ADAM6 MICE

FIELD OF INVENTION

[0001] Genetically modified mice, cells, embryos, and tissues that comprise a nucleic acid sequence encoding a functional ADAM6 locus are described. Modifications include human and/or humanized immunoglobulin loci. Mice that lack a functional endogenous ADAM6 gene but that comprise ADAM6 function are described, including mice that comprise an ectopic nucleic acid sequence that encodes an ADAM6 protein. Genetically modified male mice that comprise a modification of an endogenous immunoglobulin V_H locus that renders the mouse incapable of making a functional ADAM6 protein and results in a loss in fertility, and that further comprise ADAM6 function in the male mice are described, including mice that comprise an ectopic nucleic acid sequence that restores fertility to the male mouse.

BACKGROUND OF INVENTION

[0002] Mice that contain human antibody genes are known in the art. Pharmaceutical applications for antibodies in the last two decades have fueled a great deal of research into making antibodies that are suitable for use as human therapeutics. Early antibody therapeutics, which were based on mouse antibodies, were not ideal as human therapeutics because repeated administration of mouse antibodies to humans results in immunogenicity that can confound long-term treatment regimens. Solutions based on humanizing mouse antibodies to make them appear more human and less mouse-like were developed. Methods for expressing human immunoglobulin sequences for use in antibodies followed, mostly based on *in vitro* expression of human immunoglobulin libraries in phage, bacteria, or yeast. Finally, attempts were made to make useful human antibodies from human lymphocytes *in vitro*, in mice engrafted with human hematopoietic cells, and in transchromosomal or transgenic mice with disabled endogenous immunoglobulin loci. In the transgenic mice, it was necessary to disable the endogenous mouse immunoglobulin genes so that the randomly integrated fully human transgenes would function as the source of immunoglobulin sequences expressed in the mouse. Such mice can make human antibodies suitable for use as human therapeutics, but these mice display substantial problems with their immune systems. These problems (1) make the mice impractical for generating a sufficiently diverse antibody repertoire, (2) require the use of extensive re-engineering fixes, (3) provide a suboptimal clonal selection process likely due to incompatibility between human and mouse elements, and (4) render these mice an unreliable source of large and diverse populations of human variable sequences needed to be truly useful for making human therapeutics.

[0003] There remains a need in the art for making improved genetically modified mice that are useful in generating immunoglobulin sequences, including human antibody sequences. There also remains a need for mice that are capable of rearranging immunoglobulin gene segments to form useful rearranged immunoglobulin genes, or capable of making proteins from altered immunoglobulin loci, while at the same time reducing or eliminating deleterious changes that might result from the genetic modifications.

SUMMARY OF INVENTION

[0004] In one aspect, nucleic acid constructs, cells, embryos, mice, and methods are provided for making mice that comprise a modification that results in a nonfunctional endogenous mouse ADAM6 protein or ADAM6 gene (*e.g.*, a knockout of or a deletion in an endogenous ADAM6 gene), wherein the mice comprise a nucleic acid sequence that encodes an ADAM6 protein or ortholog or homolog or fragment thereof that is functional in a male mouse.

[0005] In one aspect, nucleic acid constructs, cells, embryos, mice, and methods are provided for making mice that comprise a modification of an endogenous mouse immunoglobulin locus, wherein the mice comprise an ADAM6 protein or ortholog or homolog or fragment thereof that is functional in a male mouse. In one embodiment, the endogenous mouse immunoglobulin locus is an immunoglobulin heavy chain locus, and the modification reduces or eliminates ADAM6 activity of a cell or tissue of a male mouse.

[0006] In one aspect, mice are provided that comprise an ectopic nucleotide sequence encoding a mouse ADAM6 or ortholog or homolog or functional fragment thereof; mice are also provided that comprise an endogenous nucleotide sequence encoding a mouse ADAM6 or ortholog or homolog or fragment thereof, and at least one genetic modification of a heavy chain immunoglobulin locus.

[0007] In one aspect, methods are provided for making mice that comprise a modification of an endogenous mouse immunoglobulin locus, wherein the mice comprise an ADAM6 protein or ortholog or homolog or fragment thereof that is functional in a male mouse. Mice according to the invention are obtainable, for example, by the methods described herein.

[0008] In one aspect, methods are provided for making mice that comprise a genetic modification of an immunoglobulin heavy chain locus, wherein application of the methods result in male mice that comprise a modified immunoglobulin heavy chain locus (or a deletion thereof), and the male mice are capable of generating offspring by mating. In one embodiment, the male mice are capable of producing sperm that can transit from a mouse uterus through a mouse oviduct to fertilize a mouse egg.

[0009] In one aspect, methods are provided for making mice that comprise a genetic modification of an immunoglobulin heavy chain locus, wherein application of the methods result in male mice that comprise a modified immunoglobulin heavy chain locus (or a deletion thereof), and the male mice exhibit a reduction in fertility, and the mice comprise a genetic modification that restores in whole or in part the reduction in fertility. In various embodiments, the reduction in fertility is characterized by an inability of the sperm of the male mice to migrate from a mouse uterus through a mouse oviduct to fertilize a mouse egg. In various embodiments, the reduction in fertility is characterized by sperm that exhibit an *in vivo* migration defect. In various embodiments, the genetic modification that restores in whole or in part the reduction in fertility is a nucleic acid sequence encoding a mouse ADAM6 gene or ortholog or homolog or fragment thereof that is functional in a male mouse.

[00010] In one embodiment, the genetic modification comprises replacing endogenous immunoglobulin heavy chain variable loci with immunoglobulin heavy chain variable loci of another species (*e.g.*, a non-mouse species). In one embodiment, the genetic modification comprises insertion of orthologous immunoglobulin heavy chain variable loci into endogenous immunoglobulin heavy chain variable loci. In a specific embodiment, the species is human. In one embodiment, the genetic modification comprises deletion of an endogenous immunoglobulin heavy chain variable locus in whole or in part, wherein the deletion results in a loss of endogenous ADAM6 function. In a specific embodiment, the loss of endogenous ADAM6 function is associated with a reduction in fertility in male mice.

[00011] In one aspect, mice are provided that comprise a modification that reduces or eliminates mouse ADAM6 expression from an endogenous ADAM6 allele such that a male mouse having the modification exhibits a reduced fertility (*e.g.*, a highly reduced ability to generate offspring by mating), or is essentially infertile, due to the reduction or elimination of endogenous ADAM6 function, wherein the mice further comprise an ectopic ADAM6 sequence or homolog or ortholog or functional fragment thereof. In one aspect, the modification that reduces or eliminates mouse ADAM6 expression is a modification (*e.g.*, an insertion, a deletion, a replacement, *etc.*) in a mouse immunoglobulin locus.

[00012] In one embodiment, the reduction or loss of ADAM6 function comprises an inability or substantial inability of the mouse to produce sperm that can travel from a mouse uterus through a mouse oviduct to fertilize a mouse egg. In a specific embodiment, at least about 95%, 96%, 97%, 98%, or 99% of the sperm cells produced in an ejaculate volume of the mouse are incapable of traversing through an oviduct in vivo following copulation and fertilizing a mouse ovum.

[00013] In one embodiment, the reduction or loss of ADAM6 function comprises an inability to form or substantial inability to form a complex of ADAM2 and/or ADAM3 and/or

ADAM6 on a surface of a sperm cell of the mouse. In one embodiment, the loss of ADAM6 function comprises a substantial inability to fertilize a mouse egg by copulation with a female mouse.

[00014] In one aspect, a mouse is provided that lacks a functional endogenous ADAM6 gene, and comprises a protein (or an ectopic nucleotide sequence that encodes a protein) that confers ADAM6 functionality on the mouse. In one embodiment, the mouse is a male mouse and the functionality comprises enhanced fertility as compared with a mouse that lacks a functional endogenous ADAM6 gene.

[00015] In one embodiment, the protein is encoded by a genomic sequence located within an immunoglobulin locus in the germline of the mouse. In a specific embodiment, the immunoglobulin locus is a heavy chain locus. In another specific embodiment, the heavy chain locus comprises at least one human V_H, at least one human D_H and at least one human J_H gene segment. In one embodiment, the ectopic protein is encoded by a genomic sequence located within a non-immunoglobulin locus in the germline of the mouse. In one embodiment, the non-immunoglobulin locus is a transcriptionally active locus. In a specific embodiment, the transcriptionally active locus is the ROSA26 locus. In a specific embodiment, the transcriptionally active locus is associated with tissue-specific expression. In one embodiment, the tissue-specific expression is present in reproductive tissues. In one embodiment, the protein is encoded by a genomic sequence randomly inserted into the germline of the mouse.

[00016] In one embodiment, the mouse comprises a human or chimeric human/mouse or chimeric human/rat light chain (*e.g.*, human variable, mouse or rat constant) and a chimeric human variable/mouse or rat constant heavy chain. In a specific embodiment, the mouse comprises a transgene that comprises a chimeric human variable/rat or mouse constant light chain gene operably linked to a transcriptionally active promoter, *e.g.*, a ROSA26 promoter. In a further specific embodiment, the chimeric human/mouse or rat light chain transgene comprises a rearranged human light chain variable region sequence in the germline of the mouse.

[00017] In one embodiment, the ectopic nucleotide sequence is located within an immunoglobulin locus in the germline of the mouse. In a specific embodiment, the immunoglobulin locus is a heavy chain locus. In one embodiment, the heavy chain locus comprises at least one human V_H, at least one human D_H and at least one human J_H gene segment. In one embodiment, the ectopic nucleotide sequence is located within a non-immunoglobulin locus in the germline of the mouse. In one embodiment, the non-immunoglobulin locus is a transcriptionally active locus. In a specific embodiment, the transcriptionally active locus is the ROSA26 locus. In one embodiment, the ectopic nucleotide sequence is positioned randomly inserted into the germline of the mouse.

[00018] In one aspect, a mouse is provided that lacks a functional endogenous ADAM6 gene, wherein the mouse comprises an ectopic nucleotide sequence that complements the loss of mouse ADAM6 function. In one embodiment, the ectopic nucleotide sequence confers upon the mouse an ability to produce offspring that is comparable to a corresponding wild-type mouse that contains a functional endogenous ADAM6 gene. In one embodiment, the sequence confers upon the mouse an ability to form a complex of ADAM2 and/or ADAM3 and/or ADAM6 on the surface of sperm cell of the mouse. In one embodiment, the sequence confers upon the mouse an ability to travel from a mouse uterus through a mouse oviduct to a mouse ovum to fertilize the ovum.

[00019] In one embodiment, the mouse lacking the functional endogenous ADAM6 gene and comprising the ectopic nucleotide sequence produces at least about 50%, 60%, 70%, 80%, or 90% of the number of litters a wild-type mouse of the same age and strain produces in a six-month time period.

[00020] In one embodiment, the mouse lacking the functional endogenous ADAM6 gene and comprising the ectopic nucleotide sequence produces at least about 1.5-fold, about 2-fold, about 2.5-fold, about 3-fold, about 4-fold, about 6-fold, about 7-fold, about 8-fold, or about 10-fold or more progeny when bred over a six-month time period than a mouse of the same age and the same or similar strain that lacks the functional endogenous ADAM6 gene and that lacks the ectopic nucleotide sequence that is bred over substantially the same time period and under substantially the same conditions.

[00021] In one embodiment, the mouse lacking the functional endogenous ADAM6 gene and comprising the ectopic nucleotide sequence produces an average of at least about 2-fold, 3-fold, or 4-fold higher number of pups per litter in a 4- or 6-month breeding period than a mouse that lacks the functional endogenous ADAM6 gene and that lacks the ectopic nucleotide sequence, and that is bred for the same period of time.

[00022] In one embodiment, the mouse lacking the functional endogenous ADAM6 gene and comprising the ectopic nucleotide sequence is a male mouse, and the male mouse produces sperm that when recovered from oviducts at about 5-6 hours post-copulation reflects an oviduct migration that is at least 10-fold, at least 20-fold, at least 30-fold, at least 40-fold, at least 50-fold, at least 60-fold, at least 70-fold, at least 80-fold, at least 90-fold, 100-fold, 110-fold, or 120-fold or higher than a mouse that lacks the functional endogenous ADAM6 gene and that lacks the ectopic nucleotide sequence.

[00023] In one embodiment, the mouse lacking the functional endogenous ADAM6 gene and comprising the ectopic nucleotide sequence when copulated with a female mouse generates sperm that is capable of traversing the uterus and entering and traversing the oviduct within about 6 hours at an efficiency that is about equal to sperm from a wild-type mouse.

[00024] In one embodiment, the mouse lacking the functional endogenous ADAM6 gene and comprising the ectopic nucleotide sequence produces about 1.5-fold, about 2-fold, about 3-fold, or about 4-fold or more litters in a comparable period of time than a mouse that lacks the functional ADAM6 gene and that lacks the ectopic nucleotide sequence.

[00025] In one aspect, a mouse comprising in its germline a non-mouse nucleic acid sequence that encodes an immunoglobulin protein is provided, wherein the non-mouse immunoglobulin sequence comprises an insertion of a mouse ADAM6 gene or homolog or ortholog or functional fragment thereof. In one embodiment, the non-mouse immunoglobulin sequence comprises a human immunoglobulin sequence. In one embodiment, the sequence comprises a human immunoglobulin heavy chain sequence. In one embodiment, the sequence comprises a human immunoglobulin light chain sequence. In one embodiment, the sequence comprises one or more V gene segments, one or more D gene segments, and one or more J gene segments; in one embodiment, the sequence comprises one or more V gene segments and one or more J gene segments. In one embodiment, the one or more V, D, and J gene segments, or one or more V and J gene segments, are not rearranged. In one embodiment, the one or more V, D, and J gene segments, or one or more V and J gene segments, are rearranged. In one embodiment, following rearrangement of the one or more V, D, and J gene segments, or one or more V and J gene segments, the mouse comprises in its genome at least one nucleic acid sequence encoding a mouse ADAM6 gene or homolog or ortholog or functional fragment thereof. In one embodiment, following rearrangement the mouse comprises in its genome at least two nucleic acid sequences encoding a mouse ADAM6 gene or homolog or ortholog or functional fragment thereof. In one embodiment, following rearrangement the mouse comprises in its genome at least one nucleic acid sequence encoding a mouse ADAM6 gene or homolog or ortholog or functional fragment thereof. In one embodiment, the mouse comprises the ADAM6 gene or homolog or ortholog or functional fragment thereof in a B cell. In one embodiment, the mouse comprises the ADAM6 gene or homolog or ortholog or functional fragment thereof in a non-B cell.

[00026] In one aspect, mice are provided that express a human immunoglobulin heavy chain variable region or functional fragment thereof from an endogenous mouse immunoglobulin heavy chain locus, wherein the mice comprise an ADAM6 activity that is functional in a male mouse.

[00027] In one embodiment, the male mice comprise a single unmodified endogenous ADAM6 allele or ortholog of homolog or functional fragment thereof at an endogenous ADAM6 locus.

[00028] In one embodiment, the male mice comprise an ectopic mouse ADAM6

sequence or homolog or ortholog or functional fragment thereof that encodes a protein that confers ADAM6 function.

[00029] In one embodiment, the male mice comprise an ADAM6 sequence or homolog or ortholog or functional fragment thereof at a location in the mouse genome that approximates the location of the endogenous mouse ADAM6 allele, *e.g.,* 3' of a final V gene segment sequence and 5' of an initial D gene segment.

[00030] In one embodiment, the male mice comprise an ADAM6 sequence or homolog or ortholog or functional fragment thereof flanked upstream, downstream, or upstream and downstream (with respect to the direction of transcription of the ADAM6 sequence) of a nucleic acid sequence encoding an immunoglobulin variable gene segment. In a specific embodiment, the immunoglobulin variable gene segment is a human gene segment. In one embodiment, the immunoglobulin variable gene segment is a human gene segment, and the sequence encoding the mouse ADAM6 or ortholog or homolog or fragment thereof functional in a mouse is between human V gene segments; in one embodiment, the mouse comprises two or more human V gene segments, and the sequence is at a position between the final V gene segment and the penultimate V gene segment; in one embodiment, the sequence is at a position following the final V gene segment and the first D gene segment.

[00031] In one aspect, a male mouse is provided that comprises a nonfunctional endogenous ADAM6 gene, or a deletion of an endogenous ADAM6 gene, in its germline; wherein sperm cells of the mouse are capable of transiting an oviduct of a female mouse and fertilizing an egg. In one embodiment, the mice comprise an extrachromosomal copy of a mouse ADAM6 gene or ortholog or homolog or functional fragment thereof that is functional in a male mouse. In one embodiment, the mice comprise an ectopic mouse ADAM6 gene or ortholog or homolog or functional fragment thereof that is functional in a male mouse.

[00032] In one aspect, mice are provided that comprise a genetic modification that reduces endogenous mouse ADAM6 function, wherein the mouse comprises at least some ADAM6 functionality provided either by an endogenous unmodified allele that is functional in whole or in part (*e.g.*, a heterozygote), or by expression from an ectopic sequence that encodes an ADAM6 or an ortholog or homolog or functional fragment thereof that is functional in a male mouse.

[00033] In one embodiment, the mice comprise ADAM6 function sufficient to confer upon male mice the ability to generate offspring by mating, as compared with male mice that lack a functional ADAM6. In one embodiment, the ADAM6 function is conferred by the presence of an ectopic nucleotide sequence that encodes a mouse ADAM6 or a homolog or ortholog or functional fragment thereof. ADAM6 homologs or orthologs or fragments

thereof that are functional in a male mouse include those that restore, in whole or in part, the loss of ability to generate offspring observed in a male mouse that lacks sufficient endogenous mouse ADAM6 activity, *e.g.*, the loss in ability observed in an ADAM6 knockout mouse. In this sense ADAM6 knockout mice include mice that comprise an endogenous locus or fragment thereof, but that is not functional, *i.e.,* that does not express ADAM6 (ADAM6a and/or ADAM6b) at all, or that expresses ADAM6 (ADAM6a and/or ADAM6b) at a level that is insufficient to support an essentially normal ability to generate offspring of a wild-type male mouse. The loss of function can be due, *e.g.,* to a modification in a structural gene of the locus (*i.e.,* in an ADAM6a or ADAM6b coding region) or in a regulatory region of the locus (*e.g.,* in a sequence 5' to the ADAM6a gene, or 3' of the ADAM6a or ADAM6b coding region, wherein the sequence controls, in whole or in part, transcription of an ADAM6 gene, expression of an ADAM6 RNA, or expression of an ADAM6 protein). In various embodiments, orthologs or homologs or fragments thereof that are functional in a male mouse are those that enable a sperm of a male mouse (or a majority of sperm cells in the ejaculate of a male mouse) to transit a mouse oviduct and fertilize a mouse ovum.

[00034] In one embodiment, male mice that express the human immunoglobulin variable region or functional fragment thereof comprise sufficient ADAM6 activity to confer upon the male mice the ability to generate offspring by mating with female mice and, in one embodiment, the male mice exhibit an ability to generate offspring when mating with female mice that is in one embodiment at least 25%, in one embodiment, at least 30%, in one embodiment at least 40%, in one embodiment at least 50%, in one embodiment at least 60%, in one embodiment at least 70%, in one embodiment at least 80%, in one embodiment at least 90%, and in one embodiment about the same as, that of mice with one or two endogenous unmodified ADAM6 alleles.

[00035] In one embodiment male mice express sufficient ADAM6 (or an ortholog or homolog or functional fragment thereof) to enable a sperm cell from the male mice to traverse a female mouse oviduct and fertilize a mouse egg.

[00036] In one embodiment, the ADAM6 functionality is conferred by a nucleic acid sequence that is contiguous with a mouse chromosomal sequence (*e.g.,* the nucleic acid is randomly integrated into a mouse chromosome; or placed at a specific location, *e.g.*, by targeting the nucleic acid to a specific location, *e.g.*, by site-specific recombinase-mediated (*e.g.*, Cre-mediated) insertion or homologous recombination). In one embodiment, the ADAM6 sequence is present on a nucleic acid that is distinct from a chromosome of the mouse (*e.g.*, the ADAM6 sequence is present on an episome, *i.e.*, extrachromosomally, *e.g.*, in an expression construct, a vector, a YAC, a transchromosome, *etc.*).

[00037] In one aspect, genetically modified mice and cells are provided that comprise a

modification of an endogenous immunoglobulin heavy chain locus, wherein the mice express at least a portion of an immunoglobulin heavy chain sequence, *e.g.,* at least a portion of a human sequence, wherein the mice comprise an ADAM6 activity that is functional in a male mouse. In one embodiment, the modification reduces or eradicates ADAM6 activity of the mouse. In one embodiment, the mouse is modified such that both alleles that encode ADAM6 activity are either absent or express an ADAM6 that does not substantially function to support normal mating in a male mouse. In one embodiment, the mouse further comprises an ectopic nucleic acid sequence encoding a mouse ADAM6 or ortholog or homolog or functional fragment thereof.

[00038] In one aspect, genetically modified mice and cells are provided that comprise a modification of an endogenous immunoglobulin heavy chain locus, wherein the modification reduces or eliminates ADAM6 activity expressed from an ADAM6 sequence of the locus, and wherein the mice comprise an ADAM6 protein or ortholog or homolog or functional fragment thereof. In various embodiments, the ADAM6 protein or fragment thereof is encoded by an ectopic ADAM6 sequence. In various embodiments, the ADAM6 protein or fragment thereof is expressed from an endogenous ADAM6 allele. In various embodiments, the mouse comprises a first immunoglobulin heavy chain allele comprises a first modification that reduces or eliminates expression of a functional ADAM6 from the first immunoglobulin heavy chain allele, and the mouse comprises a second immunoglobulin heavy chain allele that comprises a second modification that does not substantially reduce or does not eliminate expression of a functional ADAM6 from the second immunoglobulin heavy chain allele.

[00039] In one embodiment, the second modification is located 3' (with respect to the transcriptional directionality of the mouse V gene segment) of a final mouse V gene segment and located 5' (with respect to the transcriptional directionality of the constant sequence) of a mouse (or chimeric human/mouse) immunoglobulin heavy chain constant gene or fragment thereof (*e.g.*, a nucleic acid sequence encoding a human and/or mouse: C_H1 and/or hinge and/or C_H2 and/or C_H3).

[00040] In one embodiment, the modification is at a first immunoglobulin heavy chain allele at a first locus that encodes a first ADAM6 allele, and the ADAM6 function results from expression of an endogenous ADAM6 at a second immunoglobulin heavy chain allele at a second locus that encodes a functional ADAM6, wherein the second immunoglobulin heavy chain allele comprises at least one modification of a V, D, and/or J gene segment. In a specific embodiment, the at least one modification of the V, D, and or J gene segment is a deletion, a replacement with a human V, D, and/or J gene segment, a replacement with a camelid V, D, and/or J gene segment, a replacement with a humanized or camelized V, D, and/or J gene segment, a replacement of a heavy chain sequence with a light chain

sequence, and a combination thereof. In one embodiment, the at least one modification is the deletion of one or more heavy chain V, D, and/or J gene segments and a replacement with one or more light chain V and/or J gene segments (*e.g.*, a human light chain V and/or J gene segment) at the heavy chain locus.

[00041] In one embodiment, the modification is at a first immunoglobulin heavy chain allele at a first locus and a second immunoglobulin heavy chain allele at a second locus, and the ADAM6 function results from expression of an ectopic ADAM6 at a non-immunoglobulin locus in the germline of the mouse. In a specific embodiment, the non-immunoglobulin locus is the ROSA26 locus. In a specific embodiment, the non-immunoglobulin locus is transcriptionally active in reproductive tissue.

[00042] In one aspect, a mouse comprising a heterozygous or a homozygous knockout of ADAM6 is provided. In one embodiment, the mouse further comprises a modified immunoglobulin sequence that is a human or a humanized immunoglobulin sequence, or a camelid or camelized human or mouse immunoglobulin sequence. In one embodiment, the modified immunoglobulin sequence is present at the endogenous mouse heavy chain immunoglobulin locus. In one embodiment, the modified immunoglobulin sequence comprises a human heavy chain variable gene sequence at an endogenous mouse immunoglobulin heavy chain locus. In one embodiment, the human heavy chain variable gene sequence replaces an endogenous mouse heavy chain variable gene sequence at the endogenous mouse immunoglobulin heavy chain locus.

[00043] In one aspect, a mouse incapable of expressing a functional endogenous mouse ADAM6 from an endogenous mouse ADAM6 locus is provided. In one embodiment, the mouse comprises an ectopic nucleic acid sequence that encodes an ADAM6, or functional fragment thereof, that is functional in the mouse. In a specific embodiment, the ectopic nucleic acid sequence encodes a protein that rescues a loss in the ability to generate offspring exhibited by a male mouse that is homozygous for an ADAM6 knockout. In a specific embodiment, the ectopic nucleic acid sequence encodes a mouse ADAM6 protein.

[00044] In one aspect, a mouse is provided that lacks a functional endogenous ADAM6 locus, and that comprises an ectopic nucleic acid sequence that confers upon the mouse ADAM6 function. In one embodiment, the nucleic acid sequence comprises an endogenous mouse ADAM6 sequence or functional fragment thereof. In one embodiment, the endogenous mouse ADAM6 sequence comprises ADAM6a- and ADAM6b-encoding sequence located in a wild-type mouse between the 3'-most mouse immunoglobulin heavy chain V gene segment (V_H) and the 5'-most mouse immunoglobulin heavy chain D gene segment (D_H).

[00045] In one embodiment, the nucleic acid sequence comprises a sequence encoding

mouse ADAM6a or functional fragment thereof and/or a sequence encoding mouse ADAM6b or functional fragment thereof, wherein the ADAM6a and/or ADAM6b or functional fragment(s) thereof is operably linked to a promoter. In one embodiment, the promoter is a human promoter. In one embodiment, the promoter is the mouse ADAM6 promoter. In a specific embodiment, the ADAM6 promoter comprises sequence located between the first codon of the first ADAM6 gene closest to the mouse 5'-most D_H gene segment and the recombination signal sequence of the 5'-most D_H gene segment, wherein 5' is indicated with respect to direction of transcription of the mouse immunoglobulin genes. In one embodiment, the promoter is a viral promoter. In a specific embodiment, the viral promoter is a cytomegalovirus (CMV) promoter. In one embodiment, the promoter is a ubiquitin promoter.

[00046] In one embodiment, the promoter is an inducible promoter. In one embodiment, the inducible promoter regulates expression in non-reproductive tissues. In one embodiment, the inducible promoter regulates expression in reproductive tissues. In a specific embodiment, the expression of the mouse ADAM6a and/or ADAM6b sequences or functional fragments(s) thereof is developmentally regulated by the inducible promoter in reproductive tissues.

[00047] In one embodiment, the mouse ADAM6a and/or ADAM6b are selected from the ADAM6a of SEQ ID NO:1 and/or ADAM6b of sequence SEQ ID NO:2. In one embodiment, the mouse ADAM6 promoter is a promoter of SEQ ID NO:3. In a specific embodiment, the mouse ADAM6 promoter comprises the nucleic acid sequence of SEQ ID NO:3 directly upstream (with respect to the direction of transcription of ADAM6a) of the first codon of ADAM6a and extending to the end of SEQ ID NO:3 upstream of the ADAM6 coding region. In another specific embodiment, the ADAM6 promoter is a fragment extending from within about 5 to about 20 nucleotides upstream of the start codon of ADAM6a to about 0.5kb, 1kb, 2kb, or 3kb or more upstream of the start codon of ADAM6a.

[00048] In one embodiment, the nucleic acid sequence comprises SEQ ID NO:3 or a fragment thereof that when placed into a mouse that is infertile or that has low fertility due to a lack of ADAM6, improves fertility or restores fertility to about a wild-type fertility. In one embodiment, SEQ ID NO:3 or a fragment thereof confers upon a male mouse the ability to produce a sperm cell that is capable of traversing a female mouse oviduct in order to fertilize a mouse egg.

[00049] In one aspect, a mouse is provided that comprises a deletion of an endogenous nucleotide sequence that encodes an ADAM6 protein, a replacement of an endogenous mouse V_H gene segment with a human V_H gene segment, and an ectopic nucleotide sequence that encodes a mouse ADAM6 protein or ortholog or homolog or fragment thereof that is functional in a male mouse.

[00050] In one embodiment, the mouse comprises an immunoglobulin heavy chain locus that comprises a deletion of an endogenous immunoglobulin locus nucleotide sequence that comprises an endogenous ADAM6 gene, comprises a nucleotide sequence encoding one or more human immunoglobulin gene segments, and wherein the ectopic nucleotide sequence encoding the mouse ADAM6 protein is within or directly adjacent to the nucleotide sequence encoding the one or more human immunoglobulin gene segments.

[00051] In one embodiment, the mouse comprises a replacement of all or substantially all endogenous V_H gene segments with a nucleotide sequence encoding one or more human V_H gene segments, and the ectopic nucleotide sequence encoding the mouse ADAM6 protein is within, or directly adjacent to, the nucleotide sequence encoding the one or more human V_H gene segments. In one embodiment, the mouse further comprises a replacement of one or more endogenous D_H gene segments with one or more human D_H gene segments at the endogenous D_H gene locus. In one embodiment, the mouse further comprises a replacement of one or more endogenous J_H gene segments with one or more human J_H gene segments at the endogenous J_H gene locus. In one embodiment, the mouse comprises a replacement of all or substantially all endogenous V_H, D_H, and J_H gene segments and a replacement at the endogenous V_H, D_H, and J_H gene loci with human V_H, D_H, and J_H gene segments, wherein the mouse comprises an ectopic sequence encoding a mouse ADAM6 protein. In a specific embodiment, the ectopic sequence encoding the mouse ADAM6 protein is placed between the penultimate 3'-most V_H gene segment of the human V_H gene segments present, and the ultimate 3' V_H gene segment of the human V_H gene segments present. In a specific embodiment, the mouse comprises a deletion of all or substantially all mouse V_H gene segments, and a replacement with all or substantially all human V_H gene segments, and the ectopic nucleotide sequence encoding the mouse ADAM6 protein is placed downstream of human gene segment V_H1-2 and upstream of human gene segment V_H6-1.

[00052] In a specific embodiment, the mouse comprises a replacement of all or substantially all endogenous V_H gene segments with a nucleotide sequence encoding one or more human V_H gene segments, and the ectopic nucleotide sequence encoding the mouse ADAM6 protein is within, or directly adjacent to, the nucleotide sequence encoding the one or more human V_H gene segments.

[00053] In one embodiment, the ectopic nucleotide sequence that encodes the mouse ADAM6 protein is present on a transgene in the genome of the mouse. In one embodiment, the ectopic nucleotide sequence that encodes the mouse ADAM6 protein is present extrachromosomally in the mouse.

[00054] In one aspect, a mouse is provided that comprises a modification of an

endogenous immunoglobulin heavy chain locus, wherein the mouse expresses a B cell that comprises a rearranged immunoglobulin sequence operably linked to a heavy chain constant region gene sequence, and the B cell comprises in its genome (*e.g.*, on a B cell chromosome) a gene encoding an ADAM6 or ortholog or homolog or fragment thereof that is functional in a male mouse. In one embodiment, the rearranged immunoglobulin sequence operably linked to the heavy chain constant region gene sequence comprises a human heavy chain V, D, and/or J sequence; a mouse heavy chain V, D, and/or J sequence; a human or mouse light chain V and/or J sequence. In one embodiment, the heavy chain constant region gene sequence comprises a human or a mouse heavy chain sequence selected from the group consisting of a C_H1, a hinge, a C_H2, a C_H3, and a combination thereof.

[00055] In one aspect, a genetically modified mouse is provided, wherein the mouse comprises a functionally silenced immunoglobulin light chain gene, and further comprises a replacement of one or more endogenous immunoglobulin heavy chain variable region gene segments with one or more human immunoglobulin heavy chain variable region gene segments, wherein the mouse lacks a functional endogenous ADAM6 locus, and wherein the mouse comprises an ectopic nucleotide sequence that expresses a mouse ADAM6 protein or an ortholog or homolog or fragment thereof that is functional in a male mouse.

[00056] In one aspect, a mouse is provided that lacks a functional endogenous mouse ADAM6 locus or sequence and that comprises an ectopic nucleotide sequence encoding a mouse ADAM6 locus or functional fragment of a mouse ADAM6 locus or sequence, wherein the mouse is capable of mating with a mouse of the opposite sex to produce a progeny that comprises the ectopic ADAM6 locus or sequence. In one embodiment, the mouse is male. In one embodiment, the mouse is female.

[00057] In one aspect, a genetically modified mouse is provided, wherein the mouse comprises a human immunoglobulin heavy chain variable region gene segment at an endogenous mouse immunoglobulin heavy chain variable region gene locus, the mouse lacks an endogenous functional ADAM6 sequence at the endogenous mouse immunoglobulin heavy chain variable region gene locus, and wherein the mouse comprises an ectopic nucleotide sequence that expresses a mouse ADAM6 protein or an ortholog or homolog or fragment thereof that is functional in a male mouse.

[00058] In one embodiment, the ectopic nucleotide sequence that expresses the mouse ADAM6 protein is extrachromosomal. In one embodiment, the ectopic nucleotide sequence that expresses the mouse ADAM6 protein is integrated at one or more loci in a genome of the mouse. In a specific embodiment, the one or more loci include an immunoglobulin locus.

[00059] In one aspect, a mouse is provided that expresses an immunoglobulin heavy

chain sequence from a modified endogenous mouse immunoglobulin heavy chain locus, wherein the heavy chain is derived from a human V gene segment, a D gene segment, and a J gene segment, wherein the mouse comprises an ADAM6 activity that is functional in the mouse.

[00060] In one embodiment, the mouse comprises a plurality of human V gene segments, a plurality of D gene segments, and a plurality of J gene segments. In one embodiment, the D gene segments are human D gene segments. In one embodiment, the J gene segments are human J gene segments. In one embodiment, the mouse further comprises a humanized heavy chain constant region sequence, wherein the humanization comprises replacement of a sequence selected from a C_H1, hinge, C_H2, C_H3, and a combination thereof. In a specific embodiment, the heavy chain is derived from a human V gene segment, a human D gene segment, a human J gene segment, a human C_H1 sequence, a human or mouse hinge sequence, a mouse C_H2 sequence, and a mouse C_H3 sequence. In another specific embodiment, the mouse further comprises a human light chain constant sequence.

[00061] In one embodiment, the D gene segment is flanked 5' (with respect to transcriptional direction of the D gene segment) by a sequence encoding an ADAM6 activity that is functional in the mouse.

[00062] In one embodiment, the ADAM6 activity that is functional in the mouse results from expression of a nucleotide sequence located 5' of the 5'-most D gene segment and 3' of the 3'-most V gene segment (with respect to the direction of transcription of the V gene segment) of the modified endogenous mouse heavy chain immunoglobulin locus.

[00063] In one embodiment, the ADAM6 activity that is functional in the mouse results from expression of a nucleotide sequence located between two human V gene segments in the modified endogenous mouse heavy chain immunoglobulin locus. In one embodiment, the two human V gene segments are a human V_H1-2 gene segment and a V_H6-1 gene segment.

[00064] In one embodiment, the nucleotide sequence comprises a sequence selected from a mouse ADAM6b sequence or functional fragment thereof, a mouse ADAM6a sequence or functional fragment thereof, and a combination thereof.

[00065] In one embodiment, the nucleotide sequence between the two human V gene segments is placed in opposite transcription orientation with respect to the human V gene segments. In a specific embodiment, nucleotide sequence encodes, from 5' to 3' with respect to the direction of transcription of ADAM6 genes, and ADAM6a sequence followed by an ADAM6b sequence.

[00066] In one embodiment, the mouse comprises a replacement of a human ADAM6 pseudogene sequence between human V gene segments V_H1-2 and V_H6-1 with a mouse

ADAM6 sequence or a functional fragment thereof.

[00067] In one embodiment, the sequence encoding the ADAM6 activity that is functional in the mouse is a mouse ADAM6 sequence or functional fragment thereof.

[00068] In one embodiment, the mouse comprises an endogenous mouse DFL16.1 gene segment (*e.g.*, in a mouse heterozygous for the modified endogenous mouse immunoglobulin heavy chain locus), or a human D_H1-1 gene segment. In one embodiment, the D gene segment of the immunoglobulin heavy chain expressed by the mouse is derived from an endogenous mouse DFL16.1 gene segment or a human D_H1-1 gene segment.

[00069] In one aspect, a mouse is provided that comprises a nucleic acid sequence encoding a mouse ADAM6 (or homolog or ortholog or functional fragment thereof) in a DNA-bearing cell of non-rearranged B cell lineage, but does not comprise the nucleic acid sequence encoding the mouse ADAM6 (or homolog or ortholog or functional fragment thereof) in a B cell that comprise rearranged immunoglobulin loci, wherein the nucleic acid sequence encoding the mouse ADAM6 (or homolog or ortholog or functional fragment thereof) occurs in the genome at a position that is different from a position in which a mouse ADAM6 gene appears in a wild-type mouse. In one embodiment, the nucleic acid sequence encoding the mouse ADAM6 (or homolog or ortholog or functional fragment thereof) is present in all or substantially all DNA-bearing cells that are not of rearranged B cell lineage; in one embodiment, the nucleic acid sequence is present in germline cells of the mouse, but not in a chromosome of a rearranged B cell.

[00070] In one aspect, a mouse is provided that comprises a nucleic acid sequence encoding a mouse ADAM6 (or homolog or ortholog or functional fragment thereof) in all or substantially all DNA-bearing cells, including B cells that comprise rearranged immunoglobulin loci, wherein the nucleic acid sequence encoding the mouse ADAM6 (or homolog or ortholog or functional fragment thereof) occurs in the genome at a position that is different from a position in which a mouse ADAM6 gene appears in a wild-type mouse. In one embodiment, the nucleic acid sequence encoding the mouse ADAM6 (or homolog or ortholog or functional fragment thereof) is on a nucleic acid that is contiguous with the rearranged immunoglobulin locus. In one embodiment, the nucleic acid that is contiguous with the rearranged immunoglobulin locus is a chromosome. In one embodiment, the chromosome is a chromosome that is found in a wild-type mouse and the chromosome comprises a modification of a mouse immunoglobulin locus.

[00071] In one aspect, a genetically modified mouse is provided, wherein the mouse comprises a B cell that comprises in its genome an ADAM6 sequence or ortholog or homolog thereof. In one embodiment, the ADAM6 sequence or ortholog or homolog thereof is at an immunoglobulin heavy chain locus. In one embodiment, the ADAM6

sequence or ortholog or homolog thereof is at a locus that is not an immunoglobulin locus. In one embodiment, the ADAM6 sequence is on a transgene driven by a heterologous promoter. In a specific embodiment, the heterologous promoter is a non-immunoglobulin promoter. In a specific embodiment, B cell expresses an ADAM6 protein or ortholog or homolog thereof.

[00072] In one embodiment, 90% or more of the B cells of the mouse comprise a gene encoding an ADAM6 protein or an ortholog thereof or a homolog thereof or a fragment thereof that is functional in the mouse. In a specific embodiment, the mouse is a male mouse.

[00073] In one embodiment, the B cell genome comprises a first allele and a second allele comprising the ADAM6 sequence or ortholog or homolog thereof. In one embodiment, the B cell genome comprises a first allele but not a second allele comprising the ADAM6 sequence or ortholog or homolog thereof.

[00074] In one aspect, a mouse is provided that comprises a modification at one or more endogenous ADAM6 alleles.

[00075] In one embodiment, the modification renders the mouse incapable of expressing a functional ADAM6 protein from at least one of the one or more endogenous ADAM6 alleles. In a specific embodiment, the mouse is incapable of expressing a functional ADAM6 protein from each of the endogenous ADAM6 alleles.

[00076] In one embodiment, the mice are incapable of expressing a functional ADAM6 protein from each endogenous ADAM6 allele, and the mice comprise an ectopic ADAM6 sequence.

[00077] In one embodiment, the mice are incapable of expressing a functional ADAM6 protein from each endogenous ADAM6 allele, and the mice comprise an ectopic ADAM6 sequence located within 1, 2, 3, 4, 5, 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 110, or 120 or more kb upstream (with respect to the direction of transcription of the mouse heavy chain locus) of a mouse immunoglobulin heavy chain constant region sequence. In a specific embodiment, the ectopic ADAM6 sequence is at the endogenous immunoglobulin heavy chain locus (*e.g.*, in an intergenic V-D region, between two V gene segments, between a V and a D gene segment, between a D and a J gene segment, *etc.*). In a specific embodiment, the ectopic ADAM6 sequence is located within a 90 to 100 kb intergenic sequence between the final mouse V gene segment and the first mouse D gene segment. In another specific embodiment, the endogenous 90 to 100 kb intergenic V-D sequence is removed, and the ectopic ADAM6 sequence is placed between the final V and the first D gene segment.

[00078] In one aspect, an infertile male mouse is provided, wherein the mouse comprises a deletion of two or more endogenous ADAM6 alleles. In one aspect, a female

mouse is provided that is a carrier of a male infertility trait, wherein the female mouse comprises in its germline a nonfunctional ADAM6 allele or a knockout of an endogenous ADAM6 allele.

[00079] In one aspect, a mouse that lacks an endogenous immunoglobulin heavy chain V, D, and J gene segment is provided, wherein a majority of the B cells of the mouse comprise an ADAM6 sequence or ortholog or homolog thereof.

[00080] In one embodiment, the mouse lacks endogenous immunoglobulin heavy chain gene segments selected from two or more V gene segments, two or more D gene segments, two or more J gene segments, and a combination thereof. In one embodiment, the mouse lacks immunoglobulin heavy chain gene segments selected from at least one and up to 89 V gene segments, at least one and up to 13 D gene segments, at least one and up to four J gene segments, and a combination thereof. In one embodiment, the mouse lacks a genomic DNA fragment from chromosome 12 comprising about three megabases of the endogenous immunoglobulin heavy chain locus. In a specific embodiment, the mouse lacks all functional endogenous heavy chain V, D, and J gene segments. In a specific embodiment, the mouse lacks 89 V_H gene segments, 13 D_H gene segments and four J_H gene segments.

[00081] In one aspect, a mouse is provided, wherein the mouse has a genome in the germline comprising a modification of an immunoglobulin heavy chain locus, wherein the modification to the immunoglobulin heavy chain locus comprises the replacement of one or more mouse immunoglobulin variable region sequences with one or more non-mouse immunoglobulin variable region sequences, and wherein the mouse comprises a nucleic acid sequence encoding a mouse ADAM6 protein. In a preferred embodiment, the D_H and J_H sequences and at least 3, at least 10, at least 20, at least 40, at least 60, or at least 80 V_H sequences of the immunoglobulin heavy chain locus are replaced by non-mouse immunoglobulin variable region sequences. In a further preferred embodiment, the D_H, J_H, and all V_H sequences of the immunoglobulin heavy chain locus are replaced by non-mouse immunoglobulin variable region sequences. The non-mouse immunoglobulin variable region sequences can be non-rearranged. In a preferred embodiment, the non-mouse immunoglobulin variable region sequences comprise complete non-rearranged D_H and J_H regions and at least 3, at least 10, at least 20, at least 40, at least 60, or at least 80 non-rearranged V_H sequences of the non-mouse species. In a further preferred embodiment, the non-mouse immunoglobulin variable region sequences comprise the complete variable region, including all V_H, D_H, and J_H regions, of the non-mouse species. The non-mouse species can be Homo sapiens and the non-mouse immunoglobulin variable region sequences can be human sequences.

[00082] In one aspect, a mouse that expresses an antibody that comprises at least one

human variable domain/non-human constant domain immunoglobulin polypeptide is provided, wherein the mouse expresses a mouse ADAM6 protein or ortholog or homolog thereof from a locus other than an immunoglobulin locus.

[00083] In one embodiment, the ADAM6 protein or ortholog or homolog thereof is expressed in a B cell of the mouse, wherein the B cell comprises a rearranged immunoglobulin sequence that comprises a human variable sequence and a non-human constant sequence.

[00084] In one embodiment, the non-human constant sequence is a rodent sequence. In one embodiment, the rodent is selected from a mouse, a rat, and a hamster.

[00085] In one aspect, a method is provided for making an infertile male mouse, comprising rendering an endogenous ADAM6 allele of a donor ES cell nonfunctional (or knocking out said allele), introducing the donor ES cell into a host embryo, gestating the host embryo in a surrogate mother, and allowing the surrogate mother to give birth to progeny derived in whole or in part from the donor ES cell. In one embodiment, the method further comprises breeding progeny to obtain an infertile male mouse.

[00086] In one aspect, a method is provided for making a mouse with a genetic modification of interest, wherein the mouse is infertile, the method comprising the steps of (a) making a genetic modification of interest in a genome; (b) modifying the genome to knockout an endogenous ADAM6 allele, or render an endogenous ADAM6 allele nonfunctional; and, (c) employing the genome in making a mouse. In various embodiments, the genome is from an ES cell or used in a nuclear transfer experiment.

[00087] In one aspect, a mouse made using a targeting vector, nucleotide construct, or cell as described herein is provided.

[00088] In one aspect, a progeny of a mating of a mouse as described herein with a second mouse that is a wild-type mouse or genetically modified is provided.

[00089] In one aspect, a method for maintaining a mouse strain is provided, wherein the mouse strain comprises a replacement of a mouse immunoglobulin heavy chain sequence with one or more heterologous immunoglobulin heavy chain sequences. In one embodiment, the one or more heterologous immunoglobulin heavy chain sequences are human immunoglobulin heavy chain sequences.

[00090] In one embodiment, the mouse strain comprises a deletion of one or more mouse V_H, D_H, and/or J_H gene segments. In one embodiment, the mouse further comprises one or more human V_H gene segments, one or more human D_H gene segments, and/or one or more human J_H gene segments. In one embodiment, the mouse comprises at least 3, at least 10, at least 20, at least 40, at least 60, or at least 80 human V_H segments, at least 27 human D_H gene segments, and at least six J_H gene segments. In a specific embodiment, the mouse comprises at least 3, at least 10, at least 20, at least 40,

at least 60, or at least 80 human V_H segments, the at least 27 human D_H gene segments, and the at least six J_H gene segments are operably linked to a constant region gene. In one embodiment, the constant region gene is a mouse constant region gene. In one embodiment, the constant region gene comprises a mouse constant region gene sequence selected from a C_H1, a hinge, a C_H2, a C_H3, and/or a C_H4 or a combination thereof.

[00091] In one embodiment, the method comprises generating a male mouse heterozygous for the replacement of the mouse immunoglobulin heavy chain sequence, and breeding the heterozygous male mouse with a wild-type female mouse or a female mouse that is homozygous or heterozygous for the human heavy chain sequence. In one embodiment, the method comprises maintaining the strain by repeatedly breeding heterozygous males with females that are wild type or homozygous or heterozygous for the human heavy chain sequence.

[00092] In one embodiment, the method comprises obtaining cells from male or female mice homozygous or heterozygous for the human heavy chain sequence, and employing those cells as donor cells or nuclei therefrom as donor nuclei, and using the cells or nuclei to make genetically modified animals using host cells and/or gestating the cells and/or nuclei in surrogate mothers.

[00093] In one embodiment, only male mice that are heterozygous for the replacement at the heavy chain locus are bred to female mice. In a specific embodiment, the female mice are homozygous, heterozygous, or wild type with respect to a replaced heavy chain locus.

[00094] In one embodiment, the mouse further comprises a replacement of λ and/or κ light chain variable sequences at an endogenous immunoglobulin light chain locus with heterologous immunoglobulin light chain sequences. In one embodiment, the heterologous immunoglobulin light chain sequences are human immunoglobulin λ and/or κ light chain variable sequences.

[00095] In one embodiment, the mouse further comprises a transgene at a locus other than an endogenous immunoglobulin locus, wherein the transgene comprises a sequence encoding a rearranged or unrearranged heterologous λ or κ light chain sequence (*e.g.*, unrearranged V_L and unrearranged J_L, or rearranged VJ) operably linked (for unrearranged) or fused (for rearranged) to an immunoglobulin light chain constant region sequence. In one embodiment, the heterologous λ or κ light chain sequence is human. In one embodiment, the constant region sequence is selected from rodent, human, and non-human primate. In one embodiment, the constant region sequence is selected from mouse, rat, and hamster. In one embodiment, the transgene comprises a non-immunoglobulin promoter that drives expression of the light chain sequences. In a specific

embodiment, the promoter is a transcriptionally active promoter. In a specific embodiment, the promoter is a ROSA26 promoter.

[00096] In one aspect, a nucleic acid construct is provided, comprising an upstream homology arm and a downstream homology arm, wherein the upstream homology arm comprises a sequence that is identical or substantially identical to a human immunoglobulin heavy chain variable region sequence, the downstream homology arm comprises a sequence that is identical or substantially identical to a human or mouse immunoglobulin variable region sequence, and disposed between the upstream and downstream homology arms is a sequence that comprises a nucleotide sequence encoding a mouse ADAM6 protein. In a specific embodiment, the sequence encoding the mouse ADAM6 gene is operably linked with a mouse promoter with which the mouse ADAM6 is linked in a wild type mouse.

[00097] In one aspect, a targeting vector is provided, comprising (a) a nucleotide sequence that is identical or substantially identical to a human variable region gene segment nucleotide sequence; and, (b) a nucleotide sequence encoding a mouse ADAM6 or ortholog or homolog or fragment thereof that is functional in a mouse.

[00098] In one embodiment, the targeting vector further comprises a promoter operably linked to the sequence encoding the mouse ADAM6. In a specific embodiment, the promoter is a mouse ADAM6 promoter.

[00099] In one aspect, a nucleotide construct for modifying a mouse immunoglobulin heavy chain variable locus is provided, wherein the construct comprises at least one site-specific recombinase recognition site and a sequence encoding an ADAM6 protein or ortholog or homolog or fragment thereof that is functional in a mouse.

[000100] In one aspect, mouse cells and mouse embryos are provided, including but not limited to ES cells, pluripotent cells, and induced pluripotent cells, that comprise genetic modifications as described herein. Cells that are XX and cells that are XY are provided. Cells that comprise a nucleus containing a modification as described herein are also provided, *e.g.,* a modification introduced into a cell by pronuclear injection. Cells, embryos, and mice that comprise a virally introduced ADAM6 gene are also provided, *e.g.,* cells, embryos, and mice comprising a transduction construct comprising an ADAM6 gene that is functional in the mouse.

[000101] In one aspect, a genetically modified mouse cell is provided, wherein the cell lacks a functional endogenous mouse ADAM6 locus, and the cell comprises an ectopic nucleotide sequence that encodes a mouse ADAM6 protein or functional fragment thereof. In one embodiment, the cell further comprises a modification of an endogenous immunoglobulin heavy chain variable gene sequence. In a specific embodiment, the modification of the endogenous immunoglobulin heavy chain variable gene sequence

comprises a deletion selected from a deletion of a mouse V_H gene segment, a deletion of a mouse D_H gene segment, a deletion of a mouse J_H gene segment, and a combination thereof. In a specific embodiment, the mouse comprises a replacement of one or more mouse immunoglobulin V_H, D_H, and/or J_H sequences with a human immunoglobulin sequence. In a specific embodiment, the human immunoglobulin sequence is selected from a human V_H, a human V_L, a human D_H, a human J_H, a human J_L, and a combination thereof.

[000102] In one embodiment, the cell is a totipotent cell, a pluripotent cell, or an induced pluripotent cell. In a specific embodiment, the cell is a mouse ES cell.

[000103] In one aspect, a mouse B cell is provided, wherein the mouse B cell comprises a rearranged immunoglobulin heavy chain gene, wherein the B cell comprises on a chromosome of the B cell a nucleic acid sequence encoding an ADAM6 protein or ortholog or homolog or fragment thereof that is functional in a male mouse. In one embodiment, the mouse B cell comprises two alleles of the nucleic acid sequence.

[000104] In one embodiment, the nucleic acid sequence is on a nucleic acid molecule (*e.g.*, a B cell chromosome) that is contiguous with the rearranged mouse immunoglobulin heavy chain locus.

[000105] In one embodiment, the nucleic acid sequence is on a nucleic acid molecule (*e.g.*, a B cell chromosome) that is distinct from the nucleic acid molecule that comprises the rearranged mouse immunoglobulin heavy chain locus.

[000106] In one embodiment, the mouse B cell comprises a rearranged non-mouse immunoglobulin variable gene sequence operably linked to a mouse or human immunoglobulin constant region gene, wherein the B cell comprises a nucleic acid sequence that encodes an ADAM6 protein or ortholog or homolog or fragment thereof that is functional in a male mouse.

[000107] In one aspect, a somatic mouse cell is provided, comprising a chromosome that comprises a modified immunoglobulin heavy chain locus, and a nucleic acid sequence encoding a mouse ADAM6 or ortholog or homolog or fragment thereof that is functional in a male mouse. In one embodiment, the nucleic acid sequence is on the same chromosome as the modified immunoglobulin heavy chain locus. In one embodiment, the nucleic acid is on a different chromosome than the modified immunoglobulin heavy chain locus. In one embodiment, the somatic cell comprises a single copy of the nucleic acid sequence. In one embodiment, the somatic cell comprises at least two copies of the nucleic acid sequence. In a specific embodiment, the somatic cell is a B cell. In a specific embodiment, the cell is a germ cell. In a specific embodiment, the cell is a stem cell.

[000108] In one aspect, a mouse germ cell is provided, comprising a nucleic acid sequence encoding a mouse ADAM6 (or homolog or ortholog or functional fragment

thereof) on a chromosome of the germ cell, wherein the nucleic acid sequence encoding the mouse ADAM6 (or homolog or ortholog or functional fragment thereof) is at a position in the chromosome that is different from a position in a chromosome of a wild-type mouse germ cell. In one embodiment, the nucleic acid sequence is at a mouse immunoglobulin locus. In one embodiment, the nucleic acid sequence is on the same chromosome of the germ cell as a mouse immunoglobulin locus. In one embodiment, the nucleic acid sequence is on a different chromosome of the germ cell than the mouse immunoglobulin locus. In one embodiment, the mouse immunoglobulin locus comprises a replacement of at least one mouse immunoglobulin sequence with at least one non-mouse immunoglobulin sequence. In a specific embodiment, the at least one non-mouse immunoglobulin sequence is a human immunoglobulin sequence.

[000109] In one aspect, a pluripotent, induced pluripotent, or totipotent cell derived from a mouse as described herein is provided. In a specific embodiment, the cell is a mouse embryonic stem (ES) cell.

[000110] In one aspect, a tissue derived from a mouse as described herein is provided. In one embodiment, the tissue is derived from spleen, lymph node or bone marrow of a mouse as described herein.

[000111] In one aspect, a nucleus derived from a mouse as described herein is provided. In one embodiment, the nucleus is from a diploid cell that is not a B cell.

[000112] In one aspect, a nucleotide sequence encoding an immunoglobulin variable region made in a mouse as described herein is provided.

[000113] In one aspect, an immunoglobulin heavy chain or immunoglobulin light chain variable region amino acid sequence of an antibody made in a mouse as described herein is provided.

[000114] In one aspect, an immunoglobulin heavy chain or immunoglobulin light chain variable region nucleotide sequence encoding a variable region of an antibody made in a mouse as described herein is provided.

[000115] In one aspect, an antibody or antigen-binding fragment thereof (*e.g.*, Fab, $F(ab)_2$, scFv) made in a mouse as described herein is provided. In one aspect, a method for making a genetically modified mouse is provided, comprising replacing one or more immunoglobulin heavy chain gene segments upstream (with respect to transcription of the immunoglobulin heavy chain gene segments) of an endogenous ADAM6 locus of the mouse with one or more human immunoglobulin heavy chain gene segments, and replacing one or more immunoglobulin gene segments downstream (with respect to transcription of the immunoglobulin heavy chain gene segments) of the ADAM6 locus of the mouse with one or more human immunoglobulin heavy chain or light chain gene segments. In one embodiment, the one or more human immunoglobulin gene segments

replacing one or more endogenous immunoglobulin gene segments upstream of an endogenous ADAM6 locus of the mouse include V gene segments. In one embodiment, the human immunoglobulin gene segments replacing one or more endogenous immunoglobulin gene segments upstream of an endogenous ADAM6 locus of the mouse include V and D gene segments. In one embodiment, the one or more human immunoglobulin gene segments replacing one or more endogenous immunoglobulin gene segments downstream of an endogenous ADAM6 locus of the mouse include J gene segments. In one embodiment, the one or more human immunoglobulin gene segments replacing one or more endogenous immunoglobulin gene segments downstream of an endogenous ADAM6 locus of the mouse include D and J gene segments. In one embodiment, the one or more human immunoglobulin gene segments replacing one or more endogenous immunoglobulin gene segments downstream of an endogenous ADAM6 locus of the mouse include V, D and J gene segments.

[000116] In one embodiment, the one or more immunoglobulin heavy chain gene segments upstream and/or downstream of the ADAM6 gene are replaced in a pluripotent, induced pluripotent, or totipotent cell to form a genetically modified progenitor cell; the genetically modified progenitor cell is introduced into a host; and, the host comprising the genetically modified progenitor cell is gestated to form a mouse comprising a genome derived from the genetically modified progenitor cell. In one embodiment, the host is an embryo. In a specific embodiment, the host is selected from a mouse pre-morula (*e.g.,* 8- or 4-cell stage), a tetraploid embryo, an aggregate of embryonic cells, or a blastocyst.

[000117] In one aspect, a method for making a genetically modified mouse is provided, comprising replacing a mouse nucleotide sequence that comprises a mouse immunoglobulin gene segment and a mouse ADAM6 (or ortholog or homolog or fragment thereof functional in a male mouse) nucleotide sequence with a sequence comprising a human immunoglobulin gene segment to form a first chimeric locus, then inserting a sequence comprising a mouse ADAM6-encoding sequence (or a sequence encoding an ortholog or homolog or functional fragment thereof) into the sequence comprising the human immunoglobulin gene segment to form a second chimeric locus.

[000118] In one embodiment, the second chimeric locus comprises a human immunoglobulin heavy chain variable (V_H) gene segment. In one embodiment, the second chimeric locus comprises a human immunoglobulin light chain variable (V_L) gene segment. In a specific embodiment, the second chimeric locus comprises a human V_H gene segment or a human V_L gene segment operably linked to a human D_H gene segment and a human J_H gene segment. In a further specific embodiment, the second chimeric locus is operably linked to a third chimeric locus that comprises a human C_H1 sequence, or a human C_H1 and human hinge sequence, fused with a mouse C_H2 + C_H3 sequence.

[000119] In one aspect, use of a mouse that comprises an ectopic nucleotide sequence comprising a mouse ADAM6 locus or sequence to make a fertile male mouse is provided, wherein the use comprises mating the mouse comprising the ectopic nucleotide sequence that comprises the mouse ADAM6 locus or sequence to a mouse that lacks a functional endogenous mouse ADAM6 locus or sequence, and obtaining a progeny that is a female capable of producing progeny having the ectopic ADAM6 locus or sequence or that is a male that comprises the ectopic ADAM6 locus or sequence, and the male exhibits a fertility that is approximately the same as a fertility exhibited by a wild-type male mouse.

[000120] In one aspect, use of a mouse as described herein to make an immunoglobulin variable region nucleotide sequence is provided.

[000121] In one aspect, use of a mouse as described herein to make a fully human Fab or a fully human $F(ab)_2$ is provided.

[000122] In one aspect, use of a mouse as described herein to make an immortalized cell line is provided.

[000123] In one aspect, use of a mouse as described herein to make a hybridoma or quadroma is provided.

[000124] In one aspect, use of a mouse as described herein to make a phage library containing human heavy chain variable regions and human light chain variable regions is provided.

[000125] In one aspect, use of a mouse as described herein to generate a variable region sequence for making a human antibody is provided, comprising (a) immunizing a mouse as described herein with an antigen of interest, (b) isolating a lymphocyte from the immunized mouse of (a), (c) exposing the lymphocyte to one or more labeled antibodies, (d) identifying a lymphocyte that is capable of binding to the antigen of interest, and (e) amplifying one or more variable region nucleic acid sequence from the lymphocyte thereby generating a variable region sequence.

[000126] In one embodiment, the lymphocyte is derived from the spleen of the mouse. In one embodiment, the lymphocyte is derived from a lymph node of the mouse. In one embodiment, the lymphocyte is derived from the bone marrow of the mouse.

[000127] In one embodiment, the labeled antibody is a fluorophore-conjugated antibody. In one embodiment, the one or more fluorophore-conjugated antibodies are selected from an IgM, an IgG, and/or a combination thereof.

[000128] In one embodiment, the lymphocyte is a B cell.

[000129] In one embodiment, the one or more variable region nucleic acid sequence comprises a heavy chain variable region sequence. In one embodiment, the one or more variable region nucleic acid sequence comprises a light chain variable region sequence. In a specific embodiment, the light chain variable region sequence is an immunoglobulin κ

light chain variable region sequence. In one embodiment, the one or more variable region nucleic acid sequence comprises a heavy chain and a κ light chain variable region sequence.

[000130] In one embodiment, use of a mouse as described herein to generate a heavy and a κ light chain variable region sequence for making a human antibody is provided, comprising (a) immunizing a mouse as described herein with an antigen of interest, (b) isolating the spleen from the immunized mouse of (a), (c) exposing B lymphocytes from the spleen to one or more labeled antibodies, (d) identifying a B lymphocyte of (c) that is capable of binding to the antigen of interest, and (e) amplifying a heavy chain variable region nucleic acid sequence and a κ light chain variable region nucleic acid sequence from the B lymphocyte thereby generating the heavy chain and κ light chain variable region sequences.

[000131] In one embodiment, use of a mouse as described herein to generate a heavy and a κ light chain variable region sequence for making a human antibody is provided, comprising (a) immunizing a mouse as described herein with an antigen of interest, (b) isolating one or more lymph nodes from the immunized mouse of (a), (c) exposing B lymphocytes from the one or more lymph nodes to one or more labeled antibodies, (d) identifying a B lymphocyte of (c) that is capable of binding to the antigen of interest, and (e) amplifying a heavy chain variable region nucleic acid sequence and a κ light chain variable region nucleic acid sequence from the B lymphocyte thereby generating the heavy chain and κ light chain variable region sequences.

[000132] In one embodiment, use of a mouse as described herein to generate a heavy and a κ light chain variable region sequence for making a human antibody is provided, comprising (a) immunizing a mouse as described herein with an antigen of interest, (b) isolating bone marrow from the immunized mouse of (a), (c) exposing B lymphocytes from the bone marrow to one or more labeled antibodies, (d) identifying a B lymphocyte of (c) that is capable of binding to the antigen of interest, and (e) amplifying a heavy chain variable region nucleic acid sequence and a κ light chain variable region nucleic acid sequence from the B lymphocyte thereby generating the heavy chain and κ light chain variable region sequences. In various embodiments, the one or more labeled antibodies are selected from an IgM, an IgG, and/or a combination thereof.

[000133] In various embodiments, use of a mouse as described herein to generate a heavy and κ light chain variable region sequence for making a human antibody is provided, further comprising fusing the amplified heavy and light chain variable region sequences to human heavy and light chain constant region sequences, expressing the fused heavy and light chain sequences in a cell, and recovering the expressed heavy and light chain

sequences thereby generating a human antibody.

[000134] In various embodiments, the human heavy chain constant regions are selected from IgM, IgD, IgA, IgE and IgG. In various specific embodiments, the IgG is selected from an IgG1, an IgG2, an IgG3 and an IgG4. In various embodiments, the human heavy chain constant region comprises a C_H1, a hinge, a C_H2, a C_H3, a C_H4, or a combination thereof. In various embodiments, the light chain constant region is an immunoglobulin κ constant region. In various embodiments, the cell is selected from a HeLa cell, a DU145 cell, a Lncap cell, a MCF-7 cell, a MDA-MB-438 cell, a PC3 cell, a T47D cell, a THP-1 cell, a U87 cell, a SHSY5Y (human neuroblastoma) cell, a Saos-2 cell, a Vero cell, a CHO cell, a GH3 cell, a PC12 cell, a human retinal cell (*e.g.*, a PER.C6™ cell), and a MC3T3 cell. In a specific embodiment, the cell is a CHO cell.

[000135] In one aspect, a method for generating a reverse-chimeric rodent-human antibody specific against an antigen of interest is provided, comprising the steps of immunizing a mouse as described herein with the antigen, isolating at least one cell from the mouse producing a reverse-chimeric mouse-human antibody specific against the antigen, culturing at least one cell producing the reverse-chimeric mouse-human antibody specific against the antigen, and obtaining said antibody.

[000136] In one embodiment, the reverse-chimeric mouse-human antibody comprises a human heavy chain variable domain fused with a mouse or rat heavy chain constant gene, and a human light chain variable domain fused with a mouse or rat or human light chain constant gene.

[000137] In one embodiment, culturing at least one cell producing the reverse-chimeric rodent-human antibody specific against the antigen is performed on at least one hybridoma cell generated from the at least one cell isolated from the mouse.

[000138] In one aspect, a method for generating a fully human antibody specific against an antigen of interest is provided, comprising the steps of immunizing a mouse as described herein with the antigen, isolating at least one cell from the mouse producing a reverse-chimeric rodent-human antibody specific against the antigen, generating at least one cell producing a fully human antibody derived from the reverse-chimeric rodent-human antibody specific against the antigen, and culturing at least one cell producing the fully human antibody, and obtaining said fully human antibody.

[000139] In various embodiments, the at least one cell isolated from the mouse producing a reverse-chimeric rodent-human antibody specific against the antigen is a splenocyte or a B cell.

[000140] In various embodiments, the antibody is a monoclonal antibody.

[000141] In various embodiments, immunization with the antigen of interest is carried out with protein, DNA, a combination of DNA and protein, or cells expressing the antigen.

[000142] In one aspect, use of a mouse as described herein to make a nucleic acid sequence encoding an immunoglobulin variable region or fragment thereof is provided. In one embodiment, the nucleic acid sequence is used to make a human antibody or antigen-binding fragment thereof. In one embodiment, the mouse is used to make an antigen-binding protein selected from an antibody, a multi-specific antibody (*e.g.*, a bi-specific antibody), an scFv, a bi-specific scFv, a diabody, a triabody, a tetrabody, a V-NAR, a V_{HH}, a V_L, a F(ab), a $F(ab)_2$, a DVD (*i.e.*, dual variable domain antigen-binding protein), a an SVD (*i.e.*, single variable domain antigen-binding protein), or a bispecific T-cell engager (BiTE).

[000143] In one aspect, use of a mouse as described herein to introduce an ectopic ADAM6 sequence into a mouse that lacks a functional endogenous mouse ADAM6 sequence is provided, wherein the use comprises mating a mouse as described herein with the mouse that lacks the functional endogenous mouse ADAM6 sequence.

[000144] In one aspect, use of genetic material from a mouse as described herein to make a mouse having an ectopic ADAM6 sequence is provided. In one embodiment, the *use* comprises nuclear transfer using a nucleus of a cell of a mouse as described herein. In one embodiment, the use comprises cloning a cell of a mouse as described herein to produce an animal derived from the cell. In one embodiment, the use comprises employing a sperm or an egg of a mouse as described herein in a process for making a mouse comprising the ectopic ADAM6 sequence.

[000145] In one aspect, a method for making a fertile male mouse comprising a modified immunoglobulin heavy chain locus is provided, comprising fertilizing a first mouse germ cell that comprises a modification of an endogenous immunoglobulin heavy chain locus with a second mouse germ cell that comprises an ADAM6 gene or ortholog or homolog or fragment thereof that is functional in a male mouse; forming a fertilized cell; allowing the fertilized cell to develop into an embryo; and, gestating the embryo in a surrogate to obtain a mouse.

[000146] In one embodiment, the fertilization is achieved by mating a male mouse and a female mouse. In one embodiment, the female mouse comprises the ADAM6 gene or ortholog or homolog or fragment thereof. In one embodiment, the male mouse comprises the ADAM6 gene or ortholog or homolog or fragment thereof.

[000147] In one aspect, use of a nucleic acid sequence encoding a mouse ADAM6 protein or an ortholog or homolog thereof or a functional fragment of the corresponding ADAM6 protein for restoring or enhancing the fertility of a mouse having a genome comprising a modification of an immunoglobulin heavy chain locus is provided, wherein the modification reduces or eliminates endogenous ADAM6 function.

[000148] In one embodiment, the nucleic acid sequence is integrated into the genome of

the mouse at an ectopic position. In one embodiment, the nucleic acid sequence is integrated into the genome of the mouse at an endogenous immunoglobulin locus. In a specific embodiment, the endogenous immunoglobulin locus is a heavy chain locus. In one embodiment, the nucleic acid sequence is integrated into the genome of the mouse at a position other than an endogenous immunoglobulin locus.

[000149] In one aspect, use of the mouse as described herein for the manufacture of a medicament (*e.g.*, an antigen-binding protein), or for the manufacture of a sequence encoding a variable sequence of a medicament (*e.g.,* an antigen-binding protein), for the treatment of a human disease or disorder is provided.

BRIEF DESCRIPTION OF FIGURES

[000150] **FIG. 1A** shows a general illustration, not to scale, of direct genomic replacement of about three megabases (Mb) of a mouse immunoglobulin heavy chain variable gene locus (closed symbols) with about one megabase (Mb) of the human immunoglobulin heavy chain variable gene locus (open symbols).

[000151] **FIG. 1B** shows a general illustration, not to scale, of direct genomic replacement of about three megabases (Mb) of a mouse immunoglobulin κ light chain variable gene locus (closed symbols) with about 0.5 megabases (Mb) of the first, or proximal, of two nearly identical repeats of a human immunoglobulin κ light chain variable gene locus (open symbols).

[000152] **FIG. 2A** shows a detailed illustration, not to scale, of three initial steps (A–C) for direct genomic replacement of a mouse immunoglobulin heavy chain variable gene locus that results in deletion of all mouse V_H, D_H and J_H gene segments and replacement with three human V_H, all human D_H and J_H gene segments. A targeting vector for a first insertion of human immunoglobulin heavy chain gene segments is shown (3hV_H BACvec) with a 67 kb 5' mouse homology arm, a selection cassette (open rectangle), a site-specific recombination site (open triangle), a 145 kb human genomic fragment and an 8 kb 3' mouse homology arm. Human (open symbols) and mouse (closed symbols) immunoglobulin gene segments, additional selection cassettes (open rectangles) and site-specific recombination sites (open triangles) inserted from subsequent targeting vectors are shown.

[000153] **FIG. 2B** shows a detailed illustration, not to scale, of six additional steps (D–I) for direct genomic replacement of a mouse immunoglobulin heavy chain variable gene locus that results in the insertion of 77 additional human V_H gene segments and removal of a final selection cassette. A targeting vector for insertion of additional human V_H gene segments (18hV_H BACvec) to the initial insertion of human heavy chain gene segments

(3hV_H-CRE Hybrid Allele) is shown with a 20 kb 5' mouse homology arm, a selection cassette (open rectangle), a 196 kb human genomic fragment and a 62 kb human homology arm that overlaps with the 5' end of the initial insertion of human heavy chain gene segments which is shown with a site-specific recombination site (open triangle) located 5' to the human gene segments. Human (open symbols) and mouse (closed symbols) immunoglobulin gene segments and additional selection cassettes (open rectangles) inserted by subsequent targeting vectors are shown.

[000154] **FIG. 2C** shows a detailed illustration, not to scale, of three initial steps (A–C) for direct genomic replacement of a mouse immunoglobulin κ light chain variable gene locus that results in deletion of all mouse Vκ, and Jκ gene segments (Igκ-CRE Hybrid Allele). Selection cassettes (open rectangles) and site-specific recombination sites (open triangles) inserted from the targeting vectors are shown.

[000155] **FIG. 2D** shows a detailed illustration, not to scale, of five additional steps (D–H) for direct genomic replacement of a mouse immunoglobulin κ light chain variable gene locus that results in the insertion of all human Vκ and Jκ gene segments of the proximal repeat and deletion of a final selection cassette (40hVκdHyg Hybrid Allele). Human (open symbols) and mouse (closed symbols) immunoglobulin gene segments and additional selection cassettes (open rectangles) inserted by subsequent targeting vectors are shown.

[000156] **FIG. 3A** shows a general illustration, not to scale, of a screening strategy including the locations of quantitative PCR (qPCR) primer/probe sets to detect insertion of human heavy chain gene sequences and loss of mouse heavy chain gene sequences in targeted embryonic stem (ES) cells. The screening strategy in ES cells and mice for a first human heavy gene insertion is shown with qPCR primer/probe sets for the deleted region ("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 (top) and a correctly targeted chromosome (bottom).

[000157] **FIG. 3B** shows a representative calculation of observed probe copy number in parental and modified ES cells for a first insertion of human immunoglobulin heavy chain gene segments. Observed probe copy number for probes A through F were calculated as $2/2^{\Delta\Delta Ct}$. ΔΔCt is calculated as ave[ΔCt(sample) – medΔCt(control)] where ΔCt is the difference in Ct between test and reference probes (between 4 and 6 reference probes depending on the assay). The term medΔCt(control) is the median ΔCt of multiple (>60) non-targeted DNA samples from parental ES cells. Each modified ES cell clone was assayed in sextuplicate. To calculate copy numbers of IgH probes G and H in parental ES cells, these probes were assumed to have copy number of 1 in modified ES cells and a maximum Ct of 35 was used even though no amplification was observed.

[000158] **FIG. 3C** shows a representative calculation of copy numbers for four mice of each genotype calculated using only probes D and H. Wild-type mice: WT Mice; Mice heterozygous for a first insertion of human immunoglobulin gene segments: HET Mice; Mice homozygous for a first insertion of human immunoglobulin gene segments: Homo Mice.

[000159] **FIG. 4A** shows a detailed illustration, not to scale, of the three steps employed for construction of a $3hV_H$ BACvec by bacterial homologous recombination (BHR). Human (open symbols) and mouse (closed symbols) immunoglobulin gene segments, selection cassettes (open rectangles) and site-specific recombination sites (open triangles) inserted from targeting vectors are shown.

[000160] **FIG. 4B** shows pulse-field gel electrophoresis (PFGE) of three BAC clones (B1, B2 and B3) after NotI digestion. Markers M1, M2 and M3 are low range, mid range and lambda ladder PFG markers, respectively (New England BioLabs, Ipswich, MA).

[000161] **FIG. 5A** shows a schematic illustration, not to scale, of sequential modifications of a mouse immunoglobulin heavy chain locus with increasing amounts of human immunoglobulin heavy chain gene segments. Homozygous mice were made from each of the three different stages of heavy chain humanization. Open symbols indicate human sequence; closed symbols indicate mouse sequence.

[000162] **FIG. 5B** shows a schematic illustration, not to scale, of sequential modifications of a mouse immunoglobulin κ light chain locus with increasing amounts of human immunoglobulin κ light chain gene segments. Homozygous mice were made from each of the three different stages of κ light chain humanization. Open symbols indicate human sequence; closed symbols indicate mouse sequence.

[000163] **FIG. 6** shows FACS dot plots of B cell populations in wild type and VELOCIMMUNE® humanized mice. Cells from spleen (top row, third row from top and bottom row) or inguinal lymph node (second row from top) of wild type (wt), VELOCIMMUNE® 1 (V1), VELOCIMMUNE® 2 (V2) or VELOCIMMUNE® 3 (V3) mice were stained for surface IgM expressing B cells (top row, and second row from top), surface immunoglobulin containing either κ or λ light chains (third row from top) or surface IgM of specific haplotypes (bottom row), and populations separated by FACS.

[000164] **FIG. 7A** shows representative heavy chain CDR3 sequences of randomly selected VELOCIMMUNE® antibodies around the V_H-D_H-J_H (CDR3) junction, demonstrating junctional diversity and nucleotide additions. Heavy chain CDR3 sequences are grouped according to D_H gene segment usage, the germline of which is provided above each group in bold. V_H gene segments for each heavy chain CDR3 sequence are noted within parenthesis at the 5' end of each sequence (*e.g.,* 3-72 is human V_H3-72). J_H

gene segments for each heavy chain CDR3 are noted within parenthesis at the 3' end of each sequence (*e.g.,* 3 is human J_H3). SEQ ID NOs for each sequence shown are as follows proceeding from top to bottom: SEQ ID NO:21; SEQ ID NO:22; SEQ ID NO:23; SEQ ID NO:24; SEQ ID NO:25; SEQ ID NO:26; SEQ ID NO:27; SEQ ID NO:28; SEQ ID NO:29; SEQ ID NO:30; SEQ ID NO:31; SEQ ID NO:32; SEQ ID NO:33; SEQ ID NO:34; SEQ ID NO:35; SEQ ID NO:36; SEQ ID NO:37; SEQ ID NO:38; SEQ ID NO:39.

[000165] FIG. 7B shows representative light chain CDR3 sequences of randomly selected VELOCIMMUNE® antibodies around the Vκ-Jκ (CDR3) junction, demonstrating junctional diversity and nucleotide additions. Vκ gene segments for each light chain CDR3 sequence are noted within parenthesis at the 5' end of each sequence (*e.g.,* 1-6 is human Vκ1-6). Jκ gene segments for each light chain CDR3 are noted within parenthesis at the 3' end of each sequence (*e.g.,* 1 is human Jκ1). SEQ ID NOs for each sequence shown are as follows proceeding from top to bottom: SEQ ID NO:40; SEQ ID NO:41; SEQ ID NO:42; SEQ ID NO:43; SEQ ID NO:44; SEQ ID NO:45; SEQ ID NO:46; SEQ ID NO:47; SEQ ID NO:48; SEQ ID NO:49; SEQ ID NO:50; SEQ ID NO:51; SEQ ID NO:52; SEQ ID NO:53; SEQ ID NO:54; SEQ ID NO:55; SEQ ID NO:56; SEQ ID NO:57; SEQ ID NO:58.

[000166] FIG. 8 shows somatic hypermutation frequencies of heavy and light chains of VELOCIMMUNE® antibodies scored (after alignment to matching germline sequences) as percent of sequences changed at each nucleotide (NT; left column) or amino acid (AA; right column) position among sets of 38 (unimmunized IgM), 28 (unimmunized IgG), 32 (unimmunized Igκ from IgG), 36 (immunized IgG) or 36 (immunized Igκ from IgG) sequences. Shaded bars indicate the locations of CDRs.

[000167] FIG. 9A shows levels of serum immunoglobulin for IgM and IgG isotypes in wild type (open bars) or VELOCIMMUNE® mice (closed bars).

[000168] FIG. 9B shows levels of serum immunoglobulin for IgA isotype in wild type (open bars) or VELOCIMMUNE® mice (closed bars).

[000169] FIG. 9C shows levels of serum immunoglobulin for IgE isotype in wild type (open bars) or VELOCIMMUNE® mice (closed bars).

[000170] FIG. 10A shows antigen-specific IgG titers against interleukin-6 receptor (IL-6R) of serum from seven VELOCIMMUNE® (VI) and five wild type (WT) mice after two (bleed 1) or three (bleed 2) rounds of immunization with the ectodomain of IL-6R.

[000171] FIG. 10B shows anti-IL-6R-specific IgG isotype-specific titers from seven VELOCIMMUNE® (VI) and five wild type (WT) mice.

[000172] FIG. 11A shows the affinity distribution of anti-interleukin-6 receptor monoclonal antibodies generated in VELOCIMMUNE® mice.

[000173] FIG. 11B shows the antigen-specific blocking of anti-interleukin-6 receptor

monoclonal antibodies generated in VELOCIMMUNE® (VI) and wild type (WT) mice.

[000174] **FIG. 12** shows a schematic illustration, not to scale, of mouse ADAM6a and ADAM6b genes in a mouse immunoglobulin heavy chain locus. A targeting vector (mADAM6 Targeting Vector) used for insertion of mouse ADAM6a and ADAM6b into a humanized endogenous heavy chain locus is shown with a selection cassette (HYG: hygromycin) flanked by site-specific recombination sites (Frt) including engineered restriction sites on the 5' and 3' ends.

[000175] **FIG. 13** shows a schematic illustration, not to scale, of a human ADAM6 pseudogene (hADAM6Ψ) located between human heavy chain variable gene segments 1-2 (V_H1-2) and 6-1 (V_H6-1). A targeting vector for bacterial homologous recombination (hADAM6Ψ Targeting Vector) to delete a human ADAM6 pseudogene and insert unique restriction sites into a human heavy chain locus is shown with a selection cassette (NEO: neomycin) flanked by site-specific recombination sites (loxP) including engineered restriction sites on the 5' and 3' ends. An illustration, not to scale, of the resulting targeted humanized heavy chain locus containing a genomic fragment that encodes for the mouse ADAM6a and ADAM6b genes including a selection cassette flanked by site-specific recombination sites is shown.

[000176] **FIG. 14A** shows FACS contour plots of lymphocytes gated on singlets for surface expression of IgM and B220 in the bone marrow for mice homozygous for human heavy and human κ light chain variable gene loci ($H^{+/+}\kappa^{+/+}$) and mice homozygous for human heavy and human κ light chain variable gene loci having an ectopic mouse genomic fragment encoding mouse ADAM6 genes ($H^{+/+}A6^{res}\kappa^{+/+}$). Percentage of immature ($B220^{int}IgM^{+}$) and mature ($B220^{high}IgM^{+}$) B cells is noted in each contour plot.

[000177] **FIG. 14B** shows the total number of immature ($B220^{int}IgM^{+}$) and mature ($B220^{high}IgM^{+}$) B cells in the bone marrow isolated from femurs of mice homozygous for human heavy and human κ light chain variable gene loci ($H^{+/+}\kappa^{+/+}$) and mice homozygous for human heavy and human κ light chain variable gene loci having an ectopic mouse genomic fragment encoding mouse ADAM6 genes ($H^{+/+}A6^{res}\kappa^{+/+}$).

[000178] **FIG. 15A** shows FACS contour plots of $CD19^{+}$-gated B cells for surface expression of c-kit and CD43 in the bone marrow for mice homozygous for human heavy and human κ light chain variable gene loci ($H^{+/+}\kappa^{+/+}$) and mice homozygous for human heavy and human κ light chain variable gene loci having an ectopic mouse genomic fragment encoding mouse ADAM6 genes ($H^{+/+}A6^{res}\kappa^{+/+}$). Percentage of pro-B ($CD19^{+}CD43^{+}ckit^{+}$) and pre-B ($CD19^{+}CD43^{-}ckit^{-}$) cells is noted in the upper right and lower left quadrants, respectively, of each contour plot.

[000179] **FIG. 15B** shows the total number of pro-B cells ($CD19^{+}CD43^{+}ckit^{+}$) and pre-B

cells ($CD19^+CD43^-ckit^-$) in the bone marrow isolated from femurs of mice homozygous for human heavy and human κ light chain variable gene loci ($H^{+/+}\kappa^{+/+}$) and mice homozygous for human heavy and human κ light chain variable gene loci having an ectopic mouse genomic fragment encoding mouse ADAM6 genes ($H^{+/+}A6^{res}\kappa^{+/+}$).

[000180] **FIG. 16A** shows FACS contour plots of lymphocytes gated on singlets for surface expression of CD19 and CD43 in the bone marrow for mice homozygous for human heavy and human κ light chain variable gene loci ($H^{+/+}\kappa^{+/+}$) and mice homozygous for human heavy and human κ light chain variable gene loci having an ectopic mouse genomic fragment encoding mouse ADAM6 genes ($H^{+/+}A6^{res}\kappa^{+/+}$). Percentage of immature B ($CD19^+CD43^-$), pre-B ($CD19^+CD43^{int}$) and pro-B ($CD19^+CD43^+$) cells is noted in each contour plot.

[000181] **FIG. 16B** shows histograms of immature B ($CD19^+CD43^-$) and pre-B ($CD19^+CD43^{int}$) cells in the bone marrow of mice homozygous for human heavy and human κ light chain variable gene loci ($H^{+/+}\kappa^{+/+}$) and mice homozygous for human heavy and human κ light chain variable gene loci having an ectopic mouse genomic fragment encoding mouse ADAM6 genes ($H^{+/+}A6^{res}\kappa^{+/+}$).

[000182] **FIG. 17A** shows FACS contour plots of lymphocytes gated on singlets for surface expression of CD19 and CD3 in splenocytes for mice homozygous for human heavy and human κ light chain variable gene loci ($H^{+/+}\kappa^{+/+}$) and mice homozygous for human heavy and human κ light chain variable gene loci having an ectopic mouse genomic fragment encoding mouse ADAM6 genes ($H^{+/+}A6^{res}\kappa^{+/+}$). Percentage of B ($CD19^+CD3^-$) and T ($CD19^-CD3^+$) cells is noted in each contour plot.

[000183] **FIG. 17B** shows FACs contour plots for $CD19^+$-gated B cells for surface expression of Igλ and Igκ light chain in the spleen of mice homozygous for human heavy and human κ light chain variable gene loci ($H^{+/+}\kappa^{+/+}$) and mice homozygous for human heavy and human κ light chain variable gene loci having an ectopic mouse genomic fragment encoding mouse ADAM6 genes ($H^{+/+}A6^{res}\kappa^{+/+}$). Percentage of $Ig\lambda^+$ (upper left quadrant) and $Ig\kappa^+$ (lower right quadrant) B cells is noted in each contour plot.

[000184] **FIG. 17C** shows the total number of $CD19^+$ B cells in the spleen of mice homozygous for human heavy and human κ light chain variable gene loci ($H^{+/+}\kappa^{+/+}$) and mice homozygous for human heavy and human κ light chain variable gene loci having an ectopic mouse genomic fragment encoding mouse ADAM6 genes ($H^{+/+}A6^{res}\kappa^{+/+}$).

[000185] **FIG. 18A** shows FACs contour plots of $CD19^+$-gated B cells for surface expression of IgD and IgM in the spleen of mice homozygous for human heavy and human κ light chain variable gene loci ($H^{+/+}\kappa^{+/+}$) and mice homozygous for human heavy and

human κ light chain variable gene loci having an ectopic mouse genomic fragment encoding mouse ADAM6 genes ($H^{+/+}A6^{res}\kappa^{+/+}$). Percentage of mature B cells ($CD19^{+}IgD^{high}IgM^{int}$) is noted for each contour plot. The arrow on the right contour plot illustrates the process of maturation for B cells in relation to IgM and IgD surface expression.

[000186] **FIG. 18B** shows the total number of B cells in the spleen of mice homozygous for human heavy and human κ light chain variable gene loci ($H^{+/+}\kappa^{+/+}$) and mice homozygous for human heavy and human κ light chain variable gene loci having an ectopic mouse genomic fragment encoding mouse ADAM6 genes ($H^{+/+}A6^{res}\kappa^{+/+}$) during maturation from $CD19^{+}IgM^{high}IgD^{int}$ to $CD19^{+}IgM^{int}IgD^{high}$.

[000187] **FIG. 19** shows the antibody titer for first and second bleeds from mice homozygous for human heavy and human κ light chain variable gene loci ($H^{+/+}\kappa^{+/+}$; n=5) and mice homozygous for human heavy and human κ light chain variable gene loci having an ectopic mouse genomic fragment encoding mouse ADAM6 genes ($H^{+/+}A6^{res}\kappa^{+/+}$; n=5) that were immunized with a human cell surface receptor (Antigen A).

[000188] **FIG. 20** shows the antibody titer for first and second bleeds from mice homozygous for human heavy and human κ light chain variable gene loci ($H^{+/+}\kappa^{+/+}$; n=5) and mice homozygous for human heavy and human κ light chain variable gene loci having an ectopic mouse genomic fragment encoding mouse ADAM6 genes ($H^{+/+}A6^{res}\kappa^{+/+}$; n=10) that were immunized with a human antibody specific for a human receptor tyrosine-protein kinase (Antigen B).

[000189] **FIG. 21** shows the antibody titer for first and second bleeds from mice homozygous for human heavy and human κ light chain variable gene loci ($H^{+/+}\kappa^{+/+}$; n=12) and mice homozygous for human heavy and human κ light chain variable gene loci having an ectopic mouse genomic fragment encoding mouse ADAM6 genes ($H^{+/+}A6^{res}\kappa^{+/+}$; n=12) that were immunized with a secreted human protein that functions in regulation of the TGF-β signaling pathway (Antigen C).

[000190] **FIG. 22** shows the antibody titer for first and second bleeds from mice homozygous for human heavy and human κ light chain variable gene loci having an ectopic mouse genomic fragment encoding mouse ADAM6 genes ($H^{+/+}A6^{res}\kappa^{+/+}$; n=12) that were immunized with a human receptor tyrosine kinase (Antigen D).

DETAILED DESCRIPTION OF INVENTION

[000191] This invention is not limited to particular methods, and experimental conditions described, as such methods and conditions may vary. It is also to be understood that the

terminology used herein is for the purpose of describing particular embodiments only, and is not intended to be limiting, since the scope of the present invention is defined by the claims.

[000192] Unless defined otherwise, all terms and phrases used herein include the meanings that the terms and phrases have attained in the art, unless the contrary is clearly indicated or clearly apparent from the context in which the term or phrase is used. Although any methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, particular methods and materials are now described. All publications mentioned are hereby incorporated by reference.

[000193] The phrase "substantial" or "substantially" when used to refer to an amount of gene segments (*e.g.*, "substantially all" V gene segments) includes both functional and non functional gene segments and include, in various embodiments, *e.g.*, 80% or more, 85% or more, 90% or more, 95% or more 96% or more, 97% or more, 98% or more, or 99% or more of all gene segments; in various embodiments, "substantially all" gene segments includes, *e.g.*, at least 95%, 96%, 97%, 98%, or 99% of functional (*i.e.*, non-pseudogene) gene segments.

[000194] The term "replacement" includes wherein a DNA sequence is placed into a genome of a cell in such a way as to replace a sequence within the genome with a heterologous sequence (*e.g.*, a human sequence in a mouse), at the locus of the genomic sequence,. The DNA sequence so placed may include one or more regulatory sequences that are part of source DNA used to obtain the sequence so placed (*e.g.*, promoters, enhancers, 5'- or 3'-untranslated regions, appropriate recombination signal sequences, *etc.*). For example, in various embodiments, the replacement is a substitution of an endogenous sequence for a heterologous sequence that results in the production of a gene product from the DNA sequence so placed (comprising the heterologous sequence), but not expression of the endogenous sequence; the replacement is of an endogenous genomic sequence with a DNA sequence that encodes a protein that has a similar function as a protein encoded by the endogenous genomic sequence (*e.g.*, the endogenous genomic sequence encodes an immunoglobulin gene or domain, and the DNA fragment encodes one or more human immunoglobulin genes or domains). In various embodiments, an endogenous gene or fragment thereof is replaced with a corresponding human gene or fragment thereof. A corresponding human gene or fragment thereof is a human gene or fragment that is an ortholog of, a homolog of, or is substantially identical or the same in structure and/or function, as the endogenous gene or fragment thereof that is replaced.

[000195] The mouse as a genetic model has been greatly enhanced by transgenic and knockout technologies, which have allowed for the study of the effects of the directed over-

expression or deletion of specific genes. Despite all of its advantages, the mouse still presents genetic obstacles that render it an imperfect model for human diseases and an imperfect platform to test human therapeutics or make them. First, although about 99% of human genes have a mouse homolog (Waterston *et al.* 2002, Initial sequencing and comparative analysis of the mouse genome, *Nature* 420:520-562), potential therapeutics often fail to cross-react, or cross-react inadequately, with mouse orthologs of the intended human targets. To obviate this problem, selected target genes can be "humanized," that is, the mouse gene can be eliminated and replaced by the corresponding human orthologous gene sequence (*e.g.,* US 6,586,251, US 6,596,541 and US 7,105,348, incorporated herein by reference). Initially, efforts to humanize mouse genes by a "knockout-plus-transgenic humanization" strategy entailed crossing a mouse carrying a deletion (*i.e.*, knockout) of the endogenous gene with a mouse carrying a randomly integrated human transgene (see, *e.g.,* Bril *et al.,* 2006, Tolerance to factor VIII in a transgenic mouse expressing human factor VIII cDNA carrying an Arg(593) to Cys substitution, *Thromb Haemost* 95:341-347; Homanics *et al.,* 2006, Production and characterization of murine models of classic and intermediate maple syrup urine disease, BMC Med Genet 7:33; Jamsai *et al.,* 2006, A humanized BAC transgenic/knockout mouse model for HbE/beta-thalassemia, *Genomics* 88(3):309-15; Pan *et al.,* 2006, Different role for mouse and human CD3delta/epsilon heterodimer in preT cell receptor (preTCR) function:human CD3delta/epsilon heterodimer restores the defective preTCR function in CD3gamma- and CD3gammadelta-deficient mice, *Mol Immunol* 43:1741-1750). But those efforts were hampered by size limitations; conventional knockout technologies were not sufficient to directly replace large mouse genes with their large human genomic counterparts. A straightforward approach of direct homologous replacement, in which an endogenous mouse gene is directly replaced by the human counterpart gene at the same precise genetic location of the mouse gene (*i.e.,* at the endogenous mouse locus), is rarely attempted because of technical difficulties. Until now, efforts at direct replacement involved elaborate and burdensome procedures, thus limiting the length of genetic material that could be handled and the precision with which it could be manipulated.

[000196] Exogenously introduced human immunoglobulin transgenes rearrange in precursor B cells in mice (Alt *et al.,* 1985, Immunoglobulin genes in transgenic mice, *Trends Genet* 1:231-236). This finding was exploited by engineering mice using the knockout-plus-transgenic approach to express human antibodies (Green *et al.,* 1994, Antigen-specific human monoclonal antibodies from mice engineered with human Ig heavy and light chain YACs, *Nat Genet* 7:13-21; Lonberg *et al.,* 1994, Antigen-specific human antibodies from mice comprising four distinct genetic modifications, *Nature* 368:856-859; Jakobovits *et al.,* 2007, From XenoMouse technology to panitumumab, the first fully human

antibody product from transgenic mice, *Nat Biotechnol* 25:1134-1143). The mouse immunoglobulin heavy chain and κ light chain loci were inactivated in these mice by targeted deletion of small but critical portions of each endogenous locus, followed by introducing human immunoglobulin gene loci as randomly integrated large transgenes, as described above, or minichromosomes (Tomizuka *et al.,* 2000, Double trans-chromosomic mice: maintenance of two individual human chromosome fragments containing Ig heavy and kappa loci and expression of fully human antibodies, *PNAS USA* 97:722-727). Such mice represented an important advance in genetic engineering; fully human monoclonal antibodies isolated from them yielded promising therapeutic potential for treating a variety of human diseases (Gibson *et al.,* 2006, Randomized phase III trial results of panitumumab, a fully human anti-epidermal growth factor receptor monoclonal antibody, in metastatic colorectal cancer, *Clin Colorectal Cancer* 6:29-31; Jakobovits *et al.,* 2007; Kim *et al.,* 2007, Clinical efficacy of zanolimumab (HuMax-CD4): two Phase II studies in refractory cutaneous T-cell lymphoma, *Blood* 109(11):4655-62; Lonberg, 2005, Human antibodies from transgenic animals, *Nat Biotechnol* 23:1117-1125; Maker *et al.,* 2005, Tumor regression and autoimmunity in patients treated with cytotoxic T lymphocyte-associated antigen 4 blockade and interleukin 2: a phase I/II study, *Ann Surg Oncol* 12:1005-1016; McClung *et al.,* 2006, Denosumab in postmenopausal women with low bone mineral density, *New Engl J Med* 354:821-831). But, as discussed above, these mice exhibit compromised B cell development and immune deficiencies when compared to wild type mice. Such problems potentially limit the ability of the mice to support a vigorous humoral response and, consequently, generate fully human antibodies against some antigens. The deficiencies may be due to: (1) inefficient functionality due to the random introduction of the human immunoglobulin transgenes and resulting incorrect expression due to a lack of upstream and downstream control elements (Garrett *et al.,* 2005, Chromatin architecture near a potential 3' end of the IgH locus involves modular regulation of histone modifications during B-Cell development and in vivo occupancy at CTCF sites, *Mol Cell Biol* 25:1511-1525; Manis *et al.,* 2003, Elucidation of a downstream boundary of the 3' IgH regulatory region, *Mol Immunol* 39:753-760; Pawlitzky *et al.,* 2006, Identification of a candidate regulatory element within the 5' flanking region of the mouse IgH locus defined by pro-B cell-specific hypersensitivity associated with binding of PU.1, Pax5, and E2A, *J Immunol* 176:6839-6851); (2) inefficient interspecies interactions between human constant domains and mouse components of the B-cell receptor signaling complex on the cell surface, which may impair signaling processes required for normal maturation, proliferation, and survival of B cells (Hombach *et al.,* 1990, Molecular components of the B-cell antigen receptor complex of the IgM class, *Nature* 343:760-762); and (3) inefficient interspecies interactions between soluble human immunoglobulins and mouse Fc

receptors that might reduce affinity selection (Rao *et al.,* 2002, Differential expression of the inhibitory IgG Fc receptor FcgammaRIIB on germinal center cells: implications for selection of high-affinity B cells, *J Immunol* 169:1859-1868) and immunoglobulin serum concentrations (Brambell *et al.,* 1964, A Theoretical Model of Gamma-Globulin Catabolism, *Nature* 203:1352-1354; Junghans and Anderson, 1996, The protection receptor for IgG catabolism is the beta2-microglobulin-containing neonatal intestinal transport receptor, *PNAS USA* 93:5512-5516; Rao *et al.*, 2002; Hjelm *et al.,* 2006, Antibody-mediated regulation of the immune response, *Scand J Immunol* 64:177-184; Nimmerjahn and Ravetch, 2007, Fc-receptors as regulators of immunity, *Adv Immunol* 96:179-204). These deficiencies can be corrected by *in situ* humanization of only the variable regions of the mouse immunoglobulin loci within their natural locations at the endogenous heavy and light chain loci. This would effectively result in mice that make "reverse chimeric" (*i.e.*, human V:mouse C) antibodies which would be capable of normal interactions and selection with the mouse environment based on retaining mouse constant regions. Further such reverse chimeric antibodies may be readily reformatted into fully human antibodies for therapeutic purposes.

[000197] Genetically modified animals that comprise a replacement at the endogenous immunoglobulin heavy chain locus with heterologous (*e.g.*, from another species) immunoglobulin sequences can be made in conjunction with replacements at endogenous immunoglobulin light chain loci or in conjunction with immunoglobulin light chain transgenes (*e.g.*, chimeric immunoglobulin light chain transgenes or fully human fully mouse, etc.). The species from which the heterologous immunoglobulin heavy chain sequences are derived can vary widely; as with immunoglobulin light chain sequences employed in immunoglobulin light chain sequence replacements or immunoglobulin light chain transgenes.

[000198] Immunoglobulin variable region nucleic acid sequences, *e.g.*, V, D, and/or J segments, are in various embodiments obtained from a human or a non-human animal. Non-human animals suitable for providing V, D, and/or J segments include, for example bony fish, cartilaginous fish such as sharks and rays, amphibians, reptiles, mammals, birds (e.g., chickens). Non-human animals include, for example, mammals. Mammals include, for example, non-human primates, goats, sheep, pigs, dogs, bovine (*e.g.*, cow, bull, buffalo), deer, camels, ferrets and rodents and non-human primates (*e.g.*, chimpanzees, orangutans, gorillas, marmosets, rhesus monkeys baboons). Suitable non-human animals are selected from the rodent family including rats, mice, and hamsters. In one embodiment, the non-human animals are mice. As clear from the context, various non-human animals can be used as sources of variable domains or variable region gene segments (*e.g.*, sharks, rays, mammals (e.g., camels, rodents such as mice and rats).

[000199] According to the context, non-human animals are also used as sources of constant region sequences to be used in connection with variable sequences or segments, for example, rodent constant sequences can be used in transgenes operably linked to human or non-human variable sequences (*e.g.*, human or non-human primate variable sequences operably linked to, *e.g.*, rodent, *e.g.*, mouse or rat or hamster, constant sequences). Thus, in various embodiments, human V, D, and/or J segments are operably linked to rodent (*e.g.*, mouse or rat or hamster) constant region gene sequences. In some embodiments, the human V, D, and/or J segments (or one or more rearranged VDJ or VJ genes) are operably linked or fused to a mouse, rat, or hamster constant region gene sequence in, *e.g.*, a transgene integrated at a locus that is not an endogenous immunoglobulin locus.

[000200] In a specific embodiment, a mouse is provided that comprises a replacement of V_H, D_H, and J_H segments at an endogenous immunoglobulin heavy chain locus with one or more human V_H, D_H, and J_H segments, wherein the one or more human V_H, D_H, and J_H segments are operably linked to an endogenous immunoglobulin heavy chain gene; wherein the mouse comprises a transgene at a locus other than an endogenous immunoglobulin locus, wherein the transgene comprises an unrearranged or rearranged human V_L and human J_L segment operably linked to a mouse or rat or human constant region.

[000201] A method for a large *in situ* genetic replacement of the mouse germline immunoglobulin variable gene loci with human germline immunoglobulin variable gene loci while maintaining the ability of the mice to generate offspring is described. Specifically, the precise replacement of six megabases of both the mouse heavy chain and κ light chain immunoglobulin variable gene loci with their human counterparts while leaving the mouse constant regions intact is described. As a result, mice have been created that have a precise replacement of their entire germline immunoglobulin variable repertoire with equivalent human germline immunoglobulin variable sequences, while maintaining mouse constant regions. The human variable regions are linked to mouse constant regions to form chimeric human-mouse immunoglobulin loci that rearrange and express at physiologically appropriate levels. The antibodies expressed are "reverse chimeras," *i.e.*, they comprise human variable region sequences and mouse constant region sequences. These mice having humanized immunoglobulin variable regions that express antibodies having human variable regions and mouse constant regions are called VELCOIMMUNE® mice.

[000202] VELOCIMMUNE® humanized mice exhibit a fully functional humoral immune system that is essentially indistinguishable from that of wild-type mice. They display normal cell populations at all stages of B cell development. They exhibit normal lymphoid

organ morphology. Antibody sequences of VELOCIMMUNE® mice exhibit normal V(D)J rearrangement and normal somatic hypermutation frequencies. Antibody populations in these mice reflect isotype distributions that result from normal class switching (*e.g.,* normal isotype *cis*-switching). Immunizing VELOCIMMUNE® mice results in robust humoral immune responses that generate a large, diverse antibody repertoires having human immunoglobulin variable domains suitable for use as therapeutic candidates. This platform provides a plentiful source of naturally affinity-matured human immunoglobulin variable region sequences for making pharmaceutically acceptable antibodies and other antigen-binding proteins.

[000203] It is the precise replacement of mouse immunoglobulin variable sequences with human immunoglobulin variable sequences that allows for making VELOCIMMUNE® mice. Yet even a precise replacement of endogenous mouse immunoglobulin sequences at heavy and light chain loci with equivalent human immunoglobulin sequences, by sequential recombineering of very large spans of human immunoglobulin sequences, may present certain challenges due to divergent evolution of the immunoglobulin loci between mouse and man. For example, intergenic sequences interspersed within the immunoglobulin loci are not identical between mice and humans and, in some circumstances, may not be functionally equivalent. Differences between mice and humans in their immunoglobulin loci can still result in abnormalities in humanized mice, particularly when humanizing or manipulating certain portions of endogenous mouse immunoglobulin heavy chain loci. Some modifications at mouse immunoglobulin heavy chain loci are deleterious. Deleterious modifications can include, for example, loss of the ability of the modified mice to mate and produce offspring.

[000204] A precise, large-scale, *in situ* replacement of six megabases of the variable regions of the mouse heavy and light chain immunoglobulin loci (V_H-D_H-J_H and $V\kappa$-$J\kappa$) with the corresponding 1.4 megabases human genomic sequences was performed, while leaving the flanking mouse sequences intact and functional within the hybrid loci, including all mouse constant chain genes and locus transcriptional control regions (FIG. 1A and FIG. 1B). Specifically, the human V_H, D_H, J_H, $V\kappa$ and $J\kappa$ gene sequences were introduced through stepwise insertion of 13 chimeric BAC targeting vectors bearing overlapping fragments of the human germline variable loci into mouse ES cells using VELOCIGENE® genetic engineering technology (see, *e.g.,* US Pat. No. 6,586,251 and Valenzuela *et al.,* 2003, High-throughput engineering of the mouse genome coupled with high-resolution expression analysis, *Nat Biotechnol* 21:652-659).

[000205] Humanization of the mouse immunoglobulin genes represents the largest genetic modification to the mouse genome to date. While previous efforts with randomly

integrated human immunoglobulin transgenes have met with some success (discussed above), direct replacement of the mouse immunoglobulin genes with their human counterparts dramatically increases the efficiency with which fully-human antibodies can be efficiently generated in otherwise normal mice. Further, such mice exhibit a dramatically increased diversity of fully human antibodies that can be obtained after immunization with virtually any antigen, as compared with mice bearing disabled endogenous loci and fully human antibody transgenes. Multiple versions of replaced, humanized loci exhibit completely normal levels of mature and immature B cells, in contrast to mice with randomly integrated human transgenes, which exhibit significantly reduced B cell populations at various stages of differentiation. While efforts to increase the number of human gene segments in human transgenic mice have reduced such defects, the expanded immunoglobulin repertoires have not altogether corrected reductions in B cell populations as compared to wild-type mice.

[000206] Notwithstanding the near wild-type humoral immune function observed in mice with replaced immunoglobulin loci (*i.e.,* VELOCIMMUNE® mice), there are other challenges encountered when employing a direct replacement of the immunoglobulin that is not encountered in some approaches that employ randomly integrated transgenes. Differences in the genetic composition of the immunoglobulin loci between mice and humans has lead to the discovery of sequences beneficial for the propagation of mice with replaced immunoglobulin gene segments. Specifically, mouse ADAM genes located within the endogenous immunoglobulin locus are optimally present in mice with replaced immunoglobulin loci, due to their role in fertility.

Genomic Location and Function of Mouse ADAM6

[000207] Male mice that lack the ability to express any functional ADAM6 protein surprisingly exhibit a defect in the ability of the mice to mate and to generate offspring. The mice lack the ability to express a functional ADAM6 protein by virtue of a replacement of all or substantially all mouse immunoglobulin variable region gene segments with human variable region gene segments. The loss of ADAM6 function results because the ADAM6 locus is located within a region of the endogenous mouse immunoglobulin heavy chain variable region gene locus, proximal to the 3' end of the V_H gene segment locus that is upstream of the D_H gene segments. In order to breed mice that are homozygous for a replacement of all or substantially all endogenous mouse heavy chain variable gene segments with human heavy chain variable gene segments, it is generally a cumbersome approach to set up males and females that are each homozygous for the replacement and await a productive mating. Successful litters are low in frequency and size. Instead, males heterozygous for the replacement have been employed to mate with females homozygous

for the replacement to generate progeny that are heterozygous for the replacement, then breed a homozygous mouse therefrom. The inventors have determined that the likely cause of the loss in fertility in the male mice is the absence in homozygous male mice of a functional ADAM6 protein.

[000208] In various aspects, male mice that comprise a damaged (*i.e.*, nonfunctional or marginally functional) ADAM6 gene exhibit a reduction or elimination of fertility. Because in mice (and other rodents) the ADAM6 gene is located in the immunoglobulin heavy chain locus, the inventors have determined that in order to propagate mice, or create and maintain a strain of mice, that comprise a replaced immunoglobulin heavy chain locus, various modified breeding or propagation schemes are employed. The low fertility, or infertility, of male mice homozygous for a replacement of the endogenous immunoglobulin heavy chain variable gene locus renders maintaining such a modification in a mouse strain difficult. In various embodiments, maintaining the strain comprises avoiding infertility problems exhibited by male mice homozygous for the replacement.

[000209] In one aspect, a method for maintaining a strain of mouse as described herein is provided. The strain of mouse need not comprise an ectopic ADAM6 sequence, and in various embodiments the strain of mouse is homozygous or heterozygous for a knockout (*e.g.*, a functional knockout) of ADAM6.

[000210] The mouse strain comprises a modification of an endogenous immunoglobulin heavy chain locus that results in a reduction or loss in fertility in a male mouse. In one embodiment, the modification comprises a deletion of a regulatory region and/or a coding region of an ADAM6 gene. In a specific embodiment, the modification comprises a modification of an endogenous ADAM6 gene (regulatory and/or coding region) that reduces or eliminates fertility of a male mouse that comprises the modification; in a specific embodiment, the modification reduces or eliminates fertility of a male mouse that is homozygous for the modification.

[000211] In one embodiment, the mouse strain is homozygous or heterozygous for a knockout (*e.g.*, a functional knockout) or a deletion of an ADAM6 gene.

[000212] In one embodiment, the mouse strain is maintained by isolating from a mouse that is homozygous or heterozygous for the modification a cell, and employing the donor cell in host embryo, and gestating the host embryo and donor cell in a surrogate mother, and obtaining from the surrogate mother a progeny that comprises the genetic modification. In one embodiment, the donor cell is an ES cell. In one embodiment, the donor cell is a pluripotent cell, *e.g.*, an induced pluripotent cell.

[000213] In one embodiment, the mouse strain is maintained by isolating from a mouse that is homozygous or heterozygous for the modification a nucleic acid sequence comprising the modification, and introducing the nucleic acid sequence into a host nucleus,

and gestating a cell comprising the nucleic acid sequence and the host nucleus in a suitable animal. In one embodiment, the nucleic acid sequence is introduced into a host oocyte embryo.

[000214] In one embodiment, the mouse strain is maintained by isolating from a mouse that is homozygous or heterozygous for the modification a nucleus, and introducing the nucleus into a host cell, and gestating the nucleus and host cell in a suitable animal to obtain a progeny that is homozygous or heterozygous for the modification.

[000215] In one embodiment, the mouse strain is maintained by employing *in vitro* fertilization (IVF) of a female mouse (wild-type, homozygous for the modification, or heterozygous for the modification) employing a sperm from a male mouse comprising the genetic modification. In one embodiment, the male mouse is heterozygous for the genetic modification. In one embodiment, the male mouse is homozygous for the genetic modification.

[000216] In one embodiment, the mouse strain is maintained by breeding a male mouse that is heterozygous for the genetic modification with a female mouse to obtain progeny that comprises the genetic modification, identifying a male and a female progeny comprising the genetic modification, and employing a male that is heterozygous for the genetic modification in a breeding with a female that is wild-type, homozygous, or heterozygous for the genetic modification to obtain progeny comprising the genetic modification. In one embodiment, the step of breeding a male heterozygous for the genetic modification with a wild-type female, a female heterozygous for the genetic modification, or a female homozygous for the genetic modification is repeated in order to maintain the genetic modification in the mouse strain.

[000217] In one aspect, a method is provided for maintaining a mouse strain that comprises a replacement of an endogenous immunoglobulin heavy chain variable gene locus with one or more human immunoglobulin heavy chain sequences, comprising breeding the mouse strain so as to generate heterozygous male mice, wherein the heterozygous male mice are bred to maintain the genetic modification in the strain. In a specific embodiment, the strain is not maintained by any breeding of a homozygous male with a wild-type female, or a female homozygous or heterozygous for the genetic modification.

[000218] The ADAM6 protein is a member of the ADAM family of proteins, where ADAM is an acronym for A Disintegrin And Metalloprotease. The ADAM family of proteins is large and diverse, with diverse functions including cell adhesion. Some members of the ADAM family are implicated in spermatogenesis and fertilization. For example, ADAM2 encodes a subunit of the protein fertilin, which is implicated in sperm-egg interactions. ADAM3, or cyritestin, appears necessary for sperm binding to the zona pellucida. The absence of

either ADAM2 or ADAM3 results in infertility. It has been postulated that ADAM2, ADAM3, and ADAM6 form a complex on the surface of mouse sperm cells. The human ADAM6 gene, normally found between human V_H gene segments V_H1-2 and V_H6-1, appears to be a pseudogene (Figure 12). In mice, there are two ADAM6 genes—ADAM6a and ADAM6b—that are found in an intergenic region between mouse V_H and D_H gene segments, and in the mouse the ADAM6a and ADAM6b genes are oriented in opposite transcriptional orientation to that of the surrounding immunoglobulin gene segments (FIG. 12). In mice, a functional ADAM6 locus is apparently required for normal fertilization. A functional ADAM6 locus or sequence, then, refers to an ADAM6 locus or sequence that can complement, or rescue, the drastically reduced fertilization exhibited in male mice with missing or nonfunctional endogenous ADAM6 loci.

[000219] The position of the intergenic sequence in mice that encodes ADAM6a and ADAM6b renders the intergenic sequence susceptible to modification when modifying an endogenous mouse heavy chain. When V_H gene segments are deleted or replaced, or when D_H gene segments are deleted or replaced, there is a high probability that a resulting mouse will exhibit a severe deficit in fertility. In order to compensate for the deficit, the mouse is modified to include a nucleotide sequence that encodes a protein that will complement the loss in ADAM6 activity due to a modification of the endogenous mouse ADAM6 locus. In various embodiments, the complementing nucleotide sequence is one that encodes a mouse ADAM6a, a mouse ADAM6b, or a homolog or ortholog or functional fragment thereof that rescues the fertility deficit.

[000220] The nucleotide sequence that rescues fertility can be placed at any suitable position. It can be placed in the intergenic region, or in any suitable position in the genome (*i.e.*, ectopically). In one embodiment, the nucleotide sequence can be introduced into a transgene that randomly integrates into the mouse genome. In one embodiment, the sequence can be maintained episomally, that is, on a separate nucleic acid rather than on a mouse chromosome. Suitable positions include positions that are transcriptionally permissive or active, *e.g.*, a ROSA26 locus (Zambrowicz *et al.,* 1997, *PNAS USA* 94:3789-3794), a BT-5 locus (Michael *et al.*, 1999, *Mech. Dev.* 85:35-47), or an Oct4 locus (Wallace *et al.*, 2000, *Nucleic Acids Res.* 28:1455-1464). Targeting nucleotide sequences to transcriptionally active loci are described, *e.g.,* in US 7,473,557, herein incorporated by reference.

[000221] Alternatively, the nucleotide sequence that rescues fertility can be coupled with an inducible promoter so as to facilitate optimal expression in the appropriate cells and/or tissues, *e.g.,* reproductive tissues. Exemplary inducible promoters include promoters activated by physical (*e.g.,* heat shock promoter) and/or chemical means (*e.g.,* IPTG or Tetracycline).

[000222] Further, expression of the nucleotide sequence can be linked to other genes so as to achieve expression at specific stages of development or within specific tissues. Such expression can be achieved by placing the nucleotide sequence in operable linkage with the promoter of a gene expressed at a specific stage of development. For example, immunoglobulin sequences from one species engineered into the genome of a host species are place in operable linkage with a promoter sequence of a CD19 gene (a B cell specific gene) from the host species. B cell-specific expression at precise developmental stages when immunoglobulins are expressed is achieved.

[000223] Yet another method to achieve robust expression of an inserted nucleotide sequence is to employ a constitutive promoter. Exemplary constitutive promoters include SV40, CMV, UBC, EF1A, PGK and CAGG. In a similar fashion, the desired nucleotide sequence is placed in operable linkage with a selected constitutive promoter, which provides high level of expression of the protein(s) encoded by the nucleotide sequence.

[000224] The term "ectopic" is intended to include a displacement, or a placement at a position that is not normally encountered in nature (*e.g.*, placement of a nucleic acid sequence at a position that is not the same position as the nucleic acid sequence is found in a wild-type mouse). The term, in various embodiments, is used in the sense of its object being out of its normal, or proper, position. For example, the phrase "an ectopic nucleotide sequence encoding..." refers to a nucleotide sequence that appears at a position at which it is not normally encountered in the mouse. For example, in the case of an ectopic nucleotide sequence encoding a mouse ADAM6 protein (or an ortholog or homolog or fragment thereof that provides the same or similar fertility benefit on male mice), the sequence can be placed at a different position in the mouse's genome than is normally found in a wild-type mouse. In such cases, novel sequence junctions of mouse sequence will be created by placing the sequence at a different position in the mouse's genome than in a wild-type mouse. A functional homolog or ortholog of mouse ADAM6 is a sequence that confers a rescue of fertility loss (*e.g.,* loss of the ability of a male mouse to generate offspring by mating) that is observed in an ADAM6$^{-/-}$ mouse. Functional homologs or orthologs include proteins that have at least about 89% identity or more, *e.g.*, up to 99% identity, to the amino acid sequence of ADAM6a and/or to the amino acid sequence of ADAM6b, and that can complement, or rescue ability to successfully mate, of a mouse that has a genotype that includes a deletion or knockout of ADAM6a and/or ADAM6b.

[000225] The ectopic position can be anywhere (*e.g.,* as with random insertion of a transgene containing a mouse ADAM6 sequence), or can be, *e.g.,* at a position that approximates (but is not precisely the same as) its location in a wild-type mouse (*e.g.*, in a modified endogenous mouse immunoglobulin locus, but either upstream or downstream of its natural position, *e.g.,* within a modified immunoglobulin locus but between different gene

segments, or at a different position in a mouse V-D intergenic sequence). One example of an ectopic placement is placement within a humanized immunoglobulin heavy chain locus. For example, a mouse comprising a replacement of one or more endogenous V_H gene segments with human V_H gene segments, wherein the replacement removes an endogenous ADAM6 sequence, can be engineered to have a mouse ADAM6 sequence located within a sequence that contains the human V_H gene segments. The resulting modification would generate a (ectopic) mouse ADAM6 sequence within a human gene sequence, and the (ectopic) placement of the mouse ADAM6 sequence within the human gene sequence can approximate the position of the human ADAM6 pseudogene (*i.e.*, between two V segments) or can approximate the position of the mouse ADAM6 sequence (*i.e.,* within the V-D intergenic region). The resulting sequence junctions created by the joining of a (ectopic) mouse ADAM6 sequence within or adjacent to a human gene sequence (*e.g.,* an immunoglobulin gene sequence) within the germline of the mouse would be novel as compared to the same or similar position in the genome of a wild-type mouse.

[000226] In various embodiments, non-human animals are provided that lack an ADAM6 or ortholog or homolog thereof, wherein the lack renders the non-human animal infertile, or substantially reduces fertility of the non-human animal. In various embodiments, the lack of ADAM6 or ortholog or homolog thereof is due to a modification of an endogenous immunoglobulin heavy chain locus. A substantial reduction in fertility is, *e.g.*, a reduction in fertility (*e.g.*, breeding frequency, pups per litter, litters per year, etc.) of about 50%, 60%, 70%, 80%, 90%, or 95% or more. In various embodiments, the non-human animals are supplemented with a mouse ADAM6 gene or ortholog or homolog or functional fragment thereof that is functional in a male of the non-human animal, wherein the supplemented ADAM6 gene or ortholog or homolog or functional fragment thereof rescues the reduction in fertility in whole or in substantial part. A rescue of fertility in substantial part is, *e.g.*, a restoration of fertility such that the non-human animal exhibits a fertility that is at least 70%, 80%, or 90% or more as compared with an unmodified (*i.e.*, an animal without a modification to the ADAM6 gene or ortholog or homolog thereof) heavy chain locus.

[000227] The sequence that confers upon the genetically modified animal (*i.e.*, the animal that lacks a functional ADAM6 or ortholog or homolog thereof, due to, *e.g.*, a modification of a immunoglobulin heavy chain locus) is, in various embodiments, selected from an ADAM6 gene or ortholog or homolog thereof. For example, in a mouse, the loss of ADAM6 function is rescued by adding, in one embodiment, a mouse ADAM6 gene. In one embodiment, the loss of ADAM6 function in the mouse is rescued by adding an ortholog or homolog of a closely related specie with respect to the mouse, *e.g.*, a rodent, *e.g.*, a mouse of a different strain or species, a rat of any species, a rodent; wherein the addition

of the ortholog or homolog to the mouse rescues the loss of fertility due to loss of ADAM6 function or loss of an ADAM6 gene. Orthologs and homologs from other species, in various embodiments, are selected from a phylogenetically related species and, in various embodiments, exhibit a percent identity with the endogenous ADAM6 (or ortholog) that is about 80% or more, 85% or more, 90% or more, 95% or more, 96% or more, or 97% or more; and that rescue ADAM6-related or (in a non-mouse) ADAM6 ortholog-related loss of fertility. For example, in a genetically modified male rat that lacks ADAM6 function (*e.g.*, a rat with an endogenous immunoglobulin heavy chain variable region replaced with a human immunoglobulin heavy chain variable region, or a knockout in the rat immunoglobulin heavy chain region), loss of fertility in the rat is rescued by addition of a rat ADAM6 or, in some embodiments, an ortholog of a rat ADAM6 (*e.g.*, an ADAM6 ortholog from another rat strain or species, or, in one embodiment, from a mouse).

[000228] Thus, in various embodiments, genetically modified animals that exhibit no fertility or a reduction in fertility due to modification of a nucleic acid sequence encoding an ADAM6 protein (or ortholog or homolog thereof) or a regulatory region operably linked with the nucleic acid sequence, comprise a nucleic acid sequence that complements, or restores, the loss in fertility where the nucleic acid sequence that complements or restores the loss in fertility is from a different strain of the same species or from a phylogenetically related species. In various embodiments, the complementing nucleic acid sequence is an ADAM6 ortholog or homolog or functional fragment thereof. In various embodiments, the complementing ADAM6 ortholog or homolog or functional fragment thereof is from a non-human animal that is closely related to the genetically modified animal having the fertility defect. For example, where the genetically modified animal is a mouse of a particular strain, an ADAM6 ortholog or homolog or functional fragment thereof can be obtained from a mouse of another strain, or a mouse of a related species. In one embodiment, where the genetically modified animal comprising the fertility defect is of the order Rodentia, the ADAM6 ortholog or homolog or functional fragment thereof is from another animal of the order Rodentia. In one embodiment, the genetically modified animal comprising the fertility defect is of a suborder Myomoropha (*e.g.*, jerboas, jumping mice, mouse-like hamsters, hamsters, New World rats and mice, voles, true mice and rats, gerbils, spiny mice, crested rats, climbing mice, rock mice, white-tailed rats, malagasy rats and mice, spiny dormice, mole rats, bamboo rats, zokors), and the ADAM6 ortholog or homolog or functional fragment thereof is selected from an animal of order Rodentia, or of the suborder Myomorpha.

[000229] In one embodiment, the genetically modified animal is from the superfamily Dipodoidea, and the ADAM6 ortholog or homolog or functional fragment thereof is from the superfamily Muroidea. In one embodiment, the genetically modified animal is from the

superfamily Muroidea, and the ADAM6 ortholog or homolog or functional fragment thereof is from the superfamily Dipodoidea.

[000230] In one embodiment, the genetically modified animal is a rodent. In one embodiment, the rodent is selected from the superfamily Muroidea, and the ADAM6 ortholog or homolog is from a different species within the superfamily Muroidea. In one embodiment, the genetically modified animal is from a family selected from Calomyscidae (*e.g.*, mouse-like hamsters), Cricetidae (*e.g.*, hamster, New World rats and mice, voles), Muridae (true mice and rats, gerbils, spiny mice, crested rats), Nesomyidae (climbing mice, rock mice, with-tailed rats, Malagasy rats and mice), Platacanthomyidae (*e.g.*, spiny dormice), and Spalacidae (*e.g.*, mole rates, bamboo rats, and zokors); and the ADAM6 ortholog or homolog is selected from a different species of the same family. In a specific embodiment, the genetically modified rodent is selected from a true mouse or rat (family Muridae), and the ADAM6 ortholog or homolog is from a species selected from a gerbil, spiny mouse, or crested rat. In one embodiment, the genetically modified mouse is from a member of the family Muridae, and the ADAM6 ortholog or homolog is from a different species of the family Muridae. In a specific embodiment, the genetically modified rodent is a mouse of the family Muridae, and the ADAM6 ortholog or homolog is from a rat, gerbil, spiny mouse, or crested rat of the family Muridae.

[000231] In various embodiments, one or more rodent ADAM6 orthologs or homologs or functional fragments thereof of a rodent in a family restores fertility to a genetically modified rodent of the same family that lacks an ADAM6 ortholog or homolog (*e.g.*, Cricetidae (*e.g.*, hamsters, New World rats and mice, voles); Muridae (*e.g.*, true mice and rats, gerbils, spiny mice, crested rats)).

[000232] In various embodiments, ADAM6 orthologs, homologs, and fragments thereof are assessed for functionality by ascertaining whether the ortholog, homolog, or fragment restores fertility to a genetically modified male non-human animal that lacks ADAM6 activity (*e.g.*, a rodent, *e.g.*, a mouse or rat, that comprises a knockout of ADAM6 or its ortholog). In various embodiments, functionality is defined as the ability of a sperm of a genetically modified animal lacking an endogenous ADAM6 or ortholog or homolog thereof to migrate an oviduct and fertilize an ovum of the same specie of genetically modified animal.

[000233] In various aspects, mice that comprise deletions or replacements of the endogenous heavy chain variable region locus or portions thereof can be made that contain an ectopic nucleotide sequence that encodes a protein that confers similar fertility benefits to mouse ADAM6 (*e.g.*, an ortholog or a homolog or a fragment thereof that is functional in a male mouse). The ectopic nucleotide sequence can include a nucleotide sequence that encodes a protein that is an ADAM6 homolog or ortholog (or fragment

thereof) of a different mouse strain or a different species, *e.g.,* a different rodent species, and that confers a benefit in fertility, *e.g.,* increased number of litters over a specified time period, and/or increased number of pups per litter, and/or the ability of a sperm cell of a male mouse to traverse through a mouse oviduct to fertilize a mouse egg.

[000234] In one embodiment, the ADAM6 is a homolog or ortholog that is at least 89% to 99% identical to a mouse ADAM6 protein (e.g., at least 89% to 99% identical to mouse ADAM6a or mouse ADAM6b). In one embodiment, the ectopic nucleotide sequence encodes one or more proteins independently selected from a protein at least 89% identical to mouse ADAM6a, a protein at least 89% identical to mouse ADAM6b, and a combination thereof. In one embodiment, the homolog or ortholog is a rat, hamster, mouse, or guinea pig protein that is or is modified to be about 89% or more identical to mouse ADAM6a and/or mouse ADAM6b. In one embodiment, the homolog or ortholog is or is at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical to a mouse ADAM6a and/or mouse ADAM6b.

Ectopic ADAM6 in Humanized Heavy Chain Mice

[000235] Developments in gene targeting, *e.g.,* the development of bacterial artificial chromosomes (BACs), now enable the recombination of relatively large genomic fragments. BAC engineering has allowed for the ability to make large deletions, and large insertions, into mouse ES cells.

[000236] Mice that make human antibodies have been available for some time now. Although they represent an important advance in the development of human therapeutic antibodies, these mice display a number of significant abnormalities that limit their usefulness. For example, they display compromised B cell development. The compromised development may be due to a variety of differences between the transgenic mice and wild-type mice.

[000237] Human antibodies might not optimally interact with mouse pre B cell or B cell receptors on the surface of mouse cells that signal for maturation, proliferation, or survival during clonal selection. Fully human antibodies might not optimally interact with a mouse Fc receptor system; mice express Fc receptors that do not display a one-to-one correspondence with human Fc receptors. Finally, various mice that make fully human antibodies do not include all genuine mouse sequences, *e.g.,* downstream enhancer elements and other locus control elements, which may be required for wild-type B cell development.

[000238] Mice that make fully human antibodies generally comprise endogenous immunoglobulin loci that are disabled in some way, and human transgenes that comprise variable and constant immunoglobulin gene segments are introduced into a random

location in the mouse genome. As long as the endogenous locus is sufficiently disabled so as not to rearrange gene segments to form a functional immunoglobulin gene, the goal of making fully human antibodies in such a mouse can be achieved—albeit with compromised B cell development.

[000239] Although compelled to make fully human antibodies from the human transgene locus, generating human antibodies in a mouse is apparently an unfavored process. In some mice, the process is so unfavored as to result in formation of chimeric human variable/mouse constant heavy chains (but not light chains) through the mechanism of *trans*-switching. By this mechanism, transcripts that encode fully human antibodies undergo isotype switching in *trans* from the human isotype to a mouse isotype. The process is in *trans*, because the fully human transgene is located apart from the endogenous locus that retains an undamaged copy of a mouse heavy chain constant region gene. Although in such mice *trans*-switching is readily apparent the phenomenon is still insufficient to rescue B cell development, which remains frankly impaired. In any event, *trans*-switched antibodies made in such mice retain fully human light chains, since the phenomenon of *trans*-switching apparently does not occur with respect to light chains; *trans*-switching presumably relies on switch sequences in endogenous loci used (albeit differently) in normal isotype switching in *cis.* Thus, even when mice engineered to make fully human antibodies select a *trans*-switching mechanism to make antibodies with mouse constant regions, the strategy is still insufficient to rescue normal B cell development.

[000240] A primary concern in making antibody-based human therapeutics is making a sufficiently large diversity of human immunoglobulin variable region sequences to identify useful variable domains that specifically recognize particular epitopes and bind them with a desirable affinity, usually—but not always—with high affinity. Prior to the development of VELOCIMMUNE® mice (described herein), there was no indication that mice expressing human variable regions with mouse constant regions would exhibit any significant differences from mice that made human antibodies from a transgene. That supposition, however, was incorrect.

[000241] VELOCIMMUNE® mice, which contain a precise replacement of mouse immunoglobulin variable regions with human immunoglobulin variable regions at the endogenous mouse loci, display a surprising and remarkable similarity to wild-type mice with respect to B cell development. In a surprising and stunning development, VELOCIMMUNE® mice displayed an essentially normal, wild-type response to immunization that differed only in one significant respect from wild-type mice—the variable regions generated in response to immunization are fully human.

[000242] VELOCIMMUNE® mice contain a precise, large-scale replacement of germline variable regions of mouse immunoglobulin heavy chain (IgH) and immunoglobulin light

chain (e.g., κ light chain, Igκ) with corresponding human immunoglobulin variable regions, at the endogenous loci. In total, about six megabases of mouse loci are replaced with about 1.5 megabases of human genomic sequence. This precise replacement results in a mouse with hybrid immunoglobulin loci that make heavy and light chains that have a human variable regions and a mouse constant region. The precise replacement of mouse V_H-D_H-J_H and Vκ-Jκ segments leave flanking mouse sequences intact and functional at the hybrid immunoglobulin loci. The humoral immune system of the mouse functions like that of a wild-type mouse. B cell development is unhindered in any significant respect and a rich diversity of human variable regions is generated in the mouse upon antigen challenge.

[000243] VELOCIMMUNE® mice are possible because immunoglobulin gene segments for heavy and κ light chains rearrange similarly in humans and mice, which is not to say that their loci are the same or even nearly so—clearly they are not. However, the loci are similar enough that humanization of the heavy chain variable gene locus can be accomplished by replacing about three million base pairs of contiguous mouse sequence that contains all the V_H, D_H, and J_H gene segments with about one million bases of contiguous human genomic sequence covering basically the equivalent sequence from a human immunoglobulin locus.

[000244] In some embodiments, further replacement of certain mouse constant region gene sequences with human gene sequences (*e.g.*, replacement of mouse C_H1 sequence with human C_H1 sequence, and replacement of mouse C_L sequence with human C_L sequence) results in mice with hybrid immunoglobulin loci that make antibodies that have human variable regions and partly human constant regions, suitable for, *e.g.*, making fully human antibody fragments, *e.g.*, fully human Fab's. Mice with hybrid immunoglobulin loci exhibit normal variable gene segment rearrangement, normal somatic hypermutation frequencies, and normal class switching. These mice exhibit a humoral immune system that is indistinguishable from wild type mice, and display normal cell populations at all stages of B cell development and normal lymphoid organ structures—even where the mice lack a full repertoire of human variable region gene segments. Immunizing these mice results in robust humoral responses that display a wide diversity of variable gene segment usage.

[000245] The precise replacement of mouse germline variable region gene segments allows for making mice that have partly human immunoglobulin loci. Because the partly human immunoglobulin loci rearrange, hypermutate, and class switch normally, the partly human immunoglobulin loci generate antibodies in a mouse that comprise human variable regions. Nucleotide sequences that encode the variable regions can be identified and cloned, then fused (*e.g.*, in an *in vitro* system) with any sequences of choice, *e.g.*, any

immunoglobulin isotype suitable for a particular use, resulting in an antibody or antigen-binding protein derived wholly from human sequences.

[000246] Large-scale humanization by recombineering methods were used to modify mouse embryonic stem (ES) cells to precisely replace up to three megabases of the mouse heavy chain immunoglobulin locus that included essentially all of the mouse V_H, D_H, and J_H gene segments with equivalent human gene segments with up to a one megabase human genomic sequence containing some or essentially all human V_H, D_H, and J_H gene segments. Up to a one-half megabase segment of the human genome comprising one of two repeats encoding essentially all human Vκ and Jκ gene segments was used to replace a three megabase segment of the mouse immunoglobulin κ light chain locus containing essentially all of the mouse Vκ and Jκ gene segments.

[000247] Mice with such replaced immunoglobulin loci can comprise a disruption or deletion of the endogenous mouse ADAM6 locus, which is normally found between the 3'-most V_H gene segment and the 5'-most D_H gene segment at the mouse immunoglobulin heavy chain locus. Disruption in this region can lead to reduction or elimination of functionality of the endogenous mouse ADAM6 locus. If the 3'-most V_H gene segments of the human heavy chain repertoire are used in a replacement, an intergenic region containing a pseudogene that appears to be a human ADAM6 pseudogene is present between these V_H gene segments, i.e., between human V_H1-2 and V_H1-6. However, male mice that comprise this human intergenic sequence exhibit a reduction in fertility.

[000248] Mice are described that comprise the replaced loci as described above, and that also comprise an ectopic nucleic acid sequence encoding a mouse ADAM6, where the mice exhibit essentially normal fertility. In one embodiment, the ectopic nucleic acid sequence comprises a mouse ADAM6a and/or a mouse ADAM6b sequence or functional fragments thereof placed between a human V_H1-2 and a human V_H6-1 at a modified endogenous heavy chain locus. In one embodiment, the ectopic nucleic acid sequence is SEQ ID NO:3, placed between a human V_H1-2 and a human V_H6-1 at a modified endogenous heavy chain locus. The direction of transcription of the ADAM6 genes of SEQ ID NO:3 are opposite with respect to the direction of transcription of the surrounding human V_H gene segments. Although examples herein show rescue of fertility by placing the ectopic sequence between the indicated human V_H gene segments, skilled persons will recognize that placement of the ectopic sequence at any suitable transcriptionally-permissive locus in the mouse genome (or even extrachromosomally) will be expected to similarly rescue fertility in a male mouse.

[000249] The phenomenon of complementing a mouse that lacks a functional ADAM6 locus with an ectopic sequence that comprises a mouse ADAM6 gene or ortholog or

homolog or functional fragment thereof is a general method that is applicable to rescuing any mice with nonfunctional or minimally functional endogenous ADAM6 loci. Thus, a great many mice that comprise an ADAM6-disrupting modification of the immunoglobulin heavy chain locus can be rescued with the compositions and methods of the invention. Accordingly, the invention comprises mice with a wide variety of modifications of immunoglobulin heavy chain loci that compromise endogenous ADAM6 function. Some (non-limiting) examples are provided in this description. In addition to the VELOCIMMUNE® mice described, the compositions and methods related to ADAM6 can be used in a great many applications, *e.g.*, when modifying a heavy chain locus in a wide variety of ways.

[000250] In one aspect, a mouse is provided that comprises an ectopic ADAM6 sequence that encodes a functional ADAM6 protein (or ortholog or homolog or functional fragment thereof), a replacement of all or substantially all mouse V_H gene segments with one or more human V_H gene segments, a replacement of all or substantially all mouse D_H gene segments and J_H gene segments with human D_H and human J_H gene segments; wherein the mouse lacks a C_H1 and/or hinge region. In one embodiment, the mouse makes a single variable domain binding protein that is a dimer of immunoglobulin chains selected from: (a) human V_H – mouse C_H1 – mouse C_H2 – mouse C_H3; (b) human V_H – mouse hinge – mouse C_H2 – mouse C_H3; and, (c) human V_H – mouse C_H2 – mouse C_H3.

[000251] In one aspect, the nucleotide sequence that rescues fertility is placed within a human immunoglobulin heavy chain variable region sequence (*e.g.,* between human V_H1-2 and V_H1-6 gene segments) in a mouse that has a replacement of one or more mouse immunoglobulin heavy chain variable gene segments (mV_H's, mD_H's, and/or mJ_H's) with one or more human immunoglobulin heavy chain variable gene segments (hV_H's, hD_H's, and/or hJ_H's), and the mouse further comprises a replacement of one or more mouse immunoglobulin κ light chain variable gene segments (mVκ's and/or mJκ's) with one or more human immunoglobulin κ light chain variable gene segments (hVκ's and/or hJκ's).

[000252] In one embodiment, the one or more mouse immunoglobulin heavy chain variable gene segments comprises about three megabases of the mouse immunoglobulin heavy chain locus. In one embodiment, the one or more mouse immunoglobulin heavy chain variable gene segments comprises at least 89 V_H gene segments, at least 13 D_H gene segments, at least four J_H gene segments or a combination thereof of the mouse immunoglobulin heavy chain locus. In one embodiment, the one or more human immunoglobulin heavy chain variable gene segments comprises about one megabase of a human immunoglobulin heavy chain locus. In one embodiment, the one or more human immunoglobulin heavy chain variable gene segments comprises at least 80 V_H gene

segments, at least 27 D_H gene segments, at least six J_H gene segments or a combination thereof of a human immunoglobulin heavy chain locus.

[000253] In one embodiment, the one or more mouse immunoglobulin κ light chain variable gene segments comprises about three megabases of the mouse immunoglobulin κ light chain locus. In one embodiment, the one or more mouse immunoglobulin κ light chain variable gene segments comprises at least 137 Vκ gene segments, at least five Jκ gene segments or a combination thereof of the mouse immunoglobulin κ light chain locus. In one embodiment, the one or more human immunoglobulin κ light chain variable gene segments comprises about one-half megabase of a human immunoglobulin κ light chain locus. In a specific embodiment, the one or more human immunoglobulin κ light chain variable gene segments comprises the proximal repeat (with respect to the immunoglobulin κ constant region) of a human immunoglobulin κ light chain locus. In one embodiment, the one or more human immunoglobulin κ light chain variable gene segments comprises at least 40Vκ gene segments, at least five Jκ gene segments or a combination thereof of a human immunoglobulin κ light chain locus.

[000254] In one embodiment, the nucleotide sequence is place between two human immunoglobulin gene segments. In a specific embodiment, the two human immunoglobulin gene segments are heavy chain gene segments. In one embodiment, the nucleotide sequence is placed between a human V_H1-2 gene segment and a human V_H1-6 gene segment in a VELOCIMMUNE® mouse (US 6,596,541 and US 7,105,348, incorporated herein by reference). In one embodiment, the VELOCIMMUNE® mouse so modified comprises a replacement of mouse immunoglobulin heavy chain variable gene segments with at least 80 human V_H gene segments, 27 human D_H gene segments and six human J_H gene segments, and a replacement of mouse immunoglobulin κ light chain variable gene segments with at least 40 human Vκ gene segments and five human Jκ gene segments.

[000255] In one aspect, a functional mouse ADAM6 locus (or ortholog or homolog or functional fragment thereof) is present in the midst of human V_H gene segments that replace endogenous mouse V_H gene segments. In one embodiment, at least 89 mouse V_H gene segments are removed and replaced with one or more human V_H gene segments, and the mouse ADAM6 locus is present immediately adjacent to the 3' end of the human V_H gene segments, or between two human V_H gene segments. In a specific embodiment, the mouse ADAM6 locus is present between two V_H gene segments within about 20 kilo bases (kb) to about 40 kilo bases (kb) of the 3' terminus of the inserted human V_H gene segments. In a specific embodiment, the mouse ADAM6 locus is present between two V_H gene segments within about 29 kb to about 31 kb of the 3' terminus of the inserted human

V_H gene segments. In a specific embodiment, the mouse ADAM6 locus is present within about 30 kb of the 3' terminus of the inserted human V_H gene segments. In a specific embodiment, the mouse ADAM6 locus is present within about 30,184 bp of the 3' terminus of the inserted human VH gene segments. In a specific embodiment, the replacement includes human V_H gene segments V_H1-2 and V_H6-1, and the mouse ADAM6 locus is present downstream of the V_H1-2 gene segment and upstream of the V_H6-1 gene segment. In a specific embodiment, the mouse ADAM6 locus is present between a human V_H1-2 gene segment and a human V_H6-1 gene segment, wherein the 5' end of the mouse ADAM6 locus is about 13,848 bp from the 3' terminus of the human V_H1-2 gene segment and the 3' end of the ADAM6 locus is about 29,737 bp 5' of the human V_H6-1 gene segment. In a specific embodiment, the mouse ADAM6 locus comprises SEQ ID NO:3 or a fragment thereof that confers ADAM6 function within cells of the mouse. In a specific embodiment, the arrangement of human V_H gene segments is then the following (from upstream to downstream with respect to direction of transcription of the human V_H gene segments): human V_H1-2 – mouse ADAM6 locus – human V_H6-1. In a specific embodiment, the ADAM6 pseudogene between human V_H1-2 and human V_H6-1 is replaced with the mouse ADAM6 locus. In one embodiment, the orientation of one or more of mouse ADAM6a and mouse ADAM6b of the mouse ADAM6 locus is opposite with respect to direction of transcription as compared with the orientation of the human V_H gene segments. Alternatively, the mouse ADAM6 locus is present in the intergenic region between the 3'-most human V_H gene segment and the 5'-most D_H gene segment. This can be the case whether the 5'-most D_H segment is mouse or human.

[000256] Similarly, a mouse modified with one or more human V_L gene segments (*e.g.,* Vκ or Vλ segments) replacing all or substantially all endogenous mouse V_H gene segments can be modified so as to either maintain the endogenous mouse ADAM6 locus, as described above, *e.g.,* by employing a targeting vector having a downstream homology arm that includes a mouse ADAM6 locus or functional fragment thereof, or to replace a damaged mouse ADAM6 locus with an ectopic sequence positioned between two human V_L gene segments or between the human V_L gene segments and a D_H gene segment (whether human or mouse, *e.g.,* Vλ + m/hD_H), or a J gene segment (whether human or mouse, *e.g.,* Vκ + J_H). In one embodiment, the replacement includes two or more human V_L gene segments, and the mouse ADAM6 locus or functional fragment thereof is present between the two 3'-most V_L gene segments. In a specific embodiment, the arrangement of human V_L gene segments is then the following (from upstream to downstream with respect to direction of transcription of the human gene segments): human V_L3'-1 – mouse ADAM6 locus – human V_L3'. In one embodiment, the orientation of one or more of mouse

ADAM6a and mouse ADAM6b of the mouse ADAM6 locus is opposite with respect to direction of transcription as compared with the orientation of the human V_L gene segments. Alternatively, the mouse ADAM6 locus is present in the intergenic region between the 3'-most human V_L gene segment and the 5'-most D_H gene segment. This can be the case whether the 5'-most D_H segment is mouse or human.

[000257] In one aspect, a mouse is provided with a replacement of one or more endogenous mouse V_H gene segments, and that comprises at least one endogenous mouse D_H gene segment. In such a mouse, the modification of the endogenous mouse V_H gene segments can comprise a modification of one or more of the 3'-most V_H gene segments, but not the 5'-most D_H gene segment, where care is taken so that the modification of the one or more 3'-most V_H gene segments does not disrupt or render the endogenous mouse ADAM6 locus nonfunctional. For example, in one embodiment the mouse comprises a replacement of all or substantially all endogenous mouse V_H gene segments with one or more human V_H gene segments, and the mouse comprises one or more endogenous D_H gene segments and a functional endogenous mouse ADAM6 locus.

[000258] In another embodiment, the mouse comprises the modification of endogenous mouse 3'-most V_H gene segments, and a modification of one or more endogenous mouse D_H gene segments, and the modification is carried out so as to maintain the integrity of the endogenous mouse ADAM6 locus to the extent that the endogenous ADAM6 locus remains functional. In one example, such a modification is done in two steps: (1) replacing the 3'-most endogenous mouse V_H gene segments with one or more human V_H gene segments employing a targeting vector with an upstream homology arm and a downstream homology arm wherein the downstream homology arm includes all or a portion of a functional mouse ADAM6 locus; (2) then replacing and endogenous mouse D_H gene segment with a targeting vector having an upstream homology arm that includes a all or a functional portion of a mouse ADAM6 locus.

[000259] In various aspects, employing mice that contain an ectopic sequence that encodes a mouse ADAM6 protein or an ortholog or homolog or functional homolog thereof are useful where modifications disrupt the function of endogenous mouse ADAM6. The probability of disrupting endogenous mouse ADAM6 function is high when making modifications to mouse immunoglobulin loci, in particular when modifying mouse immunoglobulin heavy chain variable regions and surrounding sequences. Therefore, such mice provide particular benefit when making mice with immunoglobulin heavy chain loci that are deleted in whole or in part, are humanized in whole or in part, or are replaced (*e.g.,* with $V\kappa$ or $V\lambda$ sequences) in whole or in part. Methods for making the genetic modifications described for the mice described below are known to those skilled in the art.

[000260] Mice containing an ectopic sequence encoding a mouse ADAM6 protein, or a

substantially identical or similar protein that confers the fertility benefits of a mouse ADAM6 protein, are particularly useful in conjunction with modifications to a mouse immunoglobulin heavy chain variable gene locus that disrupt or delete the endogenous mouse ADAM6 sequence. Although primarily described in connection with mice that express antibodies with human variable regions and mouse constant regions, such mice are useful in connection with any genetic modifications that disrupt endogenous mouse ADAM6 genes. Persons of skill will recognize that this encompasses a wide variety of genetically modified mice that contain modifications of mouse immunoglobulin heavy chain variable gene loci. These include, for example, mice with a deletion or a replacement of all or a portion of mouse immunoglobulin heavy chain gene segments, regardless of other modifications. Non-limiting examples are described below.

[000261] In some aspects, genetically modified mice are provided that comprise an ectopic mouse, rodent, or other ADAM6 gene (or ortholog or homolog or fragment) functional in a mouse, and one or more human immunoglobulin variable and/or constant region gene segments. In various embodiments, other ADAM6 gene orthologs or homologs or fragments functional in a mouse may include sequences from bovine, canine, primate, rabbit or other non-human sequences.

[000262] In one aspect, a mouse is provided that comprises an ectopic ADAM6 sequence that encodes a functional ADAM6 protein, a replacement of all or substantially all mouse V_H gene segments with one or more human V_H gene segments; a replacement of all or substantially all mouse D_H gene segments with one or more human D_H gene segments; and a replacement of all or substantially all mouse J_H gene segments with one or more human J_H gene segments.

[000263] In one embodiment, the mouse further comprises a replacement of a mouse C_H1 nucleotide sequence with a human C_H1 nucleotide sequence. In one embodiment, the mouse further comprises a replacement of a mouse hinge nucleotide sequence with a human hinge nucleotide sequence. In one embodiment, the mouse further comprises a replacement of an immunoglobulin light chain variable locus (V_L and J_L) with a human immunoglobulin light chain variable locus. In one embodiment, the mouse further comprises a replacement of a mouse immunoglobulin light chain constant region nucleotide sequence with a human immunoglobulin light chain constant region nucleotide sequence. In a specific embodiment, the V_L, J_L, and C_L are immunoglobulin κ light chain sequences. In a specific embodiment, the mouse comprises a mouse C_H2 and a mouse C_H3 immunoglobulin constant region sequence fused with a human hinge and a human C_H1 sequence, such that the mouse immunoglobulin loci rearrange to form a gene that encodes a binding protein comprising (a) a heavy chain that has a human variable region, a human C_H1 region, a human hinge region, and a mouse C_H2 and a mouse C_H3 region;

and (b) a gene that encodes an immunoglobulin light chain that comprises a human variable domain and a human constant region.

[000264] In one aspect, a mouse is provided that comprises an ectopic ADAM6 sequence that encodes a functional ADAM6 protein, a replacement of all or substantially all mouse V_H gene segments with one or more human V_L gene segments, and optionally a replacement of all or substantially all D_H gene segments and/or J_H gene segments with one or more human D_H gene segments and/or human J_H gene segments, or optionally a replacement of all or substantially all D_H gene segments and J_H gene segments with one or more human J_L gene segments.

[000265] In one embodiment, the mouse comprises a replacement of all or substantially all mouse V_H, D_H, and J_H gene segments with one or more V_L, one or more D_H, and one or more J gene segments (*e.g.,* Jκ or Jλ), wherein the gene segments are operably linked to an endogenous mouse hinge region, wherein the mouse forms a rearranged immunoglobulin chain gene that contains, from 5' to 3' in the direction of transcription, human V_L – human or mouse D_H – human or mouse J – mouse hinge – mouse C_H2 – mouse C_H3. In one embodiment, the J region is a human Jκ region. In one embodiment, the J region is a human J_H region. In one embodiment, the J region is a human Jλ region. In one embodiment, the human V_L region is selected from a human Vλ region and a human Vκ region.

[000266] In specific embodiments, the mouse expresses a single variable domain antibody having a mouse or human constant region and a variable region derived from a human Vκ, a human D_H and a human Jκ; a human Vκ, a human D_H, and a human J_H; a human Vλ, a human D_H, and a human Jλ; a human Vλ, a human D_H, and a human J_H; a human Vκ, a human D_H, and a human Jλ; a human Vλ, a human D_H, and a human Jκ. In specific embodiment, recombination recognition sequences are modified so as to allow for productive rearrangements to occur between recited V, D, and J gene segments or between recited V and J gene segments.

[000267] In one aspect, a mouse is provided that comprises an ectopic ADAM6 sequence that encodes a functional ADAM6 protein (or ortholog or homolog or functional fragment thereof), a replacement of all or substantially all mouse V_H gene segments with one or more human V_L gene segments, a replacement of all or substantially all mouse D_H gene segment and J_H gene segments with human J_L gene segments; wherein the mouse lacks a C_H1 and/or hinge region.

[000268] In one embodiment, the mouse lacks a sequence encoding a C_H1 domain. In one embodiment, the mouse lacks a sequence encoding a hinge region. In one embodiment, the mouse lacks a sequence encoding a C_H1 domain and a hinge region.

[000269] In a specific embodiment, the mouse expresses a binding protein that comprises a human immunoglobulin light chain variable domain (λ or κ) fused to a mouse C_H2 domain that is attached to a mouse C_H3 domain.

[000270] In one aspect, a mouse is provided that comprises an ectopic ADAM6 sequence that encodes a functional ADAM6 protein (or ortholog or homolog or functional fragment thereof), a replacement of all or substantially all mouse V_H gene segments with one or more human V_L gene segments, a replacement of all or substantially all mouse D_H and J_H gene segments with human J_L gene segments.

[000271] In one embodiment, the mouse comprises a deletion of an immunoglobulin heavy chain constant region gene sequence encoding a C_H1 region, a hinge region, a C_H1 and a hinge region, or a C_H1 region and a hinge region and a C_H2 region.

[000272] In one embodiment, the mouse makes a single variable domain binding protein comprising a homodimer selected from the following: (a) human V_L – mouse C_H1 – mouse C_H2 – mouse C_H3; (b) human V_L – mouse hinge – mouse C_H2 – mouse C_H3; (c) human V_L – mouse C_H2 – mouse C_H3.

[000273] In one aspect, a mouse is provided with a disabled endogenous heavy chain immunoglobulin locus, comprising a disabled or deleted endogenous mouse ADAM6 locus, wherein the mouse comprises a nucleic acid sequence that expresses a human or mouse or human/mouse or other chimeric antibody. In one embodiment, the nucleic acid sequence is present on a transgene integrated that is randomly integrated into the mouse genome. In one embodiment, the nucleic acid sequence is on an episome (*e.g.*, a chromosome) not found in a wild-type mouse.

[000274] In one embodiment, the mouse further comprises a disabled endogenous immunoglobulin light chain locus. In a specific embodiment, the endogenous immunoglobulin light chain locus is selected from a kappa (κ) and a lambda (λ) light chain locus. In a specific embodiment, the mouse comprises a disabled endogenous κ light chain locus and a disabled λ light chain locus, wherein the mouse expresses an antibody that comprises a human immunoglobulin heavy chain variable domain and a human immunoglobulin light chain domain. In one embodiment, the human immunoglobulin light chain domain is selected from a human κ light chain domain and a human λ light chain domain.

[000275] In one aspect, a genetically modified animal is provided that expresses a chimeric antibody and expresses an ADAM6 protein or ortholog or homolog thereof that is functional in the genetically modified animal.

[000276] In one embodiment, the genetically modified animal is selected from a mouse and a rat. In one embodiment, the genetically modified animal is a mouse, and the ADAM6

protein or ortholog or homolog thereof is from a mouse strain that is a different strain than the genetically modified animal. In one embodiment, the genetically modified animal is a rodent of family Cricetidae (e.g., a hamster, a New World rat or mouse, a vole), and the ADAM6 protein ortholog or homolog is from a rodent of family Muridae (e.g., true mouse or rat, gerbil, spiny mouse, crested rat). In one embodiment, the genetically modified animal is a rodent of the family Muridae, and the ADAM6 protein ortholog or homolog is from a rodent of family Cricetidae.

[000277] In one embodiment, the chimeric antibody comprises a human variable domain and a constant region sequence of a rodent. In one embodiment, the rodent is selected from a rodent of the family Cricetidae and a rodent of family Muridae, In a specific embodiment, the rodent of the family Cricetidae and of the family Muridae is a mouse. In a specific embodiment, the rodent of the family Cricetidae and of the family Muridae is a rat. In one embodiment, the chimeric antibody comprises a human variable domain and a constant domain from an animal selected from a mouse or rat; in a specific embodiment, the mouse or rat is selected from the family Cricetidae and the family Muridae. In one embodiment, the chimeric antibody comprises a human heavy chain variable domain, a human light chain variable domain and a constant region sequence derived from a rodent selected from mouse and rat, wherein the human heavy chain variable domain and the human light chain are cognate. In a specific embodiment, cognate includes that the human heavy chain and the human light chain variable domains are from a single B cell that expresses the human light chain variable domain and the human heavy chain variable domain together and present the variable domains together on the surface of an individual B cell.

[000278] In one embodiment, the chimeric antibody is expressed from an immunoglobulin locus. In one embodiment, the heavy chain variable domain of the chimeric antibody is expressed from a rearranged endogenous immunoglobulin heavy chain locus. In one embodiment, the light chain variable domain of the chimeric antibody is expressed from a rearranged endogenous immunoglobulin light chain locus. In one embodiment, the heavy chain variable domain of the chimeric antibody and/or the light chain variable domain of the chimeric antibody is expressed from a rearranged transgene (*e.g.*, a rearranged nucleic acid sequence derived from an unrearranged nucleic acid sequence integrated into the animal's genome at a locus other than an endogenous immunoglobulin locus). In one embodiment, the light chain variable domain of the chimeric antibody is expressed from a rearranged transgene (*e.g.*, a rearranged nucleic acid sequence derived from an unrearranged nucleic acid sequence integrated into the animal's genome at a locus other than an endogenous immunoglobulin locus).

[000279] In a specific embodiment, the transgene is expressed from a transcriptionally

active locus, *e.g.*, a ROSA26 locus, *e.g.*, a murine (*e.g.*, mouse) ROSA26 locus.

[000280] In one aspect, a non-human animal is provided, comprising a humanized immunoglobulin heavy chain locus, wherein the humanized immunoglobulin heavy chain locus comprises a non-human ADAM6 sequence or ortholog or homolog thereof.

[000281] In one embodiment, the non-human animal is a rodent selected from a mouse, a rat, and a hamster.

[000282] In one embodiment, the non-human ADAM6 ortholog or homolog is a sequence that is orthologous and/or homologous to a mouse ADAM6 sequence, wherein the ortholog or homolog is functional in the non-human animal.

[000283] In one embodiment, the non-human animal is selected from a mouse, a rat, and a hamster and the ADAM6 ortholog or homolog is from a non-human animal selected from a mouse, a rat, and a hamster. In a specific embodiment, the non-human animal is a mouse and the ADAM6 ortholog or homolog is from an animal that is selected from a different mouse species, a rat, and a hamster. In specific embodiment, the non-human animal is a rat, and the ADAM6 ortholog or homolog is from a rodent that is selected from a different rat species, a mouse, and a hamster. In a specific embodiment, the non-human animal is a hamster, and the ADAM6 ortholog or homolog is form a rodent that is selected from a different hamster species, a mouse, and a rat.

[000284] In a specific embodiment, the non-human animal is from the suborder Myomorpha, and the ADAM6 sequence is from an animal selected from a rodent of superfamily Dipodoidea and a rodent of the superfamily Muroidea. In a specific embodiment, the rodent is a mouse of superfamily Muroidea, and the ADAM6 ortholog or homolog is from a mouse or a rat or a hamster of superfamily Muroidea.

[000285] In one embodiment, the humanized heavy chain locus comprises one or more human V_H gene segments, one or more human D_H gene segments and one or more human J_H gene segments. In a specific embodiment, the one or more human V_H gene segments, one or more human D_H gene segments and one or more human J_H gene segments are operably linked to one or more human, chimeric and/or rodent (e.g., mouse or rat) constant region genes. In one embodiment, the constant region genes are mouse. In one embodiment, the constant region genes are rat. In one embodiment, the constant region genes are hamster. In one embodiment, the constant region genes comprise a sequence selected from a hinge, a C_H2, a C_H3, and a combination thereof. In specific embodiment, the constant region genes comprise a hinge, a C_H2, and a C_H3 sequence.

[000286] In one embodiment, the non-human ADAM6 sequence is contiguous with a human immunoglobulin heavy chain sequence. In one embodiment, the non-human ADAM6 sequence is positioned within a human immunoglobulin heavy chain sequence. In a specific embodiment, the human immunoglobulin heavy chain sequence comprises a V,

D and/or J gene segment.

[000287] In one embodiment, the non-human ADAM6 sequence is positioned between two V gene segments. In one embodiment, the non-human ADAM6 sequence is juxtaposed between a V and a D gene segment. In one embodiment, the mouse ADAM6 sequence is positioned between a V and a J gene segment. In one embodiment, the mouse ADAM6 sequence is juxtaposed between a D and a J gene segment.

[000288] In one aspect, a genetically modified non-human animal is provided, comprising a B cell that expresses a human V_H domain cognate with a human V_L domain from an immunoglobulin locus, wherein the non-human animal expresses a non-immunoglobulin non-human protein from the immunoglobulin locus. In one embodiment, the non-immunoglobulin non-human protein is an ADAM protein. In a specific embodiment, the ADAM protein is an ADAM6 protein or homolog or ortholog or functional fragment thereof.

[000289] In one embodiment the non-human animal is a rodent (*e.g.*, mouse or rat). In one embodiment, the rodent is of family Muridae. In one embodiment, the rodent is of subfamily Murinae. In a specific embodiment, the rodent of subfamily Murinae is selected from a mouse and a rat.

[000290] In one embodiment, the non-immunoglobulin non-human protein is a rodent protein. In one embodiment, the rodent is of family Muridae. In one embodiment, the rodent is of subfamily Murinae. In a specific embodiment, the rodent is selected from a mouse, a rat, and a hamster.

[000291] In one embodiment, the human V_H and V_L domains are attached directly or through a linker to an immunoglobulin constant domain sequence. In a specific embodiment, the constant domain sequence comprises a sequence selected from a hinge, a C_H2 a C_H3, and a combination thereof. In a specific embodiment, the human V_L domain is selected from a $V\kappa$ or a $V\lambda$ domain.

[000292] In one aspect, a genetically modified non-human animal is provided, comprising in its germline a human immunoglobulin sequence, wherein the sperm of a male non-human animal is characterized by an *in vivo* migration defect. In one embodiment, the *in vivo* migration defect comprises an inability of the sperm of the male non-human animal to migrate from a uterus through an oviduct of a female non-human animal of the same species. In one embodiment, the non-human animal lacks a nucleotide sequence that encodes and ADAM6 protein or functional fragment thereof. In a specific embodiment, the ADAM6 protein or functional fragment thereof includes an ADAM6a and/or an ADAM6b protein or functional fragments thereof. In one embodiment, the non-human animal is a rodent. In a specific embodiment, the rodent is selected from a mouse, a rat, and a hamster.

[000293] In one aspect, a non-human animal is provided, comprising a human

immunoglobulin sequence contiguous with a non-human sequence that encodes an ADAM6 protein or ortholog or homolog or functional fragment thereof. In one embodiment, the non-human animal is a rodent. In a specific embodiment, the rodent is selected from a mouse, a rat, and a hamster.

[000294] In one embodiment, the human immunoglobulin sequence is an immunoglobulin heavy chain sequence. In one embodiment, the immunoglobulin sequence comprises one or more V_H gene segments. In one embodiment, the human immunoglobulin sequence comprises one or more D_H gene segments. In one embodiment, the human immunoglobulin sequence comprises one or more J_H gene segments. In one embodiment, the human immunoglobulin sequence comprises one or more V_H gene segments, one or more D_H gene segments and one or more J_H gene segments.

[000295] In one embodiment, the immunoglobulin sequence comprises one or more V_H gene segments have a high frequency in natural human repertoires. In a specific embodiment, the one or more V_H gene segments comprise no more than two V_H gene segments, no more than three V_H gene segments, no more than four V_H gene segments, no more than five V_H gene segments, no more than six V_H gene segments, no more than seven VH gene segments, no more than eight V_H gene segments, no more than nine V_H gene segments, no more than 10 V_H gene segments, no more than 11 V_H gene segments, no more than 12 V_H gene segments, no more than 13 V_H gene segments, no more than 14 V_H gene segments, no more than 15 V_H gene segments, no more than 16, V_H gene segments, no more than 17 V_H gene segments, no more than 18 V_H gene segments, no more than 19 V_H gene segments, no more than 20 V_H gene segments, no more than 21 V_H gene segments, no more than 22 V_H gene segments or no more than 23 V_H gene segments.

[000296] In a specific embodiment, the one or more V_H gene segments comprise five V_H gene segments. In a specific embodiment, the one or more V_H gene segments comprise 10 V_H gene segments. In a specific embodiment, the one or more V_H gene segments comprise 15 V_H gene segments. In a specific embodiment, the one or more V_H gene segments comprise 20 V_H gene segments.

[000297] In various embodiments, the V_H gene segments are selected from V_H6-1, V_H1-2, V_H1-3, V_H2-5, V_H3-7, V_H1-8, V_H3-9, V_H3-11, V_H3-13, V_H3-15, V_H3-16, V_H1-18, V_H3-20, V_H3-21, V_H3-23, V_H1-24, V_H2-26, V_H4-28, V_H3-30, V_H4-31, V_H3-33, V_H4-34, V_H3-35, V_H3-38, V_H4-39, V_H3-43, V_H1-45, V_H1-46, V_H3-48, V_H3-49, V_H5-51, V_H3-53, V_H1-58, V_H4-59, V_H4-61, V_H3-64, V_H3-66, V_H1-69, $_{VH}$2-70, V_H3-72, V_H3-73 and V_H3-74.

[000298] In various embodiments, the V_H gene segments are selected from V_H1-2, V_H1-8, V_H1-18, V_H1-46, V_H1-69, V_H3-7, V_H3-9, V_H3-11, V_H3-13, V_H3-15, V_H3-21, V_H3-23, V_H3-30,

V_H3-33, V_H3-43, V_H3-48, V_H4-31, V_H4-34, V_H4-39, V_H4-59, V_H5-51 and V_H6-1.

[000299] In various embodiments, the V_H gene segments are selected from V_H1-18, V_H1-46, V_H1-69, V_H3-7, V_H3-11, V_H3-15, V_H3-21, V_H3-23, V_H3-30, V_H3-33, V_H3-48, V_H4-34, V_H4-39, V_H4-59 and V_H5-51.

[000300] In various embodiments, the V_H gene segments are selected from V_H1-18, V_H1-69, V_H3-7, V_H3-11, V_H3-15, V_H3-21, V_H3-23, V_H3-30, V_H3-43, V_H3-48, V_H4-39, V_H4-59 and V_H5-51.

[000301] In various embodiments, the V_H gene segments are selected from V_H1-18, V_H3-11, V_H3-21, V_H3-23, V_H3-30, V_H4-39 and V_H4-59.

[000302] In various embodiments, the V_H gene segments are selected from V_H1-18, V_H3-21, V_H3-23, V_H3-30 and V_H4-39.

[000303] In various embodiments, the V_H gene segments are selected from V_H1-18, V_H3-23 and V_H4-39.

[000304] In various embodiments, the V_H gene segments are selected from V_H3-21, V_H3-23 and V_H3-30.

[000305] In various embodiments, the V_H gene segments are selected from V_H3-23, V_H3-30 and V_H4-39.

[000306] In a specific embodiment, human immunoglobulin sequence comprises at least 18 V_H gene segments, 27 D_H gene segments and six J_H gene segments. In a specific embodiment, the human immunoglobulin sequence comprises at least 39 V_H gene segments, 27 D_H gene segments and six J_H gene segments. In a specific embodiment, the human immunoglobulin sequence comprises at least 80 V_H gene segments, 27 D_H gene segments and six J_H gene segments.

[000307] In one embodiment, the non-human animal is a mouse, and the mouse comprises a replacement of endogenous mouse V_H gene segments with one or more human V_H gene segments, wherein the human V_H gene segments are operably linked to a mouse C_H region gene, such that the mouse rearranges the human V_H gene segments and expresses a reverse chimeric immunoglobulin heavy chain that comprises a human V_H domain and a mouse C_H. In one embodiment, 90-100% of unrearranged mouse V_H gene segments are replaced with at least one unrearranged human V_H gene segment. In a specific embodiment, all or substantially all of the endogenous mouse V_H gene segments are replaced with at least one unrearranged human V_H gene segment. In one embodiment, the replacement is with at least 19, at least 39, or at least 80 or 81 unrearranged human V_H gene segments. In one embodiment, the replacement is with at least 12 functional unrearranged human V_H gene segments, at least 25 functional unrearranged human V_H gene segments, or at least 43 functional unrearranged human V_H gene segments. In one embodiment, the mouse comprises a replacement of all mouse D_H

and J_H segments with at least one unrearranged human D_H segment and at least one unrearranged human J_H segment. In one embodiment, the at least one unrearranged human D_H segment is selected from 1-1, 1-7, 1-26, 2-8, 2-15, 3-3, 3-10, 3-16, 3-22, 5-5, 5-12, 6-6, 6-13, 7-27, and a combination thereof. In one embodiment, the at least one unrearranged human J_H segment is selected from 1, 2, 3, 4, 5, 6, and a combination thereof. In a specific embodiment, the one or more human V_H gene segment is selected from a 1-2, 1-8, 1-24, 1-69, 2-5, 3-7, 3-9, 3-11, 3-13, 3-15, 3-20, 3-23, 3-30, 3-33, 3-48, 3-53, 4-31, 4-39, 4-59, 5-51, a 6-1 human V_H gene segment, and a combination thereof.

[000308] In various embodiments, the human immunoglobulin sequence is in operable linkage with a constant region in the germline of the non-human animal (*e.g.*, the rodent, *e.g.*, the mouse, rat, or hamster). In one embodiment, the constant region is a human, chimeric human/mouse or chimeric human/rat or chimeric human/hamster, a mouse, a rat, or a hamster constant region. In one embodiment, the constant region is a rodent (*e.g.*, mouse or rat or hamster) constant region. In a specific embodiment, the rodent is a mouse or rat. In various embodiments, the constant region comprises at least a C_H2 domain and a C_H3 domain.

[000309] In one embodiment, the human immunoglobulin heavy chain sequence is located at an immunoglobulin heavy chain locus in the germline of the non-human animal (*e.g.*, the rodent, *e.g.*, the mouse or rat or hamster). In one embodiment, the human immunoglobulin heavy chain sequence is located at a non-immunoglobulin heavy chain locus in the germline of the non-human animal, wherein the non-heavy chain locus is a transcriptionally active locus. In a specific embodiment, the non-heavy chain locus is a ROSA26 locus.

[000310] In various aspects, the non-human animal further comprises a human immunoglobulin light chain sequence (*e.g.*, one or more unrearranged light chain V and J sequences, or one or more rearranged VJ sequences) in the germline of the non-human animal. In a specific embodiment, the immunoglobulin light chain sequence is an immunoglobulin κ light chain sequence. In one embodiment, the human immunoglobulin light chain sequence comprises one or more V_L gene segments. In one embodiment, the human immunoglobulin light chain sequence comprises one or more J_L gene segments. In one embodiment, the human immunoglobulin light chain sequence comprises one or more V_L gene segments and one or more J_L gene segments. In a specific embodiment, the human immunoglobulin light chain sequence comprises at least 16 $V\kappa$ gene segments and five $J\kappa$ gene segments. In a specific embodiment, the human immunoglobulin light chain sequence comprises at least 30 $V\kappa$ gene segments and five $J\kappa$ gene segments. In a specific embodiment, the human immunoglobulin light chain sequence comprises at least

40 Vκ gene segments and five Jκ gene segments. In various embodiments, the human immunoglobulin light chain sequence is in operable linkage with a constant region in the germline of the non-human animal (e.g., rodent, e.g., mouse or rat or hamster). In one embodiment, the constant region is a human, chimeric human/rodent, mouse, rat, or hamster constant region. In a specific embodiment, the constant region is a mouse or rat constant region. In a specific embodiment, the constant region is a mouse κ constant (mCκ) region or a rat κ constant (rCκ) region.

[000311] In one embodiment, the non-human animal is a mouse and the mouse comprises a replacement of all or substantially all Vκ and Jκ gene segments with at least six human Vκ gene segments and at least one Jκ gene segment. In one embodiment, all or substantially all Vκ and Jκ gene segments are replaced with at least 16 human Vκ gene segments (human Vκ) and at least one Jκ gene segment. In one embodiment, all or substantially all Vκ and Jκ gene segments are replaced with at least 30 human Vκ gene segments and at least one Jκ gene segment. In one embodiment, all or substantially all Vκ and Jκ gene segments are replaced with at least 40 human Vκ gene segments and at least one Jκ gene segment. In one embodiment, the at least one Jκ gene segment comprises two, three, four, or five human Jκ gene segments.

[000312] In one embodiment, the human Vκ gene segments comprise Vκ4-1, Vκ5-2, Vκ7-3, Vκ2-4, Vκ1-5, and Vκ1-6. In one embodiment, the Vκ gene segments comprise Vκ3-7, Vκ1-8, Vκ1-9, Vκ2-10, Vκ3-11, Vκ1-12, Vκ1-13, Vκ2-14, Vκ3-15 and Vκ1-16. In one embodiment, the human Vκ gene segments comprise Vκ1-17, Vκ2-18, Vκ2-19, Vκ3-20, Vκ6-21, Vκ1-22, Vκ1-23, Vκ2-24, Vκ3-25, Vκ2-26, Vκ1-27, Vκ2-28, Vκ2-29, and Vκ2-30. In one embodiment, the human Vκ gene segments comprise Vκ3-31, Vκ1-32, Vκ1-33, Vκ3-34, Vκ1-35, Vκ2-36, Vκ1-37, Vκ2-38, Vκ1-39, and Vκ2-40.

[000313] In a specific embodiment, the Vκ gene segments comprise contiguous human immunoglobulin κ gene segments spanning the human immunoglobulin κ light chain locus from Vκ4-1 through Vκ2-40, and the Jκ gene segments comprise contiguous gene segments spanning the human immunoglobulin κ light chain locus from Jκ1 through Jκ5.

[000314] In one embodiment, the human immunoglobulin light chain sequence is located at an immunoglobulin light chain locus in the germline of the non-human animal. In a specific embodiment, the immunoglobulin light chain locus in the germline of the non-human animal is an immunoglobulin κ light chain locus. In one embodiment, the human immunoglobulin light chain sequence is located at a non-immunoglobulin light chain locus in the germline of the non-human animal that is transcriptionally active. In a specific embodiment, the non-immunoglobulin locus is a ROSA26 locus.

[000315] In one aspect, a method of making a human antibody is provided, wherein the human antibody comprises variable domains derived from one or more variable region nucleic acid sequences encoded in a cell of a non-human animal as described herein.

[000316] In one aspect, a pharmaceutical composition is provided, comprising a polypeptide that comprises antibody or antibody fragment that is derived from one or more variable region nucleic acid sequences isolated from a non-human animal as described herein. In one embodiment, the polypeptide is an antibody. In one embodiment, the polypeptide is a heavy chain only antibody. In one embodiment, the polypeptide is a single chain variable fragment (e.g., an scFv).

[000317] In one aspect, use of a non-human animal as described herein to make an antibody is provided. In various embodiments, the antibody comprises one or more variable domains that are derived from one or more variable region nucleic acid sequences isolated from the non-human animal. In a specific embodiment, the variable region nucleic acid sequences comprise immunoglobulin heavy chain gene segments. In a specific embodiment, the variable region nucleic acid sequences comprise immunoglobulin light chain gene segments.

EXAMPLES

[000318] The following examples are provided so as to describe how to make and use methods and compositions of the invention, and are not intended to limit the scope of what the inventors regard as their invention. Unless indicated otherwise, temperature is indicated in Celsius, and pressure is at or near atmospheric.

Example 1

Humanization of Mouse Immunoglobulin Genes

[000319] Human and mouse bacterial artificial chromosomes (BACs) were used to engineer 13 different BAC targeting vectors (BACvecs) for humanization of the mouse immunoglobulin heavy chain and κ light chain loci. Tables 1 and 2 set forth descriptions of the steps performed for construction of all BACvecs employed for the humanization of mouse immunoglobulin heavy chain and κ light chain loci, respectively.

[000320] Identification of human and mouse BACs. Mouse BACs that span the 5' and 3' ends of the immunoglobulin heavy chain and κ light chain loci were identified by hybridization of filters spotted with BAC library or by PCR screening mouse BAC library DNA pools. Filters were hybridized under standard conditions using probes that corresponded to the regions of interest. Library pools were screened by PCR using unique primer pairs that flank the targeted region of interest. Additional PCR using the same

primers was performed to deconvolute a given well and isolate the corresponding BAC of interest. Both BAC filters and library pools were generated from 129 SvJ mouse ES cells (Incyte Genomics/Invitrogen). Human BACs that cover the entire immunoglobulin heavy chain and κ light chain loci were identified either by hybridization of filters spotted with BAC library (Caltech B, C, or D libraries & RPCI-11 library, Research Genetics/Invitrogen) through screening human BAC library pools (Caltech library, Invitrogen) by a PCR-based method or by using a BAC end sequence database (Caltech D library, TIGR).

[000321] Construction of BACvecs by bacterial homologous recombination and ligation. Bacterial homologous recombination (BHR) was performed as described (Valenzuela *et al.*, 2003; Zhang *et al.,* 1998, A new logic for DNA engineering using recombination in Escherichia coli, *Nat Genet* 20:123-128). In most cases, linear fragments were generated by ligating PCR-generated homology boxes to cloned cassettes followed by gel isolation of ligation products and electroporation into BHR-competent bacteria harboring the target BAC. After selection on appropriate antibiotic petri dishes, correctly recombined BACs were identified by PCR across both novel junctions followed by restriction analysis on pulsed-field gels (Schwartz and Cantor, 1984, Separation of yeast chromosome-sized DNAs by pulsed field gradient gel electrophoresis, *Cell* 37:67-75) and spot-checking by PCR using primers distributed across the human sequences.

[000322] A 3hV_H BACvec was constructed using three sequential BHR steps for the initial step of humanization of the immunoglobulin heavy chain locus (FIG. 4A and Table 1). In the first step (Step 1), a cassette was introduced into a human parental BAC upstream from the human V_H1-3 gene segment that contains a region of homology to the mouse immunoglobulin heavy chain locus (HB1), a gene that confers kanamycin resistance in bacteria and G418 resistance in animals cells (kanR) and a site-specific recombination site (*e.g.*, *lox*P). In the second step (Step 2), a second cassette was introduced just downstream from the last J_H segment that contains a second region of homology to the mouse immunoglobulin heavy chain locus (HB2) and a gene that confers resistance in bacteria to spectinomycin (specR). This second step included deleting human immunoglobulin heavy chain locus sequences downstream from J_H6 and the BAC vector chloramphenicol resistance gene (cmR). In the third step (Step 3), the doubly modified human BAC (B1) was then linearized using I-CeuI sites that had been added during the first two steps and integrated into a mouse BAC (B2) by BHR through the two regions of homology (HB1 and HB2). The drug selections for first (cm/kan), second (spec/kan) and third (cm/kan) steps were designed to be specific for the desired products. Modified BAC clones were analyzed by pulse-filed gel electrophoresis (PFGE) after digestion with restriction enzymes to determine appropriate construction (FIG. 4B).

[000323] In a similar fashion, 12 additional BACvecs were engineered for humanization of the heavy chain and κ light chain loci. In some instances, BAC ligation was performed in lieu of BHR to conjoin two large BACs through introduction of rare restriction sites into both parental BACvecs by BHR along with careful placement of selectable markers. This allowed for the survival of the desired ligation product upon selection with specific drug marker combinations. Recombinant BACs obtained by ligation after digestion with rare restriction enzymes were identified and screened in a similar fashion to those obtained by BHR (as described above).

Table 1

BACvec	Step	Description	Process
$3hV_H$	1	Insert upstream mouse homology box into human proximal BAC CTD-2572o2	BHR
	2	Insert downstream mouse homology box into human proximal BAC CTD-2572o2	BHR
	3	Insert $3hV_H/27hD_H/9hJ_H$ into mouse proximal BAC CT7-302a07 to create $3hV_H$ BACvec	BHR
DC	1	Insert cassette at distal end of mouse IgH locus using mouse BAC CT7-253i20	BHR
$18hV_H$	1	Insert specR marker at downstream end of $3hV_H$ insertion using human BAC CTD-2572o2	BHR
	2	Insert I-CeuI and Not sites flanking puroR at upstream end of $3hV_H$ insertion	BHR
	3	Insert Not site at downstream end of Rel2-408p02 BAC (≈10 kb downstream of V_H2-5)	BHR
	4	Insert I-CeuI site at upstream end of Rel2-408p02 BAC (≈23 kb upstream of V_H1-18)	BHR
	5	Ligate 184kb fragment from step 4 into 153kb vector from step 2	Ligation
	6	Trim human homology from CTD-2572o2 BAC deleting ≈85kb and leaving 65kb homology to $3hV_H$	BHR
	7	Insert cassette and Not site at distal end of mouse IgH locus in CT7-253i20 BAC	BHR
	8	Subclone mouse distal homology arm for insertion upstream from human BACs	Ligation
	9	Insert 20 kb mouse arm upstream of Rel2-408p02	BHR
	10	Swap selection cassette from hygR to neoR to create $18hV_H$ BACvec	BHR
$39hV_H$	1	Insert I-CeuI and PI-SceI sites flanking hygR into distal end of human BAC CTD-2534n10	BHR
	2	Insert CmR at proximal end of CTD-2534n10 BAC to allow for selection for ligation to RP11-72n10 BAC	BHR
	3	Insert PI-SceI site into RP11-72n10 BAC for ligation to CTD-2534n10 BAC	BHR

	4	Insert I-CeuI and AscI sites flanking puroR at distal end of RP11-72n10 BAC	BHR
	5	Ligate 161kb fragment from construct of step 4 into construct of step 2 replacing hygR	Ligation
	6	Insert neoR and AscI site at proximal end of mouse distal homology arm using CT7-253i20 BAC	BHR
	7	Insert specR and I-CeuI site at distal end of mouse distal homology arm	BHR
	8	Ligate mouse distal homology arm onto human insert from step 5	Ligation
	9	Swap selection cassette from neo to hyg using UbCp and pA as homology boxes to create 39hV_H BACvec	BHR
53hV_H	1	Insert specR at proximal end of human CTD-3074b5 BAC	BHR
	2	Insert AscI site at distal end of human CTD-3074b5 BAC	BHR
	3	Insert hygR and AscI site at proximal end of mouse distal homology arm using CT7-253i20 BAC	BHR
	4	Ligate mouse distal homology arm onto construct from step 2	Ligation
	5	Swap selection cassette from hyg to neo using UbCp and pA as homology boxes to create 53hV_H BACvec	BHR
70hV_H	1	Insert PI-SceI and I-CeuI sites flanking spec at distal end of human CTD-2195p5 BAC	BHR
	2	Insert I-CeuI site at proximal end of RP11-926p12 BAC for ligation to CTD-2195p5 BAC	BHR
	3	Insert PI-SceI and AscI sites at distal end of RP11-926p12 BAC for ligation of mouse arm	BHR
	4	Ligate mouse distal homology arm onto construct from step 3	Ligation
	5	Ligate mouse distal homology arm and hIgH fragment from RP11-926p12 BAC onto CTD-2195p5 BAC to create 70 hV_H BACvec	Ligation
80hV_H	1	Insert I-CeuI and AscI sites flanking hygR at distal end of CTD-2313e3 BAC	BHR
	2	Ligate mouse distal homology arm onto human CTD-2313e3 BAC from step 1 to create 80hV_H BACvec	Ligation

Table 2

BACvec	Step	Description	Process
Igκ-PC	1	Insert *lox*P site within mouse J-C intron using CT7-254m04 BAC	BHR
Igκ-DC	1	Insert *lox*P site at distal end of mouse Igκ locus using CT7-302g12 BAC	BHR
6hVκ	1	Insert PI-SceI site ≈400 bp downstream from hJκ5 in CTD-2366j12 BAC	BHR
	2	Insert I-CeuI and AscI sites flanking hygR at distal end of CTD-2366j12 BAC	BHR
	3	Insert I-CeuI and PI-SceI sites flanking puroR downstream from mJκ using CT7-254m04 BAC	BHR
	4	Insert hIgVκ/Jκ upstream from mouse Enhκ/Cκ using construct from step 3	Ligation
	5	Replace cmR in construct of step 4 with specR	BHR
	6	Insert Neo selection cassette at distal end of mouse Igκ locus using CT7-302g12 BAC	BHR
	7	Ligate mouse distal homology arm upstream of human insert in construct of step 6 to create 6hVκ BACvec	Ligation
16hVκ	1	Insert NeoR at distal end of RP11-1061b13 BAC	BHR
	2	Replace cmR in construct of step 1 with specR	BHR
	3	Insert Hyg selection cassette at distal end of mouse Igκ locus using CT7-302g12 BAC	BHR
	4	Ligate mouse distal homology arm upstream of human insert from construct of step 2 to create 16hVκ BACvec	Ligation
30hVκ	1	Insert HygR at distal end of RP11-99g6 BAC	BHR
	2	Replace cmR in construct of step 1 with specR	BHR
	3	Insert Neo selection cassette at distal end of mouse Igκ locus using CT7-302g12 BAC	BHR
	4	Ligate mouse distal homology arm upstream of human insert from construct of step 2 to create 30hVκ BACvec	Ligation
40hVκ	1	Insert NeoR at distal end of hIgH locus in CTD-2559d6 BAC	BHR
	2	Replace cmR in construct of step 1 with specR	BHR
	3	Ligate mouse distal homology arm upstream of human insert in construct of step 2 to create 40hVκ BACvec	Ligation

[000324] Modification of embryonic stem (ES) cells and generation of mice. ES cell (F1H4) targeting was performed using the VELOCIGENE® genetic engineering method as described (Valenzuela *et al.,* 2003). Derivation of mice from modified ES cells by either blastocyst (Valenzuela *et al.,* 2003) or 8-cell injection (Poueymirou *et al.,* 2007, F0 generation mice fully derived from gene-targeted embryonic stem cells allowing immediate phenotypic analyses, *Nat Biotechnol* 25:91-99) was as described. Targeted ES cells and mice were confirmed by screening DNA from ES cells or mice with unique sets of probes and primers in a PCR based assay (*e.g.,* FIG. 3A, 3B and 3C). All mouse studies were overseen and approved by Regeneron's Institutional Animal Care and Use Committee (IACUC).

[000325] Karyotype Analysis and Fluorescent *in situ* Hybridization (FISH). Karyotype Analysis was performed by Coriell Cell Repositories (Coriell Institute for Medical Research, Camden, NJ). FISH was performed on targeted ES cells as described (Valenzuela *et al.,* 2003). Probes corresponding to either mouse BAC DNA or human BAC DNA were labeled by nick translation (Invitrogen) with the fluorescently labeled dUTP nucleotides spectrum orange or spectrum green (Vysis).

[000326] Immunoglobulin Heavy Chain Variable Gene Locus. Humanization of the variable region of the heavy chain locus was achieved in nine sequential steps by the direct replacement of about three million base pairs (Mb) of contiguous mouse genomic sequence containing all V_H, D_H and J_H gene segments with about one Mb of contiguous human genomic sequence containing the equivalent human gene segments (FIG. 1A and Table 1) using VELOCIGENE® genetic engineering technology (see, *e.g.,* US Pat. No. 6,586,251 and Valenzuela *et al.,* 2003).

[000327] The intron between J_H gene segments and constant region genes (the J-C intron) contains a transcriptional enhancer (Neuberger, 1983, Expression and regulation of immunoglobulin heavy chain gene transfected into lymphoid cells, *EMBO J* 2:1373-1378) followed by a region of simple repeats required for recombination during isotype switching (Kataoka *et al.,* 1980, Rearrangement of immunoglobulin gamma 1-chain gene and mechanism for heavy-chain class switch, *PNAS USA* 77:919-923). The junction between human V_H-D_H-J_H region and the mouse C_H region (the proximal junction) was chosen to maintain the mouse heavy chain intronic enhancer and switch domain in order preserve both efficient expression and class switching of the humanized heavy chain locus within the mouse. The exact nucleotide position of this and subsequent junctions in all the replacements was possible by use of the VELOCIGENE® genetic engineering method (*supra*), which employed bacterial homologous recombination driven by synthesized oligonucleotides. Thus, the proximal junction was placed about 200 bp downstream from the last J_H gene segment and the distal junction was placed several hundred upstream of

the most 5' V_H gene segment of the human locus and about 9 kb downstream from the mouse V_H1-86 gene segment, also known as J558.55. The mouse V_H1-86 (J558.55) gene segment is the most distal heavy chain variable gene segment, reported to be a pseudogene in C57BL/6 mice, but potentially active, albeit with a poor RSS sequence, in the targeted 129 allele. The distal end of the mouse heavy chain locus reportedly may contain control elements that regulate locus expression and/or rearrangement (Pawlitzky *et al.,* 2006).

[000328] A first insertion of human immunoglobulin DNA sequence into the mouse was achieved using 144 kb of the proximal end of the human heavy chain locus containing 3 V_H, all 27 D_H and 9 J_H human gene segments inserted into the proximal end of the mouse IgH locus, with a concomitant 16.6 kb deletion of mouse genomic sequence, using about 75 kb of mouse homology arms (Step A, FIG. 2A; Tables 1 and 3, 3hV_H). This large 144kb insertion and accompanying 16.6 kb deletion was performed in a single step (Step A) that occurred with a frequency of 0.2% (Table 3). Correctly targeted ES cells were scored by a loss-of-native-allele (LONA) assay (Valenzuela *et al.,* 2003) using probes within and flanking the deleted mouse sequence and within the inserted human sequence, and the integrity of the large human insert was verified using multiple probes spanning the entire insertion (FIG. 3A, 3B and 3C). Because many rounds of sequential ES cell targeting were anticipated, targeted ES cell clones at this, and all subsequent, steps were subjected to karyotypic analysis (*supra*) and only those clones showing normal karyotypes in at least 17 of 20 spreads were utilized for subsequent steps.

[000329] Targeted ES cells from Step A were re-targeted with a BACvec that produced a 19 kb deletion at the distal end of the heavy chain locus (Step B, FIG. 2A). The Step B BACvec contained a hygromycin resistance gene (hyg) in contrast to the neomycin resistance gene (neo) contained on the BACvec of Step A. The resistance genes from the two BACvecs were designed such that, upon successful targeting to the same chromosome, approximately three Mb of the mouse heavy chain variable gene locus containing all of the mouse V_H gene segments other than V_H1-86 and all of the D_H gene segments other than DQ52, as well as the two resistance genes, were flanked by loxP sites; DQ52 and all of the mouse J_H chain gene segments were deleted in Step A. ES cell clones doubly targeted on the same chromosome were identified by driving the 3hV_H proximal cassette to homozygosity in high G418 (Mortensen *et al.,* 1992, Production of homozygous mutant ES cells with a single targeting construct, *Mol Cell Biol* 12:2391-2395) and following the fate of the distal hyg cassette. Mouse segments up to four Mb in size, having been modified in a manner to be flanked by loxP sites, have been successfully deleted in ES cells by transient expression of CRE recombinase with high efficiencies (up to ≈11%) even in the absence of drug selection (Zheng *et al.,* 2000, Engineering mouse

chromosomes with Cre-loxP: range, efficiency, and somatic applications, *Mol Cell Biol* 20:648-655). In a similar manner, the inventors achieved a three Mb deletion in 8% of ES cell clones following transient CRE expression (Step C, FIG. 2A; Table 3). The deletion was scored by the LONA assay using probes at either end of the deleted mouse sequence, as well as the loss of neo and hyg and the appearance of a PCR product across the deletion point containing the sole remaining loxP site. Further, the deletion was confirmed by fluorescence *in situ* hybridization (data not shown).

[000330] The remainder of the human heavy chain variable region was added to the $3hV_H$ allele in a series of 5 steps using the VELOCIGENE® genetic engineering method (Steps E–H, FIG. 2B), with each step involving precise insertion of up to 210 kb of human gene sequences. 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. The BACvecs of steps D, F and H contained neo selection cassettes, whereas those of steps E and G contained hyg selection cassettes, thus selections were alternated between G418 and hygromycin. Targeting in Step D was assayed by the loss of the unique PCR product across the distal loxP site of $3hV_H$ Hybrid Allele. Targeting for Steps E through I was assayed by loss of the previous selection cassette. In the final step (Step I, FIG. 2B), the neo selection cassette, flanked by Frt sites (McLeod *et al.,* 1986, Identification of the crossover site during FLP-mediated recombination in the *Saccharomyces cerevisiae* plasmid 2 microns circle, *Mol Cell Biol* 6:3357-3367), was removed by transient FLPe expression (Buchholz *et al.,* 1998, Improved properties of FLP recombinase evolved by cycling mutagenesis, *Nat Biotechnol* 16:657-662). The human sequences of the BACvecs for Steps D, E and G were derived from two parental human BACs each, whereas those from Steps F and H were from single BACs. Retention of human sequences was confirmed at every step using multiple probes spanning the inserted human sequences (as described above, *e.g.,* FIG. 3A, 3B and 3C). Only those clones with normal karyotype and germline potential were carried forward in each step. ES cells from the final step were still able to contribute to the germline after nine sequential manipulations (Table 3). Mice homozygous for each of the heavy chain alleles were viable, appeared healthy and demonstrated an essentially wild-type humoral immune system (see Example 3).

Table 3

Hybrid Allele	Human sequence	Targeting construct	Targeting efficiency	% usage	Total V_H	Functional V_H
$3hV_H$	144 kb	240 kb	0.2%	5	3	3
$3hV_H$/DC	144 kb	110 kb	0.1%	5	3	3
$3hV_H$-CRE	144 kb	-	8%	5	3	3
$18hV_H$	340 kb	272 kb	0.1%	25	18	12
$39hV_H$	550 kb	282 kb	0.2%	60	39	25
$53hV_H$	655 kb	186 kb	0.4%	65	53	29
$70hV_H$	850 kb	238 kb	0.5%	90	70	39
$80hV_H$	940 kb	124 kb	0.2%	100	80	43
$80hV_H$dNeo	940 kb	-	2.6%	100	80	43

[000331] Immunoglobulin κ Light Chain Variable Gene Locus. The κ light chain variable region was humanized in eight sequential steps by the direct replacement of about three Mb of mouse sequence containing all Vκ and Jκ gene segments with about 0.5 Mb of human sequence containing the proximal human Vκ and Jκ gene segments in a manner similar to that of the heavy chain (FIG. 1B; Tables 2 and 4).

[000332] The variable region of the human κ light chain locus contains two nearly identical 400 kb repeats separated by an 800 kb spacer (Weichhold *et al.,* 1993, The human immunoglobulin kappa locus consists of two copies that are organized in opposite polarity, *Genomics* 16:503-511). Because the repeats are so similar, nearly all of the locus diversity can be reproduced in mice by using the proximal repeat. Further, a natural human allele of the κ light chain locus missing the distal repeat has been reported (Schaible *et al.,* 1993, The immunoglobulin kappa locus: polymorphism and haplotypes of Caucasoid and non-Caucasoid individuals, *Hum Genet* 91:261-267). The inventors replaced about three Mb of mouse κ light chain variable gene sequence with about 0.5 Mb of human κ light chain variable gene sequence to effectively replace all of the mouse Vκ and Jκ gene segments with the proximal human Vκ and all of the human Jκ gene segments (FIG. 2C and 2D; Tables 2 and 4). In contrast to the method described in Example 1 for the heavy chain locus, the entire mouse Vκ gene region, containing all Vκ and Jκ gene segments, was deleted in a three-step process before any human sequence was added. First, a neo cassette was introduced at the proximal end of the variable region (Step A, FIG. 2C). Next, a hyg cassette was inserted at the distal end of the κ locus (Step B, FIG. 2C). Recombinase recognition sites (*e.g.,* loxP) were again situated within each

selection cassette such that CRE treatment induced deletion of the remaining 3 Mb of the mouse Vκ region along with both resistance genes (Step C, FIG. 2C).

[000333] A human genomic fragment of about 480 kb in size containing the entire immunoglobulin κ light chain variable region was inserted in four sequential steps (FIG. 2D; Tables 2 and 4), with up to 150 kb of human immunoglobulin κ light chain sequence inserted in a single step, using methods similar to those employed for the heavy chain (see Example 1). The final hygromycin resistance gene was removed by transient FLPe expression. As with the heavy chain, targeted ES cell clones were evaluated for integrity of the entire human insert, normal karyotype and germ-line potential after every step. Mice homozygous for each of the κ light chain alleles were generated and found to be healthy and of normal appearance.

Table 4

Hybrid Allele	Human sequence	Targeting construct	Targeting efficiency	% usage	Total Vκ	Functional Vκ
Igκ-PC	0	132 kb	1.1%	-	-	-
Igκ-PC/DC	0	90 kb	0.4%	-	-	-
Igκ-CRE	0	-	1%	-	-	-
6hVκ	110 kb	122 kb	0.3%	14	6	4
16hVκ	240 kb	203 kb	0.4%	47	16	11
30hVκ	390 kb	193 kb	0.1%	70	30	18
40hVκ	480 kb	185 kb	0.2%	100	40	25
40hVκdHyg	480 kb	-	0.7%	100	40	25

Example 2
Generation of Fully Humanized Mice by Combination of Multiple Humanized Immunoglobulin Alleles

[000334] At several points, ES cells bearing a portion of the human immunoglobulin heavy chain or κ light chain variable repertoires as described in Example 1 were microinjected and the resulting mice bred to create multiple versions of VELOCIMMUNE® mice with progressively larger fractions of the human germline immunoglobulin repertoires (Table 5; FIG. 5A and 5B). VELOCIMMUNE® 1 (V1) mice possess eighteen human V_H gene segments and all of the human D_H and J_H gene segments combined with sixteen human Vκ gene segments and all the human Jκ gene segments. VELOCIMMUNE® 2 (V2) and VELOCIMMUNE® (V3) mice have increased variable repertoires bearing a total of thirty-nine V_H and thirty Vκ, and eighty V_H and forty Vκ, respectively. Since the genomic

regions encoding the mouse V_H, D_H and J_H gene segments, and Vκ and Jκ gene segments, have been completely replaced, antibodies produced by all versions of VELOCIMMUNE® mice contain human variable regions linked to mouse constant regions. The mouse λ light chain loci remain intact in various embodiments of the VELOCIMMUNE® mice and serve as a comparator for efficiency of expression of the various VELOCIMMUNE® κ light chain loci.

[000335] Mice doubly homozygous for both immunoglobulin heavy chain and κ light chain humanizations were generated from a subset of the alleles described in Example 1. All genotypes observed during the course of breeding to generate the doubly homozygous mice occurred in roughly Mendelian proportions. Male progeny homozygous for each of the human heavy chain alleles demonstrated reduced fertility, which resulted from loss of mouse ADAM6 activity. The mouse heavy chain variable gene locus contains two embedded functional ADAM6 genes (ADAM6a and ADAM6b). During humanization of the mouse heavy chain variable gene locus, the inserted human genomic sequence contained an ADAM6 pseudogene. Mouse ADAM6 may be required for fertility, and thus lack of mouse ADAM6 genes in humanized heavy chain variable gene loci might lead to a reduction in fertility notwithstanding the presence of the human pseudogene. Examples 7-11 describe the reengineering of mouse ADAM6 genes into a humanized heavy chain variable gene locus, and restoration of wild-type level fertility in mice with a humanized heavy chain immunoglobulin locus.

Table 5

Version of VELOCIMMUNE® Mouse	Heavy Chain			κ Light Chain		
	Human V_H	Allele	5' V_H gene	Human Vκ	Allele	5' Vκ gene
V1	18	18hV_H	V_H1-18	16	16hVκ	Vκ1-16
V2	39	39hV_H	V_H4-39	30	30hVκ	Vκ2-29
V3	80	80hV_H	V_H3-74	40	40hVκ	Vκ2-40

Example 3

Lymphocyte Populations in Mice with Humanized Immunoglobulin Genes

[000336] Mature B cell populations in the three different versions of VELOCIMMUNE® mice were evaluated by flow cytometry.

[000337] Briefly, cell suspensions from bone marrow, spleen and thymus were made using standard methods. Cells were resuspended at $5x10^5$ cells/mL in BD Pharmingen FACS staining buffer, blocked with anti-mouse CD16/32 (BD Pharmingen), stained with the

appropriate cocktail of antibodies and fixed with BD CYTOFIX™ all according to the manufacturer's instructions. Final cell pellets were resuspended in 0.5 mL staining buffer and analyzed using a BD FACSCALIBUR™ and BD CELLQUEST PRO™ software. All antibodies (BD Pharmingen) were prepared in a mass dilution/cocktail and added to a final concentration of 0.5 mg/10^5 cells.

[000338] Antibody cocktails for bone marrow (A–D) staining were as follows: A: anti-mouse IgM^b-FITC, anti-mouse IgM^a-PE, anti-mouse CD45R(B220)-APC; B: anti-mouse CD43(S7)-PE, anti-mouse CD45R(B220)-APC; C: anti-mouse CD24(HSA)-PE; anti-mouse CD45R(B220)-APC; D: anti-mouse BP-1-PE, anti-mouse CD45R(B220)-APC.

[000339] Antibody cocktails for spleen and inguinal lymph node (E–H) staining were as follows: E: anti-mouse IgM^b-FITC, anti-mouse IgM^a-PE, anti-mouse CD45R(B220)-APC; F: anti-mouse Ig, λ1, λ2, λ3 Light Chain-FITC, anti mouse Igκ Light Chain-PE, anti-mouse CD45R(B220)-APC; G: anti-mouse Ly6G/C-FITC, anti-mouse CD49b(DX5)-PE, anti-mouse CD11b-APC; H: anti-mouse CD4(L3T4)-FITC, anti-mouse CD45R(B220)-PE, anti-mouse CD8a-APC. Results are shown in FIG. 6.

[000340] 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 (FIG. 6). The sizes of the $B220^+$ IgM^+ mature B cell populations in all versions of VELOCIMMUNE® mice tested were virtually identical to those of wild type mice, regardless of the number of V_H gene segments they contained. In addition, mice containing homozygous hybrid humanized immunoglobulin heavy chain loci, even those with only 3 V_H gene segments but normal mouse immunoglobulin κ light chain loci or mice containing homozygous hybrid humanized κ light chain loci with normal mouse immunoglobulin heavy chain loci, also had normal numbers of $B220^+$ IgM^+ cells in their peripheral compartments (not shown). These results indicate that chimeric loci with human variable gene segments and mouse constant regions can fully populate the mature B cell compartment. Further, the number of variable gene segments at either the heavy chain or κ light chain loci, and thus the theoretical diversity of the antibody repertoire, does not correlate with the ability to generate wild type populations of mature B cells. In contrast, mice with randomly integrated fully-human immunoglobulin transgenes and inactivated mouse immunoglobulin loci have reduced numbers of B cells in these compartments, with the severity of the deficit depending on the number of variable gene segments included in the transgene (Green and Jakobovits, 1998, Regulation of B cell development by variable gene complexity in mice reconstituted with human immunoglobulin yeast artificial chromosomes, *J Exp Med* 188:483-495). This demonstrates that the "*in situ* genetic humanization" strategy results in a fundamentally different functional outcome than the

randomly integrated transgenes achieved in the "knockout-plus-transgenic" approach.

[000341] Allelic Exclusion and Locus Choice. The ability to maintain allelic exclusion was examined in mice heterozygous for different versions of the humanized immunoglobulin heavy chain locus.

[000342] The humanization of the immunoglobulin loci was carried out in an F1 ES line (F1H4, Valenzuela *et al.,* 2003), derived from 129S6/SvEvTac and C57BL/6NTac heterozygous embryos. The human heavy chain germline variable gene sequences are targeted to the 129S6 allele, which carries the IgM^a haplotype, whereas the unmodified mouse C576BL/6N allele bears the IgM^b haplotype. These allelic forms of IgM can be distinguished by flow cytometry using antibodies specific to the polymorphisms found in the IgM^a or IgM^b alleles. As shown in FIG. 6 (bottom row), the B cells identified in mice heterozygous for each version of the humanized heavy chain locus only express a single allele, either IgM^a (the humanized allele) or IgM^b (the wild type allele). This demonstrates that the mechanisms involved in allelic exclusion are intact in VELOCIMMUNE® mice. In addition, the relative number of B cells positive for the humanized allele (IgM^a) is roughly proportional to the number of V_H gene segments present. The humanized immunoglobulin locus is expressed in approximately 30% of the B cells in VELOCIMMUNE® 1 heterozygote mice, which have 18 human V_H gene segments, and in 50% of the B cells in VELOCIMMUNE® 2 and 3 (not shown) heterozygote mice, with 39 and 80 human V_H gene segments, respectively. Notably, the ratio of cells expressing the humanized versus wild type mouse allele (0.5 for VELOCIMMUNE® 1 mice and 0.9 for VELOCIMMUNE® 2 mice) is greater than the ratio of the number of variable gene segments contained in the humanized versus wild type loci (0.2 for VELOCIMMUNE® 1 mice and 0.4 for VELOCIMMUNE® 2 mice). This may indicate that the probability of allele choice is intermediate between a random choice of one or the other chromosome and a random choice of any particular V segment RSS. Further, there may be a fraction of B-cells, but not all, in which one allele becomes accessible for recombination, completes the process and shuts down recombination before the other allele becomes accessible. In addition, the even distribution of cells that have surface IgM (sIgM) derived from either the hybrid humanized heavy chain locus or the wild type mouse heavy chain locus is evidence that the hybrid locus is operating at a normal level. In contrast, randomly integrated human immunoglobulin transgenes compete poorly with wild type mouse immunoglobulin loci (Bruggemann *et al.,* 1989, A repertoire of monoclonal antibodies with human heavy chains from transgenic mice, *PNAS* 86:6709-6713; Green *et al.,* 1994; Tuaillon *et al.,* 1993, Human immunoglobulin heavy-chain minilocus recombination in transgenic mice: gene-segment use in mu and gamma transcripts, *PNAS USA* 90:3720-3724). This further demonstrates the immunoglobulins produced by VELOCIMMUNE® mice are functionally

different than those produced by randomly integrated transgenes in mice made by "knockout-plus-transgenic" approaches.

[000343] Polymorphisms of the Cκ regions are not available in 129S6 or C57BL/6N to examine allelic exclusion of humanized versus non-humanized κ light chain loci. However, VELOCIMMUNE® mice all possess wild type mouse λ light chain loci, therefore, it is possible to observe whether rearrangement and expression of humanized κ light chain loci can prevent mouse λ light chain expression. The ratio of the number of cells expressing the humanized κ light chain relative to the number of cells expressing mouse λ light chain was relatively unchanged in VELOCIMMUNE® mice compared with wild type mice, regardless of the number of human Vκ gene segments inserted at the κ light chain locus (FIG. 6, third row from top). In addition there was no increase in the number of double positive (κ plus λ) cells, indicating that productive recombination at the hybrid κ light chain loci results in appropriate suppression of recombination of the mouse λ light chain loci. In contrast, mice containing randomly integrated κ light chain transgenes with inactivated mouse κ light chain loci—but wild type mouse λ light chain loci—exhibit dramatically increased λ/κ ratios (Jakobovits, 1998), implying that the introduced κ light chain transgenes do not function well in such mice. This further demonstrates the different functional outcome observed in immunoglobulins made by VELOCIMMUNE® mice as compared to those made by "knockout-plus-transgenic" mice.

[000344] **B cell Development.** Because the mature B cell populations in VELOCIMMUNE® mice resemble those of wild type mice (described above), it is possible that defects in early B cell differentiation are compensated for by the expansion of mature B cell populations. The various stages of B cell differentiation were examined by analysis of B cell populations using flow cytometry. Table 6 sets forth the ratio of the fraction of cells in each B cell lineage defined by FACs, using specific cell surface markers, in VELOCIMMUNE® mice compared to wild type littermates.

[000345] Early B cell development occurs in the bone marrow, and different stages of B cell differentiation are characterized by changes in the types and amounts of cell surface marker expression. These differences in surface expression correlate with the molecular changes occurring at the immunoglobulin loci inside the cell. The pro-B to pre-B cell transition requires the successful rearrangement and expression of functional heavy chain protein, while transition from the pre-B to mature B stage is governed by the correct rearrangement and expression of a κ or λ light chain. Thus, inefficient transition between stages of B cell differentiation can be detected by changes in the relative populations of B cells at a given stage.

Table 6

Version of VELOCIMMUNE® Mice	Bone Marrow				Spleen	
	pro-B	pre-B	Immature	Mature	Emerging	Mature
	$CD43^{hi}$ $B220^{lo}$	$CD24^{hi}$ $B220^{lo}$	$B220^{lo}$ IgM^{+}	$B220^{hi}$ IgM^{+}	$B220^{hi}$ IgM^{+} IgD^{+}	B220hi IgM^{+}
V1	1.1	1.0	0.9	1.0	1.1	1.0
V2	1.0	1.0	1.0	1.0	1.0	1.0
V3	1.0	1.0	1.1	1.0	1.0	1.1

[000346] No major defects were observed in B cell differentiation in any of the VELOCIMMUNE® mice. The introduction of human heavy chain gene segments does not appear to affect the pro-B to pre-B transition, and introduction of human κ light chain gene segments does not affect the pre-B to B transition in VELOCIMMUNE® mice. This demonstrates that "reverse chimeric" immunoglobulin molecules possessing human variable regions and mouse constants function normally in the context of B cell signaling and co-receptor molecules leading to appropriate B cell differentiation in a mouse environment. In contrast, the balance between the different populations during B cell differentiation are perturbed to varying extents in mice that contain randomly integrated immunoglobulin transgenes and inactivated endogenous heavy chain or κ light chain loci (Green and Jakobovits, 1998).

Example 4

Variable Gene Repertoire in Humanized Immunoglobulin Mice

[000347] Usage of human variable gene segments in the humanized antibody repertoire of VELOCIMMUNE® mice was analyzed by reverse transcriptase-polymerase chain reaction (RT-PCR) of human variable regions from multiple sources including splenocytes and hybridoma cells. Variable region sequence, gene segment usage, somatic hypermutation, and junctional diversity of rearranged variable region gene segments were determined.

[000348] Briefly, total RNA was extracted from $1x10^7$ – $2x10^7$ splenocytes or about 10^4 – 10^5 hybridoma cells using TRIZOL™ (Invitrogen) or Qiagen RNEASY™ Mini Kit (Qiagen) and primed with mouse constant region specific primers using the SUPERSCRIPT™ III One-Step RT-PCR system (Invitrogen). Reactions were carried out with 2-5 μL of RNA from each sample using the aforementioned 3' constant specific primers paired with pooled leader primers for each family of human variable regions for both the heavy chain and κ

light chain, separately. Volumes of reagents and primers, and RT-PCR/PCR conditions were performed according to the manufacturer's instructions. Primers sequences were based upon multiple sources (Wang and Stollar, 2000, Human immunoglobulin variable region gene analysis by single cell RT-PCR, *J Immunol Methods* 244:217-225; Ig-primer sets, Novagen). Where appropriate, nested secondary PCR reactions were carried out with pooled family-specific framework primers and the same mouse 3' immunoglobulin constant-specific primer used in the primary reaction. Aliquots (5 μL) from each reaction were analyzed by agarose electrophoresis and reaction products were purified from agarose using a MONTAGE™ Gel Extraction Kit (Millipore). Purified products were cloned using the TOPO™ TA Cloning System (Invitrogen) and transformed into DH10β *E. coli* cells by electroporation. Individual clones were selected from each transformation reaction and grown in 2 mL LB broth cultures with antibiotic selection overnight at 37°C. Plasmid DNA was purified from bacterial cultures by a kit-based approach (Qiagen).

[000349] Immunoglobulin Variable Gene Usage. Plasmid DNA of both heavy chain and κ light chain clones were sequenced with either T7 or M13 reverse primers on the ABI 3100 Genetic Analyzer (Applied Biosystems). Raw sequence data were imported into SEQUENCHER™ (v4.5, Gene Codes). Each sequence was assembled into contigs and aligned to human immunoglobulin sequences using IMGT V-Quest (Brochet *et al.,* 2008, IMGT/V-QUEST: the highly customized and integrated system for IG and TR standardized V-J and V-D-J sequence analysis, *Nucleic Acids Res* 36:W503-508) search function to identify human V_H, D_H, J_H and $V\kappa$, $J\kappa$ segment usage. Sequences were compared to germline sequences for somatic hypermutation and recombination junction analysis.

[000350] Mice were generated from ES cells containing the initial heavy chain modification (3hV_H-CRE Hybrid Allele, bottom of FIG. 2A) by RAG complementation (Chen *et al.,* 1993, RAG-2-deficient blastocyst complementation: an assay of gene function in lymphocyte development, *PNAS USA* 90:4528-4532), and cDNA was prepared from splenocyte RNA. The cDNA was amplified using primer sets (described above) specific for the predicted chimeric heavy chain mRNA that would arise by V(D)J recombination within the inserted human gene segments and subsequent splicing to either mouse IgM or IgG constant domains. Sequences derived from these cDNA clones (not shown) demonstrated that proper V(D)J recombination had occurred within the human variable gene sequences, that the rearranged human V(D)J gene segments were properly spliced in-frame to mouse constant domains, and that class-switch recombination had occurred. Further sequence analysis of mRNA products of subsequent hybrid immunoglobulin loci was performed.

[000351] In a similar experiment, B cells from non-immunized wild type and VELOCIMMUNE® mice were separated by flow cytometry based upon surface expression

of B220 and IgM or IgG. The $B220^+$ IgM^+ or surface IgG^+ ($sIgG^+$) cells were pooled and V_H and $V\kappa$ sequences were obtained following RT-PCR amplification and cloning (described above). Representative gene usage in a set of RT-PCR amplified cDNAs from unimmunized VELOCIMMUNE® 1 mice (Table 7) and VELOCIMMUNE® 3 mice (Table 8) was recorded (*defective RSS; †missing or pseudogene). Asterisk: gene segments with defective RSS. †: gene segment is missing or pseudogene.

Table 7

V_H	Observed
1-18	3
1-17P	0
3-16*	0
3-15	13
3-13	9
3-11	6
3-9	8
1-8	6
3-7	2
2-5	2
1-3	0
1-2	11
6-1	5

J_H	Observed
1	2
2	1
3	8
4	33
5	5
6	16

D_H	Observed
1-1	1
2-2	2
3-3	4
4-4	0
5-5	0
5-18	4
6-6	5
1-7	7
2-8	0
3-9	4
3-10	2
4-11	1
5-12	1
6-13	3
1-14	0
2-15	0
3-16	1
4-17	0
6-19	2
1-20	2
2-21	1
3-22	0
4-23	2
5-24	1
6-25	1
1-26	6
7-27	10

$V\kappa$	Observed
1-16	2
3-15	1
1-12	5
3-11	1
1-9	5
1-8	2
3-7*	0
1-6	5
1-5	8
5-2	6
4-1	8

$J\kappa$	Observed
1	12
2	10
3	5
4	10
5	0

Table 8

V_H	Observed
7-81†	0
3-74†	0
3-73	1
3-72	2
2-70	2
1-69	3
3-66	1
3-64	1
4-61	1
4-59	10
1-58	0
3-53	0
5-51	5
3-49	2
3-48	7
1-46	1
1-45	0
3-43	10
4-39	4
3-38*	0
3-35*	0
4-34	8
3-33	14
4-31	4
3-30	13
4-28	0
2-26	0
1-24	3
3-23	18
3-21	0
3-20	0
1-18	4
1-17P	1
3-16*	0
3-15	13
3-13	6
3-11	5
3-9	31
1-8	7
3-7	11
2-5	1
1-3	0

D_H	Observed
1-1	7
2-2	8
3-3	9
4-4	4
5-5	6
5-18	6
6-6	29
1-7	30
2-8	4
3-9	8
3-10	10
4-11	4
5-12	5
6-13	17
1-14	2
2-15	3
3-16	4
4-17	3
6-19	8
1-20	3
2-21	1
3-22	5
4-23	2
5-24	2
6-25	2
1-26	17
7-27	7

J_H	Observed
1	2
2	8
3	26
4	95
5	11
6	58

V_κ	Observed
2-40	1
1-39	34
1-37	2
1-33	35
2-30	8
2-29	2
2-28	7
1-27	5
2-24	7
6-21*	3
3-20	10
1-17	13
1-16	10
3-15	13
1-12	13
3-11	13
1-9	11
1-8	1
3-7*	0
1-6	6
1-5	7
5-2	0
4-1	21

J_κ	Observed
1	50
2	37
3	28
4	64
5	22

1-2	6
6-1	9

[000352] As shown in Tables 7 and 8, nearly all of the functional human V_H, D_H, J_H, $V\kappa$ and $J\kappa$ gene segments are utilized. Of the functional variable gene segments described but not detected in the VELOCIMMUNE® mice of this experiment, several have been reported to possess defective recombination signal sequences (RSS) and, thus, would not be expected to be expressed (Feeney, 2000, Factors that influence formation of B cell repertoire, *Immunol Res* 21:195-202). Analysis of several other sets of immunoglobulin sequences from various VELOCIMMUNE® mice, isolated from both naïve and immunized repertoires, has shown usage of these gene segments, albeit at lower frequencies (data not shown). Aggregate gene usage data has shown that all functional human V_H, D_H, J_H, $V\kappa$, and $J\kappa$ gene segments contained in VELOCIMMUNE® mice have been observed in various naïve and immunized repertoires (data not shown). Although the human V_H7-81 gene segment has been identified in the analysis of human heavy chain locus sequences (Matsuda *et al.,* 1998, The complete nucleotide sequence of the human immunoglobulin heavy chain variable region locus, *J Exp Med* 188:2151-2162), it is not present in the VELOCIMMUNE® mice as confirmed by re-sequencing of the entire VELOCIMMUNE® 3 mouse genome.

[000353] Sequences of heavy and light chains of antibodies are known to show exceptional variability, especially in short polypeptide segments within the rearranged variable domain. These regions, known as hypervariable regions or complementary determining regions (CDRs), create the binding site for antigen in the structure of the antibody molecule. The intervening polypeptide sequences are called framework regions (FRs). There are three CDRs (CDR1, CDR2, CDR3) and 4 FRs (FR1, FR2, FR3, FR4) in both heavy and light chains. One CDR, CDR3, is unique in that this CDR is created by recombination of both the V_H, D_H and J_H and $V\kappa$ and $J\kappa$ gene segments and generates a significant amount of repertoire diversity before antigen is encountered. This joining is imprecise due to both nucleotide deletions via exonuclease activity and non-template encoded additions via terminal deoxynucleotidyl transferase (TdT) and, thus, allows for novel sequences to result from the recombination process. Although FRs can show substantial somatic mutation due to the high mutability of the variable region as a whole, variability is not, however, distributed evenly across the variable region. CDRs are concentrated and localized regions of high variability in the surface of the antibody molecule that allow for antigen binding. Heavy chain and light chain sequences of selected antibodies from VELOCIMMUNE® mice around the CDR3 junction demonstrating junctional diversity are shown in FIG. 7A and 7B, respectively.

[000354] As shown in FIG. 7A, non-template encoded nucleotide additions (N-additions) are observed at both the V_H-D_H and D_H-J_H joint in antibodies from VELOCIMMUNE® mice, indicating proper function of TdT with the human segments. The endpoints of the V_H, D_H and J_H segments relative to their germline counterparts indicate that exonuclease activity has also occurred. Unlike the heavy chain locus, the human κ light chain rearrangements exhibit little or no TdT additions at CDR3, which is formed by the recombination of the Vκ and Jκ segments (FIG. 7B). This is expected due to the lack of TdT expression in mice during light chain rearrangements at the pre-B to B cell transition. The diversity observed in the CDR3 of rearranged human Vκ regions is introduced predominantly through exonuclease activity during the recombination event.

[000355] **Somatic hypermutation.** Additional diversity is added to the variable regions of rearranged immunoglobulin genes during the germinal center reaction by a process termed somatic hypermutation. B cells expressing somatically mutated variable regions compete with other B cells for access to antigen presented by the follicular dendritic cells. Those B cells with higher affinity for the antigen will further expand and undergo class switching before exiting to the periphery. Thus, B cells expressing switched isotypes typically have encountered antigen and undergone germinal center reactions and will have increased numbers of mutations relative to naïve B cells. Further, variable region sequences from predominantly naïve $sIgM^+$ B cells would be expected to have relatively fewer mutations than variable sequences from $sIgG^+$ B cells which have undergone antigen selection.

[000356] Sequences from random V_H or Vκ clones from $sIgM^+$ or $sIgG^+$ B cells from non-immunized VELOCIMMUNE® mice or $sIgG^+$ B cells from immunized mice were compared with their germline variable gene segments and changes relative to the germline sequence annotated. The resulting nucleotide sequences were translated *in silico* and mutations leading to amino acid changes also annotated. The data were collated from all the variable regions and the percent change at a given position was calculated (FIG. 8).

[000357] As shown in FIG. 8, human heavy chain variable regions derived from $sIgG^+$ B cells from non-immunized VELOCIMMUNE® mice exhibit many more nucleotides relative to $sIgM^+$ B cells from the same splenocyte pools, and heavy chain variable regions derived from immunized mice exhibit even more changes. The number of changes is increased in the complementarity-determining regions (CDRs) relative to the framework regions, indicating antigen selection. The corresponding amino acid sequences from the human heavy chain variable regions also exhibit significantly higher numbers of mutations in IgG versus IgM and even more in immunized IgG. These mutations again appear to be more frequent in the CDRs compared with the framework sequences, suggesting that the

antibodies were antigen-selected *in vivo*. A similar increase in the number the nucleotide and amino acid mutations are seen in the V_{κ} sequences derived from IgG^{+} B cells from immunized mice.

[000358] The gene usage and somatic hypermutation frequency observed in VELOCIMMUNE® mice demonstrate that essentially all gene segments present are capable of rearrangement to form fully functionally reverse chimeric antibodies in these mice. Further, VELOCIMMUNE® antibodies fully participate within the mouse immune system to undergo affinity selection and maturation to create fully mature human antibodies that can effectively neutralize their target antigen. VELOCIMMUNE® mice are able to mount robust immune responses to multiple classes of antigens that result in usage of a wide range of human antibodies that are both high affinity and suitable for therapeutic use (data not shown).

Example 5

Analysis of Lymphoid Structure and Serum Isotypes

[000359] The gross structures of spleen, inguinal lymph nodes, Peyer's patches and thymus of tissue samples from wild type or VELOCIMMUNE® mice stained with H&E were examined by light microscopy. The levels of immunoglobulin isotypes in serum collected from wild type and VELOCIMMUNE® mice were analyzed using LUMINEX™ technology.

[000360] Lymphoid Organ Structure. The structure and function of the lymphoid tissues are in part dependent upon the proper development of hematopoietic cells. A defect in B cell development or function may be exhibited as an alteration in the structure of the lymphoid tissues. Upon analysis of stained tissue sections, no significant difference in appearance of secondary lymphoid organs between wild type and VELOCIMMUNE® mice was identified (data not shown).

[000361] Serum Immunoglobulin Levels. The level of expression of each isotype is similar in wild type and VELOCIMMUNE® mice (FIG. 9A, 9B and 9C). This demonstrates that humanization of the variable gene segments had no apparent adverse effect upon class switching or immunoglobulin expression and secretion and therefore apparently maintain all the endogenous mouse sequences necessary for these functions.

Example 6

Immunization and Antibody Production in Humanized Immunoglobulin Mice

[000362] Different versions of VELOCIMMUNE® mice were immunized with antigen to examine the humoral response to foreign antigen challenge.

[000363] Immunization and Hybridoma Development. VELOCIMMUNE® and wild-type mice can be immunized with an antigen in the form of protein, DNA, a combination of

DNA and protein, or cells expressing the antigen. Animals are typically boosted every three weeks for a total of two to three times. Following each antigen boost, serum samples from each animal are collected and analyzed for antigen-specific antibody responses by serum titer determination. Prior to fusion, mice received a final pre-fusion boost of 5 μg protein or DNA, as desired, via intra-peritoneal and/or intravenous injections. Splenocytes are harvested and fused to Ag8.653 myeloma cells in an electrofusion chamber according to the manufacture's suggested protocol (Cyto Pulse Sciences Inc., Glen Burnie, MD). Ten days after culture, hybridomas are screened for antigen specificity using an ELISA assay (Harlow and Lane, 1988, Antibodies: A Laboratory Manual, Cold Spring Harbor Press, New York). Alternatively, antigen specific B cells are isolated directly from immunized VELOCIMMUNE® mice and screened using standard techniques, including those described here, to obtain human antibodies specific for an antigen of interest (*e.g.*, see US 2007/0280945A1, herein incorporated by reference in its entirety).

[000364] Serum Titer Determination. To monitor animal anti-antigen serum response, serum samples are collected about 10 days after each boost and the titers are determined using antigen specific ELISA. Briefly, Nunc MAXISORP™ 96 well plates are coated with 2 μg/mL antigen overnight at 4° C and blocked with bovine serum albumin (Sigma, St. Louis, MO). Serum samples in a serial 3 fold dilutions are allowed to bind to the plates for one hour at room temperature. The plates are then washed with PBS containing 0.05% Tween-20 and the bound IgG are detected using HRP-conjugated goat anti-mouse Fc (Jackson Immuno Research Laboratories, Inc., West Grove, PA) for total IgG titer, or biotin-labeled isotype specific or light chain specific polyclonal antibodies (Southern Biotech Inc.) for isotype specific titers, respectively. For biotin-labeled antibodies, following plate wash, HRP-conjugated streptavidin (Pierce, Rockford, IL) is added. All plates are developed using colorimetric substrates such as BD OPTEIA™ (BD Biosciences Pharmingen, San Diego, CA). After the reaction is stopped with 1 M phosphoric acid, optical absorptions at 450 nm are recorded and the data are analyzed using PRISM™ software from Graph Pad. Dilutions required to obtain two-fold of background signal are defined as titer.

[000365] In one experiment, VELOCIMMUNE® mice were immunized with human interleukin-6 receptor (hIL-6R). A representative set of serum titers for VELOCIMMUNE® and wild type mice immunized with hIL-6R is shown in FIG. 10A and 10B.

[000366] VELOCIMMUNE® and wild-type mice mounted strong responses towards IL-6R with similar titer ranges (FIG. 10A). Several mice from the VELOCIMMUNE® and wild-type cohorts reached a maximal response after a single antigen boost. These results indicate that the immune response strength and kinetics to this antigen were similar in the VELOCIMMUNE® and wild type mice. These antigen-specific antibody responses were

further analyzed to examine the particular isotypes of the antigen-specific antibodies found in the sera. Both VELOCIMMUNE® and wild type groups predominantly elicited an IgG1 response (FIG. 10B), suggesting that class switching during the humoral response is similar in mice of each type.

[000367] Affinity Determination of Antibody Binding to Antigen in Solution. An ELISA-based solution competition assay is typically designed to determine antibody-binding affinity to the antigen.

[000368] Briefly, antibodies in conditioned medium are premixed with serial dilutions of antigen protein ranging from 0 to 10 mg/mL. The solutions of the antibody and antigen mixture are then incubated for two to four hours at room temperature to reach binding equilibria. The amounts of free antibody in the mixtures are then measured using a quantitative sandwich ELISA. Ninety-six well MAXISORB™ plates (VWR, West Chester, PA) are coated with 1 μg/mL antigen protein in PBS solution overnight at 4°C followed by BSA nonspecific blocking. The antibody-antigen mixture solutions are then transferred to these plates followed by one-hour incubation. The plates are then washed with washing buffer and the plate-bound antibodies were detected with an HRP-conjugated goat anti-mouse IgG polyclonal antibody reagent (Jackson Immuno Research Lab) and developed using colorimetric substrates such as BD OPTEIA™ (BD Biosciences Pharmingen, San Diego, CA). After the reaction is stopped with 1 M phosphoric acid, optical absorptions at 450 nm are recorded and the data are analyzed using PRISM™ software from Graph Pad. The dependency of the signals on the concentrations of antigen in solution are analyzed with a 4 parameter fit analysis and reported as IC_{50}, the antigen concentration required to achieve 50% reduction of the signal from the antibody samples without the presence of antigen in solution.

[000369] In one experiment, VELOCIMMUNE® mice were immunized with hIL-6R (as described above). FIG. 11A and 11B show a representative set of affinity measurements for anti-hIL6R antibodies from VELOCIMMUNE® and wild-type mice.

[000370] After immunized mice receive a third antigen boost, serum titers are determined using an ELISA assay. Splenocytes are isolated from selected wild type and VELOCIMMUNE® mouse cohorts and fused with Ag8.653 myeloma cells to form hybridomas and grown under selection (as described above). Out of a total of 671 anti-IL-6R hybridomas produced, 236 were found to express antigen-specific antibodies. Media harvested from antigen positive wells was used to determine the antibody affinity of binding to antigen using a solution competition ELISA. Antibodies derived from VELOCIMMUNE® mice exhibit a wide range of affinity in binding to antigen in solution (FIG. 11A). Furthermore, 49 out of 236 anti-IL-6R hybridomas were found to block IL-6 from binding to the receptor in an in vitro bioassay (data not shown). Further, these 49 anti-IL-6R blocking

antibodies exhibited a range of high solution affinities similar to that of blocking antibodies derived from the parallel immunization of wild type mice (FIG. 11B).

Example 7

Construction of a Mouse ADAM6 Targeting Vector

[000371] Due to replacement of mouse immunoglobulin heavy chain variable gene loci with human immunoglobulin heavy chain variable gene loci, early versions of VELOCIMMUNE® mice lack expression of mouse ADAM6 genes. In particular, male VELOCIMMUNE® mice demonstrate a reduction in fertility. Thus, the ability to express ADAM6 was reengineered into VELOCIMMUNE® mice to rescue the fertility defect.

[000372] A targeting vector for insertion of mouse ADAM6a and ADAM6b genes into a humanized heavy chain locus was constructed using VELOCIGENE® genetic engineering technology (*supra*) to modify a Bacterial Artificial Chromosome (BAC) 929d24, which was obtained from Dr. Frederick Alt (Harvard University). 929d24 BAC DNA was engineered to contain genomic fragments containing the mouse ADAM6a and ADAM6b genes and a hygromycin cassette for targeted deletion of a human ADAM6 pseudogene (hADAM6Ψ) located between human V_H1-2 and V_H6-1 gene segments of a humanized heavy chain locus (FIG. 12).

[000373] First, a genomic fragment containing the mouse ADAM6b gene, ~800 bp of upstream (5') sequence and ~4800 bp of downstream (3') sequence was subcloned from the 929d24 BAC clone. A second genomic fragment containing the mouse ADAM6a gene, ~300 bp of upstream (5') sequence and ~3400 bp of downstream (3') sequence, was separately subcloned from the 929d24 BAC clone. The two genomic fragments containing the mouse ADAM6b and ADAM6a genes were ligated to a hygromycin cassette flanked by Frt recombination sites to create the targeting vector (Mouse ADAM6 Targeting Vector, Figure 12; SEQ ID NO:3). Different restriction enzyme sites were engineered onto the 5' end of the targeting vector following the mouse ADAM6b gene and onto the 3' end following the mouse ADAM6a gene (bottom of FIG. 12) for ligation into the humanized heavy chain locus.

[000374] A separate modification was made to a BAC clone containing a replacement of mouse heavy chain variable gene loci with human heavy chain variable gene loci, including the human ADAM6 pseudogene (hADAM6Ψ) located between the human V_H1-2 and V_H6-1 gene segments of the humanized locus for the subsequent ligation of the mouse ADAM6 targeting vector (FIG. 13).

[000375] Briefly, a neomycin cassette flanked by *lox*P recombination sites was engineered to contain homology arms containing human genomic sequence at positions 3' of the human V_H1-2 gene segment (5' with respect to hADAM6Ψ) and 5' of human V_H6-1

gene segment (3' with respect to hADAM6Ψ; see middle of FIG. 13). The location of the insertion site of this targeting construct was about 1.3 kb 5' and ~350 bp 3' of the human ADAM6 pseudogene. The targeting construct also included the same restriction sites as the mouse ADAM6 targeting vector to allow for subsequent BAC ligation between the modified BAC clone containing the deletion of the human ADAM6 pseudogene and the mouse ADAM6 targeting vector.

[000376] Following digestion of BAC DNA derived from both constructs, the genomic fragments were ligated together to construct an engineered BAC clone containing a humanized heavy chain locus containing an ectopically placed genomic sequence comprising mouse ADAM6a and ADAM6b nucleotide sequences. The final targeting construct for the deletion of a human ADAM6 gene within a humanized heavy chain locus and insertion of mouse ADAM6a and ADAM6b sequences in ES cells contained, from 5' to 3', a 5' genomic fragment containing ~13 kb of human genomic sequence 3' of the human V_H1-2 gene segment, ~800 bp of mouse genomic sequence downstream of the mouse ADAM6b gene, the mouse ADAM6b gene, ~4800 bp of genomic sequence upstream of the mouse ADAM6b gene, a 5' Frt site, a hygromycin cassette, a 3' Frt site, ~300 bp of mouse genomic sequence downstream of the mouse ADAM6a gene, the mouse ADAM6a gene, ~3400 bp of mouse genomic sequence upstream of the mouse ADAM6a gene, and a 3' genomic fragment containing ~30 kb of human genomic sequence 5' of the human V_H6-1 gene segment (bottom of FIG. 13).

[000377] The engineered BAC clone (described above) was used to electroporate mouse ES cells that contained a humanized heavy chain locus to created modified ES cells comprising a mouse genomic sequence ectopically placed that comprises mouse ADAM6a and ADAM6b sequences within a humanized heavy chain locus. Positive ES cells containing the ectopic mouse genomic fragment within the humanized heavy chain locus were identified by a quantitative PCR assay using TAQMAN™ probes (Lie and Petropoulos, 1998, Advances in quantitative PCR technology: 5'nuclease assays, *Curr Opin Biotechnol* 9(1):43-48). The upstream and downstream regions outside of the modified portion of the humanized heavy chain locus were confirmed by PCR using primers and probes located within the modified region to confirm the presence of the ectopic mouse genomic sequence within the humanized heavy chain locus as well as the hygromycin cassette. The nucleotide sequence across the upstream insertion point included the following, which indicates human heavy chain genomic sequence upstream of the insertion point and an I-CeuI restriction site (contained within the parentheses below) linked contiguously to mouse genomic sequence present at the insertion point:
(CCAGCTTCAT TAGTAATCGT TCATCTGTGG TAAAAAGGCA GGATTTGAAG CGATGGAAGA TGGGAGTACG GGGCGTTGGA AGACAAAGTG CCACACAGCG

CAGCCTTCGT CTAGACCCCC GGGCTAACTA TAACGGTCCT AAGGTAGCGA G) GGGATGACAG ATTCTCTGTT CAGTGCACTC AGGGTCTGCC TCCACGAGAA TCACCATGCC CTTTCTCAAG ACTGTGTTCT GTGCAGTGCC CTGTCAGTGG (SEQ ID NO:4). The nucleotide sequence across the downstream insertion point at the 3' end of the targeted region included the following, which indicates mouse genomic sequence and a PI-SceI restriction site (contained within the parentheses below) linked contiguously with human heavy chain genomic sequence downstream of the insertion point: (AGGGGTCGAG GGGGAATTTT ACAAAGAACA AAGAAGCGGG CATCTGCTGA CATGAGGGCC GAAGTCAGGC TCCAGGCAGC GGGAGCTCCA CCGCGGTGGC GCCATTTCAT TACCTCTTTC TCCGCACCCG ACATAGATAAAGCTT) ATCCCCCACC AAGCAAATCC CCCTACCTGG GGCCGAGCTT CCCGTATGTG GGAAAATGAA TCCCTGAGGT CGATTGCTGC ATGCAATGAA ATTCAACTAG (SEQ ID NO:5).

[000378] Targeted ES cells described above were used as donor ES cells and introduced into an 8-cell stage mouse embryo by the VELOCIMOUSE® mouse engineering method (see, *e.g.,* US Pat. Nos. 7,6598,442, 7,576,259, 7,294,754). Mice bearing a humanized heavy chain locus containing an ectopic mouse genomic sequence comprising mouse ADAM6a and ADAM6b sequences were identified by genotyping using a modification of allele assay (Valenzuela *et al.,* 2003) that detected the presence of the mouse ADAM6a and ADAM6b genes within the humanized heavy chain locus.

[000379] Mice bearing a humanized heavy chain locus that contains mouse ADAM6a and ADAM6b genes are bred to a FLPe deleter mouse strain (see, *e.g.,* Rodríguez *et al.,* 2000, High-efficiency deleter mice show that FLPe is an alternative to Cre-*lox*P. *Nature Genetics* 25:139-140) in order to remove any Frt'ed hygromycin cassette introduced by the targeting vector that is not removed, *e.g.,* at the ES cell stage or in the embryo. Optionally, the hygromycin cassette is retained in the mice.

[000380] Pups are genotyped and a pup heterozygous for a humanized heavy chain locus containing an ectopic mouse genomic fragment that comprises mouse ADAM6a and ADAM6b sequences is selected for characterizing mouse ADAM6 gene expression and fertility.

Example 8
Characterization of ADAM6 Rescue Mice

[000381] **Flow Cytometry**. Three mice at age 25 weeks homozygous for human heavy and human κ light chain variable gene loci ($H^{+/+}\kappa^{+/+}$) and three mice at age 18-20 weeks homozygous for human heavy and human κ light chain having the ectopic mouse genomic fragment encoding the mouse ADAM6a and ADAM6b genes within both alleles of the

human heavy chain locus ($H^{+/+}A6^{res}\kappa^{+/+}$) were sacrificed for identification and analysis of lymphocyte cell populations by FACs on the BD LSR II System (BD Bioscience). Lymphocytes were gated for specific cell lineages and analyzed for progression through various stages of B cell development. Tissues collected from the animals included blood, spleen and bone marrow. Blood was collected into BD microtainer tubes with EDTA (BD Biosciences). Bone marrow was collected from femurs by flushing with complete RPMI medium supplemented with fetal calf serum, sodium pyruvate, HEPES, 2-mercaptoethanol, non-essential amino acids, and gentamycin. Red blood cells from blood, spleen and bone marrow preparations were lysed with an ammonium chloride-based lysis buffer (*e.g.,* ACK lysis buffer), followed by washing with complete RPMI medium.

[000382] For staining of cell populations, 1 x 10^6 cells from the various tissue sources were incubated with anti-mouse CD16/CD32 (2.4G2, BD Biosciences) on ice for 10 minutes, followed by labeling with one or a combination of the following antibody cocktails for 30 minutes on ice.

[000383] Bone marrow: anti-mouse FITC-CD43 (1B11, BioLegend), PE-ckit (2B8, BioLegend), PeCy7-IgM (II/41, eBioscience), PerCP-Cy5.5-IgD (11-26c.2a, BioLegend), APC-eFluor780-B220 (RA3-6B2, eBioscience), A700-CD19 (1D3, BD Biosciences).

[000384] Peripheral blood and spleen: anti-mouse FITC-κ (187.1, BD Biosciences), PE-λ (RML-42, BioLegend), PeCy7-IgM (II/41, eBioscience), PerCP-Cy5.5-IgD (11-26c.2a, BioLegend), APC-CD3 (145-2C11, BD), A700-CD19 (1D3, BD), APC-eFluor780-B220 (RA3-6B2, eBioscience). Following incubation with the labeled antibodies, cells were washed and fixed in 2% formaldehyde. Data acquisition was performed on an LSRII flow cytometer and analyzed with FlowJo (Treestar, Inc.). Results from a representative $H^{+/+}\kappa^{+/+}$and $H^{+/+}A6^{res}\kappa^{+/+}$ mouse are shown in FIGs. 14 – 18.

[000385] The results demonstrate that B cells of $H^{+/+}A6^{res}\kappa^{+/+}$ mice progress through the stages of B cell development in a similar fashion to $H^{+/+}\kappa^{+/+}$mice in the bone marrow and peripheral compartments, and show normal patterns of maturation once they enter the periphery. $H^{+/+}A6^{res}\kappa^{+/+}$ mice demonstrated an increased $CD43^{int}CD19^{+}$ cell population as compared to $H^{+/+}\kappa^{+/+}$mice (FIG. 16B). This may indicate an accelerated IgM expression from the humanized heavy chain locus containing an ectopic mouse genomic fragment comprising the mouse ADAM6a and ADAM6b sequences in $H^{+/+}A6^{res}\kappa^{+/+}$mice. In the periphery, B and T cell populations of $H^{+/+}A6^{res}\kappa^{+/+}$mice appear normal and similar to $H^{+/+}\kappa^{+/+}$mice.

[000386] **Testis Morphology and Sperm Characterization.** To determine if infertility in mice having humanized immunoglobulin heavy chain variable loci is due to testis and/or sperm production defects, testis morphology and sperm content of the epididymis was

examined.

[000387] Briefly, testes from two groups (n=5 per group; group 1: mice homozygous for human heavy and κ light chain variable gene loci, $H^{+/+}\kappa^{+/+}$; group 2: mice heterozygous for human heavy chain variable gene loci and homozygous for κ light chain variable gene loci, $H^{+/-}\kappa^{+/+}$) were dissected with the epididymis intact and weighed. The specimens were then fixed, embedded in paraffin, sectioned and stained with hematoxylin and eosin (HE) stain. Testis sections (2 testes per mouse, for a total of 20) were examined for defects in morphology and evidence of sperm production, while epididymis sections were examined for presence of sperm.

[000388] In this experiment, no differences in testis weight or morphology was observed between $H^{+/+}\kappa^{+/+}$mice and $H^{+/-}\kappa^{+/+}$mice. Sperm was observed in both the testes and the epididymis of all genotypes. These results establish that the absence of mouse ADAM6a and ADAM6b genes does not lead to detectable changes in testis morphology, and that sperm is produced in mice in the presence and absence of these two genes. Defects in fertility of male $H^{+/+}\kappa^{+/+}$mice are therefore not likely to be due to low sperm production.

[000389] **Sperm Motility and Migration.** Mice that lack other ADAM gene family members are infertile due to defects in sperm motility or migration. Sperm migration is defined as the ability of sperm to pass from the uterus into the oviduct, and is normally necessary for fertilization in mice. To determine if the deletion of mouse ADAM6a and ADAM6b affects this process, sperm migration and motility was evaluated in $H^{+/+}\kappa^{+/+}$ mice.

[000390] Briefly, sperm was obtained from testes of (1) mice heterozygous for human heavy chain variable gene loci and homozygous for human κ light chain variable gene loci ($H^{+/-}\kappa^{+/+}$); (2) mice homozygous for human heavy chain variable gene loci and homozygous for human κ light chain variable gene loci ($H^{+/+}\kappa^{+/+}$); (3) mice homozygous for human heavy chain variable gene loci and homozygous for wild-type κ light chain ($H^{+/+}m\kappa$); and, (4) wild-type C57 BL/6 mice (WT). No significant abnormalities were observed in sperm count or overall sperm motility by inspection. For all mice, cumulus dispersal was observed, indicating that each sperm sample was able to penetrate the cumulus cells and bind the zona pellucida *in vitro*. These results establish that $H^{+/+}\kappa^{+/+}$ mice have sperm that are capable of penetrating the cumulus and binding the zona pellucida.

[000391] Fertilization of mouse ova *in vitro* (IVF) was done using sperm from mice as described above. A slightly lower number of cleaved embryos were observed for $H^{+/+}\kappa^{+/+}$ mice the day following IVF, as well as a reduced number of sperm bound to the eggs. These results establish that sperm from $H^{+/+}\kappa^{+/+}$ mice, once exposed to an ovum, are capable of penetrating the cumulus and binding the zona pellucida.

[000392] In another experiment, the ability of sperm from $H^{+/+}\kappa^{+/+}$ mice to migrate from the

uterus and through the oviduct was determined in a sperm migration assay.

[000393] Briefly, a first group of super-ovulated female mice (n=5) were set up with $H^{+/+}\kappa^{+/+}$ males (n=5) and a second group of super-ovulated female mice (n=5) were set up with $H^{+/-}\kappa^{+/+}$ males (n=5). The mating pairs were observed for copulation, and five to six hours post-copulation the uterus and attached oviduct from all females were removed and flushed for analysis. Flush solutions were checked for eggs to verify ovulation and obtain a sperm count. Sperm migration was evaluated in two different ways. First, both oviducts were removed from the uterus, flushed with saline, and any sperm identified were counted. The presence of eggs was also noted as evidence of ovulation. Second, oviducts were left attached to the uterus and both tissues were fixed, embedded in paraffin, sectioned and stained (as described above). Sections were examined for presence of sperm, in both the uterus and in both oviducts.

[000394] For the females mated with the five $H^{+/+}\kappa^{+/+}$ males, very little sperm was found in the flush solution from the oviduct. Flush solutions from oviducts of the females mated with the $H^{+/-}\kappa^{+/+}$ males exhibited a sperm level about 25- to 30-fold higher (avg, n = 10 oviducts) than present in flush solutions from the oviducts of the females mated with the $H^{+/+}\kappa^{+/+}$males. A representative breeding comparison of $H^{+/+}\kappa^{+/+}$ and $H^{+/+}A6^{res}\kappa^{+/+}$ mice is shown in Table 9.

[000395] Histological sections of uterus and oviduct were prepared. The sections were examined for sperm presence in the uterus and the oviduct (the colliculus tubarius). Inspection of histological sections of oviduct and uterus revealed that for female mice mated with $H^{+/+}\kappa^{+/+}$ mice, sperm was found in the uterus but not in the oviduct. Further, sections from females mated with $H^{+/+}\kappa^{+/+}$ mice revealed that sperm was not found at the uterotubal junction (UTJ). In sections from females mated with $H^{+/-}\kappa^{+/+}$ mice, sperm was identified in the UTJ and in the oviduct.

[000396] These results establish that mice lacking ADAM6a and ADAM6b genes make sperm that exhibit an *in vivo* migration defect. In all cases, sperm was observed within the uterus, indicating that copulation and sperm release apparently occur as normal, but little to no sperm was observed within the oviducts after copulation as measured either by sperm count or histological observation. These results establish that mice lacking ADAM6a and ADAM6b genes produce sperm that exhibit an inability to migrate from the uterus to the oviduct. This defect apparently leads to infertility because sperm are unable to cross the uterine-tubule junction into the oviduct, where eggs are fertilized. Taken together, all of these results converge to the support the hypothesis that mouse ADAM6 genes help direct sperm with normal motility to migrate out of the uterus, through the uterotubal junction and the oviduct, and thus approach an egg to achieve the fertilization

event. The mechanism by which ADAM6 achieves this may be directed by one or both of the ADAM6 proteins, or through coordinate expression with other proteins, *e.g.,* other ADAM proteins, in the sperm cell, as described below.

Table 9

Male Genotype	Breeding Animals (Male/Female)	Duration of Breeding	Litters	Offspring
$H^{+/+}\kappa^{+/+}$	6/6	6 months	2	25
$H^{+/+}A6^{res}\kappa^{+/+}$	4/8	4 months	4	198

[000397] ADAM Gene Family Expression. A complex of ADAM proteins are known to be present as a complex on the surface of maturing sperm. Mice lacking other ADAM gene family members lose this complex as sperm mature, and exhibit a reduction of multiple ADAM proteins in mature sperm. To determine if a lack of ADAM6a and ADAM6b genes affects other ADAM proteins in a similar manner, Western blots of protein extracts from testis (immature sperm) and epididymis (maturing sperm) were analyzed to determine the expression levels of other ADAM gene family members.

[000398] In this experiment, protein extracts were analyzed from groups (n=4 per group) of $H^{+/+}\kappa^{+/+}$and $H^{+/-}\kappa^{+/+}$mice. The results showed that expression of ADAM2 and ADAM3 were not affected in testis extracts. However, both ADAM2 and ADAM3 were dramatically reduced in epididymis extracts. This demonstrates that the absence of ADAM6a and ADAM6b in sperm of $H^{+/+}\kappa^{+/+}$ mice may have a direct affect on the expression and perhaps function of other ADAM proteins as sperm matures (*e.g.,* ADAM2 and ADAM3). This suggests that ADAM6a and ADAM6b are part of an ADAM protein complex on the surface of sperm, which might be critical for proper sperm migration.

Example 9

Human Heavy Chain Variable Gene Utilization in ADAM6 Rescue Mice

[000399] Selected human heavy chain variable gene usage was determined for mice homozygous for human heavy and κ light chain variable gene loci either lacking mouse ADAM6a and ADAM6b genes ($H^{+/+}\kappa^{+/+}$) or containing an ectopic genomic fragment encoding for mouse ADAM6a and ADAM6b genes ($H^{+/+}A6^{res}\kappa^{+/+}$) by a quantitative PCR assay using TAQMAN™ probes (as described above).

[000400] Briefly, $CD19^{+}$ B cells were purified from the spleens of $H^{+/+}\kappa^{+/+}$ and $H^{+/+}A6^{res}\kappa^{+/+}$ mice using mouse CD19 Microbeads (Miltenyi Biotec) and total RNA was purified using the RNEASY™ Mini kit (Qiagen). Genomic RNA was removed using an RNase-free DNase

on-column treatment (Qiagen). About 200 ng mRNA was reverse-transcribed into cDNA using the First Stand cDNA Synthesis kit (Invitrogen) and then amplified with the TAQMAN™ Universal PCR Master Mix (Applied Biosystems) using the ABI 7900 Sequence Detection System (Applied Biosystems). Relative expression of each gene was normalized to the expression of mouse κ light chain constant region (mCκ). Table 10 sets forth the sense/antisense/TAQMAN™ MGB probe combinations used in this experiment.

Table 10

Human V_H	Sequence (5'-3')	SEQ ID NO:
V_H6-1	Sense: CAGGTACAGCTGCAGCAGTCA Anti-sense: GGAGATGGCACAGGTGAGTGA Probe: TCCAGGACTGGTGAAGC	6 7 8
V_H1-2	Sense: TAGTCCCAGTGATGAGAAAGAGAT Anti-sense: GAGAACACAGAAGTGGATGAGATC Probe: TGAGTCCAGTCCAGGGA	9 10 11
V_H3-23	Sense: AAAAATTGAGTGTGAATGGATAAGAGTG Anti-sense: AACCCTGGTCAGAAACTGCCA Probe: AGAGAAACAGTGGATACGT	12 13 14
V_H1-69	Sense: AACTACGCACAGAAGTTCCAGG Anti-sense: GCTCGTGGATTTGTCCGC Probe: CAGAGTCACGATTACC	15 16 17
mCκ	Sense: TGAGCAGCACCCTCACGTT Anti-sense: GTGGCCTCACAGGTATAGCTGTT Probe: ACCAAGGACGAGTATGAA	18 19 20

[000401] In this experiment, expression of all four human V_H genes was observed in the samples analyzed. Further, the expression levels were comparable between $H^{+/+}\kappa^{+/+}$ and $H^{+/+}A6^{res}\kappa^{+/+}$ mice. These results demonstrate that human V_H genes that were both distal to the modification site (V_H3-23 and V_H1-69) and proximal to the modification site (V_H1-2 and V_H6-1) were all able to recombine to form a functionally expressed human heavy chain. These results demonstrate that the ectopic genomic fragment comprising mouse ADAM6a and ADAM6b sequences inserted into a human heavy chain genomic sequence did not affect V(D)J recombination of human heavy chain gene segments within the locus, and these mice are able to recombine human heavy chain gene segments in normal fashion to produce functional heavy chain immunoglobulin proteins.

Example 10

Humoral Immune Response in ADAM6 Rescue Mice

[000402] The humoral immune response was determined for mice homozygous for human heavy and κ light chain variable gene loci either lacking mouse ADAM6a and

ADAM6b genes ($H^{+/+}\kappa^{+/+}$) or containing an ectopic genomic fragment encoding for mouse ADAM6a and ADAM6b genes ($H^{+/+}A6^{res}\kappa^{+/+}$) by a multi-antigen immunization scheme followed by antibody isolation and characterization. Results were compared for determination of any effect on V(D)J recombination involving the human immunoglobulin gene segments, assessment of serum titer progression, production of antibodies by hybridomas and affinity for antigen.

[000403] Immunization protocol. A human cell surface receptor (Antigen A), a human antibody specific for a human receptor tyrosine-protein kinase (Antigen B), a secreted human protein that functions in regulation of the TGF-β signaling pathway (Antigen C), and a human receptor tyrosine kinase (Antigen D) were employed for comparative immunizations in groups of mice. Serum was collected from groups of mice prior to immunization with the above antigens. Each antigen (2.3 μg each) was administered in an initial priming immunization mixed with 10 μg of CpG oligonucleotide as adjuvant (Invivogen). The immunogen was administered via footpad (f.p.) in a volume of 25 μl per mouse. Subsequently, mice were boosted via f.p. with 2.3 μg of antigen along with 10 μg CpG and 25 μg Adju-Phos (Brenntag) as adjuvants on days 3, 6, 11, 13, 17, and 20 for a total of six boosts. Mice were bled on days 15 and 22 after the fourth and sixth boosts, respectively, and antisera were assayed for antibody titer to each specific antigen.

[000404] Antibody titers were determined in sera of immunized mice using an ELISA assay. Ninety six-well microtiter plates (Thermo Scientific) were coated with the respective antigen (2 μg/ml) in phosphate-buffered saline (PBS, Irvine Scientific) overnight at 4°C. The following day, plates were washed with phosphate-buffered saline containing 0.05% Tween 20 (PBS-T, Sigma-Aldrich) four times using a plate washer (Molecular Devices). Plates were then blocked with 250 μl of 0.5% bovine serum albumin (BSA, Sigma-Aldrich) in PBS and incubated for one hour at room temperature. The plates were then washed four times with PBS-T. Sera from immunized mice and pre-immune sera were serially diluted three-fold in 0.5% BSA-PBS starting at 1:300 or 1:1000 and added to the blocked plates in duplicate and incubated for one hour at room temperature. The last two wells were left blank to be used as secondary antibody control. The plates were again washed four times with PBS-T in a plate washer. A 1:5000/1:10,000 dilution of goat anti-mouse IgG-Fc-Horse Radish Peroxidase (HRP, Jackson Immunoresearch) or goat anti-mouse IgG-kappa-HRP (Southern Biotech) conjugated secondary antibody was added to the plates and incubated for one hour at room temperature. Plates were again washed eight times with PBS-T and developed using TMB/H_2O_2 as substrate. The substrate was incubated for twenty minutes and the reaction stopped with 2 N H_2SO_4 (VWR) or 1 N H_3PO_4 (JT Baker). Plates were read on a spectrophotometer (Victor, Perkin Elmer) at 450

nm. Antibody titers were calculated using Graphpad PRISM software.

[000405] Serum titer was calculated as serum dilution within experimental titration range at the signal of antigen binding equivalent to two times above background. Results for the humoral immune response are shown in FIG. 19 (Antigen A), FIG. 20 (Antigen B), FIG. 21 (Antigen C), and FIG. 22 (Antigen D). Antigen positive score of hybridomas made using two spleens isolated from mice from each group of selected immunizations is shown in Table 11 (Antigen score is equal to 2X/background).

[000406] As shown in this Example, antibody titers generated in Adam6 rescue mice ($H^{+/+}A6^{res}\kappa^{+/+}$) were comparable to those generated in mice lacking ADMA6a and ADAM6b and having humanized heavy chain ($H^{+/+}\kappa^{+/+}$). Further, spleens from $H^{+/+}A6^{res}\kappa^{+/+}$ mice yielded antigen-positive hybridomas for all antigens tested, including antibodies of high affinity, at levels comparable to $H^{+/+}\kappa^{+/+}$ mice. Thus, no impairment of V(D)J recombination of human immunoglobulin gene segments in Adam6 rescue mice is believed to exist given the production of antibodies with high affinity containing human immunoglobulin genes.

Table 11

Antigen	Mouse Strain	Antigen Score
A	$H^{+/+}A6^{res}\kappa^{+/+}$	76
A	$H^{+/+}A6^{res}\kappa^{+/+}$	32
B	$H^{+/+}\kappa^{+/+}$	4
B	$H^{+/+}\kappa^{+/+}$	12
B	$H^{+/+}A6^{res}\kappa^{+/+}$	41
B	$H^{+/+}A6^{res}\kappa^{+/+}$	95

Example 11

Antigen Binding Affinity Determination

[000407] Binding affinities of antibodies showing specific binding to Antigen B were screened using a real-time surface plasmon resonance biosensor (BIAcore 2000). Conditioned media from hybridomas isolated from two strains of mice immunized with Antigen B ($H^{+/+}\kappa^{+/+}$ and $H^{+/+}A6^{res}\kappa^{+/+}$) were used during BIAcore screening. BIAcore sensor surface was first derivatized with polyclonal rabbit anti-mouse antibody (GE) to capture anti-Antigen B antibodies from conditioned media. During the entire screening method, HBST (0.01M HEPES pH 7.4, 0.15M NaCl, 3mM EDTA, 0.005% v/v Surfactant P20) was used as the running buffer. Fab fragment of Antigen B was injected over the anti-Antigen B antibody captured surface at a flow rate of 50 µl/minute at 100nM concentration. Antibody-antigen association was monitored for three minutes while the dissociation of antigen from the captured antibody was monitored for five minutes in HBST running buffer.

The experiment was performed at 25°C. Kinetic association (ka) and dissociation (kd) rate constants were determined by processing and fitting the data to a 1:1 binding model using Scrubber 2.0 curve fitting software. Binding dissociation equilibrium constants (K_D) and dissociative half-lives ($T_{½}$) were calculated from the kinetic rate constants as: K_D (M) = kd / ka; and T½ (min) = (ln2/(60*kd). Results for selected anti-Antigen B antibodies are shown in Table 12.

Table 12

Antibody	Mouse strain	K_D (M)	$T_{½}$ (min)
5D6	$H^{+/+}\kappa^{+/+}$	1.62E-08	3
8G10	$H^{+/+}\kappa^{+/+}$	1.20E-08	5
10F10	$H^{+/+}\kappa^{+/+}$	1.09E-08	3
1F5	$H^{+/+}\kappa^{+/+}$	1.00E-07	0.3
10G8	$H^{+/+}\kappa^{+/+}$	1.47E-07	0.3
1B11	$H^{+/+}A6^{res}\kappa^{+/+}$	1.98E-08	6
2D9	$H^{+/+}A6^{res}\kappa^{+/+}$	9.40E-10	51
4D11	$H^{+/+}A6^{res}\kappa^{+/+}$	5.60E-08	0.8
6C5	$H^{+/+}A6^{res}\kappa^{+/+}$	1.10E-09	188
6F4	$H^{+/+}A6^{res}\kappa^{+/+}$	1.35E-08	3
7C4	$H^{+/+}A6^{res}\kappa^{+/+}$	2.00E-06	0.05
8G12	$H^{+/+}A6^{res}\kappa^{+/+}$	2.31E-09	19
9B12	$H^{+/+}A6^{res}\kappa^{+/+}$	3.47E-09	13
10B4	$H^{+/+}A6^{res}\kappa^{+/+}$	3.60E-09	23
11E7	$H^{+/+}A6^{res}\kappa^{+/+}$	3.06E-08	2
11E12	$H^{+/+}A6^{res}\kappa^{+/+}$	2.70E-07	0.1
1E4	$H^{+/+}A6^{res}\kappa^{+/+}$	7.00E-10	58
4D2	$H^{+/+}A6^{res}\kappa^{+/+}$	5.80E-10	150
5H6	$H^{+/+}A6^{res}\kappa^{+/+}$	2.60E-09	3
5H10	$H^{+/+}A6^{res}\kappa^{+/+}$	6.00E-09	70
9A9	$H^{+/+}A6^{res}\kappa^{+/+}$	3.80E-09	12
11C11	$H^{+/+}A6^{res}\kappa^{+/+}$	1.55E-09	38
12C10	$H^{+/+}A6^{res}\kappa^{+/+}$	5.90E-09	16
12G7	$H^{+/+}A6^{res}\kappa^{+/+}$	9.00E-08	7
12G9	$H^{+/+}A6^{res}\kappa^{+/+}$	3.32E-09	12

[000408] In a similar experiment, kinetics of different monoclonal antibodies present in hybridoma-conditioned media binding to Antigen A was determined using a real-time surface plasmon resonance biosensor (BIAcore 4000) assay. All hybridoma clones used in this assay were produced in $H^{+/+}A6^{res}\kappa^{+/+}$ mice.

[000409] Briefly, to capture the Antigen A-specific antibodies, polyclonal rabbit anti-mouse antibody (GE Catalog# BR-1008-38) was first immobilized on the sensor chip. BIAcore screening was performed in two different buffers – PBSP, pH7.2 and PBSP, pH6.0. Both the buffers were supplemented with 0.1 mg/ml BSA. Following the capture of anti-Antigen A antibodies from the conditioned media, 1 μM of Antigen A monomer (prepared in respective running buffer) was injected over the captured antibody surface for 1.5 minutes at 30 μl/minute and the dissociation of bound Antigen A monomer was monitored for 1.5 minutes in the respective running buffer at 25°C. Kinetic association (ka) and dissociation (kd) rate constants were determined by processing and fitting the data to a 1:1 binding model using Scrubber 2.0 curve fitting software. Binding dissociation equilibrium constants (K_D) and dissociative half-lives ($T_{½}$) were calculated from the kinetic rate constants as: K_D (M) = kd / ka; and $T_{½}$ (min) = (ln2/(60*kd). Table 13 sets forth the binding kinetics parameters for selected anti-Antigen A antibody binding to Antigen A monomer at pH7.2 and pH6.0. NB: no binding detected under current experimental conditions.

Table 13

Antibody	pH7.2		pH6.0	
	K_D (M)	$T_{½}$ (min)	K_D (M)	$T_{½}$ (min)
1D7	3.89E-10	25	9.45E-10	17
2B4	NB	NB	NB	NB
2B7	3.90E-09	1.2	2.98E-09	2
2F7	2.36E-10	144	2.06E-11	1882
3A7	NB	NB	6.42E-10	17
3F6	NB	NB	NB	NB
4A6	1.91E-09	2	2.12E-09	2
4C4	NB	NB	NB	NB
4E12	2.69E-10	16	2.03E-10	18
5C11	1.68E-09	3	2.31E-09	3
5D10	NB	NB	4.56E-09	2
5E7	NB	NB	NB	NB
5F10	NB	NB	NB	NB
5F11	8.18E-10	8	6.79E-10	7
5G4	3.55E-10	15	7.42E-11	53

5G9	6.39E-10	15	4.31E-10	21
5H8	4.73E-10	15	NB	NB
6D2	NB	NB	NB	NB
6D3	2.88E-10	14	8.82E-11	39
6E4	NB	NB	2.67E-09	4
6E6	1.37E-09	10	1.30E-09	14
6H6	NB	NB	NB	NB
7A12	NB	NB	NB	NB
7C3	NB	NB	NB	NB
7E8	4.38E-10	22	2.63E-10	34
7F10	NB	NB	NB	NB
7G9	NB	NB	NB	NB
8B8	NB	NB	NB	NB
8B11	NB	NB	NB	NB
8C3	NB	NB	NB	NB
8E9	NB	NB	NB	NB
8G3	NB	NB	NB	NB
8H3	NB	NB	NB	NB
8H4	3.70E-07	0.1	NB	NB
8H8	NB	NB	NB	NB
1A8	2.30E-09	4	7.40E-10	6
1B6	NB	NB	NB	NB
1C6	NB	NB	NB	NB
1C12	NB	NB	NB	NB
1D2	NB	NB	NB	NB
1E2	1.17E-09	42	3.08E-09	29
1E3	5.05E-10	89	8.10E-10	57
1E6	1.97E-08	3	1.84E-08	3
1E9	1.14E-09	30	1.14E-09	25
1H6	2.93E-09	14	9.87E-10	25
2H9	2.30E-08	2	1.91E-08	2
3A2	1.15E-10	44	1.25E-10	33
3A4	1.70E-10	31	1.44E-10	30
3D11	NB	NB	1.58E-08	1
3H10	2.82E-09	20	2.59E-09	15
4B6	7.79E-10	6	6.36E-10	7
4H6	9.18E-11	62	1.20E-10	43
5A2	NB	NB	7.04E-10	12
5C5	8.71E-11	49	7.02E-11	48
5F6	6.16E-11	114	5.46E-11	121

[000410] As shown above, high affinity antibodies were obtained from both $H^{+/+}A6^{res}\kappa^{+/+}$ and $H^{+/+}\kappa^{+/+}$ mice in a comparable manner. Among the twenty-five antibodies represented in Table 12, twenty produced in $H^{+/+}A6^{res}\kappa^{+/+}$ mice demonstrated an affinity range of 0.5 nM

to 1 μM, while the five generated in $H^{+/+}\kappa^{+/+}$ mice demonstrated an affinity range of 10 nM to 150 nM. Further, the fifty-five antibodies shown in Table 13 demonstrated an affinity range of 20 pM to 350 nM for binding to Antigen A monomer.

[000411] As demonstrated in this Example, the reinsertion of mouse Adam6 genes into a humanized immunoglobulin heavy chain locus does not impair the ability of the mouse to mount a robust immunize response to multiple antigens characterized by repertoires of human antibodies having diverse affinities in the subnanomolar range, which are derived from human gene segments rearranged from a engineered germline.

CLAIMS

1. A mouse whose genome comprises:

(a) an insertion of one or more human V_H, one or more human D_H, and one or more human J_H gene segments upstream of an endogenous heavy chain constant region gene; and

(b) an insertion of one or more human V_L and one or more human J_L gene segments upstream of an endogenous light chain constant region gene; and

(c) an ectopic nucleic acid sequence that encodes an Adam6 protein that is functional in the mouse,

so that the mouse is characterized in that:

(i) it is fertile; and

(ii) when it is immunized with an antigen, it generates antibodies comprising:

(A) heavy chain variable domains encoded by the one or more human V_H, one or more human D_H, and one or more human J_H gene segments, linked to heavy chain constant domains encoded by the heavy chain constant region gene; and

(B) light chain variable domains encoded by the one or more human V_L and one or more human J_L gene segments linked to light chain constant domains encoded by the endogenous light chain constant region gene,

wherein the antibodies specifically bind the antigen.

2. The mouse of claim 1, wherein the ectopic nucleic acid sequence is present at the endogenous heavy chain locus.

3. The mouse of claim 2, wherein the ectopic nucleic acid sequence is positioned within a human immunoglobulin heavy chain sequence.

4. The mouse of claim 3, wherein the ectopic nucleic acid sequence is positioned between two human V_H gene segments.

5. The mouse of claim 1, wherein the ectopic nucleic acid sequence is present at a position other than the endogenous heavy chain locus.

6. The mouse of claim 5, wherein the ectopic nucleic acid sequence is positioned between a V_H and a D_H gene segment.

7. The mouse of claim 1, wherein the insertion comprises at least 18 human V_H, at least one and up to 27 human D_H, and at least one and up to 6 human J_H gene segments.

8. The mouse of claim 1, wherein the insertion comprises at least 39 human V_H, at least one and up to 27 human D_H, and at least one and up to 6 human J_H gene segments.

9. The mouse of claim 1, wherein the insertion comprises 80 human V_H, at least one and up to 27 human D_H, and at least one and up to 6 human J_H gene segments.

10. The mouse of claim 1, wherein the mouse lacks endogenous V_H, D_H, and J_H gene segments.

11. The mouse of claim 1, wherein the one or more human V_L and one or more human J_L gene segments are Vκ and Jκ gene segments.

12. The mouse of claim 11, wherein the endogenous light chain constant region is a mouse Cκ region.

13. The mouse of claim 11, wherein the insertion comprises at least 16 human Vκ and at least one and up to 5 human Jκ gene segments.

14. The mouse of claim 11, wherein the insertion comprises at least 30 human Vκ and at least one and up to 5 human Jκ gene segments.

15. The mouse of claim 11, wherein the insertion comprises at least 40 human Vκ and at least one and up to 5 human Jκ gene segments.

16. The mouse of claim 11, wherein the mouse lacks endogenous Vκ and Jκ gene segments.

17. An isolated mouse cell whose genome comprises:

(a) an insertion of one or more human V_H, one or more human D_H, and one or more human J_H gene segments upstream of an endogenous heavy chain constant region; and

(b) an insertion of one or more human V_L and one or more human J_L gene segments upstream of an endogenous light chain constant region; and

(c) an ectopic nucleic acid sequence that encodes an Adam6 protein that is functional in the mouse.

18. The isolated cell of claim 17, wherein the cell is an embryonic stem (ES) cell.

19. A method of making a mouse comprising introducing the ES cell of claim 18 into a host embryo, gestating the host embryo in a surrogate mother, and allowing the surrogate mother to give birth to progeny derived in whole or in part from the ES cell.

20. The isolated cell of claim 17, wherein the cell is a B cell.

21. A hybridoma made from the B cell of claim 20.

22. A genetically modified mouse whose genome:

(i) lacks a functional endogenous ADAM6 locus; and

(ii) includes an integrated nucleic acid sequence encoding a mouse ADAM6 protein.

23. The genetically modified mouse of claim 22, wherein the integrated nucleic acid sequence encoding a mouse ADAM6 protein is not found between the two mouse immunoglobulin variable region sequences in which it is found in a wild-type mouse.

24. A genetically modified mouse whose genome:

(i) lacks a functional endogenous ADAM6 locus; and

(ii) includes an extrachromosomal nucleic acid sequence encoding a mouse ADAM6 protein.

25. The genetically modified mouse of claim 22 or claim 24, whose genome comprises a deletion of an endogenous immunoglobulin variable region sequence.

26. The genetically modified mouse of claim 25, wherein the deletion comprises a deletion of a mouse V_H gene segment, a deletion of a mouse D_H gene segment, a deletion of a mouse J_H gene segment, or a combination thereof.

27. A method of increasing mouse fertility by:

integrating into a mouse cell a nucleic acid sequence encoding a mouse ADAM6 protein.

28. The method of claim 27, wherein the mouse cell comprises a deletion of an endogenous heavy chain variable region.

29. The method of claim 27, wherein the mouse cell comprises a replacement of an endogenous heavy chain variable region.

30. The method of claim 27, wherein the mouse comprises human immunoglobulin V_H segments.

31. The method of claim 30, wherein the mouse comprises a humanized immunoglobulin heavy chain locus in that the human immunoglobulin V_H segments are at the endogenous mouse immunoglobulin heavy chain locus.

32. The method of claim 31, wherein the integrated nucleic acid sequence encoding a mouse ADAM6 protein is within the humanized immunoglobulin heavy chain locus.

33. The method of claim 32, wherein the humanized immunoglobulin heavy chain locus comprises replacement of mouse germline variable region segments with the human immunoglobulin V_H segments.

34. A genetically modified mouse produced by the method of claim 27.

35. A genetically modified mouse whose genome:

(i) lacks a functional endogenous ADAM6 locus; and

(ii) includes an integrated or extrachromosomal nucleic acid sequence encoding a mouse ADAM6 protein.

36. The genetically modified mouse of claim 35, wherein the mouse has a genome that comprises an endogenous ADAM6 locus or fragment thereof, which endogenous ADAM6 locus or fragment thereof does not express ADAM6.

37. The genetically modified mouse of claim 35, wherein the mouse has a genome that comprises an endogenous ADAM6 locus or fragment thereof, which endogenous ADAM6 locus or fragment thereof expresses ADAM6 at a level that is insufficient to support essentially normal fertility of a male mouse.

38. The mouse of any one of claims 36 or 37, wherein the endogenous ADAM6 locus or fragment thereof has a modification in a structural gene selected from the group consisting of ADAM6a, ADAM6b, and combinations thereof.

39. The mouse of any one of claims 36 or 37, wherein the endogenous ADAM6 locus or fragment thereof has a modification in a regulatory region.

40. The modified mouse of claim 39, wherein the regulatory region is selected from the group consisting of a region 5′ to the ADAM6a gene, a region 3′ of the ADAM6a coding region, a region 3′ of the ADAM6b coding region, and combinations thereof.

41. The genetically modified mouse of claim 35, wherein the integrated or extrachromosomal nucleic acid sequence is not found between the two mouse immunoglobulin variable region sequences in which it is found in a wild-type mouse.

42. A genetically modified mouse whose genome comprises a replacement of one or more endogenous V_H gene segments with human V_H gene segments, wherein the replacement

removes an endogenous ADAM6 sequence, which mouse is engineered to have a mouse ADAM6 sequence located within the sequence that contains the human V_H gene segments.

43. The genetically modified mouse of claim 42, wherein the mouse ADAM6 sequence is placed between two human V_H gene segments, where the human V_H gene segments are operably linked to D_H and J_H gene segments.

44. The genetically modified mouse of claim 43, wherein the D_H segments are human.

45. The genetically modified mouse of claim 43, wherein the D_H segments are mouse.

46. The genetically modified mouse of claim 43, wherein the J_H segments are human.

47. The genetically modified mouse of claim 43, wherein the J_H segments are mouse.

48. The genetically modified mouse of 43, wherein the human V_H gene segments are operably linked to D_H and J_H gene segments and to an immunoglobulin constant region.

49. The genetically modified mouse of claim 48, wherein the constant region is human.

50. The genetically modified mouse of claim 48, wherein the constant region is mouse.

51. A mouse immunoglobulin heavy chain locus that lacks an endogenous mouse ADAM6 sequence, which locus is humanized in that it contains a sequence that includes human V_H gene segments, wherein the mouse immunoglobulin heavy chain locus includes a mouse ADAM6 gene within the sequence that includes human V_H gene segments.

52. The mouse immunoglobulin heavy chain locus of claim 51, wherein the sequence that includes human V_H gene segments comprises a replacement one or more endogenous V_H gene segments with human V_H gene segments.

53. The mouse immunoglobulin heavy chain locus of claim 52, wherein the replacement removes the endogenous ADAM6 sequence.

54. The mouse immunoglobulin heavy chain locus of claim 52, wherein the mouse ADAM6 sequence is placed between two human V_H gene segments.

55. The mouse immunoglobulin heavy chain locus of claim 52, wherein the humanized immunoglobulin heavy chain locus comprises human V_H gene segments operably linked to human or mouse D_H and J_H gene segments, and to a human or mouse immunoglobulin constant region.

56. A genetically modified mouse whose genome comprises the mouse immunoglobulin heavy chain locus of claim 51.

57. A mouse whose genome includes an immunoglobulin heavy chain locus that lacks any mouse ADAM6 sequence, which locus is humanized in that it contains a sequence that includes human V_H gene segments, wherein the mouse genome includes an integrated mouse ADAM6 gene.

58. A mouse immunoglobulin heavy chain locus that comprises a nonfunctional endogenous mouse ADAM6 sequence or regulatory region, and the locus comprises an ectopic nucleotide sequence that encodes a mouse ADAM6 protein or a homolog or ortholog or a functional fragment thereof.

59. In a genetically modified mouse comprising a humanized immunoglobulin heavy chain locus that comprises human V_H gene segments operably linked to a human or mouse immunoglobulin constant region, the improvement comprising:

a genetic modification that introduces into the mouse a functional gene selected from the group consisting of mouse ADAM6a, mouse ADAM6b, and combinations thereof.

60. The improvement of claim 59, wherein the functional gene is introduced in a transgene that randomly integrates into the mouse genome.

61. The improvement of claim 59, wherein the functional gene is maintained episomally.

62. In a genetically modified mouse in which endogenous heavy chain immunoglobulin gene segments selected from the group consisting of V gene segments and D gene segments are deleted or replaced, the improvement comprising:

a genetic modification that introduces into the mouse a functional gene selected from the group consisting of mouse ADAM6a, mouse ADAM6b, and combinations thereof.

63. The improvement of claim 62, wherein the functional gene is introduced in a transgene that randomly integrates into the mouse genome.

64. The improvement of claim 62, wherein the functional gene is maintained episomally.

65. In a genetically modified mouse in which an endogenous immunoglobulin variable gene segment and ADAM6 nucleotide sequence has been replaced with a sequence comprising a human immunoglobulin variable region gene segment to form a first chimeric locus, the improvement comprising:

insertion of a mouse ADAM6-encoding sequence into the sequence comprising the human immunoglobulin variable region gene segment to form a second chimeric locus.

66. The improvement of claim 65, wherein the second chimeric locus comprises a human immunoglobulin heavy chain variable (V_H) segment.

67. The improvement of claim 65 or claim 66, wherein the second chimeric locus comprises a human immunoglobulin light chain variable (V_L) segment.

68. The improvement of claim 65, wherein the second chimeric locus comprises a human V_H segment or a human V_L segment operably linked to a human D_H segment and a human J_H segment.

69. The improvement of claim 65, wherein the second chimeric locus is operably linked to a third chimeric locus that comprises a human C_H1 sequence, or a human C_H1 and human hinge sequence, fused with a mouse C_H2 and C_H3 sequence.

70. The improvement of claim 65, wherein the improvement comprises increased fertility of the genetically modified mouse.

71. A genetically modified mouse whose genome comprises a human immunoglobulin heavy chain variable region gene segment at an endogenous mouse immunoglobulin heavy chain variable region gene locus and comprises a nucleotide sequence that expresses mouse ADAM6, which nucleotide sequence is extrachromosomal or is integrated in the genome of the mouse.

72. A genetically engineered mouse from which heavy chain V gene segments are deleted or replaced, or from which heavy chain D gene segments are deleted or replaced, which mouse has been modified to include a nucleotide sequence that encodes a mouse ADAM6 protein selected from the group consisting of mouse ADAM6a, mouse ADAM6b, and combinations thereof.

73. The genetically engineered mouse of claim 72, wherein the included nucleotide sequence is or comprises a transgene that randomly integrates into the mouse genome.

74. The genetically engineered mouse of claim 72, wherein the included nucleotide sequence is maintained episomally.

75. A genetically engineered mouse whose genome comprises:

a) endogenous immunoglobulin loci that are disabled;

b) a human transgene that comprises variable and constant immunoglobulin gene segments; and further comprise a nucleic acid sequence encoding a mouse ADAM6, which nucleic acid sequence is ectopic in that it is extrachromosomal or is integrated in a transcriptionally-permissive locus that is not between the two mouse immunoglobulin variable region sequences in which it is found in a wild-type mouse.

76. A genetically engineered mouse whose genome comprises hybrid immunoglobulin loci that make light and heavy chains that have a human variable region and a mouse constant region or that have a human variable regions and partly human/partly mouse constant regions, which genetically engineered mouse further comprises an extrachromosomal or integrated nucleic acid encoding a mouse ADAM6.

77. The genetically engineered mouse of claim 75 or claim 76, wherein the human variable gene segments comprise V_H1-2 and V_H6-1.

78. The genetically engineered mouse of claim 77, wherein the nucleic acid sequence encoding the mouse ADAM6 is placed between the human V_H1-2 and the human V_H6-1 gene segments.

79. The genetically engineered mouse of claim 78, wherein the nucleic acid sequence encoding the mouse ADAM6 has a direction of transcription opposite of that of the surrounding human variable gene segments.

80. The genetically engineered mouse of claim 75 or 76, wherein the nucleic acid sequence encoding a mouse ADAM6 is positioned at a transcriptionally-permissive locus in the mouse genome.

81. A genetically engineered mouse whose genome comprises a disruption of an endogenous mouse ADAM6 locus and further comprises an extrachromosomal nucleotide sequence encoding a mouse ADAM6 protein.

82. A genetically engineered mouse whose genome comprises a deletion of an endogenous mouse ADAM6 locus and further comprises an integrated nucleotide sequence encoding a mouse ADAM6 protein.

83. The genetically engineered mouse of claim 82, wherein the nucleotide sequence encoding a mouse ADAM6 protein is integrated at a transcriptionally-permissive locus in the mouse genome.

84. A genetically engineered mouse whose genome comprises a modification of an endogenous heavy chain immunoglobulin locus that compromises endogenous ADAM6 function which genetically engineered mouse further comprises an extrachromosomal or integrated nucleic acid encoding a mouse ADAM6.

85. A genetically engineered mouse whose genome comprises:

a) a heavy chain immunoglobulin locus that is modified to be deleted in whole or in part, humanized in whole or in part, or replaced in whole or in part; and

b) a nucleotide sequence encoding a mouse ADAM6 protein that is ectopic in that it is extrachromosomal or is integrated in a transcriptionally-permissive locus that is not between the two mouse immunoglobulin variable region sequences in which it is found in a wild-type mouse.

86. A nucleic acid construct comprising mouse ADAM6a and ADAM6b genes and further comprising sites engineered onto the 5′ and 3′ ends of the construct for integration into a humanized immunoglobulin heavy chain locus.

87. A genetically engineered mouse whose genome comprises mouse ADAM6a and ADAM6b genes integrated within a humanized immunoglobulin chain locus.

88. A method of increasing fertility in a mouse, the method comprising:

integrating into the genome of a mouse ES cell a targeting vector comprising mouse ADAM6a and ADAM6b genes, and

introducing the ES cell into an 8-cell-stage mouse embryo, so that a mouse that expresses

functional mouse ADAM6 protein develops.

89. A method of making a genetically engineered mouse, the method comprising steps of:

gestating in a surrogate mother a mouse embryo comprising an integrated mouse ADAM6 gene.

90. The method of claim 89, wherein the step of gestating comprises:

integrating into the mouse embryo a nucleic acid sequence encoding a mouse ADAM6 protein.

91. The method of claim 90, wherein the step of integrating into the mouse embryo comprises:

integrating into a donor mouse ES cell or a mouse embryo a nucleic acid sequence encoding a mouse ADAM6 protein; and

introducing the donor mouse ES cell into the embryo.

92. The method of claim 89, wherein the mouse into which the mouse ADAM6 gene is integrated lacks an endogenous ADAM6 gene at an endogenous immunoglobulin heavy chain locus.

93. A method of manufacturing a nucleic acid construct comprising mouse ADAM6a and ADAM6b genes by positioning the genes between 5’ and 3’ sequences that target the mouse ADAM6a and ADAM6b genes for integration into one or more loci in the mouse genome.

94. The method of claim 93, wherein the sequences target the mouse ADAM6a and ADAM6b genes for integration into an immunoglobulin heavy chain locus.

95. The method of claim 93, wherein the sequences target the mouse ADAM6a and ADAM6b genes for integration into a humanized immunoglobulin heavy chain locus.

96. A method of manufacturing a nucleic acid construct comprising mouse ADAM6a and ADAM6b genes by positioning the genes between 5′ and 3′ sequences that do not include a complete mouse immunoglobulin heavy chain locus and that direct random integration of the mouse ADAM6a and ADAM6b genes into the mouse genome.

97. A genetically engineered mouse cell comprising an integrated mouse ADAM6 gene, which ADAM6 gene was integrated separate from the intact mouse immunoglobulin heavy chain locus from which it originated.

98. The genetically engineered mouse cell of claim 97, comprising integrated ADAM6a and ADAM6b genes.

99. A mouse B cell whose genome comprises:
a rearranged human immunoglobulin variable region gene sequence; and
an integrated mouse ADAM6 gene.

100. The mouse B cell of claim 99, wherein the rearranged human immunoglobulin variable gene sequence is selected from the group consisting of a heavy chain variable gene sequence and a light chain variable gene sequence.

101. The mouse B cell of claim 99 or 100, wherein the rearranged human immunoglobulin variable region is operably linked with a mouse constant region gene sequence.

102. The mouse of any one of claims 22, 24, 34, 35, 42, 56, 57, 59, 62, 65, 71, 72, 75, 76, 81, 82, 84, 85, 87 and 97, which mouse has B cells that comprise a rearranged human immunoglobulin variable region gene sequence.

103. The mouse of claim 102, wherein the rearranged human immunoglobulin variable gene sequence is selected from the group consisting of a heavy chain variable gene sequence and a light chain variable gene sequence.

104. The mouse of claim 103, wherein the rearranged human immunoglobulin variable region is operably linked with a mouse constant region gene sequence.