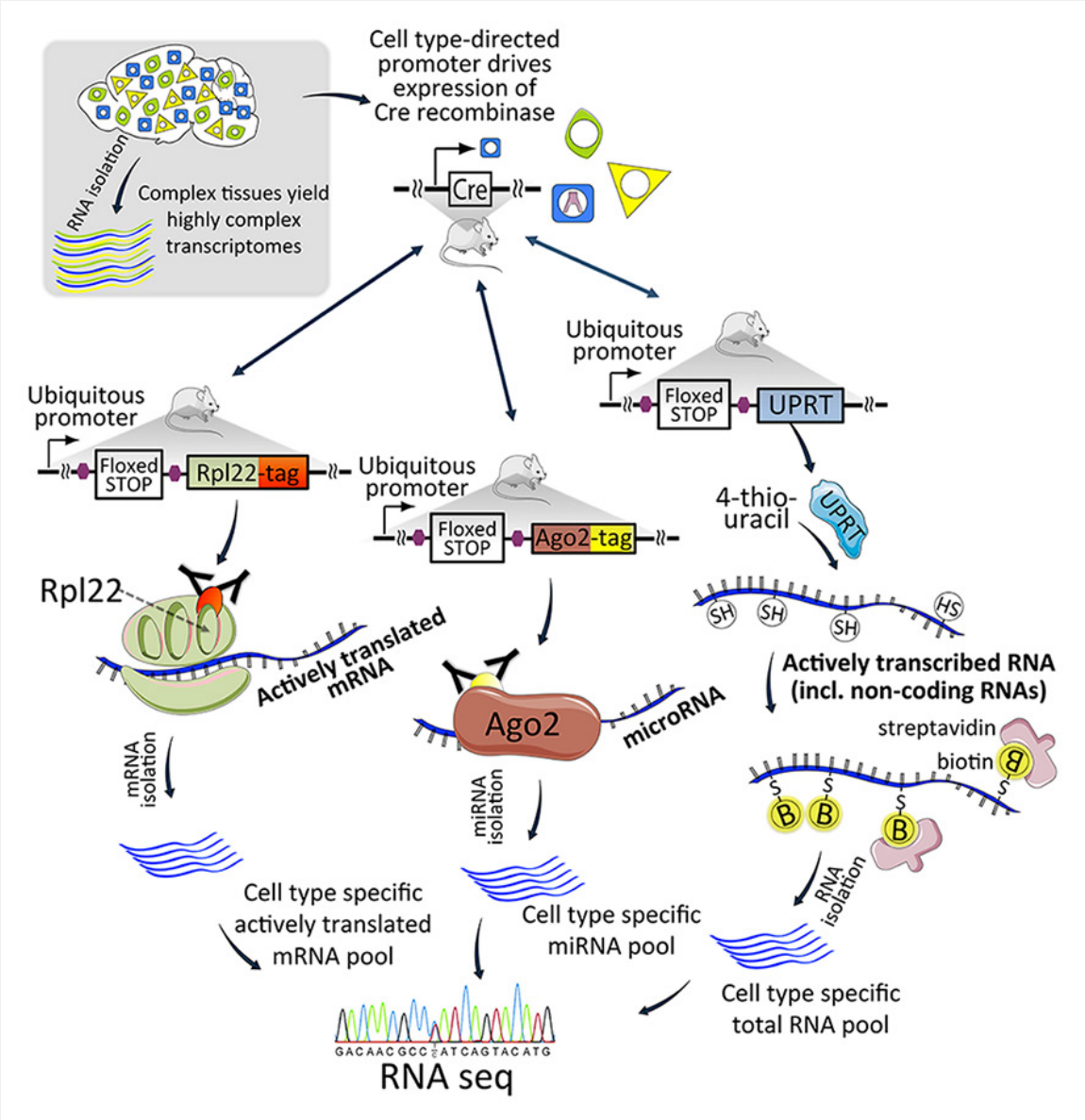

Figure 4

Transgenic techniques for cell type-specific RNA capture. A mouse expressing Cre recombinase is bred with a mouse encoding transgenes for RNA capture components. Each transgene is activated by Cre to drive cell type-specificity. The Rpl22 (ribosomal protein L22) protein captures ribosomes and ribosome-bound mRNAs during translation (the Ribotag method). The Ago2 protein captures the RISC complex and associated miRNAs. The UPRT enzyme converts 4-thiouracil into a nucleotide that is incorporated into newly transcribed RNA.
Ago2 = Argonaute2; miRNA = microRNA; RISC = RNA-induced silencing complex; UPRT = uracil phosphoribosyltransferase

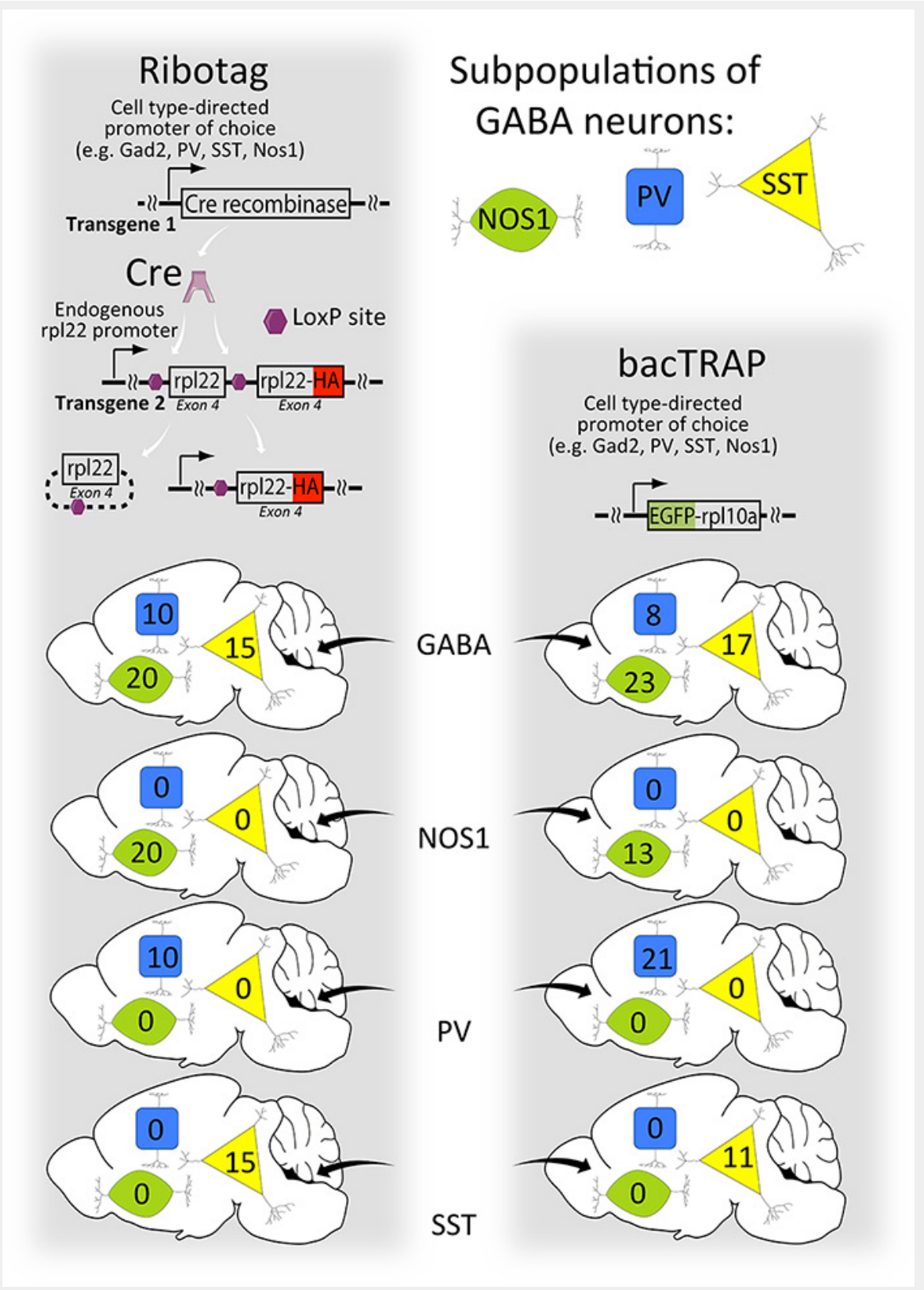

Figure 5

A comparison of Ribotag and bacTRAP methods. In the left pathway, a ubiquitous promoter drives expression of the Ribotag (rpl22-HA) protein only after Cre recombinase activity releases the inhibition of Ribotag expression. The endogenous rpl22 exon 4 functions as a 'floxed STOP' for the Ribotag method, as shown in fig. 4. The cell type specificity is determined by the pattern of Cre expression. Therefore, the same cell targeted by different Cre activators will have the same expression level of the Ribotag protein because the same ubiquitous promoter drives its expression from the same place in the genome. In contrast (right pathway), different promoters drive the expression of bacTRAP resulting in different expression levels in this hypothetical example. An important advantage of bacTRAP is that one mouse line is needed versus two for Ribotag.