Biotechnology 10:20; moreover, epigenetic effects having an influence on replication of such vectors were identified (Haase et al., (013), PLOS One 8(11):e79262). Nonetheless, S/MAR based vectors being stable enough for use in gene therapy are needed.

Suboptimal expression level, gene silencing and low establishment rate represent the major limitations of S/MAR based vectors described in the art.

There is, therefore a need for improved means and methods for stable transfection of cells, in particular using S/MAR elements and avoiding the risks involved with integration of the transgene into the genome of the host cell. This problem is solved by the means and methods disclosed herein.

The present invention relates to vectors useful for non-integrative episomal gene therapy and stem cell therapy, and more particularly, for improving transgene expression and vector establishment efficiency of a self-replicating non-integrative episomal S/MAR expression vector, and for eliminating antibiotic resistance marker gene transfer by non-viral vectors.

Improved vector methods and compositions that improve the expression and establishment efficiency of a self-replicating non-integrative episomal S/MAR expression vector in a target vertebrate cell are disclosed.

One object of the invention is to provide improved expression of a self-replicating non-integrative episomal S/MAR expression vector in a target vertebrate cell.

Another object of the invention is to provide improved establishment efficiency of a self-replicating non-integrative episomal S/MAR expression vector in a target vertebrate cell.

SUMMARY

In one aspect, the present disclosure refers to a method for improving the expression and establishment efficiency of a self-replicating non- integrative episomal S/MAR expression vector in a target vertebrate cell comprising the following steps: a. providing an episomal

S/MAR expression vector comprising: i. a bacterial replication-selection region comprising a bacterial origin of replication and a selectable marker; ii. a transcription unit for expression of a transgene in a vertebrate cell, comprising a promoter, a 5' UTR, a transgene, and a 3' UTR; iii. an S/MAR insert located within said 3' UTR; and b. modifying the episomal S/MAR expression vector such that the S/MAR is flanked by a 5' splice donor site and a 3' splice acceptor site within said 3' UTR, whereby the resultant self-replicating non-integrative episomal S/MAR expression vector has improved expression and establishment efficiency after transfection of a vertebrate cell.

In another aspect, the present disclosure refers to a polynucleotide comprising at least one promoter and an S/MAR element, wherein said S/MAR element is located downstream of said promoter and wherein the nucleic acid sequence of said S/MAR element (S/MAR sequence) comprises at least 3 sequence motifs ATTA per 100 nucleotides over a stretch of at most 200 nucleotides, wherein said S/MAR element is flanked by a splice donor and a splice acceptor, and wherein said polynucleotide further comprises an R6K gamma replication origin with at least 95% sequence identity to a sequence selected from the group consisting of SEQ ID NO: 1, SEQ ID NO:2, SEQ ID NO: 3 and SEQ ID NO: 4.

In yet another aspect, the present disclosure refers to a covalently closed circular recombinant DNA molecule comprising: a. an transcription unit for expression of a transgene in a vertebrate cell, comprising a promoter, a 5' UTR, a transgene, and a 3' UTR; b. an S/MAR located within said 3' UTR wherein said S/MAR is flanked by a 5' splice donor site and a 3' splice acceptor site; c. an R6K gamma replication origin with at least 95% sequence identity to a sequence selected from the group consisting of SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, and SEQ ID NO: 4; and d. an RNA-OUT RNA selectable marker comprising an RNA-IN regulating RNA-OUT functional variant with at least 95% sequence identity to a sequence selected from the group consisting of SEQ ID NO: 5, and SEQ ID NO: 7.

In one example, the present technology provides a method for improving the expression and establishment efficiency of a self-replicating non-integrative episomal S/MAR expression vector in a target vertebrate cell comprising the following steps: a) providing a episomal S/MAR expression vector comprising: i) a bacterial replication-selection region comprising a bacterial origin of replication and a selectable marker; ii) a transcription unit for expression of a transgene in a vertebrate cell, comprising a promoter, a 5' UTR, a transgene, and a 3' UTR;

iii) an S/MAR insert located within said 3' UTR; and b) modifying the episomal S/MAR expression vector such that the S/MAR is flanked by a 5' splice donor site and a 3' splice acceptor site within said 3' UTR, whereby the resultant self-replicating non-integrative episomal S/MAR expression vector has improved the expression and establishment efficiency after transfection of a vertebrate cell. In a further example said S/MAR contains internal AATAAA transcription termination motifs. In a further example said AATAAA transcription termination motifs in said S/MAR are replaced with AATATT motifs. In a further example said S/MAR is selected from the group consisting of human Interferon beta S/MAR, M18 S/MAR, ApoL1 S/MAR. In a further example said SMAR flanked by a 5' splice donor site and a 3' splice acceptor site has at least 95% sequence identity to a sequence selected from the group consisting of SEQ ID NO: 19, SEQ ID NO: 20, SEQ ID NO: 21, SEQ ID NO: 22, and SEQ ID NO: 23. In a further example said bacterial origin of replication is an R6K gamma replication origin. In a further example said bacterial origin of replication is an R6K gamma replication origin with at least 95% sequence identity to a sequence selected from the group consisting of SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, and SEQ ID NO: 4. In a further example said selectable marker is an RNA-IN regulating RNA-OUT functional variant with at least 95% sequence identity to a sequence selected from the group consisting of SEQ ID NO: 5, and SEQ ID NO: 7. In a further example said selectable marker is an RNA-OUT RNA selectable marker that encodes an RNA-IN regulating RNA-OUT RNA with at least 95% sequence identity to SEQ ID NO: 6. In a further example said bacterial replication-selection region comprising a bacterial origin of replication and a selectable marker is a R6K origin-RNA-OUT RNA selectable marker bacterial replication-selection region with at least 95% sequence identity to a sequence selected from the group consisting of SEQ ID NO: 8, SEQ ID NO: 9, SEQ ID NO: 10, SEQ ID NO: 11, SEQ ID NO: 12, SEQ ID NO: 13, SEQ ID NO: 14, SEQ ID NO: 15, SEQ ID NO: 16, and SEQ ID NO: 17. In a further example said 5' UTR further encodes an intron. In a further example said transcription unit further encodes an expression enhancer positioned upstream of the promoter. In a further example said expression enhancer has at least 95% sequence identity to a sequence selected from the group consisting of SEQ ID NO: 27, and SEQ ID NO: 28. In a further example said splice donor site has at least 95% sequence identity to SEQ ID NO:25. In a further example said splice acceptor site has at least 95% sequence identity to SEQ ID NO: 26. In a further example said self-replicating non-integrative episomal S/MAR expression vector is selected from the group consisting of plasmid vector, Nanoplasmid vector, Integration-Deficient Lentivirus vector, and Non-integrating Lentiviral vectors.

In another example, the present technology provides an antibiotic marker free covalently closed circular recombinant DNA molecule comprising: a) an antibiotic marker free transcription unit for expression of a transgene in a vertebrate cell, comprising a promoter, a 5' UTR, a transgene, and a 3' UTR; b) an S/MAR located within said 3' UTR wherein said S/MAR is flanked by a 5' splice donor site and a 3' splice acceptor site; c) an R6K gamma replication origin with at least 95% sequence identity to a sequence selected from the group consisting of SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, and SEQ ID NO: 4; and d) an RNA-OUT RNA selectable marker comprising an RNA-IN regulating RNA-OUT functional variant with at least 95% sequence identity to a sequence selected from the group consisting of SEQ ID NO: 5, and SEQ ID NO: 7. In a further example said R6K gamma replication origin and said RNA-OUT RNA selectable marker comprise a R6K origin-RNA-OUT RNA selectable marker bacterial replication-selection region with at least 95% sequence identity to a sequence selected from the group consisting of SEQ ID NO: 8, SEQ ID NO: 9, SEQ ID NO: 10, SEQ ID NO: 11, SEQ ID NO: 12, SEQ ID NO: 13, SEQ ID NO: 14, SEQ ID NO: 15, SEQ ID NO: 16, and SEQ ID NO: 17. In a further example said S/MAR is selected from the group consisting of human Interferon beta S/MAR, M18 S/MAR, ApoL1 S/MAR. In a further example said S/MAR contains internal AATAAA transcription termination motifs. In a further example said AATAAA transcription termination motifs in said S/MAR are replaced with AATATT motifs. In a further example said S/MAR is selected from the group consisting of human Interferon beta S/MAR, M18 S/MAR, ApoL1 S/MAR. In a further example said SMAR flanked by a 5' splice donor site and a 3' splice acceptor site has at least 95% sequence identity to a sequence selected from the group consisting of SEQ ID NO: 19, SEQ ID NO: 20, SEQ ID NO: 21, SEQ ID NO: 22, and SEQ ID NO: 23. In a further example said 5' UTR further encodes an intron. In a further example said transcription unit further encodes an expression enhancer positioned upstream of the promoter. In a further example said expression enhancer has at least 95% sequence identity to a sequence selected from the group consisting of SEQ ID NO: 27, and SEQ ID NO: 28. In a further example said splice donor site has at least 95% sequence identity to SEQ ID NO:25. In a further example said splice acceptor site has at least 95% sequence identity to SEQ ID NO: 26.

In another example, the present technology provides an covalently closed circular recombinant DNA molecule comprising: a) an transcription unit for expression of a transgene in a vertebrate cell, comprising a promoter, a 5' UTR, a transgene, and a 3' UTR; b) an S/MAR located within said 3' UTR wherein said S/MAR is flanked by a 5' splice donor site

and a 3' splice acceptor site; c) an R6K gamma replication origin with at least 95% sequence identity to a sequence selected from the group consisting of SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, and SEQ ID NO: 4; and d) an RNA-OUT RNA selectable marker comprising an RNA-IN regulating RNA-OUT functional variant with at least 95% sequence identity to a sequence selected from the group consisting of SEQ ID NO: 5, and SEQ ID NO: 7. In a further example said R6K gamma replication origin and said RNA-OUT RNA selectable marker comprise a R6K origin-RNA-OUT RNA selectable marker bacterial replication-selection region with at least 95% sequence identity to a sequence selected from the group consisting of SEQ ID NO: 8, SEQ ID NO: 9, SEQ ID NO: 10, SEQ ID NO: 11, SEQ ID NO: 12, SEQ ID NO: 13, SEQ ID NO: 14, SEQ ID NO: 15, SEQ ID NO: 16, and SEQ ID NO: 17. In a further example said S/MAR is selected from the group consisting of human Interferon beta S/MAR, M18 S/MAR, ApoL1 S/MAR. In a further example said S/MAR contains internal AATAAA transcription termination motifs. In a further example said AATAAA transcription termination motifs in said S/MAR are replaced with AATATT motifs. In a further example said S/MAR is selected from the group consisting of human Interferon beta S/MAR, M18 S/MAR, ApoL1 S/MAR. In a further example said SMAR flanked by a 5' splice donor site and a 3' splice acceptor site has at least 95% sequence identity to a sequence selected from the group consisting of SEQ ID NO: 19, SEQ ID NO: 20, SEQ ID NO: 21, SEQ ID NO: 22, and SEQ ID NO: 23. In a further example said 5' UTR further encodes an intron. In a further example said transcription unit further encodes an expression enhancer positioned upstream of the promoter. In a further example said expression enhancer has at least 95% sequence identity to a sequence selected from the group consisting of SEQ ID NO: 27, and SEQ ID NO: 28. In a further example said splice donor site has at least 95% sequence identity to SEQ ID NO:25. In a further example said splice acceptor site has at least 95% sequence identity to SEQ ID NO: 26.

The resultant plasmids with a S/MAR flanked by a 5' splice donor site and a 3' splice acceptor site within the 3' UTR have surprisingly improved establishment and transgene expression than plasmids with a S/MAR within the 3' UTR without flanking splice donor and acceptor sites.

Claims

1. A method for improving the expression and establishment efficiency of a self-replicating non- integrative episomal S/MAR expression vector in a target vertebrate cell comprising the following steps:

a. providing an episomal S/MAR expression vector comprising:

i. a bacterial replication-selection region comprising a bacterial origin of replication and a selectable marker;

ii. a transcription unit for expression of a transgene in a vertebrate cell, comprising a promoter, a 5' UTR, a transgene, and a 3' UTR;

iii. an S/MAR insert located within said 3' UTR; and

b. modifying the episomal S/MAR expression vector such that the S/MAR is flanked by a 5' splice donor site and a 3' splice acceptor site within said 3' UTR, whereby the resultant self-replicating non-integrative episomal S/MAR expression vector has improved expression and establishment efficiency after transfection of a vertebrate cell.

2. The method of claim 1, wherein said S/MAR insert contains internal AATAAA transcription termination motifs.

3. The method of claim 2, wherein said AATAAA transcription termination motifs in said S/MAR are replaced with AATATT motifs.

4. The method of claim 1, wherein said S/MAR is selected from the group consisting of human Interferon beta S/MAR, M18 S/MAR, Apolipoprotein B S/MAR.

5. The method of claim 1, wherein said SMAR flanked by a 5' splice donor site and a 3' splice acceptor site has at least 95% sequence identity to a sequence selected from the group consisting of SEQ ID NO: 19, SEQ ID NO: 20, SEQ ID NO: 21, SEQ ID NO: 22, and SEQ ID NO: 23.

6. The method of claim 1, wherein said bacterial origin of replication is an R6K gamma replication origin.

7. The method of claim 1, wherein said selectable marker is an RNA-IN regulating RNA-OUT functional variant with at least 95% sequence identity to a sequence selected from the group consisting of SEQ ID NO: 5, and SEQ ID NO: 7.

8. The method of claim 1, wherein said selectable marker is an RNA-OUT RNA selectable marker that encodes an RNA-IN regulating RNA-OUT RNA with at least 95% sequence identity to SEQ ID NO: 6.

9. The method of claim 1, wherein said bacterial replication-selection region comprising a bacterial origin of replication and a selectable marker is a R6K origin-RNA-OUT RNA selectable marker bacterial replication-selection region with at least 95% sequence identity to a sequence selected from the group consisting of SEQ ID NO: 8, SEQ ID NO: 9, SEQ ID NO: 10, SEQ ID NO: 11, SEQ ID NO: 12, SEQ ID NO: 13, SEQ ID NO: 14, SEQ ID NO: 15, SEQ ID NO: 16, and SEQ ID NO: 17.

10. The method of claim 1, wherein said self-replicating non-integrative episomal S/MAR expression vector is selected from the group consisting of plasmid vector, Nanoplasmid vector, Integration-Deficient Lentivirus vector, and Non-integrating Lentiviral vectors.

11. A polynucleotide comprising at least one promoter and an S/MAR element, wherein said S/MAR element is located downstream of said promoter and wherein the nucleic acid sequence of said S/MAR element (S/MAR sequence) comprises at least 3 sequence motifs ATTA per 100 nucleotides over a stretch of at most 200 nucleotides, wherein said S/MAR element is flanked by a splice donor and a splice acceptor, and wherein said polynucleotide further comprises an R6K gamma replication origin with at least 95% sequence identity to a sequence selected from the group consisting of SEQ ID NO: 1, SEQ ID NO:2, SEQ ID NO: 3 and SEQ ID NO: 4.

12. The polynucleotide of claim 11, wherein said promoter is comprised in a transcription unit for expression of a cargo polypeptide and/or a selectable marker in a host cell.

13. The polynucleotide according to claim 12, wherein said transcription unit comprises a promoter, a 5’ UTR, a transgene, and a 3’ UTR.

14. The polynucleotide of claim 13, wherein said S/MAR is located within said 3’ UTR.

15. A covalently closed circular recombinant DNA molecule comprising:

a. an transcription unit for expression of a transgene in a vertebrate cell, comprising a promoter, a 5’ UTR, a transgene, and a 3’ UTR;

b. an S/MAR located within said 3’ UTR wherein said S/MAR is flanked by a 5’ splice donor site and a 3’ splice acceptor site;

c. an R6K gamma replication origin with at least 95% sequence identity to a sequence selected from the group consisting of SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, and SEQ ID NO: 4; and

d. an RNA-OUT RNA selectable marker comprising an RNA-IN regulating RNA-OUT functional variant with at least 95% sequence identity to a sequence selected from the group consisting of SEQ ID NO: 5, and SEQ ID NO: 7.

16. The polynucleotide of any one of claims 11 to 14 or the recombinant DNA molecule of claim 15, wherein said R6K gamma replication origin and said RNA-OUT RNA selectable marker comprise a R6K origin-RNA-OUT RNA selectable marker bacterial replication-selection region with at least 95% sequence identity to a sequence selected from the group consisting of SEQ ID NO: 8, SEQ ID NO: 9, SEQ ID NO: 10, SEQ ID NO: 11, SEQ ID NO: 12, SEQ ID NO: 13, SEQ ID NO: 14, SEQ ID NO: 15, SEQ ID NO: 16, and SEQ ID NO: 17.

17. The polynucleotide of any one of claims 11 to 14 or the recombinant DNA molecule of claim 15, wherein said S/MAR is selected from the group consisting of human Interferon beta S/MAR, M18 S/MAR, Apolipoprotein B S/MAR.

18. The polynucleotide of any one of claims 11 to 14 or the recombinant DNA molecule of claim 15, wherein said SMAR flanked by a 5’ splice donor site and a 3’ splice acceptor site has at least 95% sequence identity to a sequence selected from the group consisting of SEQ ID NO: 19, SEQ ID NO: 20, SEQ ID NO: 21, SEQ ID NO: 22, and SEQ ID NO: 23.

19. The polynucleotide of any one of claims 11 to 14 or the recombinant DNA molecule of claim 15, where said transcription unit further encodes an expression enhancer positioned upstream of the promoter.

20. The polynucleotide of any one of claims 11 to 14 or the recombinant DNA molecule of claim 15, wherein said splice donor site has at least 95% sequence identity to SEQ ID NO:25 and/or wherein said splice acceptor site has at least 95% sequence identity to SEQ ID NO: 26.