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10 April 2014

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(54) **Title**: METHODS AND COMPOSITIONS FOR GENERATING CONDITIONAL KNOCK-OUT ALLELES

(57) **Abstract**: The disclosure provides methods and compositions for generating conditional knock-out alleles using donor constructs together with sequence-specific nucleases to generate conditional knock-out alleles. Specifically, the donor construct comprises a 5' homology region, a 5' recombinase recognition site, a donor sequence, a 3' recombinase recognition site, and a 3' homology region. Further disclosed are the donor sequences each comprises a target sequence having at least one neutral mutation. Different sequence-specific nucleases can be used with the donor constructs are further disclosed.

INTERNATIONAL SEARCH REPORT

013/045382 19.0

International application No.

PCT/US 13/45382

A. CLASSIFICATION OF SUBJECT MATTER

IPC(8) - A01K 67/00; A01K 67/027; C12N 15/00 (2013.01)

USPC - 800/13; 800/14, 800/18; 435/455

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC(8) - A01K 67/00; A01K 67/027; C12N 15/00 (2013.01)
USPC - 800/13; 800/14, 800/18; 435/455; 800/15, 800/16, 800/17

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
IPC(8) - A01K 67/00; A01K 67/027; C12N 15/00 (2013.01) - see keyword below
USPC - 800/13; 800/14, 800/18; 435/455; 800/15, 800/16, 800/17 - see keyword below

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
PubWEST(USPT,PGPB,EPAB,JPAB); PatBase; Medline, Google: conditional knock-out, KO, homology, 5', 3', recognition site, recombinase, recombination, inducible, nuclease, cleavage, tissue-specific, promoter, zinc finger, ZFN, dimer, Cre, loxP, neutral, silent, mutation, stem cell, pluripotent, Cas9, TALEN, upstream, downstream, flank, allele, Lrp5,

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y --- A	US 2011/0023143 A1 (WEINSTEIN et al.) 27 January 2011 (27.01.2011), para [0005], [0006], [0013], [0014], [0019], [0056], [0060], [0064], [0066], [0067], [0069], [0078], [0079], [0085], [0088], [0089], [0091], [0100], [0104], and [0108]	1-2, 4, 7-11,13-22, 25-27, 30 --- 12, 28-29
Y -- A	XIE et al. Cloning-Independent and Counterselectable Markerless Mutâgenesis System in Streptococcus mutans. Appl Environ Microbiol. 2011, Vol. 77(22), p. 8025-33. pg 8026, col 2, para 4; pg 8030, Fig 5, and pg 8032, col 1, top para	1-2, 4, 7-11,13-22, 25-27, 30 --- 12, 28-29
Y	US 2009/0136507 A1 (ALLEN et al.) 28 May 2009 (28.05.2009), para [0001], [0045], [0115], [0468], and [0589]	14
A	US 2009/0092578 A1 (SU et al.) 09 April 2009 (09.04.2009), para [0700], and SEQ ID NO: 86(3171 nt), the region between nucleotides 1-2974	12, 28-29
A	US 2007/0218071 A1 (MORRIS et al.) 20 September 2007 (20.09.2007), Abstract, para [0020], and SEQ ID NO: 21(108566 nt), the region between nucleotides 25612-28284	12, 28-29
A	GenBank_AC120144, Mus musculus chromosome 8, clone RP24-427K8, complete sequence, 15 October 2004 [online]. [Retrieved on 2012.11.21]. Retrieved from the Internet: <URL: http://www.ncbi.nlm.nih.gov/nuccore/AC120144> Source, and Origin: sequence the region between nucleotides 95807-93750	12, 28-29

Further documents are listed in the continuation of Box C.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
22 January 2014 (22.01.2014)	19 FEB 2014

Name and mailing address of the ISA/US

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C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	GenBank_AC113548, Mus musculus chromosome 9, clone RP23-268F15, complete sequence, 28 July 2004 [online]. [Retrieved on 2012.11.21]. Retrieved from the Internet: <URL: http://www.ncbi.nlm.nih.gov/nuccore/AC113548> Source, and Origin: sequence the region between nucleotides 59239-57476	12, 28-29
A	GenBank_AC090975, Mus musculus strain C57BL6/J chromosome 17 clone RP23-290I19, WORKING DRAFT SEQUENCE, 15 unordered pieces, 15 May 2002 [online]. [Retrieved on 2012.11.21]. Retrieved from the Internet: <URL: http://www.ncbi.nlm.nih.gov/nuccore/AC090975> Source, and Origin: sequence the region between nucleotides 93683-92321	12, 28-29
A	FRIEDEL et al. Generating conditional knockout mice. Methods Mol Biol. 2011, Vol. 693, p. 205-31. Abstract	1-2, 4, 7-22, 25-30

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing filed or furnished:

   a. (means)
      - [ ] on paper
      - [x] in electronic form

   b. (time)
      - [x] in the international application as filed
      - [ ] together with the international application in electronic form
      - [ ] subsequently to this Authority for the purposes of search

2. [ ] In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that in the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments:

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:
This application contains the following inventions or groups of inventions which are not so linked as to form a single general inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees must be paid.

Group I+, claims 1-30, drawn to methods of and compositions for generating a knock-out/conditional knock-out allele in a cell and animal. The first invention is restricted to the first named sequence-specific nuclease that is a zinc finger nuclease (ZFN)(claim 2), the first named recombinase recognition site that is a loxP site (claim 7), the first named recombinase cre (claim 22). Group I+ will be searched to the extent that it reads on ZFN, Cre/loxP, and the donor construct comprises the sequence of SEQ ID NO: 30, 31, 44, 45, or 46, without fee. It is believed that claims 1-2, 4, 7-17, 18-22, 25-30 read on this first named invention. Applicants must indicate, if applicable, the claims which read on the first named invention if different than what was indicated above for this group. Failure to clearly identify how any paid additional invention fees are to be applied to the "+" group(s) will result in only the first claimed invention to be searched/examined. An exemplary election for (an) additional claim(s) to be searched would be: wherein the sequence-specific nuclease is a transcription activator-like effector nuclease (TALEN) (claim 3) and wherein the recombinase recognition site is an frt site and wherein the recombinase is FLP recombinase (claims 23), for paying an additional fee for the pair of election. **see extra sheet***

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
1-2, 4, 7-22, 25-30

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.

Continuation of:
Box No III (unity of invention is lacking)

The inventions listed as Group I+ do not relate to a single general inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons:

Special Technical Feature
Among Group I+, zinc finger nuclease (ZFN) in the first named invention is structurally and functionally different from all other nucleases as it targets to different sequence-specific sites than TALEN or Cas9; and loxP recoombinase regognition site in the first named invention is also structurally different from all other recombination sites including frt and rox, each requires a different specific recombinase for catalyzing the recombination.

Common Technical Features
The inventions of Group I+ share the technical feature of (claim 20) a method of generating a knock-out /conditional knock-out animal, the method comprising the steps of:
a. introducing into the cell a donor construct, wherein the donor construct comprises a 5' homology region, a 5' recombinase recognition site, a donor sequence, a 3' recombinase recognition site, and a 3' homology region, wherein the donor sequence comprises a target sequence having at least one neutral mutation;
b. introducing into the cell a sequence-specific nuclease that cleaves a sequence within the target gene,
c. introducing the cell into a carrier animal to produce a transgenic animal from the transfected cell; and
d. breeding the conditional knock-out animal with a transgenic animal having a trans gene encoding a recombinase protein that catalyzes recombination at the 5' and 3' recombinase recognition site.

However, these shared technical features do not represent a contribution over prior art as being obvious over US 2011/0023143 A1 to WEINSTEIN et al. (hereinafter 'Weinstein'), in view of an article entitled 'Cloning-Independent and Counterselectable Markerless Mutagenesis System in Streptococcus mutans' by XIE et al. (hereinafter 'Xie'; Appl Environ Microbiol. 2011, Vol. 77(22), p. 8025-33) as follows:

Weinstein teaches a method of generating a knock-out or conditional knock-out animal (para [0013]- "knock out" or a "conditional knock out." [0014]- [0019]), comprising generating a conditional knock-out allele in a cell comprising a target gene (para [0013] - 'a genetically modified ... animal cell ...edited chromosomal sequence encoding a neurodevelopmental protein- 'conditional knock out', wherein 'edited chromosomal sequence' is 'a target gene'; para [0014] - 'the chromosomal sequence may be modified ... mutation'; para [0006] - 'The genetic modifications ... include ... temporal-specific knockouts using loxP-flanked ("floxed") alleles ... Cre-recombinase......or over-expression of alleles'), the method comprising the steps of:
a. introducing into the cell a donor construct, wherein the donor construct comprises a 5' homology region, a donor sequence, and a 3' homology region, wherein the donor sequence comprises a target sequence (para [0013]; para [0085] - 'editing chromosomal sequences ...introducing ...donor polynucleotide ... encoding a neurodevelopmental protein into... cell. A donor ... three components ...sequence encoding the protein is flanked by the upstream and downstream sequence, wherein the upstream and downstream sequences share sequence similarity with either side of the site of integration in the chromosome', wherein 'sequence encoding the protein is flanked by the upstream and downstream sequence...share sequence similarity with either side' are ' the donor construct comprises a 5' homology region, a donor sequence, and a 3' homology region', and wherein 'upstream and downstream sequences' are ' 5' homology region' and 'a 3' homology region', respectively; para [0091] - 'to construct a donor polynucleotide ...well-known standard recombinant techniques'); and
b. introducing into the cell a sequence-specific nuclease that cleaves a sequence within the target gene, thereby producing a conditional knock-out allele in the cell (para [0067] - 'the genetically modified ...cell ... using a zinc finger nuclease-mediated genome editing process. ... comprises:(a) introducing into ... cell at least one nucleic acid encoding a zinc finger nuclease that recognizes a target sequence ... able to cleave a site in the chromosomal sequence, and ... (i) at least one donor polynucleotide comprising a sequence for integration flanked by an upstream sequence and a downstream sequence that share substantial sequence identity with either side of the cleavage site'; para [0013] - 'a genetically modified ... cell ...edited chromosomal sequence...conditional knock out').

Weinstein further discloses the system used for generating a conditional knock-out allele in a cell is a zinc-finger system (para [0067] - 'the genetically modified ...cell ... using a zinc finger nuclease-mediated genome editing process.... cleavage site' and [0013]). Weinstein further discloses methods of generating a conditional knock-out allele in a cell include Cre-lox recombination system comprising a donor construct comprises a 5' recombinase recognition site, a donor sequence, a 3' recombinase recognition site (para [0019] - 'A Cre-lox recombination system comprises a Cre recombinase enzyme ... to produce temporal and tissue specific expression ... generated with lox sites flanking a chromosomal sequence, such as a chromosomal sequence encoding a neurodevelopmental protein'; 'lox sites flanking a chromosomal sequence' is a sequence comprising 'a 5' recombinase recognition site, a donor sequence, a 3' recombinase recognition site'; para [0006] - 'The genetic modifications ... include ... temporal-specific knockouts using loxP-flanked ("floxed") alleles ... Cre-recombinase......or over-expression of alleles').

One of ordinary skill in the art at the time the invention was made would have been motivated to combine both Zinc-finger system and Cre-lox recmination system to generate a donor construct, wherein the donor construct comprises a 5' homology region, a 5' recombinase recognition site, a donor sequence, a 3' recombinase recognition site, and a 3' homology region, because both systems are featured by flanking a donor sequence with sequences that specific for the respective system, that is to have 5' and 3' homology regions for Zinc-finger system, and 5' and 3' recombinase recognition sites for Cre-lox recombination system, in order to use a donor construct that can be regulated by two different systems for facilitating generating a conditional knock-out allele in a cell comprising target gene with the possibility to compare both systems in the same set of cells for achieving a desired result with expected success without undue experimentation.

Weinstein further discloses 5' homology region and 3' homology region sequences may share different percentage of homology with the targeted chromosomal sequence (para [0088] - 'The upstream and downstream sequences in the donor polynucleotide may share about 75%...100% sequence identity with the targeted chromosomal sequence').

********************Continued in the next extra sheet************************

Continuation of:
The previous extra sheet - Box No III (unity of invention is lacking)

Weinstein does not specifically teach wherein the donor sequence comprises a target sequence having at least one neutral mutation. Xie discloses a method of solving a problem caused by high rate of recombination of donor sequence and targeted chromosomal sequence by introducing at least one neutral mutation into the donor sequence comprising a target sequence to reduce the homology of a donor sequence with a chromosomal sequence (Pg 8029, col 2, para 1: By engineering a series of silent mutations in the remaining codons after the pheS314AG mutation site, we created a new pheS* cassette (mpheS) that still retained the amino acid sequence of the original cassette but exhibited much lower homology at the nucleotide level downstream of pheS314AG (Fig. 5A and B); pg 8030, Fig 5: a series of silent mutations were engineered in the region downstream of codon 314 to create the new mpheS negative-selection cassette. Three overlapping oligonucleotides containing the desired pheS silent mutations were synthesized. The oligonucleotides were mixed and subjected to overlapping PCR to produce an amplicon carrying the 3' portion of pheS; pg 8032, col 1, top para - 'high rate of recombination between the pheS* cassette and the chromosomal copy of pheS....The problem was finally solved by introducing a series of silent mutations downstream of the pheS* point mutation site to reduce the overall homology of the cassette with the wild-type pheS', wherein 'silent mutations' are 'neutral mutations'; Specification: pg 9, ln 15 - ' Examples of neutral mutations include silent mutations').
It would have been obvious to one of ordinary skill in the art at the time the invention was made to combine the teachings of Weinstein and Xie, to obtain a method of generating a conditional knock-out allele in a cell comprising a target gene, comprising the steps of: a. introducing into the cell a donor construct, wherein the donor construct comprises a 5' homology region, a 5' recombinase recognition site, a donor sequence, a 3' recombinase recognition site, and a 3' homology region, wherein the donor sequence comprises a target sequence; and b. introducing into the cell a sequence-specific nuclease that cleaves a sequence within the target gene, thereby producing a conditional knock-out allele in the cell, based on the teaching of Weinstein, and further wherein the donor sequence comprises a target sequence having at least one neutral mutation, based on the combination of Xie and Weinstein, in order to use the method taught by Xie to produce a series of donor constructs with different degree of reduced homology for selecting a desired homology of the donor sequence with the target sequence to obtain a donor construct that will produce a desired result with expected success without undue experimentation.

Weinstein further discloses c. introducing the cell into a carrier animal to produce a transgenic animal from the transfected cell (para [0066] - 'the cell may be a stem cell... embryonic stem cells'; para [0104] - 'an embryo may be cultured in vivo by transferring the embryo into the uterus of a female host? result in a live birth of an animal derived from the embryo'; para [0100] - 'methods of introducing the nucleic acids to the embryo or cell include ... calcium phosphate-mediated transfection, cationic transfection'; [0019]- 'a genetically modified animal is generated with lox sites flanking a chromosomal sequence, such as a chromosomal sequence encoding a neurodevelopmental protein'; Specification: pg 17: ln 19-20 - 'The cell is then introduced into a female carrier animal to produce the conditional knock-out animal from the cell').

d. breeding the conditional knock-out animal with a transgenic animal having a trans gene encoding a recombinase protein that catalyzes recombination at the 5' and 3' recombinase recognition site (para [0108]; para [0019] - 'The genetically modified animal comprising the lox-flanked chromosomal sequence encoding a neurodevelopmental protein may then be crossed with another genetically modified animal expressing Cre recombinase. Progeny animals comprising the lox-flanked chromosomal sequence and the Cre recombinase are then produced...leading to deletion or inversion of the chromosomal sequence encoding a neurodevelopmental protein', wherein 'be crossed with another genetically modified animal expressing Cre recombinase' is 'breeding the conditional knock-out animal with a transgenic animal having a trans gene encoding a recombinase protein that catalyzes recombination at the 5' and 3' recombinase recognition site'; para [0006] - 'The genetic modifications ... include ... temporal-specific knockouts using loxP-flanked ("floxed") alleles ... Cre-recombinase'; Please also see: Friedel et al.; Methods Mol Biol. 2011, Vol. 693, p. 205-31: Abstract - for how Cre works). Without a shared special technical feature, the inventions lack unity with one another.

Group I+ therefore lack unity under PCT Rule 13 because they do not share a same or corresponding special technical feature providing a contribution over prior art.