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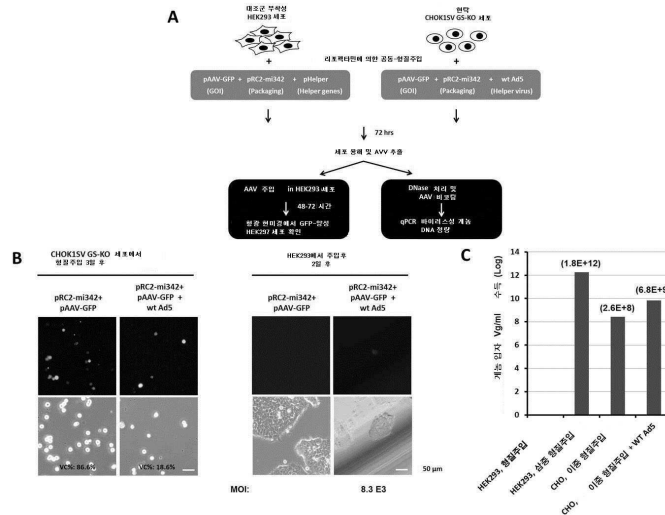
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(54) 발명의 명칭 아데노-관련 바이러스 생산을 위한 포유동물 세포

(57) 요약

본 발명은 4개 이상의 별개의 재조합 표적 부위 (RTS), E1A, E1B 또는 이들의 조합을 포함하는 아데노바이러스 (Ad) 유전자, 및 Ad 유전자에 작동 가능하게 연결된 프로모터를 포함하는 포유동물 세포에 관한 것으로, 여기서 RTS, Ad 유전자, 및 프로모터는 염색체로 통합되고, 재조합 아데노-관련 바이러스 (rAAV) 생산자 숙주 세포를 생성하기 위해 세포를 사용하는 방법; 및 rAAV 생산, 패키지 및 정제하기 위해 AAV 생산자 숙주 세포를 사용하는 방법을 제공할 수 있다.

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명세서

청구범위

청구항 1

(i) 적어도 4개의 별개의 재조합 표적 부위 (RTS), (ii) *E1A*, *E1B* 또는 이들의 조합을 포함하는 아데노바이러스 (Ad) 유전자, 및 (iii) Ad 유전자에 작동 가능하게 연결된 프로모터를 포함하고, 상기 RTS, 상기 Ad 유전자, 및 상기 프로모터는 염색체로 통합되는 것인 포유동물 세포.

청구항 2

제1항에 있어서 상기 세포는, 마우스 세포, 인간 세포, 중국 햄스터 난소 (CHO) 세포, CHO-K1 세포, CHO-DXB11 세포, CHO-DG44 세포, 모든 변이체를 포함하는 CHOK1SV™ 세포. 모든 변이체를 포함하는 CHOK1SV GS-KO™ (글루타민 합성 효소 녹아웃) 세포, 부착성 및 현탁액-적응형(adapted) 변이체를 포함하는 HEK293 세포, HeLa 세포, 또는 HT1080 세포인 포유동물 세포.

청구항 3

제1항 내지 제2항 중 어느 한 항에 있어서, 상기 세포는 4개의 RTS를 포함하는 포유동물 세포.

청구항 4

제1항 내지 제2항 중 어느 한 항에 있어서, 상기 세포는 6개의 RTS를 포함하는 포유동물 세포.

청구항 5

제1항 내지 제4항 중 어느 한 항에 있어서, 상기 세포는 하나 이상의 RTS가 서열 번호 1 내지 30으로 이루어진 군으로부터 선택되는 것인 포유동물 세포.

청구항 6

제1항 내지 제5항 중 어느 한 항에 있어서, 하나 이상의 RTS, Ad 유전자, 및 프로모터가 단일 염색체 좌위에 통합된 것인 포유동물 세포.

청구항 7

제6항에 있어서 상기 염색체 좌위는 *Fer1L4*, *ROSA26*, *HGPRT*, *DHFR*, *COSMC*, *LDHa*, *MGAT1*, *GRIK1*, *NL1*, *NL2*, X 염색체에서 *MIDI*의 첫 번째 인트론, 또는 강화된 발현 및 안정성 영역인 포유동물 세포.

청구항 8

제1항 내지 제7항 중 어느 한 항에 있어서, 부위-특이적 재조합 효소 유전자를 추가로 포함하는 포유동물 세포.

청구항 9

제8항에 있어서, 상기 부위-특이적 재조합 효소 유전자가 염색체로 통합된 것인 포유동물 세포.

청구항 10

제1항 내지 제9항 중 어느 한 항에 있어서, 제2 Ad 유전자를 추가로 포함하고, 상기 제2 Ad 유전자는 염색체로 통합된 것인 포유동물 세포.

청구항 11

제10항에 있어서, 상기 제2 Ad 유전자는 *E1A*, *E1B*, *E2A*, *E4*, *VA*, *MIR342*, 또는 이들의 조합을 포함하는 것인 포유동물 세포.

청구항 12

제11항에 있어서, 상기 제2 Ad 유전자가 아데노바이러스 유형 5 유래인 포유동물 세포.

청구항 13

제10항 내지 제12항 중 어느 한 항에 있어서, 상기 제2 Ad 유전자가 2개의 RTS 사이에 위치하는 것인 포유동물 세포.

청구항 14

제1항 내지 제13항 중 어느 한 항에 있어서, 아데노-관련 바이러스 (AAV) 유전자를 추가로 포함하고, 상기 AAV 유전자는 염색체로 통합된 것인 포유동물 세포.

청구항 15

제14항에 있어서, 상기 AAV 유전자는 *Rep*, *Cap*, 또는 이들의 조합을 포함하는 것인 포유동물 세포.

청구항 16

제16항에 있어서, 상기 AAV 유전자는 아데노-관련 바이러스 유형 2 유래인 포유동물 세포.

청구항 17

제14항 내지 제16항 중 어느 한 항에 있어서, 상기 AAV 유전자는 2개의 RTS 사이에 위치하는 것인 포유동물 세포.

청구항 18

제1항 내지 제17항 중 어느 한 항에 있어서, AAV 벡터 카세트를 추가로 포함하고, 상기 AAV 벡터 카세트는 염색체로 통합된 것인 포유동물 세포.

청구항 19

제18항에 있어서, 상기 AAV 벡터 카세트는 리포터 유전자, 선택 유전자, 치료적 관심 유전자, 또는 이들의 조합을 포함하는 것인 포유동물 세포.

청구항 20

제18항 내지 제19항 중 어느 한 항에 있어서, 상기 AAV 벡터 카세트는 2개의 RTS 사이에 위치하는 것인 포유동물 세포.

청구항 21

제1항 내지 제20항 중 어느 한 항에 있어서, 상기 세포는 헬퍼 바이러스가 실질적으로 없는 것인 포유동물 세포.

청구항 22

(i) 적어도 4개의 별개의 재조합 표적 부위 (RTS), (ii) 아데노바이러스 (Ad) 유전자 *E2A*, *E4*, *VA*, *MIR342* 또는 이들의 조합, 및 (iii) Ad 유전자에 작동 가능하게 연결된 프로모터를 포함하고, 상기 RTS, Ad 유전자 및 프로모터는 염색체로 통합되는 것인 포유동물 세포.

청구항 23

제22항에 있어서, 상기 세포는 마우스 세포, 인간 세포, 중국 햄스터 난소 (CHO) 세포, CHO-K1 세포, CHO-DXB11 세포, CHO-DG44 세포, 모든 변이체를 포함하는 CHOK1SV™ 세포, 모든 변이체를 포함하는 CHOK1SV GS-KO™ (글루타민 합성 효소 녹아웃) 세포, 부착성 및 현탁액-적응형 변이체를 포함하는 HEK293 세포, HeLa 세포, 또는 HT1080 세포인 포유동물 세포.

청구항 24

제22항 내지 제23항 중 어느 한 항에 있어서, 상기 세포는 4개의 RTS를 포함하는 포유동물 세포.

청구항 25

제22항 내지 제23항 중 어느 한 항에 있어서, 상기 세포는 6개의 RTS를 포함하는 포유동물 세포.

청구항 26

제22항 내지 제25항 중 어느 한 항에 있어서, 하나 이상의 RTS가 서열 번호: 1 내지 30으로부터 선택되는 것인 포유동물 세포.

청구항 27

제22항 내지 제26항 중 어느 한 항에 있어서, 상기 RTS, 상기 Ad 유전자, 및 상기 프로모터가 단일 염색체 좌위에 통합된 것인 포유동물 세포.

청구항 28

제27항에 있어서, 상기 염색체 좌위는 *Fer1L4*, *ROSA26*, *HGPRT*, *DHFR*, *COSMC*, *LDHa*, *MGAT1*, *GRIK1*, *NL1*, *NL2*, *X* 염색체상의 *MIDI*의 첫 번째 인트론, 또는 강화된 발현 및 안정성 영역인 포유동물 세포.

청구항 29

제22항 내지 제27항 중 어느 한 항에 있어서, 부위-특이적 재조합 효소 유전자를 추가로 포함하는 것인 포유동물 세포.

청구항 30

제29항에 있어서, 상기 부위-특이적 재조합 효소 유전자가 염색체로 통합된 것인 포유동물 세포.

청구항 31

제22항 내지 제30항 중 어느 한 항에 있어서, 제2 Ad 유전자를 추가로 포함하고, 상기 제2 Ad 유전자는 염색체로 통합된 것인 포유동물 세포.

청구항 32

제31항에 있어서, 상기 제2 Ad 유전자가 *E1A*, *E1B*, *E2A*, *E4*, *VA*, *MIR342*, 또는 이들의 조합을 포함하는 것인 포유동물 세포.

청구항 33

제32항에 있어서, 상기 제2 Ad 유전자가 아테노바이러스 유형 5 유래인 포유동물 세포.

청구항 34

제31항 내지 제33항 중 어느 한 항에 있어서, 상기 제2 Ad 유전자는 2개의 RTS 사이에 위치하는 것인 포유동물 세포.

청구항 35

제22항 내지 제34항 중 어느 한 항에 있어서 상기 세포는, 아테노-관련 바이러스 (AAV) 유전자를 추가로 포함하고, 상기 AAV 유전자는 염색체로 통합된 것인 포유동물 세포.

청구항 36

제35항에 있어서, 상기 AAV 유전자는 *Rep*, *Cap*, 또는 이들의 조합을 포함하는 것인 포유동물 세포.

청구항 37

제36항에 있어서, 상기 AAV 유전자가 아테노-관련 바이러스 유형 2로부터 유래된 것인 포유동물 세포.

청구항 38

제35항 내지 제37항 중 어느 한 항에 있어서, 상기 AAV 유전자가 2개의 RTS 사이에 위치하는 것인 포유동물 세포.

청구항 39

제22항 내지 제38항 중 어느 한 항에 있어서, AAV 벡터 카세트를 추가로 포함하고, 상기 AAV 벡터 카세트는 염색체로 통합된 것인 포유동물 세포.

청구항 40

제39항에 있어서, 상기 AAV 벡터 카세트가 리포터 유전자, 선택 유전자, 치료적 관심 유전자 또는 이들의 조합을 포함하는 것인 포유동물 세포.

청구항 41

제39항 내지 제40항 중 어느 한 항에 있어서, 상기 AAV 벡터 카세트는 2개의 RTS 사이에 위치하는 것인 포유동물 세포.

청구항 42

제20항 내지 제41항 중 어느 한 항에 있어서, 상기 세포는 헬퍼 바이러스가 실질적으로 없는 것인 포유동물 세포.

청구항 43

(i) 적어도 4개의 별개의 제조합 표적 부위 (RTS); (ii) 및 *Rep*, *Cap*, 또는 이들의 조합을 포함하는 아데노-관련 바이러스 (AAV) 유전자를 포함하고, 상기 RTS 및 AAV 유전자는 염색체로 통합되는 것인 포유동물 세포.

청구항 44

제43항에 있어서, 상기 AAV 유전자가 아데노-관련 바이러스 유형 2로부터 유래된 것인 포유동물 세포.

청구항 45

제43항 내지 제44항 중 어느 한 항에 있어서, 상기 세포는 마우스 세포, 인간 세포, 중국 햄스터 난소 (CHO) 세포, CHO-K1 세포, CHO-DXB11 세포, CHO-DG44 세포, 모든 변이체를 포함하는 CHOK1SV™ 세포, 모든 변이체를 포함하는 CHOK1SV GS-KO™ (글루타민 합성 효소 녹아웃) 세포, 부착성 및 현탁액-적응형 변이체를 포함하는 HEK293 세포, HeLa 세포 또는 HT1080 세포인 포유동물 세포.

청구항 46

제43항 내지 제45항 중 어느 한 항에 있어서, 상기 세포는 4개의 RTS를 포함하는 포유동물 세포.

청구항 47

제43항 내지 제45항 중 어느 한 항에 있어서, 상기 세포는 6개의 RTS를 포함하는 포유동물 세포.

청구항 48

제43항 내지 제47항 중 어느 한 항에 있어서, 하나 이상의 RTS가 서열 번호: 1 내지 30으로부터 선택되는 것인 포유동물 세포.

청구항 49

제43항 내지 제48항 중 어느 한 항에 있어서, 상기 RTS 및 상기 AAV 유전자가 단일 염색체 좌위에 통합된 것인 포유동물 세포.

청구항 50

제49항에 있어서, 상기 염색체 좌위는 *Fer1L4*, *ROSA26*, *HGPRT*, *DHFR*, *COSMC*, *LDHa*, *MGAT1*, *GRIK1*, *NL1*, *NL2*, X 염색체상의 *MIDI*의 첫 번째 인트론, 또는 강화된 발현 및 안정성 영역인 포유동물 세포.

청구항 51

제43항 내지 제50항 중 어느 한 항에 있어서, 부위-특이적 재조합 효소 유전자를 추가로 포함하는 것인 포유동물 세포.

청구항 52

제51항에 있어서, 상기 부위-특이적 재조합 효소 유전자가 염색체로 통합된 것인 포유동물 세포.

청구항 53

제43항 내지 제52항 중 어느 한 항에 있어서, Ad 유전자 및 Ad 유전자에 작동 가능하게 연결된 프로모터를 추가로 포함하고, 이에 의해 상기 Ad 유전자 및 상기 프로모터는 염색체로 통합되는 것인 포유동물 세포.

청구항 54

제53항에 있어서, 상기 Ad 유전자는 *E1A*, *E1B*, *E2A*, *E4*, *VA*, *MIR342*, 또는 이들의 조합을 포함하는 것인 포유동물 세포.

청구항 55

제54항에 있어서, 상기 Ad 유전자는 아데노바이러스 유형 5 유래인 포유동물 세포.

청구항 56

제43항 내지 제55항 중 어느 한 항에 있어서, 상기 AAV 유전자는 2개의 RTS 사이에 위치하는 것인 포유동물 세포.

청구항 57

제43항 내지 제56항 중 어느 한 항에 있어서, AAV 벡터 카세트를 추가로 포함하는 포유동물 세포

청구항 58

제57항에 있어서, 상기 AAV 벡터 카세트는 리포터 유전자, 선택 유전자, 치료적 관심 유전자 또는 이들의 조합을 포함하는 것인 포유동물 세포.

청구항 59

제57항 내지 제58항 중 어느 한 항에 있어서, 상기 관심 유전자는 2개의 RTS 사이에 위치하는 것인 포유동물 세포.

청구항 60

제43항 내지 제59항 중 어느 한 항에 있어서, 상기 세포는 헬퍼 바이러스가 실질적으로 없는 것인 포유동물 세포.

청구항 61

- a. 6개의 별개의 재조합 표적 부위 (RTS);
- b. *E1A* 및 *E1B*를 포함하는 아데노바이러스 (Ad) 유전자;
- c. Ad 유전자에 작동 가능하게 연결된 프로모터, 상기 RTS, 상기 Ad 유전자 및 상기 프로모터는 염색체로 통합되고;
- d. *E2A*, *E4*, *VA* 및 *MIR342*를 포함하는 제2 Ad 유전자, 상기 제2 Ad 유전자는 2개의 RTS 사이에 위치하고;
- e. *Rep* 및 *Cap*을 포함하는 아데노-관련 바이러스 (AAV) 유전자, 상기 AAV 유전자는 2개의 RTS 사이에 위치하고; 및
- f. 리포터 유전자, 선택 유전자 또는 치료적 관심 유전자를 포함하는 AAV 벡터 카세트로서, 상기 AAV 벡터 카세

트는 2개의 RTS 사이에 위치하는 AAV 벡터 카세트;를 포함하는 중국 햄스터 난소 (CHO) 세포.

청구항 62

E1A 및 *E1B*를 포함하는 아데노바이러스 (Ad) 유전자 및 Ad 유전자에 작동 가능하게 연결된 프로모터를 포함하는 중국 햄스터 난소 (CHO) 세포; *E2A*, *E4*, *VA* 및 *MIR342*를 포함하는 제2 Ad 유전자; *Rep* 및 *Cap*을 포함하는 아데노-관련 바이러스 (AAV) 유전자; 및 리포터 유전자, 선택 유전자, 치료적 관심 유전자 또는 이들의 조합을 포함하는 AAV 벡터 카세트.

청구항 63

- a. 적어도 4개의 별개의 재조합 표적 부위 (RTS), *E1A*, *E1B* 또는 이들의 조합을 포함하는 아데노바이러스 (Ad) 유전자, 및 상기 Ad 유전자에 작동 가능하게 연결된 프로모터를 포함하는 세포를 제공하는 단계로, 상기 RTS, 상기 Ad 유전자 및 상기 프로모터는 염색체로 통합되고;
 - b. (a)에서 제공된 세포를 아데노-관련 바이러스 (AAV) 유전자, 제2 Ad 유전자, AAV 벡터 카세트, 또는 이들의 조합을 인코딩하는 교환 가능한 카세트를 포함하는 벡터로 형질주입시키는 단계;
 - c. 교환 가능한 카세트를 염색체에 통합시키는 단계; 및
 - d. 염색체에 통합된 교환 가능한 카세트를 갖는 rAAV 생산자 세포를 선별하는 단계;
- 를 포함하는 재조합 아데노-관련 바이러스 (rAAV) 생산자 세포의 제조 방법.

청구항 64

제63항에 있어서, 형질주입 (b)는 2개의 벡터를 갖고, 제1벡터는 제2 Ad 유전자 및 AAV 유전자를 포함하는 교환 가능한 카세트를 포함하고, 제2 벡터는 AAV 벡터 카세트를 포함하는 교환 가능한 카세트를 포함하는 것인 방법.

청구항 65

제63항에 있어서, 형질주입 (b)는 2개의 벡터를 갖고, 제1벡터는 제2 Ad 유전자를 포함하는 교환 가능한 카세트를 포함하고, 제2 벡터는 AAV 유전자를 포함하는 교환 가능한 카세트를 포함하는 것인 방법.

청구항 66

제63항에 있어서, 상기 형질주입 (b)는 3개의 벡터를 갖고, 제1벡터는 제2 Ad 유전자를 포함하는 교환 가능한 카세트를 포함하고, 제2벡터는 AAV 유전자를 포함하는 교환 가능한 카세트를 포함하고, 제3벡터는 AAV 벡터 카세트를 포함하는 교환 가능한 카세트를 포함하는 방법.

청구항 67

제63항 내지 제66항 중 어느 한 항에 있어서, 각각의 교환 가능한 카세트는 세포의 RTS 중 2개와 일치하는 2개의 RTS를 추가로 포함하는 것인 방법.

청구항 68

제63항 내지 제67항 중 어느 한 항에 있어서, 상기 제2 Ad 유전자는 *E1A*, *E1B*, *E2A*, *E4*, *VA*, *MIR342*, 또는 이들의 조합을 포함하는 것인 방법.

청구항 69

제63항 내지 제69항 중 어느 한 항에 있어서, 상기 AAV 유전자는 *Rep*, *Cap*, 또는 이들의 조합을 포함하는 것인 방법.

청구항 70

제63항 내지 제69항 중 어느 한 항에 있어서, 상기 AAV 벡터 카세트는 리포터 유전자, 선택 유전자, 치료적 관심 유전자 또는 이들의 조합을 포함하는 것인 방법.

청구항 71

(i) rAAV로 숙주 세포를 감염시키는 단계, (ii) AAV 벡터 카세트로 패키징된 rAAV 생산 단계, 및 (iii) 상기 패키징된 rAAV를 정제하는 단계로서, 상기 숙주 세포는:

- a. 적어도 4개의 별개의 재조합 표적 부위 (RTS);
- b. *E1A*, *E1B* 또는 이들의 조합을 포함하는 아데노바이러스 (Ad) 유전자;
- c. Ad 유전자에 작동 가능하게 연결된 프로모터로, 상기 RTS, 상기 Ad 유전자 및 상기 프로모터는 염색체로 통합되고;
- d. *E1A*, *E1B*, *E2A*, *E4*, *VA*, *MIR342*, 또는 이들의 조합을 포함하는 제2 Ad 유전자;
- e. *Rep*, *Cap*, 또는 이들의 조합을 포함하는 아데노-관련 바이러스 (AAV) 유전자; 및
- f. 리포터 유전자, 선택 유전자, 치료적 관심 유전자 또는 이들의 조합을 포함하는 AAV 벡터 카세트를 포함하는;

재조합 아데노-관련 바이러스 (rAAV)의 제조 방법.

청구항 72

제71항에 있어서, rAAV 생산 및 패키징에 살아있는 야생형 헬퍼 바이러스가 요구되지 않는 것인 방법.

청구항 73

제71항 내지 제72항 중 어느 한 항에 있어서, rAAV 생산에 *E1A*의 발현이 요구되지 않는 것인 방법.

청구항 74

제71항 내지 제73항 중 어느 한 항에 있어서, 정제 후에 최소 1.0×10^9 vg/mL 활성 rAAV를 얻는 방법.

발명의 설명

기술 분야

[0001] 본 개시 내용은 4개 이상의 별개의 재조합 표적 부위 (RTS), *E1A*, *E1B* 또는 이들의 조합을 포함하는 아데노바이러스 (Ad) 유전자, 및 Ad 유전자에 작동 가능하게 연결된 프로모터를 포함하는 포유동물 세포에 관한 것이며, 여기서 RTS, Ad 유전자 및 프로모터는 염색체-통합된다. 본 발명은 또한 재조합 아데노-관련 바이러스 (rAAV) 생산자 세포를 생산하기 위해 세포를 사용하는 방법 및 살아있는 헬퍼 바이러스 없이 rAAV를 생산, 포장 및 정제하기 위해 rAAV 생산자 세포를 사용하는 방법에 관한 것이다.

배경 기술

[0002] 유전자 치료 제품의 오래 지속되고 효율적인 전달을 얻기 위해 재조합 아데노-관련 바이러스 (rAAV)의 사용이 임상 개발에서 적극적으로 추구하고 있다. rAAV 생산을 위한 바콜로바이러스 기반 플랫폼은 종종 가변적이고 시간이 많이 걸리는 원료를 필요로 한다. HEK293에서의 삼중 형질주입 및 일시적 발현은 확장성이 좋지 않아 제한될 수 있다. 기존의 확장 가능한 HeLa 세포 기반 생산자 세포주 공정은 헬퍼 바이러스로서 야생형 (wt) 아데노바이러스 5 (Ad5)를 필요로 하며, 최종 제품에서 헬퍼 바이러스의 제거는 안전 문제로 인해 중요하다. rAAV를 생산하기 위해 세포주를 구성할 때, 관심 유전자 및 보조 헬퍼 유전자는 일시적으로 발현되거나 숙주 염색체에 통합될 수 있다. 세포 계능의 변형에 사용되는 통상적인 DNA 형질도입 경로는 예측할 수 없는 변형을 겪을 수 있고 바이러스 혈청형의 상이한 요구 사항을 조정하기 위해 쉽게 상호 변경될 수 없다.

[0003] 치료적 재조합 아데노-관련 바이러스 (AAV) 구성물은 복제 결함이며 복제 및 후속 감염 단계를 위해 wt 아데노바이러스 (Ad)의 공동 감염을 필요로 한다. 치료적 재조합 아데노-관련 바이러스 (rAAV) 벡터의 생산은 일반적으로 3가지 상이한 성분이 숙주에 공급될 것을 요구한다: (i) 복제 및 패키징 요소를 제공하는 아데노-관련 바이러스 유전자 *Rep/Cap*, (ii) 바이러스 패키징을 위한 Ad 계능의 헬퍼 바이러스 기능, 및 (iii) 3' 및 5' AAV 역 말단 반복 서열 (ITR) 사이에 위치한 대상 유전자(gene of interest; GOI). 효율적인 유전자 발현, DNA 복제 및 패키징에 필요한 Ad 유전자는 *E1A*, *E1B*, *E2A*, *E4* 및 *VA RNA*를 포함하는 것으로 생각된다. *E1A*는 Ad 감염 동안 만들어진 최초의 유전자 산물 인 것으로 제안된다(Shenk, T. et al., Fields Virology, 3rd ed., 2211-

2148(1996)). *E1A*는 AAV Rep 유전자 프로모터 p5 및 p19로부터 전사를 상향 조절함으로써 및 *E1B*, *E2A* 및 *E4*에 대한 초기 아데노바이러스 프로모터를 활성화시킴으로써 바이러스 복제를 시작하는 신호로서 작용하는 것으로 여겨진다. *E1A*는 또한 AAV 인코딩 단백질 (Rep 및 Cap)이 이 기능을 수행 할 수 없기 때문에 바이러스 DNA 복제를 위해 세포주기의 S- 단계로 숙주 세포를 유도하는데 사용되는 것으로 여겨진다. *E1A*의 역효과는 p53을 안정화시켜 세포예정사(apoptosis)를 유발한다는 것이다(Low et al., *Genes & Dev.* 7:535-545 (1993)). *E1A*의 누출된 발현은 AAV Rep 유전자뿐만 아니라 Ad 유전자를 작동시킬 수 있다. 후자는 세포 증식 억제 및 세포 독성인 것으로 보고되어, *E1A*를 본질적으로 발현하는 세포로부터 안전한 세포주를 얻는 것이 어렵다. 조절된 *E1A* 유전자 발현은 헬퍼 바이러스 비함유 AAV 패키징 세포주의 성공에 결정적인 것으로 보인다.

[0004] rAAV를 생산하기 위해 이들 요소를 함께 가져 오는 다른 세포주 및 다른 접근법이 시도되어왔다. 이들 상이한 세포주는 HEK293 및 HeLa 세포주를 포함한다. 다른 접근법은 또한 바칼로바이러스 시스템의 사용을 포함하였다. 그러나 각 세포주와 접근 방식에는 문제점이 있으며 확장 가능하고 강력한 rAAV 생산 공정을 위한 이상적인 플랫폼은 없다.

[0005] 첫번째 rAAV 생산 단계는 물리적으로 전단된 Ad5 DNA를 사용하여 일차 배아 신장 세포의 형질주입에 의해 생산된 HEK293 세포를 포함한다((Graham F.L. et al., *J. Gen. Virol.* 36:59-74 (1977)). 계능 분석에 따르면, HEK293 세포는 E1 영역 및 추가 측면 서열을 함유하는 아데노바이러스 계능의 좌측 말단 (염기 1-4344)의 통합된 단편을 보유하는 것으로 입증되었다(Louis N. et al, *Virology* 233:423-9 (1997)). 일부 삼중-형질주입 공정은 나머지 필수 요소를 함유하는 플라스미드로 형질 감염하기 위해 접착 성 HEK293 세포를 사용하였다. rAAV 생산에서 HEK293을 사용할 수 있지만, 다양한 적응증 및 의존에 대한 증가하는 벡터 요구를 충족시키기 위해 확장 불가능한 부착성 세포 기반 공정은 안전한 생산자 세포주 대신 일시적 형질주입에 의존한다.

[0006] 두 번째 rAAV 생산 단계는 AAV 유전자 *Rep/Cap* 및 GOI를 모두 함유하는 안정적으로 형질주입된 HeLa 세포 클론의 생산 및 선택을 특징으로 한다. 이어서, 이들 세포주는 wt Ad5에 의해 감염되어 rAAV 입자의 패키징을 개시할 수 있다. 이 방법은 확장 가능하지만, 이 클론의 생산은 길고 노동 집약적인 단계일 수 있으며 이 단계의 최종 산물에서 wt Ad5 바이러스를 제거하면 단계에 여러 단계가 추가된다.

[0007] 세 번째 rAAV 생산 단계는 AAV 유전자 도입을 위한 재조합 바칼로바이러스 (*Rep/Cap*)에 의존하며 SF9 곤충 세포에서 rAAV 생산에 필요한 헬퍼 바이러스 기능을 직접 수행한다. 이 방법은 현탁액 배양에서 확장 가능하지만, 플라크 정제 된 재조합 바칼로바이러스 구조물의 생산을 필요로 하고, 생산물 개발에 더 오랜 시간이 걸린다.

[0008] rAAV 생산을 위한 새롭고 효율적인 플랫폼으로서, 이는 임의의 원하는 AAV 혈청형에 대해 안정적인 생산자 세포주를 생산하는 빠른 공정을 제공하여 이러한 결함을 해결하고 전임상 및 임상 연구에 사용하기 위한 고품질 벡터를 제공하기 위해서는 살아있는 헬퍼 바이러스를 첨가하지 않고도 추가로 높은 확장 가능한 생산 수율을 제공할 수 있다.

[0009] I 형 인터페론 (IFN)은 발견된 최초의 사이토카인이었으며 바이러스 복제를 방해할 수 있는 강력한 능력으로 명명되었다. 많은 바이러스 산물은 단백질 키나아제 (PKR)를 억제하는 인플루엔자 NS1 단백질 및 백시니아 바이러스 E3L 단백질 및 백시니아 바이러스-코딩된 가용성 IFN- 알파/베타 수용체 디코이드(decoys)과 같은 IFN 관련 신호를 차단할 수 있다.

[0010] 중국 햄스터 난소(CHO) 세포 (Puck, *J. Exp. Med.*, 108: 259-268 (1958))는 높은 생산성, 견고한 특성, 안전 기록 및 업계 기록(Birch and Racher, *Adv. Drug Delivery Rev.*, 58:671-685 (2006))으로 인해 포유동물 세포로부터 재조합 단백질의 상업적 생산에 종종 사용되는 잘 특성화 된 세포주이다. 이들의 그러나, CHO 세포는 rAAV의 생산에 이전에 사용되지 않았다.

[0011] 다양한 공보가 본원에 인용되어 있으며, 그 개시 내용은 그 전문이 본원에 참조로 포함된다.

발명의 내용

해결하려는 과제

[0012] 본 발명은 4개 이상의 별개의 재조합 표적 부위 (RTS), *E1A*, *E1B* 또는 이들의 조합을 포함하는 아데노바이러스 (Ad) 유전자, 및 Ad 유전자에 작동 가능하게 연결된 프로모터를 포함하는 포유동물 세포에 관한 것으로, 여기서 RTS, Ad 유전자, 및 프로모터는 염색체로 통합되는 포유동물 세포를 제공하고자 한다. 또한, 재조합 아데노-관련 바이러스 (rAAV) 생산자 숙주 세포를 생성하기 위해 세포를 사용 하는 방법; 및 rAAV 생산, 패키지 및 정제

하기 위해 AAV 생산자 숙주 세포를 사용 하는 방법을 제공하고자 한다.

과제의 해결 수단

[0013]

일부 실시예에서, 본 개시 내용은 (i) 4개 이상의 별개의 재조합 표적 부위 (RTS), (ii) *E1A*, *E1B* 또는 이들의 조합을 포함하는 아데노바이러스 (Ad) 유전자, 및 (iii) Ad 유전자에 작동 가능하게 연결된 프로모터를 포함하는 포유동물 세포를 제공하고, 상기 RTS, Ad 유전자 및 프로모터는 염색체로 통합된다. 일부 실시예에서, 상기 세포는 마우스 세포, 인간 세포, 중국 햄스터 난소 (CHO) 세포, CHO-K1 세포, CHO-DXB11 세포, CHO-DG44 세포, 모든 변이체를 포함하는 CHOK1SV™ 세포 (Lonza, Slough, UK), 모든 변이체를 포함하는 CHOK1SV GS-KO™ (글루타민 합성 효소 녹아웃) 세포 (Lonza, Slough, UK), 부착성 및 현탁액-적응형 변이체를 포함하는 HEK293 세포, HeLa 세포, 또는 HT1080 세포이다. 일부 실시 양태에서, 상기 세포는 4개의 RTS를 포함한다. 일부 실시 양태에서, 상기 세포는 6개의 RTS를 포함한다. 일부 실시 양태에서, 하나 이상의 RTS는 서열 번호 1 내지 30으로 이루어진 군으로부터 선택된다. 일부 실시 양태에서, 하나 이상의 RTS, Ad 유전자 및 프로모터는 단일 염색체 유전자 좌위에 통합된다. 일부 실시 양태에서, 염색체 유전자 좌위는 *Fer1L4*, *ROSA26*, *HGPRT*, *DHFR*, *COSMC*, *LDHa*, *MGAT1*, *GRIK1*, *NL1*, *NL2*, X 염색체상의 *MIDI*의 첫 번째 인트론, 또는 강화된 발현 및 안정성 영역이다(EESYRs, 예를 들어, 미국 특허 7,771,997를 참고한다). 일부 실시 양태에서, 상기 세포는 부위-특이적 재조합 효소 유전자를 포함한다. 일부 실시 양태에서, 상기 부위-특이적 재조합 효소 유전자는 염색체로 통합된다. 일부 실시 양태에서, 상기 세포는 제2 Ad 유전자를 포함하고, 상기 제2 Ad 유전자는 염색체로 통합된다. 일부 실시 양태에서, 제2 Ad 유전자는 *E1A*, *E1B*, *E2A*, *E4*, *VA*, *MIR342*, 또는 이들의 조합을 포함한다. 일부 실시 양태에서, 제2 Ad 유전자는 Ad5 유래의 것이다. 일부 실시 양태에서, 제2 Ad 유전자는 2개의 RTS 사이에 위치된다. 일부 실시 양태에서, 세포는 아데노-관련 바이러스 (AAV) 유전자를 추가로 포함하며, AAV 유전자는 염색체-통합된다. 일부 실시 양태에서, AAV 유전자는 *Rep*, *Cap*, 또는 이들의 조합을 포함한다. 일부 실시 양태에서, AAV 유전자는 아데노-관련 바이러스 유형 2로부터 유래된다. 일부 실시 양태에서, AAV 유전자는 2개의 RTS 사이에 위치된다. 일부 실시 양태에서, 세포는 AAV 벡터 카세트를 추가로 포함하고, 여기서 AAV 벡터 카세트는 염색체 통합된다. 일부 실시 양태에서, AAV 벡터 카세트는 리포터 유전자, 선택 유전자, 치료적 관심 유전자, 또는 이들의 조합을 포함한다. 일부 실시 양태에서, AAV 벡터 카세트는 2개의 RTS 사이에 위치된다. 일부 실시 양태에서, 세포는 헬퍼 바이러스가 실질적으로 없다.

[0014]

일부 실시 양태에서, 본 개시 내용은 (i) 4개 이상의 별개의 재조합 표적 부위 (RTS), (ii) Ad 유전자 *E2A*, *E4*, *VA*, *MIR342* 또는 이들의 조합, 및 (iii) Ad 유전자에 작동 가능하게 연결된 프로모터를 포함하는 포유동물 세포를 제공하고, 상기 RTS, Ad 유전자 및 프로모터는 염색체로 통합된다. 일부 실시 양태에서, 세포는 마우스 세포, 인간 세포, 중국 햄스터 난소 (CHO) 세포, CHO-K1 세포, CHO-DXB11 세포, CHO-DG44 세포, 모든 변이체를 포함하는 CHOK1SV™ 세포, 모든 변이체를 포함하는 CHOK1SV GS-KO™ (글루타민 합성 효소 녹아웃) 세포, 부착성 및 현탁액-적응형 변이체를 포함하는 HEK293 세포, HeLa 세포, 또는 HT1080 세포이다. 일부 실시 양태에서, 세포는 4개의 RTS를 포함한다. 일부 실시 양태에서, 세포는 6개의 RTS를 포함한다. 일부 실시 양태에서, 하나 이상의 RTS는 서열 번호 1 내지 30으로 이루어진 군으로부터 선택된다. 일부 실시 양태에서, RTS, Ad 유전자 및 프로모터는 단일 염색체 유전자 좌위에 통합된다. 일부 실시 양태에서, 염색체 유전자 좌위는 *Fer1L4*, *ROSA26*, *HGPRT*, *DHFR*, *COSMC*, *LDHa*, *MGAT1*, *GRIK1*, *NL1*, *NL2*, X 염색체 상의 *MIDI*의 첫 번째 인트론, 또는 강화된 발현 및 안정성 영역 (EESYRs)이다. 일부 구체 예에서, 세포는 부위-특이적 재조합 효소 유전자를 포함한다. 일부 실시 양태에서, 부위-특이적 재조합 효소 유전자는 염색체로 통합된다. 일부 실시 양태에서, 세포는 제2 Ad 유전자를 추가로 포함하며, 상기 제2 Ad 유전자는 염색체로 통합된다. 일부 실시 양태에서, 제2 Ad 유전자는 *E1A*, *E1B*, *E2A*, *E4*, *VA*, *MIR342*, 또는 이들의 조합을 포함한다. 일부 실시 양태에서, 제2 Ad 유전자는 Ad5 유래된다. 일부 실시 양태에서, 제2 Ad 유전자는 2개의 RTS 사이에 위치된다. 일부 실시 양태에서, 세포는 아데노-관련 바이러스 (AAV) 유전자를 추가로 포함하며, 상기 AAV 유전자는 염색체로 통합된다. 일부 실시 양태에서, AAV 유전자는 *Rep*, *Cap*, 또는 이들의 조합을 포함한다. 일부 실시 양태에서, AAV 유전자는 아데노-관련 바이러스 유형 2로부터 유래된다. 일부 실시 양태에서, AAV 유전자는 2개의 RTS 사이에 위치된다. 일부 실시 양태에서, 세포는 AAV 벡터 카세트를 추가로 포함하고, 상기 AAV 벡터 카세트는 염색체로 통합된다. 일부 실시 양태에서, AAV 벡터 카세트는 리포터 유전자, 선택 유전자, 치료적 관심 유전자 또는 이들의 조합을 포함한다. 일부 실시 양태에서, AAV 벡터 카세트는 2개의 RTS 사이에 위치된다. 일부 실시 양태에서, 세포는 헬퍼 바이러스가 실질적으로 없다.

[0015]

일부 실시 양태에서, 본 개시 내용은 (i) 4개 이상의 별개의 재조합 표적 부위 (RTS), (ii) 및 *Rep*, *Cap*, 또는 이들의 조합을 포함하는 아데노-관련 바이러스 (AAV) 유전자를 포함하는 포유동물 세포를 제공하고, 상기 RTS

및 AAV 유전자는 염색체-로 통합된다. 일부 실시 양태에서, AAV 유전자는 아데노 관련 바이러스 유형 2로부터 유래된다. 일부 실시 양태에서, 세포는 마우스 세포, 인간 세포, 중국 햄스터 난소 (CHO) 세포, CHO-K1 세포, CHO-DXB11 세포, CHO-DG44 세포, 모든 변이체를 포함하는 CHOK1SV™ 세포, 모든 변이체를 포함하는 CHOK1SV GS-KO™ 세포, 부착성 및 현탁액-적응형 변이체를 포함하는 HEK293 세포, HeLa 세포, 또는 HT1080 세포이다. 일부 실시 양태에서, 세포는 4개의 RTS를 포함한다. 일부 실시 양태에서, 세포는 6개의 RTS를 포함한다. 일부 실시 양태에서, 하나 이상의 RTS는 서열 번호 1 내지 30으로 이루어진 군으로부터 선택된다. 일부 실시 양태에서, RTS 및 AAV 유전자는 단일 염색체 유전자 좌위에 통합된다. 일부 실시 양태에서, 염색체 유전자좌는 *Fer1L4*, *ROSA26*, *HGPRT*, *DHFR*, *COSMC*, *LDHa*, *MGAT1*, *GRIK1*, *NL1*, *NL2*, X 염색체 상의 *MIDI*의 첫 번째 인트론, 또는 강화된 발현 및 안정성 영역 (EESYRs)이다. 일부 실시 양태에서, 세포는 부위-특이적 재조합 효소 유전자를 포함한다. 일부 실시 양태에서, 부위-특이적 재조합 효소 유전자는 염색체로 통합된다. 일부 실시 양태에서, 세포는 Ad 유전자 및 Ad 유전자에 작동 가능하게 연결된 프로모터를 추가로 포함하며, 이에 의해 Ad 유전자 및 프로모터는 염색체로 통합된다. 일부 실시 양태에서, Ad 유전자는 *E1A*, *E1B*, *E2A*, *E4*, *VA*, *MIR342* 또는 이들의 조합을 포함한다. 일부 실시 양태에서, Ad 유전자는 Ad5로부터 유래된다. 일부 실시 양태에서, AAV 유전자는 2개의 RTS 사이에 위치된다. 일부 실시 양태에서, 세포는 AAV 벡터 카세트를 추가로 포함하고, 상기 AAV 벡터 카세트는 염색체로 통합된다. 일부 실시 양태에서, AAV 벡터 카세트는 리포터 유전자, 선택 유전자, 치료적 관심 유전자 또는 이들의 조합을 포함한다. 일부 실시 양태에서, AAV 벡터 카세트는 2개의 RTS 사이에 위치된다. 일부 실시 양태에서, 세포는 헬퍼 바이러스가 실질적으로 없다.

[0016] 일부 실시 양태에서, 본 개시 내용은 6개의 별개의 재조합 표적 부위 (RTS), 여기서 하나 이상의 RTS는 서열 번호 1-30으로 이루어진 군으로부터 선택되고, *E1A* 및 *E1B*를 포함하는 아데노바이러스 (Ad) 유전자, Ad 유전자에 작동 가능하게 연결된 프로모터로, 상기 RTS, Ad 유전자 및 프로모터는 염색체로 통합되고, *E2A*, *E4*, *VA*, 및 *MIR342*를 포함하는 제2 Ad 유전자로, 상기 제2 Ad 유전자는 염색체로 통합되고 2개의 RTS 사이에 위치하며, *Rep* 및 *Cap*을 포함하는 아데노-관련 바이러스 (AAV) 유전자로, 상기 AAV 유전자는 염색체로 통합되고 2개의 RTS 사이에 위치하고, 리포터 유전자, 선택 유전자 또는 치료적 관심 유전자를 포함하는 AAV 벡터 카세트를 포함하고, 상기 AAV 벡터 카세트는 염색체로 통합되고 2개의 RTS 사이에 위치되는 중국 햄스터 난소 (CHO) 세포를 제공한다.

[0017] 일부 실시 양태에서, 본 개시 내용은 *E1A* 및 *E1B*를 포함하는 아데노바이러스 (Ad) 유전자 및 Ad 유전자에 작동 가능하게 연결된 프로모터, *E2A*, *E4*, *VA*, 및 *MIR342*를 포함하는 제2 Ad 유전자, *Rep* 및 *Cap*을 포함하는 아데노-관련 바이러스 (AAV) 유전자, 및 리포터 유전자, 선택 유전자, 치료적 관심 유전자 또는 이들의 조합을 포함하는 AAV 벡터 카세트를 포함하는 중국 햄스터 난소 (CHO) 세포를 제공한다.

[0018] 일부 실시 양태에서, 본 개시 내용은 하기를 포함하는 재조합 아데노-관련 바이러스 (rAAV) 생산자 세포의 제조 방법을 제공한다: 4개 이상의 별개의 재조합 표적 부위 (RTS), *E1A*, *E1B*, 또는 이들의 조합을 포함하는 아데노 바이러스 (Ad) 유전자, 및 Ad 유전자에 작동 가능하게 연결된 프로모터를 포함하는 세포를 제공하는 단계로, 상기 RTS, Ad 유전자 및 프로모터는 염색체로 통합되고, 아데노-관련 바이러스 (AAV) 유전자, 제2 Ad 유전자, AAV 벡터 카세트, 또는 이들의 조합을 인코딩하는 교환 가능한 카세트를 포함하는 벡터가 제공된 세포를 형질 감염시키는 단계, 교환 가능한 카세트를 염색체에 통합시키는 단계, 및 교환 가능한 카세트를 염색체에 통합하여 rAAV 생산자 세포를 선택하는 단계를 포함한다. 일부 실시 양태에서, 형질주입은 2개의 벡터에 의한 것이고, 제1 벡터는 제2 Ad 유전자 및 AAV 유전자를 포함하는 교환 가능한 카세트를 포함하고, 제2 벡터는 AAV 벡터 카세트를 포함하는 교환 가능한 카세트를 포함한다. 일부 실시 양태에서, 형질주입은 2개의 벡터에 의하고, 제1 벡터는 제2 Ad 유전자를 포함하는 교환 가능한 카세트를 포함하고, 제2 벡터는 AAV 유전자를 포함하는 교환 가능한 카세트를 포함한다. 일부 실시 양태에서, 형질주입은 3개의 벡터를 사용하며, 제1 벡터는 제2 Ad 유전자를 포함하는 교환 가능한 카세트를 포함하고, 제2 벡터는 AAV 유전자를 포함하는 교환 가능한 카세트를 포함하고, 제3 벡터는 AAV 벡터 카세트를 포함하는 교환 가능한 카세트를 포함한다. 일부 실시 양태에서, 각각의 교환 가능한 카세트는 세포의 RTS 중 2개와 일치하는 2개의 RTS를 추가로 포함한다. 일부 실시 양태에서, 제2 Ad 유전자는 *E1A*, *E1B*, *E2A*, *E4*, *VA*, *MIR342*, 또는 이들의 조합을 포함한다. 일부 실시 양태에서, AAV 유전자는 *Rep*, *Cap*, 또는 이들의 조합을 포함한다. 일부 실시 양태에서, AAV 벡터 카세트는 리포터 유전자, 선택 유전자, 치료적 관심 유전자 또는 이들의 조합을 포함한다.

[0019] 일부 실시 양태에서, 본 개시 내용은 (i) rAAV로 숙주 세포를 감염시키는 단계, (ii) AAV 벡터 카세트로부터 패키징된 rAAV를 생산하는 단계 및 (iii) 패키징된 rAAV로서, 상기 숙주 세포는: 적어도 4개의 별개의 재조합 표적 부위 (RTS), *E1A*, *E1B* 또는 이들의 조합을 포함하는 아데노바이러스 (Ad) 유전자, Ad 유전자에 작동 가능하게 연

결된 프로모터로, 상기 RTS, Ad 유전자 및 프로모터는 염색체로 통합되고 *E1A*, *E1B*, *E2A*, *E4*, *VA*, *MIR342*, 또는 이들의 조합을 포함하는 제2 Ad 유전자, *Rep*, *Cap*, 또는 이들의 조합을 포함하는 아데노-관련 바이러스 (AAV) 유전자, 및 리포터 유전자, 선택 유전자, 치료적 관심 유전자 또는 이들의 조합을 포함하는 AAV 벡터 카세트를 포함하는 재조합 아데노-관련 바이러스 (rAAV)의 제조 방법을 제공한다. 일부 실시예에서 rAAV 생산 및 포장에는 살아있는 야생형 헬퍼 바이러스가 필요하지 않다. 일부 실시예에서, E1A의 발현은 rAAV 생산에 필요하지 않다. 일부 실시예에서, 정제 후 최소 1.0×10^9 vg/mL 활성 AAV가 수득된다.

발명의 효과

[0020]

본 발명은 4개 이상의 별개의 재조합 표적 부위 (RTS), *E1A*, *E1B* 또는 이들의 조합을 포함하는 아데노바이러스 (Ad) 유전자, 및 Ad 유전자에 작동 가능하게 연결된 프로모터를 포함하는 포유동물 세포에 관한 것으로, 여기서 RTS, Ad 유전자, 및 프로모터는 염색체로 통합되고, 재조합 아데노-관련 바이러스 (rAAV) 생산자 숙주 세포를 생성하기 위해 세포를 사용하는 방법; 및 rAAV 생산, 패키지 및 정제하기 위해 AAV 생산자 숙주 세포를 사용하는 방법을 제공할 수 있다.

도면의 간단한 설명

[0021]

도 1은 HEK293 및 CHOK1SV GS-KO™ 세포에서 재조합 아데노-관련 바이러스 (rAAV)의 생산을 보여준다. HEK293 세포에서 플라스미드 삼중 형질주입이 완료되었다 (pAAV-GFP (AAV-400, Cell Biolabs), pRC2-mi342 (6234, Clontech) 및 pHelper (6234, Clontech)). CHOK1SV GS-KO™에서, 보조 바이러스 공동-감염(wt Ad5)으로 이중 형질주입 (pAAV-GFP (AAV-400, Cell Biolabs) 및 pRC2-mi342 (6234, Clontech))을 완료하였다. 도 1a는 실험 설계의 개략도를 도시한다. 도 1B는 형질주입된 CHOK1SV GS-KO™ 세포 (왼쪽) 및 형질주입된 CHOK1SV GS-KO™ 세포로부터의 용해물로 감염된 HEK293 세포에서 녹색 형광 단백질 (GFP) 신호의 검출을 보여준다 (오른쪽). 도 1C는 바이러스 게놈 DNA의 qPCR 분석에 의한 rAAV 역가 CHOK1SV GS-KO™ 및 HEK293 (대조군) 세포의 정량을 보여준다.

도 2는 pMF30 벡터 다이어그램을 보여준다. GS 선택 마커를 갖는 TET 유도성 벡터에서의 E1A.

도 3은 pMF23 벡터 다이어그램을 보여준다. PAC 선택 마커를 갖는 구성적 벡터에서의 E1B.

도 4는 pXC17.4_17Ad5PerProKZ 벡터 다이어그램을 보여준다. E1A 및 E1B는 Qiao et al., *J. Virol.* 76:1904-13 (2002)에 기술된 것과 같이 단일 프로모터 (E1A와 E1B 코딩 서열 사이에 위치한 2 개의 유전자와 바이러스 프로모터 영역 사이의 바이러스 조절 영역 (뉴클레오티드 498-3635)이 유지됨)에서 유지됨)로부터 발현되었다. 주석은 Qiao et al., *J. Virol.* 76:1904-13 (2002)에 설명된 좌표와 일치한다.

도 5는 헬퍼 바이러스없이 이들 세포에서 rAAV 생성을 가능하게 하기 위해 wt Ad5 E1A 및 E1B를 발현하는 CHOK1SV GS-KO™ 폴의 구조를 보여준다. 도 5A는 실험 설계의 개략도이다. 도 5B는 HEK293F, HEK293L, CHOK1SV GS-KO™ 및 CHOK1SV GS-KO™ MOCK 및 CHOK1SV GS-KO™ (E1A_E1B) 폴에서 E1A에 대한 게놈 PCR 생성물의 아가로스 겔 분석을 보여준다. 도 5C는 HEK293F, HEK293L, CHOK1SV GS-KO™ 숙주 및 CHOK1SV GS-KO™ MOCK 및 CHOK1SV GS-KO™ E1A_E1B 폴에서 E1B에 대한 게놈 PCR 생성물의 아가로스 겔 분석을 보여준다. 도 5D는 독시사이클린으로 처리된 HEK293F, HEK293L, CHOK1SV GS-KO™ 숙주 및 CHOK1SV GS-KO™ MOCK 및 CHOK1SV GS-KO™ E1A_E1B 폴에서 E1A에 대한 RT-PCR 산물의 아가로스 겔 분석을 보여준다. 도 5E는 독시사이클린으로 처리된 HEK293F, HEK293L, CHOK1SV GS-KO™ 및 CHOK1SV GS-KO™ MOCK 및 CHOK1SV GS-KO™ E1A_E1B 폴에서 E1B에 대한 RT-PCR 산물의 아가로스 겔 분석을 보여준다. 패널 F는 CHOK1SV GS-KO™ MOCK 및 CHOK1SV GS-KO™ E1A_E1B 폴에서 E1A 및 β-액틴에 대한 단백질 용해물의 웨스턴 블롯 분석을 보여준다.

도 6은 pRC2-miRNA342 (6234, Clontech) (도 6A), pHelper (6234, Clontech) (도 6B) 및 pAAV-GFP (AAV-400, Cell Biolabs) (도 6C)의 벡터 다이어그램을 보여준다.

도 7은 CHOK1SV GS-KO™ 및 HEK293 부위-특이적 통합 세포주에서 3개의 독립적으로 다룰 수 있는 부위-특이적 통합 부위 (SSIS)에 대한 개략도를 보여준다. 도 7a는 단일 또는 이중 기능보고/마커 유전자를 함유하는 랜딩 패드(landing pad) 1의 배열을 도시한다. 리포터 유전자는 SV40E 프로모터의 제어하에 있으며 SV40 PolyA 서열을 갖는다. 호환되지 않는 Frt 부위는 재조합 매개 카세트 교환 (RMCE)을 위해 SV40 프로모터와 hpt-eGFP 유전자 (Frt-A)와 SV40 폴리 A 서열 (Frt-B)의 5' 사이에 위치한다. 도 7b 및 도 7C는 후속 랜딩 패드의 배열이 호환되지 않는 Frt 부위 (C, D, E 및 F) 및 페이로드(payload) 서열의 독립적인 타겟팅을 허용하는 다른 리포터를

포함하는 것을 도시한다. 이 경우 Frt 부위 A-F는 표 2에 설명된 부위 중 하나일 수 있다.

도 8은 CHOK1SV GS-KO™-AAV 생산자 세포를 제조하는 방법에 대한 시스템 개략도를 도시한다. SSIS는 아데노 바이러스 (Ad) 유전자 (헬퍼 유전자), AAV 유전자 (*Rep/Cap*) 및 AAV 벡터 카세트 (예: ITR-GOI-ITR)를 도입하는 데 사용되고, CHOK1SV GS-KO™에서의 추가 Ad 유전자(*E1A/E1B*)는 유도성 프로모터와 함께 다른 곳에 도입된다. 이 경우 Frt 부위 A-F는 표 2에 설명된 부위 중 하나일 수 있다. 도 8a는 CHOK1SV GS-KO™ 및 HEK293 숙주의 단일 부위의 배열을 도시한다. Ad 헬퍼 유전자는 SSIS에 통합되고 *GOI/Rep/Cap* 유전자는 플라스미드에서 일시적으로 발현된다. 도 8b는 CHOK1SV GS-KO™ 및 HEK293 숙주의 이중 부위 배열을 보여준다. Ad 헬퍼 및 *Rep/Cap* 유전자는 두 개의 분리된 SSIS에 통합되며 *GOI*는 플라스미드에서 일시적으로 발현된다. 도 8c는 CHOK1SV GS-KO™ 및 HEK293 숙주의 삼중 부위 배열을 보여준다. 헬퍼, *Rep/Cap* 및 *GOI* 유전자는 세 개의 분리된 SSIS에 통합된다. 일부 경우에, *E1A* 및 *E1B* 유전자는 또한 발현을 향상시키기 위해 SSIS에 통합된다.

도 9는 상이한 부위에서 *GOI* 및 *Rep/Cap* 유전자의 부위 특이적 통합을 이용하는 CHOK1SV GS-KO™ 부위 특이적 통합 (SSI) 숙주에서의 rAAV 생산의 개략도를 보여준다. rAAV를 생성하기 위해, CHOK1SV GS-KO™ SSI 숙주를 wt Ad5로 감염시켜 rAAV 복제에 필요한 헬퍼 유전자 기능을 제공하였다.

도 10은 CHOK1SV GS-KO™ 숙주에서 부위-특이적 통합을 위한 *GOI* 벡터 및 4개의 *Rep/Cap* 유전자 디자인의 개략도를 보여준다. 모든 벡터는 2개의 Frt 부위에 측면이 붙어서 SSI가 CHOK1SV GS-KO™ SSI 숙주 세포로 유입되도록 하였다. 도 10A는 벡터 pLMC31의 구조를 개략적으로 보여준다(AAV 카세트의 어느 한 말단에서 ITR에 의해 측면을 eGFP 리포터 유전자로 rAAV 벡터를 인코딩함). 이 벡터에서 선별 마커는 네오마이신 인산전이효소 (phosphotransferase) I 유전자이고 Frt 부위는 NL1을 표적으로 하기 위해 배열된다. 도 10B는 벡터 pLMC32 (AAV2 *Rep/Cap* 유전자를 암호화하는)의 구조를 개략적으로 도시한다. 이 벡터는 *Rep*의 최소 p5 프로모터(흰색 사각형) 5', *Rep* 발현 및 rAAV 생성의 역제를 감소시키기 위해 2개의 인핸서(enhancer) 요소를 함유하고, *Cap* 발현을 향상시키기 위한 *Cap*의 완전한 p5 프로모터 (흑색 사각형) 3'를 포함한다. 도 10C는 벡터 pLMC33 (AAV2 *Rep/Cap*. 유전자를 인코딩하는)의 구조를 개략적으로 도시한다. 이 벡터는 *Rep*의 최소 p5 프로모터 (흰색 사각형) 5' 및 TATA 박스가 GGGGGG로 돌연변이된 p5mut 프로모터 (검정색 및 회색 사각형)를 포함하여 *Cap*의 프로모터 활성 3'을 감소시킨다. 도 10D는 AAV2 *Rep/Cap* 유전자를 인코딩하는 벡터 pLMC34의 구조를 개략적으로 도시한다. 벡터는 *Rep*의 야생형 p5 프로모터 (흑색 사각형) 5' 및 *Cap*의 돌연변이된 p5 프로모터 (흑색 사각형) 3'를 포함한다. 도 10E는 pLMC35의 벡터의 구조 (AAV2 *Rep/Cap* 유전자를 인코딩하는)를 개략적으로 도시한다. 이 벡터는 *Rep*의 돌연변이된 p5 프로모터 (검정 및 회색 사각형) 5' 및 *Cap*의 3'를 포함한다. pLMC32 내지 pLMC35에서의 선별 마커는 GS cDNA이고 Frt 부위는 *Fer1L4*를 표적으로 하기 위해 배열된다.

도 11은 웨스턴 블롯 분석에 의한 HEK293 및 CHOK1SV GS-KO™ 세포에서 *E1A*, *Rep* 및 *Cap*의 단백질 발현을 보여준다. HEK293 세포는 pHelper (6234, Clontech), *GOI* (pLMC31) 및 하나의 *Rep-Cap* 벡터 (pLMC32-35)로 형질주입되거나 pHelper (6234, Clontech), pRC2-mi342 (6234, Clontech) 및 pAAV-GFP (AAV-400, Cell Biolabs)로 3중 형질주입되었다. 형질주입 3일 후, 단백질 용해물을 웨스턴 블롯팅으로 분석하였다. 도 11. A는 3개의 *E1A* 동형(isoforms) : *E1A* 289R, *E1A* 243R 및 *E1A* 171R의 수준을 보여준다 (Radko et al. (2015) PLoS One. 10 : e0140124). 1 : 1000으로 희석된 마우스 항-*E1A* 항체를 사용하여 *E1A* 단백질 수준을 검출하였다 (Abcam, ab33183). *E1A*, *Rep* 및 *Cap* 단백질 동형의 분자량은 괄호 안에 표시되어 있다. 상이한 벡터로 형질주입된 HEK293 또는 CHOK1SV GS-KO™ 세포에서 *E1A* 단백질 수준의 변화는 없었지만, 각각의 *E1A* 동형의 단백질 수준은 HEK293과 CHOK1SV GS-KO™ 세포에서 상이하였다. 도 11B는 *Rep78* 및 *Rep52* 단백질의 수준을 보여준다. 1:1000으로 희석된 마우스 항 *Rep* 항체(ARP, 03-61069)를 사용하여 *Rep* 단백질 수준을 검출하였다. 괄호 안에 표시된 것은 분자량이다. 상이한 벡터로 형질주입된 HEK293 또는 CHOK1SV GS-KO™ 세포에서 *Rep52* 단백질 수준에는 변화가 없었지만, pLMC34 및 pLMC35로 형질주입된 세포에서는 *Rep78* 단백질 수준이 더 높았다. 도 11. C는 1:1000으로 희석된 마우스 항 *Cap* 항체(ARP, 03-61058)를 사용한 3가지 *Cap* 동형: *VP1*, *VP2* 및 *VP3*의 수준을 보여준다. 괄호 안에 표시된 것은 분자량이다. 상이한 벡터로 형질주입된 HEK293 세포에서 *VP3* 단백질 수준의 변화는 없었으며, *VP1* 및 *VP2*는 HEK293 세포에서 검출되지 않았다. *VP1*, *VP2* 및 *VP3*은 CHOK1SV GS-KO™ 세포에서 검출되지 않았다. 도 11. D는 1 : 1000으로 희석된 마우스 항 베타-액틴 항체(TFS, MA5-1573)를 사용한 베타-액틴 로딩 제어를 나타낸다.

도 12는 HEK293 및 CHOK1SV GS-KO™ 세포에 의해 생성된 rAAV의 감염성을 나타낸다. HEK293 세포를 *Rep/Cap*, *GOI* 및 pHelper(6234, Clontech)로 3중 형질주입시켰다. CHOK1SV GS-KO™ 세포를 *Rep/Cap* 및 *GOI*로 형질주입시키고, wt Ad5로 감염시켰다. 형질주입 후 3일에, 동일한 부위의 미정제 rAAV 수확물을 사용하여 HEK293 세포를 감염시켰다. 형질주입 2일 후, 녹색 형광 및 명 시야 이미지를 얻었다. 녹색 형광은 *GOI*-패키지된 rAAV에 의

해 생성된다. 제시된 눈금 막대는 50 μm 을 나타낸다. 3중 형질주입된 HEK293 세포 (pHelper (6234, Clontech), pRC2-mi342 (6234, Clontech), pAAV-GFP (AAV-400, Cell Biolabs)) (상단 패널)에 의해 생성된 rAAV의 감염성은 CHOK1SV GS-KO™ 세포 (제3 패널)에 의해 형질주입되어 생성된 rAAV보다 높았다. 주목할 만한 것은, pLMC32-35로 형질 감염된 HEK293 세포에 의해 생성된 rAAV의 감염성은 pRC2-mi342 (6234, Clontech) (상부 패널)로 형질주입된 HEK293 세포보다 높았다.

도 13은 SSI가 GOI이고 4개의 Rep-Cap 구성 중 하나를 갖는 CHOK1SV GS-KO™ SSI 호스트 파생 풀의 구성을 보여준다. 풀은 두 단계로 생성되었다.

1단계 1부에서 CHOK1SV GS-KO™ SSI 숙주를 FLP 재조합 효소 벡터 (pMF4)와 하나의 Rep-Cap 벡터 (pLMC32-35)로 형질주입시킨 후 글루타민이 없는 배지에서 선택했다. 1단계 2부에서 CHOK1SV GS-KO™ SSI 숙주는 FLP 재조합 효소 벡터 (pMF4), 하나의 Rep-Cap 벡터 (pLMC32-35) 및 GOI 벡터 (pLMC31)로 형질주입되었고, 그런 다음 제네티신(Geneticin) (500 $\mu\text{g}/\text{mL}$ 또는 750 $\mu\text{g}/\text{mL}$)을 함유하는 글루타민이 없는 배지에서 선택하였다. 이들이 'A' 풀(pool)이 되었다. 2단계에서, 'A' 풀 4-7을 FLP 재조합 효소 (pMF4) 및 GOI 벡터 (pLMC31)로 형질주입시킨 후, 제네티신 (400 $\mu\text{g}/\text{mL}$)을 함유하는 배지에서 선택 하였다. 이들은 'B' 풀이 되었다.

도 14는 표적(on-target) SSI 및 잔류(비-교환) 랜딩 패드를 검출하기 위한 프라이머 위치의 개략도를 도시한다. 프라이머 세트 P5는 Fer1L4 유전자에 위치한 랜딩 패드로의 특정 Rep/Cap 통합을 검출하도록 설계되었다. 예상되는 산물 크기는 1524bp이다. 프라이머 세트 P6은 잔류 랜딩 패드를 감지하도록 설계되었다. 예상되는 산물 크기는 487bp이다. 프라이머 세트 P7은 NL1 유전자 좌위에 위치한 랜딩 패드로의 특정 GOI 통합을 감지하도록 설계되었다. 예상되는 산물 크기는 1446bp이다. 프라이머 세트 P8은 잔류 NL1 랜딩 패드를 감지하도록 설계되었다. 예상되는 산물 크기는 809bp이다.

도 15는 Fer1L4 랜딩 패드로의 Rep/Cap 통합을 위한 게놈 DNA PCR 생성물의 아가로스 겔 분석을 보여준다. 게놈 DNA를 'A' 풀, 'B' 풀, CHOK1SV GS-KO™ 및 CHOK1SV GS-KO™ SSI 숙주에서 수확한 후 PCR에 의해 프라이머 세트 5 또는 6을 사용하여 증폭시켰다. PCR 생성물은 아가로스 겔 전기영동에 의해 해결되었다. 도 15A는 프라이머 세트 P5를 사용하여 Fer1L4 유전자에 위치한 랜딩 패드로의 특정 Rep/Cap 통합을 위한 게놈 DNA PCR 생성물의 아가로스 겔 분석을 보여준다. 예상되는 산물 크기는 1524bp이다. 결과는 'A' 풀 4, 5, 6, 7, 9, 11, 17, 18, 19, 21의 Fer1L4 유전자와 분석된 모든 'B' 풀에 있는 랜딩 패드에 특정 Rep/Cap 통합을 보여준다. 예상대로 CHOK1SV GS-KO™ 및 CHOK1SV GS-KO™ SSI 숙주에서 특이적 Rep/Cap 통합이 감지되지 않았다. 도 15B는 프라이머 세트 P6을 사용하여 Fer1L4 유전자에서 잔류 랜딩 패드에 대한 게놈 DNA PCR 생성물의 아가로스 겔 분석을 보여준다. 예상되는 산물 크기는 487bp이다. 결과는 일부 잔류 랜딩 패드와 18을 제외한 모든 'A' 풀 및 모든 'B' 풀의 Fer1L4 유전자에 있는 랜딩 패드에 불완전한 Rep/Cap 통합을 보여준다. 'A' 풀 19에는 더 작은 PCR 산물이 있었으며, 이는 일부 서열의 손실을 나타낸다. 예상한 바와 같이, Fer1L4 랜딩 패드는 CHOK1SV GS-KO™ 세포에서는 증폭되었지만 CHOK1SV GS-KO™ SSI 숙주에서는 검출되지 않았다.

도 16은 NL1 랜딩 패드로의 GOI 통합을 위한 게놈 DNA PCR 산물의 아가로스 겔 분석을 보여준다. 게놈 DNA를 'A' 풀, 'B' 풀, CHOK1SV GS-KO™ 및 CHOK1SV GS-KO™ SSI 숙주에서 수확한 후 PCR에 의해 프라이머 세트 7 또는 8을 사용하여 증폭하였다. PCR 산물은 아가로스 겔 전기영동에 의해 해결되었다. 도 16A는 프라이머 세트 P7을 사용하여 NL1 랜딩 패드에 특이적 GOI 통합을 위한 게놈 DNA PCR 산물의 아가로스 겔 분석을 보여준다. 예상되는 산물 크기는 1446bp이다. 결과는 모든 'B' 풀의 NL1 위치에 있지만 'A' 풀에는 없는 랜딩 패드에 특정 GOI 통합을 보여준다. 예상대로 CHOK1SV GS-KO™ 및 CHOK1SV GS-KO™ SSI 숙주에서 특이적 GOI 통합이 감지되지 않았다. 도 16B는 프라이머 세트 P8을 사용한 잔류 NL1 랜딩 패드에 대한 게놈 DNA 생성물의 아가로스 겔 분석을 보여준다. 예상되는 산물 크기는 809bp이다. 결과는 잔류 랜딩 패드와 11을 제외한 모든 'A' 풀 및 모든 'B' 풀의 NL1 유전자에 있는 랜딩 패드에 불완전한 GOI 통합을 보여준다. 결과에 따르면 NL1 랜딩 패드는 'A' 풀 11에서는 손상되지 않았지만 GOI는 랜딩 패드에 통합되지 않았다. 예상한 바와 같이, NL1 랜딩 패드는 CHOK1SV GS-KO™ 세포에서 증폭되었지만 CHOK1SV GS-KO™ SSI 숙주에서는 검출되지 않았다.

도 17은 웨스턴 블롯 분석에 의한 wt Ad5 감염된 CHOK1SV GS-KO™ 세포에서 E1A, Rep 및 Cap의 단백질 발현을 보여준다. 'A' 풀과 'B' 풀을 3일 동안 wt Ad5로 감염시킨 후 E1A, Rep, Cap 및 베타-액틴 단백질 발현 분석을 위해 수확하였다. 도 17a는 3개의 E1A 동형: E1A 289R, E1A 243R 및 E1A 171R의 수준을 나타낸다. 1:1000으로 희석된 마우스 항-E1A 항체(Abcam, ab33183)를 사용하여 E1A 단백질 수준을 검출하였다. E1A, Rep 및 Cap 단백질 동형의 분자량은 괄호 안에 표시되어 있다. 3개의 모든 E1A 동형의 수준은 모든 풀에서 동일하였고, CHOK1SV GS-KO™ 세포에서보다 약간 더 높았다. 도 17. B는 Rep78 및 Rep52 단백질의 수준을 보여준다. 1:1000

으로 희석된 마우스 항 Rep 항체(ARP, 03-61069)를 사용하여 Rep 단백질 수준을 검출하였다. 괄호 안에 표시된 것은 분자량이다. Rep78은 검출되지 않았다. Rep52는 'A' 풀 13 및 'B' 풀 6, 7, 8 및 10에서 *로 표시되었다. 도 11. C는 1:1000으로 희석된 마우스 항 Cap 항체를 사용한 3가지 Cap 동형: VP1, VP2 및 VP3의 수준을 보여 준다(ARP, 03-61058). 괄호 안에 표시된 것은 분자량이다. VP1, Vp2 및 VP3이 검출되지 않았다. 도 17. D는 1:1000으로 희석된 마우스 항 베타-액틴 항체(TFS, MA5-1573)를 사용한 베타-액틴 로딩 제어를 나타낸다.

도 18은 AAV2 Rep 및 Cap mRNA의 검출을 위한 프라이머 위치의 개략도를 보여준다. Rep78 및 Rep68에 대한 프라이머 세트 P1. Rep78, Rep68, Rep52 및 Rep40에 대한 프라이머 세트 P2. Rep78 및 Rep52에 대한 프라이머 세트 P3. 총 Rep 및 Cap에 대한 프라이머 세트 P4. *는 VP3 생산에 사용된 대체 ACG 코돈을 나타낸다. AAV2 계놈 구조는 Samulski and Muzyczka., *Annu. Rev. Virol.* 1:427-451 (2014)로부터 변형되었다.

도 19는 형질주입된 HEK293 및 CHOK1SV GS-KOTM 세포로부터의 Rep-Cap 유전자 및 Ad5 헬퍼 유전자에 대한 mRNA 발현 분석을 보여준다. HEK293 세포를 pRC2-mi342 (6234, Clontech), pAAV-GFP (AAV-400, Cell Biolabs) 벡터 (검은색 막대)로 3중 형질주입시켰다. CHOK1SV GS-KOTM 세포를 pRC2-mi342 및 pAAV-GFP로 형질주입시키고, wt Ad5 (흰색 막대)로 감염시켰다. 대조군에는 pRC2-mi342 (왼쪽 막대)로 형질주입된 HEK293 또는 CHOK1SV GS-KOTM 세포가 포함된다. cDNA는 Rep/Cap 및 Ad 헬퍼 유전자에 대한 RT-qPCR 분석을 위해 준비되었다. 발현 데이터를 베타-액틴으로 내부적으로 표준화한 후 HEK293 대조군 샘플과 비교하였다. 프라이머 서열은 도 21에 포함되어 있다. 도 19A는 Rep78 및 Rep68 mRNA 수준을 보여준다. 3중 형질주입 후 HEK293 및 CHOK1SV GS-KOTM 세포에서 ~6- 및 ~5-배 유도가 관찰되었다. 도 19b는 Rep78, Rep68, Rep52 및 Rep40 mRNA 수준을 보여준다. ~11-배 및 ~5-배 유도가 3중 형질주입 후 HEK293 및 CHOK1SV GS-KOTM 세포에서 각각 관찰되었다. 도 19.C는 Rep78 및 Rep52 mRNA 수준을 보여준다. 3중 형질주입 후 HEK293 및 CHOK1SV GS-KOTM 세포에서 ~17- 및 ~4-배 유도가 관찰되었다. 도 19D는 총 Rep 및 Cap mRNA 수준을 나타낸다. ~20-배 및 ~7-배 유도가 (각각) 삼중 형질주입 후 HEK293 및 CHOK1SV GS-KOTM 세포에서 관찰되었다. 도 19E는 E1A mRNA 수준을 보여준다. 삼중 형질주입 후 HEK293에서 ~1-배 유도가 관찰되었다. E1A mRNA는 대조군 CHOK1SV GS-KOTM 세포에서 검출되지 않았으나, 형질주입 및 wt Ad5로의 감염 후에 검출되었다. 도 19.F는 E1B-19K mRNA 수준을 보여준다. 삼중 형질주입 후 HEK293에서 ~1-배 유도가 관찰되었다. E1B-19K mRNA는 대조군 CHOK1SV GS-KOTM 세포에서 검출되지 않았으나, 형질주입 및 wt Ad5로의 감염 후에 검출되었다. 도 19.G는 E1B-55K mRNA 수준을 보여준다. 삼중 형질주입 후 HEK293에서 ~1-배 유도가 관찰되었다. E1B-55K mRNA는 대조군 CHOK1SV GS-KOTM 세포에서 검출되지 않았으나, 형질주입 및 wt Ad5로의 감염 후에 검출되었다. 도 19H는 E2A mRNA 수준을 보여준다. E2A mRNA는 대조군 샘플에서 검출될 수 없었으며, 삼중 형질주입 후에 존재하였다. 도 19.I는 E4 mRNA 수준을 나타낸다. E4 mRNA는 대조군 샘플에서 검출될 수 없었으며, 삼중 형질주입 후에 존재하였다. 도 19.J는 VAI mRNA 수준을 보여준다. VAI mRNA는 대조군 샘플에서 검출될 수 없었으며, 삼중 형질주입 후에 존재하였다. 도 19.K는 VAII mRNA 수준을 나타낸다. VAII mRNA는 대조군 샘플에서 검출될 수 없었으며, 삼중 형질주입 후에 존재하였다. E4를 제외한 모든 관찰된 mRNA 수준은 HEK293 세포와 비교하여 CHOK1SV GS-KOTM에서 더 낮았다(도 19.A-K). 이들 데이터는 Rep-Cap mRNA 유도 및 발현이 HEK293 세포와 비교하여 CHOK1SV GS-KOTM 세포에서 더 낮다는 것을 보여준다.

도 20은 삼중 형질주입된 HEK293 및 CHOK1SV GS-KOTM 세포로부터의 rAAV의 역가를 보여준다. HEK293 세포를 pHelper (6234, Clontech) 및 하나의 pLMC32-35 또는 pRC-mi342 (6234, Clontech) 및 pLMC31 또는 pAAV-GFP (AAV-400, Cell Biolabs) 벡터로 형질주입시켰다. CHOK1SV GS-KOTM 세포를 wt Ad5 및 하나의 pLMC32-35 또는 pRC-mi342 (6234, Clontech) 및 pLMC31 또는 pAAV-GFP (AAV-400, Cell Biolabs) 벡터로 감염시켰다. 형질주입 3일 후, AAV 발현에 대한 TaqMan-qPCR 분석을 위해 DNA를 추출하였다. CHOK1SV GS-KOTM 세포에 의해 생성된 AAV 역가는 HEK293 세포에서보다 낮았고, pHelper (6234, Clontech), pLMC31 및 pLMC32-35를 사용하여 삼중 형질주입된 HEK293 세포에 의해 생성된 역가는 Clontech 벡터 (pHelper (6234, Clontech), pRC2-mi342 (6234, Clontech) 및 pAAV-GFP (AAV-400, Cell Biolabs))를 사용하여 삼중 형질주입된 HEK293 세포에서보다 유의하게 높았다.

도 21은 TaqMan-qPCR 및 계놈 DNA PCR 분석에 사용된 프라이머 서열을 보여준다.

발명을 실시하기 위한 구체적인 내용

[0022] 청구 범위 및/또는 명세서에서 "포함하는" 이라는 용어와 함께 사용될 때 "하나" 라는 단어의 사용은 "하나"를 의미 할 수 있으나, "하나 또는 그 이상", "적어도 하나" 및 "하나 이상"의 의미와 일치한다.

[0023] 본 출원 전체에서, "약" 이라는 용어는 값을 결정하기 위해 사용되는 방법/장치에 대한 고유의 오차 변동 또는 연구 대상자 사이에 존재하는 변동을 포함하는 값을 나타내는데 사용된다. 일반적으로 이 용어는 대략적으로 상

황에 따른 1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9%, 10%, 11%, 12%, 13%, 14%, 15%, 16%, 17%, 18%, 19% 또는 20% 이하의 변동성을 의미하는 것을 포함하는 것으로 의도된다.

- [0024] 청구 범위에서 용어 "또는"은 특별히 선택적인 대안을 단독으로 인용하거나 이러한 선택적 대안이 서로에 대해 독립적인 경우를 제외하고 "및/또는"을 지칭하는데 사용하며, 다만 본 명세서에는 선택적 대안을 단독으로 인용하는 정의 및 " 및/또는" 을 모두 뒷받침한다.
- [0025] 본 명세서 및 청구 범위에 사용된 바와 같이, "포함하는" (및 "포함하는(comprise)" 및 "포함하는(comprises)"과 같은 임의의 형태), "갖는"(및 "갖는다(have)" 및 "갖는다(has)"와 같은 임의의 형태), "포함하는" (및 "포함하다(includes)"및 "포함하다(include)"와 같은 임의의 형태) 또는 "함유하다" (및 "함유하다(contains)" 및 "포함하다(contain)"과 같은 함유의 임의의 형태)의 단어들은 포함하는 또는 개방형이며 추가, 언급되지 않은, 요소 또는 방법 단계를 배제하지 않는다. 본 명세서에서 논의된 임의의 실시예에는 본 발명의 임의의 방법, 시스템, 숙주 세포, 발현 벡터 및/또는 조성물과 관련하여 구현될 수 있는 것으로 고려된다. 또한, 본 발명의 조성물, 시스템, 세포, 및/또는 벡터는 본원에 기재된 임의의 방법을 달성하기 위해 사용될 수 있다.
- [0026] "예를 들어" 라는 용어와 그에 해당하는 약어 "예" (이탤릭체로 표시되든 그렇지 않든)은 언급된 특정 용어가 달리 명시적으로 언급되지 않는 한 참조되거나 인용된 특정예에 제한되지 않는 본 개시의 대표적인 예 및 실시예임을 의미한다.
- [0027] 본 개시 내용은 (i) 적어도 4개의 별개의 재조합 표적 부위 (RTS), (ii) *E1A*, *E1B* 또는 이들의 조합을 포함하는 아데노바이러스 (Ad) 유전자, 및 (iii) Ad 유전자에 작동 가능하게 연결된 프로모터를 포함하는 포유동물 세포를 제공하고, 여기서 RTS, Ad 유전자 및 프로모터는 염색체로 통합된다.
- [0028] "핵산", "핵산 분자", 또는 "올리고뉴클레오티드" 는 공유 연결된 뉴클레오티드를 포함하는 중합체 화합물을 의미한다. 용어 "핵산"은 폴리 리보핵산(RNA) 및 폴리 데옥시리보핵산(DNA)을 포함하고, 이들 둘은 단일 가닥 또는 이중 가닥일 수 있다. DNA는 상보적 DNA(cDNA), 게놈 DNA, 플라스미드 또는 벡터 DNA, 및 합성 DNA를 포함하지만 이에 제한되지는 않는다. RNA는 mRNA, tRNA, rRNA, snRNA, 마이크로 RNA, miRNA 또는 MIRNA를 포함하지만 이에 제한되지는 않는다.
- [0029] 용어 "재조합"은 핵산 분자, 펩타이드, 폴리펩타이드 또는 단백질과 관련하여 사용될 때 자연에 존재하지 않는 것으로 알려진 새로운 유전 물질의 조합을 의미하거나 그로부터 생성된다. 재조합 분자는 재조합 기술 분야에서 이용 가능한 임의의 공지된 기술에 의해 생성될 수 있으며, 중합 효소 연쇄 반응 (PCR), 유전자 절단 (예를 들어, 제한 효소(restriction endonucleases) 사용), 및 핵산 분자, 펩티드 또는 단백질의 고상(solid state) 합성을 포함하나, 이에 제한되지는 않는다. 일부 실시예에서, "재조합"은 자연에 존재하지 않는 것으로 알려진 바이러스 벡터 또는 바이러스를 지칭하며, 예를 들어 바이러스 벡터 또는 바이러스에 하나 이상의 돌연변이, 핵산 삽입, 또는 상동 유전자(heterologous gene)를 갖는 바이러스 벡터 또는 바이러스이다. 일부 실시예에서, "재조합"은 예를 들어 자연에 존재하지 않는
- [0030] 것으로 공지된 세포 또는 숙주 세포를 지칭하며, 예를 들어 세포 또는 숙주 세포에서 하나 이상의 돌연변이, 핵산 삽입, 또는 상동 유전자를 갖는 세포 또는 숙주 세포이다.
- [0031] "단리된" 폴리펩티드, 단백질, 펩티드, 또는 핵산은 이의 본래의(natural) 환경에서 제거된 분자이다. "단리된" 폴리펩티드, 단백질, 펩티드, 또는 핵산은 부형 제, 예컨대 희석제 또는 보조제와 함께 제제화될 수 있으며 여전히 단리된 것으로 간주된다는 것을 이해해야 한다.
- [0032] 핵산 서열 또는 아미노산 서열과 관련하여 용어 "서열 동일성" 또는 "% 동 일성"은 서열이 특정된 비교 창(window)에 걸쳐 정렬될 때 동일한 비교된 서열의 잔기의 백분율을 지칭한다. 비교 창은 서열이 정렬되고 비교될 수 있는 10개 내지 1000개 이상의 잔기의 단편(segment) 일 수 있다. 서열 동일성을 결정하기 위한 정렬 방법은 당 업계에 공지되어 있으며 BLAST (blast.ncbi.nlm.nih.gov/Blast.cgi) 와 같은 공개적으로 이용 가능한 데이터베이스를 사용하여 수행될 수 있다.
- [0033] 일부 실시예에서, 폴리펩티드 또는 핵산 분자는 기준 폴리펩티드 또는 핵산 분자(또는 기준 폴리펩티드 또는 핵산 분자의 단편)와 각각, 약 70% 이상, 약 75% 이상, 약 80% 이상, 약 85% 이상, 약 90% 이상, 약 95% 이상, 약 97% 이상, 약 98% 이상, 약 99% 이상 또는 약 100% 서열 동일성을 갖는다. 본 개시의 특정 실시예에서, 폴리펩티드 또는 핵산 분자는 기준 폴리펩티드 또는 핵산 분자(또는 기준 폴리펩티드 또는 핵산 분자의 단편)와 각각, 약 70% 이상, 약 75% 이상, 약 80% 이상, 약 85% 이상, 약 90% 이상, 약 95% 이상, 약 97% 이상, 약 98% 이상, 또는 약 99% 이상 또는 100%의 서열 동일성을 갖는다. 일부 실시예에서, 폴리펩티드 또는 핵산 분자는 기

준 폴리펩티드 또는 핵산 분자와 각각, 약 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, 99.5% 서열 동일성을 갖는다.

- [0034] 본원에 사용된 "프로모터", "프로모터 서열" 또는 "프로모터 영역"은 RNA 폴리머라제에 결합할 수 있고 다운 스트림 코딩 또는 비-코딩 서열의 전사를 개시하는데 관여하는 DNA 조절 영역/서열을 지칭한다. 본 개시 내용의 일부 실시예에서, 프로모터 서열은 전사 개시 부위를 포함하고, 배경 위에서 검출 가능한 수준에서 전사를 개시하는데 필요한 최소 수의 염기 또는 요소를 포함하도록 업스트림으로 연장된다. 일부 실시예에서, 프로모터 서열은 전사 개시 부위뿐만 아니라 RNA 폴리머라제의 결합을 담당하는 단백질 결합 도메인을 포함한다. 진핵 생물 프로모터는 종종, 항상 그런 것은 아니지만, "TATA"박스 및 "CAT"박스를 포함한다. 유도성 프로모터를 포함하는 다양한 프로모터가 예를 들어, 본 발명의 숙주 세포 또는 박터에서 유전자 발현을 유도하는데 사용될 수 있다. 일부 실시예에서, 프로모터는 누출(leaky) 프로모터가 아니며, 즉, 프로모터는 본원에 기재된 바와 같은 유전자 생성물 중 어느 하나를 구성적으로 발현하지 않는다.
- [0035] 본원에 언급된 바와 같이, 용어 "포유동물 세포"는 포유 동물의 임의의 구성원으로부터의 세포, 예를 들어, 인간 세포, 마우스 세포, 랫트(rat) 세포, 원숭이 세포, 햄스터 세포 등을 포함한다. 일부 실시예에서, 세포는 마우스 세포, 인간 세포, 중국 햄스터 난소(CHO) 세포, CHO-K1 세포, CHO-DXB11 세포, CHO-DG44 세포, 모든 변이체를 포함하는 CHOK1SV™ 세포(예를 들어, CHOK1SV POTELLIGENT®, Lonza, Slough, UK), 모든 변이체를 포함하는 CHOK1SV GS-KO™(XCeed®, Lonza, Slough, UK) 세포, 부착성 및 현탁액-적응형-변이체를 포함하는 HEK293 세포, HeLa 세포, 또는 HT1080 세포이다.
- [0036] 본원에 언급된 바와 같이, 용어 "염색체로-통합된" 또는 "염색체 통합"은 핵산 서열이 숙주 세포, 예를 들어 포유동물 세포의 염색체에 안정적으로 혼입되는 것을 의미한다. 즉, 숙주 세포, 예를 들어 포유동물 세포의 게놈 DNA (gDNA)에 염색체로 통합된 핵산 서열이다. 일부 실시예에서, 염색체로 통합된 핵산 서열은 안정적이다. 일부 실시예에서, 염색체로 통합된 핵산 서열은 플라스미드 또는 박터 상에 위치하지 않는다. 일부 실시예에서, 염색체로 통합된 핵산 서열은 절단(excise d) 되지 않는다. 일부 실시예에서, 염색체 통합은 크리스퍼 (CRISPR; clustered regularly interspaced short palindromic repeats) 및 CRISPR 관련 단백질 (Cas) 유전자 편집 시스템(CRISPR/CAS)에 의해 매개된다.
- [0037] 본원에 사용된 용어 "재조합 표적 부위-특이적 통합" 또는 "부위-특이적 통합"은 상호 교환 가능하고 하나 이상의 유전자를 숙주 세포 염색체로 도입하는데 사용될 수 있다. 예를 들어, Bode et al., *Biol. Chem.* 381:801-813 (2000), Kolb, *Cloning and Stem Cells* 4:381-392 (2002) 및 Coates et al., *Trends in Biotech.* 23:407-419 (2005), 이들 각각은 참고로 포함된다. 일부 실시예에서, "재조합 표적 부위-특이적 통합" 또는 "부위-특이적 통합"은 특정 부위에서 염색체 내로 핵산 서열의 통합을 지칭한다. 일부 실시예에서, 부위-특이적 통합은 부위-특이적 재조합을 통해 이루어진다. 일부 실시예에서, "부위-특이적 재조합"은 그의 동족 쌍의 서열 또는 표적 부위에서 재조합을 수행하는 특이적 효소에 의한 2개의 DNA 파트너 분자의 재배열을 지칭한다. 상동 재조합과 대조적으로, 부위-특이적 재조합 및 부위-특이적 통합은 파트너 DNA 분자 사이의 DNA 상동성이 거의 필요하지 않으며, RecA-의존적이며, 어떠한 단계에서도 DNA 복제를 포함하지 않는다. 일부 실시예에서, 부위 특이적 재조합은 숙주 세포, 예를 들어, 포유동물 세포에서 핵산의 부위 특이적 통합을 달성하기 위해 부위 특이적 재조합 효소 시스템을 사용한다. 재조합 효소 시스템은 전형적으로 3개의 요소, 즉 2개의 특정 DNA 서열 (재조합 표적 부위) 및 특정 효소 (재조합 효소)로 구성된다. 재조합 효소는 특정 재조합 표적 부위 사이의 재조합 반응을 촉매한다.
- [0038] 당업자에게 공지된 다른 수단을 사용하여 하나 이상의 원하는 핵산 서열 (예를 들어, 유전자)을 삽입할 수 있다. 일부 실시예에서, 하나 이상의 핵산 서열 (예를 들어, 유전자)은 상동 재조합을 사용하여 염색체에 삽입된다. 상동 재조합은 뉴클레오티드 서열이 2개의 유사하거나 동일한 DNA 분자 사이에서 교환되는 유전자 재조합을 지칭한다. 일부 실시예에서, 결실/삽입을 표적으로 하는 영역(예를 들어, 유전자)은 상동 재조합에 의해 결실/삽입된다.
- [0039] 숙주 염색체에서 결실이 절단, 또는 (ii) 염색체에 삽입이 첨가되는 것을 위한 표적화된 영역을 정렬할 수 있다. 일부 실시예에서, 상동 재조합은 재조합 표적 부위, 아데노바이러스 (Ad), 예를 들어, *E1A*, *E1B*, *E2A*, *E4*, *VA*, *MIR342*, 또는 이들의 조합, 및/또는 Ad 유전자에 작동 가능하게 연결된 프로모터를 삽입하기 위해 사용되지 않는다.
- [0040] 재조합 효소(recombinase enzyme) 또는 재조합 효소(recombinase)는 부위-특이적 재조합에서의 재조합을 촉매하는 특정 효소이다. 본 개시 내용의 일부 실시예에서, 부위-특이적 재조합에 사용되는 재조합 효소는 비-포유

류 시스템으로 부터 유래된다. 일부 실시예에서, 재조합 효소는 박테리아, 박테리오파지 또는 효모로부터 유래된다.

[0041] 일부 실시예에서, 재조합 효소를 암호화하는 핵산 서열은 예를 들어 숙주 세포, 예를 들어 포유동물 세포 내로 통합된다. 일부 실시예에서, 재조합 효소를 코딩하는 핵산 서열은 분자 생물학에 공지된 다른 방법에 의해 숙주 세포로 전달된다. 일부 실시예에서, 재조합 효소 폴리펩티드 서열은 세포로 직접 전달 될 수 있다. 일부 실시예에서, 재조합 효소는 Cre 재조합 효소, Dre 재조합 효소, KD 재조합 효소, B2B3 재조합 효소, Hin 재조합 효소, Tre 재조합 효소, λ 인테그라제 (integrase), HK022 인테그라제, HP1 인테그라제, γ δ 레졸베이즈/인버테이스, (resolvase/invertase), a ParA 레졸베이즈/인버테이스, Tn3 레졸베이즈/인버테이스, Gin 레졸베이즈/인버테이스, Φ C31 인테그라제, BxB1 인테그라제, R4 인테그라제 또는 다른 기능적 재조합 효소이다. 예를 들어, Thorpe & Smith, *Proc. Nat'l. Acad. Sci. USA* 95: 5505-5510 (1998); Kuhstoss & Rao, *J. Mol. Biol.* 222: 897-890 (1991); U.S. Pat. No. 5,190,871; Ow & Ausubel, *J. Bacteriol.* 155: 704-713 (1983); Matsuura et al., *J. Bacteriol.* 178: 3374-3376 (1996); Sato et al., *J. Bacteriol.* 172: 1092-1098 (1990); Stragier et al., *Science* 243: 507-512 (1989); Carrasco et al., *Genes Dev.* 8: 74-83 (1994); Bannam et al., *Mol. Microbiol.* 16: 535-551 (1995); Crelin & Rood, *J. Bacteriol.* 179: 5148-5156 (1997).를 참고한다.

[0042] 일부 실시예에서, 본원에 기술된 재조합 효소는 FLP 재조합 효소이다. FLP 재조합 효소는 DNA 복제 동안 사카로마이세스 세레비지에(*Saccharomyces cerevisiae*)의 2 μ m 플라스미드의 카피(copy) 수를 증폭 시키는데 관련된 부위-특이적 재조합 반응을 촉매하는 단백질이다. 일부 실시예에서, 본 개시 내용의 FLP 재조합 효소는 사카로마이세스(*Saccharomyces*) 속의 종으로부터 유래된다. 일부 실시예에서, FLP 재조합 효소는 사카로마이세스 세레비지 애로부터 유래된다. 일부 실시예에서, FLP 재조합 효소는 사카로마이세스 세레비지에 균주로부터 유래된다. 일부 실시예에서, FLP 재조합 효소는 열 안정성 돌연변이체 FLP 재조합 효소이다. 일부 실시예에서, FLP 재조합 효소는 FLP1 또는 FLPe이다. 일부 실시예에서, FLP 재조합 효소를 코딩하는 핵산 서열은 인간 최적화된 코돈을 포함한다.

[0043] 일부 실시예에서, 재조합 효소는 Cre 재조합 효소이다. Cre (재조합을 야기함)는 Int 계열 재조합 효소(Argos et al. (1986) EMBO J 5:433)의 구성원이며 lox 좌위 (X-ing의 위치)의 효율적인 재조합을 수행하는 것으로 나타났다 박테리아뿐만 아니라 진핵 세포에서도 마찬가지로이다(Sauer (1987) Mol. Cell. Biol. 7:2087; Sauer and Henderson (1988) Proc. Natl Acad. Sci. 85:5166). 일부 실시예에서, 본원에 기술되고 사용된 Cre 재조합 효소는 박테리오파지로부터 유래된다. 일부 실시예에서, Cre 재조합 효소는 P1 박테리오파지로부터 유래된다.

[0044] 본원에 언급된 바와 같이, 용어 "재조합 표적 부위", "RTS" 및 "부위-특이적 재조합 효소 표적 부위"는 짧은 부위, 예를 들어, 부위-특이적 재조합 효소에 의해 인식되는 60개 이하의 염기쌍, 핵산 부위 또는 서열을 지칭하고, 이는 부위-특이적 재조합 상황 동안 교차 영역이 된다. 약어 "RTS"는 단일 재조합 표적 부위, 또는 복수의 재조합 표적 부위를 지칭할 수 있다. 일부 실시예에서, RTS는 약 60개 이하 염기쌍, 약 55개 이하 염기쌍, 약 50개 이하 염기쌍, 약 45개 이하 염기쌍, 약 40개 이하 염기쌍, 약 35개 이하 염기쌍, 또는 약 30개 이하의 염기쌍이다. 일부 실시예에서, RTS는 약 30 내지 약 60개의 염기쌍, 약 30 내지 약 55개의 염기쌍, 약 32 내지 약 52개의 염기쌍, 약 34 내지 약 44개의 염기쌍, 약 32개의 염기쌍, 약 34개의 염기쌍, 또는 약 52개의 염기쌍이다. RTS의 예는, 이에 제한되지 않지만, lox 부위, rox 부위, Frt 부위, att 부위 및 dif 부위를 포함한다. 일부 실시예에서, RTS는 표 1, 2, 3 또는 4의 임의의 서열에 제시된 것과 실질적으로 동일한 서열을 갖는 핵산이다.

[0045] 일부 실시예에서, RTS는 표 1로부터 선택된 lox 부위이다. 본원에 언급된 바와 같이, 용어 "lox 부위"는 Cre 재조합 효소가 부위-특이적 재조합을 촉매할 수 있는 뉴클레오티드 서열을 지칭한다. 다양한 비-동일 lox 부위가 당 업계에 공지되어 있다. 다양한 lox 부위의 서열은 이들이 재조합이 일어나는 8-염기쌍 비대칭 코어 영역의 측면에 위치한 동일한 13-염기쌍 반전된 반복물을 모두 포함한다는 점에서 유사하다. 비대칭 코어 영역은 부위의 방향성과 상이한 lox 부위 사이의 변이를 담당한다. 이들의 예시적인(비-제한적인) 예는 자연적으로 발생한 loxP(P1 계통에서 발견된 서열), loxB, loxL 및 loxR (이들은 대장균 염색체에서 발견됨)뿐만 아니라 loxP511, lox Δ 86, lox Δ 117, loxC2, loxP2, loxP3, loxP23, loxC2, loxP2, 및 loxP3와 같은 여러 돌연변이체 또는 변이된 lox 부위를 포함한다. 일부 실시예에서, RTS는 lox Δ 86, lox Δ 117, loxC2, loxP2, loxP3 및 loxP23로부터 선택된 lox 부위이다. 일부 실시예에서, lox RTS는 표 1에서 발견된 서열과 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% 이상, 또는 100% 서열 동일성을 갖는 핵산이다.

표 1

이름	식별자	서열
loxP	SEQ ID NO.: 1	ataacttcgtataatgtatgctatacgaagttat
loxP511	SEQ ID NO.: 2	ataacttcgtataatgtataactatacgaagttat
loxP2272	SEQ ID NO.: 3	ataacttcgtataaagatcctatacgaagttat
loxP5171	SEQ ID NO.: 4	ataacttcgtataatgtactatacgaagttat
loxP2272(V)	SEQ ID NO.: 5	ATAACTTCGTATAGGATACTTTATACGAAGTTAT
pLox2+	SEQ ID NO.: 6	ATAACTTCGTATAATGTATGCTATACGAAGTTAT
loxC2	SEQ ID NO.: 28	ACAACTTCGTATAATGTATGCTATACGAAGTTAT
loxP2	SEQ ID NO.: 29	TACCGTTTCGTATAGTATAGTATATACGAAGTTAT
loxP3	SEQ ID NO.: 30	TACCGTTTCGTATAGTATAGTATATACGAACGGTA

[0047]

일부 실시예에서, RTS는 표 2로부터 선택된 Frt 부위이다. 본원에 언급된 바와 같이, 용어 "Frt 부위"는 효모 2 μm 플라스미드의 FLP 유전자의 생성물인 FLP 재조합 효소는 부위-특이적 재조합을 촉진한다. 다양한 비-동일 Frt 부위는 당 업계에 공지되어 있다. 다양한 Frt 부위의 서열은 이들이 재조합이 일어나는 8-염기쌍 비대칭 코어 영역의 측면에 위치한 동일한 13-염기쌍 반전된 반복물을 모두 포함한다는 점에서 유사하다. 부위의 방향성과 다른 Frt 부위 간의 변화를 담당하는 것은 비대칭 핵심영역이다. 이들의 예시적인 (비-제한적인) 예는 자연적으로 발생하는 Frt, 및 Frt1, Frt2와 같은 몇몇 돌연변이체 또는 변이체 Frt 부위를 포함한다. 일부 실시예에서, RTS는 Frt (F), Frt F1 (F1), Frt F2 (F2), Frt F3 (F3), Frt F4 (F4) 또는 Frt F5 (F5)로부터 선택된 Frt 부위이다. 일부 실시예에서, RTS는 Frt F6 (F6), Frt F7 (F7), Frt F14 (F14), Frt F15 (F15) 또는 Ff61 로부터 선택된 Frt 부위이다. 일부 실시예에서, RTS는 F2 151, Fw2, F2161 및 F2262로부터 선택된 Frt 부위이다. 일부 실시예에서, Frt 재조합 표적 부위는 표 2에 있는 서열과 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% 이상, 또는 100% 서열 동일성을 갖는 핵산이다.

표 2

이름	식별자	서열
F	SEQ ID NO.: 7	gaagttactattccgaagttcctattctctagaagtaggaacttc
F1	SEQ ID NO.: 8	GAAGTTACTATTCCGAAGTTCCTATTCTctagaTagtataggaacttc
F2	SEQ ID NO.: 9	GAAGTTACTATTCCGAAGTTCCTATTCTctacttagtataggaacttc
F3	SEQ ID NO.: 10	GAAGTTACTATTCCGAAGTTCCTATTCTtcaaagtagtataggaacttc
F4	SEQ ID NO.: 11	GAAGTTACTATTCCGAAGTTCCTATTCTctagaagtagtataggaacttc
F5	SEQ ID NO.: 12	gaagttcctattccgaagttcctattcttcaaaagtagtataggaacttc
F6	SEQ ID NO.: 13	gaagttcctattccgaagttcctattctTCaAaaagtagtataggaacttc
F7	SEQ ID NO.: 14	gaagttcctattccgaagttcctattctTCaATaagtagtataggaacttc
F14	SEQ ID NO.: 15	gaagttcctattccgaagttcctattctAtCAGaagtagtataggaacttc
F15	SEQ ID NO.: 16	gaagttcctattccgaagttcctattcttATAgGagtagtataggaacttc
Ff61	SEQ ID NO.: 17	GAAGTTACTATTCCGAAGTTCCTATACTTTCTGGAGAATAGGAAC TTC
F2151	SEQ ID NO.: 18	GAAGTTACTATTCCGAAGTTCCTATACTCTCCAGAGAATAGGAAC TTC
Fw2	SEQ ID NO.: 19	GAAGTTACTATTCCGAAGTTCCTATACTATCTACAGAATAGGAAC TTC
F2161	SEQ ID NO.: 20	GAAGTTACTATTCCGAAGTTCCTATACTCTCTGGAGAATAGGAAC TTC
F2262	SEQ ID NO.: 21	GAAGTTACTATTCCGAAGTTCCTATACTATCTTGAGAATAGGAAC TTC

[0049]

일부 실시예에서, RTS는 표 3으로부터 선택된 rox 부위이다. 본원에 언급된 바와 같이, 용어 "rox 부위"는 Dre 재조합 효소가 부위-특이적 재조합을 촉매할 수 있는 뉴클레오티드 서열을 지칭한다, 다양한 비-동일 rox 부위가 당 업계에 공지되어 있다. 이들의 예시적인 (비-제한적인) 예는 roxR 및 roxF를 포함한다. 일부 실시예에서, 완전 재조합 표적 부위는 표 3에 있는 서열과 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% 이상, 또는 100% 서열 동일성을 갖는 핵산이다.

표 3

이름	식별자	서열
roxF	SEQ ID NO.: 22	TAAC TTAAATAATGCCAATTATTTAAAGTTA
roxR	SEQ ID NO.: 23	TAAC TTAAATAATTGGCATTATTTAAAGTTA

[0050]

[0051] 일부 실시예에서, RTS는 표 4로부터 선택된 att 부위이다. 본원에 언급된 바와 같이, 용어 "att 부위"는 λ 인테그라제 또는 ϕ C31 인테그라제가 부위-특이적 재조합을 촉매할 수 있는 뉴클레오티드 서열을 지칭한다. 동일하지 않은 다양한 att 부위가 당 업계에 알려져 있다. 이들의 예시적인(비-제한적인) 예는 attP, attB, proB, trpC, galT, thrA, 및 rmB를 포함한다. 일부 실시예에서, att 재조합 표적 부위는 표 4에 있는 서열과 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% 이상, 또는 100% 서열 동일성을 갖는 핵산이다.

표 4

[0052]

이름	식별자	서열
att B	SEQ ID NO.: 24	CATCAGGGCGGTCAGGCCGTAGATGTGGAAGAACGGCAGCACGGCGAGGACG
att P	SEQ ID NO.: 25	ATGTGGTCCTTTAGATCCACTGACGTGGGTCAGTGTCTCTAAAGGACTCGCG
att L	SEQ ID NO.: 26	CATCAGGGCGGTCAGGCCGTAGATGTGGGTCAGTGTCTCTAAAGGACTCGCG
att R	SEQ ID NO.: 27	ATGTGGTCCTTTAGATCCACTGACGTGGAAGAACGGCAGCACGGCGAGGACG

[0053] 본 명세서에 언급된 바와 같이, 용어 "별개의 재조합 표적 부위"는 동일하지 않거나 이중- 특이적인 재조합 표적 부위를 지칭한다. 예를 들어 여러 변이된 Frt 부위가 존재하지만 재조합은 일반적으로 두 개의 동일한 Frt 부위 사이에서만 발생할 수 있다. 일부 실시예에서, 별개의 재조합 표적 부위는 동일한 재조합 시스템 (예를 들어, LoxP 및 LoxR)으로부터의 동일하지 않은 재조합 표적 부위를 지칭한다. 일부 실시예에서, 별개의 재조합 표적 부위는 상이한 재조합 시스템 (예를 들어, LoxP 및 Frt)으로부터의 동일하지 않은 재조합 표적 부위를 지칭한다. 일부 실시예에서, 별개의 재조합 표적 부위는 동일한 재조합 시스템으로부터의 재조합 표적 부위 및 동일하지 않은 재조합 시스템 (예를 들어, LoxP, LoxR, Frt 및 Frt1)으로부터의 재조합 표적 부위의 조합을 지칭한다.

[0054] 다양한 RTS 조합을 사용할 수 있다. 일부 실시예에서, 상기 세포는 4개의 RTS를 포함한다. 일부 실시예에서, 상기 세포는 6개의 RTS를 포함한다. 일부 실시예에서, 하나 이상의 RTS는 서열 번호: 1-30으로부터 선택된다. 일부 실시예에서, 하나 이상의 RTS는 서열번호: 1-6으로부터 선택된다. 일부 실시예에서, 하나 이상의 RTS는 서열번호: 28-30으로부터 선택된다. 일부 실시예에서, 하나 이상의 RTS는 서열 번호: 7-21로부터 선택된다. 일부 실시예에서, 적어도 하나의 RTS는 서열 번호: 22-23에서 선택된다. 일부 실시예에서, 하나 이상의 RTS는 서열 번호: 24- 27로부터 선택된다. 일부 실시예에서, 하나 이상의 RTS는 서열 번호: 28-30으로부터 선택된다. 일부 실시예에서, 적어도 4개의 별개의 RTS를 포함하는 세포는 다음 RTS: LoxP_511, Frt_F5, Frt 및 LoxP를 포함한다. 일부 실시예에서, 적어도 4개의 별개의 RTS를 포함하는 세포는 다음 RTS Frt_F14 및 Frt_F15를 포함한다. 일부 실시예에서, 적어도 4개의 별개의 RTS를 포함하는 세포는 다음 RTS: LoxP_511, Frt_F5, Frt, LoxP, Frt_F14 및 Frt_F15를 포함한다. 일부 실시예에서, 적어도 4개의 별개의 RTS를 포함하는 상기 세포는 다음 RTS: Frt_1, Frt_2, Frt_3, Frt_4를 포함한다. 일부 실시예에서, 적어도 4개의 별개의 RTS를 포함하는 세포는 다음의 RTS: Frt_m5, Frt_wt, Frt_m14, Frt_m15, Frt_m7 및 Frt_m6을 포함한다.

[0055] 본원에 언급된 바와 같이, 용어 "랜딩 패드 (landing pad)"는 숙주세포 내로 염색체 로-통합된 제1 재조합 표적 부위를 포함하는 핵산 서열을 지칭한다. 일부 실시예에서, 랜딩 부위는 숙주세포 내로 염색체로 통합된 2개 이상의 재조합 표적 부위를 포함한다. 일부 실시예에서, 세포는 1, 2, 3, 4, 5, 6, 7, 또는 8개의 랜딩 패드를 포함한다. 일부 실시예에서, 랜딩 패드는 최대 1, 2, 3, 4, 5, 6, 7, 8개의 별개의 염색체 좌위에 통합된다.

[0056] 본원에서 언급된 바와 같이, 용어 "아데노바이러스"는 아데노바이러스과 계열의 이중 가닥 DNA를 함유하는 이십면체(icosahedral) 뉴클레오캡시드를 갖는 비-봉입 바이러스를 지칭한다. 50종 이상의 아데노바이러스 아형이 인간으로부터 단리되었고, 많은 추가 아형이 다른 포유동물 및 조류로부터 단리되었다. 조류, 예를 들어, Ishibashi et al., "Adenoviruses of animals," In *The Adenoviruses*, Ginsberg, ed., Plenum Press, New York, N.Y., pp. 497-562 (1984); Strauss, "Adenovirus infections in humans, In *The Adenoviruses*, Ginsberg, ed., Plenum Press, New York, N.Y., pp. 451-596 (1984)를 참고한다. 이들 아형은 현재 아데노바이러스 과에 속하며, 현재 두 가지 속, 즉 포유류아데노바이러스(Mastadenovirus)와 아비아데노바이러스

(Aviadenovirus)로 나뉜다. 모든 아데노바이러스는 형태학적 및 구조적으로 유사하다. 그러나, 인간에서, 아데노바이러스는 다양한 면역학적 특성을 나타내므로 혈청형으로 나뉜다. 두가지 인간 혈청형인 아데노바이러스, 즉 Ad2 및 Ad5는 집중적으로 연구되었으며 아데노바이러스에 대한 대부분의 일반 정보를 제공했다.

[0057] "유전자"는 폴리펩티드를 코딩하는 뉴클레오티드의 어셈블리(assembly)를 말하며, cDNA 및 게놈 DNA 핵산 분자를 포함한다. "유전자"는 또한 코딩 서열의 선행 (5' 비-코딩서열) 및 후속 (3' 비-코딩서열)의 조절 서열로서 작용할 수 있는 핵산 단편을 지칭한다. 일부 실시예에서, 유전자는 다중 카피(copies)와 통합된다. 일부 실시예에서, 유전자는 미리 정의된 카피 수로 통합된다.

[0058] 본원에 언급된 바와 같이, 용어 "아데노바이러스 유전자" 또는 "AV 유전자"는 하나 이상의 아데노바이러스 아형 또는 혈청형으로부터 유래된 하나 이상의 핵산 서열로 구성된 유전자를 지칭한다. 일부 실시예에서, Ad 유전자는 아데노-관련 바이러스 복제 및 패키징에 기여하는 유전자이다. 일부 실시예에서, Ad 유전자는 E1A, E1B, E2A, E4, VA, MIR342, 또는 이들의 조합 또는 임의의 다른 Ad 유전자이다. 일부 실시예에서, 본 개시 내용은 하나 이상의 Ad 유전자가 세포내로 염색체로-통합된 세포를 제공한다. 일부 실시예에서, 본 개시 내용은 E1A, E1B, E2A, E4, VA, MIR342 중 하나 이상이 세포 내로 염색체로-통합된 세포를 제공한다. 일부 실시예에서, Ad 유전자는 E1A를 포함한다. 일부 실시예에서, Ad 유전자는 E1A 및 E1B를 포함한다. 일부 실시예에서, Ad 유전자는 E1A, E1B 및 E2A를 포함한다. 일부 실시예에서, Ad 유전자는 E1A, E1B, E2A 및 E4를 포함한다. 일부 실시예에서, Ad 유전자는 E1A, E1B, E2A, E4 및 VA를 포함한다. 일부 실시예에서, Ad 유전자는 E1A, E1B, E2A, E4, VA 및 MIR342를 포함한다. 일부 실시예에서, Ad 유전자는 E1A 및 E2A를 포함한다. 일부 실시예에서, Ad 유전자는 E1A, E2A 및 E4를 포함한다. 일부 실시예에서, Ad 유전자는 E1A, E2A, E4 및 VA를 포함한다. 일부 실시예에서, Ad 유전자는 E1A, E2A, E4, VA 및 MIR 342를 포함한다. 일부 실시예에서, Ad 유전자는 E1A 및 E4를 포함한다. 일부 실시예에서, Ad 유전자는 E1A, E4 및 VA를 포함한다. 일부 실시예에서, Ad 유전자는 E1A, E4, VA 및 MIR342를 포함한다. 일부 실시예에서, Ad 유전자는 E1A 및 VA를 포함한다. 일부 실시예에서, Ad 유전자는 E1A, VA 및 MIR342를 포함한다. 일부 실시예에서, Ad 유전자는 E1A 및 MIR342를 포함한다. 일부 실시예에서, Ad 유전자는 E1B를 포함한다. 일부 실시예에서, Ad 유전자는 E1B 및 E2A를 포함한다. 일부 실시예에서, Ad 유전자는 E1B, E2A 및 E4를 포함한다. 일부 실시예에서, Ad 유전자는 E1B, E2A, E4 및 VA를 포함한다. 일부 실시예에서, Ad 유전자는 E1B, E2A, E4, VA 및 MIR342를 포함한다. 일부 실시예에서, Ad 유전자는 E1B 및 E4를 포함한다. 일부 실시예에서, Ad 유전자는 E1B, E4 및 VA를 포함한다. 일부 실시예에서, Ad 유전자는 E1B, E4, VA 및 MIR342를 포함한다. 일부 실시예에서, Ad 유전자는 E1B 및 VA를 포함한다. 일부 실시예에서, Ad 유전자는 E1B, VA 및 MIR342를 포함한다. 일부 실시예에서, Ad 유전자는 E2A를 포함한다. 일부 실시예에서, Ad 유전자는 E2A 및 E4를 포함한다. 일부 실시예에서, Ad 유전자는 E2A, E4 및 VA를 포함한다. 일부 실시예에서, Ad 유전자는 E2A, E4, VA 및 MIR342를 포함한다. 일부 실시예에서, Ad 유전자는 E2A 및 VA를 포함한다. 일부 실시예에서, Ad 유전자는 E2A, VA 및 MIR342를 포함한다. 일부 실시예에서, Ad 유전자는 E2A 및 MIR342를 포함한다. 일부 실시예에서, Ad 유전자는 E4를 포함한다. 일부 실시예에서, Ad 유전자는 E4 및 VA를 포함한다. 일부 실시예에서, Ad 유전자는 E4, VA 및 MIR342를 포함한다. 일부 실시예에서, Ad 유전자는 E4 및 MIR342를 포함한다. 일부 실시예에서, Ad 유전자는 VA를 포함한다. 일부 실시예에서, Ad 유전자는 VA 및 MIR342를 포함한다. 일부 실시예에서, Ad 유전자는 MIR342를 포함한다. 일부 실시예에서, 본 발명자들은 Ad 유전자가 E1A, E1B, E2A, E4, VA 및 MIR342를 포함할 때 상기 Ad 유전자가 염색체로 통합되어 보다 일관된 Ad 유전자 발현이 관찰된다는 것을 발견하였다. 일부 실시예에서, Ad 유전자는 E1A, E1B, E2A, E4, VA 및 MIR342로부터 선택된 하나 이상을 포함한다.

[0059] 본원에 언급된 바와 같이, 용어 "조절 요소"는 핵산 서열의 발현의 일부 측면을 제어하는 유전자 요소를 지칭한다. 본원에 사용된 "프로모터"는 작동 가능하게 연결된 코딩 영역의 전사 개시를 용이하게 하는 조절 요소이다. 일부 실시예에서, 프로모터는 Ad 프로모터이다. 본원에 사용된 용어 "AV 프로모터"는 아데노바이러스 시스템으로부터 유래된 조절 요소를 지칭한다. 일부 실시예에서, 프로모터는 AAV 프로모터이다. 본원에 사용된 용어 "AAV 프로모터"는 아데노-관련 바이러스 시스템으로부터 유래된 조절 요소를 지칭한다. 일부 실시예에서, 본 개시 내용은 하나 이상의 AAV 프로모터가 세포 내로 염색체로 통합된 세포를 제공한다. 본원에 사용된 용어 "비-AV 프로모터"는 비-아데노바이러스 시스템으로부터 유래된 조절 요소를 지칭한다. 일부 실시예에서, 비-AV 프로모터는 원핵생물 시스템으로부터 유래된다. 일부 실시예에서, 비-AV 프로모터는 진핵생물 시스템으로부터 유래된다. 일부 실시예에서, 본 개시 내용은 하나 이상의 프로모터가 세포 내로 염색체로 통합되는 세포를 제공한다. 일부 실시예에서, 본 개시 내용은 하나 이상의 비-AV 프로모터가 세포 내로 염색체로 통합된 세포를 제공한다. 일부 실시예에서, 본 개시 내용은 하나 이상의 Ad 프로모터가 세포 내로 염색체로 통합된 세포를 제공한다. 일부 실시예에서, 프로모터는 비-AVR 프로모터이다. 본원에 사용된 용어 "비-AAV 프로모터"는 비 아데노-관련

바이러스 시스템으로부터 유래된 조절 요소를 지칭한다. 일부 실시예에서, 비-AAV 프로모터는 원핵 생물 시스템으로부터 유래된다. 일부 실시예에서, 비-AAV 프로모터는 진핵 생물 시스템으로부터 유래된다. 일부 실시예에서, 본 개시 내용은 하나 이상의 비-AAV 프로모터가 세포 내로 염색체로 통합된 세포를 제공한다.

[0060] 본원에서 언급된 바와 같이, 용어 “조작 가능한 조합으로”, “조작 가능한 순서로” 및 “조작 가능하게 연결된”은 주어진 유전자의 전사 및/또는 원하는 단백질 분자의 합성을 지시할 수 있는 핵산 분자가 생성되는 방식으로 핵산 서열의 연결을 지칭한다. 상기 용어는 또한 기능성 단백질이 생성되는 방식으로 아미노산 서열의 연결을 지칭한다. 일부 실시예에서, Ad 유전자는 프로모터에 작동 가능하게 연결되며, 상기 Ad 유전자는 숙주 세포 계놈 내로 염색체로 통합된다. 일부 실시예에서, Ad 유전자는 비-Ad 프로모터에 작동 가능하게 연결되며, 상기 Ad 유전자는 숙주 세포 계놈으로 염색체로 통합된다. 일부 실시예에서, Ad 유전자는 Ad 프로모터에 작동 가능하게 연결되며, 상기 Ad 유전자는 숙주 세포 계놈 내로 염색체로 통합된다. 일부 실시예에서, AAV 유전자는 프로모터에 작동 가능하게 연결되며, 상기 AAV 유전자는 숙주 세포 계놈 내로 염색체로 통합된다. 일부 실시예에서, AAV 유전자는 비-AAV 프로모터에 작동 가능하게 연결되며, 상기 AAV 유전자는 숙주 세포 계놈 내로 염색체로 통합된다. 일부 실시예에서, AAV 유전자는 AAV 프로모터에 작동 가능하게 연결되며, 상기 AAV 유전자는 숙주 세포 계놈으로 염색체로 통합된다. 일부 실시예에서, GOI는 숙주 세포 계놈 내로 염색체로 통합된다. 일부 실시예에서, GOI 유전자는 비-GOI 프로모터에 작동 가능하게 연결되며, 상기 GOI는 숙주 세포 계놈 내로 염색체로 통합된다. 일부 실시예에서, 재조합 효소 유전자는 프로모터에 작동 가능하게 연결되며, 상기 재조합 효소 유전자는 숙주 세포 계놈으로 염색체로 통합된다. 일부 실시예에서, 재조합 효소 유전자는 프로모터에 작동 가능하게 연결되며, 상기 재조합 효소 유전자는 숙주 세포 계놈에 통합되지 않는다. 일부 실시예에서, 재조합 효소 유전자는 프로모터에 작동 가능하게 연결되며, 상기 재조합 효소 유전자는 숙주 세포 계놈으로 염색체로 통합되지 않는다. 일부 실시예에서, 재조합 효소 유전자는 프로모터에 작동 가능하게 연결되며, 상기 재조합 효소 유전자는 숙주 세포 계놈으로 염색체로 통합되지 않는다.

[0061] 일부 실시예에서, 조절 요소는 유전자 발현을 외인성의(exogenously) 공급된 리간드의 존재에 작동 가능하게 연결시킨다. 일부 실시예에서, Ad 유전자는 프로모터에 작동 가능하게 연결되며, 상기 프로모터는 유전자 발현을 외인성의 공급된 리간드의 존재에 작동 가능하게 연결시키고, Ad 유전자는 숙주 세포 계놈에 염색체로 통합된다. 일부 실시예에서, AAV 유전자는 프로모터에 작동 가능하게 연결되며, 상기 프로모터는 유전자 발현을 외인성의 공급된 리간드의 존재에 작동 가능하게 연결시키고 AAV 유전자는 숙주 세포 계놈에 염색체로 통합된다. 일부 실시예에서, GOI는 프로모터에 작동 가능하게 연결되며, 상기 프로모터는 유전자 발현을 외인성의 공급된 리간드의 존재에 작동 가능하게 연결하고, GOI는 숙주 세포 계놈에 염색체로 통합된다. 일부 실시예에서, 재조합 효소 유전자는 프로모터에 작동 가능하게 연결되며, 상기 프로모터는 유전자 발현을 외인성의 공급된 리간드의 존재에 작동 가능하게 연결시키고, 재조합 효소 유전자는 숙주 세포 계놈에 염색체로 통합된다. 일부 실시예에서, 재조합 효소 유전자는 프로모터에 작동 가능하게 연결되며, 상기 프로모터는 유전자 발현을 외인성의 공급된 리간드의 존재에 작동 가능하게 연결시키고, 재조합 효소 유전자는 숙주 세포 계놈에 염색체로 통합되지 않는다. 일부 실시예에서, E1A 발현은 프로모터에 작동 가능하게 연결되며, 프로모터는 유전자 발현을 rAAV의 생산에 작동 가능하게 연결시킨다. 일부 실시예에서, E1A 발현은 프로모터에 작동 가능하게 연결되며, 여기서 프로모터는 유전자 발현을 외인성의 공급된 리간드의 존재와 작동 가능하게 연결시키고, 유전자 발현은 외인성의 공급된 리간드의 존재를 rAAV의 생산에 작동 가능하게 연결시킨다.

[0062] 일부 실시예에서, 하나 이상의 RTS, Ad 유전자 및 프로모터는 단일 염색체 유전자 좌위에 통합된다. 일부 실시예에서, 제1 Ad 유전자 *E1A* 및 *E1B*, 및 *E2A*, *E4*, *VA* 및 *MIR 342*를 포함하는 제2 Ad 유전자는 2개의 상이한 유전자 좌위상에 존재한다. 일부 실시예에서, 제1 Ad 유전자 *E1A* 및 *E1B*, 및 *E2A*, *E4*, *VA* 및 *MIR 342*를 포함하는 제2 Ad 유전자는 동일한 유전자 좌위상에 존재한다. 일부 실시예에서, 제1 Ad 유전자 *E1A* 및 *E1B*, 및 *E2A*, *E4*, *VA* 및 *MIR 342*를 포함하는 제2 Ad 유전자는 동일한 유전자 좌위이고, 아데노-관련 바이러스 (AAV) 유전자는 다른 유전자 좌위에 있다. 일부 실시예에서, 제1 Ad 유전자 *E1A* 및 *E1B*, 및 *E2A*, *E4*, *VA* 및 *MIR 342*를 포함하는 제2 Ad 유전자는 동일한 유전자 좌위이고, 아데노-관련 바이러스 (AAV) 유전자 및 AAV 벡터 카세트는 상이한 유전자 좌위 상에 있다.

[0063] 본원에 언급된 바와 같이, 용어 “염색체 유전자 좌위”는 하나 이상의 유전자를 포함할 수 있는 세포의 염색체상의 핵산의 정의된 위치를 지칭한다. 일부 실시예에서, 염색체 유전자 좌위는 약 500 염기쌍 내지 약 100,000 염기쌍, 약 5,000 염기쌍 내지 약 75,000 염기쌍, 약 5,000 염기쌍 내지 약 60,000 염기쌍, 약 20,000 염기쌍 내지 약 50,000 염기쌍, 약 30,000 염기쌍 내지 약 50,000 염기쌍, 또는 약 45,000 염기쌍 내지 약 49,000 염기쌍이다. 일부 실시예에서, 염색체 유전자 좌위는 정의된 핵산 서열의 5' 또는 3' 말단까지 약 100 염기쌍,

약 250 염기쌍, 약 500 염기쌍, 약 750 염기쌍 또는 약 1000 염기쌍까지 연장된다. 일부 실시예에서, 염색체 유전자 좌위는 내인성(endogenous) 핵산 서열을 포함한다. 일부 실시예에서, 염색체 유전자 좌위는 분자생물학 분야의 당업자에게 공지된 방법을 사용하여 염색체에 통합된 외인성 뉴클레오티드 서열을 포함한다. 일부 실시예에서, 염색체 유전자 좌위는 염색체 유전자 좌위 내에 통합된 유전자에 의해 암호화된 단백질의 강력하고 안정적인 생산을 제공하는 숙주 세포의 게놈에서 뉴클레오티드 서열을 포함한다. 일부 실시예에서, 염색체 유전자 좌위는 강력하고 안정적인 바이러스 유전자 발현을 제공하는 숙주 세포의 게놈에서 뉴클레오티드 서열을 포함한다. 일부 실시예에서, 염색체 유전자 좌위는 효율적인 부위 특이적 재조합을 제공하는 숙주 세포의 게놈에서 뉴클레오티드 서열을 포함한다. 일부 실시예에서, 염색체 좌위는 Fer1L4(예를 들어, 미국 특허 출원번호 14/409,283 참조), *ROSA26*, *HGPRT*, *DHFR*, *COSMC*, *LDHA* 또는 *MGAT1* 을 포함한다. 일부 실시예에서, 염색체 유전자 좌위는 *NL1* 또는 *NL2*를 포함한다. 일부 실시예에서, 염색체 유전자 좌위는 X 염색체에서 *MIDI*의 첫 번째 인트론이다. 일부 실시예에서, 염색체 유전자 좌위는 향상된 발현 및 안정성 영역(Regeneron, Tarrytown, NY EESYR, 예를 들어, 미국 특허 7,771,997 참조)이다. 일부 실시예에서, 염색체 유전자 좌위 내의 핵산의 적어도 일부가 결실된다.

[0064]

일부 실시예에서, 염색체 유전자 좌위는 표 5에 기재된 바와 같이 NL1 유전자 좌위, NL2 유전자 좌위, NL3 유전자 좌위, NL4 유전자 좌위, NL5 유전자 좌위 또는 NL6 유전자 좌위를 포함한다. 일부 실시예에서, 본 개시 내용은 하나 이상의 RTS, Ad 유전자 및 프로모터가 NL1 유전자 좌위, NL2 유전자 좌위, NL3 유전자 좌위, NL4 유전자 좌위, NL5 유전자 좌위 또는 NL6 유전자 좌위 내에 통합된 2개 이상의 별개의 재조합 표적 부위 (RTS)를 포함하는 포유동물 세포에 관한 것이다. 일부 실시예에서, 제1 Ad 유전자 *E1A* 및 *E1B*, 및 *E2A*, *E4*, 및 *MIR 342*를 포함하는 제2 Ad 유전자는 2개의 상이한 유전자 좌위 상에 있으며, 그 중 하나는 NL1 유전자 좌위, NL2 유전자 좌위, NL3 유전자 좌위, NL4 유전자 좌위, NL5 유전자 좌위 또는 NL6 유전자 좌위 내에 있다. 일부 실시예에서, 제1 Ad 유전자 *E1A* 및 *E1B*, 및 *E2A*, *E4*, *VA* 및 *MIR 342*를 포함하는 제2 Ad 유전자는 NL1 유전자 좌위, NL2 유전자 좌위, NL3 유전자 좌위, NL4 유전자 좌위, NL5 유전자 좌위 또는 NL6 유전자 좌위 내에 있다. 일부 실시예에서, 제1 Ad 유전자 *E1A* 및 *E1B*, 및 *E2A*, *E4*, *VA* 및 *MIR 342*를 포함하는 제2 Ad 유전자는 NL1 유전자 좌위, NL2 유전자 좌위, NL3 유전자 좌위, NL4 유전자 좌위, NL5 유전자 좌위 또는 NL6 유전자 좌위 내에 있다. 일부 실시예에서, 제1 Ad 유전자 *E1A* 및 *E1B*, 및 *E2A*, *E4*, *VA* 및 *MIR342*를 포함하는 제2 Ad 유전자는 NL1 유전자 좌위, NL2 유전자 좌위, NL3 유전자 좌위, NL4 유전자 좌위, NL5 유전자 좌위 또는 NL6 유전자 좌위 내에 있다. 일부 실시예에서, 본 개시 내용은 3개 이상의 별개의 재조합 표적 부위 (RTS)를 포함하는 포유동물 세포에 관한 것으로 여기서 적어도 하나의 RTS, Ad 유전자 및 프로모터는 NL1 유전자 좌위, NL2 유전자 좌위, NL3 유전자 좌위, NL4 유전자 좌위, NL5 유전자 좌위 또는 NL6 유전자 좌위 내에 통합된다. 일부 실시예에서, 본 개시 내용은 4개 이상의 별개의 재조합 표적 부위 (RTS)를 포함하는 포유동물 세포에 관한 것으로 여기서 적어도 하나의 RTS, Ad 유전자 및 프로모터는 NL1 유전자 좌위, NL2 유전자 좌위, NL3 유전자 좌위, NL4 유전자 좌위, NL5 유전자 좌위 또는 NL6 유전자 좌위 내에 통합된다. 일부 실시예에서, 본 개시 내용은 5개 이상의 별개의 재조합 표적부위(RTS)를 포함하는 포유동물 세포에 관한 것으로, 여기서 적어도 하나의 RTS, Ad 유전자 및 프로모터는 NL1 유전자 좌위, NL2 유전자 좌위, NL3 유전자 좌위, NL4 유전자 좌위, NL5 유전자 좌위 또는 NL6 유전자 좌위 내에 통합된다. 일부 실시예에서, 본 개시 내용은 6개 이상의 별개의 재조합 표적 부위 (RTS)를 포함하는 포유동물 세포에 관한 것으로 여기서 적어도 하나의 RTS, Ad 유전자 및 프로모터는 NL1 유전자 좌위, NL2 유전자 좌위, NL3 유전자 좌위, NL4 유전자 좌위, NL5 유전자 좌위 또는 NL6 유전자 좌위 내에 통합된다.

표 5

새로운 유전자 좌위	유전자 좌위 주석			<i>Cricetulus griseus</i> 에서의 유전자 좌위 위치			<i>Homo sapien</i> 에서의 유전자 좌위 위치		
	5' 유전자	유전자 내	3' 유전자	NCBI 참조 서열 (<i>Cricetulus griseus</i>)	백터 위치	랜딩 패드 위치	염색체	백터 위치	랜딩 패드 위치
Fer1	Ergic3	Fer1L4	Spag4	gi 351517716 ref NW_003613833 Scaffold 1492	N/A	24894-26755	Chr 20	33613757-33614117	33630003-33629572
NL1	Col5a2 (pseudo)	주석 달린 유전자 없음	Col5a2	SEQ ID NO: 31 gi 351515650 ref NW_003615899.1 Scaffold 2552	143287	133598	Chr 2	189725696-189726735	189725180-189727852
NL2	LOC103162114	Naa15 (엑손 5&6 사이의 인트론)	Rab33b	SEQ ID NO: 32 gi 351517715 ref NW_003613834.1 Scaffold2422	1303708 to 1308873	1315999	Chr 4	140474660-140474867	140483934-140483457
NL3	CJO004127.1	주석 달린 유전자 없음	Mthfd2	SEQ ID NO: 33 gi 351516540 ref NW_003615009.1 Scaffold3922	279558	N/A	Chr 2	74277198-74297932	N/A
NL4	MORF4L1	주석 달린 유전자 없음	No annotated gene	SEQ ID NO: 34 gi 351516248 ref NW_003615301.1 Scaffold502	173850	N/A	Chr 15	76952458	N/A
NL5	ZNHIT1	주석 달린 유전자 없음	Tatc1	SEQ ID NO: 35 gi 351517397 ref NW_003614152.1 Scaffold3667	779010	N/A	Chr 7	100648176-100653983	N/A
NL6	Ahgef28	Utp15	Ankra	SEQ ID NO: 36 gi 351516857 ref NW_003614592.1 Scaffold7019	225021	N/A	Chr 5	73084488-73084783	N/A
				SEQ ID NO: 37					

[0065]

[0066]

당업자는 "NL1 유전자 좌위 내에 통합된" 또는 "NL2 유전자 좌위 내에 통합된" 이 라는 용어는 유전자 좌위의 임의의 부분으로의 통합을 포함할 것이며, 이는 단지 표시된 게놈 좌표로 제한되지 않는다는 것을 인식할 것이다. 당업자는 "NL1 유전자 좌위 내에 통합된" 또는 "NL2 유전자 좌위 내에 통합된"이라는 용어는 또한 상응하는 유기체에 상응하는 유전자 좌위를 포함할 것이라는 것을 인식할 것이다. 따라서, 일부 실시예에서, 용어 "NL1 유전자 좌위 내에 통합된" 또는 "NL2 유전자 좌위 내에 통합된" 은 표시된 게놈 좌표의 약 50,000 bp 내, 약 40,000 bp 내, 약 30,000 bp 내, 20,000 bp 내 또는 10,000 bp 내 통합을 포함할 것이다.

[0067]

일부 실시예에서, 세포는 부위-특이적 재조합 효소 유전자를 포함한다. 일부 실시예에서, 부위-특이적 재조합 효소 유전자는 염색체로 통합된다.

[0068]

본 명세서에서 언급된 바와 같이, 용어 "2개의 RTS 사이에 위치한"은 2개의 RTS 사이에 위치한 유전자, 즉 RTS 중 하나가 유전자의 5 '에 위치하고 다른 RTS가 3' 에 있는 것을 의미한다. 일부 실시예에서, RTS는 그들 사이에 위치한 유전자에 직접 인접하여 위치한다. 일부 실시예에서, RTS는 그들 사이에 위치한 유전자로부터 정의된 거리에 위치한다. 일부 실시예에서, RTS는 방향성 서열이다. 일부 실시예에서, 이들 사이에 위치한 유전자의 RTS 5 ' 및 3' 은 직접 배향(oriented)된다(즉, 동일한 방향으로 배향된다). 일부 실시예에서, 그들 사이에 위치한 유전자의 RTS 5 ' 및 3' 은 역으로 배향된다(즉, 반대 방향으로 배향된다).

[0069]

일부 실시예에서, 세포는 아데노-관련 바이러스 (AAV) 유전자를 추가로 포함하며, 상기 AAV 유전자는 염색체로 통합된다. AAV 유전자는 임의의 AAV 혈청형으로부터의 임의의 유전자를 포함한다. 일부 실시예에서, AAV 유전자는 *Rep*, *Rep78*, *Rep68*, *Rep52*, *Rep40*, *Cap*, *VP1*, *VP2*, *VP3*, 또는 이들의 조합이다. 일부 실시예에서, AAV 유전자는 아데노 관련 바이러스 유형 2로부터 유래된다. 일부 실시예에서, AAV 유전자는 아데노 관련 바이러스 Anc80 으로부터 유래된다. 일부 실시예에서, AAV 유전자는 ANC80 *Rep* 및 *Cap* 유전자를 포함한다. 일부 실시예에서, AAV 유전자는 2개의 RTS 사이에 위치된다.

[0070]

본원에 언급된 바와 같이, 용어 "아데노-관련 바이러스(AAV)"는 파르보바이러스(*Parvoviridae*) 과 계열 및 의존도파르보바이러스(*Dependoparvovirus*) 속의 단일 가닥 DNA를 포함하는 작은 크기의 복제-결합 비외피 바이러스를 지칭한다. 지금까지 10가지가 넘는 아데노-관련 바이러스 혈청형이 확인되었으며, 혈청형 AAV2가 가장 특징적이다. 다른(비-제한적) AAV 혈청형의 예는 ANC80, AAV1, AAV3, AAV4, AAV5, AAV6, AAV7, AAV8, AAV9, AAV10 및 AAV11 이다. 이들 혈청형에 더하여, AAV 위형(pseudotype)이 개발되었다. AAV 위형은 제1 혈청형의 캡시드 및 제2 혈청형의 게놈을 함유한다(예를 들어, 위형 AAV2/5는 혈청형 AAV2의 게놈 및 AAV5의 캡시드를 갖는 AAV에 상응할 것이다).

- [0071] 본원에 사용된 용어 "재조합 AAV" 또는, "rAAV" 는 상동 유전자를 단백질막으로 싸는(encapsidating) (즉, 단백질 코트로 둘러싸는) AAV 단백질 껍질로 구성된 감염성, 복제-결함 바이러스로, 이는 AAV ITR에 의해 5' 와 3' 이 측면에 있다.
- [0072] 본원에 사용된 용어 복제 가능 아데노바이러스 (RCA)는 재조합 아데노바이러스 벡터와 생산자 세포 계놈에 존재하는 아데노바이러스 유전자 사이의 상동 재조합에 의해 rAAV 생산 동안 때때로 생성되는 활성 아데노바이러스 입자의 바람직하지 않은 오염물을 지칭한다.
- [0073] 본원에 언급된 바와 같이, 용어 "아데노-관련 바이러스 유전자" 는 하나 이상의 아데노-관련 바이러스 혈청형으로부터 유래된 하나 이상의 핵산 서열로 구성된 유전자를 지칭한다. 일부 실시예에서, AAV 유전자는 AAV 복제 및 패키징에 기여하는 유전자를 포함한다.
- [0074] 본원에 언급된 바와 같이, 용어 "Rep" 유전자는 바이러스 계놈의 복제에 총체적으로 필요한 바이러스의 복제 단백질을 암호화하는 AAV 계놈의 당 업계에 인식된 영역을 의미하거나, 또는 AAV-2 DNA 복제를 매개하는 것으로 알려진 인간 헤르페스바이러스 6 (HHV-6) rep 유전자와 같은 이의 기능적 상동체를 의미한다. 따라서, rep 코딩 영역은 적어도 AAV Rep78 및 Rep68("Rep의 긴 형태"), 및 Rep52 및 Rep40("Rep의 짧은 형태"), 또는 이들의 기능적 상동체를 암호화하는 유전자를 포함한다. AAV rep 코딩 영역에 대한 추가 설명. 본원에 사용된 rep 코딩 영역은 임의의 바이러스 혈청형, 예컨대 상기 기재된 AAV 혈청형으로부터 유래될 수 있다. 상기 영역은 모든 야생형 유전자를 포함할 필요는 없지만, rep 유전자가 적합한 표적 세포에서 발현될 때 충분한 통합 기능을 제공하는 한(예를 들어, 뉴클레오티드의 삽입, 결실 또는 치환에 의해) 변경될 수 있다. 예를 들어, Muzyczka, N., *Current Topics in Microbiol, and Immunol.* 158:97-129 (1992); 및 Kotin, R. M., *Human Gene Therapy* 5: 793-801 (1994)를 참고한다.
- [0075] 본원에 언급된 바와 같이, 용어 "Cap'" 유전자는 바이러스의 캡시드 단백질을 코딩하는 AAV 계놈의 당업계에 인식된 영역을 지칭한다. 이들 캡시드 단백질의 예시적인 (비-제한적인) 예는 AAV 캡시드 단백질 VP1, VP2 및 VP3이다. 본 개시에 사용된 Cap 유전자는 임의의 AAV 혈청형 또는 AAV 혈청형의 조합으로부터 유래될 수 있다.
- [0076] 일부 실시예에서, 세포는 제2 Ad 유전자를 포함하고, 상기 제2 Ad 유전자는 염색체 통합된다. 일부 실시예에서, 제2 Ad 유전자는 *E1A, E1B, E2A, E4, VA, MIR342*, 또는 이들의 조합이다. 일부 실시예에서, 제2 Ad 유전자는 Ad5로부터의
- [0077] 것이다. 일부 실시예에서, 제2 Ad 유전자는 2개의 RTS 사이에 위치된다.
- [0078] 일부 실시예에서, 세포는 AAV 벡터 카세트를 추가로 포함하고, 상기 AAV 벡터 카세트는 염색체로 통합된다. 일부 실시예에서, AAV 벡터 카세트는 2개의 RTS 사이에 위치된다.
- [0079] "벡터" 또는 "발현벡터"는 플라스미드, 파지, 바이러스 또는 코스미드(cosmid)와 같은 레플리콘(replicon)으로, 다른 DNA 단편이 부착되어 세포에서 부착된 DNA 단편의 복제 및/또는 발현을 야기할 수 있다. "벡터" 는 에피솜(episomal) (예를 들어, 플라스미드) 및 비 에피솜 벡터를 포함한다. 본 개시 내용의 일부 실시예에서, 벡터는 에피솜 벡터이며, 이는 다수의 세포 생성 후 세포 집단으로부터 제거/손실되며, 예를 들어, 비대칭 분할에 의한다. 용어 "벡터" 는 시험관 내, 생체 내 또는 생체 외에서 핵산 분자를 세포 내로 도입하기 위한 바이러스 및 비-바이러스 수단을 모두 포함한다. 용어 벡터는 합성 벡터를 포함할 수 있다. 벡터는 형질주입, 형질도입, 세포 융합, 및 리포펙션(lipofection)을 포함하지만 이에 제한되지 않는 공지된 방법에 의해 원하는 숙주 세포 내로 도입될 수 있다. 벡터는 프로모터를 포함하는 다양한 조절 요소를 포함할 수 있다.
- [0080] 본원에 사용된 "형질주입"은 벡터를 포함하는 외인성 핵산 분자의 세포로의 도입을 의미한다. "형질 감염된" 세포는 세포 내부의 외인성 핵산 분자를 포함하고 "형질 전환된" 세포는 세포 내의 외인성 핵산 분자가 세포에서 표현형 변화를 유도하는 세포이다. 형질주입된 핵산 분자는 숙주 세포의 계놈 DNA에 통합될 수 있고/또는 세포에 의해, 일시적으로 또는 장기간, 염색체 외적으로 유지될 수 있다. 외인성 핵산 분자 또는 단편을 발현하는 숙주 세포 또는 유기체는 "재조합체", "형질 전환된" 또는 "형질전환의" 유기체로 지칭된다. 다수의 형질주입 기술이 당 업계에 일반적으로 공지되어 있다. 예를 들어, Graham et al., *Virology*, 52:456 (1973); Sambrook et al., *Molecular Cloning, a laboratory manual*, Cold Spring Harbor Laboratories, New York (1989); Davis et al., *Basic Methods in Molecular Biology*, Elsevier (1986); 및 Chu et al., *Gene* 13:197 (1981)를 참조한다. 이러
- [0081] 한 기술은 AAV 벡터 카세트, AAV 헬퍼 구조물 및 기타 핵산 분자와 같은 하나 이

- [0083] 상의 외인성 DNA 일부를 적합한 숙주 세포 내로 도입하는데 사용될 수 있다.
- [0084] 본원에 사용된 용어 “교환 가능한 카세트” 또는 “카세트”는 유전자 및 재조합 부위를 함유하는 한 유형의 이동성 유전 요소이다. 일부 실시예에서, 교환 가능한 카세트는 2개 이상의 RTS를 포함한다. 일부 실시예에서, 교환 가능한 카세트는 리포터 유전자 또는 선택 유전자를 포함한다. 일부 실시예에서, 카세트는 재조합효소-매개 카세트-교환(RMCE)을 통해 교환된다.
- [0085] 본원에 언급된 바와 같이, 용어 “AAV 벡터 카세트”는 아데노-관련 바이러스 혈청형 벡터로부터 유래된 카세트를 지칭한다. AAV 벡터 카세트는 예를 들어, 전체 또는 부분적으로 결실된 하나 이상의 AAV 야생형 유전자를 가질 수 있고, 예를 들어, *Rep* 및/또는 *Cap* 유전자이며, 그러나 기능적 측면 반전 말단 반복부(ITR; inverted terminal repeat) 서열은 유지한다.
- [0086] 본 명세서에서 언급된 바와 같이, 용어 “기능적 측면 반전 말단 반복” 또는 “기능적 측면 ITR” 서열은 145 염기쌍 ITR을 지칭하고, 처음 125 염기쌍은 AAV 벡터 카세트에서 관심 유전자 (GOI)의 5' 및 3' 끝에 위치한 Y 또는 T 형 이중 구조를 형성할 수 있다. ITR 서열은 AAV 게놈의 복제, 구출(rescue), 패키징 및 통합에 필요한 최소 서열을 나타낸다.
- [0087] 본원에 언급된 바와 같이, 용어 “보조 유전자”, “헬퍼 유전자” 및 “보조 헬퍼 유전자”는 rAAV의 복제 또는 패키징을 돕는 제1 유전자를 지칭하도록 상호 교환 가능하게 사용된다. 일부 실시예에서, 헬퍼 유전자는 바이러스 유전자를 포함한다. 일부 실시예에서, 헬퍼 유전자는 Ad 유전자를 포함한다. 일부 실시예에서, 헬퍼 유전자는 AAV 유전자를 포함한다. 일부 실시예에서, 헬퍼 유전자는 AAV 유전자 *rep*, *cap* 또는 이들의 조합을 포함한다. 일부 실시예에서, 헬퍼 유전자는 원핵 생물 유전자를 포함한다. 일부 실시예에서, 헬퍼 유전자는 진핵 생물 유전자를 포함한다. 일부 실시예에서, 헬퍼 유전자는 RNA를 코딩하는 유전자를 포함한다. 일부 실시예에서, 헬퍼 유전자는 인터페론 (IFN) 길항제(antagonist)를 포함한다. 일부 실시예에서, 헬퍼 유전자는 단백질 키나제 R (PKR) 억제제인 단백질을 암호화한다. 일부 실시예에서, 헬퍼 유전자는 인플루엔자 NS1 단백질을 암호화하는 유전자를 포함한다. 일부 실시예에서, 헬퍼 유전자는 백신시아 바이러스(Vaccinia virus) E3L 단백질을 암호화하는 유전자를 포함하고, 예를 들어, de Vries et al, *Gene Ther.* 15:545-52, (2008)를 참조한다. 일부 실시예에서, 헬퍼 유전자는 감염된 세포에서 IFN 반응에 대응하는 바이러스-암호화된 가용성 IFN-알파/베타 수용체 디코이를 암호화한다. 일부 실시예에서, 헬퍼 유전자는 NS1, E3L, 또는 유사한 단백질을 인코딩한다. 일부 실시예에서, 헬퍼 유전자는 NS1, E3L 또는 상동성 서열 또는 모티프를 갖는 단백질을 암호화한다. 본원에서 언급된 바와 같이, 용어 “1 관심 유전자(gene of interest)” 또는 “GOI”는 이중 유전자를 기술하기 위해 사용된다. 본원에서 언급된 바와 같이, 용어 “이중 유전자(heterologous gene)” 또는 “HG”는 코딩 서열 또는 제어 서열과 같은 핵산 서열에 관한 것으로서 핵산 서열을 나타내고, 예를 들어 유전자1, 이는 정상적으로 함께 연결되지 않고, 및/또는 일반적으로 특정 세포와 관련되지 않는다. 일부 실시예에서, 이중 유전자는 코딩 서열 자체가 자연에서 발견되지 않는 구조물 (예를 들어, 본래의 유전자와 상이한 코돈을 갖는 합성 서열)이다. 대립 유전자 변이 또는 자연 발생 돌연변이 사건은 본원에 사용된 이중 DNA를 발생시키지 않는다. 일부 실시예에서, 관심 유전자는 리포터 유전자, 선택 유전자, 치료적 관심 유전자, 또는 이들의 조합을 포함한다.
- [0088] 본원에 언급된 바와 같이, “리포터 유전자”는 발현이 세포 상에 표현형을 부여하여 쉽게 식별 및 측정될 수 있는 유전자이다. 일부 실시예에서, 리포터 유전자는 형광 단백질 유전자를 포함한다. 일부 실시예에서, 리포터 유전자는 선택 유전자를 포함한다.
- [0089] 본원에 언급된 바와 같이, 용어 “선택 유전자”는 필수 영양소가 아닌 배지에서 성장하는 능력을 부여하는 효소 활성을 암호화하는 유전자의 사용을 지칭하고; 또한, 선택 유전자는 선택 유전자가 발현되는 세포에 항생제 또는 약물에 대한 내성을 부여할 수 있다. 선택 유전자는 숙주 세포 상에 특정 표현형을 부여하기 위해 사용될 수 있다. 숙주 세포가 선택 배지에서 성장하기 위해 선택 유전자를 발현해야 할 때, 유전자는 양성 선택 유전자라고 한다. 선택 유전자는 또한 특정 유전자를 함유하는 숙주세포를 선별하는데 사용될 수 있으며; 이러한 방식으로 사용된 선택 유전자는 음성 선택 유전자로 지칭된다.
- [0090] 본원에 언급된 바와 같이, 용어 “치료적 관심 유전자”는 기능적으로 관련된 임의의 뉴클레오티드 서열을 지칭한다. 따라서, 본 개시 내용의 치료적 관심 유전자는 표적 세포 게놈에서 결함 또는 결실된 단백질을 암호화하거나 또는 원하는 생물학적 또는 치료 효과를 갖는 비-천연 단백질을 암호화하는 임의의 원하는 유전자를 포함할 수 있고(예를 들어, 항 바이러스 기능), 또는 서열은 안티센스 또는 리보자임 기능을 갖는 분자에 해당할 수 있다. 적합한 치료적 관심 유전자의 대표적인(비-제한적인) 예는 염증성 질환, 자가 면역, 만성 및 감염성 질환, AIDS, 암, 신경계 질환, 심혈관 질환, 고콜레스테롤혈증을 포함하고; 다양한 빈혈, 지중해 빈혈 및 혈우병을 비

롯한 다양한 혈액 장애; 낭포성 섬유증, 고세병, 아데노신 탈아미노효소 결핍증(ADA), 폐기종 등과 같은 유전적 결함의 치료에 사용되는 것들을 포함한다. 암 및 바이러스 성 질환에 대한 안티센스 요법에 유용한 몇몇 안티센스 올리고뉴클

- [0091] 레오티드 (예를 들어, mRNA의 번역 개시 부위 (AUG 코돈) 주위의 서열에 상보적
- [0092] 인 짧은 올리고 뉴클레오티드)가 공지되어 있고 또한 치료적 관심 유전자의 적합한
- [0093] 예이다.
- [0094] 일부 실시예에서, 세포는 헬퍼 바이러스가 실질적으로 없다. 본 명세서에서 언급된 바와 같이, "헬퍼 바이러스"는 아데노-관련 바이러스의 복제 및 패키징을 가능하게 하기 위해 추가되는 AAV 이외의 바이러스이다. 헬퍼 바이러스의 대표적인(비-제한적인) 예는 아데노바이러스 및 헤르페스 바이러스이다. 일부 실시예에서, 헬퍼 바이러스가 실질적으로 없는 이라는 용어는 세포 당 100 미만, 10 미만, 또는 1 미만의 헬퍼 바이러스를 갖는 세포를 지칭한다. 일부 실시예에서, 헬퍼 바이러스가 실질적으로 없는 이라는 용어는 헬퍼 바이러스가 존재하지 않는 세포 또는 헬퍼 바이러스가 존재하지 않는 세포 집단을 지칭하며, 당업자에게 공지된 검출 방법을 사용한다. 일부 실시예에서, 세포에는 야생형-헬퍼 바이러스가 없다. 일부 실시예에서, 용어 야생형-바이러스는 임의의 다른 바이러스와 독립적으로 세포에서 복제될 수 있는 완전한-비-AAV 바이러스를 지칭한다.
- [0095] 일부 실시예에서, 본 개시 내용은 (i) 4개 이상의 별개의 재조합 표적 부위(RTS), (ii) 아데노바이러스 (Ad) 유전자 *E2A*, *E4*, *VA*, *MIR342* 또는 이들의 조합; 및 (iii) Ad 유전자에 작동 가능하게 연결된 프로모터를 포함하는 포유동물 세포를 제공하고, 상기 RTS, Ad 유전자 및 프로모터는 염색체로 통합된다. 일부 실시예에서, 세포는 마우스 세포, 인간 세포, CHO 세포, CHO-K1 세포, CHO-DXB11 세
- [0096] 포, CHO-DG44 세포, 모든 변이체(예를 들어, CHOK1SV™ POTELLIGENT®)를 포함하는 CHOK1SV™ 세포, 모든 변이체 (예를 들어, XCEED™)를 포함하는 CHOK1SV GS-KO™ 세포, 부착성 및 현탁액-적응형 변이체를 포함하는 HEK293
- [0097] 세포, HeLa 세포 또는 HT1080 세포이다.
- [0098] 일부 실시예에서, 세포는 4개의 RTS를 포함한다. 일부 실시예에서, 세포는 6개의 RTS를 포함한다. 일부 실시예에서, 적어도 하나의 RTS는 서열 번호 1-30으로부터 선택된다. 일부 실시예에서, RTS, Ad 유전자 및 프로모터는 단일 염색체 유전자 좌위에 통합된다. 일부 실시예에서, 염색체 유전자 좌위는 *Fer1L4*, *ROSA26*, *HGPRT*, *DHFR*, *COSMC*, *LDHa*, *MGAT1*, *GRIK1*, *NL1*, *NL2*, *NL3*, *NL4*, *NL5*, *NL6*, X 염색체에서 *MIDI*의 첫 번째 인트론, 또는 강화된 발현 및 안정성 영역(EESYR)이다. 일부 실시예에서, 세포는 부위-특이적 재조합 효소 유전자를 포함한다. 일부 실시예에서, 부위-특이적 재조합 효소 유전자는 염색체로 통합된다. 일부 실시예에서, 세포는 제2 Ad 유전자를 추가로 포함하며, 여기서 제2 Ad 유전자는 염색체로 통합된다. 일부 실시예에서, 제2 Ad 유전자는 *E1A*, *E1B*, *E2A*, *E4*, *VA*, *MIR342*, 또는 이들의 조합을 포함한다. 일부 실시예에서, 제2 Ad 유전자는 Ad5로부터 유래된다. 일부 실시예에서, 제2 Ad 유전자는 2개의 RTS 사이에 위치된다. 일부 실시예에서, 세포는 아데노-관련 바이러스 (AAV) 유전자를 추가로 포함하며, 여기서 AAV 유전자는 염색체로 통합된다. 일부 실시예에서, AAV 유전자는 *Rep*, *Cap*, 또는 이들의 조합을 포함한다. 일부 실시예에서, AAV 유전자는 아데노-관련 바이러스 유형 2로부터 유래된다. 일부 실시예에서, AAV 유전자는 2 개의 RTS 사이에 위치된다. 일부 실시예에서, 세포는 AAV 벡터 카세트를 추가로 포함하고, 상기 AAV 벡터 카세트는 염색체로 통합된다. 일부 실시예에서, AAV 벡터 카세트는 리포터 유전자, 선택 유전자, 치료적 관심 유전자 또는 이들의 조합이다. 일부 실시예에서, AAV 벡터 카세트는 2 개의 RTS 사이에 위치된다. 일부 실시예에서, 세포는 헬퍼 바이러스가 실질적으로 없다.
- [0099] 일부 실시예에서, 본 개시 내용은 (i) 적어도 4개의 별개의 재조합 표적 부위 (RTS); 및 (ii) Rep, Cap, 또는 이들의 조합을 포함하는 아데노-관련 바이러스(AAV) 유전자를 포함하는 포유동물 세포를 제공하고, 상기 RTS 및 AAV 유전자는 염색체로 통합된다. 일부 실시예에서, 세포는 마우스 세포, 인간 세포, CHO 세포, CHO-K1 세포, CHO-DXB11 세포, CHO-DG44 세포, 모든 변이체(예를 들어, CHOK1SV™ POTELLIGENT®)를 포함하는 CHOK1SV™ 세포, 모든 변이체를 포함하는 CHOK1SV GS-KO™ 세포, 부착성 및 현탁액-적응형 변이체를 포함하는 HEK293 세포, HeLa 세포, 또는 HT1080 세포이다. 일부 실시예에서, 세포는 4개의 RTS를 포함한다. 일부 실시예에서, 세포는 6 개의 RTS를 포함한다. 일부 실시예에서, 적어도 하나의 RTS는 서열 번호 1-30으로부터 선택된다. 일부 실시예에서, RTS 및 AAV 유전자는 단일 염색체 유전자 좌위에 통합된다. 일부 실시예에서, 염색체 유전자 좌위는 *Fer1L4*, *ROSA26*, *HGPRT*, *DHFR*, *COSMC*, *LDHa*, *MGAT1*, *GRIK1*, *NL1*, *NL2*, X 염색체에서 *MIDI* 첫 번째 인트론, 또

는 강화된 발현 및 안정성 영역(EESYR)이다. 일부 실시예에서, 세포는 부위-특이적 재조합 효소 유전자를 포함한다. 일부 실시예에서, 부위-특이적 재조합 효소 유전자는 염색체로 통합된다. 일부 실시예에서, 세포는 Ad 유전자 및 Ad 유전자에 작동 가능하게 연결된 프로모터를 추가로 포함하며, 이에 의해 Ad 유전자 및 프로모터는 염색체로 통합된다. 일부 실시예에서, Ad 유전자는 *E1A*, *E1B*, *E2A*, *E4*, *VA*, *MIR342* 또는 이들의 조합을 포함한다. 일부 실시예에서, Ad 유전자는 Ad5로부터 유래된다. 일부 실시예에서, AAV 유전자는 2개의 RTS 사이에 위치된다. 일부 실시예에서, 세포는 AAV 벡터 카세트를 추가로 포함한다. 일부 실시예에서, AAV 벡터 카세트는 리포터 유전자, 선택 유전자, 치료적 관심 유전자 또는 이들의 조합을 포함한다. 일부 실시예에서, 관심 유전자는 2개의 RTS 사이에 위치된다. 일부 실시예에서, 세포는 헬퍼 바이러스가 실질적으로 없다. 일부 실시예에서, 세포는 헬퍼 바이러스가 완전히 없다.

[0100] 일부 실시예에서, 본 개시 내용은 하나 이상의 RTS가 서열 번호 1-30으로 부터 선택되는 6개의 별개의 재조합 표적 부위 (RTS); *E1A* 및 *E1B*를 포함하는 아데노바이러스 (Ad) 유전자; RTS, Ad 유전자 및 프로모터가 염색체로 통합된 Ad 유전자에 작동 가능하게 연결된 프로모터; *E2A*, *E4*, *VA* 및 *MIR 342*를 포함하는 제2 Ad 유전자 (여기서, 제2 Ad 유전자는 2개의 RTS 사이에 위치함); Rep 및 Cap을 포함하는 아데노-관련 바이러스 (AAV) 유전자로, 상기 AAV 유전자는 2개의 RTS 사이에 위치하며; 및 리포터 유전자, 선택 유전자 또는 치료적 관심 유전자를 포함하는 AAV 벡터 카세트를 포함하고, AAV 벡터 카세트는 2개의 RTS 사이에 위치하는 것을 포함하는 중국 햄스터 난소 (CHO) 세포를 제공한다.

[0101] 일부 실시예에서, 본 개시 내용은 *E1A* 및 *E1B*를 포함하는 아데노바이러스(Ad) 유전자 및 Ad 유전자에 작동 가능하게 연결된 프로모터, Rep 및 Cap을 포함하는 아데노-관련 바이러스 (AAV) 유전자인 *E2A*, *E4*, *VA* 및 *MIR 342*를 포함하는 제2 Ad 유전자; 및 리포터 유전자, 선택 유전자, 치료적 관심 유전자 또는 이들의 조합을 포함하는 AAV 벡터 카세트를 포함하는 중국 햄스터 난소 (CHO) 세포를 제공한다.

[0102] 일부 실시예에서, 본 개시 내용은 4개 이상의 별개의 재조합 표적 부위(RTS), *E1A*, *E1B*, 또는 이들의 조합을 포함하는 아데노바이러스 (Ad) 유전자, 및 Ad 유전자에 작동 가능하게 연결된 프로모터로, 상기 RTS, Ad 유전자 및 프로모터는 염색체로 통합되는 세포를 제공하는 단계, 아데노-관련 바이러스 (AAV) 유전자, 제2 Ad 유전자, AAV 벡터 카세트, 또는 이들의 조합을 암호화하는 교환 가능한 카세트를 포함하는 벡터가 제공된 세포의 형질주입 단계, 교환 가능한 카세트를 염색체에 통합시키는 단계, 및 교환 가능한 카세트를 염색체에 통합하여 rAAV 생산자 세포를 선택하는 단계를 포함하는 재조합 아데노-관련 바이러스 (rAAV) 생산자 세포의 제조 방법을 제공한다. 일부 실시예에서, 형질주입은 2개의 벡터에 의하고, 제1 벡터는 제2 Ad 유전자 및 AAV 유전자를 포함하는 교환 가능한 카세트를 포함하고, 제2 벡터는 AAV 벡터 카세트를 포함하는 교환 가능한 카세트를 포함한다. 일부 실시예에서, 형질 감염은 2개의 벡터로 이루어지며, 제1 벡터는 제2 Ad 유전자를 포함하는 교환 가능한 카세트를 포함하고, 제2 벡터는 AAV 유전자를 포함하는 교환 가능한 카세트를 포함한다. 일부 실시예에서, 형질 감염은 3개의 벡터를 사용하며, 제1 벡터는 제2 Ad 유전자를 포함하는 교환 가능한 카세트를 포함하고, 제2 벡터는 AAV 유전자를 포함하는 교환 가능한 카세트를 포함하고, 제3 벡터는 AAV 벡터 카세트를 포함하는 교환 가능한 카세트를 포함한다. 일부 실시예에서, 각각의 교환 가능한 카세트는 세포의 RTS 중 2개와 일치하는 2개의 RTS를 추가로 포함한다. 일부 실시예에서, 본 발명자들은 SSI의 사용은 세포가 유전자 조성이 상동(homogenous)하기 때문에 형질주입된 세포로부터 세포를 클로닝할 필요가 없다는 것을 발견하였다.

[0103] 용어 "일치(matching)"는 2개의 RTS 서열과 관련하여 재조합 효소에 의해 결합될 수 있고 2개의 서열 사이의 부위-특이적 재조합에 영향을 줄 수 있는 능력을 갖는 2개의 서열을 지칭한다. 일부 실시예에서, 세포의 RTS와 일치하는 교환 가능한 카세트의 RTS는 세포의 RTS와 실질적으로 동일한 서열을 갖는 카세트의 RTS를 지칭한다. 일부 실시예에서, 교환 가능한 카세트는 세포에 염색체로 통합된 RTS 중 하나 또는 둘과 실질적으로 동일한 서열을 함유한다.

[0104] 일부 실시예에서, 통합(integrating) 이라는 용어는 통합(integration)을 지칭하고, 예를 들어, 염색체 내로 교환 가능한 카세트의 삽입이다. 일부 실시예에서, 통합은 부위-특이적 재조합에 의해 매개된다.

[0105] 일부 실시예에서, 용어 "선택(selecting)"은 염색체로 통합된 마커를 함유하는 세포를 식별하는 것을 지칭한다. 일부 실시예에서, 선택은 당업자에게 공지된 방법을 사용하여 마커의 존재의 검출을 통해 이루어진다. 일부 실시예에서, 선택은 당업자에게 공지된 방법을 사용하여 마커의 부재의 검출을 통해 이루어진다.

[0106] 일부 실시예에서, 제2 Ad 유전자는 *E1A*, *E1B*, *E2A*, *E4*, *VA*, *MIR342*, 또는 이들의 조합을 포함한다. 일부 실시예에서, AAV 유전자는 Rep, Cap, 또는 이들의 조합이다. 일부 실시예에서, AAV 벡터 카세트는 리포터 유전자, 선택 유전자, 치료적 관심 유전자 또는 이들의 조합을 포함한다.

- [0107] 일부 실시예에서, 본 개시 내용은 (i) 숙주 세포를 AAV로 감염시키는 단계, (ii) AAV 벡터 카세트에 패키징된 rAAV를 생성하는 단계, 및 (iii) 패키징된 rAAV를 정제하는 단계를 포함하고, 상기 숙주 세포는 적어도 4개의 별개의 제조함 표적 부위 (RTS), *E1A*, *E1B* 또는 이들의 조합을 포함하는 아데노바이러스 (Ad) 유전자, Ad 유전자에 작동 가능하게 연결된 프로모터를 포함하고, 상기 RTS, Ad 유전자 및 프로모터는 염색체로 통합되고, *E1A*, *E1B*, *E2A*, *E4*, *VA*, *MIR342*, 또는 이들의 조합을 포함하는 제2 Ad 유전자, *Rep*, *Cap*, 또는 이들의 조합을 포함하는 아데노-관련 바이러스 (AAV) 유전자, 및 리포터 유전자, 선택 유전자, 치료적 관심 유전자 또는 이들의 조합을 포함하는 AAV 벡터 카세트를 포함하는 제조함 아데노-관련 바이러스 (rAAV)의 제조 방법을 제공한다. 일부 실시예에서, rAAV 생산 및 패키징에는 살아있는 야생형 헬퍼 바이러스가 필요하지 않다. 일부 실시예에서, E1A의 발현은 rAAV 생산에 필요하지 않다. 일부 실시예에서, 정제 후 최소 1.0×10^9 vg/mL 활성 rAAV가 수득된다. 본 개시 내용의 일부 실시예에서, rAAV는 복제 경쟁 아데노바이러스 (RCA replication competent adenovirus)가 실질적으로 없는 상태로 얻어진다. 예를 들어, 본 개시 내용의 일부 실시예에서, RCA는 정제 전 검출 한계 미만이다. 바이러스 입자의 정량 방법은 당업자에게 공지되어 있으며, 정량적 중합 효소 연쇄 반응 (qPCR) 분석, 감염 적정 분석, 종말점 회색 분석 및 바이러스성 플라크 분석을 포함할 수 있다. 일부 실시예에서, 본 발명자들은 rAAV 생산자 세포를 제조하기 위한 SSI의 사용이 생산자 세포의 풀이 그의 유전자 구성에서 균질하다는 것을 보장한다는 것을 발견하였다. 일부 실시예에서, 본 발명자들은 rAAV 생산자 세포를 제조하기 위해 SSI의 사용이 생산자 세포의 풀이 그의 효율이 균질하다는 것을 보장한다는 것을 발견하였다. 일부 실시예에서, 본 발명자들은 rAAV 생산자 세포를 제조하기 위한 SSI의 사용이 생산자 세포의 풀이 제1 헬퍼 유전자 대 제2 헬퍼 유전자의 배급(ration)에서 균질하다는 것을 보장한다는 것을 발견하였다. 일부 실시예에서, 본 발명자들은 rAAV 생산자 세포를 제조하기 위한 SSI의 사용이 생산자 세포의 풀이 헬퍼 유전자 대 치료적 관심 유전자의 비에서 균질하다는 것을 발견하였다. 일부 실시예에서, 본 발명자들은 rAAV 생산자 세포를 제조하기 위해 SSI의 사용이보다 일관된 rAAV 생산물 품질을 보장한다는 것을 발견하였다.
- [0108] 본원에 사용된 "아미노산"은 카르복실(-COOH) 및 아미노(-NH₂)기를 모두 함유하는 화합물을 지칭한다. "아미노산"은 천연 및 비천연, 즉 합성 아미노산을 모두 지칭한다. 3 글자 및 단일 문자 약어를 갖는 천연 아미노산은 알라닌 (Ala; A); 아르기닌 (Arg, R); 아스파라긴 (Asn; N); 아스파르트산 (Asp; D); 시스테인 (Cys; C); 글루타민 (Gln; Q); 글루탐산 (Glu; E); 글리신 (Gly; G); 히스티딘 (His; H); 이소류신 (Ile; I); 류신 (Leu; L); 라이신 (Lys; K); 메티오닌 (Met; M); 페닐알라닌(Phe; F); 프롤린 (Pro; P); 세린 (Ser; S); 트레오닌 (Thr; T); 트립토판 (Trp; W); 티로신 (Tyr; Y); 및 발린 (Val; V)을 포함한다.
- [0109] 용어 "펩티드", "폴리펩티드" 및 "단백질"은 본원에서 상호 교환적으로 사용되며, 화학적 또는 생화학적으로 변형되거나 유도체화된 아미노산을 코딩하고 코딩되지 않은 아미노산을 포함할 수 있는 임의의 길이의 아미노산의 중합체 형태, 및 변형된 펩티드 골격을 갖는 폴리펩티드를 지칭한다.
- [0110] 원핵 생물 및/또는 진핵 생물 세포주를 포함하는 원하는 세포주는 본원에 기술된 임의의 적합한 장치, 시설 및 방법을 사용하여 배양될 수 있다. 또한, 실시예에서, 장치, 시설 및 방법은 현탁 세포 또는 고정-의존성 (점착성) 세포를 배양하기에 적합하고, 제약 및 생물약제 제품, 예컨대 폴리펩티드 제품, 핵산 제품 (예를 들어 DNA 또는 RNA), 또는 포유동물 또는 미생물 세포 및/또는 바이러스, 예를 들어 세포 및/또는 바이러스 및 미생물 요법에 사용되는 바이러스를 생산하기에 적합하다.
- [0111] 일부 실시예에서, 세포는 제조함 치료 또는 진단 제품과 같은 제품을 발현하거나 생산한다. 아래에 보다 상세히 기술된 바와 같이, 세포에 의해 생산된 제품의 예는 다음을 포함하고, 이에 제한되지는 않지만, 항체 분자 (예를 들어, 단일 클론 항체, 이중 특이적 항체), 항체 모방체 (항원에 특이적으로 결합하지만 항체와 구조적으로 관련되지 않은 폴리펩타이드 분자, 예를 들어 DARPin, 애피바디(affibodies), 아드넥틴, 또는 IgNAR), 융합 단백질 (예를 들어, Fc 융합 단백질, 키메라 사이토카인), 다른 제조함 단백질 (예를 들어, 글리코실화된 단백질, 효소, 호르몬), 바이러스 치료제 (예를 들어, 항암 종양방괴성(oncolytic) 바이러스, 유전자 치료를 위한 바이러스 벡터 및 바이러스 면역 치료), 세포 치료제 (예를 들어, 다능성 줄기 세포, 중간엽 줄기 세포 및 성체 줄기 세포), 백신 또는 지질 캡슐화된 입자 (예를 들어, 엑소좀, 바이러스 유사 입자), RNA (예를 들어, siRNA) 또는 DNA (예를 들어, 플라스미드 DNA), 항생제 또는 아미노산을 포함한다. 실시예에서, 장치, 시설 및 방법은 바이오시밀러를 생산하는데 사용될 수 있다.
- [0112] 언급한 바와 같이, 실시예에서, 장치, 시설 및 방법은 진핵 세포, 예를 들어 포유 동물 세포 또는 하부 진핵 세포, 예를 들어 효모세포 또는 사상 진균 세포, 또는 원핵 세포, 예컨대 그람 양성 또는 그람 음성 세포 및/또는 진핵 세포 또는 원핵 세포의 생성물, 예를 들어, 진핵 세포에 의해 합성된 단백질, 펩티드, 항생제, 아미노

산, 핵산 (예를 들어, DNA 또는 RNA) 대규모로 생산하는 것을 허용한다. 일부 실시예에서, 미생물 치료요법에 사용되는 미생물 유기체 및 이의 포자의 사용이

- [0113] 또한 개시되어있다. 본 명세서에서 달리 언급되지 않는 한, 장치, 설비 및 방법은
- [0114] 벤치-규모, 파일럿-규모 및 전체 생산 규모 용량을 포함하지만 이에 제한되지 않는 임의의 원하는 부피 또는 생산 능력을 포함할 수 있다.
- [0115] 또한, 본 명세서에서 달리 언급되지 않는 한, 장치, 설비 및 방법은 제한되는 것은 아니지만, 임의의 적합한 반응기(들)를 포함하고 교반 탱크, 에어리프트, 섬유, 마이크로 섬유, 중공섬유, 세라믹 매트릭스, 무동층, 고정층 및/또는 스파우트층 생물 반응기를 포함한다. 본원에 사용된 "반응기"는 발효기 또는 발효 유닛, 또는 임의의 다른 반응 용기를 포함할 수 있으며, "반응기"라는 용어는 "발효기"와 상호 교환적으로 사용된다. 발효기 또는 발효라는 용어는 미생물 및 포유동물 배양 둘 다를 지칭한다. 예를 들어, 일부 측면에서, 예시적인 생물 반응기 유닛은 다음 중 하나 이상 또는 전부를 수행할 수 있다: 영양소 및/또는 탄소 공급원 공급, 적합한 기체 (예: 산소) 주입, 발효 또는 세포 배양 배지의 유입 및 배출 흐름, 가스 및 액체상의 분리, 온도 유지, 산소 및 CO2 수준 유지, pH 수준, 교반(agitation) (예: 교반(stirring)) 및/또는 세척/살균을 수행할 수 있다. 발효 유닛과 같은 예시적인 반응기 유닛은 유닛내에 다수의 반응기를 함유할 수 있고, 예를 들어, 장치는 각각 1, 2, 3, 4, 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 60, 70, 80, 90 또는 100개 이상의 생물 반응기를 가질 수 있고 및/또는 설비는 설비 내에 단일 또는 다수의 반응기를 갖는 다수의 유닛을 포함할 수 있다. 다양한 실시예에서, 생물 반응기는 बै치(batch), 반유가식(semi fed-batch), 유가식, 관류, 및/또는 연속 발효 공정에 적합할 수 있다.
- [0116] 임의의 적합한 반응기 직경이 사용될 수 있다. 실시예에서, 생물 반응기는 약 100mL 내지 약 50,000L의 부피를 가질 수 있다. 비-제한적인 예는 100mL, 250mL, 500 mL, 750 mL, 1 리터, 2 리터, 3 리터, 4 리터의 부피, 5 리터, 6 리터, 7 리터, 8 리터, 9 리터, 10 리터, 15 리터, 20 리터, 25 리터, 30 리터, 40 리터, 50 리터, 60 리터, 70 리터, 80 리터, 90 리터, 100 리터, 150 리터, 200 리터, 250 리터, 300 리터, 350 리터, 400 리터, 450 리터, 500 리터, 550 리터, 600 리터, 650 리터, 700 리터, 750 리터, 800 리터, 850 리터, 900 리터, 950 리터, 1000 리터, 1500 리터, 2000 리터, 2500 리터, 3000 리터, 3500 리터, 4000 리터, 4 500 리터, 5000 리터, 6000 리터, 7000 리터, 8000 리터, 9000 리터, 10,000 리터, 15,000 리터, 20,000 리터, 및/또는 50,000 리터를 포함한다. 또한, 적합한 반응기는 다용도, 일회용, 일회용 또는 비-일회용일 수 있으며 다음을 포함하는 임의의 적합한 재료로 형성될 수 있고, 스테인레스 스틸 (예: 316L 또는 기타 적합한 스테인레스 스틸) 및 인코넬, 플라스틱 및/또는 유리와 같은 금속 합금을 포함한다.
- [0117] 본 명세서에서 달리 언급되지 않는 한, 실시예에서, 본 명세서에 기술된 장치, 설비 및 방법은 달리 언급되지 않은 생성물의 분리, 정제, 및 격리를 위한 작동 및/또는 장비와 같은 임의의 적절한 유닛 작동 및/또는 장비를 포함할 수 있다. 전통적인 스틱-빌트 시설, 모듈식, 이동 및 임시 시설, 또는 다른 적합한 구조물, 시설 및/또는 레이아웃과 같은 임의의 적합한 시설 및 환경이 사용될 수 있다. 예를 들어, 일부 실시예에서 모듈식 청소된-방이 사용될 수 있다. 추가로 그리고 달리 언급되지 않는 한, 본원에 기술된 장치, 시스템 및 방법은 단일 위치 또는 시설에서 수용 및/또는 수행될 수 있거나, 또는 별개의 또는 다수의 위치 및/또는 시설에서 수용 및/또는 수행될 수 있다.
- [0118] 비-제한적인 예 및 제한없는 예로써, 미국 출원 공개번호 2013/0280797; 2012/0077429; 2011/0280797; 2009/0305626; 및 미국 특허 번호 8,298,054; 7,629,167; 및 5,656,491, 여기에 그 전체 내용이 참조로 포함되며, 적합한 시설, 장비 및/또는 시스템의 예를 설명한다.
- [0119] 실시예에서, 세포는 진핵 세포, 예를 들어 포유동물 세포이다. 포유동물 세포는 예를 들어 인간 또는 설치류 또는 소 세포주 또는 세포 균주일 수 있다. 이러한 세포, 세포주 또는 세포 균주의 예는 예를 들어, 마우스 골수종(NSO)-세포주, 중국 햄스터 난소 (CHO)-세포주, HT1080, H9, HepG2, MCF7, MDBK Jurkat, NIH3T3, PC12, BHK (새끼 햄스터 신장 세포), VERO, SP2/0, YB2/0, YO, C127, L 세포, COS, 예를 들어, COS1 및 COS7, QC1-3, HEK293, VERO, PER.C6, HeLa, EB1, EB2, EB3, 중앙봉괴성 또는 하이브리도마-세포주이다. 바람직하게는 포유동물 세포는 CHO 세포주이다. 일 실시예에서, 세포는 CHO 세포이다. 일 실시예에서, 세포는 CHO-K1 세포, CHOK1SV™ 세포, DG44 CHO 세포, DUXB11 CHO 세포, CHOS, CHO GS 녹-아웃 세포, CHO FUT8 GS 녹-아웃 세포, CHOZN 또는 CHO 유래 세포이다. CHO GS 녹-아웃 세포 (예를 들어, GSKO 세포)는 예를 들어 CHOK1SV GS-KO (Lonza's Xceed®, Expression System, Lonza, Slough, UK의 일부)이다. CHO FUT8 녹아웃 세포는 예를 들어 CHOK1SV™ Potelligent® (Lonza, Slough, UK)이다. 진핵 세포는 또한 조류(avian) 세포, 세포주 또는 세포 균주, 예를

들어 EBx® 세포, EB14, EB24, EB26, EB66 또는 EBv13일 수 있다.

- [0120] 일부 실시예에서, 진핵 세포는 줄기 세포이다. 줄기 세포는 예를 들어, 배아 줄기 세포 (ESC), 성체 줄기 세포, 유도된 다능성 줄기 세포 (iPSC), 조직 특이적 줄기 세포 (예를 들어, 조혈 줄기 세포) 및 중간엽 줄기 세포 (MSC)를 포함하는 다능성 줄기 세포일 수 있다.
- [0121] 일 실시예에서, 세포는 본원에 기술된 임의의 세포의 분화된 형태이다. 일 실시예에서, 세포는 배양에서 임의의 1차 세포로부터 유래된 세포이다. 일부 실시예에서, 세포는 줄기 세포로부터 유래되지 않는다. 예를 들어, 일부 실시예에서, 세포는 개별 환자로부터 또는 확립된 세포 은행으로부터 추출되고 단리된 면역 요법(예를 들어, 림프구)에 사용된다. 일부 실시예에서, 세포는 유전적으로 조작된 세포(즉, CAR-T 등)를 포함할 수 있다.
- [0122] 실시예에서, 세포는 간세포, 예컨대 인간 간세포, 동물 간세포 또는 비-조직질실(parenchymal) 세포이다. 예를 들어, 세포는 판형의(plateable) 대사 가능한 인간 간세포, 판형 유도 적격의 (qualified) 인간 간세포, plateable Qualyst Transporter Certified™ 인간 간세포, 현탁액 적격의 인간 간세포 (10-기증자 및 20-기증자 풀 간세포 포함), 인간 간 쿠퍼 세포, 인간 간 정상 세포, 개 간세포 (단일 및 합동된(pooled) 비글 간세포 포함), 마우스 간세포 (CD-1 및 C57BI/6 간세포 포함), 쥐 간세포 (Sprague-Dawley, Wistar Han 및 Wistar 간세포 포함), 원숭이 간세포 (사이노몰구스 또는 붉은 털 원숭이 간세포 포함), 고양이 간세포 (국내쇼트헤어 간세포 포함), 및 토끼 간세포 (뉴질랜드 화이트 간세포 포함)일 수 있다. 예시적인 간세포는 미국 노스 캐롤라이나 주 27709의 6 Davis Drive Research Triangle Park의 Triangle Research Labs, LLC로부터 시판되고 있다.
- [0123] 일 실시예에서 진핵 세포는 예를 들어, 효모 세포(예: 피키아(*Pichia*) 속(예: 피키아 파스토리스(*Pichia pastoris*), 피키아 메타놀리카(*Pichia methanolica*), 피키아 클루이베리(*Pichia kluyveri*), 및 피키아 안구스타(*Pichia angusta*)), 코마가타엘라(*Komagataella*) 속(예: 코마가타엘라 파스토리스(*Komagataella pastoris*), 코마가타엘라 슈도파스토리스(*Komagataella pseudopastoris*) 또는 코마가타엘라 파피(*Komagataella phaffii*)), 사카로마이세스(*Saccharomyces*) 속(예: 사카로마이세스 세레비지에 (*Saccharomyces cerevisiae*), 사카로 마이세스 클루이베리(*Saccharomyces kluyveri*), 사카로마이세스 우바룸 (*Saccharomyces uvarum*)), 클루이베로마이세스(*Kluyveromyces*) 속(예: 클루이베로마이세스 락티스 (*Kluyveromyces lactis*), 클루이베로마이세스 막시아누스 (*Kluyveromyces marxianus*)), 칸디다(*Candida*) 속(예: 칸디다 우틸리스(*Candida utilis*), 칸디다 카카오이 (*Candida cacaoi*), 칸디다 보이디니 (*Candida boidinii*)), 게오트리쿰 (*Geotrichum*) 속(예: 게오트리쿰 퍼멘탄스 (*Geotrichum fermentans*)), 한세놀라 폴리모르파(*Hansenula polymorpha*), 야로위아 리폴리티카 (*Yarrowia lipolytica*), 또는 쉬조사카로마이세스 폼브 (*Schizosaccharomyces pombe*)와 같은 하류 진핵 세포이다. 바람직하게는 피키아 파스토리스이다. 피키아 파스토리스 균주의 예는 X33, GS115, KM71, KM71H; 및 CBS7435이다.
- [0124] 일 실시예에서, 진핵 세포는 진균 (fungal) 세포(예: 아스퍼질러스 (*Aspergillus*) (아스퍼질러스 니게르 (*A. niger*), 아스퍼질러스 푸미가투스(*A. fumigatus*), 아스퍼질러스 오리재 (*A. oryzae*), 아스퍼질러스 니둘라(*A. nidula*)와 같은), 아크레모늄(*Acremonium*)(예: 아크레모늄 써모필리움(*A. thermophilum*)과 같은), 카에토미움 (*Chaetomium*)(예: 카에토미움 써모필리움(*C. thermophilum*)과 같은), 크리소스포리움(*Chrysosporium*)(예: 크리소스포리움 써모필(*C. thermophile*)과 같은), 동충하초(*Cordyceps*)(예: 번데기동충하초(*C. militaris*)와 같은), 코리나스쿠스 (*Corynascus*), 씨테노마이세스 (*Ctenomyces*), 푸사리움 (*Fusarium*) (예: 푸사리 움 옥시스포룸(*F. oxysporum*)과 같은), 글로메렐라(*Glomerella*)(글로메렐라 그라미니콜라(*G. graminicola*)와 같은), 하이포크레아(*Hypocrea*(하이포크레아 제코리나(*H. jecorina*)와 같은), 마그나포르테(*Magnaporthe*)(마그나포르테 오리재 (*M. oryzae*)와 같은), 마이셀리오프쏘라 (*Myceliophthora*)(마이셀리오프쏘라 써모필(*M. thermophile*)와 같은), 넥트리아(*Nectria*) (넥트리아 헤마토코코아(*N. heamatococca*)와 같은), 뉴로스포라(*Neurospora*)(뉴로스포라 크라싸(*N. crassa*)와 같은), 페니실리움(*Penicillium*), 스포로트리쿰(*Sporotrichum*)(스포로트리쿰 써모필(*S. thermophile*)과 같은), 티엘라비아(*Thielavia*)(티엘라비아 테르스트리스(*T. terrestris*)와 같은), 티엘라비아 헤테로할리카(*heteroyhallica*)와 같은), 트리코더마(*Trichoderma*)(트리코더마 레세이 (*T. reesei*)와 같은), 또는 버티실룸(*Verticillium*) (버티실룸 달리아(*V. dahlia*)와 같은))이다.
- [0125] 일 실시예에서, 진핵 세포는 곤충 세포(예: Sf9, Mimic™ Sf9, Sf21, High Five™(BT1-TN-5B1-4), 또는 BT1-Ea88 세포), 조류 (algae) 세포(예: 암포라(Amphora), 규조류(*Bacillariophyceae*), 두날리엘라(*Dunaliella*, *Chlorella*), 클라미도모나스(*Chlamydomonas*), 시아노피타 (Cyanophyta, 시아노박테리아), 난노클로롭시스 (Nannochloropsis), 스피루리나(Spirulina), 또는 오크로모나스(*Ochromonas*)속의), 또는 식물 세포(예: 단자엽 식물 유래의(예: 옥수수, 쌀, 밀, 또는 강아지풀(*Setaria*)), 또는 쌍떡잎 식물 유래의(예: 카사바(cassava), 감

자, 대두, 토마토, 담배, 자주개자리(alfalfa), 이끼(*Physcomitrella patens*) 또는 애기장대(*Arabidopsis*)이다.

[0126] 일 실시예에서, 세포는 박테리아 또는 원핵 세포이다. 일부 실시예에서, 원핵 세포는 바실러스(*Bacillus*), 스트렙토마이세스 스트렙토코커스(*Streptomyces Streptococcus*), 스태필로코커스(*Staphylococcus*) 또는 락토바실러스(*Lactobacillus*)와 같은 그람-양성 세포이다. 사용될 수 있는 바실러스는 예를 들어 바실러스 서브틸리스(*B. subtilis*), 바실러스 아밀로리퀴파시엔스(*B. amyloliquefaciens*), 바실러스 리체니포르미스(*B. licheniformis*), 바실러스 나토(*B. natto*) 또는 바실러스 메가테리움(*B. megaterium*)이다. 실시예에서, 세포는 바실러스 서브틸리스, 예컨대 바실러스 서브틸리스 3NA 및 바실러스 서브틸리스 168이다. 바실러스는 예를 들어, *Bacillus* Genetic Stock Center, Biological Sciences 556, 484 West 12th Avenue, Columbus OH 43210-1214에서 얻을 수 있다.

[0127] 일 실시예에서, 원핵 세포는 살모넬라 종(*Salmonella spp.*) 또는 대장균(*Escherichia coli*)와 같은 그람 음성 세포이고, 예를 들어, TG1, TG2, W3110, DH1, DHB4, DH5a, HMS 174, HMS174 (DE3), NM533, C600, HB101, JM109, MC4100, XL1-Blue 및 Origami와 같고, 예를 들어, BL-21 또는 BL21 (DE3)과 같은 대장균 B-군주 유래뿐만 아니라, 이들은 모두 시판되고 있다. 적합한 숙주 세포는 예를 들어, DSMZ (Deutsche Sammlung von Mikroorganismen and Zellkulturen GmbH, Braunschweig, Germany) 또는 American Type Culture Collection (ATCC)과 같은 배양물 수집처로부터 상업적으로 입수할 수 있다. 일부 실시예에서, 세포는 치료제로서 사용되는 다른 미생물 총(microbiota)을 포함한다. 여기에는 필라 후벽균(Firmicutes), 의간균류(*Bacteroidetes*), 프로테오박테리아, 베루미크로미아(*Verrumicrobia*), 방선균류(*Actinobacteria*), 푸소박테리움균류(*Fusobacteria*) 및 시아노박테리아에 속하는 인간 마이크로바이옴(microbiome)에 존재하는 미생물 총이 포함된다. 미생물총은 호기성, 엄격한 혐기성 또는 기능성 혐기성 둘 다를 포함할 수 있고 세포 또는 포자를 포함할 수 있다. 치료적 미생물 총은 또한 유전자 조작 유기체 및 그의 변형에 이용된 벡터를 포함할 수 있다. 다른 마이크로바이옴-관련 치료적 유기체는 고세균, 진균 및 바이러스를 포함할 수 있다. 예를 들어, The Human Microbiome Project Consortium. *Nature* 486, 207-214 (14 June 2012); Weinstock, *Nature*, 489 (7415): 250-256 (2012); Lloyd-Price, *Genome Medicine* 8:51 (2016)를 참고한다.

[0128] 일부 실시예에서, 배양된 세포는 치료 용도를 위해 단백질, 예를 들어 항체, 예를 들어 모노클로날 항체 및/또는 재조합 단백질을 생성하는데 사용된다. 실시예에서, 배양된 세포는 펩티드, 아미노산, 지방산 또는 다른 유용한 생화학 중간체 또는 대사산물을 생성한다. 예를 들어, 실시예에서, 약 4000 달톤 내지 약 140,000 달톤 초과 분자량을 갖는 분자가 생성될 수 있다. 실시예에서, 이들 분자는 다양한 복잡성을 가질 수 있고 글리코실화를 포함하는 번역-후 변형을 포함할 수 있다.

[0129] 실시예에서, 단백질은, 예를 들어, 보톡스, 마이오블록(Myobloc), 뉴로블록(Neurobloc), 디스포트(Dysport) (또는 보툴리눔 뉴로톡신의 다른 혈청형), 알글루코시다제 알파(alglucosidase alpha), 닙토마이신(daptomycin), YH-16, 코리오고나 도트로핀 알파(choriogonadotropin alpha), 필그라스티움(filgrastim), 세트로렐릭스(cetrorelix), 인터류킨-2, 알데스류킨(aldesleukin), 테셀류틴(teceleulin), 데니류킨 디프티톡스(denileukin diftotox), 인터페론 알파-n3(주입), 인터페론 알파-n1, DL-8234, 인터페론, 설토리(감마-1a), 인터페론 감마, 티모신 알파 1, 타소너민(tasonermin), DigiFab, 비페라랩 (ViperaTAB), 에치랩(EchiTAB), 크로랩(CroFab), 네시리타이드 (nesiritide), 아바타셉트 (abatacept), 알레파셉트 (alefacept), 레비프 (Rebif), 엡토터미날파(eptoterminalfa), 테리파라타이드(teriparatide) (골다공증), 칼시토닌 주사용(뼈질환), 칼시토닌(비강, 골다공증), 에타너셉트(etanercept), 헤모글로빈 글루타머(glutamer) 250 (소), 드로트레코진 알파, 콜라게나제, 카르페리티드, 재조합 인간 표피 성장 인자(국소 겔, 상처 치유), DWP401, 달베포이틴 알파, 에포에틴 오메가, 에포에틴 베타, 에포에틴 알파, 데시루딘, 레피루딘, 비발리 루딘, 노나코그 알파, 모노닌, 엡타코그(eptacog) 알파 (활성화된), 재조합 인자(recombinant Factor) VIII+VWF, 리콤비네이트(Recombinate) VIII, 인자 VIII (재조합체), 알픈메이트(Alphnmate), 옥토코그 알파, 인자 VIII, 팔리퍼민(palifermin), 인디키나제(Indikinase), 테넥테플라제(tenecteplase), 알테플라제(alteplase), 파미테플라제(pamiteplase), 레테플라제 (reteplase), 나테플라제 (nateplase), 몬테플라제(montepilase), 폴리트로핀(follitropin) 알파, rFSH, hpFSH, 미카펙긴(micafungin), 페그필그라스티움 (pegfilgrastim), 레노그라스티움(lenograstim), 나르토그라스티움 (nartograstim), 세르모레린(sermorelin), 글루카곤, 엑세나타이드, 프람린타이드, 이니글루세라제 (iniglucerase), 갈설투라제 (galsulfase), 류코트로핀 (Leucotropin), 몰그라모스티르(molgramostirn), 트립토렐린 아세테이트 (triptorelin acetate), 히스트렐린 (histrelin) (피하 이식, Hydron), 데슬로렐린(deslorelin), 히스트렐린(histrelin), 나파렐린 (nafarelin), 류프롤리드 서방형 저장소

(leuprolide sustained release depot)(ATRIGEL), 뉴프롤리드 이식 (DUROS), 고세렐린(goserelin), 유트로핀 (Eutropin), KP-102 프로그램, 소마트로핀(somatropin), 메카세르민(mecasermin) (성장 실패), 엔파비르타이드 (enfavirtide), Org-33408, 인슐린 글라진(glargine), 인슐린 글루리신(glulisine), 인슐린(흡입), 인슐린 리스프로(lispro), 인슐린 디터니르(deternir), 인슐린 (구강, 래피드미스트 흡입기(RapidMist)), 메카세르민 린파베이트(mecasermin rinfabate), 아나킨라(anakinra), 셀모류킨(celmoleukin), 99 mTc-알시타이드(alpcitide) 주사, 미엘로피드 (myelopid), 베타세론 (Betaseron), 글라티라머 아세테이트 (glatiramer acetate), 제폰 (Gepon), 사그라모스팀(sargramostim), 오펠베킨(oprelvekin), 인간 백혈구-유래 알파 인터페론, 빌리브 (Bilive), 인슐린 (재조합체), 재조합 인간 인슐린, 인슐린 아스파르트(aspart), 메카세닌(mecasenin), 로페론 (Roferon)-A, 인터페론-알파 2, 알파페론(Alfaferone), 인터페론 알파콘(alfacon)-1, 인터페론 알파, 아보넥스 (Avonex)의 재조합 인간 루테인화 호르몬, 도르나제(dornase) 알파, 트라페민(trafermin), 지코노타이드 (ziconotide), 탈티렐린 (taltirelin), 디보터미날파(diboterminalfa), 아토시반 (atosiban), 베카플러민 (becaplermin), 엡티파타이드(eptifibatide), 제마이라(Zemaira), CTC-111, 샨박(Shanvac)-B, HPV 백신 (4가), 옥트레오타이드(octreotide), 란레오타이드(lanreotide), 안세스틴 (ancestirn), 아갈시다제 (agalsidase) 베타, 아갈시다제 알파, 라로니다제(laronidase), 프레자타이드 카퍼(prezatide copper) 아세테이트 (국소 겔), 라스부리카제(rasburicase), 라니비주맙(ranibizumab), 악티문(Actimmune), PEG-인트론, 트리코민(Tricomin), 재조합 집먼지 진드기 알러지 탈감작 주사, 재조합 인간 부갑상선 호르몬 (PTH) 1-84(sc, 골다공증), 에포에틴 델타, 형질전환 항트롬빈 III, 그란디트로핀(Granditropin), 비트라제(Vitrase), 재조합 인슐린, 인터페론-알파 (구강 로젠지), GEM-21S, 바프레오타이드(vapreotide), 이두르술포제(idursulfase), 옴나파트릴랏(omnapatrilat), 재조합 혈청 알부민, 세르토리주맙 페골 (certolizumab pegol), 글루카르피다제 (glucarpidase), 인간 재조합 C1 에스테라제 억제제 (혈관부종), 라노테플라제(lanoteplase), 재조합 인간 성장 호르몬, 엔푸비르타이드(enfuvirtide)(주사침 없는 주사제, Biojector 2000), VGV-1, 인터페론 (알파), 루시안크탄트(lucinactant), 아비브타딜(aviptadil) (흡입제, 폐질환), 이카티반트(icatibant), 에칼란타이드 (ecallantide), 오미가난(omiganan), 아우로그랩(Aurograb), 펙시가난아세테이트(pexigananacetate), ADI-PEG-20, LDI-200, 데가렐릭스(degarelix), 신트레델린베수도톡스(cintredelinbesudotox), 파블드(Favld), MDX-1379, ISAtx-247, 리라글루타이드(liraglutide), 테리파라타이드(teriparatide) (골다공증), 티파코긴 (tifacogin), AA4500, T4N5 리포솜 로션, 카투막소맙(catumaxomab), DWP413, ART-123, 크라이살린 (Chrysalin), 데스모테플라제(desmoteplase), 아메디플라제(amediplase), 코리폴리트로핀 알파 (corifollitropin alpha), TH-9507, 테두글루타이드(teduglutide), 다이아미드(Diamyd), DWP-412, 성장 호르몬 (서방형 주사제), 재조합체 G-CSF, 인슐린 (흡입형, AIR), 인슐린 (흡입형, 테크노스피어), 인슐린 (흡입성, AERx), RGN-303, DiaPep277, 인터페론 베타(C형 간염 바이러스 감염 (HCV)), 인터페론 알파-n3 (경구용), 벨라타셉트(belatacept), 경피형 인슐린 패치, AMG-531, MBP-8298, 제레셉트(Xerecept), 오오베바칸(opebacan), AIDSVAX, GV-1001, 림포스칸(LymphoScan), 란피르나제(ranpirnase), 리폭시산(Lipoxysan), 루수풀타이드 (lusupultide), MP52(베타-트리칼슘 포스페이트 담체, 골 재생용), 흑색종 백신, 시풀레우셀(sipuleucel)-T, CTP-37, 인세지아(Insegia), 비테스텐(vitespen), 인간 트롬빈 (동결, 수술시 출혈), 트롬빈, 트랜스MID, 알피메프라제(alfimeprase), 퓨리카제(Puricase), 테리리프레신(terlipressin) (정맥주사, 간장 신장형 증후군), EUR-1008M, 재조합 FGF-I (주사제, 혈관 질환), BDM-E, 로티갑타이드(rotigaptide), ETC-216, P-113, MBI-594AN, 듀라마이신(duramycin) (흡입형, 낭포성 섬유증), SCV-07, OPI-45, 엔도스타틴(Endostatin), 안지오스타틴(Angiostatin), ABT-510, 보우만 비르크(Bowman Birk) 저해제 농축물, XMP-629, 99 mTc-하이닉-아넥신 V, 카할라라이드(kahalalide) F, CTCE-9908, 테베렐릭스(teverelix) (연장 방출), 오자렐릭스(ozarelix), 로르니덱신(rornidepsin), BAY-504798, 인터류킨 4, PRX-321, 펩스칸(Pepscan), 이보크타데킨(iboctadekin), 락토페린(rhlactoferrin), TRU-015, IL-21, ATN-161, 실렌지타이드(cilengitide), 알부페론(Albuferon), 비파식스 (Biphaxis), IRX-2, 오메가 인터페론, PCK-3145, CAP-232, 파시레오타이드(pasireotide), huN901-DMI, 난소암 면역치료용 백신, SB-249553, 온코박스(Oncovax)-CL, 온코박스-P, BLP-25, 세르박스-16, 다중-에피토프 펩티드 흑색종 백신 (MART-1, gp100, 티로시나제), 님피타이드(nemifitide), rAAT (흡입용), rAAT (피부용), CGRP (흡입용, 천식), 페그수네르셉트(pegsunercept), 티모신베타4, 플리타덱신(plitidepsin), GTP-200, 라모플라닌 (ramoplanin), GRASPA, OBI-1, AC-100, 연어 칼시토닌 (경구용, 엘리젠), 칼시토닌(경구용, 골다공증), 엑사모렐린(examorelin), 카프로모렐린(capromorelin), 카르데바(Cardeva), 벨라페르민(velafermin), 131I-TM-601, KK-220, T-10, 울라리타이드(ularitide), 데펠레스타트(depelestat), 헤마타이드(hematide), 크리살린 (Chrysalin) (국소용), rNAPc2, 재조합 인자 V111 (PEG화 리포솜성), bFGF, PEG화 재조합 스태필로키나제 (staphylokinase) 변이체, V-10153, 소노라이시스 프롤라이제(SonoLysis Prolyse), 뉴로박스(NeuroVax), CZEN-002, 취도 세포(islet cell) 신생 요법, rGLP-1, BIM-51077, LY-548806, 텍세나타이드(exenatide) (조절방출,

메디소브), AVE-0010, GA-GCB, 아보렐린(avorelin), ACM-9604, 리나클로티드 o에아세테이트(naclotid eacetate), CETi-1, 헤모스판(Hemospan), VAL (주사제), 신속-작용 인슐린 (주사용, Viadel), 비강내 인슐린, 인슐린 (흡입용), 인슐린 경구용, 엘리젠), 제조합 메티오닐 인간 렙틴, 프피트라킨라(trakinra) (피하주사, 슝진), 피트라킨라 (흡입형 건조 분말, 천식), 멀티킨(Multikine), RG-1068, MM-093, NBI-6024, AT-001, PI-0824, Org-39141, Cpn10 (자가면역 질환/염증), 탈락토페린(talactoferrin) (국소용), rEV-131 (안과용), rEV-131 (호흡기 질환), 경구용 제조합 인간 인슐린 (당뇨병), RPI-78M, 오프렐베킨(oprelvekin) (경구용), CYT-99007 CTLA4-Ig, DTY-001, 발라테그라스트(valategrast), 인터페론 알파-n3 (국소용), IRX-3, RDP-58, 타우페론(Tauferon), 담즙 염 자극 리파아제, 메리스파제(Merispase), 알라닌 포스파타제, EP-2104R, 멜라노탄-II, 브레멜라노타이드(bremelanotide), ATL-104, 제조합 인간 마이크로플라스민(microplasmin), AX-200, SEMAX, ACV-1, Xen-2174, CJC-1008, 다이노르핀(dynorphin) A, SI-6603, LAB GHRH, AER-002, BGC-728, 말라리아 백신 (바이로솜, PeviPRO), ALTU-135, 파르보바이러스 B19 백신, 인플루엔자 백신 (제조합 뉴라미니다제), 말라리아 /HBV 백신, 안트락스 백신, Vacc-5q, Vacc-4x, HIV 백신 (경구용), HPV 백신, 타트 독소이드(Tat Toxoid), YPSL, CHS-13340, PTH(1-34) 리포솜 크립 (노바솜), 오스탈린-(Ostabolin)-C, PTH 유사체 (국소용, 건선), MBRI-93.02, MTB72F 백신 (폐결핵), MVA-Ag85A 백신 (폐결핵), FARA04, BA-210, 제조합 플라그 FIV 백신, AG-702, OxSODrol, rBetV1, Der-p1/Der-p2/Der-p7 알레르겐-표적화 백신 (집먼지 진드기 알레르기), PR1 펩티드 항원 (백혈병), 돌연변이 ras 백신, HPV-16 E7 리포솜티드 백신, 라비린틴(labyrinthin) 백신 (선암; adenocarcinoma), CML 백신, WT1-펩티드 백신 (암), IDD-5, CDX-110, 펜트리스(Pentrys), 노렐린(Norelin), CytoFab, P-9808, VT-111, 이크로캡티드(icrocaptide), 텔베르민(telbermin)(피부과, 당뇨발 궤양), 루핀트리 비르(rupintrivir), 레티쿨로스(reticulose), rGRF, HA, 알파-갈락토시다제 A, ACE-011, ALTU-140, CGX-1160, 안지오텐신 치료 백신, D-4F, ETC-642, APP-018, rhMBL, SCV-07 (경구용, 폐결핵), DRF-7295, ABT-828, ErbB2-특이적 이뮤노톡신 (항암제), DT3SSIL-3, TST-10088, PRO-1762, 콤보톡스(Combotox), 콜레사이스토키닌-B/가스 트린-수용체 결합 펩티드, 111In-hEGF, AE-37, 트라스니주맙(trasnizumab)-DM1, 길항제 G, IL-12 (제조합체), PM-02734, IMP-321, rhIGF-BP3, BLX-883, CUV-1647 (국소용), L-19 계 방사면역치료제 (암), Re-188-P-2045, AMG-386, DC/1540/KLH 백신 (암), VX-001, AVE-9633, AC-9301, NY-ESO-1 백신 (펩티드), NA17.A2 펩티드, 흑색 종 백신 (펄스 항원 치료제), 전립선암 백신, CBP-501, 제조합 인간 락토페린 (안구건조증), FX-06, AP-214, WAP-8294A (주사제), ACP-HIP, SUN-11031, 펩티드 YY [3-36] (비만, 비강내 투여), FGLL, 아타시셉트 (atacicept), BR3-Fc, BN-003, BA-058, 인간 부갑상선 호르몬 1-34 (비강, 골다공증), F-18-CCR1, AT-1100 (복강 질환/당뇨병), JPD-003, PTH(7-34) 리포솜 크립 (노바솜), 듀라마이신 (안과용, 안구건조증), CAB-2, CTCE-0214, 글리코PEG화 에리트르톨트로포이틴, EPO-Fc, CNTO-528, AMG-114, JR-013, 인자 XIII, 아미노칸딘 (aminocandin), PN-951, 716155, SUN-E7001, TH-0318, BAY-73-7977, 테베렐릭스 (즉시 방출), EP-51216, hGH (조절 방출, Biosphere), OGP-I, 시푸비르타이드(sifuvirtide), TV4710, ALG-889, Org-41259, rhCC10, F-991, 티모펜틴(thymopentin) (폐질환), r(m)CRP, 간 선택적 인슐린, 수발린(subalin), L19-IL-2 융합 단백질, 엘라 핀(elafin), NMK-150, ALTU-139, EN-122004, rhTPO, 트롬보포이에틴 수용체 작용제 (혈소판 감소성 장애), AL-108, AL-208, 신경 성장인자 길항제 (통증), SLV-317, CGX-1007, INNO-105, 경구형 테리파라타이드 (엘리젠), GEM-OS1, AC-162352, PRX-302, LFn-p24 융합 백신 (테라포어), EP-1043, S 페럼 소아 백신, 말라리아 백신, 나 이세리아 수막염 B군 백신, 신생아 B군 스트렙토코쿠스 백신, 탄저병 백신, HCV 백신 (gpE1+gpE2+MF-59), 중이 염 치료, HCV 백신 (코어 항원+ISCOMATRIX), hPTH(1-34) (경피성, ViaDerm), 768974, SYN-101, PGN-0052, 아비 스쿰닌(aviscumnine), BIM-23190, 폐결핵 백신, 다중-에피토프 티로시나제 펩티드, 암 백신, 엔카스팀, APC-8024, GI-5005, ACC-001, TTS-CD3, 혈관-표적 TNF (고형 종양), 테스모프레신(desmopressin) (경구 조절-방 출), 오너셉트(onercept), 및 TP-9201 등이 있다.

[0130] 일부 실시예에서, 폴리펩티드는 아달리무맙 (HUMIRA), 인플릭시맙 (REMICADE™), 리툽시맙 (RITUXAN™ /MABTHERA™) 에타너셉트(ENBREL™), 베바시주맙(AVASTIN™), 트라스투주맙(HERCEPTIN™), 페그릴라스트림 (NEULASTA™), 또는 바이오시밀러 및 바이오베터(biobetter)를 포함하는 임의의 다른 적합한 폴리펩티드이다.

[0131] 다른 적합한 폴리펩티드는 하기 표 6 및 US2016/0097074의 표 1에 열거된 것들 이다. 당업자는 본 발명의 개시 내용이 본원에 기재된 바와 같은 생산물 및/또는 접합체의 조합을 포함할 수 있음을 이해할 수 있다((즉, 다-단 백질, 변형 단백질 (PEG, 독소, 기타 활성 성분과 결합)).

표 6

[0132]	단백질 산물 인터페론 감마-1b	참조약물 목록 악티뮴(Actimmune) [®]
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알테플라제, 조직 플라스미노겐 활성화제	악티바제(Activase) [®] /카트플로(Cathflo) [®]
재조합 항혈우병 인자	아드베이트(Advate)
인간 알부민	알부테인(Albutein) [®]
라로니다제	알두라자임(Aldurazyme) [®]
인터페론 알파-N3, 인간 백혈구 유래	알페론(Alferon) N [®]
인간 항혈우병 인자	알파네이트(Alphanate) [®]
바이러스-여과된 인간 응집 인자 IX	알파나인(AlphaNine) [®] SD
알레파셉트; 재조합 이량체 융합 단백질 LFA3-Ig	아메비브(Amevive) [®]
비발리루딘	안지오맥스(Angiomax) [®]
다르베포에틴 알파	아라네스프(Aranesp) [™]
베바씨주맵	아바스틴(Avastin) [™]
인터페론 베타-1a; 재조합체	아보넥스(Avonex) [®]
응집 인자 IX	베네피크스(BeneFix) [™]
인터페론 베타-1b	베타세론(Betaseron) [®]
토시투모맵	벡사르(BEXXAR) [®]
항혈우병 인자	바이오클레이트(Bioclate) [™]
인간 성장 호르몬	바이오토포핀(BioTropin) [™]
보툴리눔 독소 A형	보톡스(BOTOX) [®]
알렘투주맵	캄파스(Campath) [®]
아크리투모맵; 테크네튬-99 표지됨	CEA-스칸(Scan) [®]
알글루세라제; 변성 형태의 베타-글루코세레브로시다제	세레다제(Ceredase) [®]
이미글루세라제; 재조합 형태의 베타-글루코세레브로시다제	세레자임(Cerezyme) [®]
크로탈리다제 다가 면역 과브 [양]	크로팜(CroFab) [™]
디곡신 면역 과브 [양]	디지팜(DigiFab) [™]
라스부리카제	엘리텍(Elitek) [®]
에타네르셉트	엔브렐(ENBREL) [®]
에포이에틴 알파	에포젠(Epogen) [®]
세톡시맵	에르비톡스(Erbix) [™]
알가시다제 베타	파브라자임(Fabrazyme) [®]
우르폴리트로핀	페리닉스(Ferinx) [™]
폴리트로핀 베타	폴리스티움(Follistim) [™]
테리파라타이드	포르테오(FORTEO) [®]
인간 소마트로핀	게노트로핀(GenoTropin) [®]
글루카곤	글루카젠(GlucaGen) [®]
폴리트로핀 알파	고날(Gonal)-F [®]
항혈우병 인자	헬릭사이트(Helixate) [®]

항혈우병 인자; 인자(XIII)	헤모필(HEMOFIL)
아데포비르 디피복실	헵세라(Hepsera) TM
트라스투주맙	헤르셉틴(Herceptin) [®]
인슐린	휴말로그(Humalog) [®]
항혈우병 인자/본 빌레브랜트 인자 복합체-인간	휴메이트(Humate)-P [®]
소마트로핀	휴마트로페(Humatrope) [®]
아달리무맙	휴미라(HUMIRA) TM
인간 인슐린	휴물린(Humulin) [®]
재조합 인간 히알루로니다제	하일레넥스(Hylenex) TM
인터페론 알파콘-1	인페르젠(Infergen) [®]
에피티피바타이드	인테그릴린(Integrilin) TM
알파-인터페론	인트론(Intron) A [®]
팔리페민	케피반스(Kepivance)
아나킨라	키네렛(Kineret) TM
항혈우병 인자	코게네이트(Kogenate) [®] FS
인슐린 글라진	란투스(Lantus) [®]
과립구 마크로파지 콜로니-모사 인자	류킨(Leukine) [®] / 류킨(Leukine) [®] 액상제
루트로핀 알파 주사제	루베리스(Luveris)
OspA 리포단백질	라이메릭스(LYMERix) TM
라니비주맙	루센티스(LUCENTIS) [®]
갬투주맙 오조가미신	마일로타르그(Mylotarg) TM
갈수과제	나글라자임(Naglazyme) TM
네시리타이드	나트레코르(Natrecor) [®]
페그필그라스티م	네우라스타(Neulasta) TM
오프렐베킨	네우메가(Neumega) [®]
필그라스티م	네우포젠(Neupogen) [®]
파놀레소맙	뉴트로스펙(NeutroSpec) TM (옛이름, 레우테크(LeuTech) [®])
소마트로핀 [rDNA]	노르디트로핀(Norditropin) [®] / 노르디트로핀 노르디플렉스 (Norditropin Nordiflex) [®]
미톡산트론	노반트론(Novantrone) [®]
인슐린; 아연 현탁액	노볼린(Novolin) L [®]
인슐린; 이소판 현탁액	노볼린(Novolin) N [®]
인슐린, 일반형	노볼린(Novolin) R [®]
인슐린	노볼린(Novolin) [®]
응집 인자 VIIa	노보세븐(NovoSeven) [®]
소마트로핀	누트로핀(Nutropin) [®]

면역글로불린 정맥주사제	옥타감(Octagam) [®]
PEG-L-아스파라기나제	온카스파르(Oncaspar) [®]
아바타셉트, 완전 용해형 인간 융합 단백질	오렌시아(Orencia) [™]
무로모맵-CD3	오르토클론(Orthoclone) OKT3 [®]
고분자량 히알루론산	오르토비스크(Orthovisc) [®]
인간 융모막 고나드로핀	오비드렐(Ovidrel) [®]
체내 감퇴 바실러스 칼메트-구에린	파시스(Pacis) [®]
페그 인터페론 알파-2a	페가시스(Pegasys) [®]
인터페론 알파-2b의 페길레이트화 버전	PEG-인트론(Intron) [™]
아바렐릭스(주사용 현탁액); 고나도트로핀-방출 호르몬 길항제	플레낙시스(Plenaxis) [™]
에포이에틴 알파	프로크리트(Procrit) [®]
알데스류킨	프롤류킨(Proleukin), IL-2 [®]
소마트렘	프로트로핀(Protropin) [®]
도르나제 알파	플모자임(Pulmozyme) [®]
에팔리주맵; 선택형 가역성 T-세포 차단제	랍티바(RAPTIVA) [™]
리바비린과 알파 인터페론의 조합물	레벤투론(Rebetron) [™]
인터페론 베타 1a	레비프(Rebif) [®]
항혈우병 인자	리콤비네이트(Recombinate) [®] rAHF
항혈우병 인자	레팩토(ReFacto) [®]
레피루딘	레플루단(Refludan) [®]
인플릭시맵	레미케이드(Remicade) [®]
아비식시맵	레오프로(ReoPro) [™]
레테플라제	레타바세(Retavase) [™]
리투시마	리투산(Rituxan) [™]
인터페론 알파-2 ^a	로페론(Roferon)-A [®]
소마트로핀	세이젠(Saizen) [®]
합성 돼지 세크레틴	세크로플로(SecreFlo) [™]
바실릭시맵	시물렉트(Simlect) [®]
에쿨리주맵	솔리리스(SOLIRIS) [®]
페그비소만트	소마베르트(SOMAVERT) [®]
팔리비주맵; 재조합 생성, 인간화 mAb	사이나기스(Synagis) [™]
타이로트로핀 알파	타이로젠(Thyrogen) [®]
테네크테플라제	TNK카제(TNKase) [™]
나탈리주맵	타이사브리(TYSABRI) [®]
인간 면역글로불린 정맥주사용 5% 및 10% 용액	베노글로불린(Venoglobulin)-S [®]
인터페론 알파-n1, 림프아구	웰페론(Wellferon) [®]

드로트레코긴 알파	자이그리스(Xigris) TM
오말루주맵; 재조합 DNA-유래의 인간화 단클론 항체 표적화 면역글로불린-E	졸라이르(Xolair) [®]
다칼리주맵	제나팍스(Zenapax) [®]
이브리투모맵 티옥세탄	제발린(Zevalin) TM
소마토트로핀	조르브티브(Zorbtive) TM (세로스팀 (Serostim) [®])

[0133] 실시예에서, 폴리펩티드는 표 7에 나타난 바와 같은 호르몬, 혈액 응고/응고 인자, 사이토카인/성장 인자; 항체 분자, 융합 단백질, 단백질 백신, 또는 펩티드이다.

표 7

예시적인 제품

[0134]

치료제 종류	제품	상표명
호르몬	에리트르포이에틴, 에포에인-α 다르베포이에틴-α 성장 호르몬(Growth hormone, GH), 소마토트로핀 인간 각질-자극 호르몬(follicle-stimulating hormone, FSH) 인간 융모막 고나도트로핀 루트로핀-α 글루카곤 성장 호르몬 방출 호르몬 (Growth hormone releasing hormone, GHRH) 세크레틴 갑상선 자극 호르몬 (Thyroid stimulating hormone, TSH), 타이로트로핀	에포젠, 프로크리트 아라네스프 게노트로핀, 휴마트로프, 노르디트로핀, 노브IV비트로핀, 뉴트로핀, 움니트로프, 프로트로핀, 시아젠, 세로스팀, 발트로핀, 고날-F, 폴리스팀 오비드렐, 루베리스 GlcaGen 제레프 치로스팀 (인간 펩티드), 세크레플로 (돼지 펩티드) 타이로젠
혈전/응집 인자	인자 VIIa 인자 VIII 인자 IX 항트롬빈 III (AT-III) 단백질 C 농축물	노보세븐 바이오클레이트, 헬릭세이트, 코제네이트, 리콤비네이트, 레팍토 베네픽스 트롬베이트 III 세프로틴
사이토카인/성장 인자	제 1형 알파-인터페론 인터페론-αn3 (IFN αn3) 인터페론-β1a (rIFN-β) 인터페론-β1b (rIFN-β) 인터페론-γ1b (IFN γ) 알데스류킨 (인터류킨 2(IL2), 표피 흉선세포 활성화 인자; ETAF) 팔리페르민 (각질세포 성장 인자; KGF) 베카플레민 (혈소판-유래 성장 인자; PDGF) 아나킨라 (재조합체 IL1 길항제)	인페르젠 알페론 N 아보넥스, 레비프 베타세론 악티뮴 프로류킨 케피반스 레그라넥스 안틸, 키네렛

<p>항체 분자</p>	<p>베바시주맵 (VEGFA mAb) 세특시맵 (EGFR mAb) 파니투무맵 (EGFR mAb) 알렘투주맵 (CD52 mAb) 리톡시맵 (CD20 키메릭 Ab) 트라스투주맵 (HER2/Neu mAb) 아바타셉트 (CTLA Ab/Fc 융합물) 아달리무맵 (TNF α mAb) 에타네르셉트 (TNF 수용체/Fc 융합물) 인플릭시맵 (TNF α 키메릭 mAb) 알레과셉트 (CD2 융합 단백질) 에팔리주맵 (CD11a mAb) 나탈리주맵 (인테그린 α4 서브유닛 mAb) 에쿨리주맵 (C5mAb) 무로모넵-CD3</p>	<p>아바스틴, 에르비투스 벡티빅스 캄파스 리톡산 헤르셉틴, 오렌시아 휴미라, 엔브렐 레미케이드 아메비브 랍티바, 타이사브리 솔리리스, 오르토클론, OKT3</p>
<p>기타: 융합 단백질/단백질 백신/펩티드</p>	<p>인슐린 헤파티티스 B 표면 항원 (HBsAg) HPV 백신 OspA 항-붉은털 원숭이(Rh) 면역글로불린 G 엔푸비르타이드 스파이더 실크, 가령, 피브리온</p>	<p>휴몰린, 노볼린 엔제릭스, 레콤비박스 HB 가르다실 라임에릭스 로파일락 푸제온 QMONOS</p>

[0135] 실시예에서, 단백질은 다중특이적 단백질, 예를 들어, 표 8에 나타난 바와 같은 이중특이적 항체이다.

[0139] 이러한 접근법에서 mAb 유전자는 랜딩 패드 A 및 B 둘 다를 표적으로 할 수 있다. 폴의 균질성은 단백질 A HPLC에 의한 상청액의 FACS 분류 및 분석에 의해 결정될 수 있다.

[0140] 2 단계: 대다수의 차세대 항체 (예: 4가 이중특이적 항체)의 경우 여러 중쇄 또는 경쇄의 조립은 반복되는 문제이다. 원치 않는 불순물이 거의 없는 최적의 생산물 품질을 얻으려면 안정하고 재현 가능한 4개의 항체 사슬을 발현하는 적절한 CHO클론의 선택이 유리하다. 결과적으로 클론 선택시 ELISA, RP-HPLC 또는 CD-SDS를 활용하는 대량의 제품 분석이 종종 필요하다. 그러나, 다수의 RTS를 포함하는 세포주에서, 다중 사슬 단백질을 암호화하는 유전자는 개별 프로모터로 공간적으로 2개의 부위에 걸쳐 분리된다. 이것은 개개의 사슬의 카피 수 및 상대적인 발현이 형질주입체 풀만큼 일찍 일관되고 엔코딩 전사 단위의 프로모터 강도 또는 카피 수 조작을 통해 개별 폴리펩타이드 쇄의 이용 가능성을 실험적으로 미세 조정할 수 있게 한다. 이것의 장점은 생산물 품질이 보다 일관되어 SSI 생성 풀과 세포주에서 생산된 잘못 접힌 다중 사슬 재조합 단백질의 비율을 줄임으로써 초기 평가의 필요성을 극적으로 회복한다는 것이다. 테스트 분자: 세르구투맙 아무나류킨(cergutumab amunaleukin).

[0141] 3 단계: CHOK1SV GS-KO™ 세포주의 분비 능력을 확장시키는 내인성 단백질의 발현은 제품 역가를 증가시키는 입증된 접근법이다. 후보물질은 PCT 출원 WO2015018703 A1로부터 확인될 수 있고, 표 9 및 10의 후보는 다수의 별개의 재조합 표적 부위 (RTS)를 포함하는 세포주에서 평가될 수 있다. 생성물 역가는 단백질 A HPLC에 의해 측정될 수 있고 항체 어셈블리는 SDS-PAGE 및 IEF에 의해 평가될 수 있다. 테스트 분자: 리톡시맙, cB72.3, 인플릭시맙 및 에타너셉트.

표 9

단계	근거	치료 단백질	보조 유전자
1	qP를 증가시키기 위한 다중 SSI의 mAb 유전자	리톡시맙, cB72.3 및 .H3IK5 등	N/A
2	SSIS로부터 발현된 이중 특이적 mAb 및 > 2 사슬 DtE 유전자	세르구투주맙 아무나류킨 등.	N/A
3	DtE의 제품 품질을 높이기 위한 보조 유전자 발현	인플릭시맙 등.	본원에 참고로 포함된 미국 특허 제 62 / 322,621 호에서 확인된 후보자로부터의 지질 유전자, 도 20에서 발견되는 miRNA 스폰지 벡터, 및 임의의 혈액 인자 프로테아제.

표 10

miR	표적 유전자 (3'UTR에서의 miR 결합 부위)	스폰지 서열 (유전자 3'UTR에서의 miR 표적 서열)	3'UTR 표적 부위의 복제
miR-15b and miR-16-1	CPEB2A	5'-aggggcaacacagctctgctgcta-3'	1, 3, 6, 9 및 12
miR-15b and miR-16-1	CPEB2B	5'-aagctgtattagctttgctgcta-3'	
miR-34c	SRPR α	5'-taatcatgttacaatcactgcc-3'	
miR-708	CNTFR	5'-caccatcagattataagctcctg-3'	
miR-186	EIF3A	5'-cagtcctaaattgaattcttta-3'	

[0144] 실시예

[0145] 실시예1: CHK1SV GS-KO™에서 아데노바이러스 5 바이러스 공동-감염을 통한 일시적 AAV 생산

[0146] CHOK1SV GS-KO™ (Lonza, Slough, UK) 세포가 생물학적으로 rAAV 벡터 단백질을 생산하고 조립할 수 있는지를 결정하기 위해, CHOK1SV GS-KO™ 세포를 2개의 플라스미드-pRC2-mi342 (6234, Clontech) 및 pAAV-GFP (AAV-400, Cell Biolabs), 대조군 벡터(카탈로그 번호: AAV-400 Cell Biolabs, 미국 샌디에고) (도 6), wt Ad5 바이러스(미국 카탈로그 번호: ATCC® VR-1516™, American Type Culture Collection, Manassas, 미국) 존재 하에 (AAV-400, Cell Biolabs) 형질주입시켰다. 자연 조건 하에서, CHOK1SV GS-KO™ 세포는 양이온성 형질주입 시약 (리포펙타민 또는 PEI)의 존재 하에 바이러스 수용체가 없기 때문에 아데노바이러스를 수용하지 못한다; 음으로 하전된 아데노바이러스 5는 CHOK1SV GS-KO™ 세포로 들어갈 수 있어 모든 헬퍼 요소를 제공한다. 이 개념은

CHOK1SV GS-KO™ 세포에 모든 요소 (형질주입에 의한 Rep-Cap 및 GOI 및 감염에 의한 Ad5 요소)가 제공되는 이 평가 실험을 설계하는데 사용되었다. 형질주입/감염 72시간 후에 세포를 수거하고 4회 동결/해동주기를 거친 후 용해시키고, 이어서 임의의 플라스미드 DNA를 용해시키는(digest) 벤조나아제 처리를 수행하였다. 용해물을 AAV 역가에 대해 qPCR에 의해 시험하고, 전이유전자 (녹색 형광 단백질-GFP) 발현에 대한 HEK293의 형질도입 효율에 대해 시험하였다. 이 실험을 위한 대조군으로서, 부착성 HEK293 세포가 사용되었고 HEK293 삼중 형질주입 (pHelper (6234, Clontech), pRC2-mi342 (6234, Clontech), pAAV-GFP (AAV-400, Cell Biolabs)로부터의 세포 용해물 (도 6)은 pRC2-mi342 (6234, Clontech), pAAV-GFP로 형질주입되고 wt Ad5로 감염된 CHOK1SV GS-KO™ 세포와 동일하게 분석되었다. 이 실험의 실험 개요와 결과는 도 1에 도시되었다. 형질주입 후 CHOK1SV GS-KO™ 세포에서 녹색 형광이 검출되어 성공적인 형질주입을 나타냈다(도 1B, 왼쪽 패널). 형질주입 후 CHOK1SV GS-KO™ 세포 용해물을 사용하여 HEK293 세포를 형질도입하였고, 녹색 형광은 비록 낮지만 (도 1B, 오른쪽 패널) HEK293 세포에서 검출되었으며, AAV의 낮은 수준의 생성을 시사한다. qPCR 데이터는 6.8E+09 vg/mL의 AAV 역가를 보여 준다(도 1C). 삼중 형질주입 갖는 양성 대조군 HEK293은 예상대로 1.8e+12의 더 높은 AAV 역가를 생성하였고 음성 대조군인 HEK293 GFP 형질주입에서 AAV가 생성되지 않았다. 요약하면, 이것은 CHOK1SV GS-KO™ 세포가 AAV 패키징에 필요한 모든 알려진 계놈 요소가 제공될 때 AAV를 생산할 수 있음을 보여주는 최초의 알려진 실험이다.

[0147] 실시예2: 바이러스 공동-감염없이 CHOK1SV GS-KO™에서 일시적인 rAAV 생성 CHOK1SV GS-KO™ 세포가 wt Ad5 바이러스가 없는 상태에서 rAAV를 생산할 수 있는지를 결정하기 위해 세포는 Howe *et al.*, 2005와 유사한 접근법으로 Ad5 E1A 및 E1B를 발현하도록 조작되었다. TET-on 프로모터 (pMF30, 도 2)의 제어 하에 E1A, 구성적인 인간 CMV 프로모터 (pMF23, 도 3) 및 Ad5 E1A-E1B 오픈 리딩 프레임(pXC17.4_17AV5PerProKZ, 도 4)의 제어하에 E1B를 발현하는 플라스미드 구조물이 생성되었다. CHOK1SV GS-KO™ 유래 풀은 pMF30으로 형질주입시키고 50 μM L-MSX가 보충된 글루타민이 없는 배지에서 생존할 수 있는 세포를 선택하여 생성되었다 (도 5A). 이 CHOK1SV GS-KO™ E1A를 pMF23으로 형질주입시키고 50 μM L-MSX 및 10 μg/mL 프로마이신(Puromycin)이 보충된 글루타민이 없는 배지에서 생존할 수 있는 세포를 선택했다(도 5A). 물 제어는 모든 계놈 DNA PCR에서 오염되었으나, E1A PCR 생성물은 HEK293F 및 HEK293L 세포 및 pXC17.4_17AV5PerProKZ에서 증폭된 것이 분명하다(도 1B). CHOK1SV GS-KO™ MOCK (MOCK) 및 CHOK1SV GS-KO™ E1A E1B (E1A E1B) 풀을 독시사이클린 (2 μg/ml)을 0 (처리되지 않음), 2, 3, 4 및 7 일 처리하여 TET 프로모터 및 E1A 전사를 활성화했고, 그리고 나서 E1A (도 1D) 및 E1B (도 1E) 용 프라이머를 사용하여 역전사 PCR을 위해 mRNA를 분리하였다. 결과는 독시사이클린으로 처리된 CHOK1SV GS-KO™ E1A E1B 풀 및 HEK293L 세포에서 더 높은 수준의 E1A 243R 및 E1A 171R을 보여준다. 독시사이클린 처리 후 E1B mRNA 생성물의 양은 변하지 않았지만, HEK293L 세포에서는 현저하게 더 높았다. 독시사이클린으로 7일 동안 처리된 CHOK1SV GS-KO™ E1A E1B 풀 (E1A 리리에서 E1A 단백질이 검출되었으며, CHOK1SV GS-KO™ MOCK (MOCK) 단백질 용해물에서는 검출되지 않았다(도 5F). 이 풀 CHOK1SV GS-KO™ E1A_E1B는 독시사이클린 (2 μg/ml)의 유무에 관계없이 rAAV를 생성하는 능력에 대해 시험되었다. E1A 및 E1B 단백질의 높은 발현을 보장하기 위해, CHOK1SV GS-KO™ 세포에서 E1A-E1B 플라스미드 (pXC17.4_17AV5PerProKZ, 도 4)의 일시적 형질주입도 수행하였다. 이들 데이터는 E1A 단백질 발현이 wt Ad 감염에 대한 요구없이 CHO에서 달성될 수 있음을 보여준다. E1B 통합 및 발현 확인은 더 어려웠다.

[0148] 실시예3: 바이러스 공동-감염없이 CHOK1SV GS-KO™ 및 HEK293에서 영구 rAAV 생산.

[0149] 제조합 CHOK1SV™ 세포주(Fer1L4, NL1, 2, 3, 4, 5 및 6(예를 들어, WO2013190032A1 및 EP2711428A1 참조))로부터 확인된 유전자 좌위로서 RMCE 뒤에 적합한 랜딩 패드를 CHOK1SV GS-KO™ 및 HEK293 계놈에 통합시켰다. 이 랜딩 패드 내의 요소 배열의 개략도는 그림 7A, B 및 C에 표시되어 있다. SV40E 프로모터와 선택 마커 사이에 Frt 부위를 배치하면 후속 라운드의 RMCE에서 사용할 수 있다. 호환되지 않는 Frt 부위 (A, B, C, D, E 및 F)와 다른 리포트/선택 마커를 사용하면 부위 주소를 독립적으로 지정할 수 있다. 도 8은 1 (도 8A), 2 (도 8B) 및 3 (도 8C) 부위 CHO- 및 HEK293- 유래 SSI 세포주에서 RMCE 후 AAV, Ad5 및 GOI 유전자의 다양한 배열을 보여준다. HEK293 SSI 숙주를 생성하기 위해, CHOK1SV 유래 벡터 통합 부위 및 랜딩 패드 위치는 캘리포니아 산타크루즈 대학교(UCSC) 인간 계놈 데이터베이스의 독립형 사본을 사용하여 인간 계놈 (버전: Mar. 2006 (NCBI36/hg18))에 대해 BLAT 검색하였다. 이는 CHOK1SV 서열의 25개 이상의 염기쌍에서 95% (및 그 이상) 유사성의 서열을 식별한다. 유사 지역은 IGV 뷰어 (Broad Institute 버전 2.4)에서 시각화되었다. Crispr-Cas9 gRNA는 자사의 Crispr-Cas9 디자인 도구를 사용하여 디자인되었다. CHOK1SV 및 HEK293 유전자 좌위가 표 5에 요약되어 있다.

[0150] 실시예4: CHOK1SV GS-KO™에서 rAAV 생산을 향상시키기 위한 보조 유전자 발현

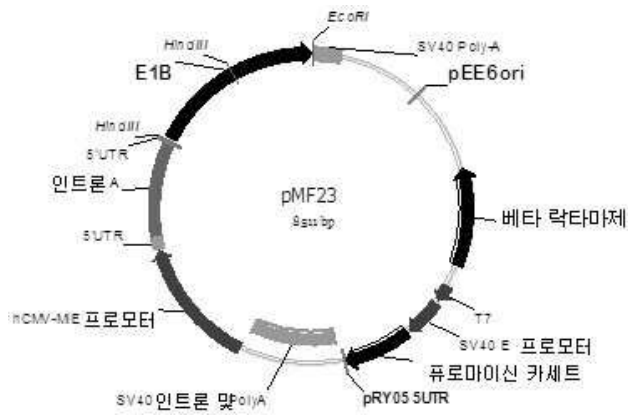
- [0151] 포유동물 선천 면역 시스템은 바이러스 특징을 인식함으로써 바이러스 감염을 감지하고 강력한 항 바이러스 반응 (예: RIG-1 IFN α 반응)을 활성화 시킨다. RNA 침묵 또는 RNA 간섭 (RNAi)이 포유동물 세포에서 항 바이러스 메커니즘으로 작용한다는 증거가 축적되고 있다. 포유동물 바이러스는 인플루엔자 NS1 단백질 및 백시니아 바이러스 E3L 단백질과 같은 IFN 길항제를 암호화한다(예를 들어, de Vries et al, *Gene Ther.*, 15:545-52 (2008), 이는 PKR을 억제하고, 백시니아 바이러스-인코딩된 가용성 IFN-알파/베타 수용체 디코이는 감염된 세포에서 IFN 반응을 방해한다. IFN 길항제의 공동-발현은 실시예 2 및 3에서 관찰된 불량한 Ad5 및 AAV 유전자 발현을 극복하기 위한 가능한 접근법으로서 조사될 수 있다.
- [0152] 실시예5: 부위 별 통합을 사용하여 CHOK1SV GS-KOTM에서 rAAV 생산
- [0153] 1절: 이중 랜딩패드 CHOK1SV GS-KOTM SSI 숙주의 구성
- [0154] CHOK1SV GS-KOTM SSI 숙주는 CHOK1SV GS-KOTM 숙주의 게놈에 2개의 랜딩 패드를 삽입하여 생성되었다(도 9). 랜딩 패드 A는 Fer1 L4 유전자에 위치하고 (표 11 참조) SV40 초기 프로모터 (SV40E), 하이그로마이신 포스포트랜스퍼라제-eGFP 융합 유전자 (Hpt-eGFP) 및 SV40 폴리아데닐화 서열 (pA)로 구성된다. Hpt-eGFP 유전자 및 SV40 pA는 호환되지 않는 Frt 부위 (각각 Frt₅ 및 Frt_{wt}) 에 의해 측면에 위치하여 재조합 유전자의 RMCE를 CHOK1SV GS-KOTM 게놈으로 촉진시킨다. 랜딩 패드 B는 NL1 유전자 좌위에 위치하고 (표 5 참조) SV40 초기 프로모터 (SV40E), 퓨로마이신(puromycin) N-아세틸트랜스퍼라제-DsRed 융합 유전자 (PAC-DsRed) 및 SV40 폴리아데닐화 서열 (pA)로 구성된다. PAC-DsRed 유전자 및 SV40 pA는 호환되지 않는 Frt 부위 (각각 Frt₁₄ 및 Frt₁₅)에 의해 측면에 위치된다. 두 랜딩 패드에서 SV40E 프로모터와 선택 마커 사이에 Frt 부위를 배치하면 후속 라운드의 RMCE에서 사용할 수 있다. CHOK1SV GS-KOTM SSI 숙주에서 RMCE를 위해 설계된 표적화 벡터에는 대상 랜딩 패드와 호환되는 Frt 사이트의 3'에 즉시 배치된 양성 선택 마커 (예: GS)가 포함되어 있다 (도 9). 벡터의 나머지 부분은 GOI에 대한 전사 단위를 포함하고, 랜딩 패드의 제2 Frt 부위와 호환되는 Frt 부위를 포함한다. 표적화 벡터 DNA (도 10)는 Flp 재조합 효소를 발현하는 벡터와 함께 형질주입된다. 형질주입된 세포를 24 시간 동안 배양한 후, 선택 압력을 가한다(예를 들어, 배양 배지로부터 글루타민의 제거). 성공적인 RMCE는 Hpt-eGFP 또는 PAC-DsRed 유전자의 손실 및 양성 선택 마커 유전자로의 대체에 의해 나타난다. 형광 현미경 또는 유세포 분석기로 세포가 어떻게 나타난다. FACS는 양성 선택에서 회복되는 비-교환 세포를 쉽게 제거한다.
- [0155] 2절: CHOK1SV GS-KOTM SSI 숙주와 호환되는 Rep-Cap 및 GOI 발현 벡터 제작
- [0156] Rep-Cap 및 GOI 유전자를 모두 발현하는 CHOK1SV GS-KOTM SSI 숙주 유래 풀을 생성하기 위해(도 9), 5개의 벡터가 설계되었다(pLMC31-35, 도 10A 내지 E). pLMC31 (도 10A)은 CHOK1SV GS-KOTM SSI 숙주에서 GOI (eGFP) 생산 세포주를 생성하기 위한 표적 벡터이다. eGFP의 전사는 hCMV 주요 중간 초기 유전자 1(hCMV)의 프로모터에 의해 구동되고 β -글로빈 폴리아데닐화 (pA) 서열을 사용한다. 전체 전사 카세트는 바이러스 복제 및 캡슐화에 필요한 유일한시스-작용 요소인 역 말단 반복 (ITR)에 의해 측면에 위치한다. 네오마이신 포스포트랜스퍼라제 I(npt1) 유전자는 Frt₁₄의 3'에 즉시 배열되고 NL1 유전자 좌위의 랜딩 패드에 위치한 SV40E 프로모터에 의해 성공적으로 RMCE 전사된다. pLMC32 내지 35(도 10B 내지 E)는 CHOK1SV GS-KOTM SSI 숙주에서 AAV Rep-Cap 생산 세포주를 생산하기 위한 AAV p5 프로모터의 상이한 형태를 갖는 Rep-Cap 발현 카세트를 함유하는 벡터를 표적화하고 있다(도 10B 내지 E). 야생형 p5 프로모터에 의해 유도된 정상 Rep78 발현은 AAV 생성을 억제하는 것으로 나타났다. 다른 형태의 목표는 AAV 생산을 최대화하기 위해 Cap 발현을 유지하면서 Rep78 발현을 감소시키는 것이다. 글루타민 합성 효소 (GS) cDNA는 Frt₅의 3'에 즉시 배열되고 성공적인 RMCE 전사는 Fer1L4 유전자 좌위의 랜딩 패드에 위치한 SV40E 프로모터에 의해 구동된다. pLMC32(도 10B)에는 두 개의 인헨서 요소가 제거된 최소 p5 (흰색 사각형)가 포함되어 있다. 전체 p5 (흑색 사각형)는 3'의 Cap 유전자에 위치하여 Cap 발현을 향상시킨다. pLMC33(도 10C)에는 최소 p5 프로모터 (흰색 사각형) (PLMC32와 유사)가 있지만 Cap 유전자 3'의 p5^{mut} 프로모터 (흑색 및 회색 사각형)에는 돌연변이 (GGGGGG) TATA 상자가 포함되어 있다. pLMC34 (도 10D)에는 p5 프로모터 (흑색 사각형)가 5'에서 Rep로, TATA 상자가 GGGGGG로 돌연변이된 p5^{mut} 프로모터 (흑색 및 회색 사각형)가 Cap의 3' 끝에 있다. pLMC35(도 10E)는 p5 프로모터 (흑색 및 회색 사각형) 모두에서 TATA 돌연변이를 갖는다. pLMC32-35는 GS cDNA 선택 마커를 사용한다.
- [0157] 3절: 4개의 Rep-Cap 구성으로 삼중 형질주입하여 CHOK1SV GS-KOTM 및 HEK293 세포에서 rAAV 생산
- [0158] wt Ad5 바이러스로 공동-감염된 pOK1SV GS-KOTM 또는 pHelper (6234, Clontech) 벡터로 공동-형질주입된 HEK293에서 rAAV 생산을 지원하기 위한 GOI (pLMC31) 및 Rep-Cap (pLMC32-35) 발현 벡터의 능력. 웨스턴 블롯 분석은 E1A 단백질 발현에 의해 CHOK1SV GS-KOTM 세포에서 wt Ad5의 형질도입을 확인하였고(도 11A), HEK293

세포에서 pLMC31-35 벡터를 사용한 높은 수준의 Rep 및 Cap 단백질 발현을 나타낸 반면, 낮은 수준의 Rep 단백질 발현 및 Cap 단백질은 CHOK1SV GS-KO™ 세포에서 검출되지 않았다(도 11B 및 11C). TaqMan-qPCR 분석 결과 HEK293 세포에서 Clontech (5#, pRC2-mi342 (6234, Clontech) 및 pAAV-GFP)의 원래 플라스미드와 비교하여 이러한 pLMC32-35 벡터로 AAV 역가가 3-5배 증가한 것으로 나타난 반면, CHOK1SV GS-KO™ 세포의 생산량은 더 낮았다(도 20). 감염성 분석은 qPCR 역가를 확인하여 HEK293 샘플에 대한 높은 수준의 바이러스 형질 도입과 CHOK1SV GS-KO™ 샘플에 대한 낮은 수준의 감염을 보여준다(도 12). 요약하면, 이들 데이터는 HEK293 및 CHO 세포에서 AAV 생산을 위한 pLMC32-35 벡터의 기능성을 검증하였으며, 이들은 일단 안정하게 발현된 CHO 세포에서 rAAV 생산이 가능하다는 것을 시사한다.

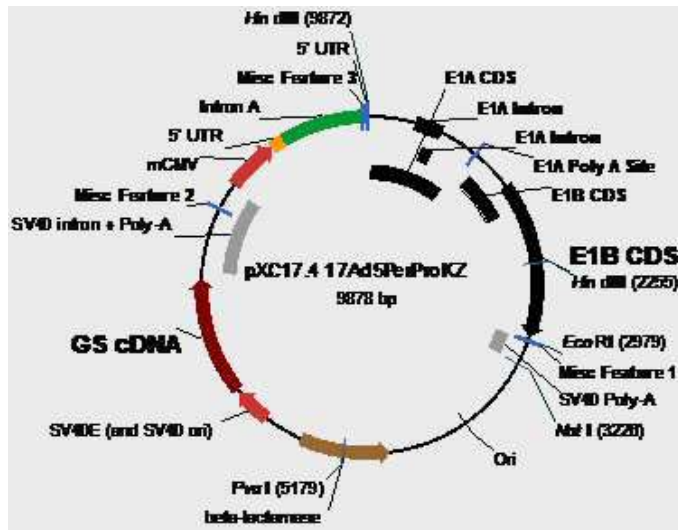
[0159] 4절: 부위 특이적 통합(SSI; Site Specific Integration)에 의해 Rep-Cap 및 GOI가 안정적으로 발현되는 CHO 세포의 생성.

[0160] GOI와 Rep/Cap을 안정적으로 통합한 CHOK1SV GS-KO™ SSI 숙주가 rAAV 생산을 지원할 수 있는지 확인하기 위해 GOI (pLMC31) 및 각각의 Rep-Cap 벡터 (PLMC32-35)를 FLP 재조합 효소-발현 벡터 (pMF4)로 CHOK1SV GS-KO™ SSI 숙주 내로 형질주입시켰다. Rep-Cap과 GOI를 동시에 또는 순차적으로 통합하여 두 세트의 풀을 생성했다(도 13). Rep-Cap, Fer1L 랜딩 패드, GOI 또는 NL1 랜딩 패드에 어닐링(anneal)하도록 설계된 프라이머를 사용한 PCR 분석(도 14)은 대부분의 풀에서 Fer1L4 유전자에 위치한 랜딩 패드에 Rep/Cap이 성공적으로 통합되었음을 보여준다(도 15A). 2개의 풀 ('A' 풀 18 및 19)을 제외한 나머지 Fer1L4 랜딩 패드의 PCR 산물이 모두 감지되었다. 이 풀에서 완전한 SSI를 나타낸다(도15B). 그러나 GOI는 순차적으로 표적화하여 생성된 풀 (NL) 풀의 NL1 위치에 있는 랜딩 패드에만 통합되었으며('B' 풀) (도 16A) 모든 풀에서 잔류 랜딩 패드가 감지되었다(도 16B). 웨스턴 블롯 분석은 Rep가 안정적으로 발현되는 5개의 풀을 확인하였으나, 반면, Cap 단백질 발현은 감지할 수 없었다(도 17). CHOK1SV GS-KO™ 세포에서 Rep-Cap 발현을 입증하기 위해, 이들 유전자의 mRNA 발현을 TaqMan-qPCR 분석에 의해 분석하였고, Rep 및 Cap의 다른 동형을 검출하기 위해 프라이머를 사용하였다(도 18 및 도 21). Rep-Cap2, GOI 및 pHelper (6234, Clontech) 벡터를 사용한 CHOK1SV GS-KO™ 세포의 형질주입은 GOI (pLMC31) 및 Rep-Cap (pLMC32-35) 벡터와 비교하여(도 19A-C) ~5배 더 높은 Rep mRNA수준 (도 19A-C) 및 Rep 및 Cap mRNA 수준을 ~7배 유도하였다(도 19D). CHOK1SV GS-KO™ 세포가 Rep-Cap2, GOI 및 pHelper (6234, Clontech)로 형질주입된 경우에만 E1A, E1B, E2A, E4 및 VA I mRNA 수준을 검출할 수 있었고, GOI (pLMC31) 및 Rep-Cap (pUV!C32-35)으로 형질주입된 경우는 그렇지 않았다(도 19E-J). VA II mRNA 수준은 CHOK1SV GS-KO™ 세포에서 검출되지 않았다(도 19K). E4를 제외한 모든 관찰된 mRNA 수준은 HEK293 세포와 비교하여CHOK1SV GS-KO™에서 더 낮았다(도 19A-K). 이들 데이터는 Rep-Cap mRNA 유도 및 발현이 HEK293 세포와 비교하여 CHOK1SV GS-KO™ 세포에서 더 낮다는 것을 보여준다. 요약하면, CHOK1SV GS-KO™ 세포는 Ad5 헬퍼 유전자 및 Rep-Cap을 발현할 수 있지만, 이들 유전자의 발현 수준은 추가로 최적화될 필요가 있다.

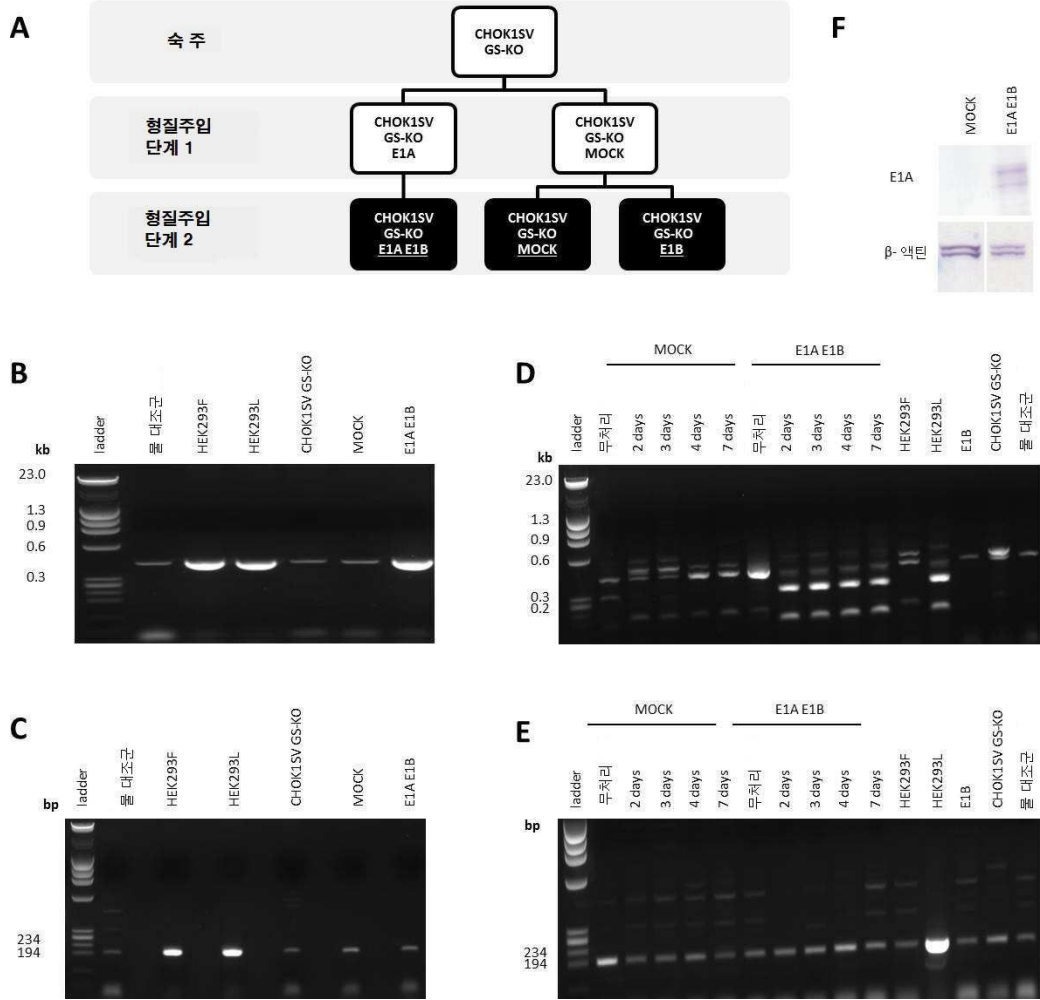
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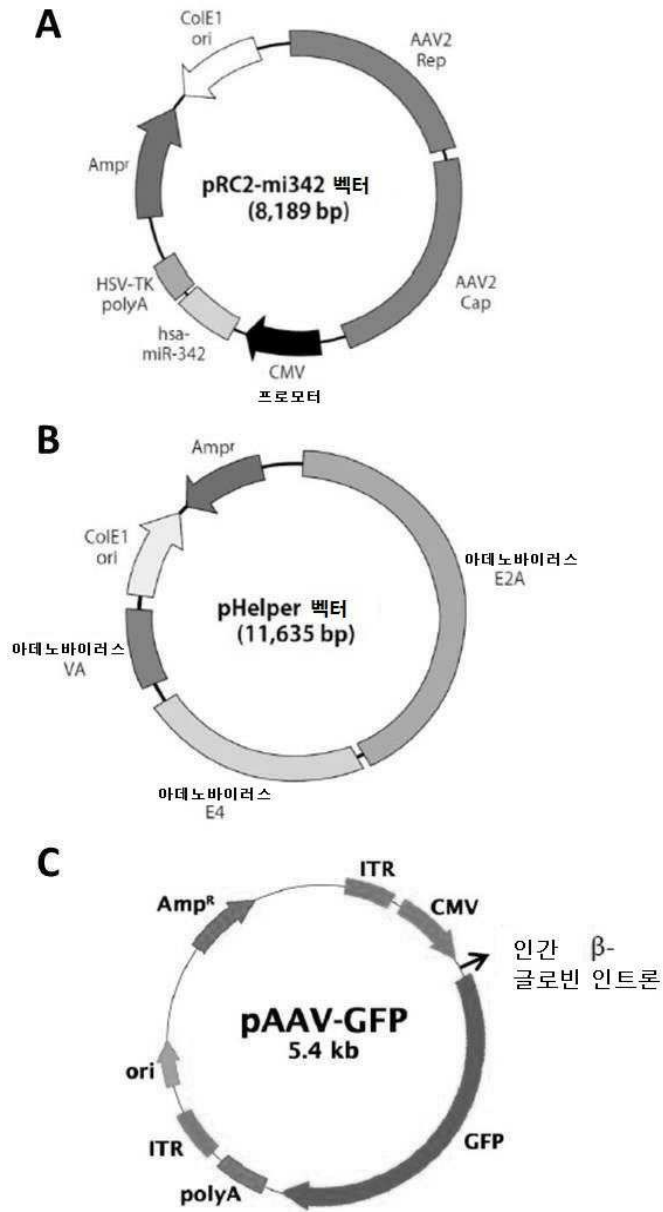
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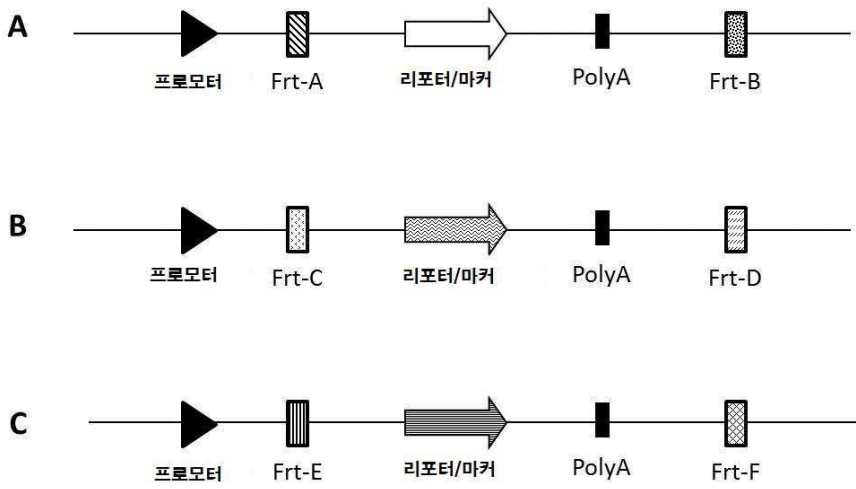
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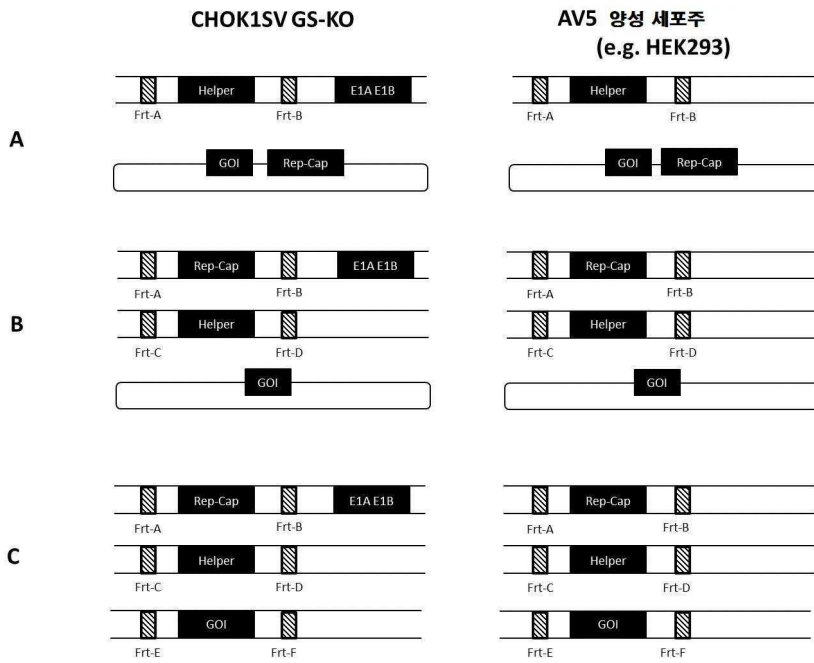
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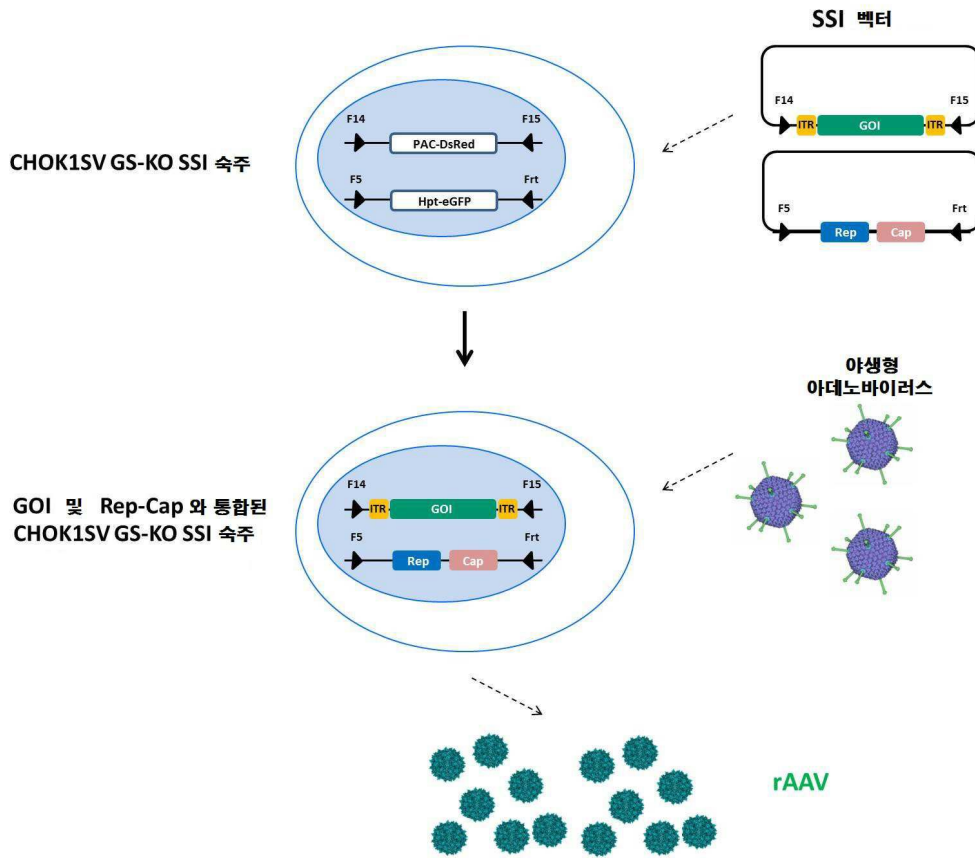
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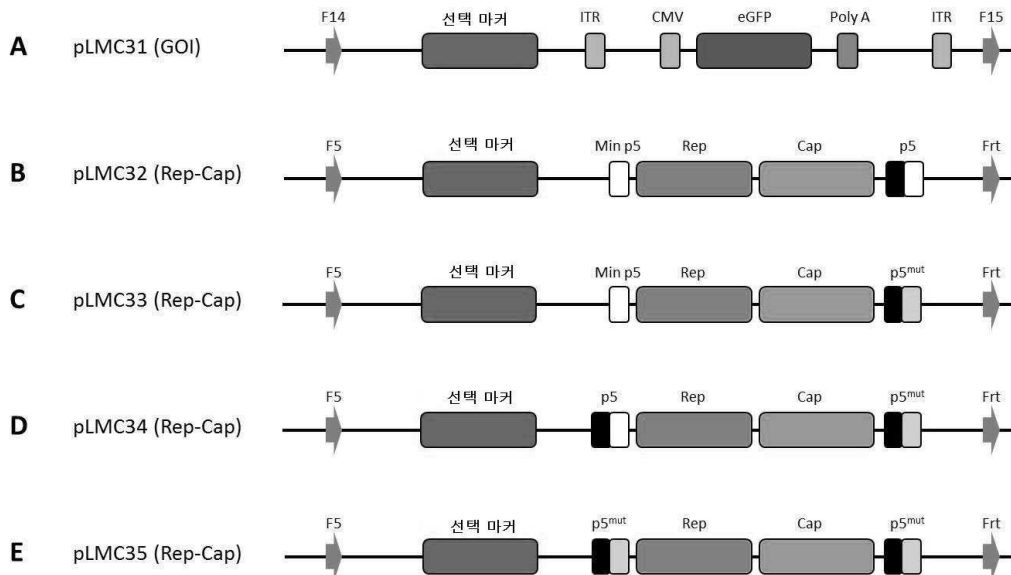
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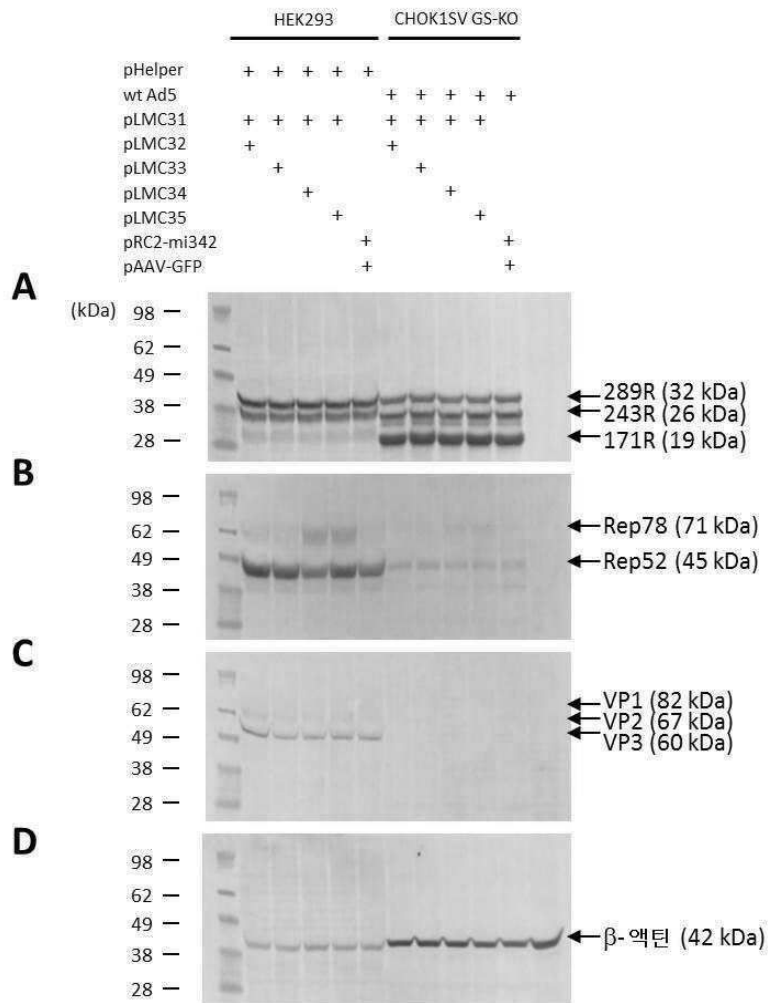
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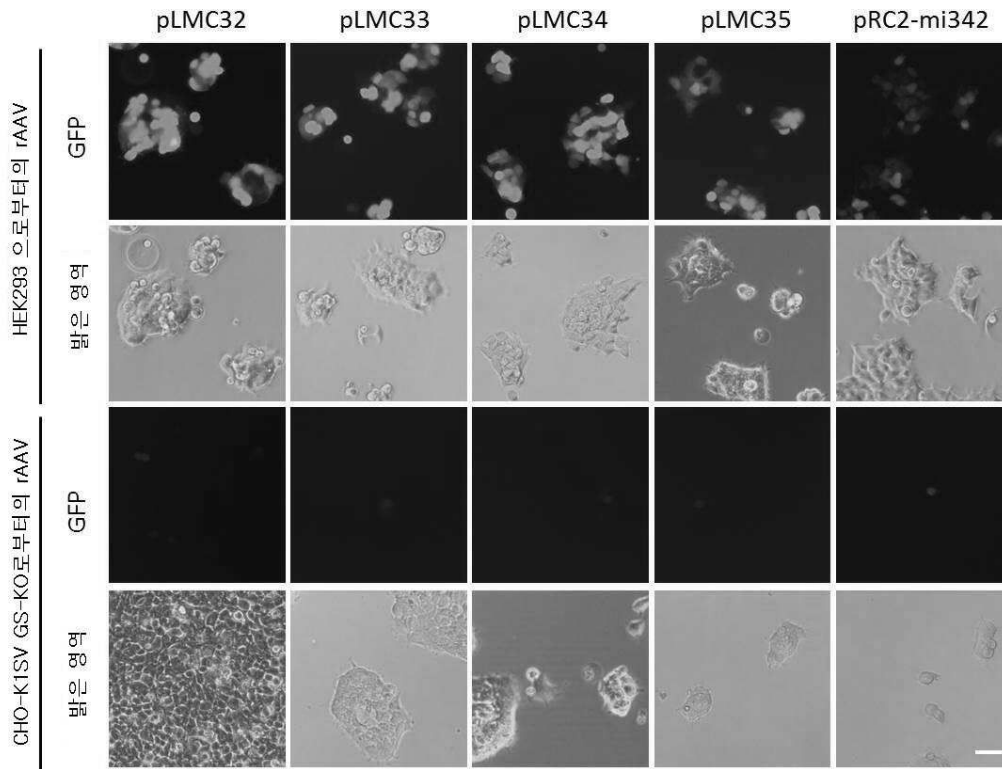
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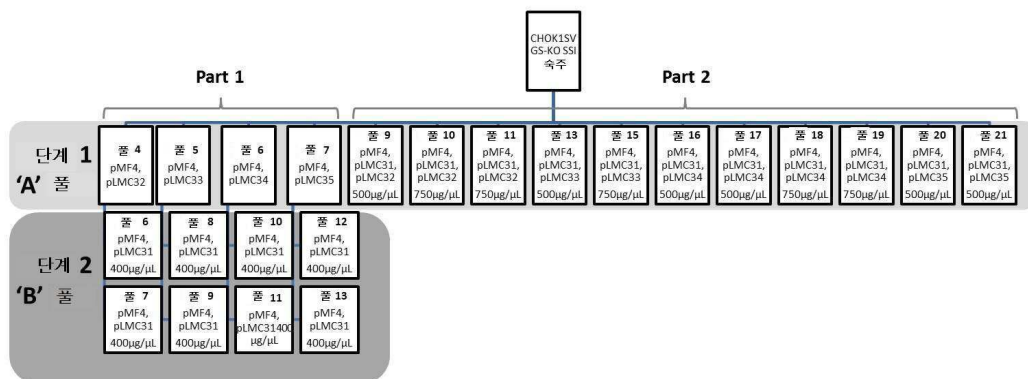
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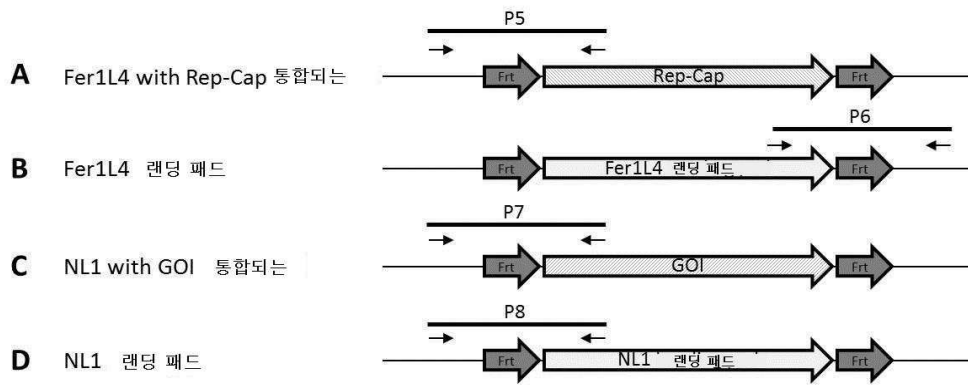
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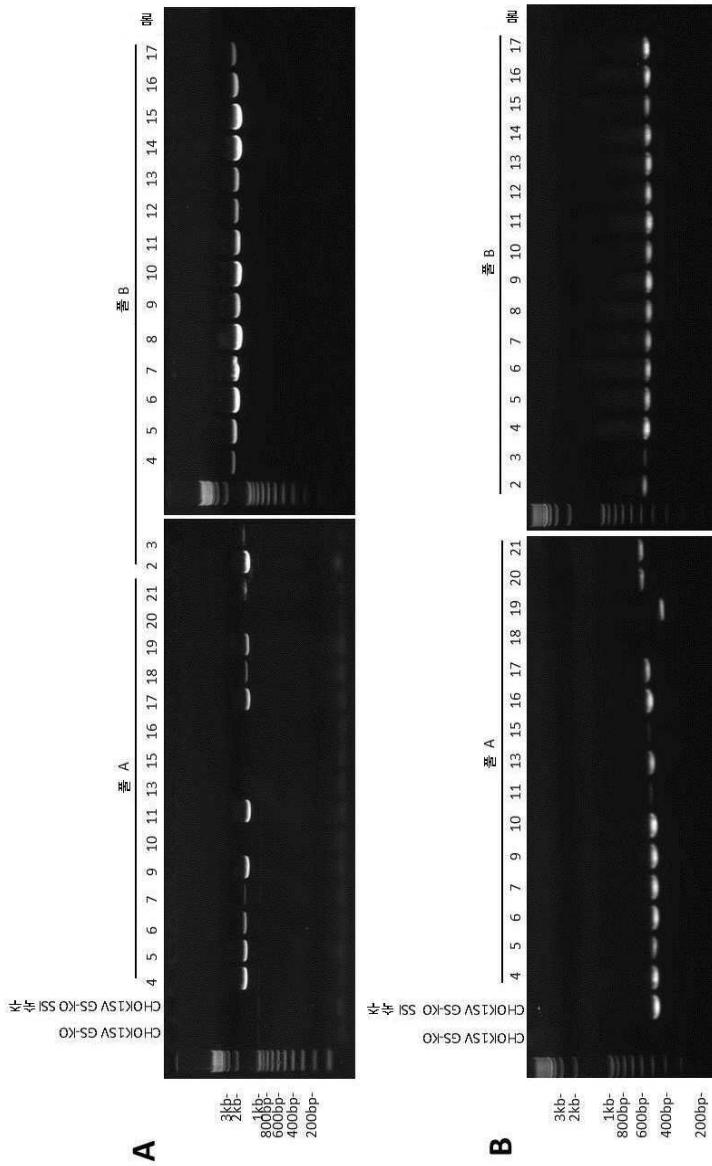
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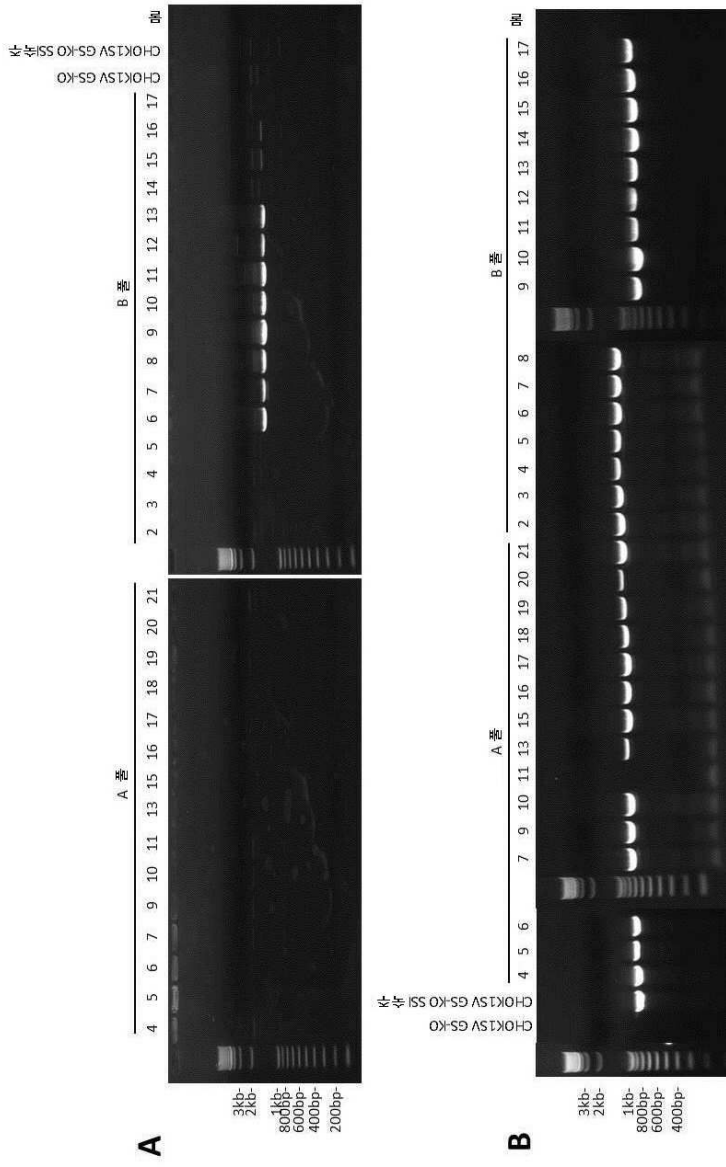
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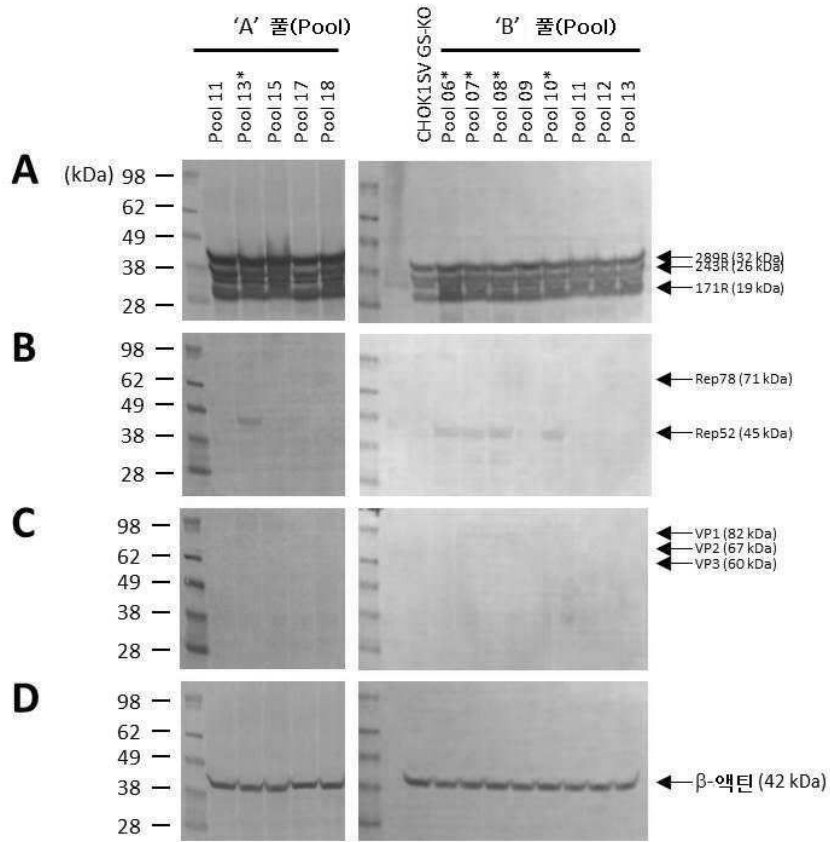
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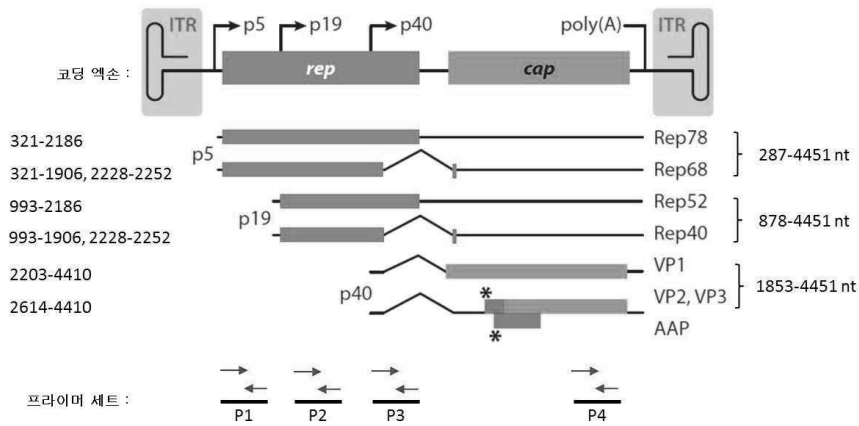
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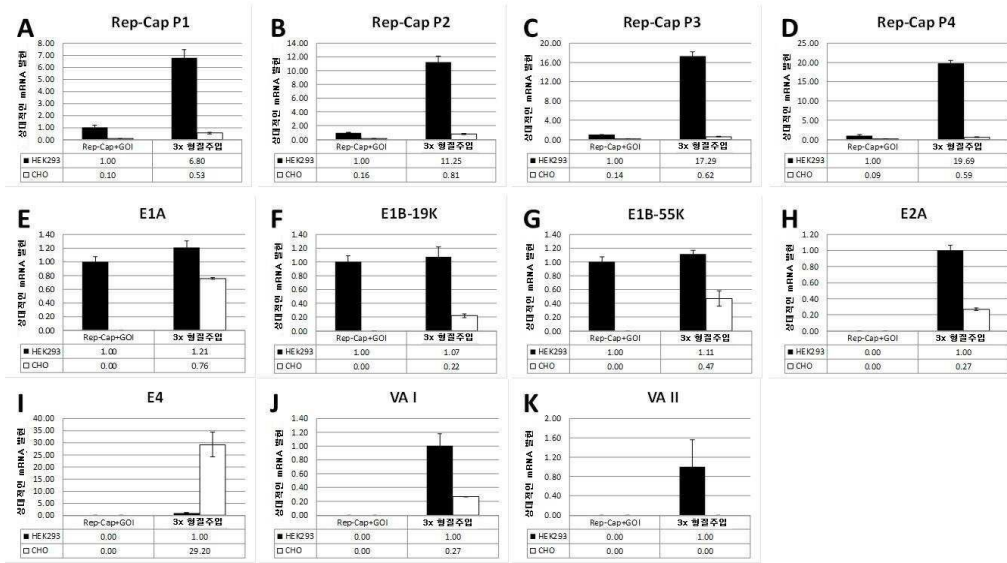
도면17



도면18



도면19



도면20

	1#	2#	3#	4#	5#
HEK293	p헬퍼				
CHOK1SV GS-KO	wt Ad5 (500 MOI)				
Rep-Cap 벡터	pLMC32	pLMC33	pLMC34	pLMC35	pRC2-mi342
GOI 벡터	pLMC31				pAAV-GFP
역가/ HEK293(vg/ml)	4.9E+09	2.9E+09	2.9E+09	2.2E+09	8.4E+08
역가/ CHO (vg/ml)	1.4E+07	1.3E+07	1.8E+07	2.3E+07	3.7E+07

도면21

올리고(Oligo) 이름	서열 (5' to 3')
Rep-Cap-P1-F	TTTGGGACGTTTCCTGAGTC
Rep-Cap-P1-R	CTCATCCACCACCTTGTTCC
Rep-Cap-P2-F	GCAAGACCGGATGTTCAAAT
Rep-Cap-P2-R	CCTCAACCACGTGATCCTTT
Rep-Cap-P3-F	CTGCAGACAATGCGAGAGAA
Rep-Cap-P3-R	CGACAGAAAACGGGTTGAGAT
Rep-Cap-P4-F	GAGTGACATTCGGGACCAGT
Rep-Cap-P4-R	GTCTCTGCCATTGAGGTGGT
E1A-F	GAGGACCTGTGGCATGTTTG
E1A-R	CATTTTAGGACGGCGGGTAG
E1B-19K-F	GAAGTCCTGTGGTGAGCTGT
E1B-19K-R	TGGCCAGAAAATCCAGCAGG
E1B-55K-F	CTGGAAGGTGCTGAGGTACG
E1B-55K-R	CTCAGCTCCTCGGTCACATC
E2A-F	CTTTGGTAGCTGCCTTCCCA
E2A-R	GTATCCTAACGCCAGACCG
E4-F	TAATAAACTCCCCGGGCAGC
E4-R	ACAACGCGTGGACTTCTC
VAI-F	CACTCTCCGTGGTCTGGTG
VAI-R	GTTGTCTGACGTGCGACAC
VAlI-F	TTTCCAAGGGTTGAGTCGCA
VAlI-R	GAATTTGCAAGCGGGGTCTT
Actin-F	CGACATCCGCAAAGACCTCT
Actin-R	AGGATGCTTAGCTCACCTTGA
Fer1L4 with Rep-Cap integrated-P5-F	GGAGTTCAAAGCCAGCTTATACCAACATG
Fer1L4 with Rep-Cap integrated-P5-R	GCTAAAGTTGGTATGGCAGCCTGCACC
Fer1L4 landing pad-P6-F	GGATCACTCTCGGCATGGACGAG
Fer1L4 landing pad-P6-R	CCTCTTCTTCAGCCACAGTCACTTC
NL1 with GOI integrated-P7-F	GCAGCACGAACAAGAGTCAC
NL1 with GOI integrated-P7-R	GAACCTGCGTGAATCCATC
NL1 landing pad-P8-F	GGACATATCTGGGTGAAGGGAGCTG
NL1 landing pad-P8-R	CAGCCGCACGGTAGGCTGTACTCGGTCAT

서열 목록

SEQUENCE LISTING

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<130> PI-19K1058US

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