UniversitätsSpital Zürich

Genetic humanization and the generation of human antibodies from transgenic animals

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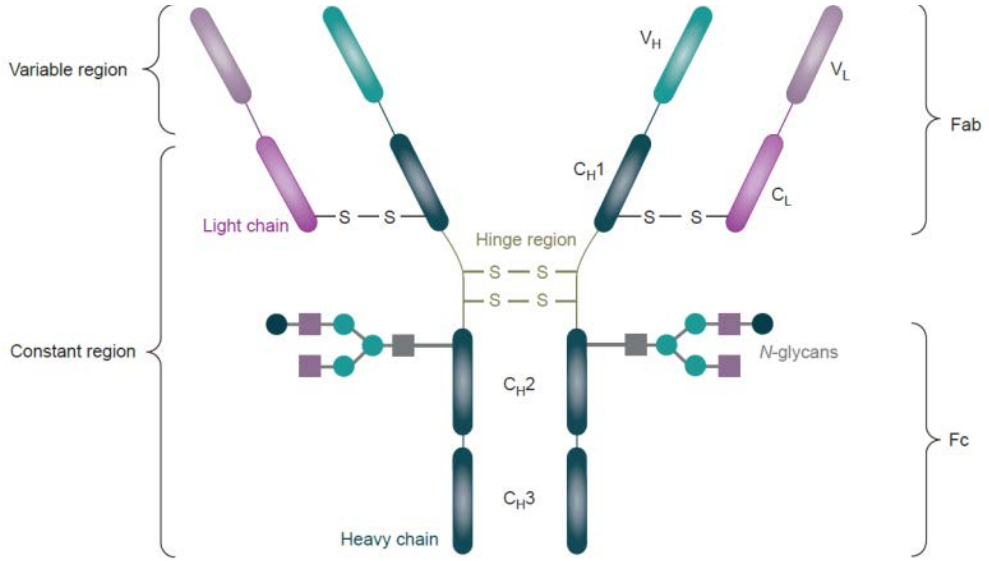
January 10th 2017

Outline

- Immunoglobulin loci and B cell development: basic concepts
- Focus on techniques for genetic humanization of mice for the generation of human antibodies.
- Example of how these engineered animals allows for human antibody responses within a mouse background and provide a valuable platform for the generation of fully human antibodies as therapeutics.

Immunoglobulin structure



Naturally produced immunoglobulins or antibodies are macromolecular Y-shaped proteins of approximately 150 kDa.

In the animal, antibodies are produced primarily by plasma cells, a type of terminally differentiated B lymphocyte upon activation of the immune system.

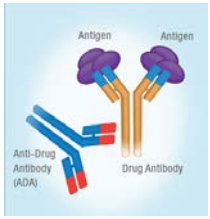
They can be generated to selectively target a specific antigen binding partner and are universal weapons against pathogenic threats.

- **Diagnostic**
- **Research**
- **Therapy**

How therapeutic antibodies can be produced?

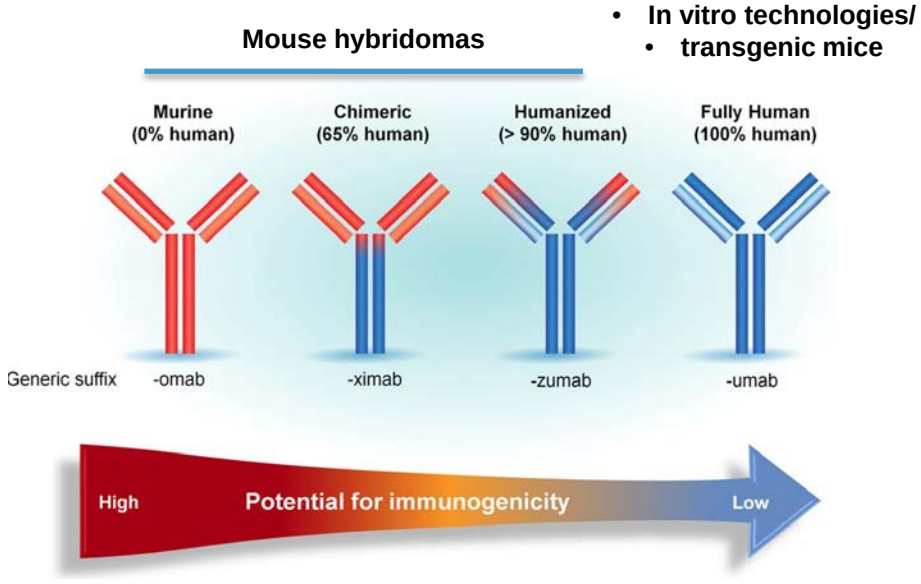
Humanization of antibodies for therapy

- Mice can readily be immunized with any antigen of interest and are not tolerant to most human proteins (mAbs with picomolar affinity for target antigens with high (>90%) identity in the amino acid sequence between mouse and human).
- Tolerance issues: anti-drug-antibody (ADA;HAMA)

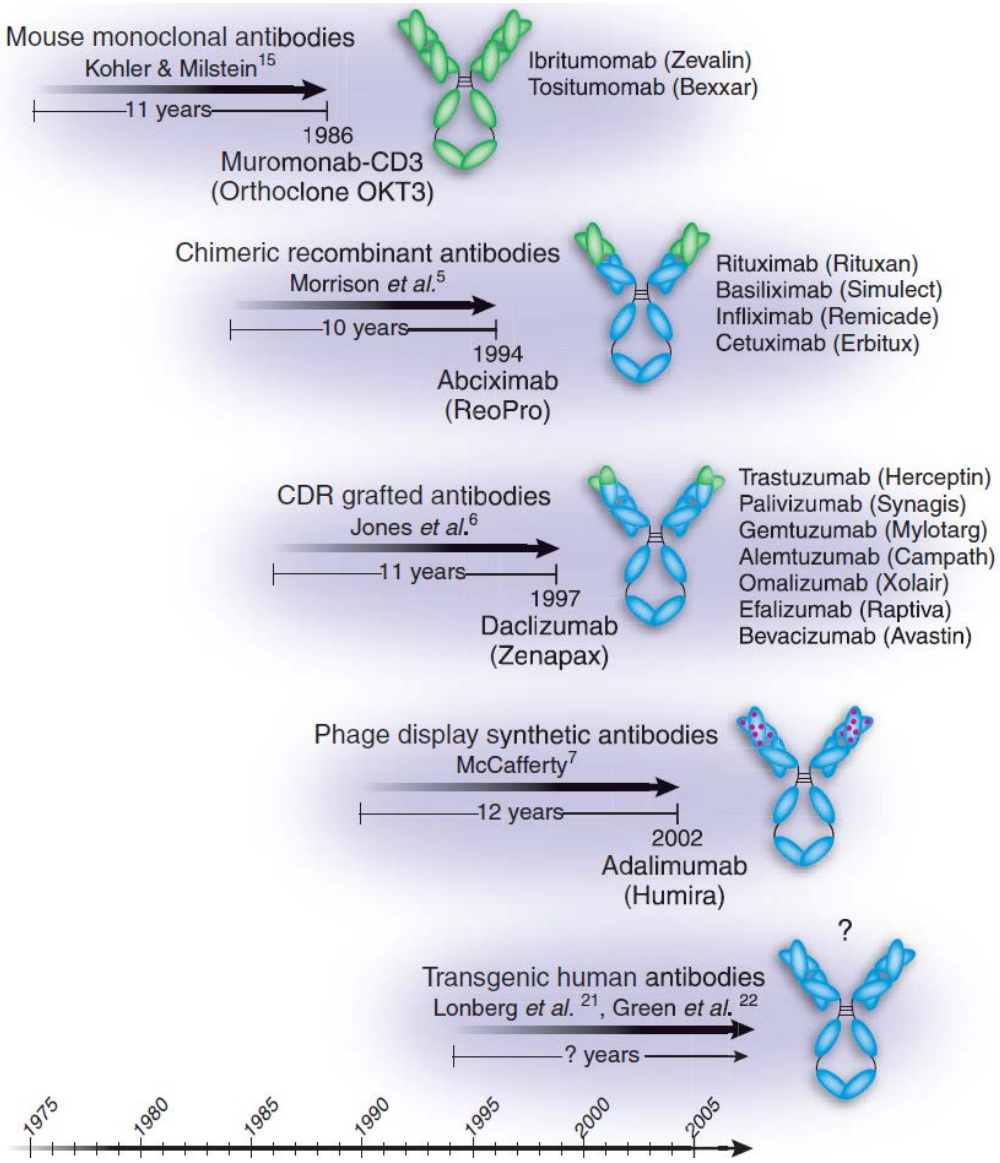


- Production of chimeric, humanized and fully human antibodies

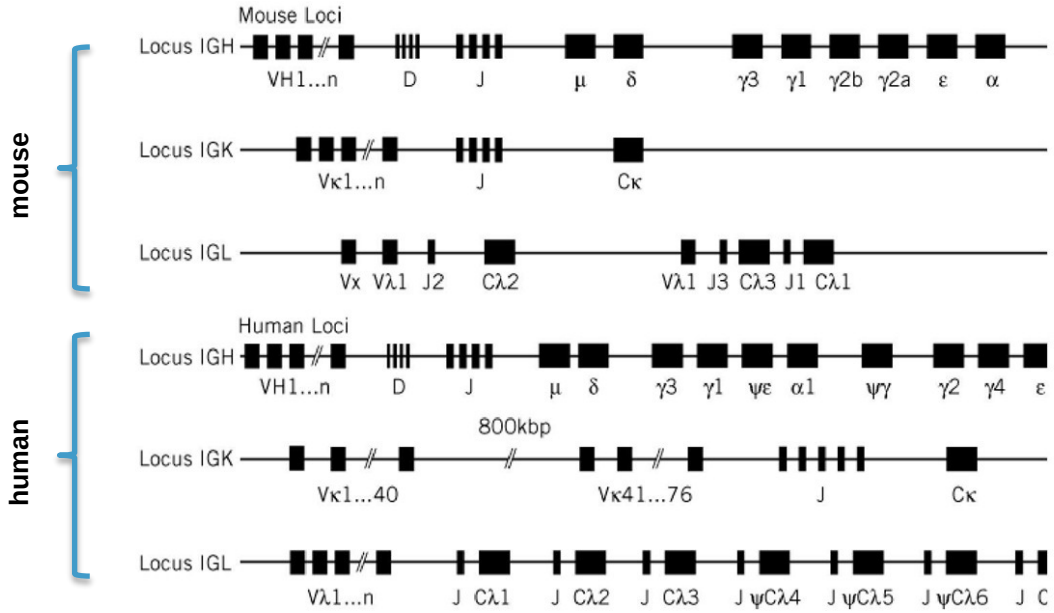
- Beside in vitro technologies, production of human antibodies in genetically modified mice is a highly attractive approach for the generation of human mAbs against human antigens



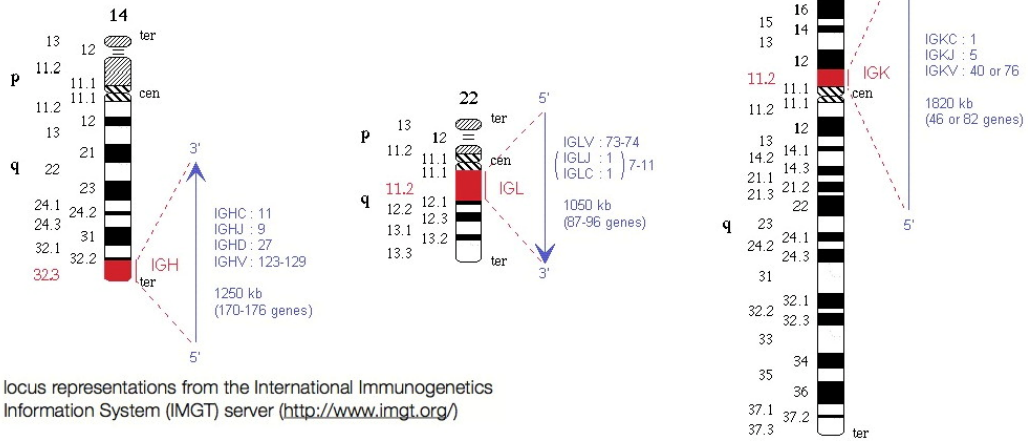
Evolution of therapeutic antibody technology and progress to the clinic



Schematic organization of the three Ig gene loci in mice and humans

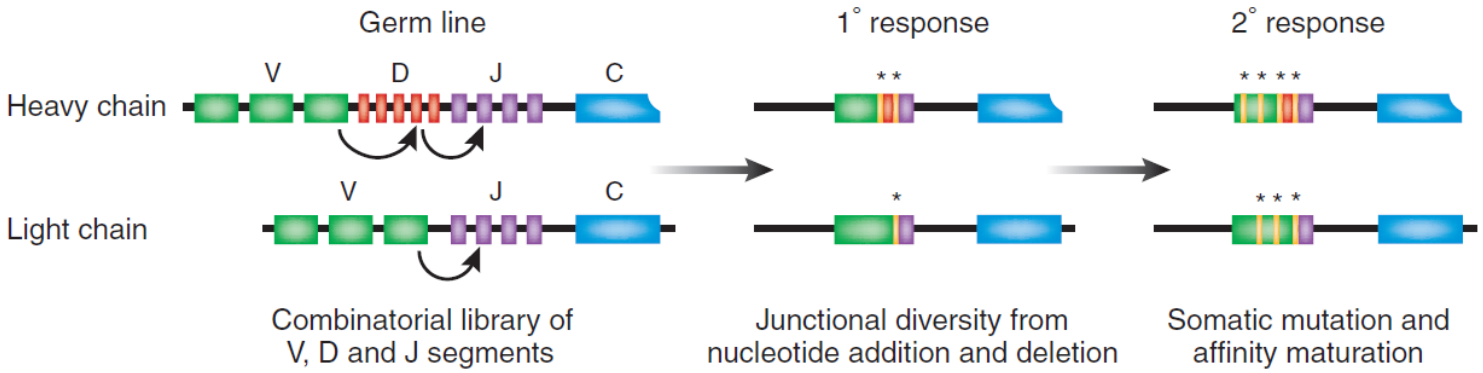


The IGK, IGL, and IGH loci are located on mouse chromosomes 6, 16, and 12 an human chromosomes 2, 22, and 14, respectively



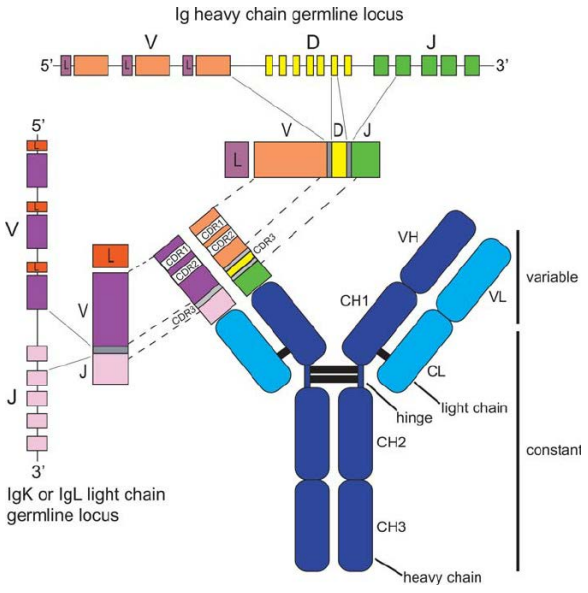
locus representations from the International Immunogenetics Information System (IMGT) server (http://www.imgt.org/)

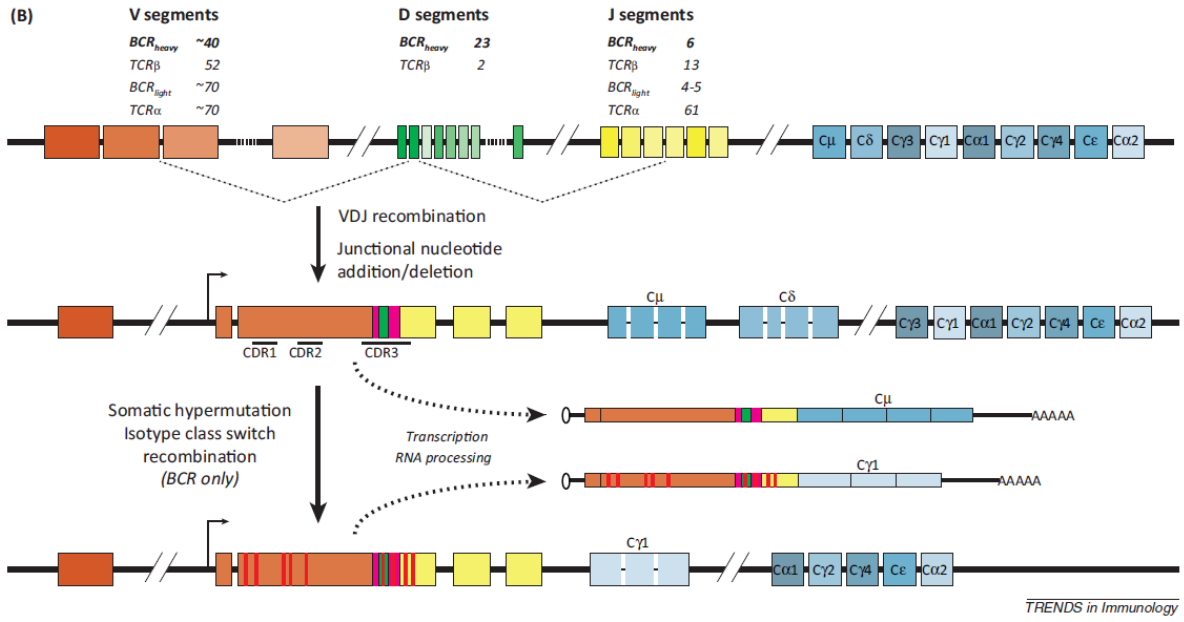
Three sources of diversity contribute to antibody repertoires: combinatorial, junctional and somatic



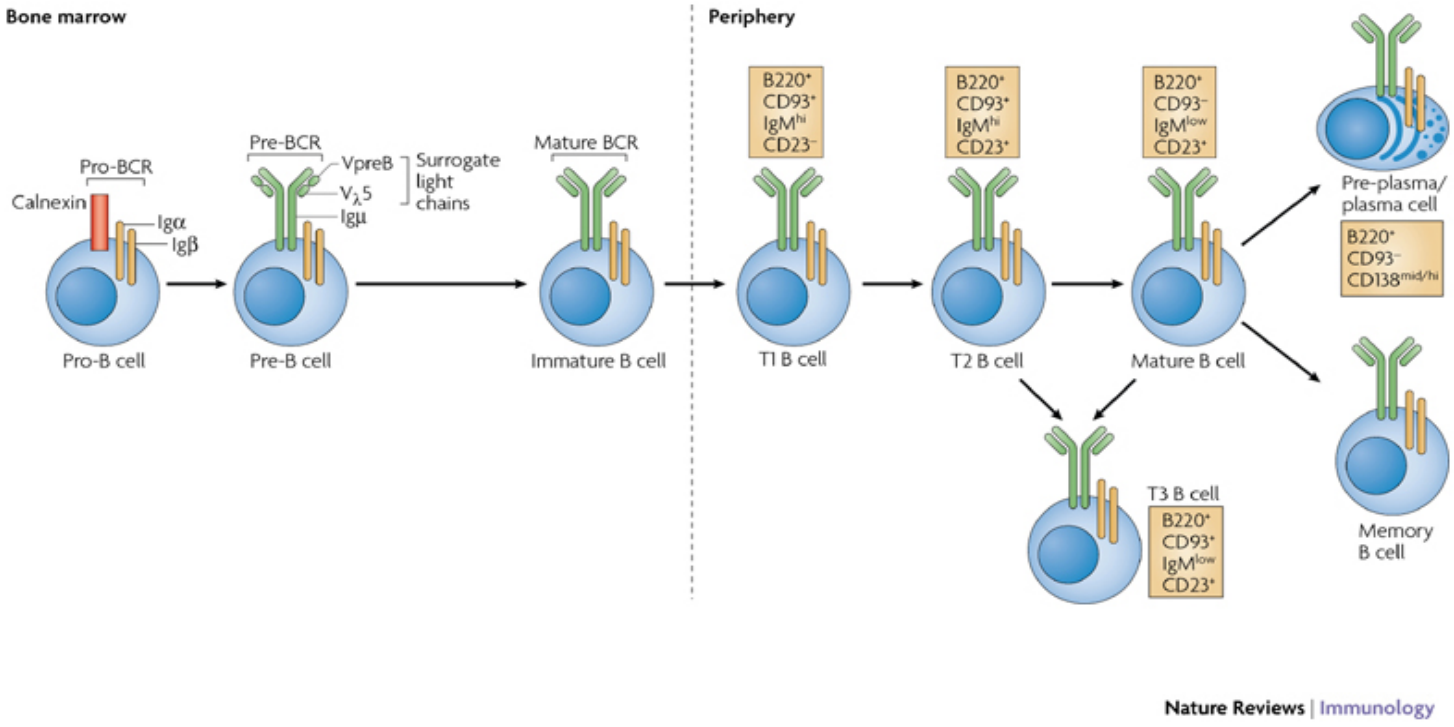
Lonberg Nature Biotech2005

Three sources of diversity contribute to antibody repertoires: combinatorial, junctional and somatic





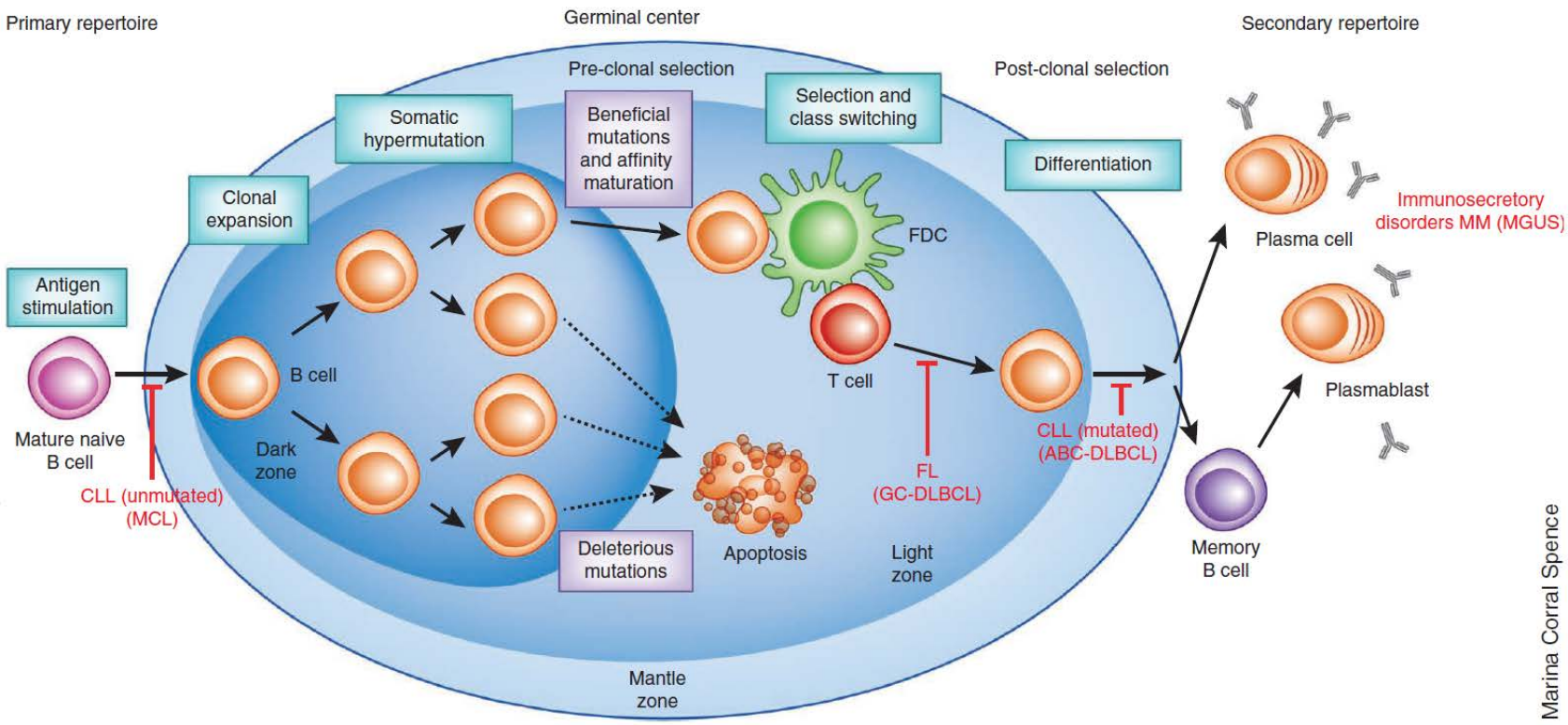
B-cell development occurs in both the bone marrow and peripheral lymphoid tissues



In the bone marrow, development progresses through the pro-B-cell, pre-B-cell and immature-B-cell stages. During this differentiation, rearrangements at the immunoglobulin locus result in the generation and surface expression of the pre-B-cell receptor (pre-BCR, Igμ heavy chain and surrogate light chains (VpreB or Vλ5)) and finally a mature BCR (comprised of rearranged heavy- and light-chain genes) that is capable of binding antigen. At this immature stage of development, B cells undergo a selection process to prevent any further development of self-reactive cells.

Cells successfully completing this checkpoint leave the bone marrow as transitional B cells, eventually maturing into mature follicular B cells (or marginal-zone B cells). Following an immune response, antigen-specific B cells develop into either plasma (antibody-secreting) cells or memory B cells. Transitional 3 (T3) B cells, are thought to represent primarily self-reactive anergic B cells (also known as An1 B cells).

Antigen stimulation and development of antigen-specific B cells in the germinal center



The first mice genetically engineered to produce fully human monoclonal antibodies

- Mice from Brüggemann et al. (1989) only expressed a human heavy chain mini locus in the presence of the endogenous mouse heavy and light chain loci.

- In 1994, in two different laboratories, mice were generated that combined disruptions of the endogenous mouse heavy and kappa light chain loci and transgenes encoding human heavy and kappa light chain loci

- The endogenous mouse lambda light chain locus was left unmodified by both groups because it only contributes to 5–15% of the antibody repertoire

- The human transgenes in such mice had undergone V_HDJ_H and $V_\kappa J_\kappa$ rearrangements with N region diversification, i.e., additions or deletions of nucleotides at the V_H-D-J_H and V_κ-J_κ junctions, class switch recombination and somatic hypermutation

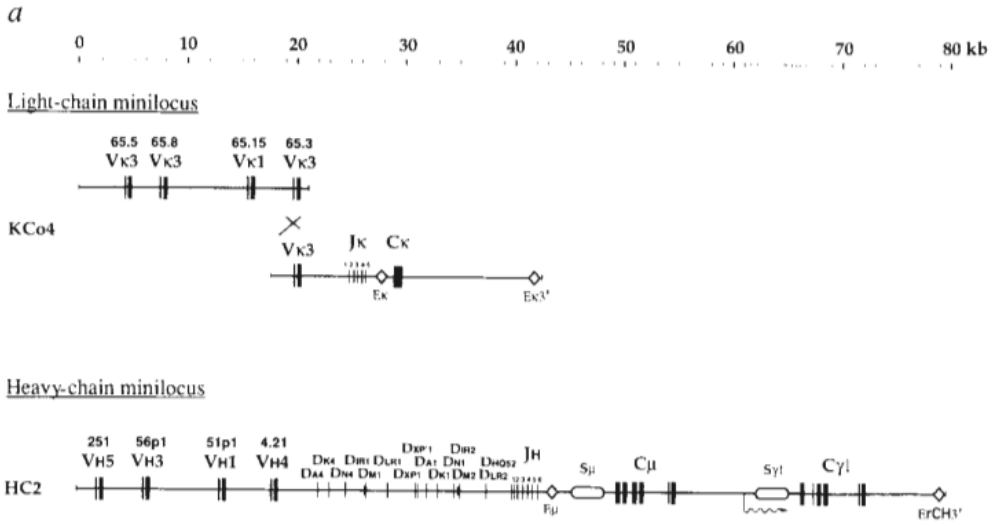
- Despite the limited repertoire present in both transgenic mouse strains, immunization led to the generation of specific human antibodies against several antigens that were accessible by conventional hybridoma technology.

First generation of humanized Ig mice: Double transgenic/double deletion

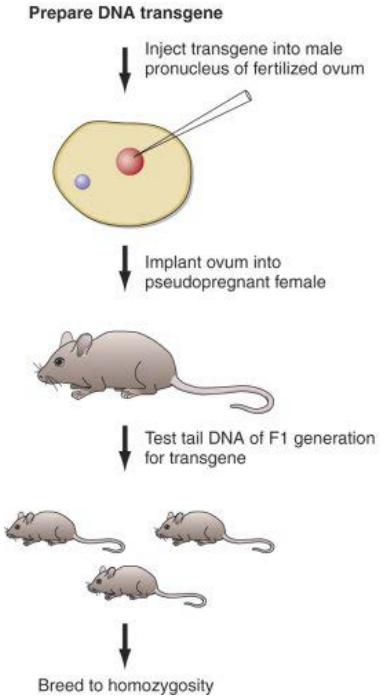
Antigen-specific human antibodies from mice comprising four distinct genetic modifications

Nils Lonberg, Lisa D. Taylor, Fiona A. Harding, Mary Trounstine, Kay M. Higgins, Stephen R. Schramm, Chiung-Chi Kuo, Roshanak Mashayekh, Kathryn Wymore, James G. McCabe, Donna Munoz-O'Regan, Susan L. O'Donnell, Elizabeth S. G. Lapachet, Tasha Bengoechea, Dianne M. Fishwild, Condie E. Carmack, Robert M. Kay* & Dennis Huszar

HuMabMouse®

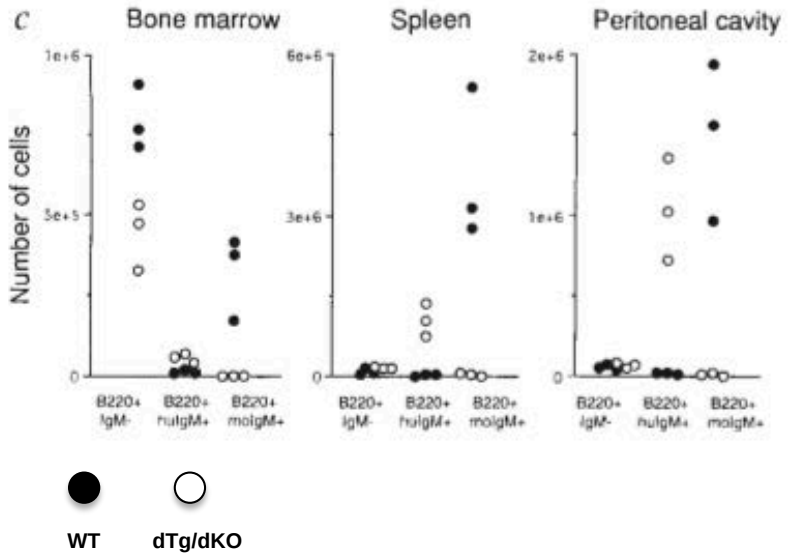


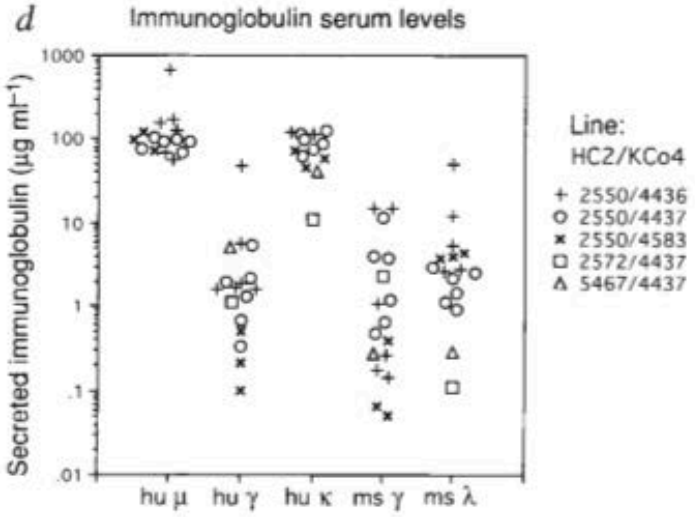
- the human heavy chain locus: 3 V_H, 16 D, all 6 J_H and the C_μ and $C_{\gamma 1}$ constant region gene segments.
- the light chain locus: 4 V_κ, all 5 J_κ and the C_κ gene segments (subcloned into plasmid vector from a human placental genomic DNA phage library)
- Tg mice bred with Ig KO mice
  - JHD (neo cassette in the J segment)
  - JCKD (deletion Jk and Ck)



Genpharm Inc (later Medarex, Inc. and now part of Bristol-Meyers Squibb, Inc. after the acquisition of Medarex)

B cells and Ig in Double transgenic/double deletion mice



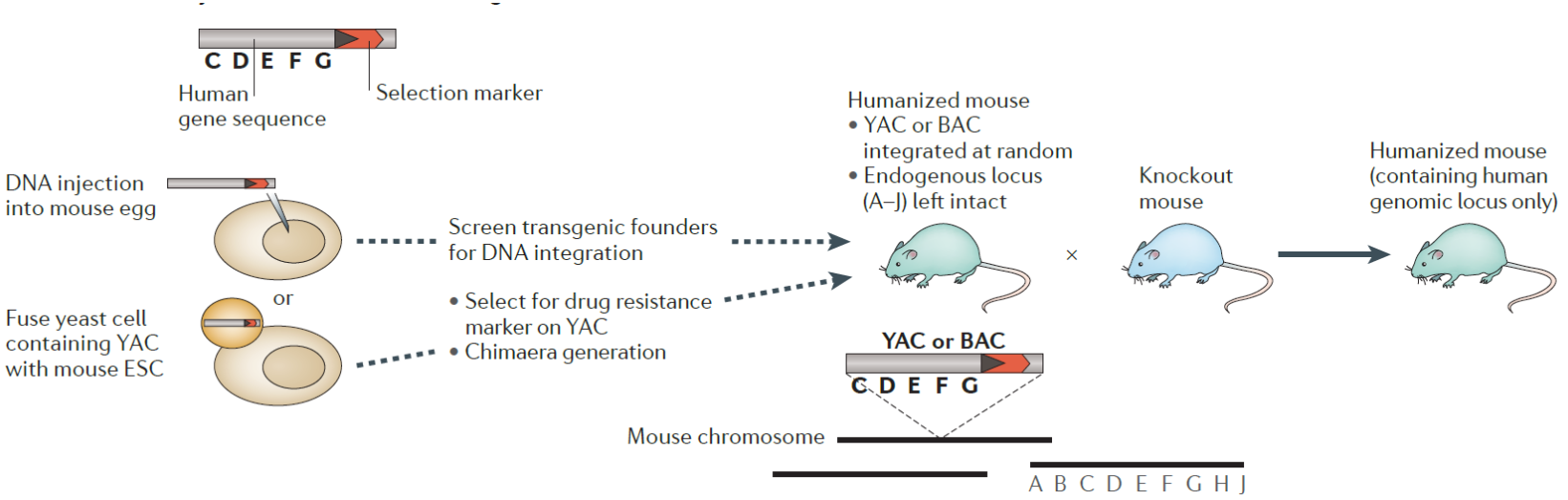


- Comparison between pre-B and B cells
- The number of Tg B-cell is 10-50% of WT in Bone marrow and spleen

- DTg/dKO express fully human antibody in the serum
- Hu γ derives from class switch

- dTg/dKO mice responded to immunization and hybridomas could be isolated that secreted IgG with Kd of ≈ 700 nM

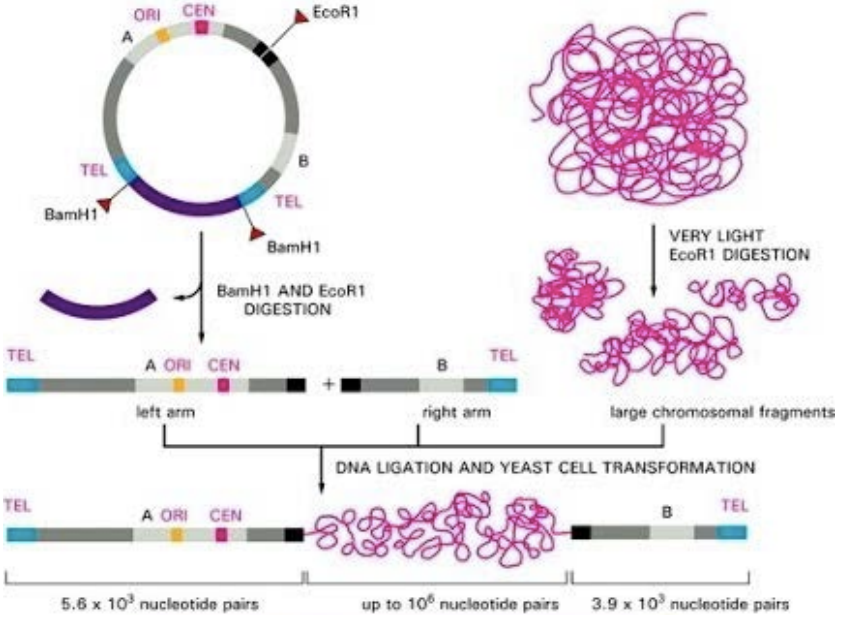
Humanization by YAC (or BAC) additive transgenesis



- Transgenic lines can be created YAC (and BAC) DNAs, and these DNAs will integrate at random chromosomal positions
- The key advantage of BACs and YACs is their size: up to 300 kb for BACs and up to Mb for YACs
- Inclusion of all or some of the upstream and downstream *cis*-sequences regulating expression of a gene of interest
- YAC and BAC transgenic insertions show position-independent and copy-number-dependent expression
- The extent of humanization that is achievable with YACs can be increased by exploiting homologous recombination in yeast to join two existing YACs via a region of shared homology into a single larger recombinant YAC.
- The recombinant YAC can then be transferred into ESCs by fusion with yeast spheroplasts carrying the YAC, followed by selection for a drug-resistance selection marker.
- ESCs can then be used to generate chimaeras to achieve germline transmission.

YAC: yeast artificial chromomosome

YAC: genetically engineered chromosomes derived from the DNA of the yeast



Essential components of YAC vectors

- Centromers (CEN), telomeres (TEL) and autonomous replicating sequence (ARS) for proliferation in the host cell.
- *amp*^{r} for selective amplification and markers such as TRP1 and URA3 for identifying cells containing the YAC vector.
- Recognition sites of restriction enzymes (e.g., EcoRI and BamHI)
- Capable of carrying a large DNA fragment (up to 2 Mb)
- Low transformation efficiency

Procedure for YAC libraries

The target DNA is partially digested by EcoRI and the YAC vector is cleaved by EcoRI and BamHI.
Ligation of the cleaved vector segments with a digested DNA fragment to form an artificial chromosome.
Transformation of yeast cells to make a large number of copies.

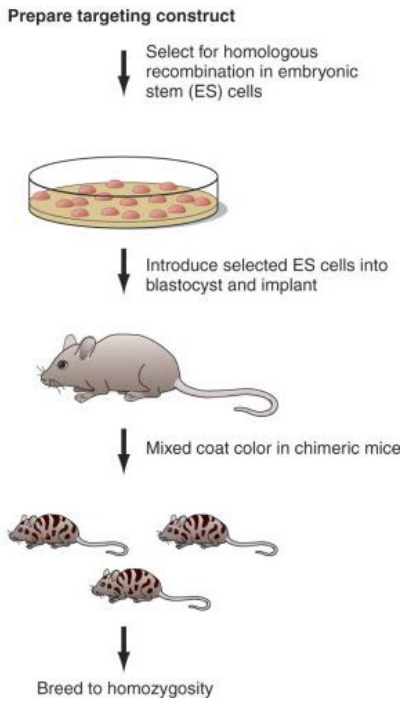
http://www.web-books.com/MoBio/Free/Ch9A4.htm

First generation of humanized Ig mice: YAC/double deletion

Antigen-specific human monoclonal antibodies from mice engineered with human Ig heavy and light chain YACs

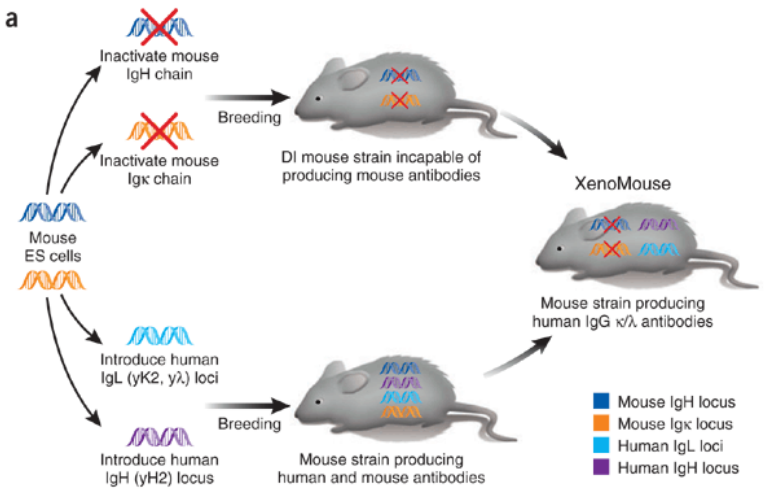
L.L. Green, M.C. Hardy, C.E. Maynard-Currie, H. Tsuda, D.M. Louie, M.J. Mendez, H. Abderrahim, M. Noguchi, D.H. Smith, Y. Zeng, N.E. David, H. Sasai, D. Garza, D.G. Brenner, J.F. Hales, R.P. McGuinness, D.J. Capon, S. Klapholz & A. Jakobovits

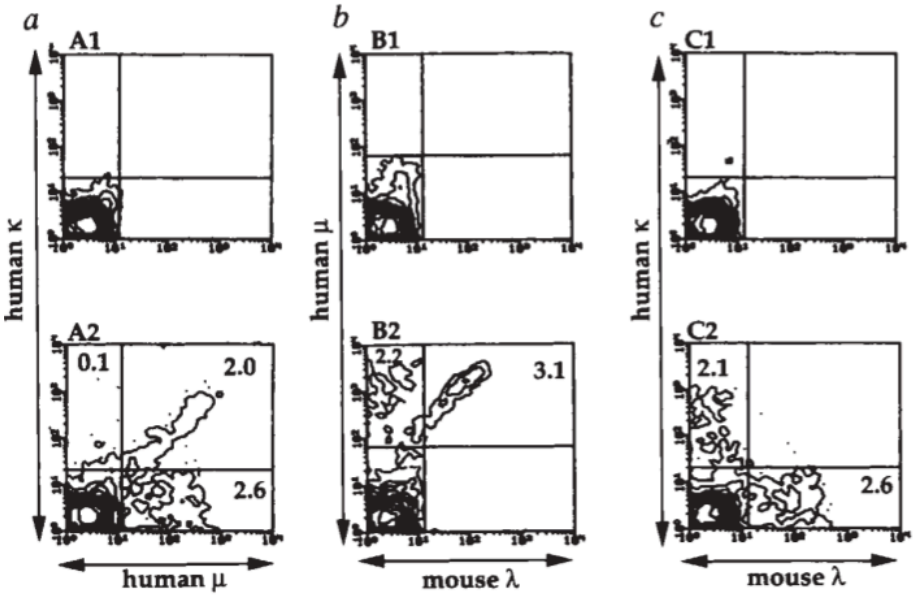
- yH1: Heavy chain locus: 5 V_H, all 25 D, all 6 J_H and the C_μ and C_δ constant region gene segments from YAC library
- yK1: Light chain locus: 2 V_κ, all 5 J_κ and the C_κ gene segments from YAC library
- HPRT gene in the right arm of the YAC
- Introduction in HPRT deficient ES cell line by yeast-spheroplast-ES cell fusion
- Single intact YAC containing chimeric mice were identified
- HuAb: yH1 x yK1
- Diverse adult-like human Ig repertoire in mice
  - V representation;
  - N addition



Cell Genesys, Inc. (later Abgenix, Inc. and now part of Amgen, Inc. after the acquisition of Abgenix).

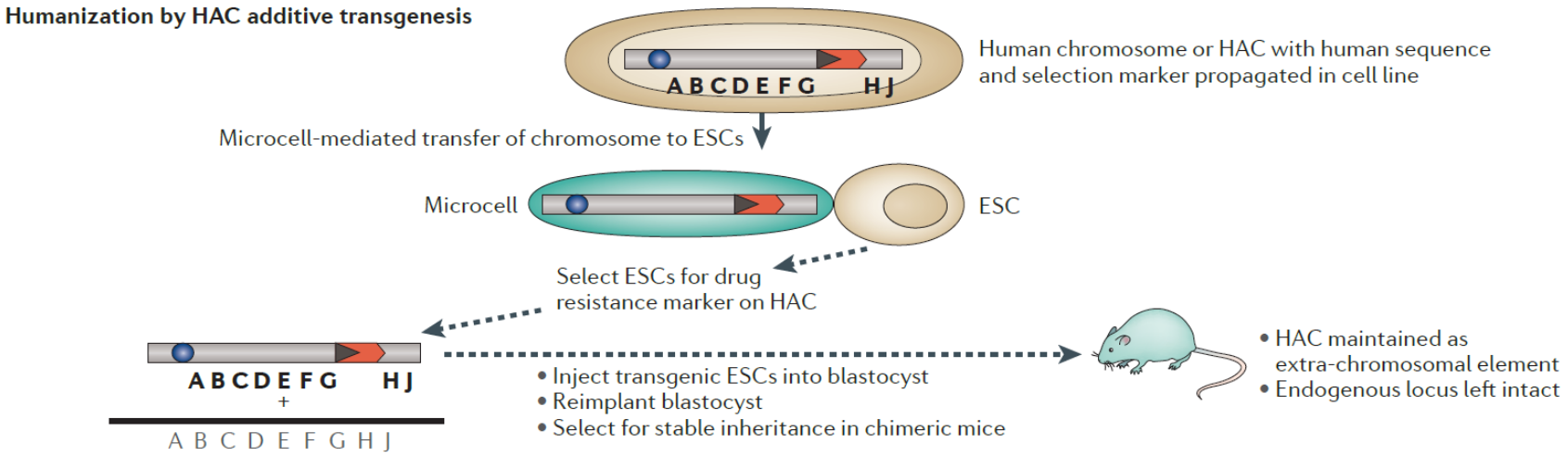
XenoMouse®





- γH1 and γK1 Tg mice bred with mouse Ig KO mice
  - ΔJ (mouse heavy chain KO)
  - ΔC (mouse light K chain KO)
- Human YACs restore B cell production but less efficient than in WT mice (10% of WT): limited number of h V genes
- Fully human hμ/hλκ were detected

Non-integrative approach through the introduction of a human artificial chromosome (HAC) into ESCs by microcell-mediated chromosome transfer (MMCT).



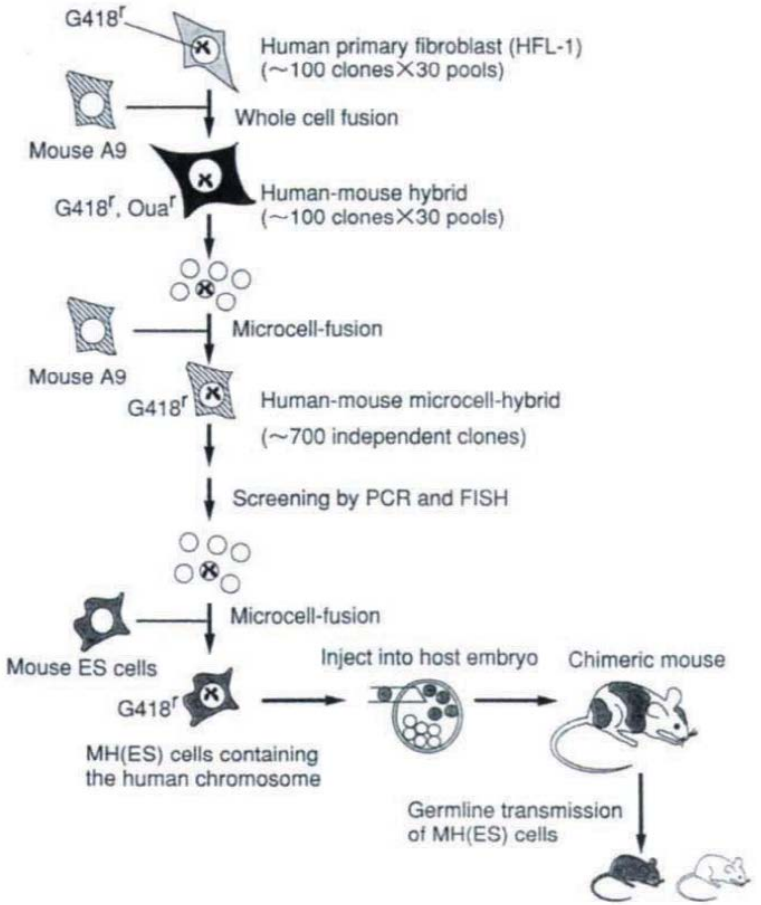
First generation of humanized Ig mice: MMCT/double deletion

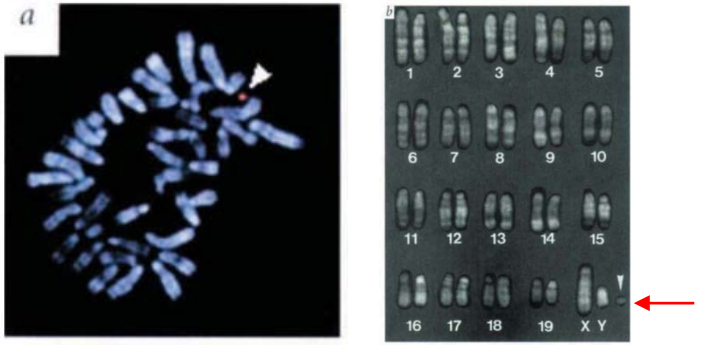
Functional expression and germline transmission of a human chromosome fragment in chimaeric mice

Kazuma Tomizuka[1], Hitoshi Yoshida[1], Hiroshi Uejima[2], Hiroyuki Kugoh[2], Kaoru Sato[1], Atsuko Ohguma[1], Michiko Hayasaka[3], Kazunori Hanaoka[3], Mitsuo Oshimura[2] & Isao Ishida[1]

TC Mouse

- Fraction of the human germline repertoire was introduced by microcell-mediated chromosome transfer.
- MMCT basic procedure:
  - donor cell are induced to multinucleate by mitotic arrest (colcemid).
  - Nuclei removed by cytoscheleton disruption and density gradient centrifugation
  - Fusion of the microcell to a recipient cell line
- Transchromosomal mice were generated that express the complete heavy chain and kappa light chain repertoire in absence of mouse heavy and kappa light chains.
- Serum concentration of human Igs was higher than in YAC mice
- Responsive to immunization
- However, instability of the chromosome fragment containing the kappa light chain locus led to a substantial reduction in the generation of hybridomas, a problem that was solved by crossing Ig_H transchromosomal mice with $Ig_κ$ transgenic mice.





Limitation of the first generation of human Ig transgenic mice

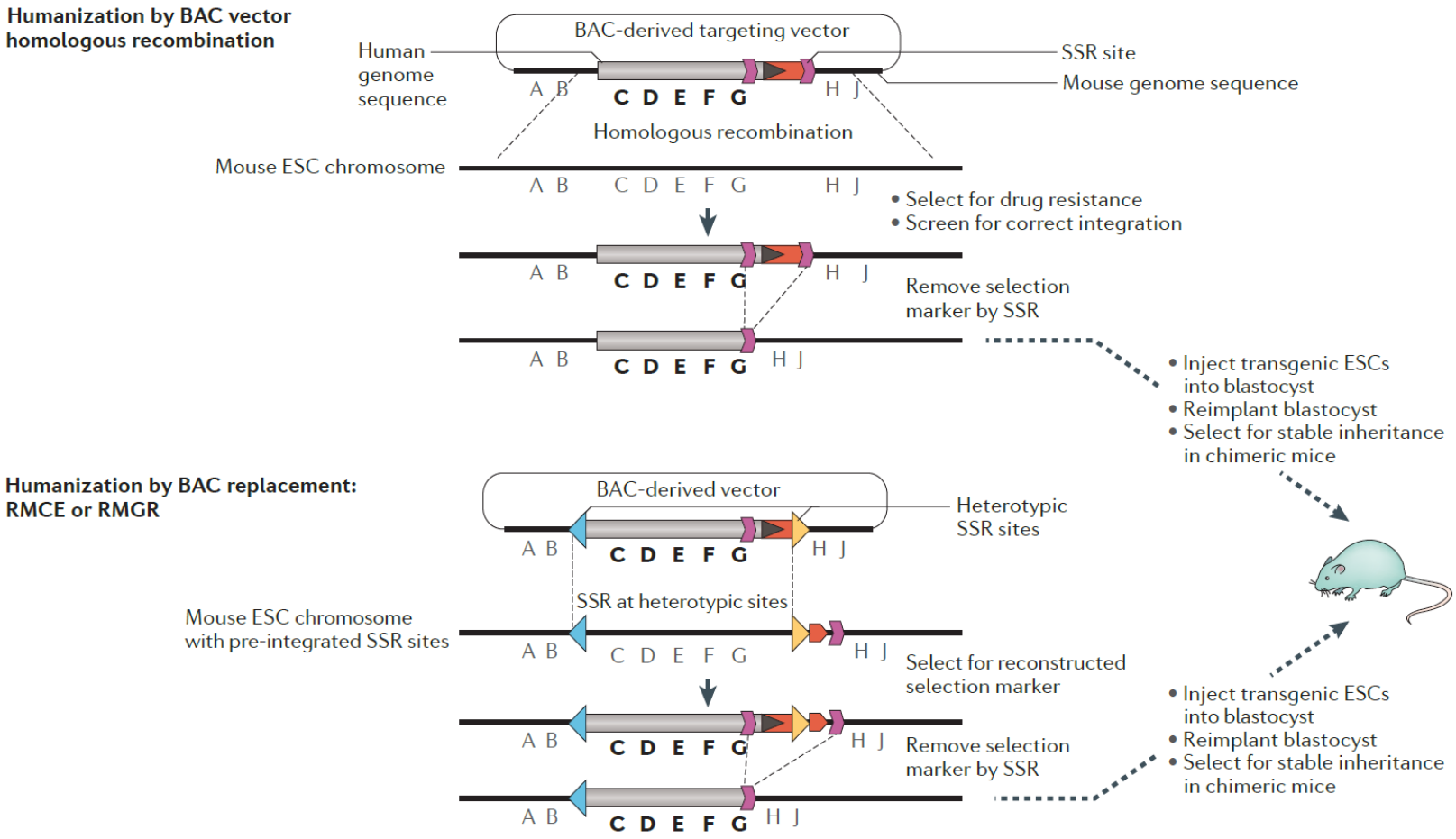
- Transgenes randomly inserted into the genome are influenced by their location, and only rarely will they fully recapitulate normal gene expression.
- The size limit of conventional transgenes means that substantial portions of the human antibody repertoire are not represented in first-generation human immunoglobulin transgenic mice.
- This limits the diversity of antibodies these mice could produce.
- A subsequent generation of transgenic animals carry the entire human heavy-chain locus on a minichromosome. Although these mice offer the potential advantage of a full heavy-chain repertoire, the minichromosome is mitotically and meiotically unstable.
- Divergence of human and mouse constant regions. This divergence may influence the interaction of BCRs containing human constant regions with mouse signaling proteins such as Igα and Igβ, as well the interactions between antibodies and mouse Fc receptors.
- This would affect many aspects of B-cell development, function and differentiation.

A solution is to retain the mouse constant regions while inserting human variable regions into the mouse genome.

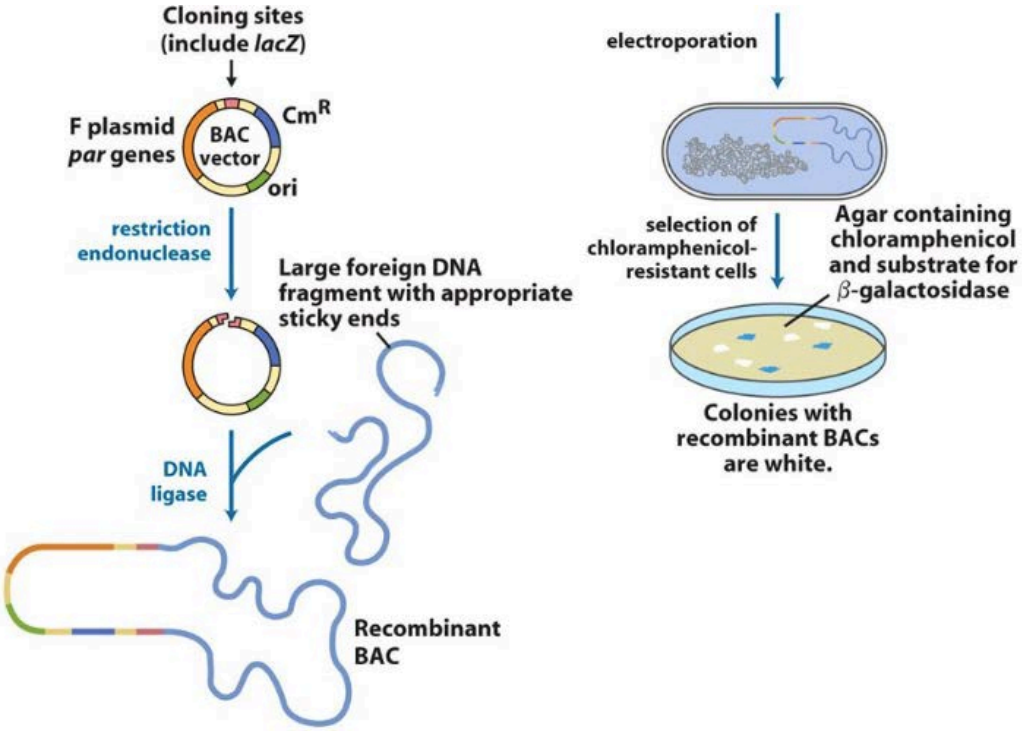
Variable: human

Constant: mouse

In situ humanization:
Specific targeting and replacement of genomic loci using BACs



BAC: bacterial artificial chromosome



Common Gene Components

- ***oriS, repE•F***: regulates plasmid replication and copy number
- ***parA and parB***: **partitions F plasmid** DNA into daughter cells during division and ensures stable maintenance of the BAC
- ***A* selectable markers**: antibiotic resistance (some BACs include lacZ at the cloning site for blue/white selection)
- ***T7 and Sp6***: phage promoters for transcription of inserted genes
- **They can carry up to 300 kb of DNA**

http://www.web-books.com/MoBio/Free/Ch9A4.htm

Precise and in situ genetic humanization of 6 Mb of mouse immunoglobulin genes

Lynn E. Macdonald[a], Margaret Karow[a,1], Sean Stevens[a,2], Wojtek Auerbach[a], William T. Poueymirou[a], Jason Yasenchak[a], David Frendewey[a], David M. Valenzuela[a], Cosmas C. Giallourakis[b,c,d], Frederick W. Alt[c,d], George D. Yancopoulos[a,3], and Andrew J. Murphy[a,3]

[a]Regeneron Pharmaceuticals, Inc., Tarrytown, NY 10591; [b]Gastrointestinal Unit, Massachusetts General Hospital, Boston, MA 02115; [c]The Howard Hughes Medical Institute, The Children's Hospital, Boston, MA 02115; and [d]The Immune Disease Institute and the Department of Genetics, Harvard Medical School, Boston, MA 02115

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Direct replacement of large mouse genes with their large human genomic counterpart:

- up to 210 kb of human sequences were inserted in a single step using LC-BACvecs and normal drug selections, allowing efficient replacement of 6 Mb of the mouse genome with the corresponding human genetic sequences.

The methodologies used here rely on robust established technologies of:

- VelociGene,
- bacterial homologous recombination
- Cre recombinase-mediated large deletions,
- a variation of the original VelociGene technology involving the creation of LC-BACvecs.

Generation of mice making "reverse chimeric" (i.e., human V: mouse C) antibodies that undergo normal interactions and selections with the mouse environment based on retaining mouse constant regions.

These reverse chimeric antibodies should be readily reformattable into fully human antibodies for therapy.

ES targeting vectors based on BACs for homologous recombination

High-throughput engineering of the mouse genome coupled with high-resolution expression analysis

David M Valenzuela[1], Andrew J Murphy[1,3], David Frendewey[1,3], Nicholas W Gale[1,3], Aris N Economides[1,3], Wojtek Auerbach[1], William T Poueymirou[1], Niels C Adams[1], Jose Rojas[1], Jason Yasenchak[1], Rostislav Chernomorsky[1], Marylene Boucher[1], Andrea L Elsasser[1], Lakeisha Esau[1], Jenny Zheng[1], Jennifer A Griffiths[1], Xiaorong Wang[1], Hong Su[1], Yingzi Xue[1], Melissa G Dominguez[1], Irene Noguera[1], Richard Torres[1], Lynn E Macdonald[1], A Francis Stewart[2], Thomas M DeChiara[1] & George D Yancopoulos[1]

VOLUME 21 NUMBER 6 JUNE 2003 NATURE BIOTECHNOLOGY

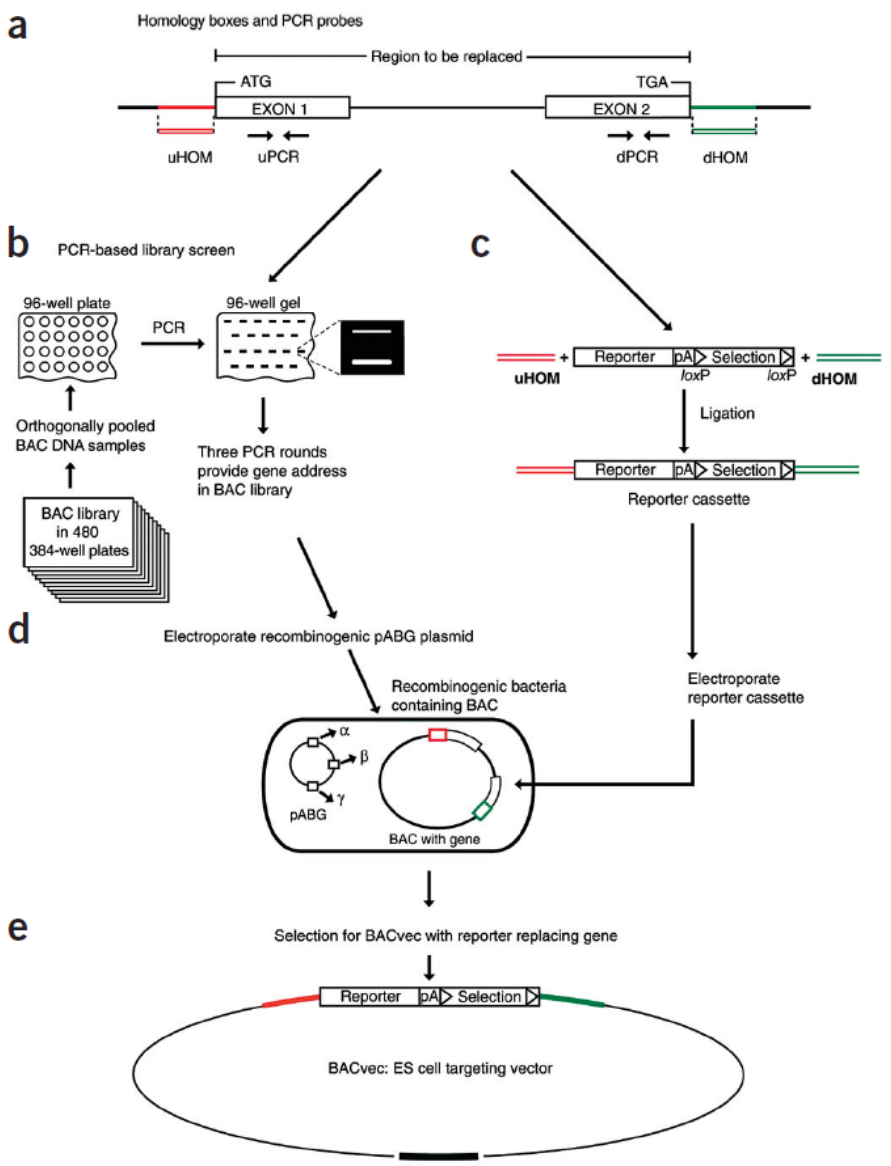
VelociGene: High-throughput and largely automated process that uses targeting vectors based on BACs.

VelociGene permits genetic alteration with:

- Relies on very long homology arms
- Nucleotide precision with a targeting frequency of 3.8%
- Allows large deletions
- Does not depend on isogenicity between the BAC vector and the ES cell line.
- Can precisely replace the gene of interest with a reporter that allows for high-resolution localization of target-gene expression.

Velocigene technnology (1):
BAC-based vectors generation to target ES cells

- 50-200 bp double stranded oligos are synthesized matching the gene of interest upstream the ATG (uHOM) and downstream the TGA (dHOM).
- 17-24 bp single stranded gene specific primers
- BACs containing the gene of interest are isolated from a BAC library.
- uHOM and dHOM that target homologous recombination in bacteria are ligated to the ends of the reporter(beta-gal)-selection cassette (neomycin). The selection gene is flanked by loxP sites and driven by dual promoter for selection in bacteria and in mammalian cells.
- Recombinogenic bacteria are prepared that contain the pABG plasmid encoding recombination enzymes and the BAC containing the gene of interest.
- The linear uHOM-reporter-loxP-selection-loxP-dHOM cassette is introduced in recombination competent bacteria by elecroporation.
- The recombination enzyme pABG is induced by arabinoside and bacteria in which the BAC has incorporated the reporter cassette are selected.
- After linearization, this modified BACvec is ready for introduction into ES cells by elecroporation and G418 selection.
- BACvecs contain 100 kb flanking DNA and allow deletion of at least 75 kb by HR in ES cells.

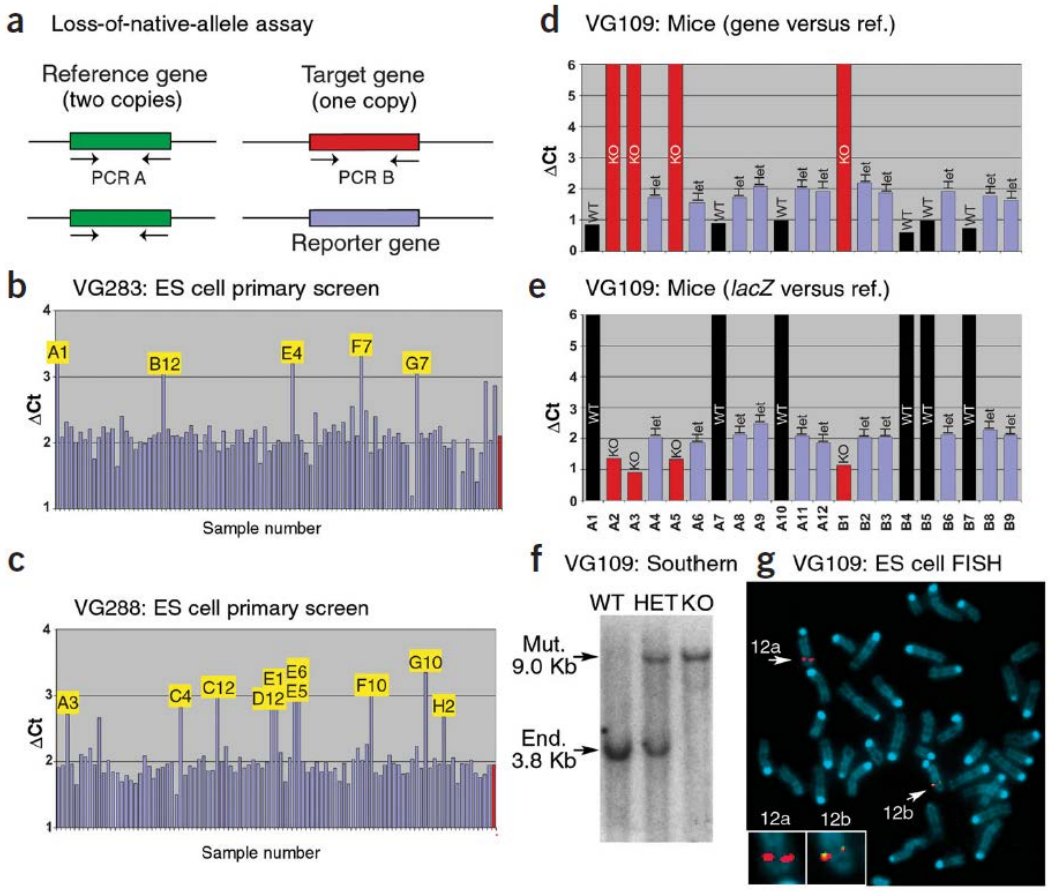


Velocigene (2):
Screening ESCs targeted with BACvecs by Loss-Of-Native-Allele-Assay

- Optimized variation of real-time PCR that detects ES clones in which loss of one native allele has occurred.
- The assay employs the uPCR and dPCR amplification primers (marking the ends of the desired replacement) in combination with a fluorescent TaqMan probe that is quantitatively activated as amplified product accumulates.
- The assay is performed robotically and the data are analyzed digitally.
- The assay screens for correctly targeted ES clones but can also be used to screen the resulting mice

ESC where the native gene is successfully altered loses one copy of the sequence, while ESCs with unsuccessful targeting events (for example, random integration of the BACvec) retain all copies of the sequence.

Two primary screens of 96 ES cell clones, in each of which a single uPCR or dPCR probe is compared to a single reference gene to yield a ΔCt value.



KO have infinite ΔCt when the gene of interest is compared to reference gene

WT have infinite ΔCt when the reporter gene is compared to reference gene

FISH confirmation of the corrected ES clones:
Red: BAC probe
Green: reporter probe

Construction of LC-BACvec

To perform the Ig large genetic humanizations human and mouse BACs were combined to create large compound BACvecs (LC-BACvecs).

A total of 13 different LC-BACvecs were used to humanize the variable region genes of both the heavy and κ light chain loci.

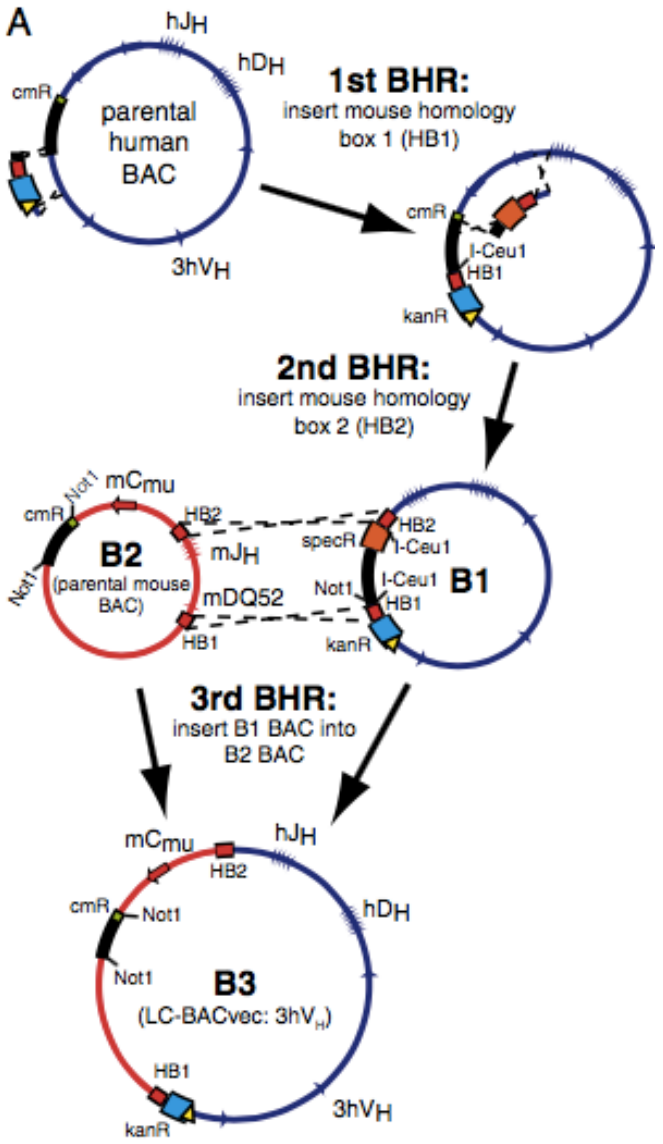
Mouse BAC library and human BAC library were screened to identify the IgH and Igk loci

The 3hVH LC-BACvec was constructed using **three sequential bacterial homologous recombination (BHR) steps**.

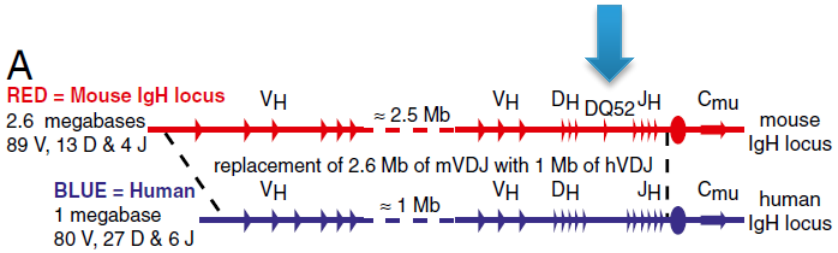
- In step 1, a cassette was introduced upstream from VH1–3 that contains a region of homology to the mouse IgH locus (HB1), a gene that confers kanamycin resistance in bacteria and G418 resistance in animals cells (kanR) and a loxP site. Drug selection for step 1: chloramphenicol/ kanamycin (cm/kan).

- In step 2, a second cassette was introduced just downstream from the last JH segment that contains a second region of homology to the mouse IgH locus (HB2) and a gene that confers resistance in bacteria to spectinomycin (specR). Step 2 also deleted human IgH sequences downstream from JH6 and the BAC vector chloramphenicol resistance gene (cmR). Drug selection for step 2: spectinomycin/kanamycin

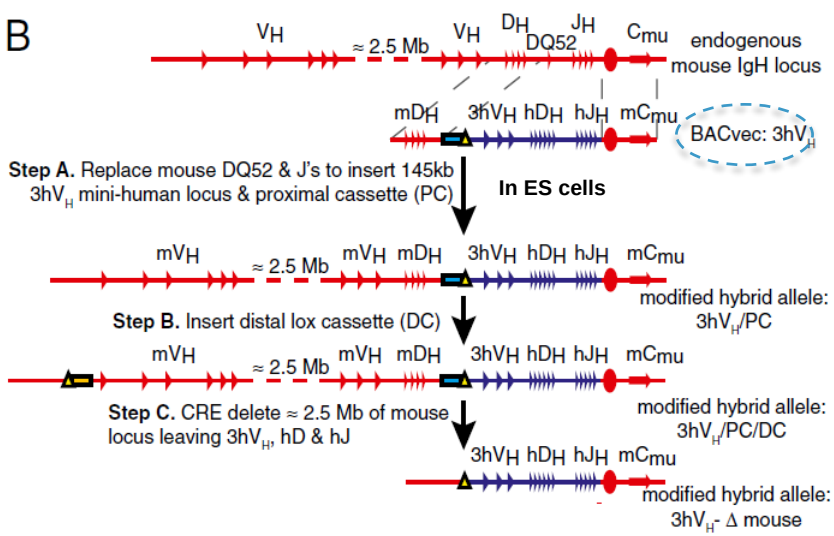
- In step 3, the doubly modified human BAC (B1) was then linearized using I-Ceu1 sites that had been added during steps 1 and 2 and integrated into a mouse BAC (B2) by BHR through the HB1 and HB2 regions of homology. Drug selection for step 3: cm/kan.

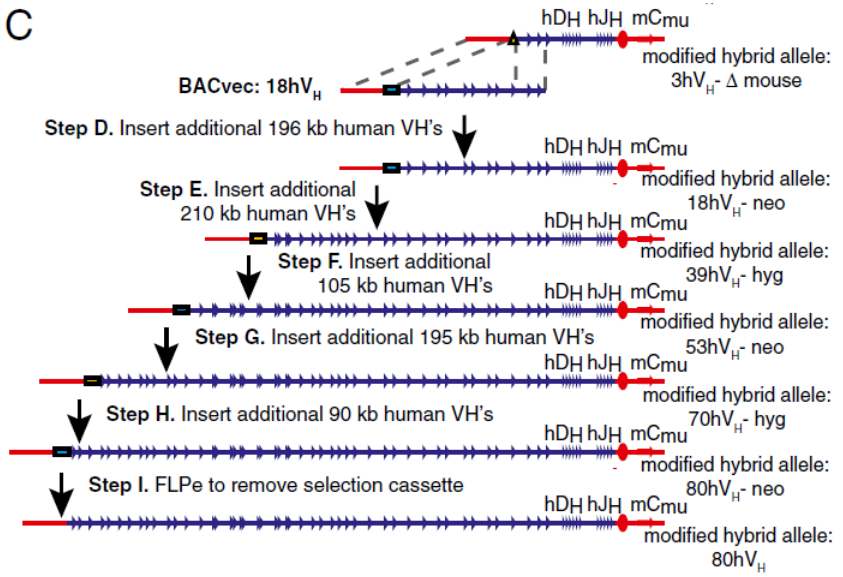


Humanization of the mouse Ig heavy chain variable locus



The junction between human V-D-J region and the mouse C was maintains the mouse intronic enhancer and switch domain to give the best chance for efficient expression and class switching in the mouse

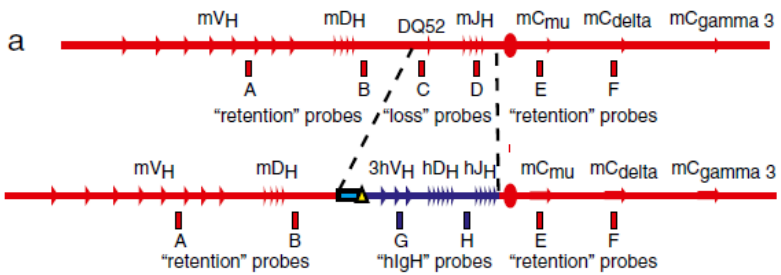




- Double targeted ES cells on the same chromosome are neo and hyg resistant
- Transient expression of **Cre**

- The rest of the human heavy chain variable region was added to the 3hVH allele in a series of five steps using the Velocigene method
- For each step, the proximal end of each new BACvec was designed to overlap the most distal human sequences of the previous step, and the distal end of each new BACvec contained the same distal region of mouse homology as used in step A.
- **ES cells with 9 sequential manipulation were used to generate mice**

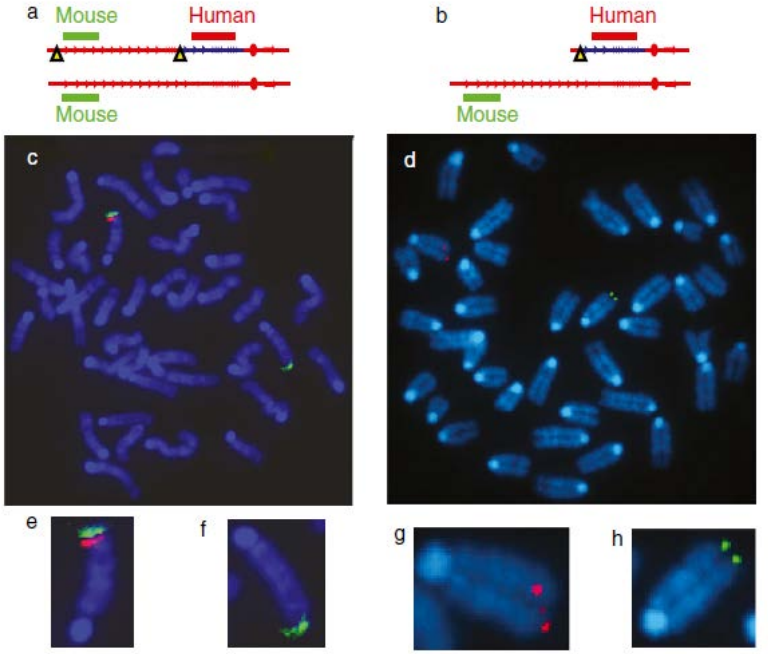
Screening in ES cells for the first human heavy gene insertion: Loss of native allele assay



Screen ES cell clones for:
Loss of targeted mouse region (probes C and D)
Introduction of human sequence (probes G and H)
Retention of flanking mouse sequences (probes A, B, E and F)

b

		A	B	C	D	E	F	G	H
Parental ES	Theoretical copy number	2	2	2	2	2	2	0	0
	Observed copy number	1.9	1.8	2.1	1.8	1.9	1.8	< 0.01	< 0.04
Modified ES	Theoretical copy number	2	2	1	1	2	2	1	1
	Observed copy number	1.9	2.4	1.0	1.0	2.0	1.9	+	+



The **locations of quantitative PCR probe/primer sets** (A) for the region deleted (loss probes C and D), the region inserted (hIgH probes G and H), and flanking regions (retention probes A, B, E, and F) on an unmodified mouse chromosome (Upper) and a correctly targeted chromosome (Lower).
Observed probe copy numbers in parental and modified ES cells (B) for probes A–F were calculated as $2/2^{\Delta\Delta Ct.}$

FISH confirmation of targeted 3hVH insertion (A) and subsequent deletion of mouse heavy chain variable region (B). Metaphase FISH results are shown for ES cell clones before (A, C, E, and F) and after (B, D, G, and H) Cre-mediated deletion of the 3-Mb mouse VH segment. Position of mouse and human bacterial artificial chromosome (BAC) probes are shown in the graphics (A and B), and close-ups of the targeted (E and G) and non targeted (F and H) chromosomes are shown.

VelocImmune mice

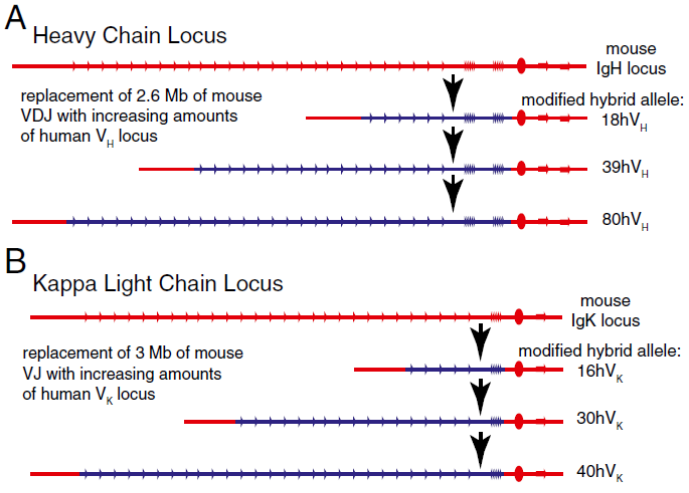
- Humanization of the κ light chain was obtained
- Generation of mice doubly homozygous for both heavy and κ light chain humanizations

PNAS

Mice with megabase humanization of their immunoglobulin genes generate antibodies as efficiently as normal mice

Andrew J. Murphy[1], Lynn E. Macdonald, Sean Stevens[2], Margaret Karow[3], Anthony T. Dore, Kevin Pobursky, Tammy T. Huang, William T. Poueymirou, Lakeisha Esau, Melissa Meola, Warren Mikulka, Pamela Krueger, Jeanette Fairhurst, David M. Valenzuela, Nicholas Papadopoulos, and George D. Yancopoulos[1]

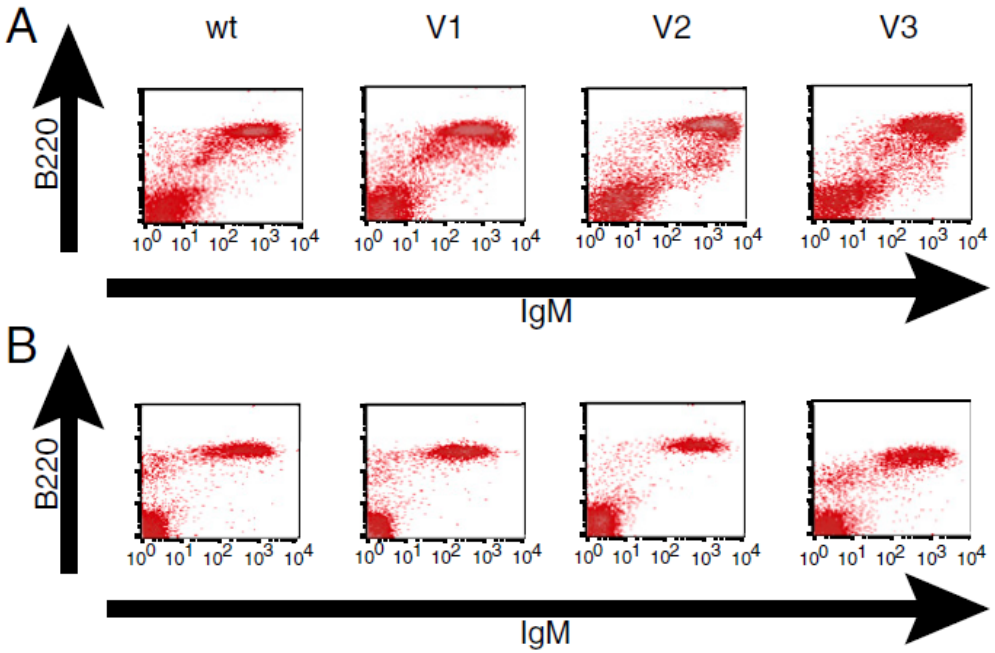
Regeneron Pharmaceuticals, Inc., Tarrytown, NY 10591



C

Version of *VelocImmune* mouse	Heavy Chain			Kappa Light Chain		
	number of human V_H's	allele name	most distal V	number of human V_K's	allele name	most distal V
V1	18	$18hV_H$	V_H1-18	16	$16hV_K$	V_K1-16
V2	39	$39hV_H$	V_H4-39	30	$30hV_K$	V_K2-29
V3	80	$80hV_H$	V_H3-74	40	$40hV_K$	V_K2-40

Mature B-Cell Populations



Spleen

Inguinal lymph node

Lymphocytes isolated from spleen or lymph node of homozygous VelocImmune mice were stained for surface expression of the markers B220 and IgM and analyzed using flow cytometry.

The sizes of the B220+ IgM+ mature B-cell populations in all versions of VelocImmune mice were virtually identical to those of WT mice, regardless of the number of VH segments they possess.

Locus Choice and Allelic Exclusion

VelocImmune mice all possess **WT mouse lambda light chain loci**. It was assessed whether rearrangement and expression of humanized IgK loci can prevent mouse lambda expression.

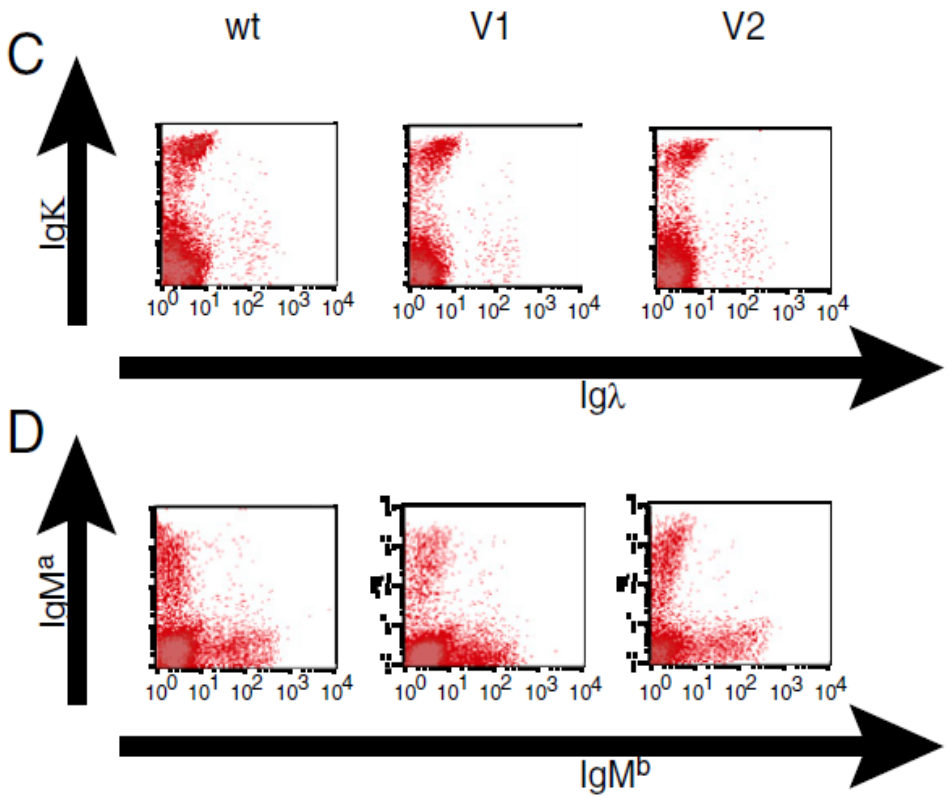
The ratio of the number of cells expressing humanized IgK relative to the number of cells expressing the mouse λ chain was relatively unchanged in VelocImmune vs WT mice.

In addition, there is no increase in the small number of double positive (kappa plus lambda) cells, indicating that productive recombination at the hybrid kappa loci results in appropriate suppression of recombination of the mouse lambda loci

Allelic exclusion in mice heterozygous for different versions of humanized IgH loci.

Humanization of the Ig loci in a 129S6:C57BL/6N F1 ES line. The human IgH germ-line variable sequences are targeted to the 129S6 allele, carrying the IgMa haplotype, whereas the unmodified mouse C576BL/6N allele bears the IgMb haplotype. The allelic forms of IgM can be distinguished by flow cytometry using specific antibodies.

B cells found in mice heterozygous for each version of the humanized IgH locus only express a single allele, either the humanized IgMa allele or the WT IgMb allele.
Therefore, the mechanisms involved in allelic exclusion are intact in VelocImmune mice.



B-cell populations in WT and VelocImmune mice**. Cells from spleen** of WT or VelocImmune 1 (V1), 2 (V2)mice were stained for surface Ig containing either kappa or lambda light chains (C), or surface IgM of specific allotypes (D); populations are separated by FACS

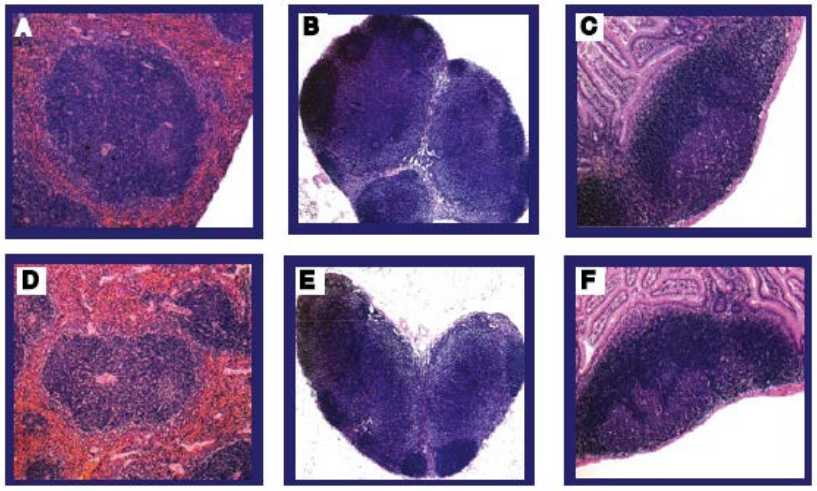
Normal B cell development and lymphoid structures

Table 1. Proportion of sizes of cell populations in *VelocImmune* mice compared with WT littermates

	Bone Marrow				Spleen	
	→			→		
Maturation	CD43^{hi} B220^{lo}	CD24^{hi} B220^{lo}	B220^{lo} IgM^{+}	B220^{hi} IgM^{+}	B220^{hi} IgM^{+}	B220hi IgM^{+} IgD^{+}
V1	1.1	1.0	0.9	1.0	1.0	1.1
V2	1.0	1.0	1.0	1.0	1.0	1.0
V3	1.0	1.0	1.1	1.0	1.1	1.0

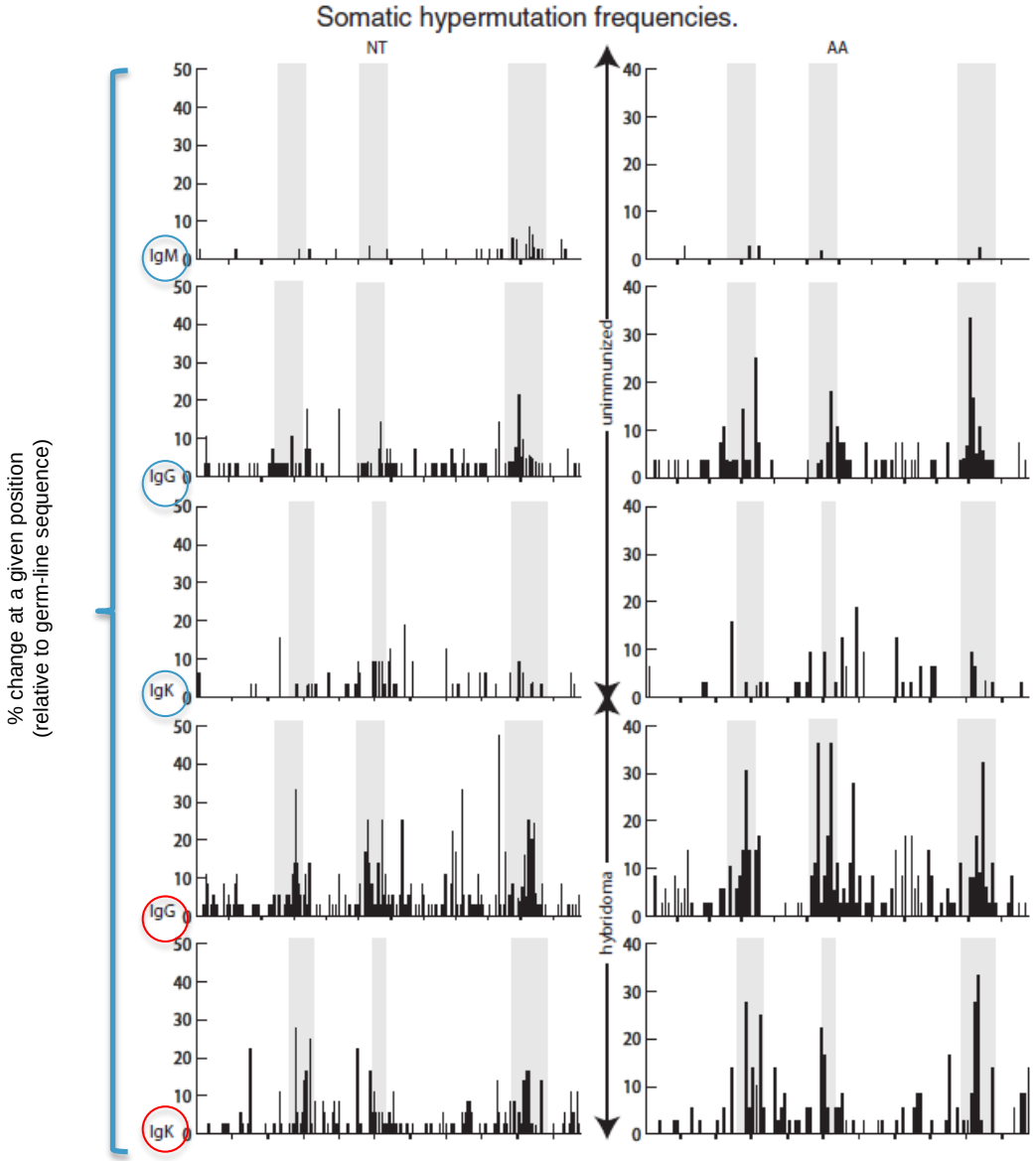
The ratio of the fraction of cells in each B-cell lineage population, defined by FACS using the indicated markers, in *VelocImmune* compared with WT mice.

Lymphoid organ histology.



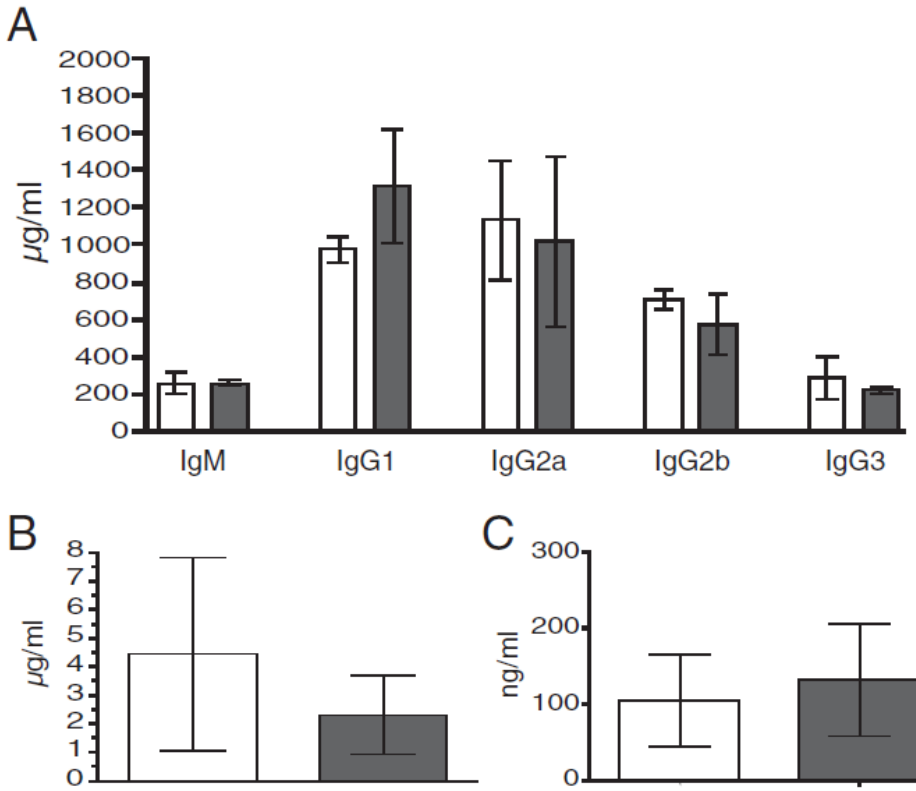
A defect in B-cell development or function may be exhibited as an alteration in the structure of the lymphoid tissues.
Gross structures of the spleen, inguinal lymph nodes, and Peyer's patches by light microscopy.
No significant difference in appearance of secondary lymphoid organs between the WT and VelocImmune mice by H&E.

Somatic Hypermutation



Somatic hypermutation frequencies by RT-PCR from splenocytes or hybridomas. Hypermutation frequencies were scored (after alignment to matching germ-line sequences) as percent of sequences changed at each nucleotide (NT) or amino acid (AA) position among sets of 38 (unimmunized IgM), 28 (unimmunized IgG), 32 (unimmunized IgK from IgG), 36 (IgG from hybridomas) or 36 (IgK from IgG hybridomas) sequences. Shading indicates CDRs.

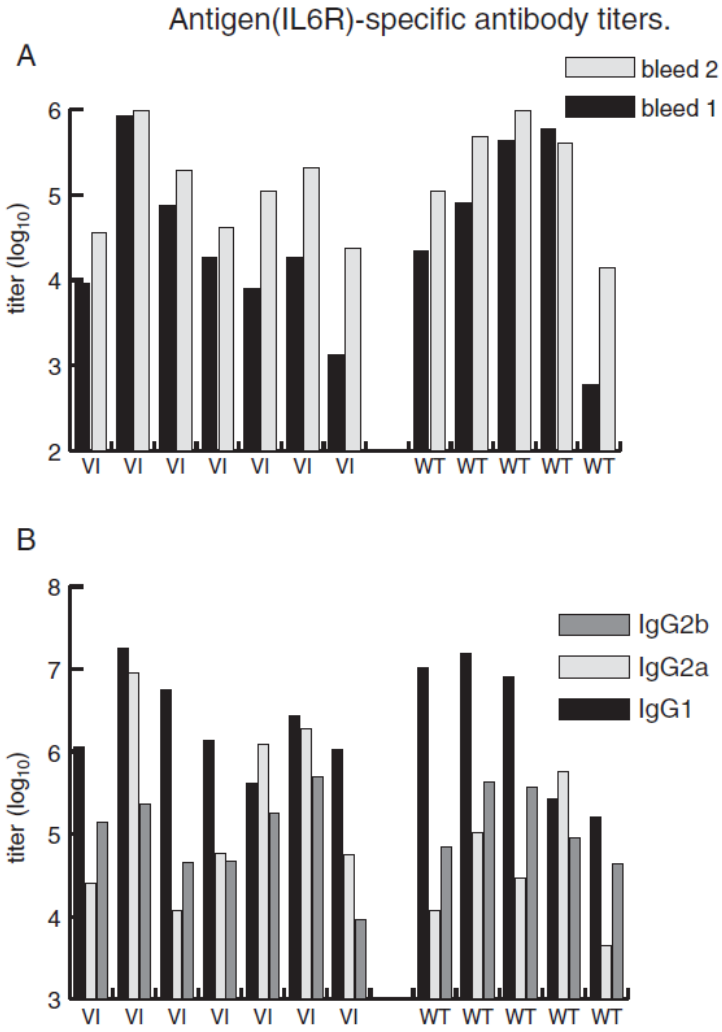
Serum Isotypes



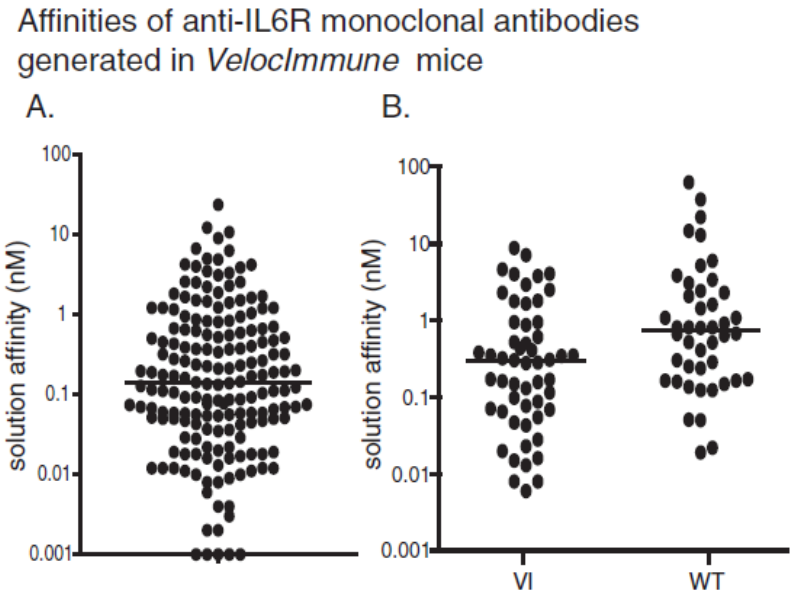
IgA **IgE**

Luminex technology.
The level of expression of each isotype is similar in WT and VelocImmune mice, suggesting that humanization of the variable segments had no effect on class switching or Ig expression and secretion and therefore maintains all of the murine sequences necessary for these functions

Immunization and Antibody Production

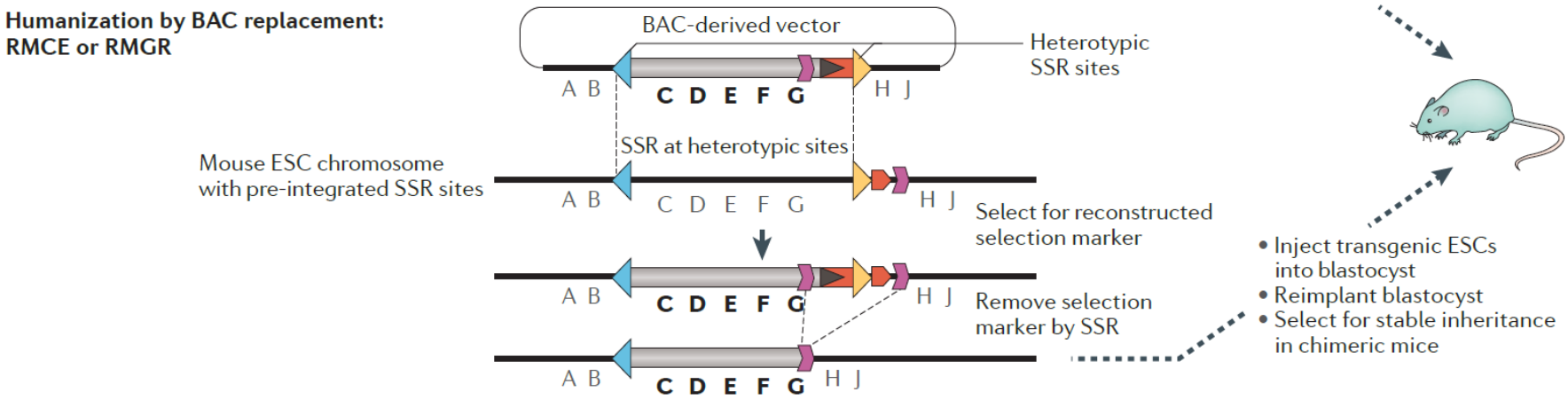


Antigen specific IgG titers against IL6R (A) were determined by ELISA of serum from seven VelocImmune (VI) and five WT mice after two (bleed 1) or three (bleed 2) rounds of immunization with IL6R ectodomain protein. IgG isotype-specific titers (B) were determined from the same mice.



Affinities of anti-IL6R monoclonal antibodies generated in VelocImmune mice. The affinity distribution of antigen-specific monoclonals (A) from VelocImmune mice and antigen-specific blocking monoclonals (B) from VelocImmune (VI) and WT mice

Specific targeting and replacement of genomic loci by RMCE



- Recombineering (recombination-mediated genetic engineering) technologies to manipulate large human genomic BAC clones in Escherichia coli. It is based on homologous recombination systems
- Recombinase Mediated Cassette Exchange RMCE insert these large segments of DNA into the ES cell genome
- Based on the features of site-specific recombination processes (SSRs).
- RMCE is achieved by the clean exchange of a preexisting gene cassette for an analogous cassette carrying the "gene of interest" (GOI).
- piggyBac transposons is used to remove all foreign nonhuman DNA, enabling us to recycle selection markers
- Permits the systematic, repeated modification of higher eukaryotic genomes by targeted integration.

Engineering of the mouse *Ig* loci by S-RMCE

Complete humanization of the mouse immunoglobulin loci enables efficient therapeutic antibody discovery

E-Chiang Lee[1], Qi Liang[1], Hanif Ali[1], Luke Bayliss[1], Alastair Beasley[1], Tara Bloomfield-Gerdes[1], Laura Bonoli[1], Richard Brown[1], Jamie Campbell[1], Adam Carpenter[1], Sara Chalk[1], Alison Davis[1], Nick England[1], Alla Fane-Dremucheva[1], Bettina Franz[1], Volker Germaschewski[1], Helen Holmes[1], Steve Holmes[1], Ian Kirby[1], Miha Kosmac[1], Anais Legent[1], Hui Lui[1], Anais Manin[1], Siobhan O'Leary[1], Jemima Paterson[1], Rocco Sciarrillo[1], Anneliese Speak[2], Dominik Spensberger[1], Laura Tuffery[1], Nikole Waddell[1], Wei Wang[2], Sophie Wells[1], Vivian Wong[1], Andrew Wood[1], Michael J Owen[1], Glenn A Friedrich[1] & Allan Bradley[1,2]

APRIL 2014 nature biotechnology

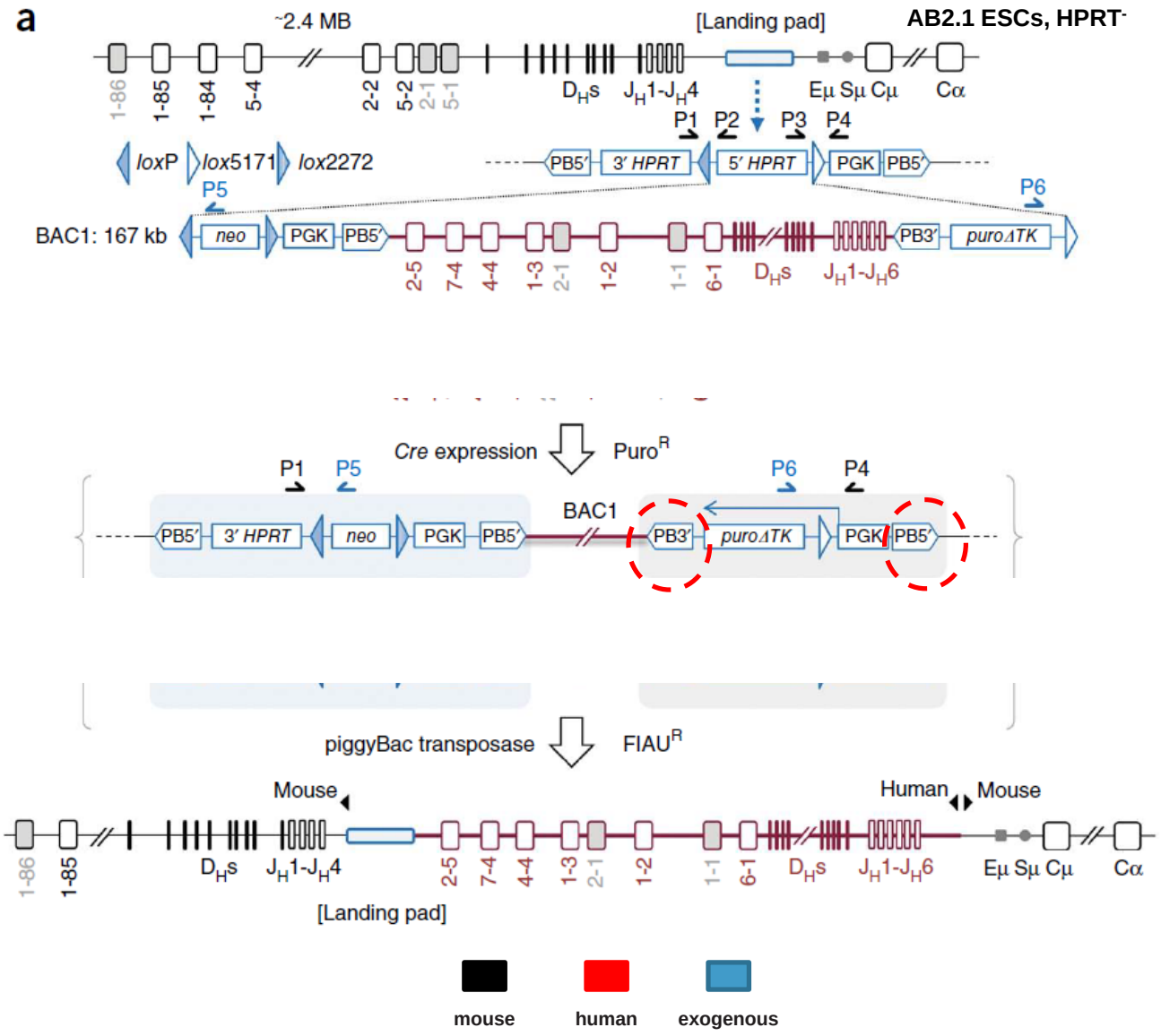
- Insertion of the full complement of variable genes from all three human immunoglobulin loci into precise locations in the corresponding loci of mouse embryonic stem (ES) cells using a technology called sequential recombinase-mediated cassette exchange (S-RMCE).
- The full repertoire of human immunoglobulin variable genes (2.7 Mb of DNA) was inserted into the mouse genome. Up to 15 successive genetic modifications per locus was required
- The endogenous mouse variable genes are silenced by large chromosomal inversion. In these mice, aside from 5.4 Mb of human sequence, no non-mouse DNA remains and none of the mouse immunoglobulin genes are deleted.
- The mice produce reverse chimeric antibodies with human variable domains and mouse constant domains
- The heavy-chain and the two light-chain loci were engineered separately starting with AB2.1 cells and only brought together through breeding of the resulting animals.

Engineering of the mouse *Igh* locus

A **landing pad** construct with human HPRT minigene flanked by inverted piggybac terminal repeats, and two incompatible target lox sites (WT loxP and mutant lox5171) is created by recombineering, elecroporated and inserted into the Igh locus in mouse ESCs by HR.

BAC1 encoding a portion of the human IGH variable region is elecroporated and inserted directionally at these lox sites in the landing pad by Cre-recombination. Correct integrations juxtapose the PGK promoter next to the puroΔTK gene, enabling positive selection in puromycin. In puromycin-resistant clones, the 3′ side of the insertion now has an intact piggyBac transposon (PB) with both 5′ and 3′ inverted terminal repeats.

Expression of piggyBac transposase results in **excision** of this element. Clones which have excised the PB transposon lose the puroΔTK gene and therefore thymidine kinase expression, facilitating negative selection in medium containing FIAU. The 5′ end of the insertion recreates a **new landing pad** with a wild-type loxP and a mutant lox2272 site (shaded blue), allowing integration of additional BACs in an **iterative process**

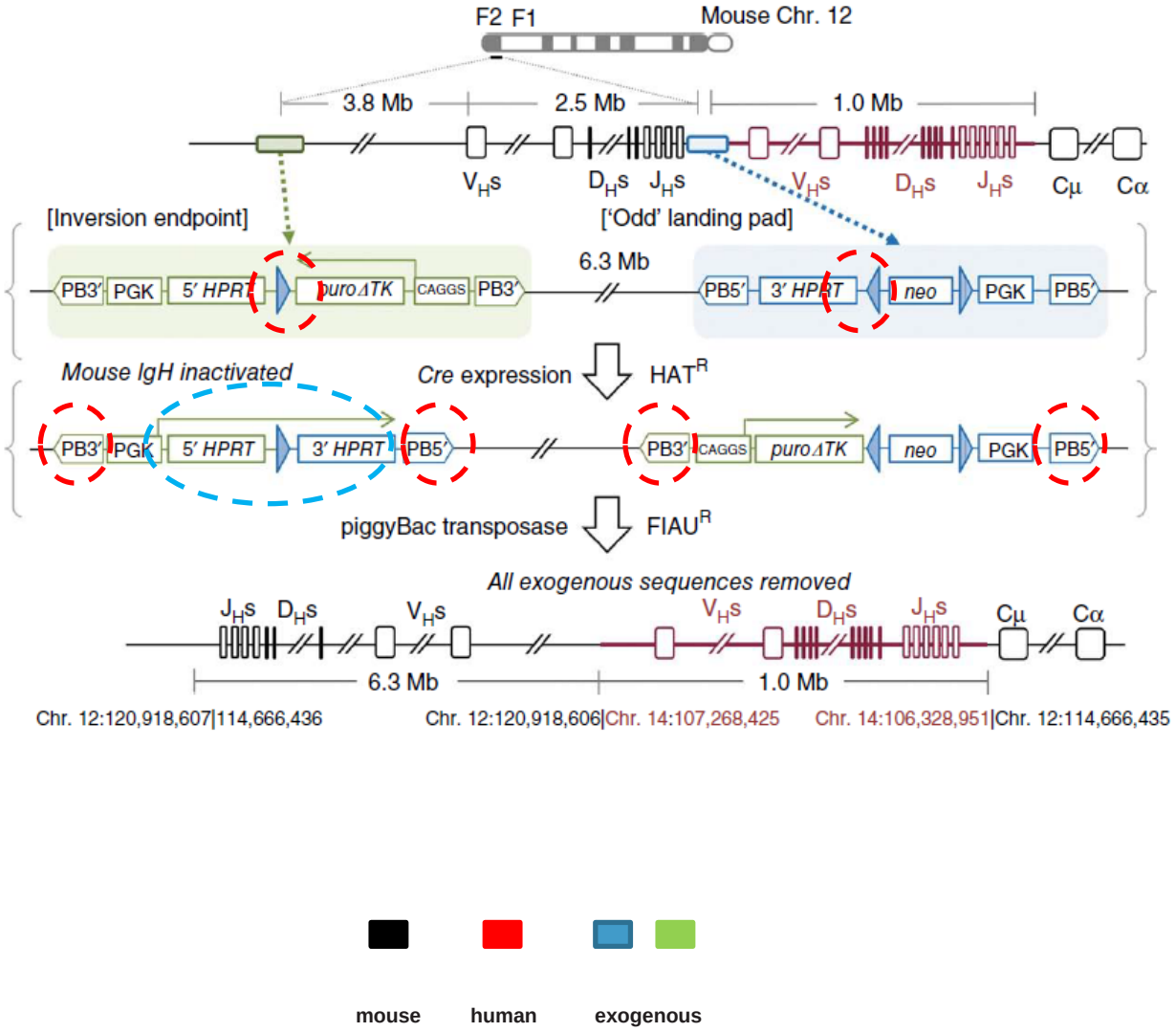


mouse V-region-inactivating inversion

An **inversion end point** is targeted onto the same chromosome several megabases distant (green-box). This provides a loxP site that is inverted relative to the loxP in the landing pad (such that expression of Cre inverts the intervening region) and a 5' HPRT.

The inversion forms an intact HPRT minigene**,** enabling positive selection for clones with these events in medium containing sodium hypoxanthine, aminopterin and thymidine (HAT). The inversion also generates two intact PB transposons.

These are simultaneously excised by piggyBac transposase expression and negatively selected in FIAU. All exogenous sequences are removed from the locus.



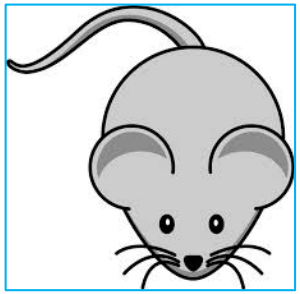
Genes such as *Adam6a* and *Adam6b*, whose deletion results in infertility, were unaffected by the inversion

Generation of mice

HK:

Homozygous human H chain
X
Homozygous human k light chain

and unmodified mouse λ light chain



HL:

Homozygous human H chain
X
Homozygous human λ light chain

KO mouse k light chain (neo cassette)

And unmodified mouse λ light chain

Genetic background: C57BL/6J and 129S7

Mice that are homozygous for either or both the heavy-chain and kappa light-chain inversions are phenotypically normal and have wild-type fertility. Genes such as *Adam6a* and *Adam6b*, whose deletion results in infertility, were unaffected by the inversion.

5’ RACE and Deep sequencing of antibody coding transcripts

B cells from Spleen or PBMC → RNA

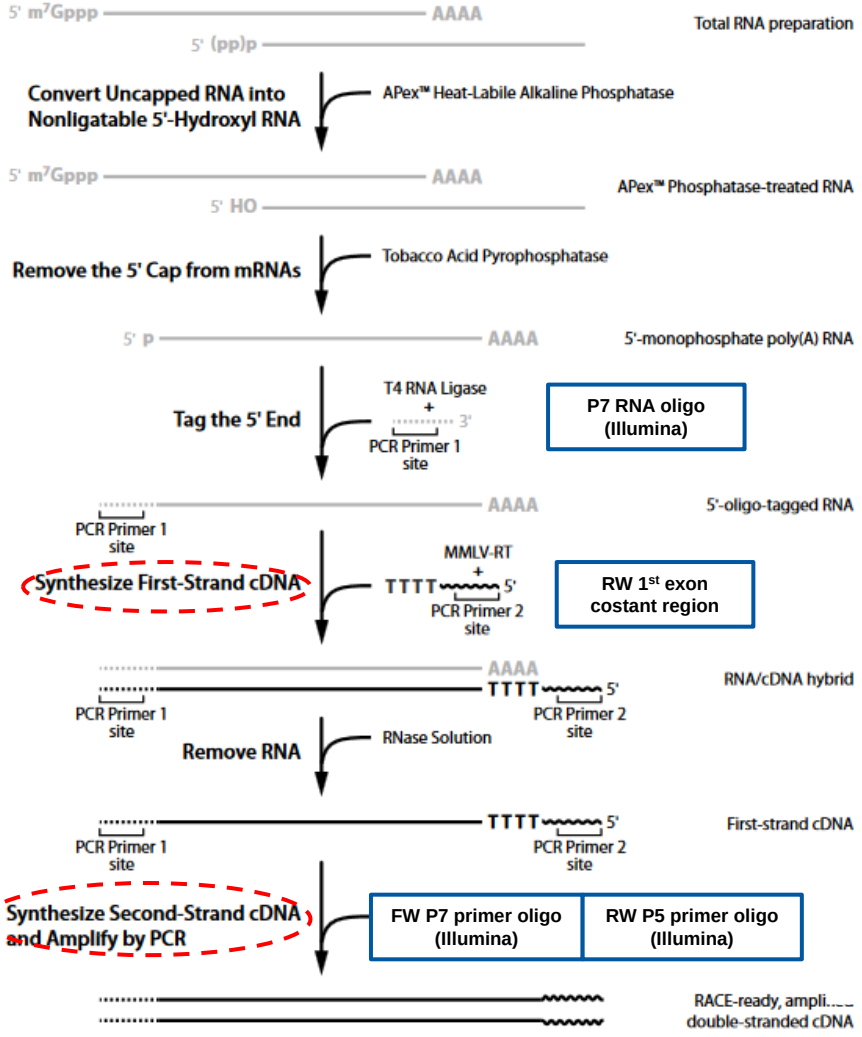


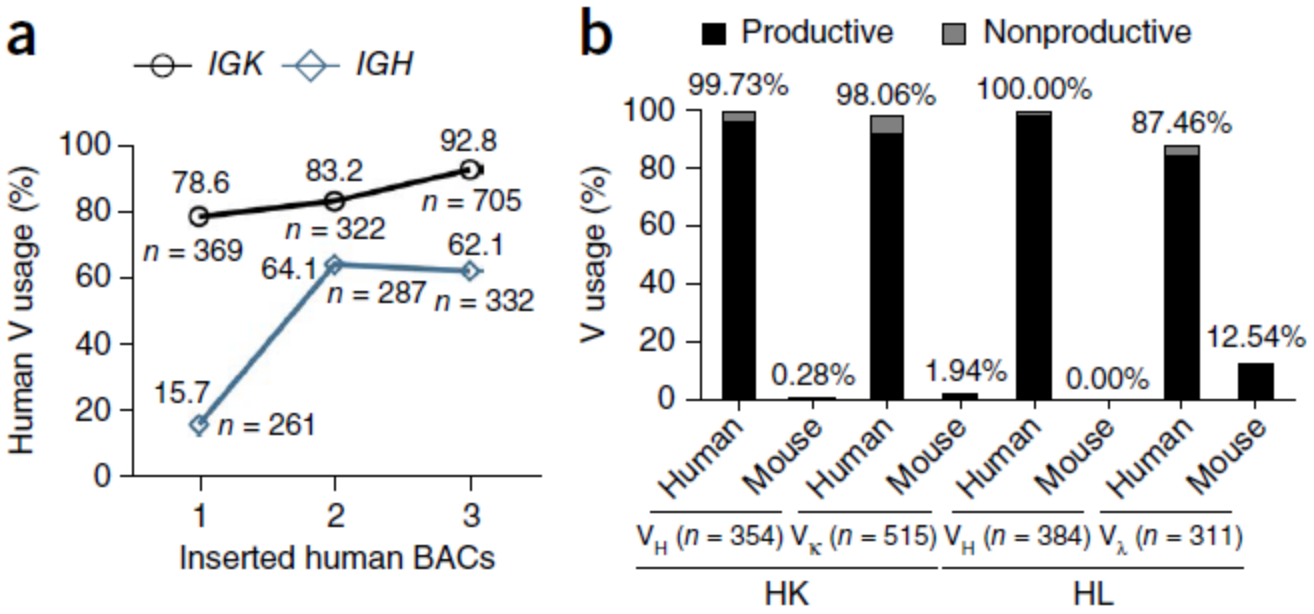
Figure 1. The ExactSTART™ Eukaryotic mRNA 5′- & 3′-RACE Kit Procedure.

PCR product purification → 2nd PCR for complete P5 and P7 illumina adaptor sequences → PCR product purification → MiSeq

Human and mouse variable region usage in transgenic mice

Without inversion of mouse loci

After inversion of mouse loci

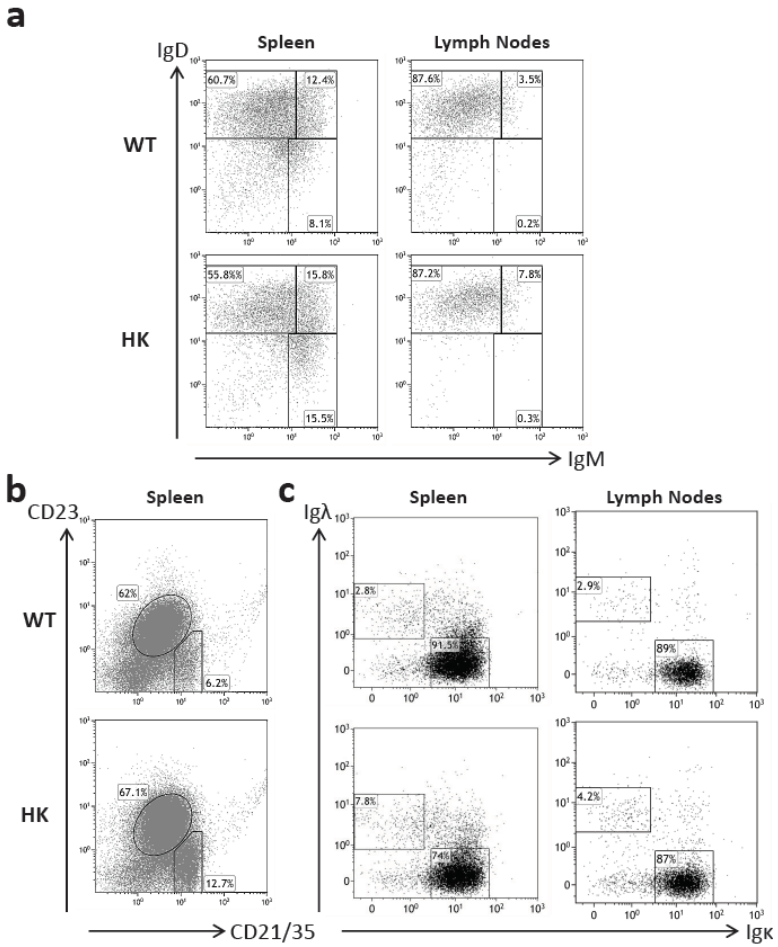


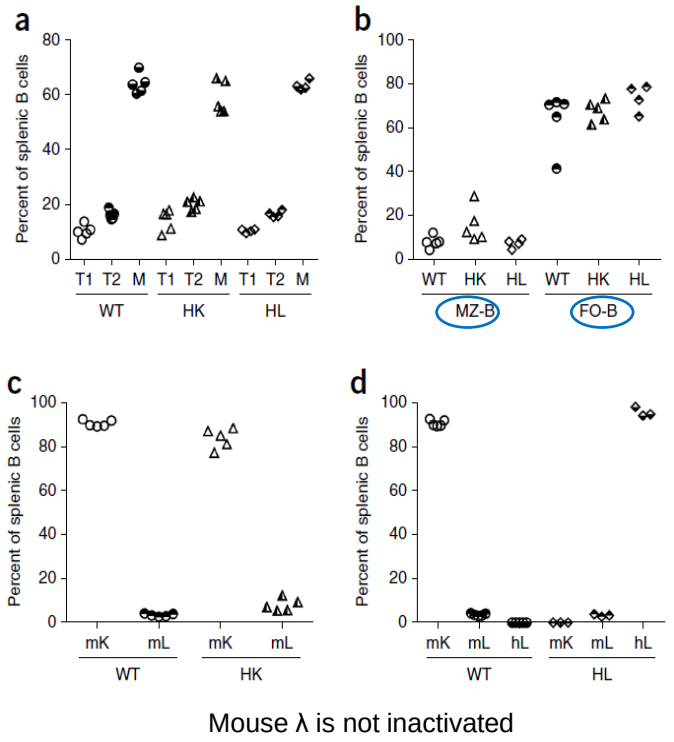
V-region usage in antibody transcripts as determined by cloning and sequencing 5′ RACE products synthesized from RNA isolated from spleens of the mice. The reverse transcription reactions were primed from Cμ-specific or Cκ-specific primers. All the heavy-chain transcripts used the human DH and JH segments, and all the kappa light-chain transcripts used human JK.

(**a**) V-region usage in mice with 1–3 *IGH* BACs or *IGK* BACs, without any inversions of endogenous immunoglobulin.

(**b**) V-region usage in HK and HL mice with inversions of the endogenous *Igh* (HK and HL) and *Igk* loci (HK). Sample size (*n*) is provided for each data point.

Analysis of B cell development in HK mice: Normal B-cell maturation



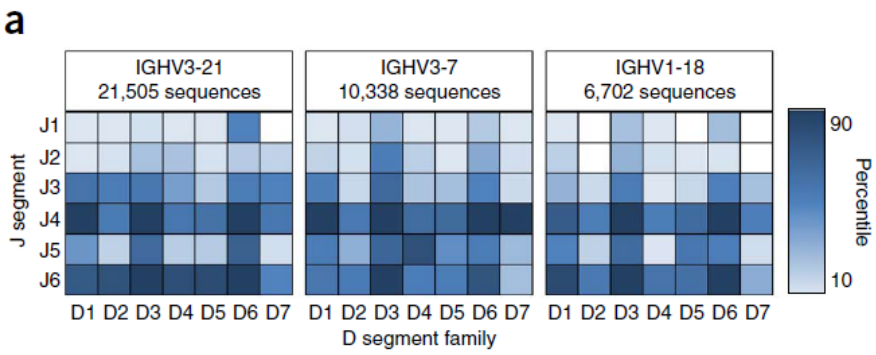


Mouse λ is not inactivated

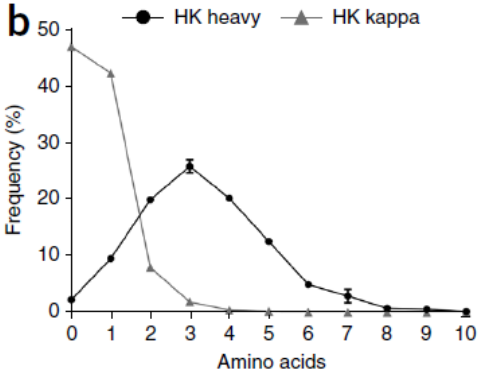
(**a**) B220+ cells from spleen and lymph nodes were characterized as B220+ IgM+IgD- (T1), B220+ IgM+IgD+ (T2), and B220+ IgMlowIgD+ (Mature).
(**b**) B220+ cells from spleen were characterized as CD21+CD23- (Marginal Zone B cells), and CD21low CD23+ (Follicular B cells).
(**c**) Expression profile of mouse kappa and mouse lambda light chain on B220+ cells was examined in spleen and lymph nodes.

Primary Ab repertoire:
Sequence analysis of antibody transcripts in HK mice

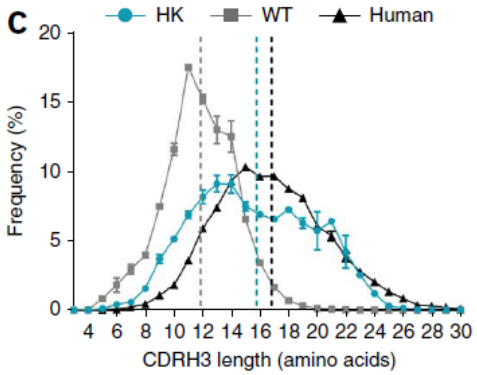
Analyis of CDRH3 diversity



Heat map of frequency of particular V-D-J combinations



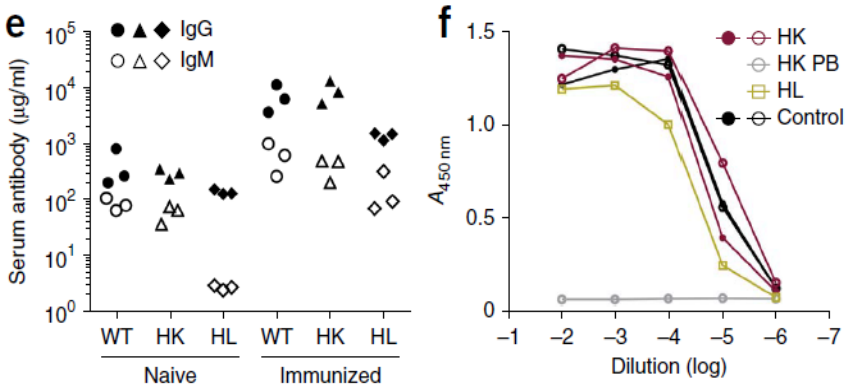
N and P-nucleotide addition
More than 50% light chain:1 or 2 aa
More than 69% of heavy chain: 3 aa



CDRH3 length

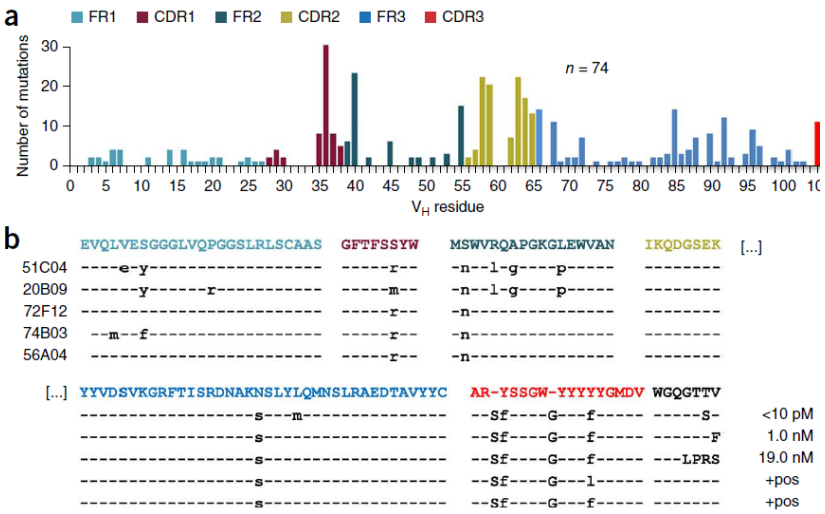
Robust immune response in HK and HL mice and analysis of V-region sequences of specific mAbs

Production of Anti-hCD40L Ab upon immunization



Polyclonal anti-CD40L IgG

Production of Anti-Ovalbumin mAb upon immunization



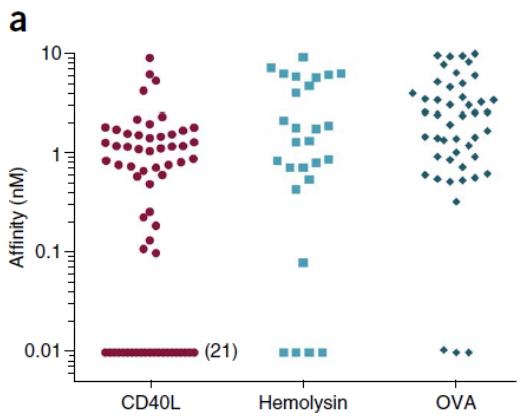
Class switch

AID dependent Somatic hypermutation in 74 mAb analyzed

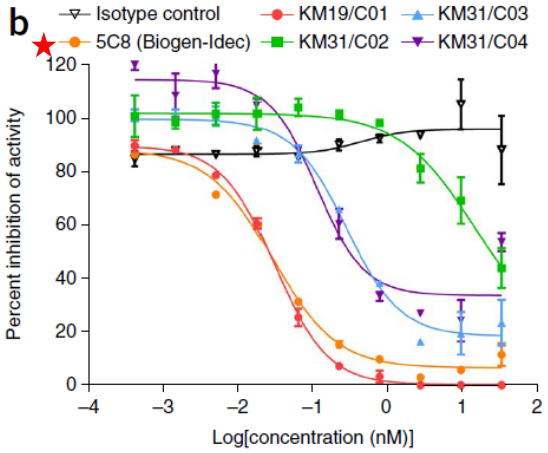
Binding and function of mAbs isolated from HK mice

Potency

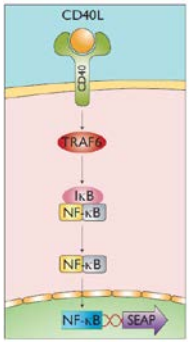
Kd by SPR



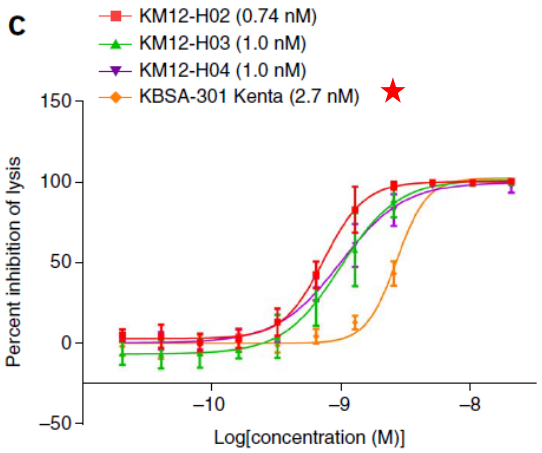
Anti-CD40L mAb



neutralization of a CD40L-induced NF-κB–mediated secreted embryonic alkaline phosphatase reporter gene

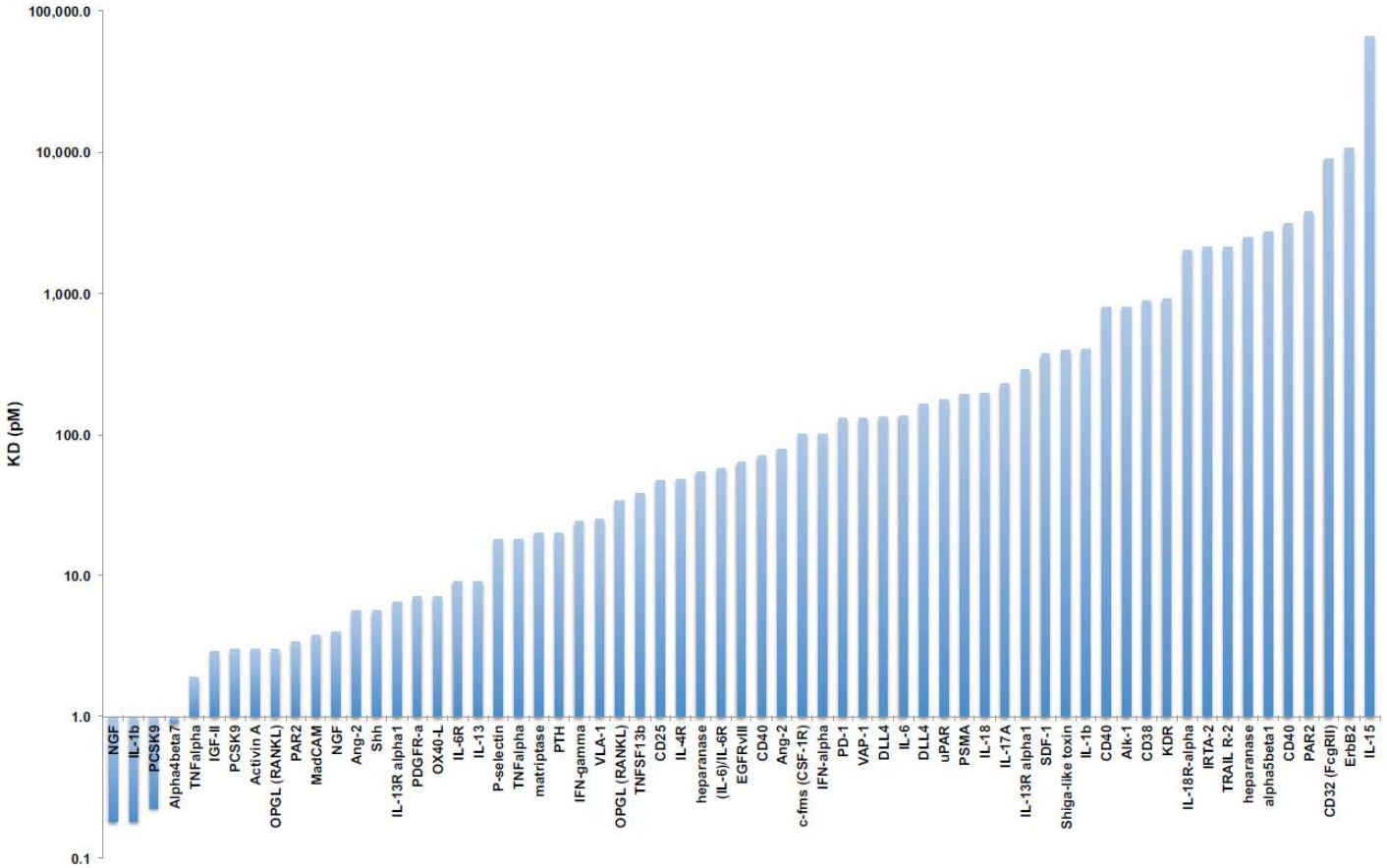


Anti-Hla mAb



blocking of Hemolysin-mediated membrane pore formation and lysis of rabbit RBCs

Antibody drug candidates from transgenic mice



Transgenic mice produce mAbs with antigen binding affinities <100 pM. The KD for binding of lead mAbs from 63 different discovery projects against 50 different antigen-targets is plotted. Binding affinity for all mAbs was measured by either Biacore

Transgenic mouse technologies have been successful as a source for innovator drugs, such as denosumab (anti-RANKL), ipilimumab (anti-CTLA-4) and ustekinumab (anti-p40 subunit of IL-12/IL-23), and for biosuperior drugs, such as panitumumab (anti-EGFR), canakinumab (anti-IL-beta), ofatumumab (anti-CD20), and golimumab (anti-TNFalpha).
Approximately 70 mAbs from transgenic mice are in clinical development.

Conclusion

- Transgenic mice are a proven technology for the discovery of high affinity antibody drug candidates with inherent qualities for successful antibody drug development.
- These therapeutic antibody drug candidates have affinities and potencies that meet stringent therapeutic design requirements (very low picomolar affinity.
- The mAbs bring high specificity
- Less risk of challenges in clinical- and commercial- scale production as well as retaining solubility at high concentrations.
- While the acquisitions of Abgenix, Inc. and Medarex, Inc have made the first generation technologies generally unavailable, new transgenic animal platforms are being developed to enable transgenic platforms remaining a key element of the discovery of human therapeutic antibodies.

Thank you for attention!