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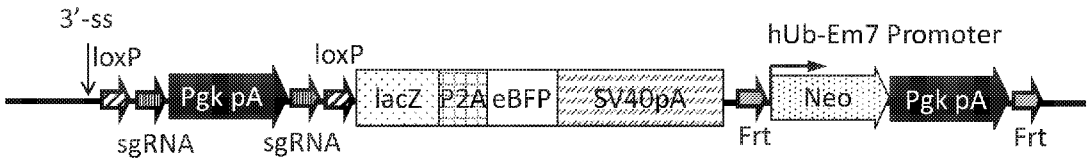

FIG. 1A

(57) Abstract: Methods and compositions are provided for assessing CRISPR/Cas-mediated non-homologous end joining (NHEJ) activity and/or CRISPR/Cas-induced recombination of a target genomic locus with an exogenous donor nucleic acid in vivo or ex vivo. The methods and compositions employ non-human animals comprising a CRISPR reporter such as a genomically integrated CRISPR reporter for detecting and measuring targeted excision of a sequence between two CRISPR/Cas nuclease cleavage sites or disruption of a sequence near a CRISPR/Cas nuclease cleavage site and/or measuring CRISPR/Cas-induced recombination of the CRISPR reporter with an exogenous donor nucleic acid to convert the coding sequence for a first reporter protein to the coding sequence for a different second reporter protein. Methods and compositions are also provided for making and using these non-human animals.

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AMENDED CLAIMS
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We claim:

1. A non-human animal comprising a CRISPR reporter for assessing CRISPR/Cas-induced excision of a nucleic acid between first and second guide RNA target sequences, wherein the CRISPR reporter is integrated at a target genomic locus and comprises a first polyadenylation signal flanked by the first and second guide RNA target sequences followed by a reporter cassette comprising a coding sequence for a first reporter protein and a coding sequence for a second reporter protein in any order, wherein the first reporter protein and the second reporter protein are different.

2. The non-human animal of claim 1, wherein the CRISPR reporter is also for assessing CRISPR/Cas-induced recombination of the CRISPR reporter with an exogenous donor nucleic acid.

3. The non-human animal of claim 2, wherein the first reporter protein comprises a third guide RNA target sequence, wherein recombination of the CRISPR reporter with the exogenous donor nucleic acid changes the coding sequence for the first reporter protein into a coding sequence for a third reporter protein.

4. The non-human animal of claim 3, wherein the coding sequence for the first reporter protein is changed into the coding sequence for the third reporter protein by changing a single codon.

5. The non-human animal of claim 3 or 4, wherein the third guide RNA target sequence overlaps with the portion of the coding sequence for the first reporter protein modified by the exogenous donor nucleic acid.

6. The non-human animal of any preceding claim, wherein one of the first and second reporter proteins comprises a fluorescent reporter protein.

7. The non-human animal of claim 6, wherein the fluorescent reporter protein comprises an enhanced green fluorescent protein (eGFP) or an enhanced blue fluorescent protein (eBFP).

8. The non-human animal of claim 6 or 7, wherein the first and second reporter proteins comprise the fluorescent reporter protein and a non-fluorescent reporter protein.

9. The non-human animal of claim 8, wherein the fluorescent reporter protein can be detected in a flow cytometry assay, and the non-fluorescent protein can be detected in a histochemical assay.

10. The non-human animal of any preceding claim, wherein one of the first and second reporter proteins comprises a beta-galactosidase protein.

11. The non-human animal of any preceding claim, wherein the first polyadenylation signal is also flanked by recombinase recognition sites for a first recombinase.

12. The non-human animal of claim 11, wherein the recombinase recognition sites for the first recombinase are loxP sequences.

13. The non-human animal of any preceding claim, wherein the reporter cassette comprises a multicistronic nucleic acid comprising the coding sequence for the first reporter protein and the coding sequence for the second reporter protein separated by an intervening internal ribosome entry site (IRES) or an intervening 2A peptide coding sequence.

14. The non-human animal of claim 13, wherein the multicistronic nucleic acid comprises a beta-galactosidase coding sequence and an enhanced blue fluorescent protein (eBFP) coding sequence or an enhanced green fluorescent protein (eGFP) coding sequence separated by an intervening P2A peptide coding sequence.

15. The non-human animal of any preceding claim, wherein the CRISPR reporter is operably linked to an endogenous promoter at the target genomic locus.

16. The non-human animal of any preceding claim, wherein the 5' end of the CRISPR reporter further comprises a 3' splicing sequence.

17. The non-human animal of any preceding claim, wherein the CRISPR reporter further comprises a selection cassette.

18. The non-human animal of claim 17, wherein the selection cassette is flanked by recombinase recognition sites for a second recombinase.

19. The non-human animal of claim 17 or 18, wherein the selection cassette comprises a drug resistance gene.

20. The non-human animal of any preceding claim, wherein the distance between the first guide RNA target sequence and the second guide RNA target sequence is less than about 500 base pairs.

21. The non-human animal of any preceding claim, wherein the first guide RNA target sequence and the second guide RNA target sequence are identical, and each comprises SEQ ID NO: 41.

22. The non-human animal of any preceding claim, wherein the non-human animal is a rat or mouse.

23. The non-human animal of claim 22, wherein the non-human animal is a mouse.

24. The non-human animal of any preceding claim, wherein the target genomic locus is a safe harbor locus.

25. The non-human animal of claim 24, wherein the safe harbor locus is a *Rosa26* locus.

26. The non-human animal of claim 25, wherein the CRISPR reporter is inserted into the first intron of the *Rosa26* locus.

27. The non-human animal of any preceding claim, wherein the non-human animal is a mouse, and

wherein the target genomic locus is the *Rosa26* locus, and

wherein the CRISPR reporter is operably linked to the endogenous *Rosa26* promoter, is inserted into the first intron of the *Rosa26* locus, and comprises from 5' to 3':

(a) a 3' splicing sequence;

(b) a first polyadenylation signal flanked by:

(i) first and second loxP sites; and

(ii) first and second guide RNA target sequences, wherein the first guide RNA target sequence and the second guide RNA target sequence are identical, and each comprises SEQ ID NO: 41; and

(c) a reporter cassette, comprising from 5' to 3':

(i) a beta-galactosidase coding sequence;

(ii) a P2A coding sequence;

(iii) an enhanced blue fluorescent protein (eBFP) coding sequence, wherein the eBFP coding sequence comprises a third guide RNA target sequence comprising SEQ ID NO: 42; and

(iv) a second polyadenylation signal, wherein the first polyadenylation signal and the second polyadenylation signal are different.

28. The non-human animal of claim 27, wherein the CRISPR reporter further comprises:

(d) a selection cassette 3' of the reporter cassette, wherein the selection cassette is flanked by FRT sites and comprises from 5' to 3':

(i) a neomycin phosphotransferase coding sequence operably linked to a human ubiquitin promoter; and

(ii) a third polyadenylation signal.

29. The non-human animal of any preceding claim, wherein the non-human animal is heterozygous for the CRISPR reporter at the target genomic locus.

30. The non-human animal of any one of claims 1-28, wherein the non-human animal is homozygous for the CRISPR reporter at the target genomic locus.

31. A method of testing the ability of a CRISPR/Cas nuclease to excise a genomic nucleic acid *in vivo*, comprising:

(a) introducing into the non-human animal of any one of claims 1-30:

(i) a first guide RNA designed to hybridize to the first guide RNA target sequence in the CRISPR reporter;

(ii) a second guide RNA designed to hybridize to the second guide RNA target sequence in the CRISPR reporter; and

(iii) a Cas protein; and

(b) measuring the activity or expression of at least one of the first and second reporter proteins.

32. The method of claim 31, wherein the Cas protein is a Cas9 protein.

33. The method of claim 31 or 32, wherein the Cas protein is introduced into the non-human animal in the form of a protein.

34. The method of claim 31 or 32, wherein the Cas protein is introduced into the non-human animal in the form of a messenger RNA encoding the Cas protein.

35. The method of claim 31 or 32, wherein the Cas protein is introduced into the non-human animal in the form of a DNA encoding the Cas protein, wherein the DNA is operably linked to a promoter active in one or more cell types in the non-human animal.

36. The method of any one of claims 31-35, wherein the first guide RNA and the second guide RNA are identical, and each comprises the sequence set forth in SEQ ID NO: 2.

37. The method of any one of claims 31-36, wherein the reporter protein measured in step (b) is a fluorescent reporter protein, and step (b) comprises a flow cytometry assay.

38. The method of any one of claims 31-36, wherein the reporter protein measured in step (b) is a beta-galactosidase protein, and step (b) comprises a histochemical staining assay.

39. The method of any one of claims 31-38, wherein the guide RNAs in step (a) are introduced in the form of RNA.

40. The method of any one of claims 31-38, wherein the guide RNAs in step (a) are each introduced into the non-human animal in the form of a DNA encoding the guide

RNA, wherein the DNA is operably linked to a promoter active in one or more cell types in the non-human animal.

41. The method of any one of claims 31-40, wherein the introducing comprises adeno-associated virus (AAV)-mediated delivery, lipid nanoparticle-mediated delivery, or hydrodynamic delivery.

42. The method of claim 41, wherein the introducing comprises AAV-mediated delivery.

43. The method of claim 42, wherein the introducing comprises AAV8-mediated delivery, and step (b) comprises measuring activity of the reporter protein in the liver of the non-human animal.

44. A method of optimizing the ability of a CRISPR/Cas nuclease to excise a genomic nucleic acid *in vivo*, comprising:

(I) performing the method of any one of claims 31-43 a first time in a first non-human animal;

(II) changing a variable and performing the method of step (I) a second time with the changed variable in a second non-human animal; and

(III) comparing the activity or expression of the reporter protein in step (I) with the activity or expression of the at least one of the reporter protein in step (II), and selecting the method resulting in the higher activity or expression of the reporter protein.

45. The method of claim 44, wherein the changed variable in step (II) is the delivery method of introducing the guide RNAs and/or the Cas protein into the non-human animal.

46. The method of claim 44, wherein the changed variable in step (II) is the route of administration of introducing the guide RNAs and/or the Cas protein into the non-human animal.

47. The method of claim 44, wherein the changed variable in step (II) is the concentration or amount of the guide RNAs and/or the Cas protein introduced into the non-human animal.

48. The method of claim 44, wherein the changed variable in step (II) is the concentration or amount of the guide RNAs introduced into the non-human animal relative to the concentration or amount of Cas protein introduced into the non-human animal.

49. The method of claim 44, wherein the changed variable in step (II) is the guide RNAs introduced into the non-human animal.

50. The method of claim 44, wherein the changed variable in step (II) is the Cas protein introduced into the non-human animal.

51. A method of testing CRISPR/Cas-induced recombination of a genomic nucleic acid with an exogenous donor nucleic acid *in vivo*, comprising:

(a) providing the non-human animal of any one of claims 1-30, wherein the CRISPR reporter is also for assessing CRISPR/Cas-induced recombination of the CRISPR reporter with an exogenous donor nucleic acid, wherein the first polyadenylation signal has been removed from the CRISPR reporter, and wherein the coding sequence for the first reporter protein comprises a third guide RNA target sequence, and introducing into the non-human animal:

(i) a guide RNA designed to hybridize to the third guide RNA target sequence in the CRISPR reporter;

(ii) a Cas protein; and

(iii) an exogenous donor nucleic acid capable of recombining with the CRISPR reporter and changing the coding sequence for the first reporter protein into a coding sequence for a third reporter protein; and

(b) measuring the activity or expression of the third reporter protein.

52. The method of claim 51, wherein the Cas protein is a Cas9 protein.

53. The method of claim 51, wherein the Cas protein is introduced into the non-human animal in the form of a protein.

54. The method of claim 51, wherein the Cas protein is introduced into the non-human animal in the form of a messenger RNA encoding the Cas protein.

55. The method of claim 51, wherein the Cas protein is introduced into the non-human animal in the form of a DNA encoding the Cas protein, wherein the DNA is operably linked to a promoter active in one or more cell types in the non-human animal.

56. The method of any one of claims 51-55, wherein the third reporter protein measured in step (b) is a fluorescent reporter protein, and step (b) comprises a flow cytometry assay.

57. The method of any one of claims 51-56, wherein the first reporter protein is an enhanced blue fluorescent protein (eBFP), and the third guide RNA comprises the sequence set forth in SEQ ID NO: 14.

58. The method of any one of claims 51-57, wherein the first reporter protein is an enhanced blue fluorescent protein (eBFP), and the third reporter protein is an enhanced green fluorescent protein (eGFP).

59. The method of claim 58, wherein the exogenous donor nucleic acid comprises the sequence set forth in SEQ ID NO: 15 or SEQ ID NO: 16.

60. The method of any one of claims 51-59, wherein the exogenous donor nucleic acid is a single-stranded deoxynucleotide.

61. The method of any one of claims 51-60, wherein the guide RNA in step (a) is introduced in the form of RNA.

62. The method of any one of claims 51-60, wherein the guide RNA in step (a) is introduced into the non-human animal in the form of a DNA encoding the guide RNA, wherein the DNA is operably linked to a promoter active in one or more cell types in the non-human animal.

63. The method of any one of claims 51-62, wherein the introducing comprises adeno-associated virus (AAV)-mediated delivery, lipid nanoparticle-mediated delivery, or hydrodynamic delivery.

64. The method of claim 63, wherein the introducing comprises AAV-mediated delivery.

65. The method of claim 64, wherein the introducing comprises AAV8-mediated delivery, and step (b) comprises measuring activity of the reporter protein in the liver of the non-human animal.

66. A method of optimizing the ability of CRISPR/Cas to induce recombination of a genomic nucleic acid with an exogenous donor nucleic acid *in vivo*, comprising:

(I) performing the method of any one of claims 51-65 a first time in a first non-human animal;

(II) changing a variable and performing the method of step (I) a second time with the changed variable in a second non-human animal; and

(III) comparing the activity or expression of the third reporter protein in step (I) with the activity or expression of the third reporter protein in step (II), and selecting the method resulting in the higher activity or expression of the third reporter protein.

67. The method of claim 66, wherein the changed variable in step (II) is the delivery method of introducing one or more of the guide RNA, the Cas protein, and the exogenous donor nucleic acid into the non-human animal.

68. The method of claim 66, wherein the changed variable in step (II) is the route of administration of introducing one or more of the guide RNA, the Cas protein, and the exogenous donor nucleic acid into the non-human animal.

69. The method of claim 66, wherein the changed variable in step (II) is the concentration or amount of one or more of the guide RNA, the Cas protein, and the exogenous donor nucleic acid introduced into the non-human animal.

70. The method of claim 66, wherein the changed variable in step (II) is the exogenous donor nucleic acid introduced into the non-human animal.

71. The method of claim 66, wherein the changed variable in step (II) is the concentration or amount of the guide RNA introduced into the non-human animal relative to the concentration or amount of Cas protein introduced into the non-human animal.

72. The method of claim 66, wherein the changed variable in step (II) is the guide RNA introduced into the non-human animal.

73. The method of claim 66, wherein the changed variable in step (II) is Cas protein introduced into the non-human animal.

74. A non-human animal cell comprising a CRISPR reporter for assessing CRISPR/Cas-induced excision of a nucleic acid between first and second guide RNA target sequences, wherein the CRISPR reporter is integrated at a target genomic locus and comprises a first polyadenylation signal flanked by the first and second guide RNA target sequences followed by a reporter cassette comprising a coding sequence for a first reporter protein and a coding sequence for a second reporter protein in any order, wherein the first reporter protein and the second reporter protein are different.

75. A non-human animal genome comprising a CRISPR reporter for assessing CRISPR/Cas-induced excision of a nucleic acid between first and second guide RNA target sequences, wherein the CRISPR reporter is integrated at a target genomic locus and comprises a first polyadenylation signal flanked by the first and second guide RNA target sequences followed by a reporter cassette comprising a coding sequence for a first reporter protein and a coding sequence for a second reporter protein in any order, wherein the first reporter protein and the second reporter protein are different.

76. A targeting vector comprising a CRISPR reporter flanked by homology arms, wherein the CRISPR reporter is for assessing CRISPR/Cas-induced excision of a nucleic acid between first and second guide RNA target sequences, wherein the CRISPR reporter comprises a first polyadenylation signal flanked by the first and second guide RNA target sequences followed by a reporter cassette comprising a coding sequence for a first reporter

protein and a coding sequence for a second reporter protein in any order, wherein the first reporter protein and the second reporter protein are different, and wherein the homology arms are suitable for directing recombination with a desired target genomic locus to facilitate genomic integration.

77. A method for making the non-human animal of any one of claims 1-30, comprising:

(a) modifying the genome of a pluripotent non-human animal cell to comprise a CRISPR reporter integrated at a target genomic locus, wherein the CRISPR reporter is for assessing CRISPR/Cas-induced excision of a nucleic acid between first and second guide RNA target sequences, wherein the CRISPR reporter comprises a first polyadenylation signal flanked by the first and second guide RNA target sequences followed by a reporter cassette comprising a coding sequence for a first reporter protein and a coding sequence for a second reporter protein in any order, wherein the first reporter protein and the second reporter protein are different;

(b) identifying or selecting the genetically modified pluripotent non-human animal cell comprising the CRISPR reporter;

(c) introducing the genetically modified pluripotent non-human animal cell into a non-human animal host embryo; and

(d) implanting and gestating the non-human animal host embryo in a surrogate mother.

78. A method for making the non-human animal of any one of claims 1-30, comprising:

(a) modifying the genome of a non-human animal one-cell stage embryo to comprise a CRISPR reporter integrated at a target genomic locus, wherein the CRISPR reporter is for assessing CRISPR/Cas-induced excision of a nucleic acid between first and second guide RNA target sequences, wherein the CRISPR reporter comprises a first polyadenylation signal flanked by the first and second guide RNA target sequences followed by a reporter cassette comprising a coding sequence for a first reporter protein and a coding sequence for a second reporter protein in any order, wherein the first reporter protein and the second reporter protein are different;

(b) selecting the genetically modified non-human animal one-cell stage embryo; and

(c) implanting and gestating the genetically modified non-human animal one-cell stage embryo in a surrogate mother.