Transgenic Animals for the Generation of Human Antibodies

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What You Will Learn in This Chapter

Currently, 28% of all monoclonal antibody (mAb) therapies and 74% of fully human mAb therapies approved by US Food and Drug Administration (FDA) are derived from transgenic animal platforms. In these platforms, the host antibody heavy chain VDJ and light chain VJ repertoire are substituted by their counterparts which are encoded exclusively by human transgenes. These platforms take advantage of the host's naturally antigen-driven antibody selection and maturation process associated with the primary and secondary immune response. Through immunisations and subsequent antibody discovery using common technologies including hybridoma and single B-cell screening, a large panel of mAbs exhibiting high affinity and specificity against the target antigen along with ideal biophysical characteristics and developability can be obtained. They can be streamlined into clinical development. This chapter gives a comprehensive overview of the transgenic strategies, methods of creating knockout of the endogenous immunoglobulin (Ig) production and generation of transgenic constructs containing human Ig heavy and light chain gene locus. Brief introductions are also given to a list of representative transgenic platforms which have originated in multiple species and can produce human antibodies in various formats. The strategies for antibody discovery in these platforms are also discussed.

5.1 The Concept of Using Transgenic Animals to Generate Human Therapeutic Antibodies

The major drawback of using rodent-derived monoclonal antibodies (mAbs) for human therapies is their immunogenicity. Upon administration of the fully xenogeneic mAbs, patients can develop anti-drug antibodies (ADAs). The ADAs can alter the pharmacokinetics of therapeutic mAbs, leading to their accelerated clearance. A sub-set of ADAs, characterised as neutralisation antibodies, can lead to the loss of efficacy of therapeutic mAbs [1, 2]. Further, life-threatening allergic reactions have been reported in some patients treated with murine-derived mAbs [1–3]. To mitigate these problems, chimeric antibodies which contain the variable domains of a xenogeneic mAb appended onto human Ig constant regions in a functional configuration have been engineered to reduce the immunogenicity and maintain or enhance the efficacy of the original mAb [4, 5]. Up to date, nine chimeric therapeutic mAbs have been approved for human therapies by FDA, accounting

for 11% of all approved mAbs in the USA (https://www.antibodysociety.org/resources/approved-antibodies/). Although the immunogenicity is reduced, the use of therapeutic chimeric antibodies can still elicit human anti-chimeric antibody response in patients [2]. To further increase the human content of therapeutic mAbs, sophisticated humanisation processes have been developed since the mid-1980s. Successful methods include grafting the complementarity-determining regions (CDRs) of the variable domains of a xenogeneic mAb into the framework regions (FRs) of a selected human antibody, resulting in transferring the specificity and affinity to the recipient antibody [6, 7]. In recent years, with the increased number of protein structures published for antibodies from various origins, computer modelling and prediction programs have been developed to aid the decision-making during the antibody humanisation process [8]. To date, 40 humanised mAbs have been approved by FDA, accounting for half of antibody drugs on the US market. Despite this success, there are several drawbacks inherent in the humanisation process. The strategy for humanisation needs to be designed case by case, and there is still no generally applicable technology guaranteeing a positive outcome. Frequently, during the process, the humanised mAb candidates exhibit altered affinity, specificity and biophysical characteristics such as solubility, and can be prone to aggregation and decreased expression in production systems comparing to their original counterparts [9–11]. Significant amounts of time and resources are often needed to re-engineer and re-validate candidates to yield a humanised product that retains biological function as well as clinical efficacy and meets the Chemistry, Manufacturing and Control (CMC) requirements. It is usually impractical to put multiple xenogeneic mAbs into the humanisation process in parallel, which generates a bottleneck for drug development.

Another widely used technology for the discovery of fully human mAbs relies on screening large naïve phage display libraries, usually containing over 10^{10} individual phage clones displaying Ig variable regions derived from human B lymphocytes [12, 13]. In this technology, the in vitro selection process carried out in test tubes replaces the target antigen-driven primary and secondary immune response in vivo. With carefully designed panning strategies, a diverse panel of candidate mAbs can be obtained. Further rounds of mutagenesis combined with screening are often required to yield mAbs with high affinity to a given target and desired biological functions. The major drawback of this technology is that the natural pairing of Ig heavy and light chain in clonal B cells are not preserved in the phage clones because during the process of library construction, the Ig heavy chain and light chain variable domains are randomly assembled together. It has been reported that a significant number of naïve library-derived mAbs exhibit developability problems including off-target binding, reduced solubility, being prone to aggregation and unacceptable expression yields [14]. As described for the humanisation process, considerable effort is also required for re-engineering and re-validating naïve library-derived mAb candidates to generate a clinically and commercially successful product. To date, eight fully human mAbs approved by FDA have been discovered through screening naïve phage libraries, accounting for 10% of all marketed mAb drugs and just 26% of fully human mAb drugs in the USA.

It is therefore highly desirable to reconstitute the human humoral immune response in model organisms such as mouse and rat through a transgenic approach. In this system, the human genomic regions encompassing the genes encoding antibody repertoire and the relevant expression regulatory regions are stably integrated into the genome of the host animal. The resulting transgenic animals can be immunised with a target antigen similar as in the wild-type animal. The naturally target-driven antibody selection and maturation process associated with the primary and secondary immune response can generate significant numbers of antibodies encoded exclusively by the human transgenes. These fully human antibodies with high affinity and specificity to the target can be discovered using well-established technologies such as hybridoma technology. Consequently, a diverse panel of candidate mAbs can be streamlined into the pre-clinical development with minimal further engineering work. It has been suggested the numerous checkpoints during natural B-cell development may indirectly select for antibody variable domains with desirable qualities for product development such as solubility [14, 15]. So far, 23 fully human mAbs approved by FDA have been discovered using transgenic platform based in mouse, accounting for 28% all marketed mAb drugs and 74% of fully human mAb drugs in the USA.

Thirty years have passed since the first attempt to introduce human Ig genes into an animal using transgenic technology. In 1989, Brüggemann and colleagues reported the creation of a transgenic mouse line carrying a human/mouse hybrid IgH minilocus [16]. The transgene in this study included one functional human and one functional mouse V_H segment, one mouse D segment, two human-like D segments derived from mutated mouse D, one human D segment and all six human J_H segments followed by a chimeric human/mouse C_μ gene. This line of mouse rearranged the transgene locus in the lymphoid tissue, and serum expression of human IgM could be readily detected. To extend from this work, in 1991, the same team reported another transgenic mouse line carrying 100 kilobase pairs (kbps) of the human IgH locus mostly in the germline configuration [17]. The transgene in this instance was all human, including two functional V_H, seven D and all six J_H followed by the complete C_μ gene. Rearrangement involving both V_H segments was observed, and extensive junction diversity in the V_H-(D)-J_H joining was also demonstrated. In 1992, Taylor and colleagues from then GenPharm International Inc. reported a new transgenic mouse line carrying both human IgH and Igκ minilocus [18, 19]. The fully human light chain transgene consisted of one functional V_κ, all five J_κ and C_κ segment followed by a downstream regulatory sequence. One fully human heavy chain construct used in this study consisted of one functional V_H, ten D, all six J_H, μ switch region, entire C_μ gene, γ1 switch region, entire $C_{\gamma 1}$ gene and a downstream regulatory sequence. Serum analysis showed the expression of both human IgM and IgG1 in the transgenic mice. Extensive rearrangement involving all segments in the transgene and substantial diversity in both V_κ-J_κ and V_H-(D)-J_H joining were demonstrated. Importantly, the study showed that the functional gene segment in the IgH transgene could undergo antibody class switch from IgM to IgG1. These early exploratory studies demonstrated there were only minor differences between the mouse and human humoral immune system. With respect to gene rearrangement and

antibody class switch, the trans-acting factors in mouse can exert their functions on cis-elements in the human transgene loci. In all the studies described above, the transgenic constructs were microinjected into the pronuclei of fertilised mouse oocytes, resulting in the transgene loci being integrated into mouse genome in a random fashion with one or more copies. Hence, the reported data also suggest that the trans-acting factors in mouse for rearrangement and class switch can function irrelevant of chromosomal locations and varied gene copy numbers. These studies validated the concept and paved the way for developing transgenic animals for the discovery of fully human antibodies.

5.2 Strategies in Developing Transgenic Platforms for Human Antibody Discovery

To recapitulate the immensely diverse human antibody repertoire and ensure the high expression of human antibodies in transgenic animals, it is anticipated to be necessary to incorporate as many functional gene segments in the human Ig heavy and light chain genomic loci as well as the associated cis-regulatory elements into the transgenic constructs. It is also critical to inactivate the endogenous Ig heavy and light chain loci in the host to minimise the competition between endogenous antibodies and human antibodies derived from transgenes. Different aspects in transgenic strategies and designing the human transgenic constructs as well as Ig-knockout host are discussed below.

5.2.1 Transgenic Strategies

Owing to the collective efforts of the scientific community in human genome research, large human genomic regions, ranging from 10 to greater than 1000 kbps, have been cloned into cosmid, P1-derived artificial chromosome (PAC), bacterial artificial chromosome (BAC) and yeast artificial chromosome (YAC) vectors and can be acquired from commercial companies or academic laboratories. Specific clones that contain human Ig heavy and light chain gene loci can be identified using conventional molecular biology techniques including end sequencing followed by alignment to reference genome sequence and amplifying specific regions by PCR. PACs and BACs can host up to 300 kbps of mammalian DNA and be stably propagated in *Escherichia coli*. Precise modification of PACs and BACs can be achieved in *E. coli* using homologous recombination systems derived from λ phage such as the well-established Red/ET Recombination system [20–22]. The cloning capacity of YACs is open-ended and ranges from less than 100 to greater than 1000 kbps, providing an extensive coverage of human Ig gene loci. YACs can be shortened, extended and modified in *Saccharomyces cerevisiae* by utilising the robust homologous recombination system in this organism together with a range of selectable markers [23]. In addition, human artificial chromosomes (HACs) ranging from 10 to 20 megabase pairs (Mbps) were generated by truncating human chromosome 2, 14 and 22

containing Igκ-, IgH- and Igλ-gene loci, respectively, and maintained in mouse embryonic stem cells (ES cells) and homologous recombination-proficient chicken DT40 cells [24, 25]. When designing and building transgenic constructs, the target site of site-specific recombinase such as LoxP (for Cre recombinase), FRT (for flippase) and attP (for phiC31 integrase) and that for PiggyBac transposase have been frequently incorporated into the constructs [20–25]. These sites can facilitate the deletion, insertion, translocation and inversion at specific sites in the DNA of cells that carry the transgenic constructs.

One of the most commonly used methods for introducing transgenic constructs into the host genome is microinjecting purified linear DNA directly into the pronuclei of fertilised oocytes of the host [26, 27]. The transgenes can integrate into the host genome in a random fashion and usually in one or more copies. Subsequently, the microinjected oocytes are implanted into pseudo-pregnant foster mothers to produce the transgene-carrying founders at varying frequencies. The founders are typically identified by either a Southern blot or PCR assay using transgene-specific probes or primer pair, respectively, on genomic DNA extracted from ears or tail, and positives used to establish independent transgenic lines through breeding. The profile and strength in transgene expression may vary among different lines due to the nature of different transgene integration sites as well as gene copy numbers. Those transgenic lines with the most desired pattern of transgene expression can be chosen for further breeding to homozygosity. Even though it has low efficiency, this method is simple, relatively speedy and proven to be successful in many mammals including mice, rats and rabbits. Due to the physical nature of this method and the difficulties in purifying and handling large DNA fragments, the size of individual linear DNA for microinjection is usually restricted to under 300 kbps.

The well-established mouse ES cells provide an unparalleled system for generating transgenic strains. Gene targeting by the deletion of large murine genomic region and subsequent replacement with human counterparts in situ can be achieved in the ES cells [22, 28]. Using this strategy, mouse Ig heavy and light chain gene loci can be precisely humanised in situ, which renders the human Ig transgenes under the authentic temporal and spatial regulation of mouse trans-acting factors. To generate transgenic mouse ES cells, constructs as plasmids or BACs comprising designated transgenes, appropriate mammalian cell selectable markers and specially positioned LoxP or FRT sites flanked by murine genomic sequences to allow homologous integration are transfected or electroporated into the cultured cells. Upon selection, stable ES cell clones with the desired transgene integration can be identified via assays such as Southern blot, PCR including real-time quantitative PCR and fluorescence in situ hybridisation (FISH) [28]. Through the transient expression of corresponding site-specific recombinase, the desired chromosome modification such as insertion, deletion or inversion can be achieved and verified using similar assays. Subsequently, the verified ES cells are injected into recipient blastocysts which can be transplanted into surrogate mothers to yield chimeric mouse. Transgenic lines with germline transmission of the intended genetic features can then be established through several rounds of breeding from the initial chimeras. ES cell-based gene targeting and genetic engineering as described here for mouse are currently not established in rat, rabbit,

cattle and other non-human mammals. Furthermore, spheroplasts derived from yeast cells that carry a modified YAC with appropriate mammalian cell selectable markers can be fused to mouse ES cells to create yeast-ES cell hybrid [23]. These ES cells with entire human transgenes harboured in the YAC integrated into the genome can be selected and verified using Southern blot, PCR and FISH analysis. Through this technology, transgenic mouse lines carrying megabase-sized human transgenes have been established [23, 29]. In this case, the integration site is random, and there is usually just one copy of the YAC integrated into the mouse genome due to its large size. Additionally, mouse ES cells harbouring a HAC can be directly used for generating transchromosomic (Tc) lines. In this instance, the HAC behaves as an autonomously replicating entity, independent from the endogenous chromosomes. The stability during mitosis and frequency of germline transmission vary significantly among different HACs when developed into Tc mouse lines.

For large mammals such as cattle, transgenic constructs can be introduced into cultured foetal fibroblast cells via electroporation. Additionally, through microcell-mediated chromosome transfer, HACs which have been modified and maintained in DT40 cells can be introduced into foetal fibroblast cells to establish Tc cells. Subsequently, selected fibroblast cells which have been verified for carrying the transgenes can be used for whole animal cloning to yield transgenic offspring [30, 31]. In chickens, isolated primordial germ cells can be cultured and genetically modified using transgenic constructs together with traditional transfection and selection techniques. These modified cells still possess the ability to develop into sperms and eggs when injected into the recipient chicken embryos. Transgenic chickens with germline transmission of the transgenes can be established through this strategy [32, 33].

5.2.2 Animals with Knockout of Endogenous Ig Production

To guarantee the optimal expression of human Ig gene-encoded antibodies in transgenic animals and to facilitate the downstream antibody discovery with minimal interference by both endogenous Ig heavy and light chains, it is essential to inactivate the endogenous Ig production in the host. This is rather crucial when the transgenic constructs carrying human Ig gene loci are integrated randomly into the host genome or maintained on a separate chromosome. The knockout strategies for host Ig gene loci are closely associated with the transgenic strategies described above. Using mouse ES cell-based gene targeting technology, a variety of constitutive IgH knockout mice which lack B-cell development and antibody production have been generated. These include the μMT^{-} (*Ighm*^{tm1Cgn}/J) mice in which the first exon encoding the transmembrane domain of IgM was disrupted [34], J_HT (*Igh-J*^{tm1Cgn}/J) mice in which the genomic region encompassing all J_H gene segments was deleted [35] and homozygous C-gene deletion mice in which a 200 kbps genomic region accommodating all C_H genes, namely, C_μ-C_δ-$C_{\gamma3}$-$C_{\gamma1}$-$C_{\gamma2b}$-$C_{\gamma2a}$-C_ε-C_α, was deleted by targeted integration of two LoxP sites followed by Cre-loxP-mediated in vivo deletion [36].

The ES cell-based gene targeting was also applied to knockout mouse IgK and created lines with features including C_κ disruption [37] and targeted deletion of all J_κ and C_κ gene segments [38]. The mouse Igλ gene locus, spanning 200 kbps, contains two sets of V_λ, J_λ and C_λ gene segments separated by an intergenic region of approximately 100 kbps. A large deletion that removes all functional C_λ gene segments was accomplished in several stages using ES cell-based gene targeting combined with Cre-loxP-mediated in vivo deletion [39].

In large mammals such as cattle and pig, targeted gene disruption and deletion of large genomic region can be achieved initially in cultured primary fibroblast cells. Through animal cloning, the offspring with intended genotype can be derived from the engineered fibroblast cells. Using this strategy, both bovine IgM genes, b*IGHM* and b*IGHML1*, were disrupted sequentially to yield double knockout cattle in which the endogenous B-cell development was blocked. Tc cattle was established in the double knockout background using HACs comprising the entire unrearranged human IgH, Igκ and Igλ germline loci [40]. However, because in this genetic background the endogenous bovine Ig light chain gene loci were still intact, significant levels of chimeric antibodies comprising human IgH derived from transgene and the endogenous bovine Igκ or Igλ could be found. To improve fully human Ig production in Tc cattle, the entire genomic region encompassing bovine J_λ and C_λ gene segments was deleted using the fibroblast approach to yield Tc cattle in triple knockout background (b$IGHM^{-/-}$, b$IGHML1^{-/-}$ and b$IGL^{-/-}$) [41]. Furthermore, by using a similar strategy, knockout pigs with the deletion of all J_H gene segments or the C_κ gene were separately established in 2011 [42, 43]. Targeted deletions in chicken Ig heavy and light chain gene loci were also achieved using the primordial germ cells approach described above [44].

Neither ES cell-based gene targeting nor animal cloning is well established in rat and rabbit. In both species, gene disruption and deletion can be achieved using specially designed zinc finger nucleases (ZFNs). In particular, the messenger RNAs encoding a pair of engineered ZFNs that introduce double-strand break in a specific DNA region can be directly injected into fertilised oocytes. In these cells, repairment of the DNA break by the error-prone non-homologous end joining can usually introduce nucleotide insertion or deletion ranging from 1 bp to several kbps into the break point. Animals born which carry the ensuing gene disruption or deletion can be identified through PCR, sequencing and Southern blot analysis [45, 46]. Using this method, rat lines carrying deletions of the entire J_H gene segments, C_κ gene and all functional J_λ-C_λ gene segments were generated to establish the triple knockout strain which completely lacks the production of endogenous Ig heavy and light chains [47, 48]. Rabbit lines with disruption in exon 1 and 2 of IgM gene locus were also established [46].

5.2.3 Human IgH Gene Loci in Transgenic Constructs

To date, there are documented 56 functional V_H, 23 functional D and 6 functional J_H gene segments in humans (http://www.imgt.org/genedb/) [49]. In the genome, the functional V_H segments are distributed among many highly related pseudogenes with a total number of identified V_H segments reaching 159. The V_H gene locus, spanning nearly 1 Mbps, is highly polymorphic among different people. This is reflected not only in the abundance of alleles for each V_H gene but also in the variation of V_H gene counts among individuals. Duplication and deletion of genomic regions containing V_H segments are frequently observed. For instance, a study on the haplotypes of 9 human donors showed there were between 35 and 46 functional V_H segments in 18 unique haplotypes [50]. When developing transgenic animals, great efforts have been made to incorporate nearly all known functional human V_H gene segments into the host's genome. However, since the majority of transgenic constructs containing human IgH locus have been generated from cloned genomic regions from one or more donors, they inevitably represent the genetic makeup of those individuals. This makes it very difficult for any transgenic construct to capture the complete functional V_H gene repertoire of the human species. In addition, there are some issues in antibody drug discovery that argue against using the complete repertoire of human functional V_H genes. Firstly, it is known that some V_H genes are more likely to encode antibody fragments prone to misfolding or aggregation [51]. One example is VH7–4 which contains an unpaired cysteine residue in its framework 3 whose reactivity may promote aggregation [52]. Secondly, if an antibody drug were to be given to individuals who don't possess the corresponding V_H gene used in that drug, there can be serious concerns for immunogenicity due to the likely absence of tolerance in these patients. It has been suggested when designing the transgenic construct, it may be preferential to incorporate the functional V_H gene repertoire that are most common to the bulk of the human population [53]. The functional D and J_H gene segments used in humans are covered in a relatively compact genomic region. Thus, the majority of transgenic constructs have this entire region incorporated.

The first-generation transgenic constructs were designed to produce fully human antibodies in animals, including the genomic loci encoding human IgM, IgD and one of the IgG constant regions [53]. Although, upon immunisation, transgenic animals carrying this type of construct elicited pronounced immune responses, and fully human therapeutic mAbs were subsequently discovered, there have been some concerns about the suboptimal B-cell development and relatively low antibody serum titres in these animals [48, 54, 55]. This drawback is underpinned by several possible sources of inefficiencies. Firstly, when functioning as a B-cell receptor (BCR), the interspecies interaction between human IgM constant region and the host's signalling proteins such as Igα and Igβ may be inefficient [56]. This could compromise the level of signalling which are normally required for B-cell maturation, survival and proliferation in the host. Secondly, the secreted fully human immunoglobulins from B cells in transgenic animals may interact inefficiently with various host Fc receptors, further compromising the magnitude of humoral immune response

against target antigens [57]. Thirdly, even though the trans-acting molecular machinery in the host can exert its function on regulatory elements in the human IgH loci such as the recombination signal sequences and switch regions which govern gene rearrangement, antibody class switch and appropriate expression in a temporal and cell lineage-specific manner, such interactions may not be optimal [55]. Lastly, due to the size limit imposed by cloning and transgenic strategies, some of the first-generation transgenic constructs may lack the extended locus control region such as the complete 3′ enhancer located downstream of all constant region genes which might affect antibody expression and class switch [48, 58]. To overcome these inefficiencies, the second-generation transgenic constructs have been created which are chimeric, comprising the human IgH locus encoding antibody variable regions followed by the host locus encoding constant regions which provides a functional replacement to the endogenous locus especially in the knockout background [48, 59]. In particular, the host locus may contain the intronic enhancer region at the 3′ of J_H and 5′ of C_μ, the entire C_μ gene, part of or all the rest of constant region genes including one or more C_γ genes, all intact switch regions corresponding to the constant region genes and the extensive locus control region. Furthermore, in the in situ humanisation approach through ES cell manipulation in mouse, the gene replacement has been limited to the mouse genomic region encompassing V_H, D and J_H gene segments while leaving the constant region genes completely intact [22, 54, 60]. As a result, the second-generation transgenic animals can efficiently produce chimeric Ig with only the variable regions encoded exclusively by the human gene repertoire. Following the discovery process, such chimeric antibodies can be easily converted to a desired fully human format through antibody engineering.

5.2.4 Human Igκ and Igλ Gene Loci in Transgenic Constructs

Sequencing of the human Igκ gene locus has identified 41 functional V_κ genes which are found in 2 clusters arising through segmental duplication [61, 62]. The proximal cluster is located ~24 kbps upstream of 5 J_κ genes and a single C_κ gene and spans ~444 kbps. There are 23 functional V_κ genes in this cluster, with all but the 2 most 3′ V_κ genes sharing the same orientation as the J_κ and C_κ genes. The distal cluster is located almost ~1 Mbps upstream of the J_κ and C_κ genes and contains 18 functional V_κ genes. The orientation of all these V_κ genes is opposite to that of J_κ and C_κ genes. Expression analysis has shown that the distal cluster contributes little to the diversity of the human Igκ repertoire, and in some people, this cluster appears to be missing [63]. Hence, many transgenic human Igκ constructs only contain part of or the entire proximal cluster together with human J_κ and C_κ genes [22, 48, 53]. Additionally, it is crucial to incorporate the regulatory regions at the 3′ end of the C_κ gene, especially the kappa deleting element, as these regions ensure the proper expression of human Igκ in animals [64]. Further, in situ humanisation of mouse V_κ genes by replacement with the corresponding human genes has been achieved [22, 54, 60].

The human Igλ gene locus contains 33 functional V_λ genes and 5 downstream tandem cassettes, each made up of a J_λ gene followed by a C_λ gene. The majority of transgenic constructs expressing human Igλ in animals contain part of or all human V_λ genes, all J_λ-C_λ cassettes and the 3′ enhancer region [48, 60, 65]. In mammals, the proportion of serum antibodies containing κ or λ light chains varies considerably between different species. In mice and rat, the (% κ:λ) ratio can be more than 95:5; in humans, the ratio is approximately 67:33; and in cattle, the ratio can reach 1:99 (https://www.sigmaaldrich.com/technical-documents/articles/biology/antibody-basics.html) [41, 66, 67]. As mice make few antibodies with a λ light chain, the Igλ gene locus in this species is considered to be relatively silent. Therefore, since human Igκ-transgenes can achieve significantly high expression, it is regarded as preferable but not essential to include human Igλ-transgenes in transgenic mice platforms.

5.3 Examples of Currently Available Transgenic Platforms for Human Antibody Discovery

Table 5.1 contains a list of transgenic platforms in different species currently in use for human antibody discovery. Several platforms with published data in peer-reviewed journals are described in detail below.

5.3.1 HuMAb-Mouse® and XenoMouse®

Developed in the 1990s, HuMAb-Mouse® and XenoMouse® are both history-making first-generation transgenic platforms for generating fully human antibodies. HuMAb-Mouse® was originally created by Genpharm Inc. which later became Medarex and is now part of Bristol-Myers Squibb. This platform was also branded as UltiMAb® transgenic mouse technology by Medarex and licensed to Genmab and ImClone Systems (now part of Eli Lilly and Company). This platform employed fully human IgH- and Igκ-miniloci transgenes with the following distinctive features: deletion of all murine J_H gene segments as well as the entire J_κ-C_κ genomic region; a single 80 kbps IgH transgene locus containing 4 functional human V_H, 16 D, all 6 J_H, C_μ and $C_{\gamma 1}$ gene segments with associated regulatory regions such as intronic enhancer, switch regions and locus control region; and a reconstituted Igκ-transgene locus (~500 kbps) through co-integration of 3 constructs into the mouse genome including 26 functional human V_κ, all J_κ and C_κ followed by a downstream regulatory region (Fig. 5.1a) [70, 71]. The diversity of the V_H and V_κ gene repertoire of this platform was later expanded to include additional variable gene segments [53]. Even with its initially limited variable gene diversity, HuMAb-Mouse® is the most prolific transgenic platform so far, yielding 11 FDA-approved fully human mAb drugs, accounting for nearly half of those derived from transgenic animals [72].

Table 5.1 A list of currently available transgenic platforms for human antibody discovery

Platform name	Transgenic strategy	Features disclosed in scientific publications or online	Reference
Platforms producing conventional fully human or chimeric antibodies:			
5′ feature mouse (MRC Laboratory of Molecular Biology, UK)	ES cells fused to YAC-containing spheroplasts	1. Background: $\mu MT^{-/-}$ and $MoIg\kappa^{-/-}$ (disruption of Cκ) 2. IgH construct contains 5 functional human V_{Hs}, complete D_s and J_{Hs} and human C_μ-C_δ, but no C_γ gene 3. Igκ and Igλ constructs contain part of human loci	[68, 69]
HuMAb-Mouse®	Pronuclei microinjection	See Sect. 5.3.1	[70, 71]
XenoMouse®	ES cells fused to YAC-containing spheroplasts	See Sect. 5.3.1	[73–75]
Tc Mouse™	ES cells carrying HACs	See Sect. 5.3.2	[25, 76]
Tc Bovine	Engineered HACs transferred into fibroblast cells followed by animal cloning	See Sect. 5.3.2	[31, 40, 41, 55, 77]
THP rabbit (Therapeutic Human Polyclonals, now Roche)	Pronuclei microinjection	For the generation of polyclonal antibodies encoded by human Ig gene loci on transgenic constructs	[82]
VelociMouse®	ES cells, in situ humanisation	See Sect. 5.3.4	[22, 54]
H2L2 Transgenic Mice (Harbour Antibodies)	Pronuclei microinjection	1. Endogenous Ig expression inactivated 2. IgH construct contains 18 human V_{Hs}, 19 human D_s, 6 human J_{Hs}, rat C_μ, $C\gamma_{2c}$, $C\gamma_1$, $C\gamma_{2b}$, $C\gamma_\alpha$ and mouse locus control region 3. Igκ construct contains 11 human $V\kappa_s$, 5 human $J\kappa_s$, rat Cκ and mouse enhancer regions 4. No Igλ transgene	https://harbourantibodies.com/science-technology/h2l2/#.Xek7kej7SUk
OmniRat® and OmniMouse®	Pronuclei microinjection	See Sect. 5.3.3	[48, 59]

(continued)

Table 5.1 (continued)

Platform name	Transgenic strategy	Features disclosed in scientific publications or online	Reference
Kymouse™	ES cells, in situ humanisation	See Sect. 5.3.4	[60]
AlivaMab Mouse (Ablexis)	(information not available)	1. Autonomously functioning synthetic Ig transgenes were designed and synthesised 2. Curated repertoires of human V_H, V_κ and V_λ gene segments were selected for developability and lowered risk of immunogenicity 3. To better maintain the native conformation of human F(ab) fragment, the transgenes yield human/mouse chimeric antibodies with the heavy chain variable domain-CH1-upper hinge region and the entire light chain being fully human	https://www.ablexis.com/technology/
Trianni Mouse®	ES cells, in situ humanisation	See Sect. 5.3.4	https://trianni.com/technology/transgenicmouse/
OmniChicken®	Genetic modification in cultured primordial germ cells, then injection into recipient embryos	See Sect. 5.3.3	[44, 83]
Tc goat (caprine)	HACs transferred into fibroblast cells followed by animal cloning	See Sect. 5.3.2	[81]
Platforms producing chimeric antibodies with a fixed human Ig light chain:			
OmniFlic®	Pronuclei microinjection	See Sect. 5.3.5.1	[85]
OmniClic®	As in OmniChicken®	See Sect. 5.3.5.1	https://www.omniab.com/
MeMo® mouse (Merus)	(information not available)	1. Knockout of endogenous murine Ig loci 2. Transgenes contain the human IgH gene locus and a rearranged fragment encoding a single Ig light chain	https://merus.nl/technology/

(continued)

Table 5.1 (continued)

Platform name	Transgenic strategy	Features disclosed in scientific publications or online	Reference
Platforms producing chimeric heavy chain only antibodies (HCOAs):			
HCAb transgenic mice	Pronuclei microinjection	See Sect. 5.3.5.2	[100, 101]
Humabody® VH platform	ES cells fused to YAC-containing spheroplasts and pronuclei microinjection	See Sect. 5.3.5.2	[102]
UniRat®	Pronuclei microinjection	See Sect. 5.3.5.2	[103]

XenoMouse® was originally created by Cell Genesys Inc. which later became Abgenix and now part of Amgen. The technology of fusing mouse ES cells with spheroplasts carrying modified YACs which contain megabase-sized human Ig transgene locus was employed to generate this platform. XenoMouse® carries the following features: double knockout background through deleting all mouse J_H gene segments and C_κ region; stable integration into the genome of a 970 kbps fully human IgH locus encompassing in germline configuration 66 consecutive V_H gene segments of which 34 are functional; all 30 D, 6 J_H, C_μ and C_δ followed by 1 C_γ ($C_{\gamma1}$, $C_{\gamma2}$ or $C_{\gamma4}$) in conjunction with all associated regulatory regions; and stable integration of a 800 kbps human Igκ locus encompassing the entire proximal cluster of V_κ gene segments, all J_κ and C_κ followed by the downstream regulatory region (Fig. 5.1b) [73–75]. Three different XenoMouse® strains were generated, each producing fully human antibodies with only one of the three isotypes, IgG1κ, IgG2κ and IgG4κ, allowing the generation of antigen-specific mAbs with the desired effector functions for specific disease indications. Later, the entire human Igλ locus carried on a YAC was also introduced into existing XenoMouse® strains to generate three additional strains, each producing both human IgGκ and IgGλ antibodies with the corresponding isotype [53]. To date the XenoMouse® platform has yielded 7 FDA-approved fully human mAb drugs, accounting for nearly one third of those derived from transgenic animals [72].

5.3.2 Tc Mouse™, Tc Bovine and Tc Goat (Caprine)

The Tc Mouse™ which stands for 'TransChromo mouse' was engineered by Kyowa Hakko Kirin Co., Ltd. from the mid-1990s to early 2000s. This platform was generated by using mouse ES cells that harbour HACs derived from the truncated human chromosomes 2, 14 or 22 containing the entire Igκ-, IgH- and Igλ-gene locus, respectively, to establish the Tc lines which were then bred with a double knockout mouse line with the disruption of endogenous IgM and Igκ gene locus [25]. The lack of stability of the HACs was a major issue of this platform, which limited its use for antibody discovery. It appears

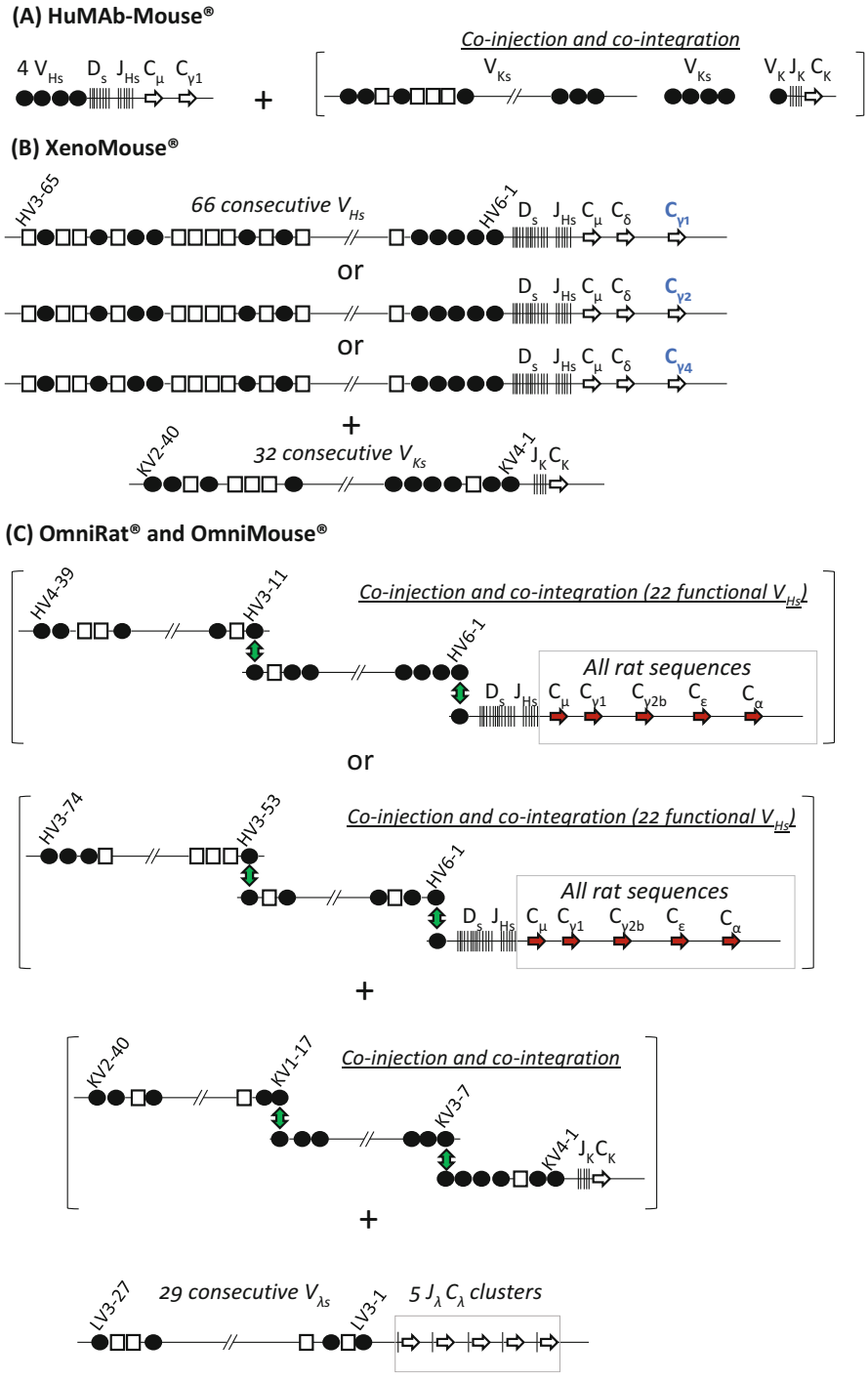

Fig. 5.1 Diagram showing the composition of transgenes in (**a**) HuMAb-Mouse®, (**b**) XenoMouse®, (**c**) OmniRat® and OmniMouse®. Functional V_H, V_K and V_λ genes are represented

that the HAC carrying the human IgH locus is relatively stable mitotically and can be transmitted through germline, whereas significant mitotic and meiotic loss is observed for Igκ- and Igλ-bearing HAC, and germline transmission have not been achieved for both. An immediate solution to this problem was to crossbreed the Tc Mouse™ with HuMAb-Mouse® to produce offspring which retains both the stable IgH-bearing HAC from the former and the Igκ locus stably integrated into the genome from the latter. This strategy yielded the KM Mouse™ [76]. Another more generally applicable solution to the problem is through further engineering of the HACs in the DT40 cell line using Cre-LoxP-mediated chromosome translocation. In particular, via homologous integration, LoxP sites were inserted into the designated sequence on IgH- and Igκ- or Igλ-bearing HAC, respectively, in DT40 cells. Through the function of transiently expressed Cre recombinase, a chimeric HAC that contains both the human IgH locus and Igκ or Igλ locus could then be generated. Such chimeric HAC combines the genetic features of both parental HACs and, most importantly, possesses the same degree of stability as the IgH-bearing HAC [25]. So far, the Tc Mouse™ has yielded one FDA-approved fully human mAb drug.

Tc Bovine was initially developed by Hematech, a subsidiary of Kyowa Hakko Kirin Co. Ltd. and later became Sanford Applied Biosciences LLC (SAB) after its acquisition by North Dakota-based Sanford Health. The key aim of this platform is to rapidly produce a substantial quantity of fully human polyclonal antibodies (pAbs) from immunised or vaccinated cattle. Continuous efforts have been made to optimise this platform since the early 2000s. Firstly, chimeric HAC via fusing human Igλ or Igκ locus with IgH locus as described above and the latest construct by combining all three loci in one HAC greatly improved its stability and efficiency of germline transmission [30, 31, 77]. Secondly, knockout of both bovine IgM genes, b*IGHM* and b*IGHML1*, and, additionally, deletion of J_λ and C_λ gene segments to disrupt the dominantly expressed bovine Igλ locus abolished the endogenous antibody production and significantly reduced the level of circulating chimeric pAbs which contain human heavy chain and bovine light chain [31, 40, 41]. Thirdly, to circumvent the human-bovine interspecies incompatibility between human IgM and bovine transmembrane signalling proteins - Igα and Igβ in the pre-BCR complex, the IgM gene segment on the HAC was partially bovinised by replacing the human sequences encoding part of CH2 and the entire CH3, CH4, TM1 as well as TM2 with their bovine counterparts [77]. The resulting chimeric IgMs in the membrane-bound form showed notably improved functions in supporting B-cell activation and proliferation, and led to a significant increase in the level of fully human pAbs in the plasma. Finally, further bovinisation to counter human-bovine interspecies incompatibility included replacing the

Fig. 5.1 (continued) by black circles, and pseudogenes are represented by blank squares. The first and last V_H, V_K and V_λ genes are labelled. Regions in continuous sequences but not shown in the diagram are represented by broken lines. All variable region, constant region genes and intergenic regions are derived from human except the rat sequences specified in (**c**). In (**c**), the overlaps between transgenic constructs are indicated as green double arrows

class switch regulatory element preceding human IgG1 gene segment with the counterpart associated with bovine IgG1 gene and the sequence encoding human IgG1 transmembrane as well as cytoplasmic domains with their counterparts from bovine IgG1 gene [55]. These species-specific modifications at the chromosome level greatly improved the fully human pAbs production, especially with a desired dominance of human IgG1, κ sub-class in the resulting Tc cattle. Plasma levels of up to 15 g/L fully human IgGs were reached in these transgenic animals, close to the serum antibody levels in a healthy human. Following immunisations or vaccinations, Tc bovine has demonstrated its remarkable potential in producing a substantial quantity of highly potent pAbs against several deadly human viral pathogens including Ebola virus and Middle East respiratory syndrome (MERS) coronavirus [78–80]. The same HAC optimised via a couple rounds of bovinisation to establish the latest Tc cattle was also used to produce Tc goat (caprine) and proven to function efficiently in the latter species due to the high similarity of genomic sequences in both ungulates [81]. It has been shown that Tc goat could work as an economical platform for producing fully human pAbs and upon immunisation, specific antibodies against Avian influenza virus subtype H7N9 could be obtained from this platform.

5.3.3 OmniRat®, OmniMouse® and OmniChicken®

OmniRat® and OmniMouse® are second-generation transgenic platforms producing chimeric antibodies comprising human variable and rodent Fc regions. Both were developed between 2008 and 2015 by Open Monoclonal Technologies Inc. (OMT) which became part of Ligand Pharmaceuticals Inc. in 2016. Technological advances in three areas underlined the rapid development of OmniRat® [48, 59]. Firstly, a circular YAC (cYAC)/BAC shuttle vector was developed which allows multiple DNA fragments with overlapping ends to be assembled as a self-replicating cYAC in a single step in *S. cerevisiae*. Following extraction, such cYAC can be transformed into *E. coli* as a BAC. The high fidelity associated with the robust homologous recombination system and the stability of highly repetitive DNA in yeast have been especially advantageous when constructing the human-rat chimeric transgene locus. This method ensures the integrity of the highly complex regulatory regions linked to the rat IgH constant gene segments which include switch regions, various intronic enhancers and the 3′ locus control region. In addition, the resulting BACs can be efficiently purified in a large quantity and high purity from *E. coli* to facilitate the transgenic process. Secondly, the gene knockout technology via specifically designed ZFNs was employed to generate a triple knockout rat strain carrying deletions of the entire J_H gene segments, C_κ gene and all functional J_λ-C_λ gene segments in a short timeframe. The endogenous antibody production is completely inactivated in this strain. Thirdly, co-injection of three large DNA fragments with overlapping ends of 6–20 kbps into the pronuclei of fertilised rat oocytes resulted in the co-integration of these fragments into the host genome in the expected configuration with relatively high efficiency. This method allows the reconstitution of a transgene locus up to 500–600 kbps in size via one round of microinjection

combined with subsequent founder identification. As shown in Fig. 5.1c, two OmniRat® strains have been generated, each harbouring a distinct chimeric IgH transgene locus composed of a series of human V_H with 22 being functional, all human D_H and J_H followed immediately by rat intronic enhancer region at the 3′ of J_H and 5′ of C_μ, the entire rat C_μ-$C_{\gamma 1}$-$C_{\gamma 2b}$-C_ε-C_α in conjunction with all associated regulatory regions and the 3′ locus control region [48, 59, 83]. Both strains also contain the reconstituted human Igκ locus encompassing all functional V_κ gene segments within the proximal cluster, all J_κ and C_κ followed by the downstream regulatory region and a human Igλ locus encompassing 16 functional V_λ genes, all 5 J_λ-C_λ cassettes and the 3′ enhancer region ([48, 59] and unpublished data). The two OmniRat® strains can be bred together to increase the diversity of the V_H gene repertoire. The same transgenic constructs for establishing OmniRat® were also used to develop OmniMouse®, and the latter was generated in the double knockout background by deleting all mouse J_H gene segments and C_κ region via gene targeting in ES cells.

OmniChicken® is also a second-generation transgenic platform producing chimeric IgGs comprising human variable and chicken IgY Fc regions. It was developed by Crystal Bioscience Inc. which later became part of Ligand Pharmaceuticals Inc. As human and chicken are much more phylogenetically distant than human and rodent, this platform potentially provides a way to raise mAbs against antigens which are highly conserved between mammals. Further, cross-reactive mAbs against a given human antigen and its mouse homolog can be obtained from this platform, which is highly desirable for pre-clinical drug development. The chicken Ig heavy chain gene locus contains a single functional V_H, a small number of highly related D_H and a single J_H segment, and its only light chain locus contains a single functional V_L and J_L segment. Rearrangement in both loci produces limited diversity in the somatic repertoire. Further diversity is accumulated by multiple rounds of gene conversion events involving a variety of upstream pseudogenes which are used to mutate the rearranged V_HDJ_H and V_LJ_L [84]. These mutations are mainly focused in the CDRs. To create OmniChicken®, the endogenous Ig heavy and light chain loci were humanised in situ in the cultured primordial germ cells prior to establishing transgenic lines. The humanisation of the heavy chain locus was achieved in a two-step process: (1) a landing pad containing selectable markers and an attP site was utilised to replace the single J_H segment by homologous recombination, producing an IgH knockout, and (2) co-transfection of phiC31 integrase along with a synthetic human locus encompassing 20 V_H pseudogenes followed by a region encoding a rearranged functional V_HDJ_H which consists of V_H3–23, D1 and J_H4, a frequently found combination in the human population [84]. The integrase facilitated the integration of human transgene into the attP site specifically, allowing the V_HDJ_H region to splice precisely to the endogenous chicken Ig constant region gene segments. The pseudogenes were carefully designed to encode the similar framework regions as in the rearranged V_HDJ_H but with very high diversity in CDRs. Using a strategy analogous to heavy chain humanisation, chicken Ig light chain locus was humanised to contain a series of V_K or V_λ pseudogenes followed by a region encoding a rearranged functional V_KJ_K or $V_\lambda J_\lambda$ which is well expressed in humans.

Robust immune responses and production of high-affinity chimeric antibodies have been demonstrated in OmniChicken® [84]. OmniRat®, OmniMouse® and OmniChicken® form the core of the multi-species OmniAb® therapeutic human antibody platforms which can be licensed from Ligand Pharmaceuticals Inc.

5.3.4 VelociMouse®, Kymouse™ and Trianni Mouse®

VelociMouse® was developed by Regeneron Pharmaceuticals from the late 1990s to early 2010s. It is the first transgenic mouse platform with a megabase of endogenous Ig gene loci being precisely humanised in situ using the ES cell-based technology. As illustrated in Fig. 5.2a, the mouse IgH locus was humanised in nine steps [22]. The initial step was to

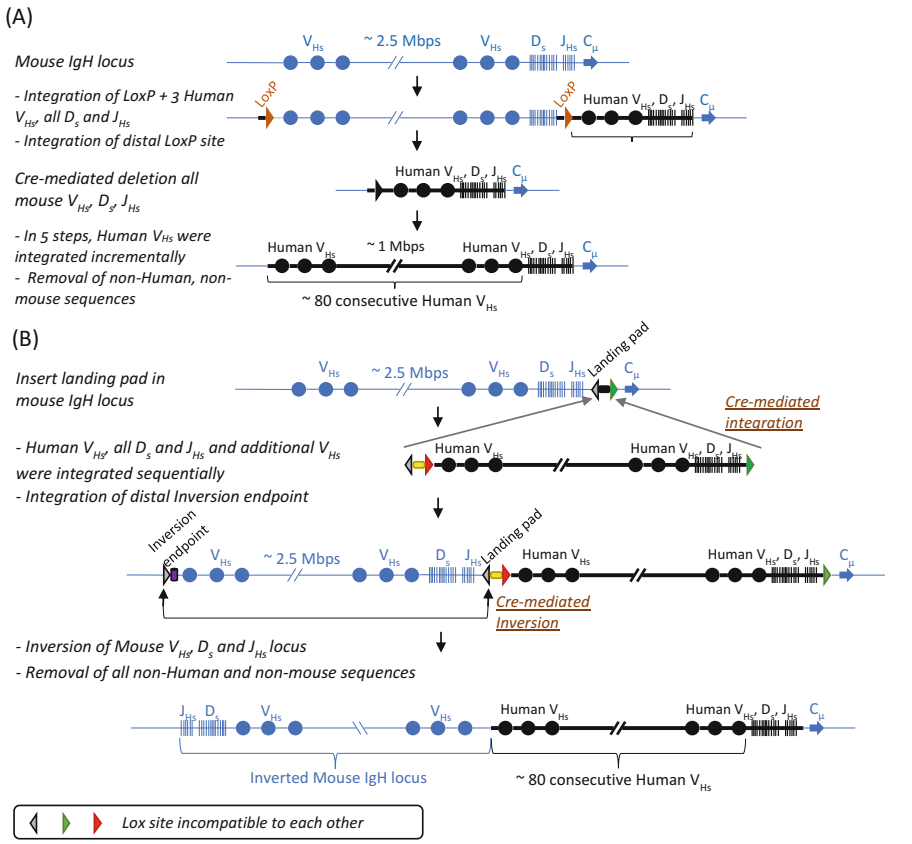

Fig. 5.2 Diagram showing the transgenic process of in situ humanisation of the mouse IgH gene locus when creating (A) VelociMouse® and (B) Kymouse™. Functional V_H genes are represented by solid circles. Regions in continuous sequences but not shown in the diagram are represented by broken lines. Mouse endogenous IgH locus is in blue, and human transgene locus is in black

integrate a 144 kbps transgenic construct derived from a modified BAC into the mouse genome to replace a 16 kbps region including all J_{Hs} via homologous recombination. The construct contains a LoxP site and a human genomic region encompassing 3 V_H, all D and J_H in germline configuration flanked by mouse genomic sequences matching those surrounding the designated insertion sites. This integration ensured the intact mouse intronic enhancer and C_μ switch region to be located immediately downstream of the human J_H segments which in turn prompted the efficient production of chimeric antibodies comprising human variable and mouse Fc region. The second step was to insert a LoxP site along with an appropriate selectable marker upstream of the most distal mouse V_H segment. In the third step, via Cre-LoxP-mediated chromosome deletion, a genomic region spanning ~2.5 Mbps and containing the entire mouse V_H, D and J_H locus was removed in the ES cells. Subsequently, in five steps, additional human V_H carried on modified BACs were inserted upstream of the existing ones through homologous recombination. In the final step, the remaining selectable markers after the final insertion step were removed through FLP-FRT-mediated deletion. As a result, the mouse V_H, D and J_H locus was precisely replaced by ~1 Mbps of human equivalent sequence consisting of 80 consecutive V_H, 27 D and 6 J_H. In addition, through a similar strategy, the mouse V_K and J_K locus spanning ~3 Mbps were deleted and subsequently replaced by a 500 kbps of human genomic region encompassing the entire proximal V_K gene cluster and all J_K segments [22]. Humanisation of the mouse Igλ locus wasn't reported in the published data of VelociMouse®. In this platform, the serum levels of antibodies of all subtypes are similar to those found in wild-type mouse pre- and post-immunisation, and the gene usage of V_H, D and J_H as well as V_K and J_K in the antibody repertoire is comparable to that in humans [54]. To date, four FDA-approved antibody drugs are derived from VelociMouse®.

Kymouse™ was developed by Kymab Ltd. and accomplished later than VelociMouse®. The creation of this platform also involved in situ humanisation of mouse IgH, IgK and, additionally, Igλ gene locus in ES cells [60]. As shown in Fig. 5.2b, in the first stage, ~1 Mbps of human V_H, D_s and J_H locus similar to that described for VelociMouse® was inserted immediately downstream of mouse J_H region in several steps. A technical advance in this work is the inventive use of three types of Lox sites (LoxP and two derivatives) which are incompatible with each other. Firstly, via homologous recombination, a landing pad containing an appropriate selectable marker flanked by two different Lox sites was integrated downstream of the most proximal mouse J_H segment. Subsequently, a series of human V_H, all D and J_H segments flanked by two Lox sites matching those in the initial landing pad were integrated into the genome facilitated by the transiently expressed Cre recombinase. Along with the integrated human transgene, a new landing pad at its 5′ end was generated which has a different makeup from the initial one. Additional human V_H gene segments were inserted upstream of existing ones through several rounds of Cre-Lox-mediated integration. The process was designed so that between rounds, non-human and non-mouse sequences except those in the newly generated landing pad were removed through transiently expressed PiggyBac transposase. In the second stage, an 'Inversion endpoint' was integrated upstream of the most distal mouse V_H

segment. The Lox site in the endpoint and the matching one in the most distal landing pad are inverted relative to each other. Using Cre-Lox-mediated inversion, the entire mouse V_H, all D and J_H locus was put into opposite orientation to the mouse Ig constant region genes, resulting in no functional endogenous antibodies being produced. Lastly, all non-human and non-mouse sequences were removed by PiggyBac transposase. The inversion strategy of the endogenous locus differentiates from the large genome deletion used in developing VelociMouse®. Using a similar process, a derivative of human IgK locus spanning ~825 kbps including an inverted distal V_K gene cluster, the proximal V_K gene cluster and all J_K segments was integrated into the mouse genome upstream of the C_K segment, and a ~ 26.3 Mbps mouse genomic region encompassing all V_K and J_K segments was inverted relative to mouse C_K gene [60]. Furthermore, a ~917 kbps human genomic region encompassing all V_λ genes, J_λ-C_λ cassettes and the 3′ enhancer region was integrated into the mouse Igλ locus [60]. Robust immune responses and an antibody repertoire with diversities as extensive as in humans were demonstrated in Kymouse™. Trianni Mouse® is a newly accomplished platform by Trianni Inc. In this platform, the in situ humanisation of mouse V_H, D, J_H, V_K, J_K, V_λ and J_λ gene segments was restricted to the coding regions only. All intronic and intergenic regions in mouse genome within and surrounding each of the gene segment were kept intact, preserving all the authentic cis-elements for gene expression control and consequently, eliminating any interspecies barrier between human and mouse. Details of the transgene design haven't been published for this platform.

5.3.5 Transgenic Platforms Expressing Different Formats of Antibodies

5.3.5.1 OmniFlic®and OmniClic®

Several transgenic platforms including OmniFlic®, OmniClic® and MeMo® mouse (Table 5.1) were created to express only a single human IgK light chain, so their antibody diversity was derived primarily from the human IgH transgene. These fixed light chain antibodies are especially useful in formulating bi-specific and multispecific antibody drugs as the undesirable mispairing between heavy and light chains of multiple different antibodies is eliminated. OmniFlic® was developed by OMT Inc. as a transgenic rat platform whose chimeric human-rat IgH transgene locus is the same as in OmniRat®. The fully human IgK transgene locus in this platform contains a genomic region encoding the rearranged IgKV3–15-J_K1 which is commonly found in the human population, followed by the C_κ gene and downstream regulatory region [85]. OmniClic® was developed by Ligand Pharmaceuticals Inc. as a transgenic chicken platform which bears the same human IgH transgene as in OmniChicken®. The human genomic region encoding the rearranged IgKV3–15-J_K1 was used to replace the endogenous functional V_L and J_L segment, and all upstream V_L pseudogenes were removed. Upon immunisation, both OmniFlic® and OmniClic® can yield diverse, specific and highly potent antibodies with

a common light chain against a variety of antigens ([85] and unpublished data). Both have been included in the OmniAb® collection of therapeutic human antibody platforms.

5.3.5.2 HCAb Transgenic Mice (Harbour Biomed), Humabody® VH Platform and UniRat®

Heavy chain only antibodies (HCAbs) devoid of any Ig light chain occur naturally in Camelidae and cartilaginous fish [86, 87]. In camelids these homodimeric IgG antibodies lack the first constant domain (CH1) [88] and use a diverse but distinct set of V_H region genes. These genes, termed V_{HH}, contain four hydrophilic amino acid substitutions at conserved positions which normally associate with the light chain and so improve single domain solubility [89]. The lack of the CH1 domain removes both a cysteine used in making a disulphide bond with the light chain constant region and a control mechanism ensuring the pairing of heavy and light chain in conventional antibodies. In the absence of light chain, the chaperonin protein BiP binds to the CH1 domain and prevents the heavy chain from being secreted from the ER [90, 91]. The specificity of camelid HCAbs is dependent on the variable domain which can be expressed separately, producing an antibody fragment (nanobody) of 12–14 kDa. These single domain antibodies (sdAb) have good biophysical properties including excellent stability and low levels of aggregation [92, 93]. They also target epitopes not readily accessible to classical antibodies [94, 95] and are predicted to penetrate tissue or tumours better than mAbs [96, 97]. A further feature is they can be easily linked together to generate multivalent or multispecific molecules [96, 98]. Several clinical trials involving nanobodies are ongoing, with the first therapeutic drug, caplacizumab, a humanised bivalent anti-von Willebrand factor nanobody, receiving approval in Europe and the USA recently [99].

The first transgenic animals to make HCAbs used a hybrid llama/human antibody construct. This contained two llama V_{HH} genes and human D, J_H and mu and/or gamma constant region genes missing CH1, in a mouse background lacking endogenous B-cell development (μMT^-). These animals also gave the first evidence that the mu/delta constant regions were not necessary to produce HCAbs and actually may be detrimental [100]. This hybrid transgenic mouse was modified to make fully human HCAbs by replacing the V_{HH} genes with, in the first instance human VH1–46, VH3–11, VH3–53 and VH3–23 [101]. These HCAb transgenic mice, developed by Harbour Antibodies (Harbour Biomed), have been modified further to include more V_H genes.

A second mouse transgenic platform, the Humabody® VH platform, otherwise known as the Crescendo Mouse, was created by Crescendo Biologics Ltd. using a 339 kbps YAC introduced into fertilised oocytes by pronuclear microinjection. The YAC comprised ten human V_H genes, all human D and J_H genes, the mouse μ enhancer, mouse Cγ1 without CH1 and murine 3′ enhancer region [102]. The background for this mouse is a triple knockout, silenced for endogenous heavy or light chain expression. These animals successfully utilised all V_H and J_H gene segments producing high-affinity VH domains with bio-therapeutic potential.

A platform producing human HCAbs in transgenic rats has been developed by TeneoBio Inc., a spinoff from OMT Inc. Similar to the OmniRat® platform, two UniRat® strains in triple knockout background were generated of which the human V_H, D and J_H loci are identical to both OmniRat® strains. The engineered rat IgH constant gene locus in both UniRat® strains contains the rat intronic enhancer at the 3′ of J_H locus and μ switch region which is directly linked to Cγ2a-Cγ1-Cγ2b, all lacking CH1 domains, followed by Cε-Cα genes in conjunction with all associated regulatory regions and the 3′ locus control region [83]. One germline change was introduced, W103R (Kabat numbering) in J_H4, a residue which is constitutively conserved and is an important contact for the light chain [103]. This change decreases the V domain hydrophobicity and was found to reduce the occurrence of lambda light chain association on screening UniRat sequences by ELISA. The UniRat® platform gave robust immune responses to a wide array of antigens, with HCAbs covering nearly the entire productive human heavy chain V(D)J repertoire being identified, producing a large panel of stable sdAbs with high affinity and specificity [83].

5.4 Strategies in Antibody Discovery from Transgenic Platforms

Hybridoma technology is commonly used for the discovery of fully human or chimeric mAbs from immunised transgenic rodents. Antigen-specific single B-cell sorting followed by antibody repertoire cloning from single cells is also widely used [104]. Encapsulating single plasma cells along with detection reagents in microdroplets combined with image analysis is especially useful in identifying antigen-specific mAbs from immunised transgenic chickens [84]. New nanotechnology by placing single plasma cells on a microchip to achieve one cell per nano-well followed by screening and antibody repertoire cloning has also been validated for mAbs discovery from transgenic rodents [105]. Moreover, from immunised transgenic mice, a phage or yeast display library of antibody fragments with native heavy and light chain pairing has been constructed, which involves partitioning single B cells into emulsion droplets containing oligo(dT)-microbeads for synthesising beads-captured cDNA and, subsequently, amplifying linked cognate heavy and light chain transcripts via emulsion PCR from single cDNA-beads [106]. The library screening provides a fast way of identifying antigen-specific mAbs. Alternatively, with platforms producing mAbs with fixed light chain or HCAbs, since the antibody diversity is derived from the heavy chain only, following immunisation, a list of candidate binders can be predicted through next-generation sequencing of the entire heavy chain V(D)J repertoire. Ranking analysis, followed by high-throughput expression of antibody fragments from the candidate list and downstream screening, enables the antigen-specific VH domains to be identified [83, 85]. After the discovery phase, all selected binders can be re-formatted into products with desired formulations and expressed in large scale in a suitable system, such as the CHO-expression system which can routinely yield 5 g/L for mAbs and their derivatives.

A big challenge in antibody discovery from transgenic platforms is to obtain specific binders to antigens that are highly conserved between human and the host species. Many strategies have been adopted to break the immune tolerance, which include the knockout or knockdown of the homolog of a given antigen in the host prior to immunisations. Another solution to this problem is to choose a suitable transgenic host other than mice which have been dominantly used. These species include transgenic rat, rabbit and chicken that were made available commercially in recent years. Moreover, developing transgenic platforms is an expensive and highly time- as well as resource-consuming process, and consequently, each platform is protected through sophisticated patents. End users usually pay a substantial amount of licensing fee in adopting transgenic platforms for their discovery projects. In addition, a hefty royalty charge ensues if an antibody drug developed from a particular platform gains FDA approval. However, the choices of different platforms (multi-species and multiple antibody formats) have been significantly broadened recently, which may in turn drive down the cost in using transgenic platforms for antibody discovery.

Take Home Messages

- In transgenic platforms for human antibody discovery, the host antibody heavy chain VDJ and light chain VJ repertoire are substituted by their counterparts which are encoded exclusively by human transgenes.
- Transgenic platforms take advantage of the host's naturally antigen-driven antibody selection and maturation process which is associated with the primary and secondary immune response following immunisations with a selected target antigen.
- In transgenic platforms, the endogenous antibody production is inactivated via gene knockout and large chromosome deletion or inversion.
- Transgenic constructs in BACs or YACs containing human germline V_{Hs}, D_s and J_{Hs} genes in conjunction with C_H gene segments and all associated regulatory regions derived from human or host species and, separately, constructs containing human IgK and/or Igλ locus can be integrated into the host genome following oocyte microinjection or spheroplast-ES cell fusion.
- Alternatively, human humoral immunity in transgenic animals can be recapitulated from HACs containing human IgH, IgK and/or Igλ locus which are maintained in ES cells or transferred into cultured foetal fibroblast cells prior to establishing transgenic lines.
- In mouse ES cells or chicken primordial germ cells, the variable region gene segments in host IgH, IgK and/or Igλ loci can be humanised in situ.
- Antibody production in transgenic animals carrying full human IgH locus may be suboptimal. This has been attributed to imperfect interaction of the human constant region with endogenous signalling components such as the Igα/β in mouse, rat or cattle. Significant improvements have been obtained when the human IgH variable region genes were linked to the endogenous C_H gene segments and associated regulatory regions.

(continued)

- The antibody discovery process using transgenic platforms can be as short as 6–8 months from the immunisations to obtaining ideal binders, which significantly reduces the development time for antibody-based therapies.

Further Reading

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