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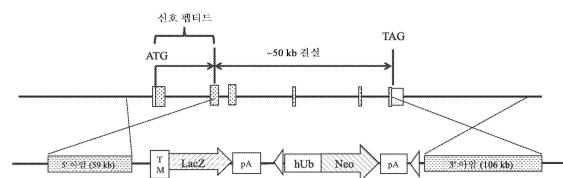
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(54) 발명의 명칭 저하된 상위 및 하위 운동 뉴런 기능 및 감각 지각을 나타내는 비-인간 동물

(57) 요약

유전자 변형 DR6 대립유전자를 포함하고 출생시 및 출생 후 몇 주 또는 몇 개월 동안 정상적인 표현형을 나타내는 유전자 변형 비-인간 동물을 포함하는 것으로서, 근 위축성 측삭 경화증(ALS)과 같은 질환의 운동 뉴런 기능 장애를 위한 동물 모델. 그러나 비-인간 동물은 나이가 들에 따라, 하나 이상의 ALS 유사 증상으로 나타나는 운동 뉴런 기능 장애가 발생하며, 이는 발병 후 빠르게 진행될 수 있다. ALS를 억제, 지연 또는 치료하는데 사용될 수 있는 후보 제제를 확인하는 방법이 또한 제공된다.

대표도 - 도1



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명세서

청구범위

청구항 1

(i) 전체 DR6 코딩 영역 또는 그 일부분의 결실을 포함하고, (ii) 막 관통 도메인에 작동가능하게 융합된 기능성 DR6 신호 펩티드를 인코딩하는 핵산 서열을 포함하는 변형 내인성 DR6 유전자좌를 게놈 내에 포함하는 유전자 변형 설치류 세포로서, 막 관통 도메인은 리포터 단백질에 작동가능하게 융합되고, 핵산 서열은 내인성 DR6 프로모터에 작동가능하게 연결되고, 내인성 DR6 유전자좌에 의해 인코딩된 임의의 폴리펩티드는 기능성 DR6 세포질 도메인이 결여되며,

설치류 세포는 래트 세포 또는 마우스 세포인, 유전자 변형 설치류 세포.

청구항 2

제1 항에 있어서,

- (a) 기능성 DR6 신호 펩티드는 내인성 DR6 신호 펩티드이고;
- (b) 막 관통 도메인은 내인성 DR6 막 관통 도메인이 아니고;
- (c) 막 관통 도메인은 ROR1 막 관통 도메인이고; 및/또는
- (d) 리포터 단백질은 베타 갈락토시다제인 것을 특징으로 하는 유전자 변형 설치류 세포.

청구항 3

제1 항에 있어서, 유전자 변형 설치류 세포는 변형 내인성 DR6 유전자좌에 대해 이형접합성인 것을 특징으로 하는 유전자 변형 설치류 세포.

청구항 4

제1 항에 있어서, 설치류 세포는 마우스 세포인 것을 특징으로 하는 유전자 변형 설치류 세포.

청구항 5

제4 항에 있어서, 내인성 마우스 DR6 유전자좌는, 내인성 DR6 프로모터에 작동가능하게 연결된, β -갈락토시다제에 작동가능하게 융합된 ROR1 막 관통 도메인에 작동가능하게 융합된 내인성 DR6 신호 펩티드를 인코딩하는 핵산 서열을 포함하는 것을 특징으로 하는 유전자 변형 설치류 세포.

청구항 6

제1 항에 있어서, 핵산 서열은 서열 번호 17로 제시되는 것을 특징으로 하는 유전자 변형 설치류 세포.

청구항 7

제1 항에 있어서, 설치류 세포는 배아 줄기 세포인 것을 특징으로 하는 유전자 변형 설치류 세포.

청구항 8

제1 항에 있어서, 설치류 세포는 운동 뉴런인 것을 특징으로 하는 유전자 변형 설치류 세포.

청구항 9

제7 항의 유전자 변형 설치류 배아 줄기 세포로부터 발생된 설치류 배상체(embryoid body).

청구항 10

제9 항의 설치류 배상체로부터 분화된 설치류 운동 뉴런.

청구항 11

제1 항 내지 제8 항 중 어느 한 항의 유전자 변형 설치류 세포, 제9 항의 설치류 배상체, 또는 제10 항의 설치류 운동 뉴런에 있어서, 설치류 세포, 설치류 배상체, 또는 설치류 운동 뉴런은 배양되는 것을 특징으로 하는, 유전자 변형 설치류 세포, 설치류 배상체, 또는 설치류 운동 뉴런.

청구항 12

삭제

청구항 13

삭제

청구항 14

삭제

청구항 15

삭제

청구항 16

삭제

청구항 17

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청구항 45

삭제

청구항 46

삭제

발명의 설명

기술 분야

- [0001] 관련 출원의 상호 참조
- [0002] 본 출원은 (2015년 3월 16일자로 출원된) 미국 가출원 제62/133,909호 및 (2015년 11월 3일자로 출원된) 미국 가출원 제62/250,229호에 대한 이익을 주장하며, 이들 각각의 출원은 본 명세서에 참고로 포함된다.
- [0003] 서열 목록
- [0004] 서열 목록의 공식 사본은 2016년 3월 16일자로 작성되고 크기가 약 685 킬로바이트인 2016-03-16-T0041W001-SEQ-LIST_ST25.txt의 파일명의 ASCII 포맷 서열 목록으로서 EFS-웹을 통해 전자적으로 제출되며, 본 명세서와 동시에 제출된다. 이러한 ASCII 포맷 문서에 포함된 서열 목록은 본 명세서의 일부이며, 전체적으로 본 명세서에 참고로 포함된다.
- [0005] 기술분야
- [0006] 본 출원은 일반적으로 시간 경과에 따라 상위 운동 뉴런, 하위 운동 뉴런 및/또는 감각 지각의 저하가 발생하는 비-인간 동물로서, 신경 퇴행성 장애, 예를 들어 근 위축성 측삭 경화증 (ALS)과 같은 운동 뉴런 질환에 유용한 모델을 제공할 수 있는 비-인간 동물에 관한 것이다.

배경 기술

- [0007] 루게릭병 (Lou Gehrig's Disease)으로 알려진 근 위축성 측삭 경화증 (ALS)은 수의 운동을 담당하는 척수, 뇌간 및 피질의 운동 뉴런의 파괴로 인한 치명적 신경 퇴행성 질환이다. 이 질환은 임상적으로 진행성 근육 약화와 위축으로 나타나, 발병 후 3 내지 5년 이내에 마비와 사망을 초래한다.
- [0008] 미국에서는 대략 2만 명이 ALS를 앓고 있으며, 매년 5,000 명이 ALS로 진단된다. ALS는 전 세계의 모든 인종 및 민족 배경의 사람들에서 공통적으로 발병된다. ALS가 발병하는 평균 연령은 40세에서 60세 사이이나, ALS는 젊은 연령층과 노년층의 남성과 여성 모두에서 발생할 수 있다. ALS 사례의 90 내지 95%에서, 상기 질환은 명백히 무작위적이다 (산발성 ALS (sALS)로 알려짐). 이러한 sALS 사례에서, 상기 질환은 가족력이 없으며, 명확하게 관련된 위험 인자가 없다. ALS 사례의 5 내지 10%에서는 선천성 유전적 연결이 존재한다 (가족성 ALS (fALS)로 알려져 있음).
- [0009] ALS와 관련된 돌연변이 중 구리 아연 초과산화물 불균등화 효소 (SOD1) 유전자의 돌연변이는 오랫동안 SOD1 효소의 항산화 기능의 손상보다는 기능의 독성 증가를 통해 ALS 질환을 일으키는 것으로 생각되어 왔다. fALS와 관련된 돌연변이를 가진 다른 유전자로는 알신 (alsin) (ALS2), 세나탁신 (senataxin) (ALS4), 소포 관련 막 단백질 (VAPB, ALS8), 안지오제닌 (Angiogenin), 및 다이낙틴 (dynactin)의 p150 하위 단위 (DCTN1)가 포함된다. 최근, 분명한 가족력이 있거나 그렇지 않은 ALS 환자에서 Tardbp의 TDP-43-코딩 영역에 30개 초과 돌연변이가 확인되었으며, 이는 대략 4%의 fALS 및 1% 미만의 sALS에 상응하였다. TDP-43 돌연변이(들)를 가진 대부분의 환자는 인지 결손이 없는 고전적인 ALS 표현형을 나타내며, 이는 ALS 발생에서의 TDP-43의 중요한 역할

을 시사한다. 또한, 연장된 GGGGCC 핵사뉴클레오타이드는 C9ORF72 유전자의 프로모터에서 반복되고, fALS 및 sALS뿐만 아니라 ALS 관련 전두측두성 치매 (ALS-FTD)의 매우 공통적인 원인으로 나타난다.

[0010] *SOD1*, *TDP43* 또는 *FUS*, ALS2-녹아웃 마우스 및 뉴로필라멘트 하위 단위를 코딩하는 유전자 조작된 유전자를 갖는 마우스에서 돌연변이를 갖는 설치류 계통을 포함하는 다수의 마우스 모델이 ALS 질환에 대해 수립되었다. 이들 중, 인간 돌연변이형 *SOD1* (m*SOD1*) 트랜스제닉 마우스 모델은, ALS 환자와 몇몇 임상적 표현형을 공유하기 때문에, 현재 가장 널리 사용되는 모델이다. m*SOD1* 마우스의 첫 번째 증상은 하나 이상의 팔다리의 미세한 "떨림 (jittering)/진전 (tremor)"으로, 대략 90일 내지 100일령에 나타난다. 후기 단계에서, 마우스는 근육 약화 및/또는 뒷다리의 불완전 마비로 임상 경과가 개시된 후, 그 다음에 앞다리가 불완전 마비되고, 마지막으로 중증 사지 마비가 유발된다. 그러나, 현재의 동물 모델은, 동물이 상위 운동 뉴런 증상, *TDP43* 및/또는 *SOD1* 응집체 및/또는 비-운동 뉴런 상실을 나타내지 않는다는 점에서 인간의 질환으로 해석되지 못한다. 따라서, 인간의 ALS를 더욱 근접하게 반영하는 동물 모델이 요구된다.

발명의 내용

[0011] 상위 운동 뉴런, 하위 운동 뉴런 및/또는 감각 지각의 기능 장애 증상의 병증이 발생하고, 이와 같이, 근 위축성 측삭 경화증 (ALS), 원발성 측삭 경화증, 원발성 측삭 경화증, 진행성 근이영양증 등과 같은 운동 뉴런에 유발된 하나 이상의 신경 퇴행성 질환의 모델로서 유용할 수 있는 유전자 변형 비-인간 동물이 본 명세서에 제공된다. 구체적으로, 변형 내인성 *DR6* 유전자좌를 포함하는 래트 또는 마우스와 같은 설치류로서, 상기 변형 *DR6* 유전자좌는, *DR6* 세포질 사멸 도메인 또는 *DR6* 세포질 도메인 전체를 인코딩하는 제1 뉴클레오타이드 (예를 들어, 제1 DNA, 제1 cDNA, 제1 게놈 DNA 등) 서열이 결여된 (예를 들어, 포함하지 않는, 부재하는 등) 설치류, 예를 들어, 상기 변형 *DR6* 유전자좌는, *DR6* 세포질 사멸 도메인 또는 *DR6* 세포질 도메인의 임의의 일부분을 인코딩하는 뉴클레오타이드 서열이 결여된 설치류가 제공된다. 상기 변형 *DR6* 유전자좌는 (i) *DR6* 막 관통 도메인 전체를 인코딩하는 제2 뉴클레오타이드 (예를 들어, 제2 cDNA, 제2 DNA, 제2 게놈 DNA) 서열, 예를 들어 *DR6* 막 관통 도메인의 임의의 일부분을 인코딩하는 제2 뉴클레오타이드 서열; (ii) *DR6* 세포 외 도메인 또는 그의 임의의 일부분을 인코딩하는 제3 뉴클레오타이드 (예를 들어, 제3 cDNA, 제3 DNA, 제3 게놈 DNA) 서열, 예를 들어, *DR6* 세포 외 도메인의 임의의 일부분을 인코딩하는 제3 뉴클레오타이드 서열; (iii) 제4 뉴클레오타이드 서열, 예를 들어, 내인성 설치류 *DR6* 유전자의 개재 인트론을 포함하여, 3번 엑손 전체에서 6번 엑손 전체에 걸친 제4 게놈 서열; 및/또는 (iv) 제5 뉴클레오타이드 서열, 예를 들어, 내인성 설치류 *DR6* 유전자의 개재 인트론을 포함하여, 2번 엑손의 일부, 예를 들어 4103번 염기에서 6번 엑손의 전체, 예를 들어 종결 코돈에 걸친 제4 게놈 서열이 추가로 결여될 수 있으며, 제5 뉴클레오타이드 서열은 전장의 성숙 내인성 설치류 *DR6* 단백질을 인코딩한다. 일부 실시 형태에서, 상기 설치류는 마우스이고, 상기 변형 *DR6* 대립유전자는 *DR6* 세포질 사멸 도메인을 인코딩하는 제1 뉴클레오타이드 서열이 결여되어 있으며, 예를 들어, 상기 변형 *DR6* 대립유전자는 서열 번호 15의 아미노산 388 내지 454 또는 그의 임의의 일부분을 인코딩하는 제1 뉴클레오타이드 서열이 결여되어 있다. 일부 실시 형태에서, 세포질 도메인, 예를 들어 서열 번호 15의 아미노산 330 내지 614로 제시된 아미노산 서열을 가지는 세포질 도메인을 인코딩하는 뉴클레오타이드 서열이 결여된 변형 *DR6* 대립유전자를 포함하는 마우스가 제공된다. 일부 실시 형태에서, 본 명세서에 제공된 마우스는, 예를 들어 서열 번호 15의 아미노산 1 내지 614로 제시된 바와 같은 성숙 *DR6* 단백질을 인코딩하는 뉴클레오타이드 서열이 결여된 변형 *DR6* 대립유전자를 포함한다. 일부 실시 형태에서, 상기 변형 *DR6* 대립유전자는 5 kb 초과인 내인성 게놈 서열이 결여되어 있다. 일부 실시 형태에서, 상기 변형 *DR6* 대립유전자는 10 kb 초과인 내인성 게놈 서열이 결여되어 있다. 일부 실시 형태에서, 상기 변형 *DR6* 대립유전자는 20 kb 초과인 내인성 게놈 서열이 결여되어 있다. 일부 실시 형태에서, 상기 변형 *DR6* 대립유전자는 30 kb 초과인 내인성 게놈 서열이 결여되어 있다. 일부 실시 형태에서, 상기 변형 *DR6* 대립유전자는 40 kb 초과인 내인성 게놈 서열이 결여되어 있다. 일부 실시 형태에서, 상기 변형 *DR6* 대립유전자는 50 kb 초과인 내인성 게놈 서열이 결여되어 있다. 일부 실시 형태에서, 상기 설치류는 내인성 *DR6* 세포 외 도메인, 내인성 막 관통 도메인 및/또는 내인성 *DR6* 세포질 도메인의 임의의 부분을 인코딩하는 뉴클레오타이드 서열이 결여된 변형 *DR6* 유전자좌에서 이형접합성 또는 동형접합성이다.

[0012] 본 명세서에 제공된 바와 같은 유전자 변형 설치류는, 예를 들어 내인성 *DR6* 유전자좌 (예를 들어, 본 명세서에 기재된 바와 같은 변형 내인성 *DR6* 유전자좌)에서 내인성 *DR6* 전사 조절 서열에 작동가능하게 연결될 수 있거나 또는 설치류 게놈에 무작위로 삽입될 수 있는 핵산을 발현할 수 있으며, 상기 핵산은 기능성 *DR6* 신호 펩티드 (예를 들어, 기능성 이중성 *DR6* 신호 펩티드, 기능성 내인성 *DR6* 신호 펩티드, 기능성 래트 *DR6* 신호 펩티드, 기능성 마우스 *DR6* 신호 펩티드 등), 막 관통 도메인 (예를 들어, *DR6* 막 관통 도메인 또는 이중성 막 관통 도메인 (예를 들어, ROR1 막 관통 도메인)), 리포터 단백질 (예를 들어, β -갈락토시다제 단백질) 또는 이들의 조

합을 포함한 폴리펩티드를 인코딩하고, 상기 폴리펩티드는 기능성 DR6 세포질 도메인 또는 그의 임의의 일부분이 결여되어 있다 (예를 들어, 작동가능하게 융합되어 있지 않거나, 포함하지 않는 등). 일부 실시 형태에서, 상기 폴리펩티드는 기능성 DR6 세포 외 도메인 또는 그의 임의의 일부분이 또한 결여되어 있다. 일부 실시 형태에서, 상기 폴리펩티드는 기능성 DR6 신호 펩티드로 본질적으로 이루어지거나 또는 그로 이루어진다. 일부 실시 형태에서, 상기 폴리펩티드는 막 관통 도메인에 작동가능하게 융합된 기능성 DR6 신호 펩티드로 본질적으로 이루어지거나 또는 그로 이루어진다. 일부 실시 형태에서, 상기 폴리펩티드는, 리포터 단백질에 작동가능하게 융합된 막 관통 도메인에 작동가능하게 융합된 기능성 DR6 신호 펩티드로 본질적으로 이루어지거나 또는 그로 이루어진다. 일부 실시 형태에서, 상기 폴리펩티드는 막 관통 도메인으로 본질적으로 이루어지거나 또는 그로 이루어진다. 일부 실시 형태에서, 상기 폴리펩티드는 리포터 단백질에 작동가능하게 융합된 막 관통 도메인으로 본질적으로 이루어지거나 또는 그로 이루어진다. 일부 실시 형태에서, 상기 폴리펩티드는 리포터 단백질로 본질적으로 이루어지거나 또는 그로 이루어진다. 일부 실시 형태에서, 상기 DR6 신호 펩티드는, 예를 들어 서열 번호 15의 아미노산 -1 내지 -41 (또는, 그의 임의의 일부분)로 제시되는 바와 같은 설치류 DR6 신호 펩티드, 예를 들어, 마우스 DR6 신호 펩티드이며, 예를 들어 서열 번호 6에 제시된 서열 또는, 동일한 아미노산 서열을 인코딩하나 단지 상기 유전자 코드의 축퇴 (degeneracy)로 인해 서열 번호 6과 상이한 그의 축퇴 변이체에 의해 인코딩된다. 일부 실시 형태에서, 본 명세서에 제공된 바와 같은 설치류는, 서열 번호 6으로 제시된 서열 또는, 동일한 아미노산 서열을 인코딩하나 단지 상기 유전자 코드의 축퇴로 인해 서열 번호 6과 상이한 그의 축퇴 변이체를 포함하거나, 이들로 본질적으로 이루어지거나 또는 이들로 이루어진 핵산을 포함하며 이를 발현시킨다. 일부 실시 형태에서, 상기 막 관통 도메인은 ROR1 막 관통 도메인이며, 예를 들어, 서열 번호 7로 제시된 서열 또는, 동일한 아미노산 서열을 인코딩하나 단지 상기 유전자 코드의 축퇴로 인해 서열 번호 7과 상이한 그의 축퇴 변이체에 의해 인코딩된다. 일부 실시 형태에서, 상기 핵산 서열은, 서열 번호 7로 제시된 서열 또는, 동일한 아미노산 서열을 인코딩하나 단지 상기 유전자 코드의 축퇴로 인해 서열 번호 7과 상이한 그의 축퇴 변이체를 포함하거나, 이들로 본질적으로 이루어지거나 또는 이들로 이루어진다. 일부 실시 형태에서, 리포터 단백질은 β -갈락토시다제이며, 예를 들어 서열 번호 8로 제시된 서열 또는, 동일한 아미노산 서열을 인코딩하나 단지 상기 유전자 코드의 축퇴로 인해 서열 번호 8과 상이한 그의 축퇴 변이체에 의해 인코딩된다. 일부 실시 형태에서, 상기 핵산 서열은, 서열 번호 8 또는, 동일한 아미노산 서열을 인코딩하나 단지 상기 유전자 코드의 축퇴로 인해 서열 번호 8과 상이한 그의 축퇴 변이체를 포함하거나, 이들로 본질적으로 이루어지거나 또는 이들로 이루어진다. 일부 실시 형태에서, 본 명세서에 제공된 바와 같은 설치류는, β -갈락토시다제에 작동가능하게 융합된 ROR1 막 관통 도메인에 작동가능하게 융합된 DR6 신호 펩티드를 인코딩하는 핵산을 포함하며 이를 발현시키고, 예를 들어, 본 명세서에 제공된 바와 같은 설치류는, 서열 번호 17로 제시된 핵산 서열 또는, 동일한 아미노산 서열을 인코딩하나 단지 상기 유전자 코드의 축퇴로 인해 서열 번호 17과 상이한 그의 축퇴 변이체를 포함하며 이를 발현시킨다.

[0013] 일부 실시 형태에서, 상기 핵산은 내인성 DR6 유전자좌에서, 하나 이상의 내인성 DR6 조절 요소, 예를 들어 내인성 전사 조절 요소에 선택적으로 작동가능하게 연결되어, 상기 설치류가, 상기 핵산을 포함하나, 적어도, 내인성 DR6 세포질 도메인 전체 또는 그의 임의의 일부분 전체 (예를 들어, DR6 세포질 사멸 도메인 전체)를 인코딩하는 뉴클레오타이드 서열이 결여되고, 선택적으로, 내인성 DR6 막 관통 도메인 또는 그의 임의의 일부분 전체 및/또는 내인성 DR6 세포 외 도메인 전체를 인코딩하는 추가의 뉴클레오타이드 서열이 추가로 결여된 변형 DR6 유전자좌에서 이형접합성 또는 동형접합성이 된다.

[0014] 일부 실시 형태에서, 본 명세서에 기재된 바와 같은 내인성 DR6 유전자좌의 변형 및/또는 상기 핵산의 발현은 뉴런, 신경교세포 또는 이들의 조합의 기능에 영향을 미친다. 일부 실시 형태에서, 상기 핵산의 발현은 동일한 유전적 배경, 예를 들어 상기 유전자 변형 설치류와 동일한 계통인 대조군 설치류와 비교하여, 예를 들어 (a) 척추후만증 (kyphosis); (b) 비정상적인 뒷다리 움켜쥐기; (c) 운동 협응과 운동 학습 능력의 결손, (d) 중량 감소 및 (e) 감각 지각의 저하로 나타날 수 있는 상위 및/또는 하위 운동 뉴런의 기능의 저하, 예를 들어 기능 장애를 초래한다. 일부 실시 형태에서, 본 명세서에 기재된 바와 같은 변형 DR6 유전자좌를 포함하고/하거나 상기 핵산을 발현하는 설치류는 운동 뉴런 기능 장애, 예를 들어 상위 운동 뉴런 및/또는 하위 운동 뉴런 기능 장애의 하나 이상의 증상을 나타낼 수 있다. 일부 실시 형태에서, 상기 증상은, 동일한 유전적 배경, 예를 들어 상기 유전자 변형 설치류와 동일한 계통인 대조군 설치류와 비교하여, (a) 척추후만증; (b) 비정상적인 뒷다리 움켜쥐기; (c) 운동 협응과 운동 학습 능력의 결손, (d) 중량 감소 및/또는 (e) 감각 지각의 저하이다. 일부 실시 형태에서, 상기 하나 이상의 증상은 진전, 경직성 마비 (강직), 비정상적인 반사 및 이들의 조합으로 이루어진 군으로부터 선택되는 상위 운동 뉴런 기능 장애를 포함한 ALS 유사 증상 및/또는 근력 약화와 소모,

근섬유다발수축, 및 이들의 조합으로 이루어진 군으로부터 선택되는 하위 운동 뉴런 기능 장애를 포함한 ALS 유사 증상이다. 이러한 ALS 유사 증상은 블라인드 주관적 ALS-TDI 신경학적 점수화(blinded subjective ALS-TDI neurological scoring), 로타로드 테스트(rotarod testing), 캣워크 테스트(catwalk testing), 오픈 필드 테스트(open field testing) 중 하나 이상을 사용하여, 확인될 수 있다. 또한, 본 명세서에 개시된 바와 같은 설치류는 8주령 내지 20주령의 대조군 야생형 동물과 비교하여 중량 증가의 감소를 나타낼 수 있다. 본 명세서에 개시된 바와 같은 설치류는, 상기 설치류가 로타로드 위에서의 시간의 결손, 보행성 운동 기능의 손상, 캣워크 능력의 저하 및 이들의 조합으로 나타나는 운동 뉴런 기능의 저하를 나타내는지 또는 그러하지 않은지에 관계없이, 통증 자극에 대한 반응 지체 시간의 결손을 또한 나타낼 수 있다.

[0015] 일 태양에서, 상기 설치류는 출생 시 지극히 정상적일 수 있으며 (예를 들어, 육안으로 볼 때, 정상적으로 보이고, 예를 들어, 운동 뉴런 기능 장애 및/또는 감각 지각의 저하가 블라인드 주관적 신경학적 점수화 검정, 로타로드 테스트, 캣워크 테스트, 오픈 필드 테스트, 중량 측정 및/또는 통증 종속 시험 (pain subjection test)에서 분명하지 않음), 나이가 들에 따라, 하나 이상의 가시적인 ALS 유사 증상이 발생하고, 예를 들어, 상기 설치류는 2주령, 3주령, 4주령, 5주령, 6주령 등 이후, 하나 이상의 ALS 유사 증상이 발생할 수 있다. 일 태양에서, 본 명세서에 기재된 바와 같은 설치류는 4주령 미만이며, 어떠한 ALS 유사 증상도 나타내지 않는다. 일 태양에서, 본 명세서에 기재된 바와 같은 설치류는 8주령 미만이며, 어떠한 ALS 유사 증상도 나타내지 않는다. 다른 태양에서, 상기 설치류는 22주령 이전에 ALS 유사 증상이 발생한다. 일 실시 형태에서, 상기 설치류는 적어도 16주령 이상이며, 대조군 설치류와 비교하여 하기 표현형 중 하나 이상을 나타낸다: (a) 척추후만증; (b) 비정상적인 뒷다리 움켜쥐기; (c) 운동 협응과 운동 학습 능력의 결손, (d) 중량 감소, 및 (e) 통증 자극에 대한 반응 지체 시간의 지연.

[0016] 본 명세서에 개시된 설치류의 유전자 발현 패턴, 예를 들어, 신경계 기관 (뇌, 척수 등)의 유전자 특징은 (1) 각각, ALS를 앓는 환자의 유전자 발현 패턴, 또는 상기 환자의 기관 (뇌, 척수 등)의 유전자 특징, 및/또는 (2) 각각, ALS의 다른 동물 모델, 예를 들어, SOD1 비-인간 동물의 유전자 발현 패턴, 또는 상기 비-인간 동물의 기관 (뇌, 척수 등)의 유전자 특징과 유사할 수 있다. 예를 들어, 본 명세서에 개시된 설치류의 뇌 및/또는 척수의 유전자 발현 패턴(들)은 인간 ALS 바이오세트 및/또는 쥐와 동물 SOD1 바이오세트와 상관관계가 존재할 수 있으며, 이는 면역 반응과 연결될 수 있다. 인간 ALS 및/또는 쥐와 동물 SOD1 바이오세트와의 상관관계는, ALS 또는 SOD1 동물 모델을 사용하여, 본 명세서에 개시된 설치류에서 관찰된 병리학이 인간에서 볼 수 있는 것과 매우 유사함을 시사한다. 일부 실시 형태에서, 본 명세서에 개시된 바와 같은 설치류는 면역 반응에 연결된 유전자 특징을 가질 수 있으나, 말초의 경우, 야생형 설치류와 비교하여 비정상적인 면역 반응을 나타내지 않을 수 있다.

[0017] 일 태양에서, 본 명세서에 개시된 바와 같은 설치류의 운동 뉴런의 수는 야생형 설치류의 운동 뉴런의 수와 유사하며, 예를 들어, 현저하게 크거나 작지 않다. 일부 실시 형태에서, 본 명세서에 개시된 바와 같은 설치류의 운동 뉴런은 야생형 설치류의 운동과 비교하여 증가된 산화 스트레스를 나타낸다.

[0018] 일 태양에서, 본 명세서에 기재된 바와 같은 설치류는 래트 또는 마우스이다. 일부 실시 형태에서, 상기 설치류는 129 계통, C57BL/6 계통, 및 혼합된 C57BL/6 x 129 계통으로 이루어진 군으로부터 선택된 계통의 마우스이다.

[0019] 또한, 본 명세서에 기재된 설치류로부터 단리되거나 유래된 조직 또는 세포, 예를 들어 배아 줄기 세포, 운동 뉴런 세포 등이 본 명세서에 제공되며, 이는 예를 들어, 조직학적으로 검사되고/되거나 배양될 수 있다. 또한, 본 명세서에 기재된 핵산이 제공된다.

[0020] 또한, 본 명세서에 기재된 설치류로부터 단리된 세포 또는 세포 집단을 배양하는 단계를 포함하는 방법이 제공되며, 이는 예를 들어, 시험관 내(*in vitro*) 조작에 유용한 운동 뉴런을 생성할 수 있다. 일부 실시 형태에서, 대조군 운동 뉴런 세포와 비교하여 증가된 산화 스트레스를 나타내는 운동 뉴런 집단을 제조하는 방법이 제공되며, 상기 방법은 본 명세서에 제공된 설치류로부터 유래된 배양체를 수립하는 단계 및 상기 배양체를 운동 뉴런으로 분화시키는 단계를 포함한다. 일부 실시 형태에서, 배양체는 내세포집단 배아 줄기 세포로부터 발생된다. 일부 실시 형태에서, 내세포집단 배아 줄기 세포를 단리하고, 예를 들어 배아 줄기 세포 배지 (ESM: DMEM + 15% 소 태아 혈청 + 페니실린/스트렙토마이신 + 글루타민 + 비 필수 아미노산 + 뉴클레오시드 + β -메르캅토에탄올 + 피루브산나트륨 + LIF)에서 2일 동안 배양하여, 배양체를 형성시킨다. 상기 배양체를, 예를 들어, 레티노산 및 평활화 작동제에서, 예를 들어 5일 동안 추가로 배양하기 전에 1 내지 3일, 예를 들어 2일 동안, 분화 배지 (최신 DMEM/F12 + 뉴런세포 기본 배지 + 10% 녹아웃 혈청 + 페니실린/스트렙토마이신 + 글루타민 + β -메르캅토

에탄올)에서 후속하여 배양할 수 있다. 본 명세서에 제공된 설치류에서 단리된 내세포집단 배아 줄기 세포로부터 발생된 분화 운동 뉴런을, 예를 들어 배아 줄기 세포-유래 운동 뉴런 배지 (ESMN: 뉴런세포 기본 배지 + 2% 말 혈청 + B27 + 글루타민 + 페니실린/스트렙토마이신 + β -메르캅토에탄올 + 10 ng/ml GDNF, BDNF, CNTF)에서 성숙시켜, 상기 변형 *DRG* 대립유전자 및/또는 핵산을 포함하지 않는 설치류에서 발생된 운동 뉴런 세포와 비교하여 증가된 산화 스트레스를 나타내는 안정한 운동 뉴런 세포주를 형성시킬 수 있다.

[0021] 또한, ALS를 치료, 억제 및/또는 저해하는 데 유용할 수 있는 것으로서, 운동 뉴런 기능 장애를 조절하기 위한 후보 체제를 확인하는 방법으로서, (a) 본 명세서에 개시된 설치류에 상기 체제를 투여하는 단계; 및 (b) 동일한 계통 구조를 가지나 상기 후보 체제가 제공되지 않은 시험 대조군 설치류와 비교하여, 상기 설치류에서의 상기 후보 체제의 효과를 결정하는 단계, 예를 들어, 대조군 설치류와 비교하여, 상기 체제가 상기 설치류에서 ALS 유사 증상 중 적어도 하나를 억제, 저해, 지연 및/또는 역전시키는지의 여부를 결정하는 단계를 포함하고; 상기 설치류에서, 하나 이상의 ALS 유사 증상, 예를 들어 척추후만증, 비정상적인 뒷다리 움켜쥐기, 운동 협응 결손, 운동 학습 능력 결손, 중량 감소 및 감각 지각 결손과 같은 운동 뉴런 기능 장애의 증상의 억제, 저해, 지연 및/또는 역전은 운동 뉴런 기능 장애의 치료, 예를 들어 ALS의 억제 및/또는 저해에 유용할 수 있는 후보 체제를 나타내는 방법이 본 명세서에 제공된다. 상기 체제는 상기 설치류에서 ALS 유사 증상의 발병 전에, 그와 동시에 또는 그 후에, 예를 들어 블라인드 주관적 ALS-TDI 신경학적 점수화, 로타로드 테스트, 캣워크 테스트, 오픈 필드 테스트, 중량 측정 또는 통증 자극에 대한 반응 지체 시간의 결정에 의한 운동 뉴런 기능 장애의 하나 이상의 증상의 검출 전에, 그와 동시에 또는 그 후에, 예를 들어 둘 이상의 상이한 시점에 투여될 수 있다.

[0022] 일부 실시 형태에서, 상기 후보 체제는 운동 뉴런 기능 장애의 적어도 하나의 증상을, 예를 들어, 10% 이상, 예를 들어, 15% 이상, 예를 들어, 20% 이상 조절한다. 다른 실시 형태에서, 상기 후보 체제의 존재는 운동 뉴런 기능 장애의 적어도 하나의 증상을, 예를 들어, 50% 이상, 예를 들어, 75% 이상, 예를 들어, 80% 이상, 예를 들어, 95% 이상, 예를 들어, 99% 이상 조절한다. 일부 실시 형태에서, 상기 후보 체제는 운동 뉴런 기능 장애의 적어도 하나의 증상을 억제한다. (1) 척추후만증 및 비정상적인 뒷다리 움켜쥐기 중 적어도 하나의 억제 및/또는 저해 및/또는 (2) 운동 협응 결손, 운동 학습 능력 결손, 중량 감소 및/또는 감각 지각 결손의 억제 및/또는 회복에 의해 상기 적어도 하나의 증상을 조절하는 후보 체제가 신경퇴행성 질환, 예를 들어 ALS의 치료에 유용할 수 있다.

[0023] 상기 체제가 상기 설치류에서, 대조군 설치류와 비교하여, ALS 유사 증상 중 적어도 하나를 억제, 저해, 지연 및/또는 역전시키는지의 여부를 결정하는 방법은 상기 설치류에서 단리된 조직 및/또는 세포를 검사하는 단계, 예를 들어, 조직화학; 세포, 조직 및/또는 기관 배양, 현미경 기법 및/또는 단백질, 예를 들어 마이엘린 결합 단백질 (MBP), 신경성장인자수용체 (NGFR), 콜린 아세틸트랜스퍼라제 (Chat), Mnx 호메오박스, 글루타메이트 [NMDA] 수용체 하위 단위 3B (Grin3b), 및 글루타메이트 수용체 2 (Gria2)의 발현의 평가에 의해, 상기 조직 및/또는 세포의 조직학, 기능 및 구조를 이해하기 위한 상기 조직 및/또는 세포의 분석 단계를 포함할 수 있다. 일 태양에서, 평가되는 세포는 뉴런, 신경교세포 또는 이들의 조합이다. 다른 태양에서, 평가되는 조직은 뇌 및/또는 척수이다. 상기 체제가 상기 설치류에서, 대조군 설치류와 비교하여, ALS 유사 증상 중 적어도 하나를 억제, 저해, 지연 및/또는 역전시키는지의 여부를 결정하는 방법은 주관적 ALS-TDI 신경학적 점수화, 로타로드 테스트, 캣워크 테스트, 오픈 필드 테스트, 중량 측정, 및/또는 통증 자극에 대한 반응 지체 시간의 결정을 또한 포함할 수 있다.

[0024] 일부 실시 형태에서, 시험되는 적어도 하나의 증상은 상위 운동 뉴런 기능 장애를 나타내며, 예를 들어 상기 적어도 하나의 증상은 진전, 경직성 마비 (강직), 비정상적인 반사 및 이들의 조합으로 이루어진 군으로부터 선택된다. 일부 실시 형태에서, 시험되는 적어도 하나의 증상은 하위 운동 뉴런 기능 장애를 나타내며, 예를 들어 상기 적어도 하나의 증상은 근육 약화 및 소모, 근섬유다발수축 및 이들의 조합으로 이루어진 군으로부터 선택된다. 일부 실시 형태에서, 평가되는 적어도 하나의 증상은 중량 증가의 감소이다. 일부 실시 형태에서, 시험되는 적어도 하나의 기능은 통각수용의 저하이다.

[0025] 또한, 운동 뉴런에서 산화 스트레스를 감소시키기 위한 후보 체제를 확인하는 방법으로서, (a) 본 명세서에 개시된 바와 같은 비-인간 동물 모델로부터 유래된 운동 뉴런을 체제의 존재 또는 부재하에 배양하는 단계; (b) 상기 체제가 상기 운동 뉴런에서, 상기 체제의 부재하에 배양된 대조군 운동 뉴런과 비교하여, 산화 스트레스를 억제, 저해 및/또는 감소시키는지의 여부를 결정하는 단계를 포함하며, 상기 운동 뉴런에서의 산화 스트레스의 억제, 저해 및/또는 감소는, 예를 들어, 운동 뉴런에서 산화 스트레스를 감소시키기 위한 후보 체제를

나타내고, ALS의 치료, 억제 및/또는 저해에 유용한 후보 제제일 수 있는 방법이 본 명세서에 제공된다.

[0026] 일부 실시 형태에서, 상기 후보 제제는 운동 뉴런의 산화 스트레스를 10% 이상, 예를 들어, 15% 이상, 예를 들어, 20% 이상 감소시킨다. 기타 실시 형태에서, 상기 후보 제제의 존재는 운동 뉴런에 의한 산화 스트레스를, 예를 들어, 50% 이상, 예를 들어, 75% 이상, 예를 들어, 80% 이상, 예를 들어, 95% 이상, 예를 들어, 99% 이상 저해한다. 일부 실시 형태에서, 상기 후보 제제는 운동 뉴런에서 산화 스트레스를 억제하며, 예를 들어, 상기 산화 스트레스 수준은 야생형 운동 뉴런의 수준과 유사하다.

[0027] 또한, (a) 내인성 *DR6* 신호 펩티드 서열의 내인성 *DR6* 유전자와 하류에 삽입하기 위한 벡터를 유도하는, 5' 표적화 아암 및 3' 표적화 아암, (b) 선택 카세트, 및 선택적으로 (c) 막 관통 도메인 인코딩 서열을 포함하는 표적화 벡터가 본 명세서에 제공된다. 일 실시 형태에서, 막 관통 도메인 인코딩 서열은 *ROR1* 막 관통 도메인을 인코딩한다. 다른 실시 형태에서, 막 관통 도메인 인코딩 서열은 리포터 유전자에 작동가능하게 연결된다. 일 실시 형태에서, 선택 카세트는 리포터 유전자, 약물 내성 유전자 또는 이들의 조합을 포함한다. 다른 실시 형태에서, 선택 카세트는 약물 저항성 유전자를 포함한다. 일부 실시 형태에서, 선택 카세트는 네오마이신 포스포트랜스퍼라제 유전자를 포함한다. 일부 실시 형태에서, 본 명세서에 제공된 바와 같은 표적화 벡터는 서열 번호 16으로 제시된 서열을 포함한다. 일부 실시 형태에서, 표적화 벡터는 선택 카세트를 포함하지 않는다. 일부 실시 형태에서, 본 명세서에 제공된 바와 같은 표적화 벡터는 서열 번호 5로 제시된 서열을 포함한다.

[0028] 배아 줄기 세포, 예를 들어 쥐와 동물 배아 줄기 세포, 예를 들어 C57BL/6NTac 배아 줄기 세포와 같은 본 명세서에 기재된 바와 같은 표적화 벡터를 포함하는 설치류 세포가 또한 본 명세서에 제공된다.

도면의 간단한 설명

[0029] 도 1은 *DR6* 유전자좌의 표적화된 분열에 대한 전략을 도시한다. 야생형 마우스 *DR6* 유전자좌가 상단 라인으로 도시되며 (비례척을 사용하지 않음), 5'에서 3' 방향으로 5' UTR (흰색 박스), 개시 코돈 (ATG), 1 내지 6번 엑손 (점 찍힌 박스), 종결 코돈 (TAG) 및 3' UTR이 제시된다. 제시된 바와 같이, 내인성 마우스 신호 서열은 1번 엑손과 2번 엑손의 일부 (개시 코돈으로부터 ~4103번 염기)에 의해 인코딩되고, 신호 펩티드를 인코딩하는 핵산 서열은 하단 라인으로 도시된 (비례척을 사용하지 않음) Zen-Ub1 카세트에 의한 재조합 후 잔존한다. 도 1에 제시된 바와 같이, ZEN-Ub1 표적화 벡터 (서열 번호 5)는 (5'에서 3' 방향으로) 5' 상동성 아암 (5' 아암; 서열 번호 1), *ROR1* 막 관통 도메인 인코딩 서열 (TM; 서열 번호 7)에 작동가능하게 연결된 이. 콜라이(*E. coli*) LacZ 리포터 유전자 (LacZ; 서열 번호 8)에 작동가능하게 연결된 폴리아데닐화 신호 (pA; 서열 번호 9)를 포함한다. Zen-Ub1 카세트는 폴리A 신호 (pA; 서열 번호 13)에 작동가능하게 연결된 네오마이신 포스포트랜스퍼라제 저항성 유전자 (Neo; 서열 번호 12)에 작동가능하게 연결된 인간 유비퀴틴 C 유전자 (hUB; 서열 번호 11)의 프로모터를 포함하는 선택 카세트를 또한 포함하며, 상기 선택 카세트는 동일한 loxP 핵산 서열 (서열 번호 10)에 의해 플랭킹되어 있다. Zen-UB1 카세트 (3' 아암)의 3' 상동성 아암은 서열 번호 14로 제시되어 있다. 상동성 재조합 시에, 생성된 대립유전자는 *ROR1* 막 관통 도메인 인코딩 서열과 LacZ 리포터 유전자에 작동가능하게 연결된 내인성 *DR6* 신호 서열을 인코딩하는 핵산 서열을 포함하며, 상기 핵산 서열은 서열 번호 17로 제시되고, 2번 엑손의 4103번 염기에서 시작하여 6번 엑손의 종결 코돈까지의 전체 내인성 게놈 서열이 결여되어 있으며, 예를 들어, 내인성 세포질 도메인, 막 관통 도메인 및 세포 외 도메인을 인코딩하는 뉴클레오티드 서열이 결여되어 있다.

도 2a 내지 도 2i는 성별에 관계없이 시간 경과에 따라 빠르게 진행되는 *DR6*^{-/-} 마우스의 근 위축성 측삭 경화증 (ALS) 유사 운동 손상을 제시한다. 도 2a는 상이한 시점 (x-축)에서의 야생형 (-●-) 및 *DR6*^{-/-} 마우스 (-■-)의 평균 운동 손상 점수 (y-축)를 제시하는 한편, 도 2b는 상이한 시점 (x-축)에서의 수컷 (-▲-) 및 암컷 (-●-) *DR6*^{-/-} 마우스의 평균 운동 손상 점수를 제시한다. 도 2c는 상이한 시점 (x-축)에서의 야생형 (-●-) 및 *DR6*^{-/-} 마우스 (-■-)의 평균 강직 점수 (y-축)를 제시하는 한편, 도 2d는 상이한 시점 (x-축)에서의 수컷 (-▲-) 및 암컷 (-●-) *DR6*^{-/-} 마우스의 평균 강직 점수를 제시한다. 도 2e는 상이한 시점 (x-축)에서의 야생형 (-●-) 및 *DR6*^{-/-} 마우스 (-■-)의 평균 진전 점수 (y-축)를 제시하는 한편, 도 2f는 상이한 시점 (x-축)에서의 수컷 (-▲-) 및 암컷 (-●-) *DR6*^{-/-} 마우스의 평균 진전 점수를 제시한다. 도 2g는 9 내지 21주령 (x-축)에서의 야생형 (-●-) 및 *DR6*^{-/-} 마우스 (-■-)의 평균 운동 손상 점수 (y-축)를 제시한다. 도 2h는 9 내지 21주령 (x-축)에서의 야생형 (-●-) 및 *DR6*^{-/-} 마우스 (-■-)의 평균 강직 점수 (y-축)를 제시한다. 도 2i는 9 내지 21주령

(x-축)에서의 야생형 (●-) 및 DR6^{-/-} 마우스 (■-)의 평균 진전 점수를 제시한다. 실험 1 (도 2a 내지 도 2f)에서, 야생형 마우스는 n=12이고, DR6^{-/-} 마우스는 n=16이고 (수컷 DR6^{-/-} 마우스 n=8, 암컷 DR6^{-/-} 마우스 n=8), 각 시점에서 야생형 동물은 각각의 시험에 대해 0점이었다. 실험 2 (도 2g 내지 도 2i)에서, 야생형 마우스는 n=14이고, DR6^{-/-} 마우스는 n=18이다. 모든 데이터는 평균 ± SEM으로 보고되어 있다. 통계 분석을 위해 양방향 ANOVA를 수행하여 야생형 마우스의 값을 녹아웃 마우스의 값과 비교하며, *는 P ≤ 0.05, **는 P ≤ 0.01, ***은 P ≤ 0.001, 및 ****는 P ≤ 0.0001이다.

도 3a 내지 도 3f는, 중량 감소 및 유의한 운동 이상을 나타낸 DR6^{-/-} 마우스의 ALS 유사 표현형을 제시한다. 도 3a: 좌측 및 우측 패널은 각각 2014년 5월 29일과 2014년 6월 11일의 야생형 (tnsfr21^{+/+}) 및 DR6^{-/-} (tnsfr21^{-/-}) 마우스의 체중 (y-축; 그래프)을 제시한다. 도 3b: 좌측 및 우측 패널은 각각 야생형 (tnsfr21^{+/+}; 줄무늬 막대) 및 DR6^{-/-} (tnsfr21^{-/-}; 단색 막대) 마우스의 로타로드에서 추락할 때까지의 최대 지체 시간과 중위 지체 시간 (y-축; 초)을 제시한다. 도 3c: 60분 동안 야생형 (tnsfr21^{+/+}; 줄무늬 막대) 및 DR6^{-/-} (tnsfr21^{-/-}; 단색 막대) 마우스의 총 부동 상태 (y-축), 기본 이동 (y-축) 및 미세 운동 이동이 제시되어 있다. 도 3d: 야생형 (tnsfr21^{+/+}; 줄무늬 막대) 및 DR6^{-/-} (tnsfr21^{-/-}; 단색 막대) 마우스의 1시간 동안의 X+Y 보행 (y-축)이 제시되어 있다. 도 3e: 좌측 및 우측 패널은 각각 야생형 (tnsfr21^{+/+}; 줄무늬 막대) 및 DR6^{-/-} (tnsfr21^{-/-}; 줄무늬 막대)의 양육 합계 (y-축) 및 총 양육 시간 (y-축; 초)을 제시한다. 도 3f: 좌측 및 우측 패널은 각각 야생형 (tnsfr21^{+/+}; 줄무늬 막대) 및 DR6^{-/-} (tnsfr21^{-/-}; 단색 막대)의 총 이동 거리 및 총 휴식 시간 (y-축; 60분의 합계)을 제시한다. * (p < 0.05)는 두 그룹 사이에 통계적으로 유의한 차이를 나타낸다.

도 4a 내지 도 4c는 9 내지 21주령의 야생형 (●-; n=14) 및 DR6^{-/-} (■-; n=18) 마우스 (x-축)의 (A) 평균 체중 (y-축; 그래프 단위 중량) (B) 로타로드에서 추락할 때까지의 중위 지체 시간 (y-축; 초) 및 (C) 로타로드에서 추락할 때까지의 최대 시간 (y-축; 초)을 제시한다. 로타로드에서 소요된 최대 시간은 180초였다. 모든 데이터는 평균 ± SEM으로 보고되어 있다. 통계 분석을 위해 양방향 ANOVA를 수행하여 야생형 마우스의 값을 녹아웃 마우스의 값과 비교하며, *는 P ≤ 0.05, **는 P ≤ 0.01, ***은 P ≤ 0.001, 및 ****는 P ≤ 0.0001이다.

도 5a 내지 도 5h는 야생형 및 DR6^{-/-} 마우스의 오픈 필드 보행성 운동 거동, 예를 들어, 10 내지 21주령의 야생형 (●-; n=14) 및 DR6^{-/-} (녹아웃; ■-; n=16) 동물의 1시간 동안의 총 거리 (도 5a), 부동 상태 (도 5b), 총 양육 시간 (도 5c), 기본 이동 (도 5d), 미세 이동 (도 5e), X+Y 보행 (도 5f), 총 휴식 (도 5g) 및 양육 (도 5h)을 제시한다. 모든 데이터는 평균 ± SEM으로 보고되어 있다. 통계 분석을 위해 양방향 ANOVA를 수행하여 야생형 마우스의 값을 녹아웃 마우스의 값과 비교하며, *는 P ≤ 0.05, **는 P ≤ 0.01, ***은 P ≤ 0.001, 및 ****는 P ≤ 0.0001이다.

도 6a 내지 도 6f는 10 내지 21주령의 야생형 (●-; n=14) 및 DR6^{-/-} (녹아웃; ■-; n=18) 동물의 캣워크 거동, 예를 들어, 하지간 협응 (interlimb coordination) (도 6a), 발 압력 (도 6b), 발자국 면적 (도 6c), 보폭 길이 (도 6d), 입각기 (stance phase) (도 6e), 유각기 (swing phase) (도 6f), 유각 속도 (swing speed) (도 6g), 및 듀티 사이클 (도 6h)을 제시한다. 모든 데이터는 평균 ± SEM으로 보고되어 있다. 통계 분석을 위해 양방향 ANOVA를 수행하여 야생형 마우스의 값을 녹아웃 마우스의 값과 비교하며, *는 P ≤ 0.05, **는 P ≤ 0.01, ***은 P ≤ 0.001, 및 ****는 P ≤ 0.0001이다.

도 7a 내지 도 7g는 9 내지 21주령의 야생형 (●-, n=14) 및 DR6^{-/-} (■-; n=18) 동물 (x 축)의 앞다리와 뒷다리의 걸음걸이 능력 (gait ability)을 제시한다. 도 7a는 평균 앞발 압력 및 평균 뒷발 압력을 제공한다. 도 7b는 평균 앞발자국 면적 및 평균 뒷발자국 면적을 제공한다. 도 7c는 평균 앞다리 보폭 길이 및 평균 뒷다리 보폭 길이를 제공한다. 도 7d는 평균 앞다리 입각기 및 평균 뒷다리 입각기를 제공한다. 도 7e는 평균 앞다리 유각 속도 및 평균 뒷다리 유각 속도를 제공한다. 도 7f는 평균 앞다리 유각기 및 평균 뒷다리 유각기를 제공한다. 도 7g는 평균 앞다리 듀티 사이클 및 평균 뒷다리 듀티 사이클을 제공한다. 모든 데이터는 평균 ± SEM으로 보고되어 있다. 통계 분석을 위해 양방향 ANOVA를 수행하여 야생형 마우스의 값을 녹아웃 마우스의 값과 비교하며, *는 P ≤ 0.05, **는 P ≤ 0.01, ***은 P ≤ 0.001, 및 ****는 P ≤ 0.0001이다.

도 8은 10 내지 21주령의 야생형 (-●-) 및 DR6^{-/-} (녹아웃; -■-) 동물의 신경학적 점수, 예를 들어, 운동 손상 점수, 진전 점수 및 강직 점수를 제시한다. 모든 데이터는 평균 $\pm$ SEM으로 보고되어 있다. 통계 분석을 위해 양방향 ANOVA를 수행하여 야생형 마우스의 값을 녹아웃 마우스의 값과 비교하며, *는 $P \leq 0.05$, **는 $P \leq 0.01$, ***은 $P \leq 0.001$, 및 ****는 $P \leq 0.0001$ 이다.

도 9는 야생형 (좌측) 또는 DR6^{-/-} (우측) 동물로부터 단리되고 헤마톡실린 및 에오신에 의해 면역염색된 뇌의 대표 슬라이드를 상단 패널에 제시한다. 또한, 하단 패널에는 야생형 (좌측 막대) 또는 DR6^{-/-} (녹아웃, 우측 막대) 동물의 척수의 운동 뉴런의 수 (y-축; 평균 운동 뉴런 수)가 제시되어 있다.

도 10은 야생형 (좌측 막대) 또는 DR6^{-/-} (녹아웃; 우측 막대) 동물의 척수 (좌측 패널)와 뇌 (우측 패널)의 MBP (상단 패널) 또는 NGFR (하단 패널)의 mRNA의 수준 (β -액틴에 대비한 평균 mRNA 수준; y-축)을 제시한다.

도 11은 야생형 (좌측 막대) 또는 DR6^{-/-} (녹아웃; 우측 막대) 동물의 척수 (좌측 패널)와 뇌 (우측 패널)의 Chat (상단 패널) 또는 Mn1 (하단 패널)의 mRNA의 수준 (β -액틴에 대비한 평균 mRNA 수준; y-축)을 제시한다.

도 12는 야생형 (좌측 막대) 또는 DR6^{-/-} (녹아웃; 우측 막대) 동물의 척수 (좌측 패널)와 뇌 (우측 패널)의 Grin3b (상단 패널) 또는 Gria2 (하단 패널)의 mRNA의 수준 (β -액틴에 대비한 평균 mRNA 수준; y-축)을 제시한다.

도 13은 야생형 (WT; x-축) 또는 DR6^{-/-} (KO; x-축) 마우스의 흥선, 비장 또는 말초로부터 수득된 모 세포의 퍼센트 (모 %; y-축)로서 상이한 세포들의 수를 제시한다.

도 14는 야생형 (WT; x-축) 또는 DR6^{-/-} (KO; x-축) 마우스의 흥선, 비장 또는 말초로부터 수득된 모 세포의 퍼센트 (모 %; y-축)로서 상이한 세포들의 수를 제시한다.

도 15는 야생형 (n=6, 흑색 막대) 또는 DR6^{-/-} (n=6, 회색 막대) 동물의 혈청에서의 상이한 사이토카인 또는 케모카인 (x-축)의 농도 (평균 관찰 농도; y-축)를 제시한다.

도 16a는 야생형 동물 (DR6^{+/+}, 흑색 막대, 축)과 DR6^{+/-} 동물 (DR6^{+/-}; 바둑판 무늬 막대; x-축)로부터 수득된 배아 줄기 세포 유래 운동 뉴런의 배양 후 수득된 생존 세포의 수 (y-축)를 제시한다. 도 16b는 배아 줄기 세포 유래 운동 뉴런 (ESMN) 배지에서, 1일 또는 7일째 (x-축)에 야생형 (DR6^{+/+}, 흑색 막대)과 이형접합 동물 (DR6^{+/-}; 바둑판 무늬 막대)로부터 수득된 배아 줄기 세포 유래 운동 뉴런의 산화 스트레스 (평균 광학 밀도; y-축)를 제시한다. ****는 $P \leq 0.0001$ 이다.

도 17a는 10 내지 21주령의 야생형 (-●-, n=27), 이형접합 DR6^{+/-} (-■-; n=14), 및 동형접합 DR6^{-/-} (-▲-; n=33) 동물의 신경학적 점수, 예를 들어, 운동 손상 점수, 진전 점수 및 강직 점수를 제시한다. 도 17b는 10 내지 21주령의 야생형 (-●-, n=27), 이형접합 DR6^{+/-} (-■-; n=14), 및 동형접합 DR6^{-/-} (-▲-; n=33) 동물의 평균 체중 (y-축; 그램 단위 중량)을 제시한다. 도 17c의 상단 패널은 야생형 (-●-; n=13), 이형접합 DR6^{+/-} (-■-; n=14), 및 동형접합 DR6^{-/-} (-▲-; n=15) 동물의 로타로드에서 추락할 때까지의 중위 지체 시간 (y-축; 초)을 제시하며; 하단 패널은 12 내지 21주령의 야생형 (-●-; n=13), 이형접합 DR6^{+/-} (-■-; n=14), 및 동형접합 DR6^{-/-} (-▲-; n=15) 동물의 로타로드에서 추락할 때까지의 최대 시간 (y-축; 초)을 제시한다. 로타로드에서 소요된 최대 시간은 180초였다. 도 17d 내지 도 17k는 야생형, 이형접합 DR6^{+/-}, 및 동형접합 DR6^{-/-} 마우스의 오픈 필드 보행성 운동 거동, 예를 들어 10 내지 21주령의 야생형 (-●-; n=27), 이형접합 DR6^{+/-} (-■-; n=14), 및 동형접합 DR6^{-/-} (-▲-; n=33) 동물의 1시간 동안의 양육 (도 17d), 총 양육 시간 (도 17e), 기본 이동 (도 17f), 부동 상태 (도 17g), 미세 이동 (도 17h), X+Y 보행 (도 17i), 총 거리 (도 17j) 및 총 휴식 (도 17k)을 제시한다. 도 17l 내지 도 17z는 10 내지 21주령의 야생형 (-●-; n=27), 이형접합 DR6^{+/-} (-■-; n=14), 및 동형접합 DR6^{-/-} (-▲-; n=33) 동물의 캣워크 거동, 예를 들어 보폭 길이 (도 17l 및 도 17m), 유각 속도 (도 17n 및 도 17o), 하지간 협응 (도 17p), 유각기 (도 17q 및 도 17r), 듀티 사이클 (도 17s 및 도 17t), 발자국

면적 (도 17u 및 도 17v), 입각기 (도 17w 및 도 17x), 및 발 압력 (도 17y 및 도 17z)을 제시한다. 모든 데이터는 평균 $\pm$ SEM으로 보고되어 있다. 통계 분석을 위해 양방향 ANOVA를 수행하여 야생형 마우스의 값을 녹아웃 마우스의 값과 비교하며, *는 $P \leq 0.05$, **는 $P \leq 0.01$, ***은 $P \leq 0.001$, 및 ****는 $P \leq 0.0001$ 이다.

도 18은 48°C, 52°C, 또는 55°C의 일정한 온도로 가온된 금속 판에 적용된 야생형 (좌측 막대; n=13), 이형접합 DR6^{+/-} (중간 막대; n=14), 및 동형접합 DR6^{-/-} (우측 막대; n=15) 동물의 통각수용 반응에 대한 지체 시간 (시간; y-축)을 제시한다. 모든 데이터는 평균 $\pm$ SEM으로 보고되어 있다. 통계 분석을 위해 양방향 ANOVA를 수행하여 야생형 마우스의 값을 녹아웃 마우스의 값과 비교하며, *는 $P \leq 0.05$, **는 $P \leq 0.01$, ***은 $P \leq 0.001$, 및 ****는 $P \leq 0.0001$ 이다.

발명을 실시하기 위한 구체적인 내용

- [0030] 루게릭병이라고도 부르는 근 위축성 측삭 경화증 ("ALS")은 2만 명에 달하는 미국인이 앓고 있으며, 미국에서 매년 5,000 건의 신규 사례가 발생하는 치명적인 신경학적 진행성 질환이다. 상기 장애는 운동 뉴런 질환으로 알려진 장애의 한 부류에 속한다. ALS는 수의 운동을 제어하는 뇌와 척수의 특정 신경 세포가 점차적으로 퇴화될 때 발생한다. 뇌와 척수 둘 모두가 신체의 근육에 메시지를 개시 및 전달하는 능력을 상실한다. 기능할 수 없는 근육은 점차적으로 위축되고 단수축 (twitch)된다.
- [0031] ALS는 근육이 먼저 약화됨에 따라 다른 방식으로 자체적으로 나타난다. 증상은 떨어지기(tripping)와 넘어지기(falling), 손과 팔의 운동 제어 상실, 말하기, 연하 및/또는 호흡 곤란, 지속적인 피로감, 및 단수축과 경련을 포함할 수 있으며, 때때로 매우 중증의 증상으로 나타난다. 결국, 횡격막과 흉부 벽의 근육이 너무 약해지면, 환자는 호흡을 위해 인공 호흡기를 필요로 하게 된다. ALS를 앓고 있는 대부분의 사람들은 보통 진단 후 3년 내지 5년 후 호흡 부전으로 사망하나; 일부 사람들은 진단 후 10년 이상 생존한다. ALS는 중년기에 발생한다. 남성이 여성보다 상기 질환에 걸릴 가능성이 약 1.5배 높다.
- [0032] ALS에 대한 완치 사례는 없으며, 상기 장애의 진행을 억제하거나 역전시키는 입증된 치료법도 존재하지 않는다. 미국 식품의약국 (FDA)은 최근 ALS 환자의 생존 기간을 연장시키는 것으로 나타난 최초의 약물인 릴루졸 (riluzole)을 승인하였다. 환자는 또한 그 증상의 일부를 다루는 지지적 치료를 받을 수 있다.
- [0033] 인간에서 ALS를 치료하는 데 유용할 수 있는 후보 제제를 찾는 데 유용할 수 있는 ALS에 대한 동물 모델이 본 명세서에 제공된다.
- [0034] 본 명세서에 기재되거나 참조된 기술 및 절차는 당업자에 의해 일반적으로 잘 이해되며, 예를 들어, 문헌 [Sambrook et al., Molecular Cloning: A Laboratory Manual 2nd. edition (1989) Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.]에 기재된 광범위하게 사용되는 분자 클로닝 방법론과 같은 종래의 방법론을 사용하여 보통 사용된다. 적절하게, 구매가능한 키트 및 시약의 사용을 포함하는 절차는, 달리 명시되지 않는 한, 일반적으로 제조업체에 의해 규정된 프로토콜 및/또는 파라미터에 따라 수행된다.
- [0035] 본 방법 및 검정에 대해 기재하기 전에, 본 발명은 기재된 특정 방법론, 프로토콜, 세포주, 동물 종 또는 속, 작제물 및 시약에 제한되지 않으며, 따라서 물론 변화될 수 있음이 이해되어야 한다. 단수 형태("a", "an" 및 "the")는, 문맥이 명확하게 달리 지시하지 않는 한, 복수의 지시 대상을 포함한다는 것을 알아야 한다. 따라서, 예를 들어, "유전자 변이"에 대한 언급은 복수의 이러한 변이를 포함하며, "프로브"에 대한 언급은 당업자에 공지된 하나 이상의 프로브 및 그 균등물 등에 대한 언급을 포함한다. 본 명세서 및 관련된 청구 범위에 인용된 모든 숫자 (예를 들어, 아미노산 22 내지 81, 1 내지 354 등)는 용어 "약"에 의해 수식되는 것으로 이해된다.
- [0036] 본 명세서에 언급된 모든 간행물은, 인용된 간행물과 연계된 방법 및/또는 재료를 개시하고 기재하기 위해 본 명세서에 참고로 포함된다. 본 명세서에 인용된 간행물들은 본 출원의 출원일 이전의 그들의 개시에 대해 인용된다. 여기서, 어떠한 것도, 더 빠른 발명 우선일 또는 선행일로 인해, 본 발명자들이 상기 간행물들에 대해 선행 권리가 없다는 것을 인정하는 것으로 해석되어서는 안 된다. 또한, 실제 공개일은 표시된 것과 상이할 수 있으며, 별도의 확인이 필요할 수 있다.
- [0037] 정의
- [0038] 용어 "배아 줄기 세포" 또는 "ES 세포"는 배아로의 도입 시에 발생 중인 배아의 임의의 조직에 기여할 수 있는

배아 유래 전능성 또는 만능성 세포를 포함한다. 용어 "만능성 세포"는 하나 초과와 분화된 세포 유형으로 발현할 능력을 갖는 미분화된 세포를 포함한다.

[0039] 일부 표적화 벡터는 "대형 표적화 벡터" 또는 "LTVEC"이며, 이는 진핵 세포에서 상동성 유전자 표적화를 수행하도록 의도된 다른 접근법에 의해 전형적으로 사용되는 것보다 큰 핵산 서열에 상응하며 이로부터 유래되는 상동성 아암을 포함하는 진핵 세포에 대한 대형 표적화 벡터를 포함한다. LTVEC의 예는 박테리아 상동 염색체(BAC) 및 효모 인공 염색체(YAC)를 포함하지만, 이에 한정되지 않는다. LTVEC를 사용하여 표적화된 유전자 변형을 생성하는 예는, 예를 들어, 각각 모든 목적을 위하여 본 명세서에 전체적으로 참고로 포함된, 국제 특허 공개 WO 2015/088643호, 미국 특허 출원 공개 제2015/0159175호, 미국 특허 출원 공개 제2015/0159174호, 미국 특허 출원 공개 제2014/0310828호, 미국 특허 출원 공개 제2014/0309487호 및 미국 특허 출원 공개 제2013-0309670호에 개시되어 있다. LTVEC는 세포에서 상동성 재조합을 수행하도록 의도된 다른 접근법에 의해 전형적으로 사용되는 것보다 큰 핵산 서열을 갖는 핵산 삽입물을 포함하는 표적화 벡터를 또한 포함한다. 예를 들어, LTVEC는, 크기 제한 때문에 전통적인 플라스미드 기반 표적화 벡터에 의해 수용될 수 없는 큰 유전자좌의 변형을 가능하게 한다. 예를 들어, 표적화된 유전자좌는, 뉴클레아제 체계(예를 들어, Cas 단백질)에 의해 유도된 Nick 또는 이중-가닥 브레이크의 부재하에서 단지 부정확하게 또는 단지 유의하게 낮은 효율로 표적화될 수 있거나 종래의 방법을 사용하여 표적화될 수 없는 세포의 유전자좌일 수 있다(즉, 5' 및 3' 상동성 아암은 그러한 유전자좌에 상응할 수 있다).

[0040] LTVEC의 예는 박테리아 인공 염색체(BAC), 인간 인공 염색체 또는 효모 인공 염색체(YAC)로부터 유래된 벡터를 포함한다. LTVEC의 비제한적인 예 및 이들을 제조하는 방법은 예를 들어, 각각 본 명세서에 참고로 포함된, 미국 특허 제6,586,251호; 미국 특허 제6,596,541호; 미국 특허 제7,105,348호; 및 국제 특허 공개 WO 2002/036789호(PCT/US01/45375호)에 기재되어 있다. LTVEC는 선형 형태 또는 원형 형태일 수 있다.

[0041] LTVEC는 예를 들어, 10 kb 이상 또는 약 50 kb 내지 약 400 kb 이상을 포함하는 임의의 길이일 수 있다. LTVEC의 크기는 너무 커서 종래의 검정, 예를 들어 서던 블롯팅(southern blotting) 및 긴 범위(long-range)(예를 들어, 1 kb 내지 5 kb) PCR에 의한 표적화 사건의 스크리닝을 가능하게 할 수 없다. 5' 상동성 아암 및 3' 상동성 아암의 총합은, 예를 들어, 10 kb 이상일 수 있다(각 상동성 아암은, 예를 들어 약 5 kb 내지 약 200 kb의 범위일 수 있다). LTVEC 및 핵산 삽입물은, 예를 들어 약 5 kb 내지 약 3 Mb(예를 들어, 약 500 kb 이상)의 길이의 표적 유전자좌에서의 결실이 허용되도록 설계될 수 있다. 마찬가지로, LTVEC 및 핵산 삽입물은, 예를 들어 약 5 kb 내지 약 400 kb 이상의 범위의 길이의 외인성 핵산 서열의 표적 유전자좌 내로의 삽입이 허용되도록 설계될 수 있다.

[0042] 용어 "재조합 부위"는, 부위-특이적 재조합효소에 의해 인식되고 재조합 사건에 대한 기질의 역할을 할 수 있는 뉴클레오타이드 서열을 포함한다.

[0043] 용어 "부위-특이적 재조합효소"는 "재조합 부위들" 사이의 재조합을 촉진할 수 있는 효소 군을 포함한다. "부위-특이적 재조합효소"의 예는 Cre, F1p, 및 Dre 재조합효소를 포함하지만 이에 한정되지 않는다.

[0044] 핵산 서열과 관련하여 용어 "생식세포계열(germline)"은 자손으로 전해질 수 있는 있는 핵산 서열을 포함한다.

[0045] 어구 "작동가능하게 연결된"은 성분들이 그들의 의도된 방식으로 함께 기능하도록 연결되어 있음을 의미한다. 한 경우에, 단백질을 인코딩하는 핵산 서열이 적당한 전사 조절을 보유하도록 조절 서열(예를 들어, 프로모터, 인핸서, 사일렌서(silencer) 서열 등)에 작동가능하게 연결될 수 있다. 다른 경우에, 신호 펩티드를 인코딩하는 핵산 서열은, 예를 들어 막 관통 도메인을 인코딩하는 핵산 서열에 작동가능하게 연결되어 있어, 상기 막 관통 도메인에 작동가능하게 융합된 신호 펩티드를 포함하는 폴리펩티드로 번역될 수 있으며, 신호 펩티드와 막 관통 도메인 둘 모두는 그들의 각각의 생물학적 기능을 보유한다.

[0046] 용어 "유전자좌"는 게놈 DNA 내의 DNA의 분절로 정의된다. 예를 들어, DR6 유전자좌는 DR6을 인코딩하는 게놈 DNA 내의 DNA의 분절이며, DR6의 발현에 관여하는 비번역 및/또는 조절 DNA를 포함한다.

[0047] 사멸 수용체 6(DR6)(문헌에서 "TR9"로 또한 지칭되며; 문헌에서 TNF 수용체 슈퍼패밀리 멤버 21 또는 TNFRS21로 또한 알려져 있다)으로 불리는 TNFR 패밀리 멤버는 4개의 세포 외 시스테인-풍부 모티프 및 세포질 사멸 도메인 구조를 가지는 I 형 막 관통 수용체로 기재되어 왔다(문헌[Pan et al., FEBS Lett., 431:351-356(1998)]; 또한 미국 특허 제 6,358,508호; 제 6,667,390호; 제 6,919,078호; 제 6,949,358호 참조). 특정 형질감염 세포주에서 DR6의 과발현은 NF- κ B와 JNK 둘 모두의 활성화 및 세포 사멸을 야기하는 것으로 보고되어 있다(문헌[Pan et al., FEBS Letter, 431:351-356(1998)]). DR6 결손 마우스 모델에서, T 세포가 JNK 활성화

시에 실질적으로 손상되었으며, DR6 (-/-) 마우스를 단백질 항원으로 챌린징한 경우, 그의 T 세포가 과증식하여, Th2 반응에 대해 온전한 편향성을 나타내는 것으로 밝혀졌다 (반면, Th1 분화는 동일한 영향을 받지 않았다) (문헌 [Zhao et al., J. Exp. Med., 194:1441-1448 (2001)]). 또한, 시험관 내에서, DR6의 표적화된 분열이 T 헬퍼 2 (Th2) 분화의 향상을 야기하는 것으로 보고되었다 (상기 Zhao 등의 문헌). B 세포 매개 병증 또는 신경 병증의 조절에서 DR6 작동제 또는 길항제의 다양한 용도가 2005년 3월 31일자로 공개된 미국 특허 출원 공개 제2005/0069540호 및 2010년 8월 12일자로 공개된 미국 특허 출원 공개 제2010/0203044호에 기재되었다.

[0048] 쥐와 동물의 DR6 수용체 유전자좌는 17번 염색체에서 발견되며, 6번 엑손을 가진다. 이는 추정 신호 서열 (아미노산 1 내지 41), 세포 외 도메인 (아미노산 42 내지 349), 막 관통 도메인 (아미노산 350 내지 370), 이어서, 세포질 도메인 (아미노산 370 내지 655)을 가지는 655개의 아미노산 단백질 (서열 번호 16)을 인코딩한다. 성숙 DR6 단백질은 그의 신호 단백질의 절단 후 번역된 폴리펩티드를 지칭하며, 예를 들어, 성숙한 쥐와 동물의 DR6 단백질은 서열 번호 16의 아미노산 42 내지 655를 지칭한다.

[0049] 서열 번호에 의해 확인되는 서열에 대한 간단한 설명이 하기 표 1에 제공된다.

[0050] [표 2]

서열의 설명.

서열 번호	유형	설명
1	DNA	도 1에 도시된 Zen-Ub1 LTVEC의 5' 상동성 아암
2	DNA	β -갈락토시다제 및 폴리-A 영역에 작동가능하게 연결된 ROR1
3	DNA	loxP에 플랭킹된(floxed) <i>neo^r</i> 카세트
4	DNA	도 1에 도시된 Zen-Ub1 LTVEC의 3' 상동성 아암
5	DNA	도 1에 도시된 전체 Zen-Ub1 LTVEC
6	DNA	<i>mDR6</i> 신호 펩티드
7	DNA	<i>mROR1</i> 막 관통 도메인
8	DNA	β -갈락토시다제 리포터 유전자
9	DNA	<i>mDR6</i> 신호/ROR1/ β -갈락토시다제 융합 유전자의 폴리-A 영역
10	DNA	<i>Flox</i> 재조합 서열
11	DNA	<i>hUbl</i> 프로모터
12	DNA	<i>neo^r</i> 유전자
13	DNA	<i>neo^r</i> 유전자의 폴리-A 영역
14	DNA	<i>mDR6</i> (신호 펩티드 인코딩 서열을 포함함)
15	아미노산	<i>mDR6</i> (추정 신호 펩티드(들)를 포함함)
16	DNA	loxP에 플랭킹된 <i>neo^r</i> 카세트가 없는 도 1에 도시된 Zen-Ub1 LTVEC
17	DNA	<i>mDR6</i> 신호/ROR1/ β -갈락토시다제 융합 유전자

[0051]

[0052] I. 적어도 하나의 DR6 유전자좌의 유전자 변형을 포함하는 조성

[0053] 내인성 DR6 세포질 도메인의 임의의 일부분을 인코딩하는 뉴클레오티드 서열이 결여되고, DR6 신호 펩티드, 막 관통 도메인 및/또는 리포터 단백질을 인코딩하는 핵산 서열을 추가로 포함할 수 있는 변형 DR6 유전자좌를 포함하는 비-인간 동물, 세포, 조직 및 배아가 제공된다. DR6 발현을 조작하기 위한 방법 및 조성이 제공된다. DR6의 변형을 유도하는 표적화 조성이 또한 제공된다. DR6 기능의 조절과 관련된 운동 뉴런 기능 장애, 예를 들어, ALS 유사 표현형을 나타내는 비-인간 동물, 세포 및 조직이 제공된다. 하기 설명은 구체적인 특정 DR6의 조사된 사항에 관련된 것이나, 상기 방법 및 조성은 임의의 DR6을 사용하여 실행될 수 있다.

[0054] DR6 유전자좌의 표적화된 유전자 변형 (예를 들어, 리포터 녹인(knock-in))을 위해 사용될 수 있거나, DR6 기능에 영향을 줄 수 있는 변형 DR6 유전자좌 및/또는 핵산을 포함하는 비-인간 동물, 세포, 조직 및 배아가 본 명세서에 제공된다. 이러한 경우, 상기 변형 DR6 유전자좌는 DR6을 인코딩하는 핵산 서열에서의 기능 상실 돌연변이를 포함한다. 또한, DR6의 기능 상실 돌연변이를 포함하는 비-인간 동물 유래의 세포, 조직 및 배아가 제공된다.

[0055] DR6과 관련된 용어 "기능 상실"은 DR6의 발현의 감소 또는 결여 및/또는 DR6의 활성/기능의 감소 또는 결여를 야기하는 DR6 유전자좌의 임의의 변형 및/또는 트랜스진의 발현을 포함할 수 있다. DR6의 발현 수준은, 예를 들어, 세포 또는 유기체에서 DR6의 수준을 검정함으로써, 직접 측정할 수 있다.

[0056] 일반적으로, DR6 발현 수준 및/또는 DR6의 활성 수준이, DR6의 발현 및/또는 활성이 저해되도록 유전자 변형되거나 돌연변이가 유발되지 않은 적절한 대조군 세포 또는 유기체의 DR6 수준보다 통계적으로 낮은 경우 ($p \leq 0.05$), DR6의 발현 수준 및/또는 DR6의 활성이 저하된다. 구체적인 실시 형태에서, DR6의 농도 및/또는 활성은

DR6의 수준 및/또는 활성을 저하시키도록 변형되지 않은 대조군 세포 또는 유기체에 비해, 적어도 1%, 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 95%, 99% 또는 그 이상 저하된다.

[0057] 다른 경우에는, DR6의 발현 수준 및/또는 활성을 저하시키는 표적화된 유전자 변형을 갖는 세포 또는 유기체는 서던 블롯 분석(Southern blot analysis), DNA 염기 서열 결정(sequencing), PCR 분석, 또는 표현형 분석을 포함하지만 이에 한정되지 않는 방법을 사용하여 선택된다. 이어서, 이러한 세포 또는 유기체는 본 명세서에 기재된 다양한 방법 및 조성에 이용된다.

[0058] "대상 세포" 또는 "대상 유기체"는 유전자 변이, 예컨대 본 명세서에 개시된 유전자 변형을 일으킨 것이거나, 이렇게 변이된 세포/유기체의 자손이며 상기 변이를 포함하는 세포/유기체이다. "대조군" 또는 "대조군 세포" 또는 "대조군 유기체"는 대상 세포 또는 유기체의 표현형 변화를 측정하는 기준점을 제공한다. 일 실시 형태에서, 대조군 세포/유기체는, 발현 및/또는 활성의 저하를 야기하는 유전자 변형 또는 돌연변이가 결여된 것을 제외하고는, DR6에서 유전자 변형을 갖는 세포/유기체와 가능한 한 거의 일치한다 (예를 들어, 각 세포는 동일한 세포주로부터 유래할 수 있음). 다른 예에서, 대조군 세포/유기체는, 예를 들어: (a) 야생형 세포/유기체, 즉, 대상 세포/유기체를 초래하는 유전자 변이를 위한 출발 물질과 동일한 유전자형의 세포/유기체; (b) 널 작제물(null construct) (즉, 마커 유전자를 포함하는 작제물과 같은, 관심 특질에 대한 알려진 효과가 없는 작제물)에 의해 유전자 변형된 것을 제외하고는, 출발 물질과 동일한 유전자형의 세포/유기체; (c) 대상 세포/유기체의 비-유전자 변형 자손인 세포/유기체 (즉, 대조군 세포 및 대상 세포가 동일 세포주로부터 유래됨); (d) 대상 세포/유기체와 유전적으로 동일하나, 관심 유전자의 발현을 유도하는 조건 또는 자극에 노출되지 않은 세포/유기체; 또는, (e) 유전자 변형이 관심 폴리뉴클레오티드의 발현에 변이를 초래하지 않는 조건 하의 대상 세포/유기체 그 자체를 포함할 수 있다.

[0059] 동물, 세포, 조직 또는 배아와 관련하여 용어 "동물"은 포유류, 어류 및 조류를 포함한다. 포유류는, 예를 들어, 인간, 비-인간 영장류, 원숭이, 유인원, 고양이, 개, 말, 황소, 사슴, 들소, 양, 설치류 (예를 들어, 마우스, 래트, 햄스터, 기니피그), 가축 (예를 들어, 소 종(bovine species), 예를 들어, 암소, 거세 황소 등; 양 종(ovine species), 예를 들어, 양, 염소 등; 및 돼지 종, 예를 들어, 피그 및 수태지)을 포함한다. 조류는 예를 들어, 닭, 칠면조, 타조, 거위, 오리 등을 포함한다. 사육된 동물 및 농업용 동물이 또한 포함된다. 동물, 세포, 조직, 또는 배아와 관련하여, 어구 "비-인간 동물"은 인간을 제외한다.

[0060] 일 실시 형태에서, 동물은 비-인간 동물이다. 다른 실시 형태에서, 비-인간 동물은 포유류이다. 다른 실시 형태에서, 포유류는 설치류이다. 추가 실시 형태에서, 설치류는 마우스, 래트 또는 햄스터이다. 추가 실시 형태에서, 설치류는 마우스 또는 래트이다. 일부 실시 형태에서, 설치류는 마우스이다.

[0061] 본 명세서에 기재된 바와 같은 유전자 변형은 DR6 유전자좌로부터의 결실, DR6 유전자좌로의 첨가, DR6 유전자좌 또는 그 일부분의 대체 및/또는 이들의 임의의 조합 중 하나 이상을 포함할 수 있다. 유전자좌는 코딩 영역 또는 비-코딩 조절 영역을 포함할 수 있다. 본 명세서에 기재된 바와 같은 유전자 변형은 또한 DR6 유전자좌 외부의 게놈에서의 트랜스진의 삽입을 포함할 수 있으며, 상기 트랜스진의 발현은 리간드 결합, 세포막 내의 배치 등에 대해 내인성 DR6 단백질을 방해하고, 예를 들어 그와 경쟁한다.

[0062] 본 명세서에 제공된 유전자 변형은 DR6 유전자좌로 표적화될 수 있다. DR6의 기능 상실은 DR6 유전자의 표적화된 유전자 변형 (즉, 조절 영역, 코딩 영역, 엑손 및/또는 인트론 등에서의 유전자 변형)으로부터 야기될 수 있다. 이러한 표적화된 변형은 하나 이상의 뉴클레오티드의 첨가, 하나 이상의 뉴클레오티드의 결실, 하나 이상의 뉴클레오티드의 치환, DR6 유전자좌의 분열, DR6 유전자좌 또는 그 일부분의 녹아웃, DR6 유전자좌 또는 그 일부분의 녹인, 내인성 DR6 핵산 서열 또는 그 일부분의 이중성 핵산 서열로의 대체, 또는 이들의 조합을 포함하지만 이에 한정되지 않는다. 구체적인 실시 형태에서, 적어도 1, 2, 3, 4, 5, 7, 8, 9, 10, 50, 100, 400개 또는 그 이상의 뉴클레오티드가 변화되어 표적화된 게놈 변형을 형성한다.

[0063] 일 실시 형태에서, 기능 상실 돌연변이는 적어도 하나의 DR6 기능의 분열 또는 녹아웃을 특징으로 한다.

[0064] DR6 유전자좌는 상기 유전자좌의 임의의 영역에서 유전자 변형되어, 변형에 의해 DR6 기능의 조절이 야기되도록 할 수 있다. 일 실시 형태에서, DR6 유전자좌의 변형은 전체 DR6 코딩 영역 또는 그 일부분의 결실을 포함한다. 일 실시 형태에서, 변형 DR6 유전자좌는 성숙 DR6 단백질 또는 그 일부분을 인코딩하는 하나 이상의 엑손의 결실을 포함한다. 다른 실시 형태에서, 상기 결실은 DR6 유전자좌의 제1, 제2, 제3, 제4, 제5 및/또는 제6 엑손에서 개시되는 DR6 유전자좌 내의 하나 이상의 엑손의 결실을 포함한다. 다른 실시 형태에서, 상기 결실은 DR6 유전자좌의 제2 엑손에서 개시되는 DR6 유전자좌 내의 하나 이상의 엑손의 결실을 포함한다.

- [0065] 일부의 경우, DR6 유전자좌 또는 그 일부분은 삽입 핵산으로 대체된다. 이러한 경우, 상기 대체는 DR6 유전자좌 또는 그 일부분의 전체 RNA 코딩 영역의 삽입 핵산으로의 대체, DR6 유전자좌의 하나 이상의 엑손의 삽입 핵산으로의 대체, DR6 유전자좌의 제1 엑손에서 개시되는 DR6 유전자좌 내의 하나 이상의 엑손의 삽입 핵산으로의 대체 또는 제2 엑손에서 개시되는 DR6 유전자좌 내의 하나 이상의 엑손의 삽입 핵산으로의 대체일 수 있다.
- [0066] 일부 예에서, 삽입 핵산은, 내인성 DR6 프로모터와 작동가능하게 연결되어 내인성 DR6 프로모터가 삽입 핵산의 발현을 유도하도록 DR6 유전자좌에 위치한다. 이러한 경우, 상기 핵산 서열의 발현은 DR6의 발현 패턴을 따른다.
- [0067] 일 실시 형태에서, DR6 유전자좌 또는 그 일부분은, 리포터를 인코딩하는 제1 핵산 서열을 포함하는 삽입 핵산으로 대체된다. 예를 들어, 상기 삽입 핵산이 리포터 유전자를 포함하고, DR6 프로모터에 작동가능하게 연결되어 DR6 유전자좌 내에 위치하는 경우, 리포터 유전자의 발현은 내인성 DR6 프로모터에 의해 유도된다. 대안적으로, 상기 삽입 핵산은 내인성 DR6 프로모터와 작동가능하게 연결되어 삽입되지 않는다. 이러한 경우, 상기 삽입 핵산은 프로모터를 포함할 수 있다. 일 실시 형태에서, 삽입 핵산은, 리포터 유전자의 발현을 유도하는 프로모터에 작동가능하게 연결된 리포터 유전자를 포함한다.
- [0068] 일부 실시 형태에서, 삽입 핵산은 내인성 DR6 신호 서열을 포함할 수 있으며, 예를 들어, 내인성 DR6 신호 서열 및 선택적으로 내인성 DR6 막 관통 도메인과 작동가능하게 연결되도록 DR6 유전자좌에 위치할 수 있다. 이러한 경우, DR6 유전자좌에 대해 외인성으로 위치하거나, 내인성 DR6 유전자좌에 위치한 삽입 핵산에 의해 인코딩되는 임의의 단백질(들) (예를 들어, 리포터)의 목적지는 DR6의 목적지와 유사하다 (예를 들어, 내인성 막 관통 도메인에 의해 막에 고정됨). 일 실시 형태에서, 삽입 핵산은 내인성 막 관통 도메인을 대체할 수 있다. 이러한 경우와 다른 경우에, 삽입 핵산은, 이종성 막 관통 도메인을 인코딩하는 제2 핵산을 포함할 수 있다. 일 실시 형태에서, 삽입 핵산은 이종성 막 관통 도메인 유전자에 작동가능하게 연결된 리포터 유전자를 포함하며, 삽입 핵산은, 내인성 DR6 프로모터에 의해 상기 삽입 핵산의 발현이 유도되도록 내인성 DR6 신호 서열에 작동가능하게 연결된 DR6 유전자에 삽입되고, 상기 삽입 핵산에 의해 인코딩되는 단백질(들)은 신호 서열에 따라 막에 고정된다.
- [0069] 일 실시 형태에서, DR6 유전자좌 또는 그 일부분은 선택가능한 마커를 인코딩하는 추가의 핵산 서열을 포함하는 삽입 핵산으로 대체된다. 이러한 경우, 상기 추가의 핵산 서열은 선택가능한 마커의 발현을 유도하는 프로모터에 작동가능하게 연결된다.
- [0070] 다른 실시 형태에서, DR6 유전자좌 또는 그 일부분은 막 관통 유전자, 리포터 유전자 및 선택가능한 마커 유전자를 포함하는 삽입 핵산으로 대체된다. 이러한 경우, 상기 리포터 유전자 및/또는 선택가능한 마커 유전자는 프로모터에 작동가능하게 연결될 수 있거나, 그러하지 않을 수 있다.
- [0071] 상기 방법 및 구성에 사용될 수 있는 다양한 프로모터가 본 명세서의 다른 부분에 제공된다.
- [0072] 이러한 유전자 변형 (표적 DR6의 발현 및/또는 활성의 저하 또는 조절을 야기하는 것을 포함함)은 또한 생식세포계열을 통해 전달될 수 있다. 구체적인 실시 형태에서, 유전자 변형은 원하는 표적 유전자좌의 녹아웃을 야기한다. 예를 들어, 이러한 비-인간 동물은 본 명세서의 다른 부분에서 논의된 바와 같이 다양한 실험 시스템에서 사용된다.
- [0073] 예를 들어, DR6 녹아웃은 DR6 기능, 발생 중 DR6의 역할, 및 다양한 세포 경로 및 질환, 특히 운동 뉴런 기능 장애, 예를 들어 ALS에서의 DR6의 역할을 연구하기 위한 동물 모델을 제공한다.
- [0074] 비-인간 동물, 세포, 조직 또는 배아에서 DR6 유전자좌의 유전자 변형을 위한 방법 및 구성이 본 명세서에 제공된다.
- [0075] DR6 유전자좌의 유전자 변형은 본 명세서의 다른 부분에 상세히 기재된 바와 같은 상기 유전자좌의 임의의 변형 (즉, 결실, 삽입, 대체 등)일 수 있다. 이러한 경우, 상기 유전자 변형은 DR6의 기능 상실을 야기한다. 일 실시 형태에서, 유전자 변형은 DR6의 분열 또는 녹아웃을 포함한다.
- [0076] A. 리포터의 녹인 대립유전자의 설계 및 제작
- [0077] 비제한적인 예로서, 삽입 핵산이 내인성 신호 서열에 작동가능하게 연결될 수 있도록 제2 엑손에 결실 개시점을 설정할 수 있다. 도 1은 DR6을 코딩하는 서열의 전부 또는 대부분의 표적화된 결실 및 *ROR1* (수용체 티로신 키나제-유사 고아 수용체 1 (receptor tyrosine kinase-like orphan receptor 1)) 유전자로부터의 막 관통 도메인 서열, β -갈락토시다제를 인코딩하는 이. 콜라이(*E. coli*) lacZ 유전자의 서열을 함유하는 카세트 및 G418-

저항성 ES 세포 콜로니의 선택을 위한 네오마이신 포스포트랜스퍼라제를 발현하는 카세트 (neo^r)로의 대체의 예를 제시한다. 표현형 분석 전에 Cre-매개 절제를 가능하게 하는 loxP 재조합효소 인식 부위는 약물 선택 카세트를 플랭킹한다.

[0078] LTVEC 표적화 벡터를 ES 세포에 도입하고, 대립유전자 변형 검정 (modification-of-allele assay) (문헌 [Frendewey, D., et al. (2010), Methods Enzymol 476, 295-307])에 의해 정확하게 표적화된 클론에 대해 스크리닝할 수 있다.

[0079] 다양한 방법을 사용하여 결실 또는 삽입과 같은 표적화된 변형을 갖는 세포를 확인할 수 있다. 이러한 방법은 표적 유전자좌에 표적화된 변형을 갖는 하나의 세포를 확인하는 단계를 포함할 수 있다. 스크리닝을 수행하여 변형된 게놈 유전자좌를 갖는 이러한 세포를 확인할 수 있다.

[0080] 스크리닝 단계는 모 염색체의 대립유전자 변형 (MOA)을 평가하는 정량적인 검정을 포함할 수 있다. 예를 들어, 실시간 PCR과 같은 정량적인 PCR (qPCR)을 통해 정량적인 검정을 수행할 수 있다. 실시간 PCR은 표적 유전자좌를 인식하는 제1 프라이머 세트 및 비표적화된 기준 유전자좌를 인식하는 제2 프라이머 세트를 이용할 수 있다. 프라이머 세트는 증폭된 서열을 인식하는 형광 프로브를 포함할 수 있다.

[0081] 적합한 정량적인 검정의 다른 예는 형광 매개 원위치 혼성화(fluorescence-mediated in situ hybridization, FISH), 비교 게놈 혼성화, 등은 DNA 증폭, 고정화된 프로브(들)에 대한 정량적인 혼성화, Invader Probes®, MMP assays®, TaqMan® 분자 비콘(Molecular Beacon) 또는 Eclipse™ 프로브 기술을 포함한다 (예를 들어, 본 명세서에 전체적으로 참고로 포함된 미국 특허 출원 공개 제2005/0144655호 참조).

[0082] VelociMouse® 방법 (문헌[Poueymirou, WT, et al. (2007), Nat Biotechnol 25, 91-99])이, 표적화된 ES 세포를, 동형접합성으로의 증식 또는 lacZ 발현 프로파일링을 위해 준비된 완전한 ES 세포-유래 F0 세대 이형접합 마우스로 전환시키기 위해 8-세포 배아 단계 주입에 적용될 수 있다. ZEN-Ub1 카세트를 보유한 마우스는, 임의의 loxP에 플랭킹된 neo^r 카세트를 제거하기 위해, Cre 결실자 마우스 계통 (예를 들어, 국제 특허 공개 WO 2009/114400호 참조)으로 증식될 수 있다.

[0083] DR6 리포터 녹인 동물을 생산하는 방법의 추가 세부 사항은 본 명세서의 실시예 1에 제공되어 있다.

[0084] *B. 리포터 발현 프로파일링*

[0085] 본 명세서의 다른 부분에 기재된 바와 같이, DR6 유전자좌의 유전자 변형은 DR6 유전자좌 또는 그 일부분의 삽입 핵산으로의 대체 또는 삽입/첨가를 포함할 수 있다. 일부의 경우, 삽입 핵산은 리포터 유전자를 포함한다. 일 실시 형태에서, 리포터 유전자는 내인성 DR6 프로모터와 작동가능하게 연결되어 DR6 유전자좌에 위치한다. 이러한 변형은 내인성 DR6 프로모터에 의해 유도되는 리포터 유전자의 발현을 가능하게 한다. 대안적으로, 리포터 유전자는 내인성 DR6 프로모터와 작동가능하게 연결되어 위치하지 않는다.

[0086] 임의의 리포터 (또는 검출가능한 모이어티(moiety))가, 잘 알려진 방법 및 조성으로 사용될 수 있다. 리포터의 비제한적인 예는, 예를 들어, β-갈락토시다제 (lacZ 유전자에 의해 인코딩됨), 녹색 형광 단백질 (GFP), 증강 녹색 형광 단백질 (eGFP), 애플럼(mPlum), 엠체리(mCherry), 티디토마토(tdTomato), 엠스트로베리(mStrawberry), 제이-레드(J-Red), 디에스레드(DsRed), 엠오렌지(mOrange), mKO, 엠시트린(mCitrine), 베누(Venu), 와이펫(YPet), 증강 황색 형광 단백질 (EYFP), 에메랄드(Emerald), 사이펫(CyPet), 시안 형광 단백질 (CFP), 세룰리안(Cerulean), T-사파이어(T-Sapphire), 루시페라제, 알칼리 포스파타제, 또는 이들의 조합을 포함한다.

[0087] 본 명세서에 기재된 방법 및 조성은 리포터 유전자의 부재하에 또는 임의의 리포터 유전자의 존재 하에 수행될 수 있다. 하기 설명은 β-갈락토시다제를 인코딩하는 lacZ 리포터 유전자를 이용하는 비제한적인 예이다.

[0088] *C. 표현형*

[0089] 내인성 DR6 유전자좌의 유전자 변형은 본 명세서에 제공된 비-인간 동물에서 다양한 표현형을 야기할 수 있다. 일 실시 형태에서, 내인성 DR6 유전자좌의 유전자 변형은 출생 시에 지극히 정상이나, 나이가 든 때에, 예를 들어, 1주령 후, 2주령 후, 3주령 후, 4주령 후, 5주령 후, 6주령 후, 7주령 후, 8주령 후 등에 ALS-유사 증상이 발생하는 비-인간 동물을 야기한다. 다른 실시 형태에서, 내인성 DR6 유전자좌의 유전자 변형은 하나 이상의 세포 유형, 예를 들어 뉴런 및/또는 신경교세포 및/또는 그 일부분, 예를 들어, 마이엘린의 비정상적인 기능을 야기한다. 뉴런은 감각 뉴런, 운동 뉴런, 및 중간 뉴런으로 보통 지칭되는 기타 모든 뉴런 유형을 포함한다.

신경교세포는 성상교세포, 희소돌기아교세포 등을 포함한다.

[0090] 용어 "ALS 유사 증상" 등은 일반적으로 "ALS와 관련된 증상" 또는 "상위 및/또는 하위 운동 뉴런 기능 장애로 인한 증상"을 의미한다. ALS 유사 증상은 뉴런, 예를 들어 운동 뉴런, 감각 뉴런 및/또는 중간 뉴런의 손상을 포함할 수 있다. 예를 들어, 상위 운동 뉴런과 관련된 ALS 유사 증상은 경직 (예를 들어, 경직성 마비, 강직), 증가되고/되거나 비정상적인 반사 (예를 들어, 바빈스키 징후), 진전 및 이들의 조합을 야기할 수 있다. 하위 운동 뉴런의 손상과 관련된 ALS 유사 증상은 근육 약화와 소모, 근섬유다발수축 및 이들의 조합, 및/또는 삼키는 동작의 불능을 야기하는 연수 손상 및 혀의 근섬유다발수축을 야기할 수 있다. ALS 유사 증상은 또한 하기 표현형 중 하나 이상을 포함할 수 있다: a) 척추후만증; b) 비정상적인 뒷다리 움켜쥐기, 끌기 또는 발가락 구부러짐; c) 운동 협응과 운동 학습 능력의 결손, 로타로드, 캣워크 및/또는 오픈 필드 테스트(들)에서의 결손; d) 척수에서의 운동 뉴런 상실; e) 척수에서의 성상세포증; f) 대조군 설치류와 비교한 중량 감소; g) 폴리-유비퀴틴화 단백질의 축적; h) ALS-TDI 신경학적 점수화 시스템을 사용한 신경학적 점수의 증가 및/또는 (i) 통증 자극에 대한 반응 지체 시간의 증가.

[0091] ALS-TDI 신경학적 점수화 시스템

- 0점: 마우스를 그의 꼬리에 의해 매달았을 때, 뒷다리가 측방향 중심선으로부터 멀리 완전히 신장되고, 2회 내지 3회 매달았을 때, 마우스가 2초 동안 이를 유지할 수 있다.
 1점: 꼬리로 매달린 동안 측방향 중심선으로의 다리 신장의 붕괴 또는 일부 붕괴 (약화) 또는 뒷다리의 전율.
 2점: 12인치 걷는 동안 발가락이 적어도 두 번 아래로 구부러지거나, 발의 임의의 일부가 사육장 바닥/테이블*을 따라 끌린다.
 3점: 뻗뻗한 마비 또는 최소한의 관절 운동, 발은 앞으로 움직이는 데 사용되지 않는다.
 4점: 마우스를 뒤집어 놓으면 그 후 30초 이내에 스스로 바로 잡지 못한다.

[0092]

[0093] II. 비-인간 동물에서 내인성 DR6 유전자좌를 변형시키는 방법

[0094] 비-인간 동물, 세포, 조직 또는 배아에서 내인성 DR6 유전자좌를 유전자 변형시키는 방법이 본 명세서에 제공된다. 일 실시 형태에서, 만능성 세포에서 DR6 유전자좌를 변형시키는 방법이 제공된다. 이러한 방법은 (a) DR6 유전자좌와의 상동성 재조합을 거칠 수 있는 5' 및 3' 상동성 아암에 의해 플랭킹된 삽입 핵산을 포함하는 표적화 작제물을 만능성 세포로 도입시키는 단계; 및 (b) DR6 유전자좌에서 표적화된 유전자 변형을 포함하는 변형 만능성 세포를 확인하는 단계를 포함한다. 이러한 방법에서, 유전자 변형은 DR6의 기능 상실을 야기한다. 일 실시 형태에서, 만능성 세포는 설치류 배아 줄기이다. 다른 실시 형태에서, 만능성 세포는 인간 iPS 세포이다.

[0095] A. 표적화 벡터 및 삽입 핵산

[0096] 본 명세서에 제공된 유전자 변형 비-인간 동물, 세포, 조직 또는 배아를 제조하는 방법에 사용되는 표적화 벡터 또는 표적화 작제물이 추가로 제공된다.

[0097] 일 실시 형태에서, DR6 유전자좌와의 상동성 재조합을 거칠 수 있는 5' 및 3' 상동성 아암에 의해 플랭킹된 삽입 핵산을 포함하는 표적화 벡터가 제공된다.

[0098] 표적화 벡터 및 상기 표적화 벡터의 성분 (즉, 삽입 핵산, 관심 폴리뉴클레오티드, 발현 카세트 등)의 예가 하기에 본 명세서에 상세하게 기재되어 있다.

[0099] i. 삽입 핵산

[0100] 용어 "삽입 핵산" 또는 "삽입 폴리뉴클레오티드"는 표적 유전자좌에 통합하기를 원하는 DNA의 분절을 포함한다. 일 실시 형태에서, 삽입 핵산은 하나 이상의 관심 폴리뉴클레오티드를 포함한다. 다른 실시 형태에서, 삽입 핵산은 하나 이상의 발현 카세트를 포함할 수 있다. 주어진 발현 카세트는 관심 폴리뉴클레오티드, 선택 마커 및/또는 리포터 유전자를 인코딩하는 폴리뉴클레오티드와 함께 발현에 영향을 미치는 다양한 조절 성분을 포함할 수 있다.

[0101] 임의의 관심 폴리뉴클레오티드가 다양한 삽입 폴리뉴클레오티드에 삽입되어 표적 게놈 유전자좌에 통합될 수 있다. 본 명세서에 개시된 방법은 표적화된 DR6 게놈 유전자좌에 통합될 적어도 1개, 2개, 3개, 4개, 5개, 6개 또는 그 이상의 관심 폴리뉴클레오티드를 제공한다.

[0102] 일 실시 형태에서, 삽입 핵산에 함유된 관심 폴리뉴클레오티드는 리포터를 인코딩한다. 다른 실시 형태에서, 관심 폴리뉴클레오티드는 선택가능한 마커를 인코딩한다.

[0103] 일 실시 형태에서, 관심 폴리뉴클레오티드는 부위-특이적 재조합 부위에 의해 플랭킹될 수 있다. 구체적인 실시 형태에서, 부위-특이적 재조합 부위는 리포터를 인코딩하는 분절 및/또는 선택가능한 마커를 인코딩하는 분

절을 플랭킹한다.

- [0104] 삽입 핵산 내에 포함될 수 있는 선택 마커 및 리포터 유전자를 포함하는 관심 폴리뉴클레오타이드의 비제한적인 예는 본 명세서의 다른 부분에서 상세히 논의된다.
- [0105] 표적 DR6 유전자좌에 통합될 때 삽입 폴리뉴클레오타이드 내의 관심 폴리뉴클레오타이드는 세포 내로 하나 이상의 유전자 변형을 도입할 수 있다. 유전자 변형은 내인성 핵산 서열의 결실 및/또는 표적 게놈 유전자좌 내로의 외인성 또는 이중성 또는 이중상동성 폴리뉴클레오타이드의 첨가를 포함할 수 있다. 일 실시 형태에서, 유전자 변형은 표적 게놈 유전자좌에서 내인성 핵산 서열의 관심 외인성 폴리뉴클레오타이드로의 대체를 포함한다. 따라서, 본 명세서에 제공되는 방법은 표적 DR6 유전자좌에서의 녹아웃, 결실, 삽입, 대체 ("녹인"), 점 돌연변이, 도메인 교체, 엑손 교체, 인트론 교체, 조절 서열 교체, 유전자 교체 또는 이들의 조합을 포함하는 유전자 변형의 생성을 가능하게 한다. 이러한 변형은 제1, 제2, 제3, 제4, 제5, 제6, 제7 또는 임의의 후속 삽입 폴리뉴클레오타이드를 표적 게놈 유전자좌로 통합 시에 발생할 수 있다.
- [0106] 삽입 폴리뉴클레오타이드 내에 있고/있거나 표적 게놈 유전자좌에 통합된 관심 폴리뉴클레오타이드는 그가 도입되는 세포에 상동성이거나 고유한 서열을 포함할 수 있거나; 관심 폴리뉴클레오타이드는 그가 도입되는 세포에 이중성일 수 있거나; 관심 폴리뉴클레오타이드는 그가 도입되는 세포에 외인성일 수 있거나; 관심 폴리뉴클레오타이드는 그가 도입되는 세포에 이중상동성일 수 있거나; 또는 관심 폴리뉴클레오타이드는 그가 도입되는 세포와 상이한 종으로부터 유래된 것일 수 있다. 서열과 관련하여, 용어 "상동성"은 세포에 고유한 서열이다. 서열과 관련하여, 용어 "이중성"은 외래종으로부터 유래된 서열이거나, 또는 동일 종으로부터 유래된 경우에는, 인간의 의도적인 개입에 의해 조성 및/또는 게놈 유전자좌에서 그의 고유한 형태로부터 실질적으로 변형된 서열이다. 서열과 관련하여, 용어 "외인성"은 외래종으로부터 유래된 서열이다. 용어 "이중상동성"은 다른 종 (즉, 종 변이체)에서의 공지된 참조 서열과 기능적으로 동등한 하나의 종으로부터 유래된 폴리뉴클레오타이드이다. 관심 폴리뉴클레오타이드는 원핵생물, 진핵생물, 비-인간, 설치류, 햄스터, 마우스, 래트, 인간, 원숭이, 조류, 농업용 포유류 또는 비농업용 포유류를 포함하지만 이에 한정되지 않는 임의의 관심 유기체로부터 유래될 수 있다. 관심 폴리뉴클레오타이드는 코딩 영역, 비코딩 영역, 조절 영역 또는 게놈 DNA를 추가로 포함할 수 있다. 따라서, 제1, 제2, 제3, 제4, 제5, 제6, 제7 및/또는 임의의 후속 삽입 폴리뉴클레오타이드는 이러한 서열을 포함할 수 있다.
- [0107] 일 실시 형태에서, 관심 폴리뉴클레오타이드는 상기에 기재된 바와 같이, 약 500개의 뉴클레오타이드 내지 약 200 kb의 범위일 수 있다. 관심 폴리뉴클레오타이드는 약 500개의 뉴클레오타이드 내지 약 5 kb, 약 5 kb 내지 약 200 kb, 약 5 kb 내지 약 10 kb, 약 10 kb 내지 약 20 kb, 약 20 kb 내지 약 30 kb, 약 30 kb 내지 약 40 kb, 약 40 kb 내지 약 50 kb, 약 60 kb 내지 약 70 kb, 약 80 kb 내지 약 90 kb, 약 90 kb 내지 약 100 kb, 약 100 kb 내지 약 110 kb, 약 120 kb 내지 약 130 kb, 약 130 kb 내지 약 140 kb, 약 140 kb 내지 약 150 kb, 약 150 kb 내지 약 160 kb, 약 160 kb 내지 약 170 kb, 약 170 kb 내지 약 180 kb, 약 180 kb 내지 약 190 kb 또는 약 190 kb 내지 약 200 kb일 수 있다.
- [0108] 폴리뉴클레오타이드 내에 있고/있거나 표적 게놈 유전자좌에 삽입된 관심 폴리뉴클레오타이드는 폴리펩티드를 인코딩할 수 있거나, RNA를 인코딩할 수 있거나, 이것은, 예를 들어 조절 서열, 프로모터 서열, 인핸서 서열, 전사 억제인자 결합 서열, Kozak 공통 분절, 개시 코돈을 포함하는 임의의 관심 조절 영역 또는 비코딩 영역, 또는 비단백질 코딩(non-protein-coding) 서열의 결실을 포함할 수 있지만, 단백질 코딩 서열의 결실은 포함하지 않는다. 게다가, 삽입 폴리뉴클레오타이드 내에 있고/있거나 표적 게놈 유전자좌에 삽입된 관심 폴리뉴클레오타이드는 신경계, 골격계, 소화계, 순환계, 근육계, 호흡계, 심혈관계, 림프계, 내분비계, 비뇨계, 생식계 또는 이들의 조합에서 발현되는 단백질을 인코딩할 수 있다.
- [0109] 일 실시 형태에서, 삽입 핵산은 내인성 유전자의 적어도 하나의 엑손의 녹인 대립유전자를 포함한다. 일 실시 형태에서, 삽입 핵산은 전체 내인성 유전자의 녹인 대립유전자 (즉, "유전자-교체 녹인")를 포함한다.
- [0110] 일 실시 형태에서, 삽입 핵산은, 예를 들어 프로모터, 인핸서 또는 전사 억제인자 결합 요소를 포함하는 조절 요소를 포함한다.
- [0111] 추가의 실시 형태에서, 삽입 핵산은 조건 대립유전자를 포함한다. 일 실시 형태에서, 조건 대립유전자는, 본 명세서에 전체적으로 참고로 포함된 미국 특허 출원 공개 제2011/0104799호에 기재된 바와 같은, 다기능 대립유전자이다. 구체적인 실시 형태에서, 조건 대립유전자는 하기를 포함한다: (a) 표적 유전자의 전사와 관련된 센스 배향의 작동 서열, 및 센스 또는 안티센스 배향의 약물 선택 카세트; (b) 안티센스 배향의 관심 뉴클레오타

드 서열 (NSI) 및 역전 모듈에 의한 조건 대립유전자 (COIN, 엑손-분할 인트론 및 가역적 유전자트랩형 모듈 사용; 예를 들어 전체적으로 참고로 포함된 미국 특허 출원 공개 제2011/0104799호 참조); 및 (c) 제1 재조합효소에 노출 시에 재조합되어, (i) 작동 서열 및 DSC가 결합되어 있고, (ii) 센스 배향의 NSI 및 안티센스 배향의 COIN을 함유하는 조건 대립유전자를 형성하는 재조합가능한 단위.

- [0112] 일 실시 형태에서, 삽입 핵산은 코딩 서열의 유전자 변형을 포함한다. 일 실시 형태에서, 유전자 변형은 코딩 서열의 결실 돌연변이를 포함한다. 일 실시 형태에서, 유전자 변형은 2개의 내인성 코딩 서열의 융합을 포함한다.
- [0113] 일 실시 형태에서, 유전자 변형은 비단백질 코딩 서열의 결실을 포함하나, 단백질 코딩 서열의 결실은 포함하지 않는다. 일 실시 형태에서, 비단백질 코딩 서열의 결실은 DR6 유전자좌 또는 그 일부분의 결실을 포함한다. 일 실시 형태에서, 비단백질 코딩 서열의 결실은 조절 요소의 결실을 포함한다. 일 실시 형태에서, 유전자 변형은 프로모터의 결실을 포함한다. 일 실시 형태에서, 유전자 변형은 프로모터 또는 조절 요소의 첨가를 포함한다. 일 실시 형태에서, 유전자 변형은 프로모터 또는 조절 요소의 대체를 포함한다.
- [0114] 일 실시 형태에서, 표적화 벡터의 핵산 서열은, 게놈에 통합되면, 포유류, 비-인간 동물 또는 비-인간 포유류 DR6 유전자좌의 영역의 유전자 변형을 생성하는 폴리뉴클레오티드를 포함할 수 있으며, DR6 유전자좌의 유전자 변형은 DR6의 기능 상실을 야기한다. 일 실시 형태에서, DR6 녹아웃 ("널 대립유전자")이 생성된다. 다른 실시 형태에서, DR6 유전자좌의 분열이 생성된다.
- [0115] 추가의 실시 형태에서, 삽입 핵산은 포유류, 비-인간 동물 또는 비-인간 포유류 DR6 유전자좌의 일부분의 삽입 핵산 서열로의 대체를 야기한다. 일 실시 형태에서, 삽입 핵산 서열은 리포터 핵산 서열이다.
- [0116] 주어진 삽입 폴리뉴클레오티드 및 대체되는 포유류, 비-인간 또는 비-인간 포유류 유전자좌의 상응하는 영역은 비-코딩 영역, 코딩 영역, 인트론, 엑손, 비번역 영역, 조절 영역, 프로모터, 또는 인핸서 또는 이들의 임의의 조합일 수 있다. 또한, 주어진 삽입 폴리뉴클레오티드 및/또는 결실되는 포유류, 비-인간 또는 비-인간 포유류 유전자좌의 영역은, 예를 들어, 10 내지 100개의 뉴클레오티드 길이, 100 내지 500개의 뉴클레오티드 길이, 500 개 내지 1 kb의 뉴클레오티드 길이, 1 kb 내지 1.5 kb의 뉴클레오티드 길이, 1.5 kb 내지 2 kb의 뉴클레오티드 길이, 2 kb 내지 2.5 kb의 뉴클레오티드 길이, 2.5 kb 내지 3 kb의 뉴클레오티드 길이, 3 kb 내지 5 kb의 뉴클레오티드 길이, 5 kb 내지 8 kb의 뉴클레오티드 길이, 8 kb 내지 10 kb의 뉴클레오티드 길이 이상을 포함하는 임의의 원하는 길이일 수 있다. 다른 예에서, 삽입 또는 대체되는 크기는 약 5 kb 내지 약 10 kb, 약 10 kb 내지 약 20 kb, 약 20 kb 내지 약 40 kb, 약 40 kb 내지 약 60 kb이다. 다른 실시 형태에서, 주어진 삽입 폴리뉴클레오티드 및/또는 결실되는 포유류, 인간 세포 또는 비-인간 포유류 유전자좌의 영역은 적어도 100, 200, 300, 400, 500, 600, 700, 800, 또는 900개의 뉴클레오티드 또는 적어도 1 kb, 2 kb, 3 kb, 4 kb, 5 kb, 6 kb, 7 kb, 8 kb, 9 kb, 10 kb, 11 kb, 12 kb, 13 kb, 14 kb, 15 kb, 16 kb, 17 kb, 18 kb, 19 kb, 20 kb, 25 kb, 30 kb, 35 kb, 40 kb, 45 kb, 50 kb 이상이다.
- [0117] 일 실시 형태에서, 삽입 핵산은, 내인성 DR6 프로모터에 작동가능하게 연결되도록 관심 DR6 유전자좌에 삽입된다. 이러한 경우, DR6 프로모터는 삽입 핵산 서열의 발현을 유도한다. 일 실시 형태에서, 삽입 핵산 서열은 리포터 핵산 서열이다.
- [0118] 일부의 경우, 삽입 핵산은 프로모터를 포함한다. 일 실시 형태에서, 삽입 핵산은, 관심 폴리뉴클레오티드의 발현을 유도하는 프로모터에 작동가능하게 연결된 관심 폴리뉴클레오티드를 포함한다. 일 실시 형태에서, 관심 폴리뉴클레오티드는 리포터 핵산 서열을 포함한다. 다른 실시 형태에서, 관심 폴리뉴클레오티드는 선택 마커 핵산 서열을 포함한다.
- [0119] 일 실시 형태에서, 프로모터는 항시적 활성 프로모터이다.
- [0120] 일 실시 형태에서, 프로모터는 유도성 프로모터이다. 일 실시 형태에서, 유도성 프로모터는 화학적 조절 프로모터이다. 일 실시 형태에서, 화학적 조절 프로모터는 알코올-조절 프로모터이다. 일 실시 형태에서, 알코올-조절 프로모터는 알코올 탈수소효소 (alcA) 유전자 프로모터이다. 일 실시 형태에서, 화학적 조절 프로모터는 테트라사이클린-조절 프로모터이다. 일 실시 형태에서, 테트라사이클린-조절 프로모터는 테트라사이클린-반응성 프로모터이다. 일 실시 형태에서, 테트라사이클린-조절 프로모터는 테트라사이클린 작동자 서열 (tetO)이다. 일 실시 형태에서, 테트라사이클린-조절 프로모터는 tet-On 프로모터이다. 일 실시 형태에서, 테트라사이클린-조절 프로모터는 tet-Off 프로모터이다. 일 실시 형태에서, 화학적 조절 프로모터는 스테로이드 조절 프로모터이다. 일 실시 형태에서, 스테로이드 조절 프로모터는 래트의 글루코코르티코이드 수용체의 프로

모터이다. 일 실시 형태에서, 스테로이드 조절 프로모터는 에스트로겐 수용체의 프로모터이다. 일 실시 형태에서, 스테로이드 조절 프로모터는 엑디손 수용체의 프로모터이다. 일 실시 형태에서, 화학적 조절 프로모터는 금속 조절 프로모터이다. 일 실시 형태에서, 금속 조절 프로모터는 금속단백질 프로모터이다. 일 실시 형태에서, 유도성 프로모터는 물리적 조절 프로모터이다. 일 실시 형태에서, 물리적 조절 프로모터는 온도 조절 프로모터이다. 일 실시 형태에서, 온도 조절 프로모터는 열 충격 프로모터이다. 일 실시 형태에서, 물리적 조절 프로모터는 광 조절 프로모터이다. 일 실시 형태에서, 광 조절 프로모터는 광 유도성 프로모터이다. 일 실시 형태에서, 광 조절 프로모터는 광 억제성 프로모터이다.

[0121] 일 실시 형태에서, 프로모터는 조직 특이적 프로모터이다. 일 실시 형태에서, 프로모터는 뉴런 특이적 프로모터이다. 일 실시 형태에서, 프로모터는 신경교 특이적 프로모터이다. 일 실시 형태에서, 프로모터는 근육 세포 특이적 프로모터이다. 일 실시 형태에서, 프로모터는 심장 세포 특이적 프로모터이다. 일 실시 형태에서, 프로모터는 신장 세포 특이적 프로모터이다. 일 실시 형태에서, 프로모터는 골 세포 특이적 프로모터이다. 일 실시 형태에서, 프로모터는 내피 세포 특이적 프로모터이다. 일 실시 형태에서, 프로모터는 면역 세포 특이적 프로모터이다. 일 실시 형태에서, 면역 세포 프로모터는 B 세포 프로모터이다. 일 실시 형태에서, 면역 세포 프로모터는 T 세포 프로모터이다.

[0122] 일 실시 형태에서, 프로모터는 발생적 조절 프로모터이다. 일 실시 형태에서, 발생적 조절 프로모터는 배아 발생 단계에서만 활성이다. 일 실시 형태에서, 발생적 조절 프로모터는 성체 세포에서만 활성이다.

[0123] 구체적인 실시 형태에서, 프로모터는 세포 유형에 기초하여 선택될 수 있다. 따라서, 다양한 프로모터가 진핵 세포, 포유류 세포, 비인간 포유류 세포, 만능성 세포, 비인간 만능성 세포, 인간 만능성 세포, 인간 ES 세포, 인간 성체 줄기 세포, 발생적으로 제한된 인간 전구 세포, 인간 iPS 세포, 인간 세포, 설치류 세포, 래트 세포, 마우스 세포, 햄스터 세포, 섬유아세포 또는 CHO 세포에서 사용된다.

[0124] 일부 실시 형태에서, 삽입 핵산은 부위-특이적 재조합 표적 서열이 플랭킹된 핵산을 포함한다. 전체 삽입 핵산이 이러한 부위-특이적 재조합 표적 서열에 의해 플랭킹될 수 있지만, 삽입 핵산 내의 임의의 영역 또는 개별 관심 폴리뉴클레오타이드가 또한 이러한 부위에 의해 플랭킹될 수 있는 것으로 인식된다. 부위-특이적 재조합효소는, 재조합효소 폴리펩티드를 세포 내로 도입하거나 부위-특이적 재조합효소를 인코딩하는 폴리뉴클레오타이드를 숙주 세포 내로 도입하는 것을 포함하는 임의의 수단에 의해 세포 내로 도입될 수 있다. 부위-특이적 재조합효소를 인코딩하는 폴리뉴클레오타이드는 삽입 핵산 내에 또는 개별 폴리뉴클레오타이드 내에 위치할 수 있다. 부위-특이적 재조합효소는, 예를 들어, 유도성 프로모터, 세포에 대해 내인성인 프로모터, 세포에 대해 이종성인 프로모터, 세포 특이적 프로모터, 조직 특이적 프로모터, 또는 발생 단계 특이적 프로모터를 포함하는, 세포에서 활성인 프로모터에 작동가능하게 연결될 수 있다. 삽입 핵산 또는 삽입 핵산 내의 임의의 관심 폴리뉴클레오타이드를 플랭킹할 수 있는 부위-특이적 재조합 표적 서열은 loxP, lox511, lox2272, lox66, lox71, loxM2, lox5171, FRT, FRT11, FRT71, attP, att, FRT, rox, 또는 이들의 조합을 포함할 수 있지만 이에 한정되지 않는다.

[0125] 일부 실시 형태에서, 부위-특이적 재조합 부위는 삽입 핵산 내에 함유된 선택 마커를 인코딩하는 폴리뉴클레오타이드 및/또는 리포터 유전자를 플랭킹한다. 그러한 경우에, 표적화된 유전자좌에서의 삽입 핵산의 통합 후에, 부위-특이적 재조합 부위들 사이의 서열이 제거될 수 있다.

[0126] 일 실시 형태에서, 삽입 핵산은 선택 마커를 인코딩하는 폴리뉴클레오타이드를 포함한다. 선택 마커는 선택 카세트에 함유될 수 있다. 이러한 선택 마커는 네오마이신 포스포트랜스퍼라제(neo^r), 하이그로마이신 B 포스포트랜스퍼라제(hyg^r), 퓨로마이신-N-아세틸트랜스퍼라제(puro^r), 블라스티사이드인 S 데아미나제(bsr^r), 잔틴/구아닌 포스포리보실 트랜스퍼라제(gpt), 또는 단순 포진 바이러스 티미딘 키나제(HSV-k), 또는 이들의 조합을 포함하지만 이에 한정되지 않는다. 일 실시 형태에서, 선택 마커를 인코딩하는 폴리뉴클레오타이드는 세포에서 활성인 프로모터에 작동가능하게 연결된다. 일 실시 형태에서, 선택 마커를 인코딩하는 폴리뉴클레오타이드에는 부위-특이적 재조합 표적 서열이 플랭킹된다.

[0127] 삽입 핵산은 프로모터에 작동가능하게 연결된 리포터 유전자를 추가로 포함할 수 있으며, 상기 리포터 유전자는 β-갈락토시다제 (lacZ 유전자에 의해 인코딩됨), GFP, 앵플럼, 엠체리, 티디토마토, 엠스트로베리, 제이-레드, 디에스레드, 엠옐렌지, mKO, 엠시트린, 베누, 와이펫, 증강 황색 형광 단백질 (EYFP), 에메랄드, 증강 녹색 형광 단백질 (EGFP), 사이펫, 시안 형광 단백질 (CFP), 세룰리안, T-사파이어, 루시퍼라제, 알칼리 포스파타제, 및/또는 이들의 조합으로 이루어지거나 이를 포함하는 군으로부터 선택되는 리포터 단백질을 인코딩한다. 이러

한 리포터 유전자는 세포에서 활성인 프로모터에 작동가능하게 연결될 수 있다. 이러한 프로모터는 유도성 프로모터, 리포터 유전자 또는 세포에 대해 내인성인 프로모터, 리포터 유전자 또는 세포에 대해 이중성인 프로모터, 세포 특이적 프로모터, 조직 특이적 프로모터 또는 발생 단계 특이적 프로모터일 수 있다.

[0128] *ii. 발현 카세트*

[0129] DR6 유전자좌를 표적화하기 위해 본 명세서에 제공된 표적화된 게놈 통합 시스템에 사용되는 다양한 성분 (즉, 뉴클레아제 제제, 인식 부위, 삽입 핵산, 관심 폴리뉴클레오티드, 리포터 서열, 표적화 벡터, 선택 마커 및 다른 성분 중 임의의 하나 또는 임의의 조합)을 포함하는 폴리뉴클레오티드 또는 핵산 분자가 본 명세서에 제공된다.

[0130] 용어 "폴리뉴클레오티드", "폴리뉴클레오티드 서열", "핵산 서열" 및 "핵산 단편"은 본 명세서에서 상호교환적으로 사용된다. 이들 용어는 뉴클레오티드 서열 등을 포함한다. 폴리뉴클레오티드는, 선택적으로 합성, 비-천연 또는 변이된 뉴클레오티드 염기를 함유하는 단일- 또는 이중-가닥인 RNA 또는 DNA의 중합체일 수 있다. DNA의 중합체 형태의 폴리뉴클레오티드는 cDNA, 게놈 DNA, 합성 DNA, 또는 이들의 혼합물의 하나 이상의 분절로 구성될 수 있다. 폴리뉴클레오티드는 천연 분자 및 합성 유사체 둘 모두를 포함하는 데옥시리보뉴클레오티드 및 리보뉴클레오티드, 및 이들의 임의의 조합을 포함할 수 있다. 본 명세서에 제공된 폴리뉴클레오티드는 또한 단일 가닥 형태, 이중 가닥 형태, 헤어핀, 스템-앤드-루프 구조(stem-and-loop structure) 등을 포함하지만 이에 한정되지 않는 모든 형태의 서열을 포함한다.

[0131] DR6 유전자좌를 표적화하기 위한 표적화된 게놈 통합 시스템의 다양한 성분을 포함하는 재조합 폴리뉴클레오티드가 추가로 제공된다. 용어 "재조합 폴리뉴클레오티드" 및 "재조합 DNA 작제물"은 본 명세서에서 상호 교환적으로 사용된다. 재조합 작제물은 핵산 서열의 인공 또는 이중성 조합, 예를 들어 자연 상태에서는 함께 발견되지 않는 조절 서열 및 코딩 서열을 포함한다. 다른 실시 형태에서, 재조합 작제물은 상이한 공급원으로부터 유래된 조절 서열 및 코딩 서열, 또는 동일한 공급원으로부터 유래되지만 자연 상태에서 발견되는 것과 상이한 방식으로 배열된 조절 서열 및 코딩 서열을 포함할 수 있다. 이러한 작제물은 그 자체로 사용될 수 있거나 벡터와 함께 사용될 수 있다. 벡터가 사용되는 경우, 벡터의 선택은 당업자에게 잘 알려진 바와 같이 숙주 세포를 형질전환시키는 데 사용되는 방법에 의존한다. 예를 들어, 플라스미드 벡터가 사용될 수 있다. 본 명세서에 제공된 단리된 핵산 단편 중 임의의 것을 포함하는 숙주 세포를 성공적으로 형질전환, 선택 및 증식시키는 데 필요한 유전적 요소가 또한 제공된다. 스크리닝은 그 중에서도 DNA의 서던 분석, mRNA 발현의 노던(Northern) 분석, 단백질 발현의 면역블롯팅 분석, 또는 표현형 분석에 의해 달성될 수 있다.

[0132] 구체적인 실시 형태에서, 본 명세서에 기재된 DR6 유전자좌를 표적화하기 위한 표적화된 게놈 통합 시스템의 하나 이상의 성분은 원핵 세포, 진핵 세포, 박테리아, 효모 세포 또는 포유류 세포 또는 다른 관심 유기체 또는 세포 유형에서의 발현을 위한 발현 카세트에 제공될 수 있다. 카세트는 본 명세서에 제공된 폴리뉴클레오티드에 작동가능하게 연결된 5' 및 3' 조절 서열을 포함할 수 있다. "작동가능하게 연결된"은, 작동가능하게 연결된 성분이 그의 의도된 방식으로 기능하는 관계를 포함한다. 예를 들어, 관심 폴리뉴클레오티드와 조절 서열 (즉, 프로모터) 사이의 작동가능한 연결은 관심 폴리뉴클레오티드의 발현을 가능하게 하는 기능적 연결이다. 작동가능하게 연결된 요소는 인접하거나 인접하지 않을 수 있다. 2개의 단백질 코딩 영역의 결합을 지칭하는 데 사용될 때, '작동가능하게 연결된'은 코딩 영역이 동일한 리딩 프레임(reading frame)에 존재함을 의미한다. 다른 경우에, 단백질을 인코딩하는 핵산 서열이 적당한 전사 조절을 보유하도록 조절 서열 (예를 들어, 프로모터, 인핸서, 사일렌서 서열 등)에 작동가능하게 연결될 수 있다. 카세트는 유기체 내로 공동 도입되도록 적어도 하나의 추가의 관심 폴리뉴클레오티드를 추가로 함유할 수 있다. 대안적으로, 추가의 관심 폴리뉴클레오티드가 다수의 발현 카세트 상에 제공될 수 있다. 이러한 발현 카세트에는 조절 영역의 전사 조절 하에 있도록 재조합 폴리뉴클레오티드의 삽입을 위한 복수의 제한 부위 및/또는 재조합 부위가 제공된다. 발현 카세트는 추가적으로 선택 마커 유전자를 함유할 수 있다.

[0133] 발현 카세트는, 5'-3'의 전사 방향으로, 관심 포유류 세포 또는 숙주 세포에서 기능성인 전사 및 번역 개시 영역 (즉, 프로모터), 본 명세서에 제공된 재조합 폴리뉴클레오티드, 및 전사 및 번역 종결 영역 (즉, 종결 영역)을 포함할 수 있다. 본 명세서에 제공된 조절 영역 (즉, 프로모터, 전사 조절 영역, Kozak 서열, 및 번역 종결 영역) 및/또는 폴리뉴클레오티드는 숙주 세포에 대해 또는 서로에 대해 고유/유사할 수 있다. 대안적으로, 본 명세서에 제공된 조절 영역 및/또는 폴리뉴클레오티드는 숙주 세포에 대해 또는 서로에 대해 이중성일 수 있다. 예를 들어, 이중 폴리뉴클레오티드에 작동가능하게 연결된 프로모터는 폴리뉴클레오티드가 유래된 종과 상이한 종으로부터 유래되거나, 동일한/유사한 종으로부터 유래된 경우, 하나 또는 둘 모두가 실질적

으로 그들의 원래 형태 및/또는 게놈 유전자좌로부터 변형되거나, 프로모터는 작동가능하게 연결된 폴리뉴클레오티드의 고유 프로모터가 아니다. 대안적으로, 본 명세서에 제공된 조절 영역 및/또는 재조합 폴리뉴클레오티드는 전체적으로 합성될 수 있다.

[0134] 종결 영역은 전사 개시 영역에 대해 고유할 수 있거나, 작동가능하게 연결된 재조합 폴리뉴클레오티드에 대해 고유할 수 있거나, 숙주 세포에 대해 고유할 수 있거나, 프로모터, 재조합 폴리뉴클레오티드, 숙주 세포, 또는 이들의 임의의 조합에 대해 다른 공급원 (즉, 외래 또는 이종성)으로부터 유래될 수 있다.

[0135] 발현 카세트를 재조합에 있어서, 다양한 DNA 단편을 조작하여 적당한 배향으로 DNA 서열을 제공할 수 있다. 이러한 목적을 위해, 어댑터(adapter) 또는 링커(linker)를 사용하여 DNA 단편들을 결합할 수 있거나 다른 조작을 포함시켜 편리한 제한 부위, 피상적 DNA의 제거, 제한 부위의 제거 등을 제공할 수 있다. 이러한 목적을 위해, 시험관내 돌연변이 유발, 프라이머 수복, 제한, 어닐링, 재치환, 예를 들어 전이(transition) 및 역위(transversion)가 포함될 수 있다.

[0136] 다수의 프로모터가 본 명세서에 제공된 발현 카세트에 사용될 수 있다. 프로모터는 원하는 결과에 기반하여 선택될 수 있다. 관심 폴리뉴클레오티드의 발현의 시기, 위치 및/또는 수준을 조절하기 위해 발현 카세트에서 다양한 프로모터를 사용하여 다양한 적용이 향상될 수 있음이 인식된다. 이러한 발현 작제물은 또한, 원하는 경우, 프로모터 조절 영역 (예를 들어, 유도성, 항시적, 환경적으로 또는 발생적으로 조절되는, 또는 세포- 또는 조직-특이적/선택적 발현을 부여하는 것), 전사 개시 부위, Kozak 공통 서열, 리보솜 결합 부위, RNA 프로세싱 신호, 전사 종결 부위 및/또는 폴리아데닐화 신호를 함유할 수 있다.

[0137] 본 명세서에 제공된 폴리뉴클레오티드를 함유하는 발현 카세트는 또한 형질전환된 세포의 선택을 위한 선택 마커 유전자를 포함할 수 있다. 선택가능한 마커 유전자는 형질전환된 세포 또는 조직의 선택을 위해 이용된다.

[0138] 적절한 경우, 본 방법 및 조성에 사용된 서열 (즉, 관심 폴리뉴클레오티드, 뉴클레아제 제제 등)은 세포에서의 발현의 증가를 위해 최적화될 수 있다. 즉, 유전자는 개선된 발현을 위해, 예를 들어 포유류 선호 코돈, 인간 선호 코돈, 설치류 선호 코돈, 마우스 선호 코돈, 래트 선호 코돈, 햄스터 선호 코돈 등을 포함하는, 주어진 관심 세포에서 선호되는 코돈을 사용하여 합성될 수 있다.

[0139] 본 명세서에 제공되는 다양한 방법 및 조성은 선택 마커를 사용할 수 있다. 다양한 선택 마커가 본 명세서에 개시된 방법 및 조성에 사용될 수 있다. 그러한 선택 마커는, 예를 들어, 항생제, 예컨대 G418, 하이그로마이신, 블라스토사이드인, 네오마이신, 또는 퓨로마이신에 대한 저항성을 부여할 수 있다. 이러한 선택 마커는 네오마이신 포스포트랜스퍼라제 (neo^r), 하이그로마이신 B 포스포트랜스퍼라제 (hyg^r), 퓨로마이신-N-아세틸트랜스퍼라제 ($puro^r$) 및 블라스티사이드인 S 데아미나제 (bsr^r)를 포함한다. 또 다른 실시 형태에서, 선택 마커는 유도성 프로모터에 작동가능하게 연결되고 선택 마커의 발현은 세포에 독성이다. 이러한 선택 마커의 비제한적인 예는 잔틴/구아닌 포스포리보실 트랜스퍼라제 (gpt), 하이포잔틴-구아닌 포스포리보실트랜스퍼라제 (HGPRT) 또는 단순 포진 바이러스 티미딘 키나제 (HSV-TK)를 포함한다. 선택 마커를 인코딩하는 폴리뉴클레오티드는 세포에서 활성인 프로모터에 작동가능하게 연결되어 있다.

[0140] *iii. 표적화 벡터*

[0141] 표적화 벡터는 진핵생물, 비-인간, 포유류, 비-인간 포유류, 인간, 설치류, 마우스, 래트 또는 햄스터 핵산의 DR6 유전자좌로 삽입 핵산을 도입하는 데 사용된다. 표적화 벡터는 삽입 핵산을 포함하고, 상기 삽입 핵산을 플랭킹하는 5' 및 3' 상동성 아암을 추가로 포함한다. 삽입 핵산을 플랭킹하는 상동성 아암은 진핵생물, 비-인간, 포유류, 비-인간 포유류, 인간, 설치류, 마우스, 래트 또는 햄스터 핵산의 표적 DR6 유전자좌 내의 영역에 상응한다. 참고하기 쉽게, 표적화된 게놈 유전자좌 내의 상응하는 동족 게놈 영역은 "표적 부위"로 지칭된다. 예를 들어, 표적화 벡터는 제1 및 제2 표적 부위에 상보적인 제1 및 제2 상동성 아암에 의해 플랭킹된 제1 삽입 핵산을 포함할 수 있다. 이와 같이, 표적화 벡터는 그에 따라 세포의 게놈 내의 상보적인 표적 부위와 상동성 아암 사이에서 일어나는 상동성 재조합 사건을 통해 표적 유전자좌 핵산으로의 삽입 핵산의 통합을 돕는다.

[0142] 일 실시 형태에서, 진핵생물, 포유류, 비-인간 포유류, 인간, 설치류, 마우스 또는 햄스터 핵산의 표적 유전자좌는, 5' 상동성 아암에 상보적인 제1 핵산 서열 및 3' 상동성 아암에 상보적인 제2 핵산 서열을 포함한다. 일 실시 형태에서, 제1 및 제2 핵산 서열은 5 kb 이상만큼 분리되어 있다. 다른 실시 형태에서, 제1 및 제2 핵산 서열은 1 kb 이상 50 kb 미만만큼 분리되어 있다. 일 실시 형태에서, 제1 및 제2 핵산 서열은 2 kb 이상만큼 분리되어 있다. 일 실시 형태에서, 제1 및 제2 핵산 서열은 3 kb 이상, 4 kb 이상, 5 kb 이상, 6 kb 이상, 7

kb 이상, 8 kb 이상, 9 kb 이상, 10 kb 이상, 15 kb 이상, 20 kb 이상, 30 kb 이상, 40 kb 이상, 또는 50 kb 이상만큼 분리되어 있다. 더욱 추가의 실시 형태에서, 제1 및 제2 핵산 서열은 1 kb 이상 2 kb 미만, 2 kb 이상 3 kb 미만, 4 kb 이상 5 kb 미만, 5 kb 이상 6 kb 미만, 6 kb 이상 7 kb 미만, 7 kb 이상 8 kb 미만, 약 8 kb 이상 9 kb 미만, 9 kb 이상 10 kb 미만, 또는 10 kb 이상 15 kb 미만, 약 15 kb 이상 약 20 kb 미만, 약 20 kb 이상 약 30 kb 미만, 또는 약 40 kb 이상 약 50 kb 미만만큼 분리되어 있다.

[0143] 표적화 벡터의 상동성 아암은, 예를 들어 적어도 5 내지 10 kb, 5 내지 15 kb, 10 내지 20 kb, 20 내지 30 kb, 30 내지 40 kb, 40 내지 50 kb, 50 내지 60 kb, 60 내지 70 kb, 70 내지 80 kb, 80 내지 90 kb, 90 내지 100 kb, 100 내지 110 kb, 110 내지 120 kb, 120 내지 130 kb, 130 내지 140 kb, 140 내지 150 kb, 150 내지 160 kb, 160 내지 170 kb, 170 내지 180 kb, 180 내지 190 kb, 190 내지 200 kb의 길이 또는 그 이상을 포함하는, 상응하는 표적 서열과의 상동성 재조합 사건을 촉진시키기에 충분한 임의의 길이를 가질 수 있다. 아래에서 더욱 상세하게 서술되는 바와 같이, 대형 표적화 벡터는 더 긴 길이의 표적화 아암을 사용할 수 있다. 구체적인 실시 형태에서, 5' 상동성 아암 및 3' 상동성 아암의 총합은 10 kb 이상이거나, 5' 상동성 아암 및 3' 상동성 아암의 총합은 적어도 약 16 kb 내지 약 100 kb 또는 약 30 kb 내지 약 100 kb이다. 다른 실시 형태에서, LTVEC의 5' 상동성 아암과 3' 상동성 아암의 총합의 크기는 약 10 kb 내지 약 150 kb, 약 10 kb 내지 약 100 kb, 약 10 kb 내지 약 75 kb, 약 20 kb 내지 약 150 kb, 약 20 kb 내지 약 100 kb, 약 20 kb 내지 약 75 kb, 약 30 kb 내지 약 150 kb, 약 30 kb 내지 약 100 kb, 약 30 kb 내지 약 75 kb, 약 40 kb 내지 약 150 kb, 약 40 kb 내지 약 100 kb, 약 40 kb 내지 약 75 kb, 약 50 kb 내지 약 150 kb, 약 50 kb 내지 약 100 kb, 또는 약 50 kb 내지 약 75 kb, 약 10 kb 내지 약 30 kb, 약 20 kb 내지 약 40 kb, 약 40 kb 내지 약 60 kb, 약 60 kb 내지 약 80 kb, 약 80 kb 내지 약 100 kb, 약 100 kb 내지 약 120 kb, 또는 약 120 kb 내지 약 150 kb이다. 일 실시 형태에서, 결실의 크기는 LTVEC의 5' 상동성 아암과 3' 상동성 아암의 총합의 크기와 동일하거나 유사하다.

[0144] 상동성 아암 및 표적 부위 (즉, 동족 게놈 영역)는, 두 영역이 상동성 재조합 반응에 대한 기질로서 작용하기에 충분한 수준의 서열 동일성을 서로 공유하는 경우, 서로 "상보성" 또는 "상보적"이다. "상동성"이란, 상응하는 또는 "상보적인" 서열과 동일하거나 서열 동일성을 공유하는 DNA 서열을 의미한다. 주어진 표적 부위와, 표적화 벡터 상에서 확인되는 상응하는 상동성 아암 사이의 서열 동일성은 상동성 재조합이 발생하도록 허용하는 임의의 정도의 서열 동일성일 수 있다. 예를 들어, 표적화 벡터 (또는 이의 단편)의 상동성 아암과 표적 부위 (또는 이의 단편)에 의해 공유되는 서열 동일성의 양은 50% 이상, 55% 이상, 60% 이상, 65% 이상, 70% 이상, 75% 이상, 80% 이상, 81% 이상, 82% 이상, 83% 이상, 84% 이상, 85% 이상, 86% 이상, 87% 이상, 88% 이상, 89% 이상, 90% 이상, 91% 이상, 92% 이상, 93% 이상, 94% 이상, 95% 이상, 96% 이상, 97% 이상, 98% 이상, 99% 이상 또는 100% 서열 동일성일 수 있어서, 이들 서열은 상동성 재조합을 거치게 된다. 더욱이, 상동성 아암과 상보적인 표적 부위 사이의 상보적인 상동성 영역은 절단된 인식 부위에서 상동성 재조합을 촉진하기에 충분한 임의의 길이를 가질 수 있다. 예를 들어, 주어진 상동성 아암 및/또는 상보적인 표적 부위는 (예컨대, 본 명세서의 다른 부분에 기재된 LTVEC 벡터에서 기재된 바와 같이) 적어도 5 내지 10 kb, 5 내지 15 kb, 10 내지 20 kb, 20 내지 30 kb, 30 내지 40 kb, 40 내지 50 kb, 50 내지 60 kb, 60 내지 70 kb, 70 내지 80 kb, 80 내지 90 kb, 90 내지 100 kb, 100 내지 110 kb, 110 내지 120 kb, 120 내지 130 kb, 130 내지 140 kb, 140 내지 150 kb, 150 내지 160 kb, 160 내지 170 kb, 170 내지 180 kb, 180 내지 190 kb, 190 내지 200 kb, 200 kb 내지 300 kb 길이 또는 그 이상의 상보적인 상동성 영역을 포함할 수 있어서, 상동성 아암이 세포의 게놈 내의 상응하는 표적 부위와의 상동성 재조합을 거치기에 충분한 상동성을 갖게 된다. 참고하기 쉽게, 상동성 아암은 본 명세서에서 5' 및 3' 상동성 아암으로 지칭된다. 이 용어는 표적화 벡터 내의 삽입 핵산에 대한 상동성 아암의 상대적인 위치에 관한 것이다.

[0145] 그러므로, 표적화 벡터의 상동성 아암은 표적화된 유전자좌를 갖는 표적 부위에 상보적일도록 설계된다. 따라서, 상동성 아암은 세포에 고유한 유전자좌에 상보적일 수 있거나, 대안적으로 이는 트랜스진, 발현 카세트, 또는 게놈 DNA의 이종성 또는 외인성 영역을 포함하지만 이에 한정되지 않는, 세포의 게놈에 통합된 DNA의 이종성 또는 외인성 분절의 영역에 상보적일 수 있다. 대안적으로, 표적화 벡터의 상동성 아암은 적절한 숙주 세포에 함유된 인간 인공 염색체의 영역 또는 임의의 다른 유전자 조작된 게놈 영역에 상보적일 수 있다. 또한, 표적화 벡터의 상동성 아암은 BAC 라이브러리, 코스미드(cosmid) 라이브러리 또는 P1 파지 라이브러리의 영역에 상보적이거나 이로부터 유래될 수 있다. 따라서, 구체적인 실시 형태에서, 표적화 벡터의 상동성 아암은 주어진 세포에 대해 고유한, 이종성인 또는 외인성인 진핵생물, 비-인간, 포유류, 비-인간 포유류, 인간, 설치류, 마우스 또는 래트 게놈 유전자좌에 상보적이다. 일 실시 형태에서, 상동성 아암은 합성 DNA로부터 유래된다.

- [0146] 표적화 벡터 (예컨대, 대형 표적화 벡터)는 또한 본 명세서의 다른 부분에서 논의된 바와 같은 선택 카세트 또는 리포터 유전자를 포함할 수 있다. 선택 카세트는 선택 마커를 인코딩하는 핵산 서열을 포함할 수 있으며, 여기서 핵산 서열은 본 명세서의 다른 부분에서 논의된 바와 같은 프로모터에 작동가능하게 연결된다. 표적화 벡터의 선택 마커 및/또는 리포터 유전자는 5' 및 3' 상동성 아암에 의해 플랭킹될 수 있거나, 상동성 아암의 5' 또는 3' 중 하나에서 발견될 수 있다.
- [0147] 일 실시 형태에서, 표적화 벡터는, 리포터를 인코딩하는 제1 뉴클레오티드 서열을 포함하는 삽입 핵산을 포함한다. 일부의 경우, DR6 유전자좌와의 상동성 재조합 후, 리포터를 인코딩하는 제1 뉴클레오티드 서열은, DR6 유전자좌에서 DR6의 발현을 유도하는 내인성 프로모터에 작동가능하게 연결된다.
- [0148] 다른 실시 형태에서, 표적화 벡터는, 리포터를 인코딩하는 제2 뉴클레오티드 서열과 작동가능하게 연결된 막 관통 도메인을 인코딩하는 제1 뉴클레오티드 서열을 포함하는 삽입 핵산을 포함한다. 일부의 경우, DR6 유전자좌와의 상동성 재조합 후, 막 관통 도메인을 인코딩하는 제1 뉴클레오티드 서열은, 막 관통 도메인 및 리포터의 목적지를 유도하는 내인성 DR6 신호 서열에 작동가능하게 연결된다.
- [0149] 다른 실시 형태에서, 표적화 벡터의 삽입 핵산은, 선택가능한 마커를 인코딩하는 추가의 뉴클레오티드 서열을 포함한다. 일부의 경우, 추가의 핵산은 프로모터에 작동가능하게 연결된다.
- [0150] 일 실시 형태에서, 삽입 핵산의 제1 및/또는 추가 뉴클레오티드 서열은 Kozak 공통 서열을 포함한다.
- [0151] 일 실시 형태에서, 표적화 벡터 (예를 들어, 대형 표적화 벡터)는 본 명세서의 다른 부분에 기재된 바와 같은 프로모터에 작동가능하게 연결된 리포터 유전자 및/또는 선택가능한 마커 유전자를 포함한다. 이러한 리포터 유전자 및/또는 선택가능한 마커 유전자는 본 명세서의 다른 부분에 기재된 바와 같은 세포에서 활성인 프로모터에 작동가능하게 연결될 수 있다.
- [0152] 일 실시 형태에서, 표적화 벡터는 부위-특이적 재조합효소 유전자를 포함한다. 일 실시 형태에서, 부위-특이적 재조합효소 유전자는 Cre 재조합효소를 인코딩한다. 일 실시 형태에서, Cre 재조합효소 유전자는 Crei이며, Cre 재조합효소를 인코딩하는 2개의 엑손이 인트론에 의해 분리되어, 원핵 세포에서 그의 발현이 억제된다. 일 실시 형태에서, 부위-특이적 재조합효소 유전자는 Dre 재조합효소를 인코딩한다.
- [0153] 일 실시 형태에서, Cre 재조합효소 유전자는 핵에 대한 Cre (또는 임의의 재조합효소 또는 뉴클레아제 제제)의 위치화를 촉진시키기 위한 핵 위치화 신호를 추가로 포함한다 (예를 들어, 상기 유전자는 NLS-Cre 유전자임). 구체적인 실시 형태에서, Cre 재조합효소 유전자는 핵 위치화 신호 (NLS) 및 인트론 (예를 들어, NLS-Crei)을 추가로 포함한다.
- [0154] 다양한 실시 형태에서, 상기에서 논의된 Cre 또는 Crei 재조합효소의 발현에 적합한 프로모터는 Prm1, Blimp1, Gata6, Gata4, Igf2, Lhx2, Lhx5, 및/또는 Pax3으로부터 선택되거나, 그를 포함한다. 구체적인 실시 형태에서, 프로모터는 Gata6 또는 Gata4 프로모터이다. 상기 다양한 프로모터는, 예를 들어, 마우스 또는 래트와 같은 설치류, 진핵생물, 비-인간 포유류, 포유류, 인간 또는 햄스터를 포함한 임의의 유기체로부터 유래된 것일 수 있다. 구체적인 다른 실시형태에서, 프로모터는 Prm1 프로모터이다. 구체적인 다른 실시형태에서, 프로모터는 Blimp1 프로모터 또는 그의 단편, 예를 들어 Blimp1 프로모터의 1 kb 또는 2 kb 단편이다. 예를 들어, 각각 본 명세서에 전체적으로 참고로 포함되는 미국 특허 제8,697,851호, 미국 특허 제9,267,152호, 미국 특허 제9,096,870호, 미국 특허 제8,354,389호, 미국 특허 제8,946,505호, 미국 특허 제8,946,504호, 미국 특허 제8,518,392를 참조한다.
- [0155] 일 실시 형태에서, 삽입 핵산은 2개의 부위-특이적 재조합 부위에 의해 플랭킹된 뉴클레오티드 서열을 포함한다. 부위-특이적 재조합 부위의 예는 loxP, lox511, lox2272, lox66, lox71, loxM2, lox5171, FRT, FRT11, FRT71, attp, att, FRT, rox, 및 이들의 조합을 포함하지만 이에 한정되지 않는다.
- [0156] *iv. 대형 표적화 벡터*
- [0157] 용어 "대형 표적화 벡터" 또는 "LTVEC"는 세포에서 상동성 표적화를 수행하도록 의도된 다른 접근법에 의해 전형적으로 사용되는 것보다 큰 핵산 서열에 상응하며 이로부터 유래되는 상동성 아암을 포함하고/하거나 세포에서 상동성 재조합 표적화를 수행하도록 의도된 다른 접근법에 의해 전형적으로 사용되는 것보다 큰 핵산 서열을 포함하는 삽입 뉴클레오티드를 포함하는 대형 표적화 벡터를 포함한다. 구체적인 실시 형태에서, LTVEC의 상동성 아암 및/또는 삽입 폴리뉴클레오티드는 진핵 세포의 게놈 서열을 포함한다. LTVEC의 크기는 너무 커서 종래의 검정, 예를 들어, 서던 블롯팅 및 긴-범위 (예를 들어, 1 kb 내지 5 kb) PCR에 의한 표적화 사건의 스크리닝

을 가능하게 할 수 없다. LTVEC의 예는 박테리아 인공 염색체 (BAC), 인간 인공 염색체 또는 효모 인공 염색체 (YAC)로부터 유래된 벡터를 포함하지만 이에 한정되지 않는다. LTVEC의 비제한적인 예 및 이들을 제조하는 방법은 예를 들어, 각각 본 명세서에 참고로 포함된, 미국 특허 제6,586,251호, 제6,596,541호, 제7,105,348호 및 국제 특허 공개 WO 2002/036789호 (PCT/US01/45375호)에 기재되어 있다.

[0158] LTVEC는 적어도 약 10 kb, 약 15 kb, 약 20 kb, 약 30 kb, 약 40 kb, 약 50 kb, 약 60 kb, 약 70 kb, 약 80 kb, 약 90 kb, 약 100 kb, 약 150 kb, 약 200 kb, 약 10 kb 내지 약 15 kb, 약 15 kb 내지 약 20 kb, 약 20 kb 내지 약 30 kb, 약 30 kb 내지 약 50 kb, 약 50 kb 내지 약 300 kb, 약 50 kb 내지 약 75 kb, 약 75 kb 내지 약 100 kb, 약 100 kb 내지 125 kb, 약 125 kb 내지 약 150 kb, 약 150 kb 내지 약 175 kb, 약 175 kb 내지 약 200 kb, 약 200 kb 내지 약 225 kb, 약 225 kb 내지 약 250 kb, 약 250 kb 내지 약 275 kb 또는 약 275 kb 내지 약 300 kb를 포함하지만 이에 한정되지 않는 임의의 길이일 수 있다.

[0159] 일 실시 형태에서, LTVEC의 상동성 아암은 BAC 라이브러리, 코스미드 라이브러리, 또는 P1 파지 라이브러리로부터 유래된다. 다른 실시 형태에서, 상동성 아암은 세포의 표적화된 DR6 게놈 유전자좌로부터 유래되며, 일부 경우에, LTVEC가 표적화하도록 설계된 표적 게놈 유전자좌는 종래의 방법을 사용하여 표적화될 수 없다. 또 다른 실시 형태에서, 상동성 아암은 합성 DNA로부터 유래된다.

[0160] 일 실시 형태에서, LTVEC의 상류 상동성 아암과 하류 상동성 아암의 총합은 10 kb 이상이다. 다른 실시 형태에서, 상류 상동성 아암은 약 5 kb 내지 약 100 kb 범위이다. 일 실시 형태에서, 하류 상동성 아암은 약 5 kb 내지 약 100 kb 범위이다. 다른 실시 형태에서, 상류 및 하류 상동성 아암의 총합은 약 5 kb 내지 약 10 kb, 약 10 kb 내지 약 20 kb, 약 20 kb 내지 약 30 kb, 약 30 kb 내지 약 40 kb, 약 40 kb 내지 약 50 kb, 약 50 kb 내지 약 60 kb, 약 60 kb 내지 약 70 kb, 약 70 kb 내지 약 80 kb, 약 80 kb 내지 약 90 kb, 약 90 kb 내지 약 100 kb, 약 100 kb 내지 약 110 kb, 약 110 kb 내지 약 120 kb, 약 120 kb 내지 약 130 kb, 약 130 kb 내지 약 140 kb, 약 140 kb 내지 약 150 kb, 약 150 kb 내지 약 160 kb, 약 160 kb 내지 약 170 kb, 약 170 kb 내지 약 180 kb, 약 180 kb 내지 약 190 kb, 또는 약 190 kb 내지 약 200 kb이다. 일 실시 형태에서, 결실의 크기는 LTVEC의 5' 상동성 아암과 3' 상동성 아암의 총합의 크기와 동일하거나 유사하다.

[0161] 일 실시 형태에서, LTVEC는 본 명세서의 다른 부분에서 논의된 바와 같은 선택 카세트 또는 리포터 유전자를 포함한다.

[0162] *III. 서열을 도입하여 트랜스제닉 동물을 생산하는 방법*

[0163] 상기에서 개괄적으로 서술된 바와 같이, DR6 유전자좌의 표적화된 유전자 변형을 가능하게 하는 방법 및 조성이 본 명세서에 제공된다. 추가의 표적화된 유전자 변형이 이루어질 수 있다는 것이 추가로 인식된다. 이러한 표적화된 유전자 변형을 가능하게 하는 이러한 시스템은 다양한 성분을 사용할 수 있으며, 참고하기 쉽게, 본 명세서에서 용어 "표적화된 게놈 통합 시스템"은 일반적으로 통합 사건에 요구되는 모든 성분 (즉, 다양한 뉴클레아제 제제, 인식 부위, 삽입 DNA 폴리뉴클레오티드, 표적화 벡터, 표적 게놈 유전자좌 및 관심 폴리뉴클레오티드)을 포함한다.

[0164] 본 명세서에 제공된 방법은 표적화된 게놈 통합 시스템의 다양한 성분을 포함하는 하나 이상의 폴리뉴클레오티드 또는 폴리펩티드 작제물을 세포에 도입하는 것을 포함한다. "도입"은 서열이 세포 내부에 접근하도록 하는 방식으로 서열 (폴리펩티드 또는 폴리뉴클레오티드)을 세포에 제공하는 것을 의미한다. 본 명세서에 제공된 방법은 폴리뉴클레오티드가 적어도 하나의 세포 내부에 접근하는 것을 제외하고는, 표적화된 게놈 통합 시스템의 임의의 성분을 세포에 도입하는 특정 방법에 의존하지 않는다. 폴리뉴클레오티드를 다양한 세포 유형에 도입하는 방법은 당해 분야에 공지되어 있으며, 안정한 형질감염 방법, 일시적 형질감염 방법 및 바이러스 매개 방법을 포함하지만 이에 한정되지 않는다.

[0165] 일부 실시 형태에서, 상기 방법 및 조성에 사용되는 세포는 이들의 게놈에 안정하게 혼입된 DNA 작제물을 갖는다. "안정하게 혼입된" 또는 "안정하게 도입된"은, 뉴클레오티드 서열이 세포의 게놈에 통합되고 그의 자손에 의해 유전될 수 있도록 폴리뉴클레오티드를 세포에 도입하는 것을 의미한다. DNA 작제물 또는 표적화된 게놈 통합 시스템의 다양한 성분의 안정한 혼입을 위해 임의의 프로토콜을 사용할 수 있다.

[0166] 형질감염 프로토콜뿐만 아니라 폴리펩티드 또는 폴리뉴클레오티드 서열을 세포 내로 도입시키는 프로토콜도 다양할 수 있다. 화학 기반 형질감염 방법을 포함한 비제한적인 형질감염 방법은 리포솜; 나노입자; 인산칼슘 (문헌[Graham *et al.* (1973). *Virology* 52 (2): 456-67], 문헌[Bacchetti *et al.* (1977) *Proc Natl Acad Sci USA* 74 (4): 1590-4] 및 문헌[Kriegler, M (1991). *Transfer and Expression: A Laboratory Manual*. New

York: W. H. Freeman and Company. pp.96-97)]; 덴드리머 (dendrimer); 또는 DEAE-텍스트란 또는 폴리에틸렌 이민과 같은 양이온성 중합체의 사용을 포함한다. 비화학적 방법은 전기천공법; 초음파천공법(Sono-poration); 및 광학 형질감염을 포함한다. 입자-기반 형질감염은 유전자 총(gene gun), 자성 보조 형질감염 (문헌 [Bertram, J. (2006) Current Pharmaceutical Biotechnology 7, 277-28])의 사용을 포함한다. 바이러스 방법 도 형질감염에 사용될 수 있다.

[0167] 비-인간 동물은 본 명세서에 개시된 다양한 방법을 사용하여 생산될 수 있다. 이러한 방법은 (1) 본 명세서에 개시된 방법을 사용하여, 비-인간 동물의 만능성 세포의 관심 표적 DR6 게놈 유전자좌에 하나 이상의 관심 폴리뉴클레오티드를 통합하여, 표적화된 DR6 게놈 유전자좌에 삽입 폴리뉴클레오티드를 포함하는 유전자 변형된 만능성 세포를 생성하는 단계; (2) 표적 DR6 게놈 유전자좌에 하나 이상의 관심 폴리뉴클레오티드를 가지는 유전자 변형된 만능성 세포를 선택하는 단계; (3) 유전자 변형된 만능성 세포를, 예를 들어 상실기 이전 단계의 비-인간 동물의 숙주 배아에 도입하는 단계; 및 (4) 유전자 변형된 만능성 세포를 포함하는 숙주 배아를 대리모에 이식하여, 유전자 변형된 만능성 세포로부터 유래된 F0 세대를 생산하는 단계를 포함한다. 켈링징 표적 염색체 유전자좌를 표적화하도록 유사한 방법이 사용될 수 있다. 비-인간 동물은 비-인간 포유류, 설치류, 마우스, 래트, 햄스터, 원숭이, 농업용 포유류 또는 사육용 포유류, 또는 어류 또는 조류일 수 있다.

[0168] 만능성 세포는 인간 ES 세포, 비-인간 ES 세포, 설치류 ES 세포, 마우스 ES 세포, 래트 ES 세포, 햄스터 ES 세포, 원숭이 ES 세포, 농업용 포유류 ES 세포 또는 사육용 포유류 ES 세포일 수 있다. 다른 실시 형태에서, 만능성 세포는 비-인간 세포, 포유류 세포, 인간 세포, 비-인간 포유류 세포, 인간 만능성 세포, 인간 ES 세포, 인간 성인 줄기 세포, 발생적으로 제한된 인간 전구 세포, 인간 iPS 세포, 설치류 세포, 래트 세포, 마우스 세포, 햄스터 세포이다. 일 실시 형태에서, 표적화된 유전자 변형은 DR6의 기능 상실을 야기한다.

[0169] 마우스 만능성 세포, 전능성 세포 또는 숙주 배아는, 예를 들어, 근친 교배 계통, 잡종 계통, 및 이계 교배 계통을 포함하는 임의의 마우스 계통으로부터 유래된 것일 수 있다. 마우스 계통의 예는 129 계통, C57BL 계통 (예를 들어, C57BL/6 계통), 129 및 C57BL/6의 혼합 (예를 들어, 50% 129 및 50% C57BL/6), BALB/c 계통, 및 스위스 웹스터(Swiss Webster) 계통을 포함한다. 129 계통의 예는 129P1, 129P2, 129P3, 129X1, 129S1 (예를 들어, 129S1/SV, 129S1/SvIm), 129S2, 129S4, 129S5, 129S9/SvEvH, 129S6 (129/SvEvTac), 129S7, 129S8, 129T1, 및 129T2를 포함한다 (예를 들어, 문헌 [Festing et al. (1999) Revised nomenclature for strain 129 mice, Mammalian Genome 10:836] 참조). C57BL 계통의 예는 C57BL/A, C57BL/An, C57BL/GrFa, C57BL/KaLwN, C57BL/6, C57BL/6J, C57BL/6ByJ, C57BL/6NJ, C57BL/10, C57BL/10ScSn, C57BL/10Cr, 및 C57BL/01a를 포함한다. 마우스는 전술한 129 계통 (예를 들어, 129S6 (129/SvEvTac) 계통) 및 전술한 C57BL/6 계통의 혼합물, 하나 이상의 전술한 129 계통의 혼합물, 또는 하나 이상의 전술한 C57BL 계통의 혼합물일 수 있다. 마우스는 또한 129 계통을 제외한 계통으로부터 유래된 것일 수 있다.

[0170] 래트 만능성 세포, 전능성 세포 또는 숙주 배아는, 예를 들어 근친 교배 계통, 잡종 계통, 및 이계 교배 계통을 포함하는 임의의 래트 계통으로부터 유래된 것일 수 있다. 래트 계통의 예는 ACI 래트 계통, 다크 아구티(Dark Agouti; DA) 래트 계통, 위스타(Wistar) 래트 계통, LEA 래트 계통, 스프라그 다울리(Sprague Dawley; SD) 래트 계통, 또는 피셔(Fischer) 래트 계통, 예를 들어 피셔 F344 또는 피셔 F6을 포함한다. 래트 만능성 세포, 전능성 세포 또는 숙주 배아는 또한 상기에 열거된 둘 이상의 계통의 혼합체로부터 유래되는 계통으로부터 얻어질 수 있다. 예를 들어, 래트 만능성 세포, 전능성 세포 또는 숙주 배아는 DA 계통 및 ACI 계통으로부터 선택되는 계통으로부터 유래될 수 있다. ACI 래트 계통은 블랙 아구티(black agouti)를 가지며 배와 발이 흰색이고 RTI^{av1} 하플로타입(haplotype)을 갖는 것을 특징으로 한다. 이러한 계통은 할란 래보러토리즈(Harlan Laboratories)를 포함하는 다양한 공급처로부터 입수가능하다. ACI 래트 유래의 래트 ES 세포주의 예는 ACI.G1 래트 ES 세포이다. 다크 아구티 (DA) 래트 계통은 아구티 털(agouti coat) 및 RTI^{av1} 하플로타입을 갖는 것을 특징으로 한다. 이러한 래트는 찰스 리버 래보러토리즈(Charles River Laboratories) 및 할란 래보러토리즈를 포함하는 다양한 공급처로부터 입수가능하다. DA 래트 유래의 래트 ES 세포주의 예는 DA.2B 래트 ES 세포주 또는 DA.2C 래트 ES 세포주이다. 래트 계통의 다른 예는, 예를 들어, 각각 모든 목적을 위하여 본 명세서에 전체적으로 참고로 포함된 미국 특허 출원 공개 제2014/0235933호, 미국 특허 출원 공개 제2014/0310828호, 및 미국 특허 출원 공개 제2014/0309487호에 제공되어 있다.

[0171] 예를 들어, N2 보충제, B27 보충제, 약 50 U/mL 내지 약 150 U/mL의 백혈병 저해 인자 (LIF) 및 MEK 저해제 및 GSK3 저해제로 이루어진 저해제의 조합을 포함하는 배지를 사용하여, 단리된 래트 ES 세포를 피더 세포 층 상에서 배양함으로써, 생식세포계열-전달가능한 래트 ES 세포를 얻을 수 있고, 상기 피더 세포 층은 LIF를 발현하고

록 변형되지 않으며, 래트 ES 세포는 (i) 래트 ES 세포의 게놈 내로의 선택 마커를 포함한 이중성 폴리뉴클레오타이드의 적어도 하나의 삽입을 포함하는 표적화된 유전자 변형을 포함하도록 변형되었으며, 생식세포계열을 통해 상기 표적화된 유전자 변형을 전달할 수 있고; (ii) 정상 핵형을 가지며; (iii) c-Myc의 발현이 결여되어 있고; (iv) 배양 시에 구형의 자유-유동 콜로니를 형성한다 (예를 들어, 각각 전체적으로 참고로 포함된 미국 특허 출원 공개 제2014-0235933 A1호 및 미국 특허 출원 공개 제2014-0310828 A1호 참조). 래트 배아 줄기 세포의 유도 및 표적화된 변형의 다른 예는, 예를 들어, 문헌[Yamamoto et al. ("Derivation of rat embryonic stem cells and generation of protease-activated receptor-2 knockout rats," Transgenic Res. 21:743-755, 2012)] 및 문헌[Kwamata and Ochiya ("Generation of genetically modified rats from embryonic stem cells," Proc. Natl. Acad. Sci. USA 107(32):14223-14228, 2010)]에 제공되어 있다.

[0172] 또한, 핵 전달 기법을 사용하여 비-인간 동물을 생산할 수 있다. 간략히 말해서, 핵 전달을 위한 방법은 하기의 단계를 포함한다: (1) 난모세포를 제핵시키는 단계; (2) 제핵된 난모세포와 조합될 공여자 세포 또는 핵을 단리하는 단계; (3) 제핵된 난모세포 내로 상기 세포 또는 핵을 삽입하여, 재구성된 세포를 형성하는 단계; (4) 재구성된 세포를 동물의 자궁에 이식하여 배아를 형성하는 단계; 및 (5) 배아가 발생하도록 하는 단계. 이러한 방법에서, 난모세포는 일반적으로 사망한 동물로부터 회수되지만, 살아있는 동물의 난관 및/또는 난소로부터도 단리될 수 있다. 난모세포는 제핵 전에 당업자에게 공지된 다양한 배지에서 성숙될 수 있다. 난모세포의 제핵은 당업자에게 잘 알려진 다수의 방식으로 수행할 수 있다. 공여자 세포 또는 핵을 제핵된 난모세포 내로 삽입하여 재구성된 세포를 형성하는 것은 보통 융합 전에 투명대 (zona pellucida) 아래의 공여자 세포의 미세주입 (microinjection)에 의한다. 융합은 접촉면/융합면(전기융합)을 가로질러 DC 전기 펄스를 인가함으로써, 폴리에틸렌 글리콜과 같은 융합 촉진 화학 물질에 세포를 노출시킴으로써, 또는 센다이 바이러스(Sendai virus)와 같은 비활성화 바이러스에 의해 유도될 수 있다. 재구성된 세포는 전형적으로 핵 공여자 및 수용자 난모세포의 융합 전, 융합 동안, 및/또는 융합 후 전기적 및/또는 비-전기적 수단에 의해 활성화된다. 활성화 방법은 전기 펄스, 화학적으로 유도된 충격, 정자에 의한 침투, 난모세포에서의 2가 양이온 수준의 증가, 및 난모세포에서의 세포 단백질의 인산화 감소 (키나제 저해제에 의한 바와 같음)를 포함한다. 활성화된 재구성된 세포, 또는 배아는 전형적으로 당업자에게 잘 알려진 배지에서 배양되고 이어서 동물의 자궁으로 전달된다. 예를 들어, 각각 본 명세서에 참고로 포함된, 미국 특허 출원 공개 제20080092249호, 국제 특허 공개 WO/1999/005266A2호, 미국 특허 출원 공개 제20040177390호, 국제 특허 공개 WO/2008/017234A1호 및 미국 특허 제7,612,250호를 참조한다.

[0173] 하기의 단계를 포함하는 것으로서, 본 명세서에 기재된 바와 같은 생식세포계열의 하나 이상의 유전자 변형을 포함하는 비-인간 동물을 제조하는 다른 방법이 제공된다: (a) 본 명세서에 기재된 다양한 방법을 사용하여, 원핵 세포에서 비-인간 동물의 표적화된 게놈 DR6 유전자좌를 변형시키는 단계; (b) 표적화된 게놈 유전자좌에서 유전자 변형을 포함하는 변형된 원핵 세포를 선택하는 단계; (c) 변형된 원핵 세포의 게놈으로부터 유전자 변형된 표적화 벡터를 단리하는 단계; (d) 유전자 변형된 표적화 벡터를 비-인간 동물의 만능성 세포에 도입하여, 표적화된 DR6 게놈 유전자좌에서 삽입 핵산을 포함하는 유전자 변형된 만능성 세포를 생성하는 단계; (e) 유전자 변형된 만능성 세포를 선택하는 단계; (f) 유전자 변형된 만능성 세포를 상실기 이전 단계에서 비-인간 동물의 숙주 배아에 도입하는 단계; 및 (g) 유전자 변형된 만능성 세포를 포함하는 숙주 배아를 대리모에 이식하여, 유전자 변형된 만능성 세포로부터 유래된 F0 세대를 생산하는 단계. 그러한 방법에서, 표적화 벡터는 대형 표적화 벡터를 포함할 수 있다. 비-인간 동물은 비-인간 포유류, 설치류, 마우스, 래트, 햄스터, 원숭이, 농업용 포유류 또는 사육용 포유류일 수 있다. 만능성 세포는 인간 ES 세포, 비-인간 ES 세포, 설치류 ES 세포, 마우스 ES 세포, 래트 ES 세포, 햄스터 ES 세포, 원숭이 ES 세포, 농업용 포유류 ES 세포 또는 사육용 포유류 ES 세포일 수 있다. 다른 실시 형태에서, 만능성 세포는 비-인간 세포, 포유류 세포, 인간 세포, 비-인간 포유류 세포, 인간 만능성 세포, 인간 ES 세포, 인간 성인 줄기 세포, 발생적으로 제한된 인간 전구 세포, 인간 iPS 세포, 인간 세포, 설치류 세포, 래트 세포, 마우스 세포, 햄스터 세포이다. 일 실시 형태에서, 표적화된 유전자 변형은 DR6의 기능 상실을 야기한다.

[0174] 추가의 방법에서, 단리하는 단계 (c)는 (c1) 유전자 변형된 표적화 벡터 (즉, 유전자 변형된 LTVEC)를 선형화하는 단계를 추가로 포함한다. 추가의 실시 형태에서, 도입 단계 (d)는 (d1) 상동성 재조합을 촉진하기 위해 만능성 세포에 뉴클레아제 체제를 도입하는 단계를 추가로 포함한다. 일 실시 형태에서, 선택 단계 (b) 및/또는 (e)는 본 명세서에 기재된 바와 같은 선택가능한 체제를 원핵 세포 또는 만능성 세포에 적용함으로써 수행된다. 일 실시 형태에서, 선택 단계 (b) 및/또는 (e)는 본 명세서에 기재된 바와 같은 대립유전자의 변형(MOA) 검정을 통해 수행된다.

- [0175] 일부 실시 형태에서, 본 명세서에 기재된 표적 게놈 유전자좌의 다양한 유전자 변형은 VELOCIGENE® 유전자 공학 기술을 사용하여 박테리아 인공 염색체(BAC) DNA로부터 유래된 LTVEC를 사용한 박테리아 세포에서의 일련의 상동성 재조합 반응(BHR)에 의해 수행될 수 있다 (예를 들어, 본 명세서에 전체적으로 참고로 포함된, 미국 특허 제6,586,251호 및 문헌[Valenzuela, D. M. et al. (2003), Nature Biotechnology 21(6): 652-659] 참조).
- [0176] 일부 실시 형태에서, 본 명세서에 기재된 바와 같은 다양한 유전자 변형을 포함하는 DR6 표적화된 만능성 및/또는 전능성 세포는 VELOCIMOUSE® 방법을 통해, 삽입 공여자 세포로서 사용되며, 상응하는 유기체 유래의 상실기 이전 단계의 배아, 예를 들어 8-세포기 마우스 배아로 도입된다 (예를 들어, 모두 본 명세서에 전체적으로 참고로 포함된 미국 특허 제7,576,259호, 미국 특허 제7,659,442호, 미국 특허 제7,294,754호 및 미국 특허 출원 제2008-0078000 A1호 참조). 유전자 변형된 만능성 및/또는 전능성 세포를 포함하는 비-인간 동물 배아는 배반포기 (blastocyst stage)까지 인큐베이션된 다음에, 대리모에 이식되어, F0 세대를 생산한다. 일부 실시 형태에서, 본 명세서에 기재된 바와 같은 다양한 유전자 변형을 포함하는 표적화된 포유류 ES 세포는 배반포기 배아에 도입된다. 유전자 변형된 게놈 유전자좌 (즉, DR6 유전자좌)를 가지는 비-인간 동물은 본 명세서에 기재된 바와 같은 대립유전자의 변형 (MOA) 검정을 통해 확인될 수 있다. 유전자 변형된 만능성 및/또는 전능성 세포로부터 유래된 생성된 F0 세대 비-인간 동물을 야생형 비-인간 동물과 교배시켜 F1 세대 자손을 얻는다. 특정 프라이머 및/또는 프로브를 사용하여 유전자형을 결정한 후, 유전자 변형된 게놈 유전자좌에서 이형접합성인 F1 비-인간 동물을 서로 교배시켜, 유전자 변형된 게놈 유전자좌에서 동형접합성인 F2 세대 비-인간 동물 자손을 생산한다.
- [0177] 일 실시 형태에서, 적어도 하나의 DR6 유전자좌에서 유전자 변형을 포함하는 비-인간 동물을 제조하는 방법이 제공된다. 이러한 방법은 하기의 단계를 포함한다: (a) 만능성 세포를 5' 및 3' 상동성 아암에 의해 플랜킹된 삽입 핵산을 포함하는 표적화 작제물과 접촉시키는 단계로서, 상기 표적화 작제물은 상기 세포의 게놈에서 DR6 유전자좌와 상동성 재조합을 거쳐서, 변형된 만능성 세포를 형성하는, 단계; (b) 변형된 만능성 세포를 숙주 배아에 도입하는 단계; 및 (c) 숙주 배아를 대리모에 임신시키는 단계로서, 상기 대리모는 변형 DR6 유전자좌를 포함하는 자손을 생산하고, 상기 유전자 변형은 적어도 하나의 DR6의 기능 상실을 야기하는, 단계.
- [0178] IV. 세포
- [0179] 본 명세서에 기재된 다양한 방법은 세포에서 DR6 유전자좌를 변형시키기 위해 게놈 유전자좌 표적화 시스템을 사용한다. 그러한 세포는 원핵 세포, 예컨대 이. 콜라이를 포함하는 박테리아 세포, 또는 진핵 세포, 예컨대 효모, 곤충, 양서류, 식물, 또는 마우스 세포, 래트 세포, 햄스터 세포, 토끼 세포, 돼지 세포, 소 세포, 사슴 세포, 양 세포, 염소 세포, 닭 세포, 고양이 세포, 개 세포, 페럿 세포, 영장류 (예를 들어, 마모셋, 붉은털 원숭이) 세포 등 및 사육된 포유류로부터의 세포 또는 농업용 포유류로부터의 세포를 포함하지만 이에 한정되지는 않는 포유류 세포를 포함한다. 일부 세포는 비-인간, 특히 비-인간 포유류 세포이다. 일부 실시 형태에서, 적합한 유전자 변형가능한 만능성 세포가 용이하게 입수가능하지 않는 포유류의 경우, 예를 들어 Oct3/4, Sox2, KLF4, Myc, Nanog, LIN28 및 Glis1을 포함하지만 이에 한정되지 않는 만능성 유도 인자의 조합을 체세포에 도입하는 것을 통해, 체세포를 만능성 세포로 다시 프로그래밍하기 위한 다른 방법이 사용된다. 이러한 방법에서, 세포는 또한 포유류 세포, 인간 세포, 비-인간 포유류 세포, 비-인간 세포, 설치류, 래트, 마우스, 햄스터 유래의 세포, 섬유아세포 또는 임의의 다른 숙주 세포일 수 있다. 다른 실시 형태에서, 세포는 만능성 세포, 유도 만능성 줄기(iPS) 세포, 비-인간 배아 줄기(ES) 세포이다. 이러한 세포는, 예를 들어, 유도 만능성 줄기(iPS) 세포, 인간 iPS 세포, 마우스 배아 줄기(ES) 세포, 래트 배아 줄기(ES) 세포, 인간 배아 줄기(ES) 세포 또는 발생적으로 제한된 인간 전구 세포, 설치류 배아 줄기(ES) 세포, 마우스 배아 줄기(ES) 세포 또는 래트 배아 줄기(ES) 세포를 포함하는, 만능성 세포를 포함한다.
- [0180] V. 동물 모델
- [0181] 또한, ALS를 치료, 억제 및/또는 저해하기 위한 후보 체제를 확인하는 방법이 본 명세서에 제공된다. 구체적인 실시 형태에서, 상기 물질의 저해 효과는, DR6 기능 상실을 가지고, 출생시 표현형은 정상이며, 8주령 후 ALS 유사 증상이 발생하는 동물에 상기 체제를 투여함으로써, 생체 내에서 결정된다.
- [0182] 임의의 편리한 경로, 예를 들어 전신 주입, 장기간 노출용 펌프 또는 직접적인 뇌내 주입에 의해, 시험될 체제를 상기 동물에 투여할 수 있다. 이들 동물을 거동 연구에 포함시켜, 상기 체제가 제공되지 않은 적절한 대조군 동물과 비교하여, 동물의 거동, 예를 들어 운동 거동에 대한 상기 물질의 영향을 결정할 수 있다. 동물 척수, 근육 및/또는 뇌 조직의 생검 또는 해부학적 평가를 또한 수행할 수 있고/있거나, 혈액 또는 CSF의 샘플을

수집할 수 있다.

[0183] 본 발명은 다수의 실시 형태를 참조하여 특별히 나타내어지고 기재되었으나, 당업자는, 본 발명의 사상 및 범주를 벗어나지 않으면서, 본 명세서에 개시된 다양한 실시 형태에 대한 형태 및 세부 사항의 변경이 이루어질 수 있으며, 본 명세서에 개시된 다양한 실시 형태는 청구범위의 범주를 제한하는 것으로 의도되지 않음을 이해할 것이다.

[0184] 실시예

[0185] 하기 실시예는 단지 예시를 목적으로 제공되며, 본 발명의 범주를 제한하는 것으로 의도되지 않는다.

[0186] **실시예 1: DR6 (*Tnfrs21*) 유전자좌의 유전자 변형**

[0187] 마우스에서 맞춤 유전자 돌연변이의 신속한 고처리율의 생성을 가능하게 하는 VelociGene® 방법을 전술한 바와 같이 사용하였다 (문헌[Valenzuela, D.M., *et al.* (2003b), *Nat Biotechnol* 21:652-659]). 간략히 말해서, 마우스 bMQ (129S7/SvEv Brd-Hprt b-m2) 또는 RP23 BAC 라이브러리 (문헌[Adams, D.J., *et al.* (2005), *Genomics* 86:753-758])로부터의 BAC 클론을 사용하여, 대형 표적화 벡터 (LTVEC)를 생성하였다. *lacZ/neo^r* 리포터/선택 카세트 (도 1)는, *lacZ* 부분의 β -갈락토시다제 코딩 서열의 아미노 말단이 *ROR1*로부터의 막 관통 도메인을 포함하도록 변형된 것을 제외하고는, NIH KOMP (World Wide Web (www)의 URL "www.VelociGene.com/komp/detail/10020"에서 인터넷을 통해 입수가 가능한 서열)에 사용된 ZEN-Ub1 카세트와 동일하였다.

[0188] 다중 웰 전기천공 장치 (미국 매사추세츠주 보스턴 소재의 Harvard Apparatu)를 사용하여, 전기천공 완충액 (Millipore)에서 0.125 ml의 부피 중 0.67 μ g DNA, 3.3×10^6 세포로 LTVEC를 C57BL/6NTac ES 세포 (문헌[Poueymirou *et al.* (2007), Valenzuela *et al.* (2003a)])로 도입한 후, 15 cm의 젤라틴화된 플레이트에서 배양하였다. 전기천공법 후 48시간 후에 G418을 함유하는 선택 배지를 첨가하고, 그 후에 매일 교체하였다. 약물-내성 콜로니를 전기천공법 후 10일 후에 떼어 내어, 트립신으로 처리하고, DNA 추출 및 정제 전에 3일 이상 동안 젤라틴화된 96-웰 플레이트에서 배양하였다. 대립유전자 변형 (MOA) 검정 (문헌[Frendewey *et al.* (2010), *Methods in enzymology* 476:295-307]; 문헌[Valenzuela *et al.* (2003a), *Nat. Biotechnol.* 21:652-659])에 의해 정확하게 표적화된 ES 세포 클론을 확인하였다.

[0189] VelociMouse® 방법 (문헌[Dechiara, T.M., (2009), *Methods Mol Biol* 530:311-324]; 문헌[Poueymirou *et al.* (2007), *Nat. Biotechnol.* 25:91-99])을 사용하여, 표적화된 ES 세포를 조밀하지 않은 8-세포기의 스위스 웹스터 배아에 주입하고, DR6 녹아웃 돌연변이를 지닌 완전히 ES 세포-유래된 F0 세대 마우스를 생성하였다. *lacZ* 발현 프로파일링을 위해, 수컷 VelociMouse®를 직접 사용하거나, 또는 C57BL/129 암컷과 교미시켜, *lacZ* 분석을 위한 배아 또는 성체를 생성하거나, F1 증식자 (breeder)를 생성하고, N2F1 마우스에서 표현형 연구를 수행하였다. 질전 (vaginal plug)을 확인한 날 아침을 0.5일 (E0.5)로 지정함으로써, 시간 지정 교미를 수행하였다.

[0190] **실시예 2: DR6 유전자좌에 돌연변이를 포함하는 동물의 표현형 분석**

[0191] N2F1 마우스의 표현형 연구는 6 내지 8주령에 개시하였다. 시간 지정 교미를 위해, 본 발명자들은 질전을 확인한 날 아침을 배아 0.5일 (E0.5)로 지정하였다. DR6 KO 및 야생형 한배 새끼들을 다양한 발달 지표 (발육 부진 (runting), 호흡, 얼굴 및 사지 이상, 피부색, 자세, 정위, 및 눈 뜨기)에 대해, 출생 시로부터 약 6 내지 8주령까지 관찰하였으며, 이때, 동물들은 연구 과정 동안 69 내지 74°F, 및 40 내지 60% 습도에서 하루에 12시간 동안 빛이 제공되는 곳에서 수용하였다. 모든 실험은 6 내지 9주령에 개시하였으며, 모든 동물 실험 절차는 리제너론 파마슈티칼스 기관 동물 관리 및 사용 위원회(Regeneron Pharmaceuticals Institutional Animal Care and Use Committee)에 의해 승인된 프로토콜을 준수하여 수행하였다.

[0192] 운동 손상 분석은 로타로드 테스트, 오픈 필드 보행성 운동 테스트 및 캣워크 테스트를 사용하여 수행하였다. 캣워크 테스트 시에는, 대상이 조명된 유리 플랫폼을 가로질러 걸어 다니는 동안, 아래로부터 비디오 카메라로 녹화한다. 걸음걸이 관련 파라미터-예를 들어, 보폭 패턴, 각 발의 유각 속도, 입각 시간 및 압력-를 각 동물에 대해 보고한다. 이 테스트를 사용하여, 트랜스제닉 계통의 마우스의 표현형을 결정하고, 운동 수행 능력에 대한 그의 효과에 대해 신규의 화학 물질을 평가한다. 캣워크 XT는 래트와 마우스의 발소리 (footfall)와 걸음걸이를 정량적으로 평가하는 시스템이다. 이것은 중추 신경계, 말초 신경계, 근육계 또는 골격계 이상의 거의 모든 종류의 실험 모델에서 설치류의 보행성 운동 능력을 평가하는 데 사용된다.

[0193] 상위 운동 뉴런 손상은 경직 (즉, 강직), 반사의 증가, 진전, 운동완만증 및 바빈스키 징후로 나타난다. 하위 운동 뉴런 손상은 근육 약화, 소모, 움켜쥐기, 구부러짐 및 발 끌기 및 근섬유다발수축으로 나타난다. 연수 손상은 연하 곤란, 불분명한 발음 및 혀의 근섬유다발수축으로 나타난다. 표 2는 테스트 중 동물의 운동 손상, 진전 및 강직과 관련된 점수화 방법론을 제공한다. 블라인드 주관적 점수화 검정을 사용하여 전반적인 운동 기능 평가를 수행하였으며, 모든 데이터는 평균 $\pm$ SEM으로 보고되어 있다.

[0194] [표 2]

	0	1	2	3
운동 손상	표현형 없음	움켜쥐기	움켜쥐기 + 끌기/발가락 구부러짐	마비
진전	없음	경증	중간	중증
강직	없음	경증	중간	중증

[0195]

[0196] 데이터는, DR6^{-/-} 마우스가 그의 야생형 상대와 비교하여 부동 상태가 증가하는 것으로 보이지는 않으나, 로타로드 위에서의 시간 및 중량의 차이가 9주령만큼 일찍 나타날 수 있고, 이의 각각은 시간 경과에 따라 꾸준히 저하됨에도 불구하고, 대략 14주령에 ALS 유사 신경학적 표현형 (예를 들어, 뒷다리 불완전 마비를 시사하는 적은 양육 활동)을 나타냄을 보여준다 (도 4 내지 도 7). 그러나, DR6^{-/-} 마우스의 운동 뉴런 상실은 운동 손상, 강직 및 진전 점수가 마비 및/또는 중증 질환 진행을 반영하기 시작하는 약 14주령에 임상 증상에 대한 한계점에 도달하며, 이는 동물의 성별에 무관하다 (도 2). 증상이 나타난 DR6^{-/-} 동물은 상위 및 하위 운동 뉴런 손상 둘 모두를 반영하는 중량 감소 및 유의한 운동 이상을 가지며 (도 3), 약 21주령에 사망한다.

[0197] **실시예 3: DR6 유전자좌에 돌연변이를 포함하는 동물의 해부학적 분석**

[0198] 약 20주령의 8마리의 야생형 및 8마리의 DR6 녹아웃 마우스를 50 mL의 식염수로 관류시킨 후에, pH 6.5의 아세트산염 완충액 중 4% 과라포름알데하이드 (PFA) 용액 50 mL와 pH 9.5의 붕산염 완충액 중 4% PFA 용액 50 mL로 관류시켰다. 동물의 관류 후, 마우스의 뇌와 척수를 수집하여, 이들이 떨어질 때까지, 붕산염 완충액 중 15% 수크로스 용액 후에, 30% 수크로스 용액에 넣는다. 그 다음, 조직을 OCT에 포매하고, 2-메틸부탄을 사용하여 동결시켰다.

[0199] 동결된 조직을 잘라내어, 슬라이드에 올려놓고, 헤마톡실린과 에오신 (H&E) 및 록솔 패스트 블루 (Luxol fast blue)로 면역염색하였다. 척수 당 4개의 H&E 염색된 슬라이드를 무작위로 선택하여, 각각의 척수의 운동 뉴런의 수를 계수하였다.

[0200] 대표 슬라이드가 도 9에 제공되어 있다. 야생형 마우스의 척수에서 슬라이드당 평균 운동 뉴런의 수는 약 18개이며, 녹아웃 마우스의 경우에는 약 2개이고, p 값은 0.0001 미만이다 (도 9). 이는, 증상이 나타난 DR6 녹아웃 마우스의 경우에 그들의 야생형 상대와 비교하여 척수에서 운동 뉴런이 유의하게 상실됨을 보여준다. 또한, 녹아웃 마우스의 척수는, 크로이츠펠트야콥병 환자의 인간 뇌 병리와 닮은 해면모양 병리 (spongiform pathology)를 나타내며, 이는 이들 동물에서의 프리온-유사 운동 뉴런 질환 표현형의 가능성을 시사한다.

[0201] **실시예 4: DR6 유전자좌에 돌연변이를 포함하는 동물에 의한 뉴런 단백질의 발현**

[0202] 약 20주령의 10마리의 야생형 및 7마리의 DR6 녹아웃 마우스의 뇌와 척수로부터 전체 mRNA를 수집하였다. 그 다음, 이 mRNA 샘플에 대해 Taqman®을 이용한 RT-PCR을 수행하였다. 이어서, 마이엘린 결합 단백질 (MBP), 신경성장인자수용체 (NGFR), 콜린 아세틸트랜스퍼라제 (Chat), Mnx 호메오박스, 글루타메이트 [NMDA] 수용체 하위 단위 3B (Grin3b), 및 글루타메이트 수용체 2(Gria2) 유전자의 평균 mRNA 수준을 샘플의 β 액틴의 발현에 의해 정규화하였다.

[0203] 도 10 내지 도 12는, DR6 녹아웃 마우스의 척수에서 MBP, Mnx 및 Grin3b의 발현이 감소하였으나, 뇌에서는 그러하지 않음을 보여주며, 이는 증상이 나타난 DR6 녹아웃 마우스의 척수에서 운동 뉴런 상실에 대한 증거를 보강하는 것이다. Ngfr, Chat 및 Gri2a의 발현은 영향을 받지 않는다.

[0204] **실시예 5: 내인성 DR6 유전자좌에 돌연변이를 포함하는 동물의 유전자 발현 패턴**

[0205] 10주령의 야생형 마우스 (n=5 수컷), 증상이 나타나기 전의 10주령의 DR6^{-/-} 마우스 (n=5 수컷), 20주령의 야생형 마우스 (n=6 수컷 및 3 암컷), 및 증상이 나타난 20주령의 DR6^{-/-} 마우스 (n=6 수컷 및 3 암컷)의 뇌와 척수 조직으로부터 추출한 RNA를 사용하여, 유전자 프로파일링을 수행하였다.

- [0206] 10주령 및 20주령의 DR6^{-/-} 마우스의 뇌와 척수는 유전자 발현이 상당히 증첩되고, 유사한 유전자 발현 특징을 공유한다 (데이터는 제시되지 않음). 10주령의 DR6^{-/-} 마우스의 뇌와 척수 둘 모두에서 주요 유전자 73개가 질환의 기전과 진행에 잠재적으로 관여하는 것으로 확인되었다. 10주령의 DR6^{-/-} 마우스의 척수의 유전자 특징은 또한 인간 ALS 및 쥐과 동물 SOD1 바이오세트와 고도로 상호 연계되며 (데이터는 제시되지 않음), 이는 DR6^{-/-} 마우스를 ALS 마우스 모델로서 추가로 확인시켜 주는 것이다. 10주령의 DR6^{-/-} 마우스의 뇌와 척수는 면역 반응과 연결된 유전자 발현 특징을 나타내었으며, 이는 상기 질환 과정이 염증 경로와 관련되어 있음을 시사한다.
- [0207] 실시예 6: DR6^{-/-} 동물은 말초 면역 세포의 항상성을 나타낸다.
- [0208] CD3, B220, CD21 및 CD2에 대한 항체 (모두 미국 캘리포니아주 새너제이 소재의 BD Biosciences 또는 미국 캘리포니아주 샌디에고 소재의 eBioscience로부터 입수하였음)를 사용하여, 유세포계측 분석에 의해 표현형을 결정하기 위해, 10주령 및 20주령의 야생형 및 DR6^{-/-} 마우스로부터 혈액, 골수, 흉선 및 비장 조직을 분리하였다. 또한, 20주령의 야생형 및 DR6^{-/-} 마우스로부터 분리된 혈청의 사이토카인 및 케모카인 농도를, FGF 기반, GM-CSF, IFN- γ , IL-1 α , IL-1 β , IL-2, IL-4, IL-5, IL-6, IL-10, IL-13, IL-12 (p40/p70), IL-17, IP-10, KC, MCP-1, MIG, MIP-1 α , TNF- α , 및 VEGF의 동시 정량 결정을 위해, 루미넥스 마우스 사이토카인 마그네틱 20-플렉스 패널(Luminex Mouse Cytokine Magnetic 20-Plex Panel)을 사용하여 평가하였다.
- [0209] 10주령 또는 20주령의 DR6^{-/-} 마우스 모두는 말초 면역 세포 항상성에 대해 강력한 효과를 나타내지 않았다 (도 13 내지 도 14). DR6^{-/-} 마우스는 골수에서 정상적인 B 세포 발생 및 초기 세포 전구 세포 항상성, 흉선에서 B 세포의 2배 증가, 비장 이행 1 B 세포의 증가 및 비장 변연부 B 세포의 감소, 말초 호중구의 감소, 및 말초 림프구의 증가를 나타내었다 (도 13). 골수에서, DR6^{-/-} 마우스는 거핵구/적혈구 전구 세포의 14% 증가, 골수 전구 세포의 20% 감소를 나타내었으나, 총 세포 수의 유의한 변화는 없었다 (도 14). DR6^{-/-} 동물은 흉선에서 정상적인 T 세포 발생 및 분화를 나타내었고, 비장 공통/성숙 수상돌기 세포의 20% 증가, 대식세포 및 호산구의 25% 증가, 비장의 호중구 및 단핵구의 75% 증가, 및 CD4⁺ T 세포의 19% 감소가 있었다. 그러나, 비장에서 총 세포 수의 유의한 변화는 없었다 (데이터는 제시되지 않음). 또한, 20주령의 DR6^{-/-} 마우스의 혈청의 사이토카인과 케모카인 수준은 야생형 동물에 비해 변화하지 않았다 (도 15). 이 데이터는, DR6^{-/-} 마우스가 질환 진행 중에 전반적인 염증 반응을 거치지 않음을 나타내며, 유전적 프로그래밍으로 나타나는 염증 반응이 국소적 신경염증일 수 있음을 시사한다.
- [0210] **실시예 7: DR6^{+/-} 마우스의 배아 줄기 세포 유래 운동 뉴런은 산화 스트레스의 증가를 나타낸다.**
- [0211] 야생형 (DR6^{+/+}) 또는 이형접합 DR6^{+/-} 동물로부터 내세포집단 배아 줄기 세포를 분리하고, 배아 줄기 세포 배지 (ESM; DMEM + 15% 소 태아 혈청 + 페니실린/스트렙토마이신 + 글루타민 + 비 필수 아미노산 + 뉴클레오타이드 + β -메르캅토에탄올 + 피루브산나트륨 + LIF)에서 2일 동안 배양하여, 배상체 (EB)를 형성하였다. EB를 분화 배지 (DM: 최신 DMEM/F12 + 뉴런세포 기본 배지 + 10% 녹아웃 혈청 + 페니실린/스트렙토마이신 + 글루타민 + β -메르캅토에탄올)에서 2일 동안 배양하였고, DM과 레티노산과 평활화 작동제에서 추가로 5일 동안 배양하여, 운동 뉴런을 수득하였다. 해리된 운동 뉴런을 배아 줄기 세포 유래 운동 뉴런 배지 (ESMN; 뉴런세포 기본 배지 + 2% 말 혈청 + B27 + 글루타민 + 페니실린/스트렙토마이신 + β -메르캅토에탄올 + 10 ng/ml GDNF, BDNF, CNTF)에 플레이팅하고, 성숙시켜, 안정한 운동 뉴런 세포주를 수립하였다. 안정한 운동 뉴런 세포주의 수립 후, 7일째에 운동 뉴런 개수를 결정하였고, 1일째와 7일째에는 산화 스트레스를 측정하였다.
- [0212] 데이터는, DR6^{+/+} 동물이 야생형 동물과 동일한 수의 운동 뉴런을 생성하나, 야생형 동물과 비교하여 산화 스트레스가 증가되었다는 것을 보여준다 (도 16).
- [0213] **실시예 8: DR6^{+/-} 및 DR6^{+/+} 동물의 표현형 비교**
- [0214] 실시예 1에 기재된 바와 같은 DR6 유전자 변형에 대해 이형접합성 또는 동형접합성인 마우스와 대조군 동물의 운동 기능을 실시예 2에 기재된 바와 같은 시험을 사용하여 비교하였다. 신경학적 점수는 도 17a에서 비교되고, 중량 증가 비교는 도 17b에 제시되어 있으며, 로타로드 테스트 비교는 도 17c에 제시되어 있고, 오픈

필드 측정은 도 17d 내지 도 17k에 제공되어 있고, 캣워크 측정은 도 17l 내지 도 17z에 제시되어 있다.

[0215] DR6 돌연변이의 유의한 "투여량 의존적" 유형의 효과는, 동물의 중량 증가 (도 17b)에서, 로타로드 테스트 동안 (도 17c), 오픈 필드 테스트에서 측정된 양육 및 양육 시간에서 (도 17d 내지 도 17e), 그리고 캣워크 테스트 동안 뒷다리의 보폭 길이 및 유각 속도 (도 17l 내지 도 17n)에서 나타났다.

[0216] 21주령에 블라인드 주관적 점수화 검정 (도 17a)에서 전체 운동 기능을 평가하였을 때, 이형접합 마우스가 야생형 동물과 신경학적으로 유사한 것으로 나타났으나, 이형접합 마우스의 신경학적 점수에서 시작하여, 동형접합 마우스의 신경학적 점수 쪽으로 향하는 경향이 있었다 (도 17a). 몇 가지 다른 측정이 동일한 패턴을 따랐으나, 이형접합 마우스의 표현형은 약 20주령 후 동형접합 마우스의 표현형 쪽으로 향하는 경향이 있었다 (예를 들어, 도 17f 내지 도 17k, 도 17o 참조).

[0217] 이 데이터는, 돌연변이에 대해 이형접합성인 마우스에서 DR6 돌연변이가 반가불충분성임을 시사한다. 이 데이터는 또한, 이형접합 DR6^{+/-} 마우스가 나이 들에 따라, 마우스가, 처음에는 정상인 것으로 나타난 측정에서 동형접합 DR6^{-/-} 마우스와 유사한 운동 및/또는 신경학적 결함을 나타내기 시작할 것임을 시사한다 (예를 들어, 도 17o 내지 도 17p, 도 17r 참조).

[0218] 실시예 9: DR6^{+/-} 및 DR6^{+/+} 동물의 감각 지각 결손.

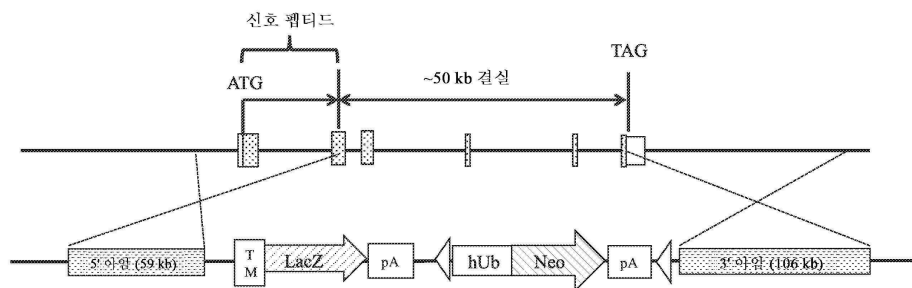
[0219] 48°C, 52°C 또는 55°C에서 유지되는 금속 표면에 동물을 배치하여, 20주령에 DR6 유전자 변형에 대해 이형접합성 또는 동형접합성인 마우스와 대조군 동물의 열적 통각수용을 시험하였다 (미국 캘리포니아 우드랜드 힐즈 소재의 IITC). 동물이 열 자극에 대해 재빨리 뒷발을 핥았을 때까지 경과된 시간으로 정의되는 반응 지체 시간을 측정하였다. 마우스가 뒷발 핥기 또는 뒷발 흔들기 두 가지의 평소와 다른 거동 중 어느 하나를 실행할 때까지, 마우스를 플레이트 상에 남겨 두었다.

[0220] 도 18에 제시된 바와 같이, DR6^{+/-} 및 DR6^{+/+} 동물 둘 모두는 감각 결손을 나타낸다. 이들 마우스는 20주령에 현저한 운동 기능 장애를 나타내지 않기 때문에, DR6^{+/-}에서 나타난 감각 결손은 정확할 가능성이 있다 (실시예 8 참조). 그러나, DR6^{-/-} 마우스의 반응 지연은 관찰된 운동 증상이 불충분하기 때문일 수 있음에 유의해야 한다.

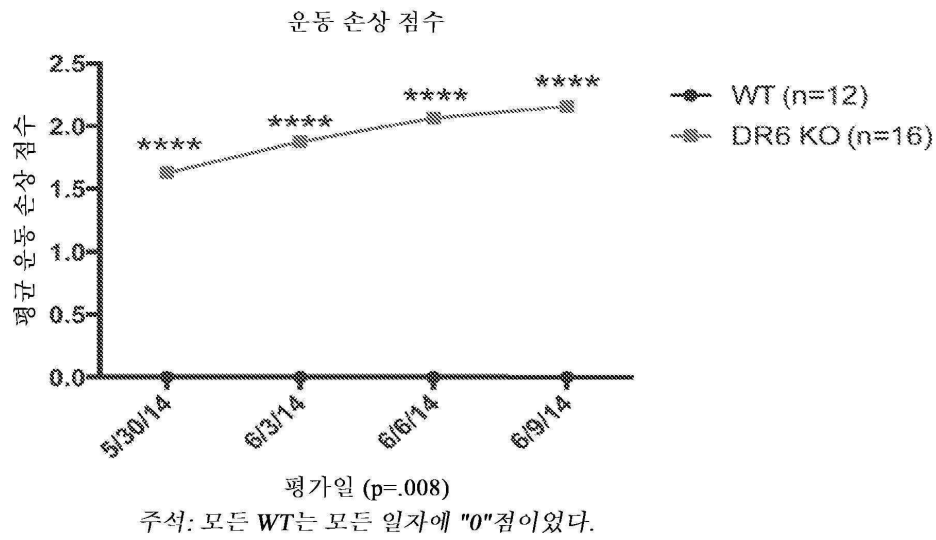
[0221] 본 명세서에 개시된 설치류는 인간에서의 ALS 진행을 보다 유사하게 따르는 새로운 동물 모델을 제공한다. 이론에 구애되고자 함이 없이, 몇 가지 가능성 중 하나는, 설치류가 발생 중에 정상적인 가지치기 (pruning)를 나타내는 데 실패할 수 있으며, 이와 같이, 증가된 연결성과 경증의 과다흥분성을 가진 설치류가 발생하고, 시간 경과에 따라 낮은 수준의 흥분독성이 축적될 수 있다는 것이다. 특히, 성체 DR6 녹아웃 동물은 발생 중에 혈액뇌관문 (BBB) 투과성의 증가 및 BBB 마커의 발생의 손상을 나타낼 수 있으나, 본 명세서에 기재된 마우스의 ALS-유사 표현형은, 혈액뇌관문의 결손이 일반적으로 운동 손상이 아닌 인지 손상으로 나타나므로, 뇌 혈관 발생 중에 DR6 역할의 결과일 가능성은 거의 없다.

도면

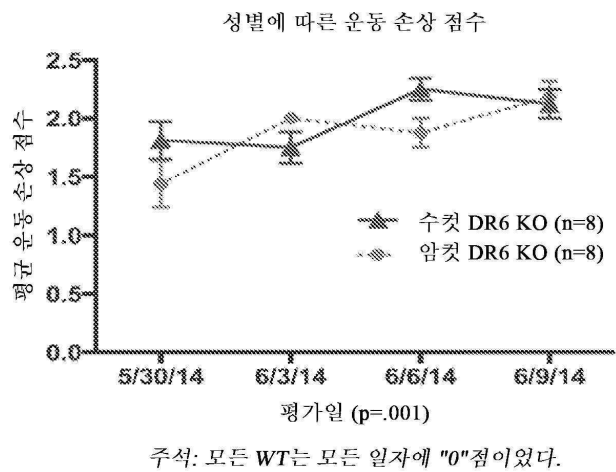
도면1



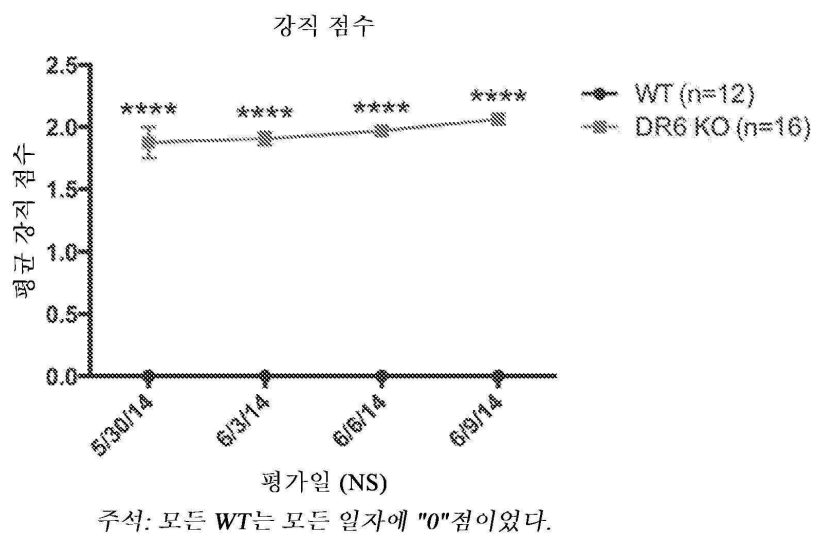
도면2a



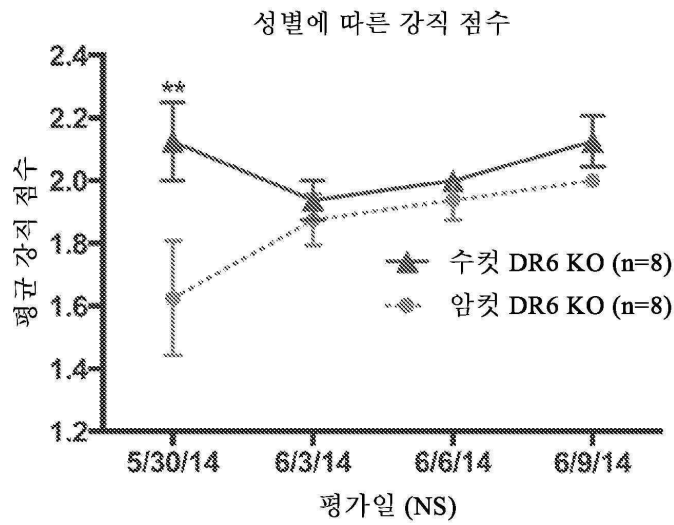
도면2b



도면2c

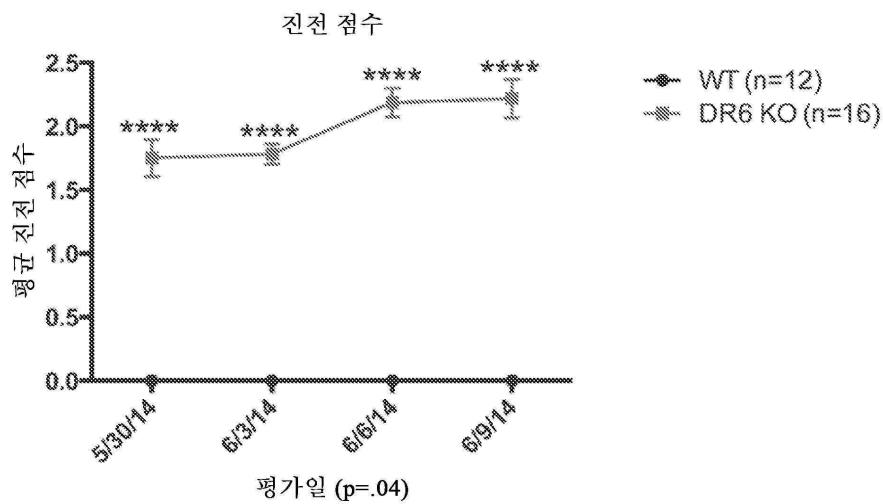


도면2d



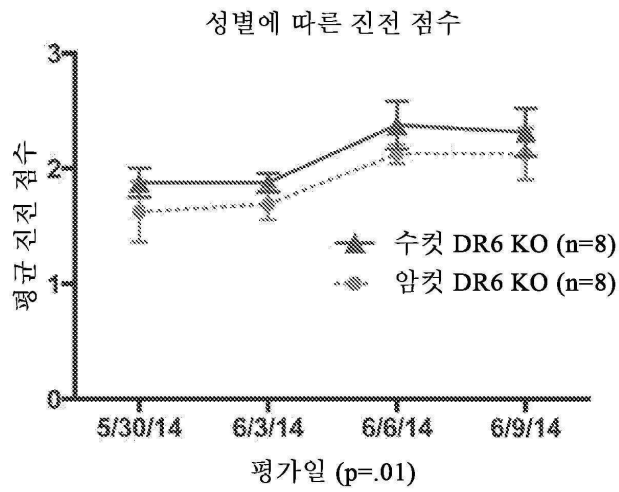
주석: 모든 WT는 모든 일자에 "0"점이었다.

도면2e



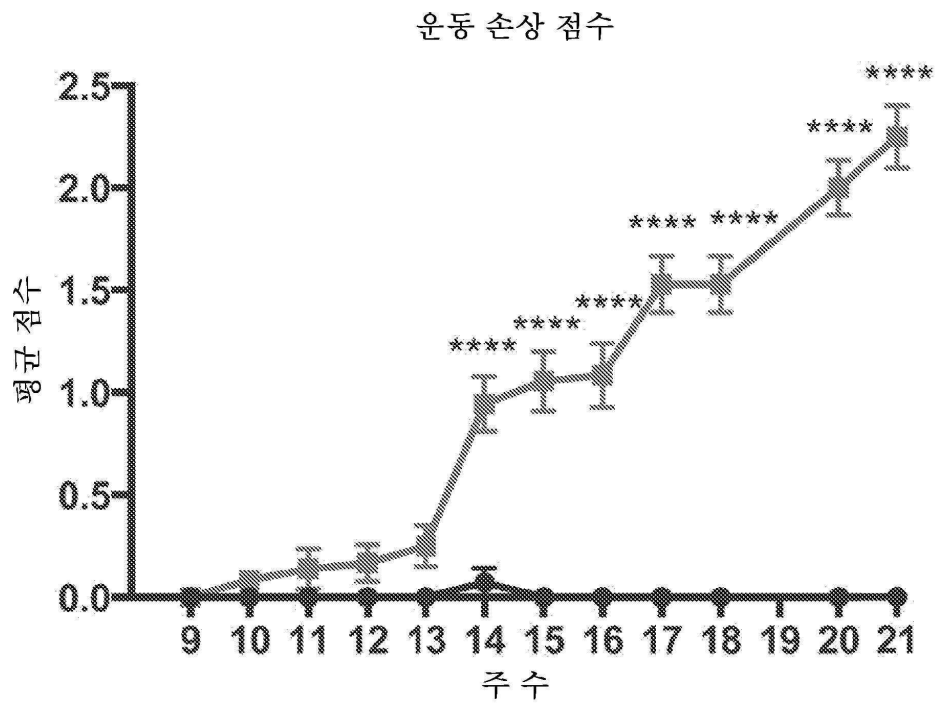
주석: 모든 WT는 모든 일자에 "0"점이었다.

도면2f

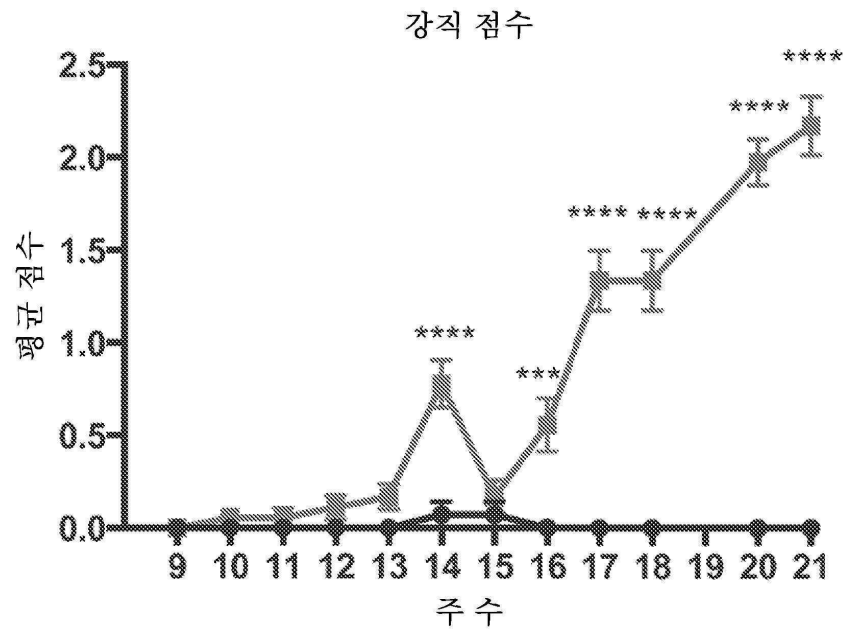


주석: 모든 WT는 모든 일자에 "0"점이였다.

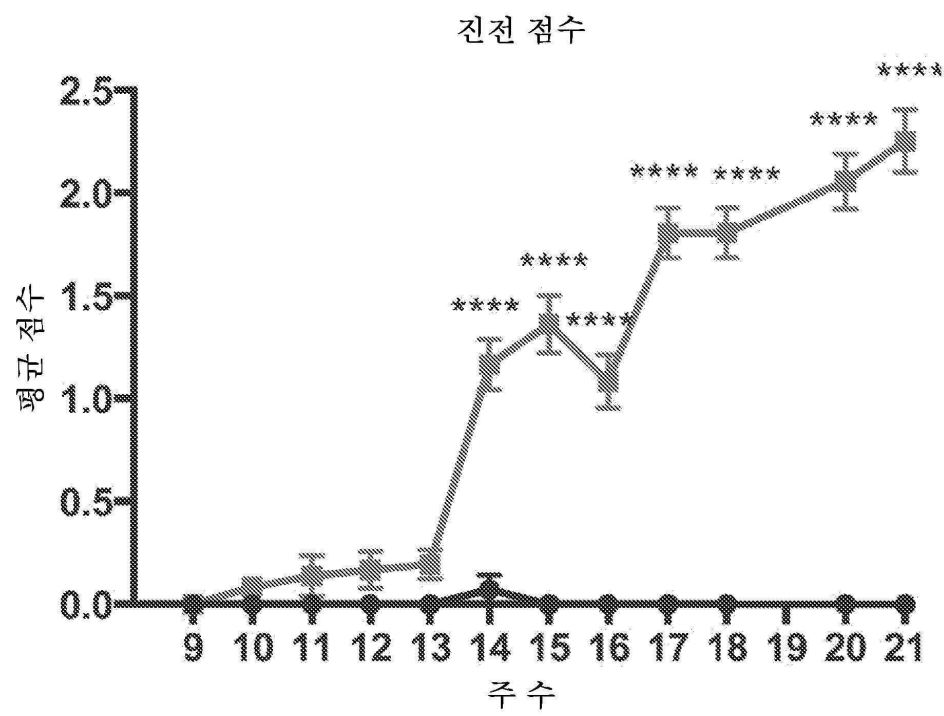
도면2g



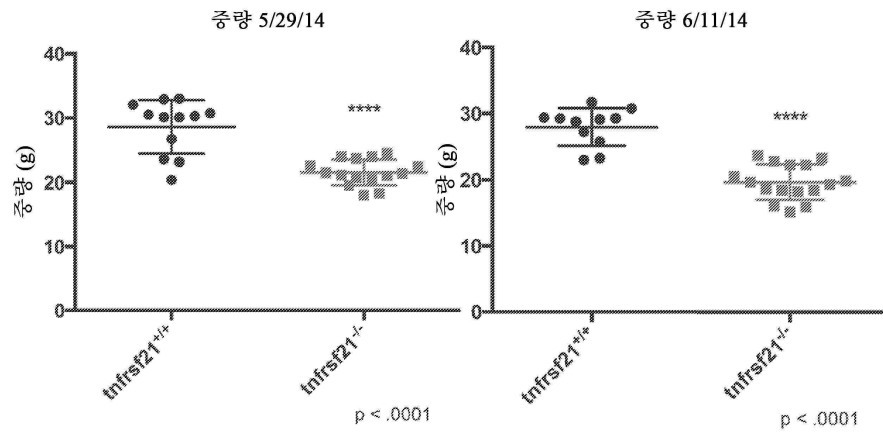
도면2h



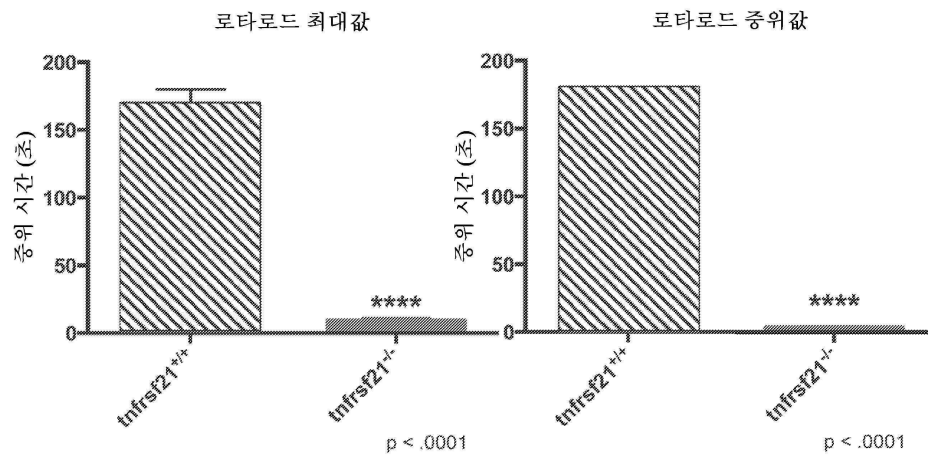
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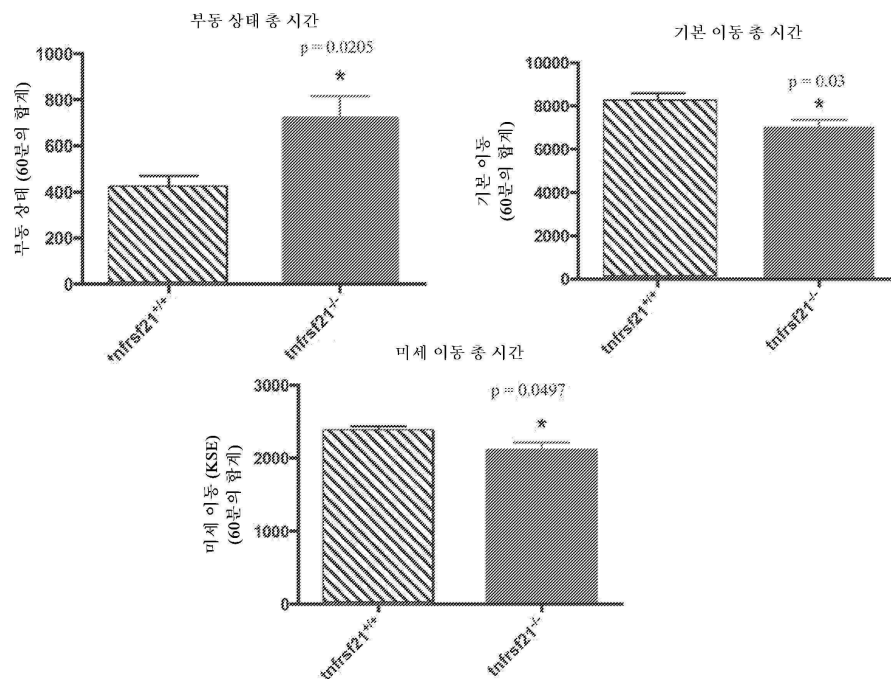
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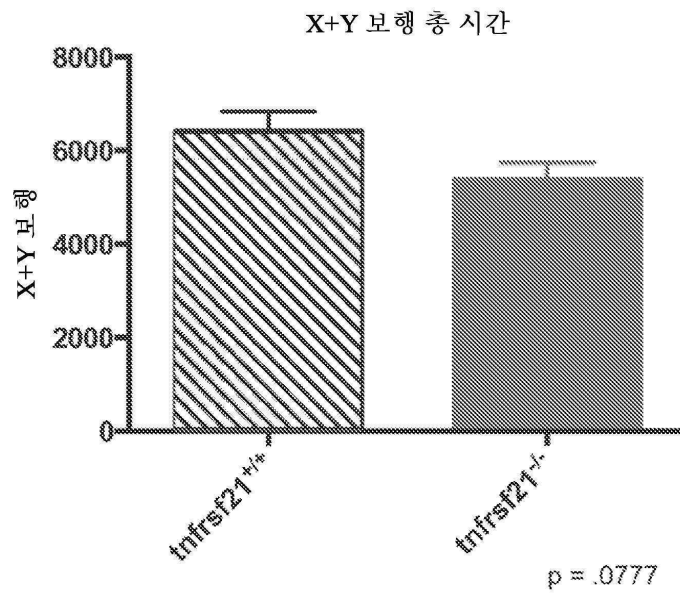
도면3b



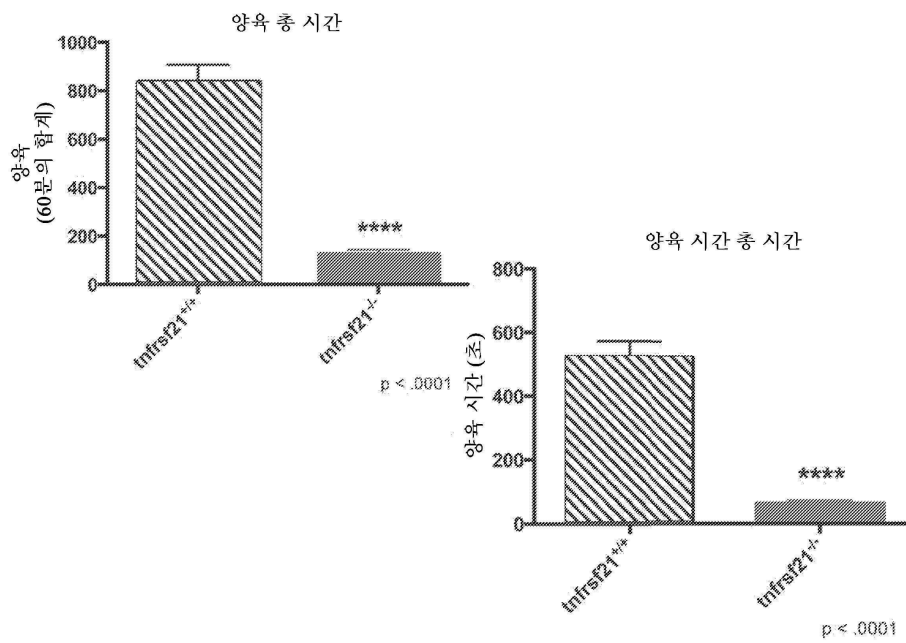
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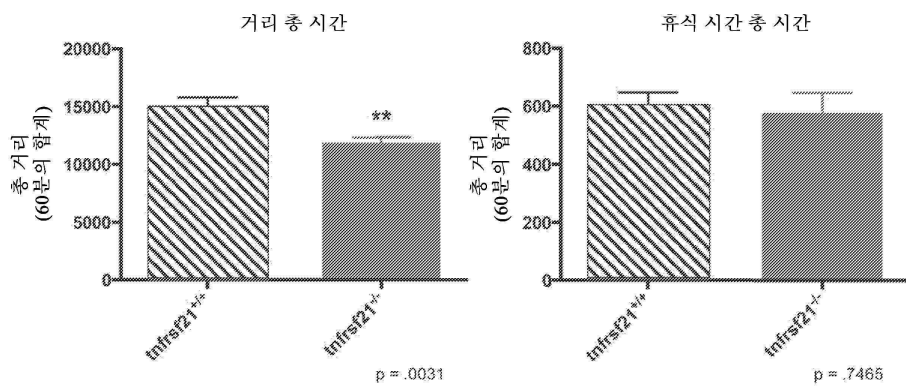
도면3d



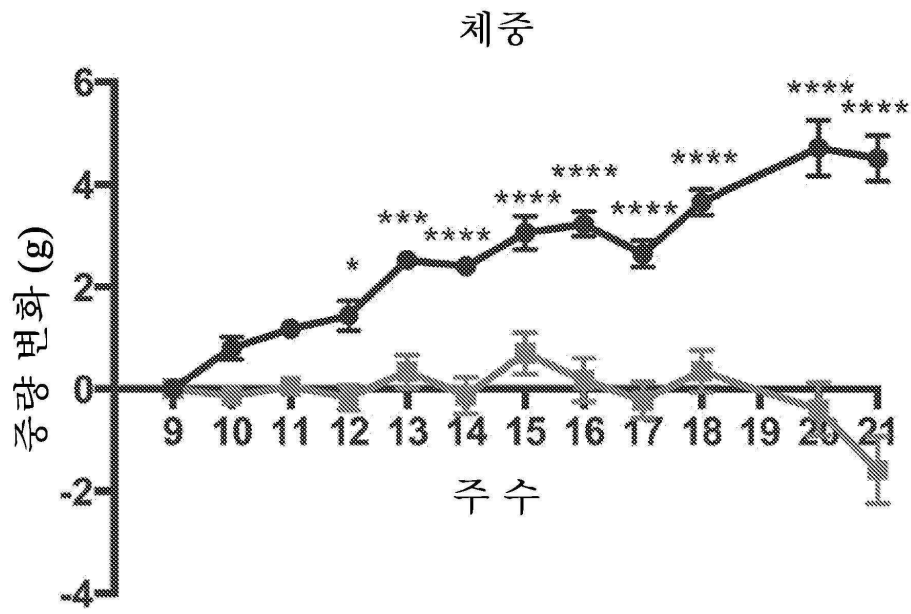
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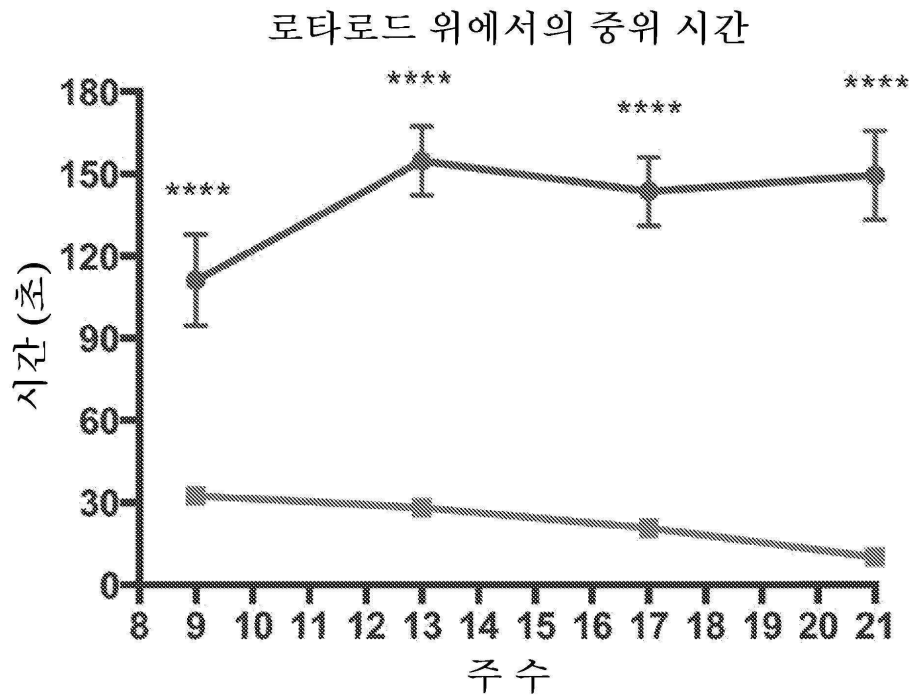
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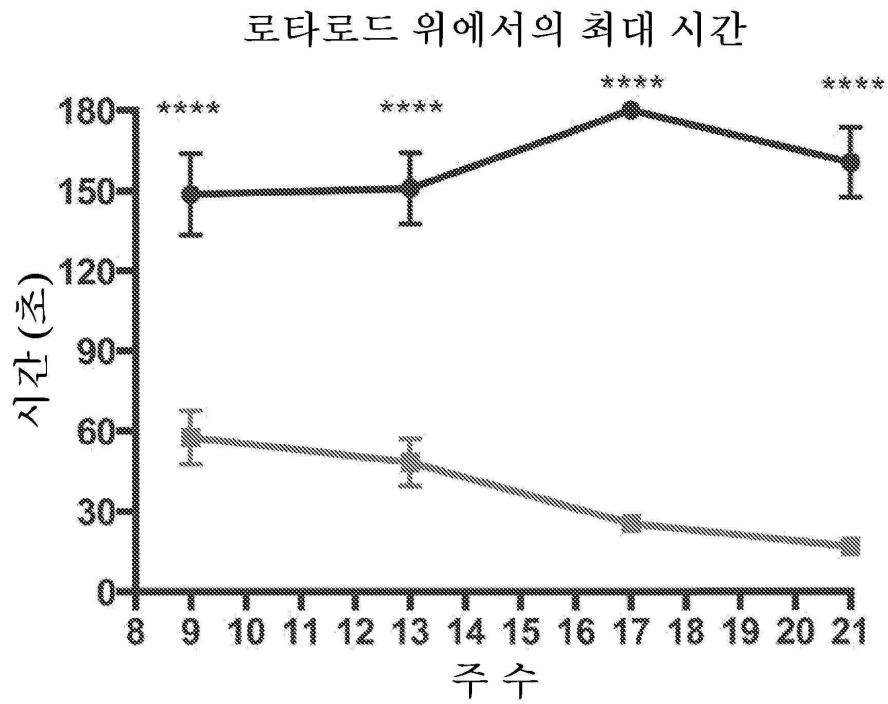
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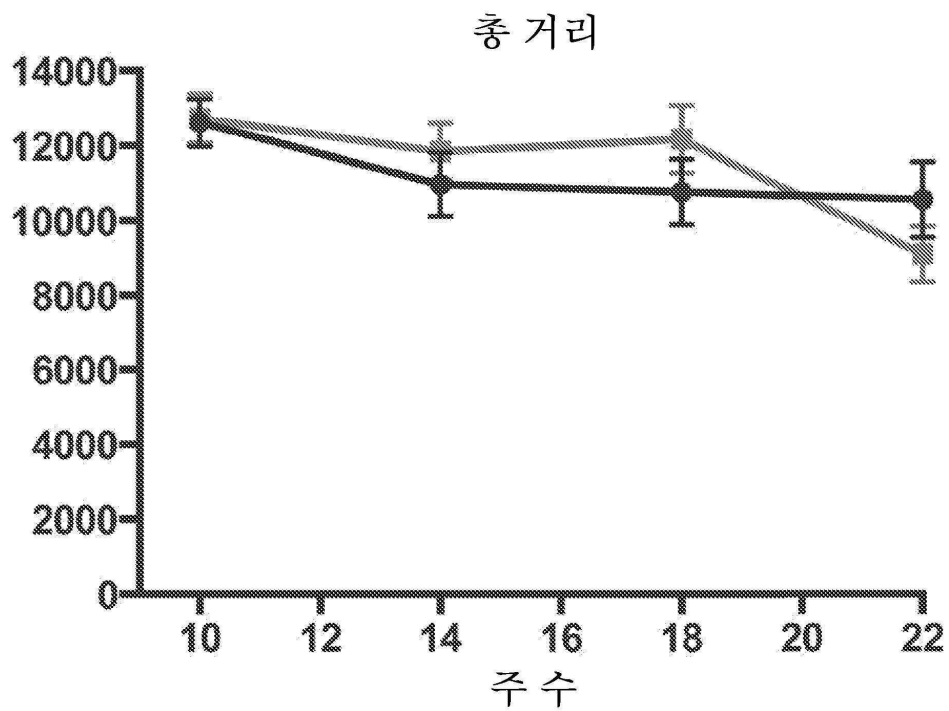
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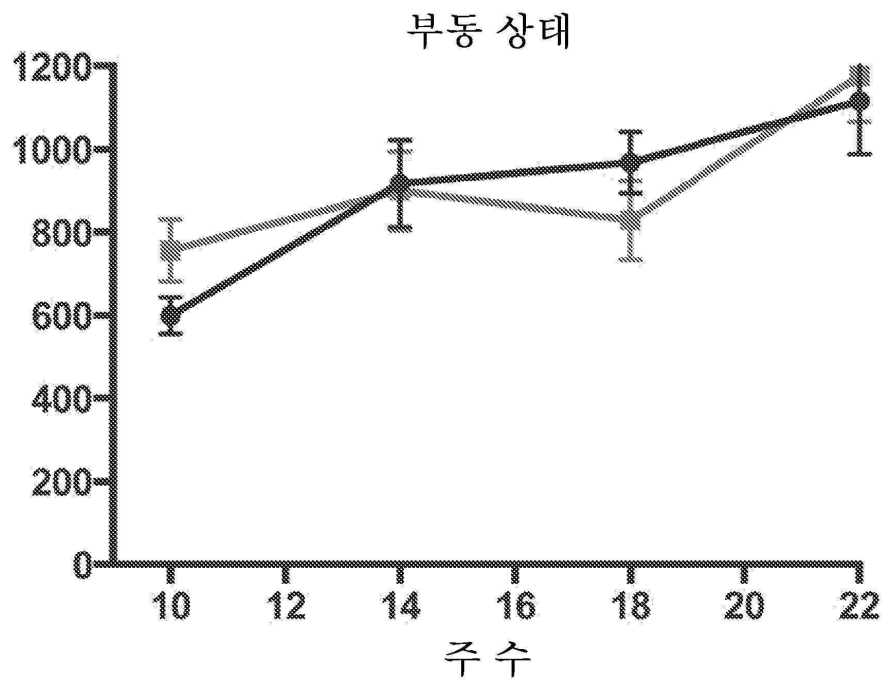
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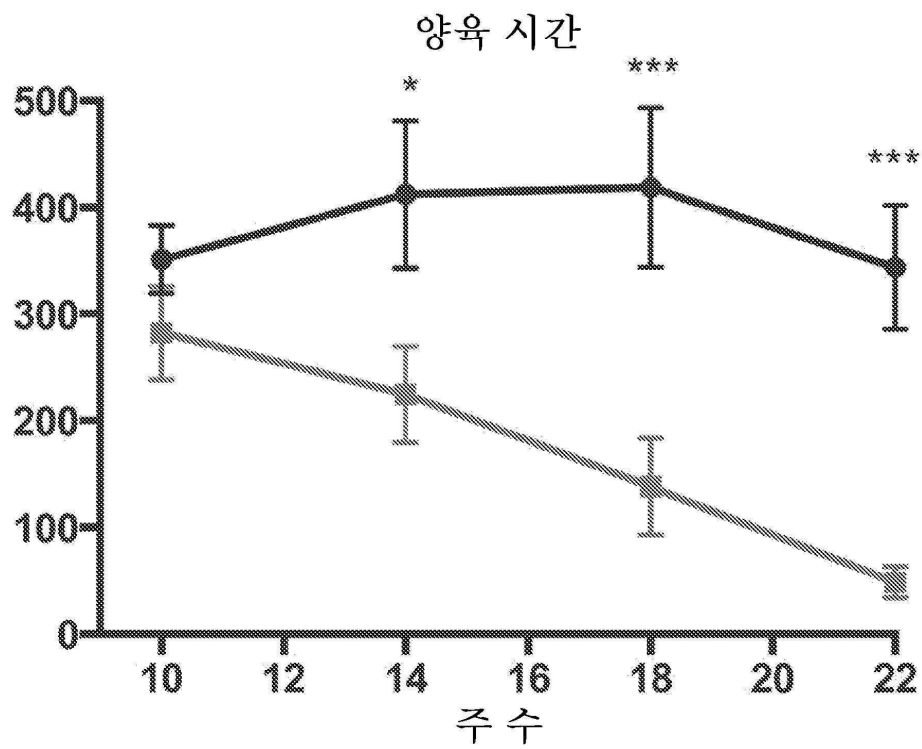
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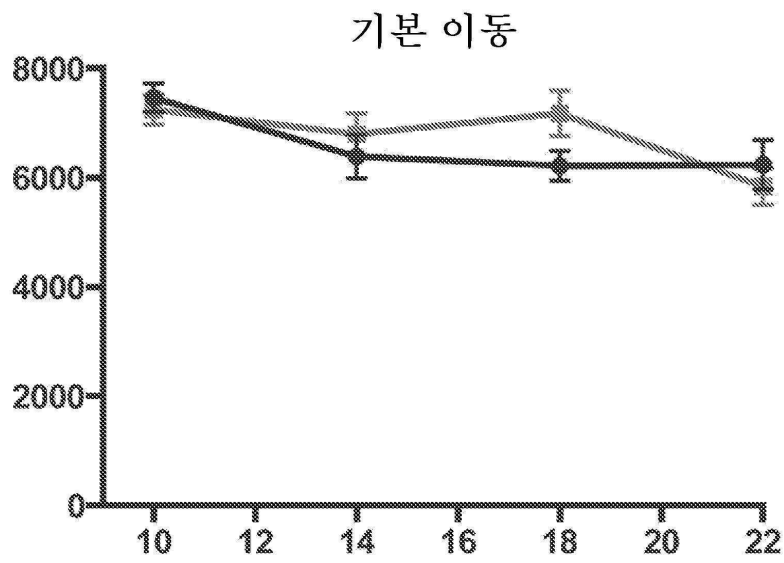
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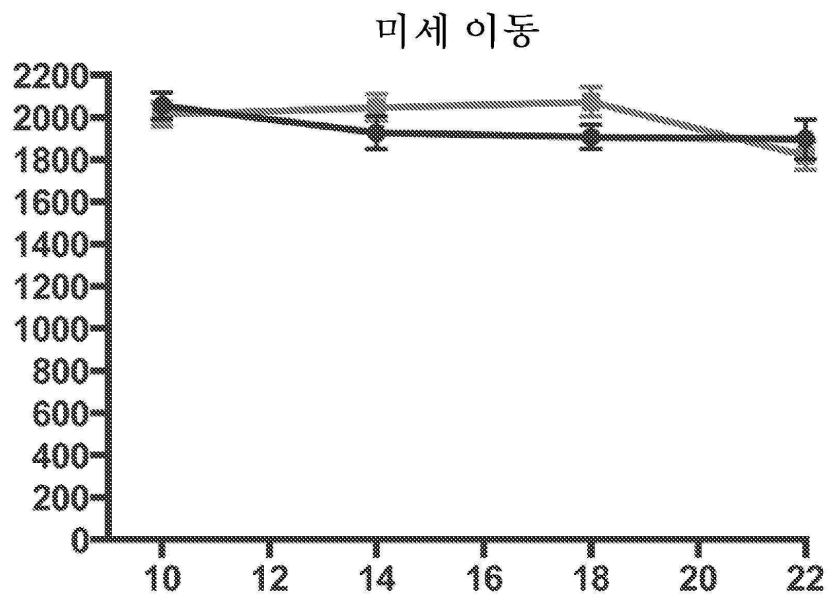
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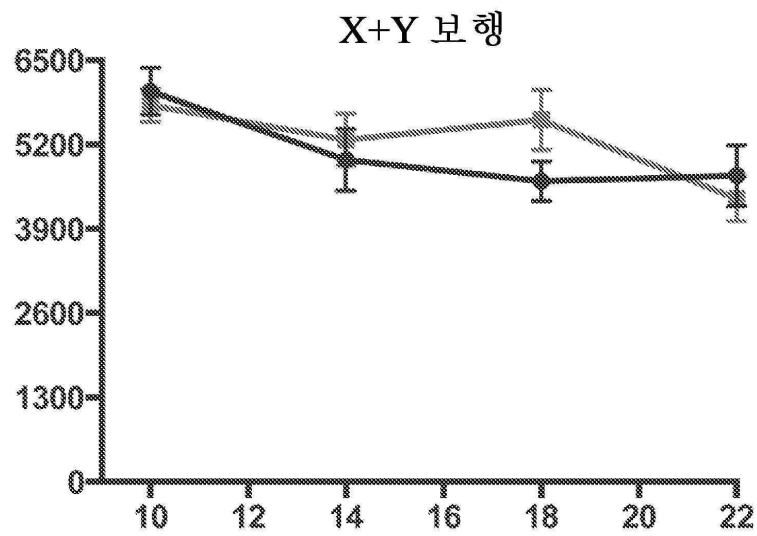
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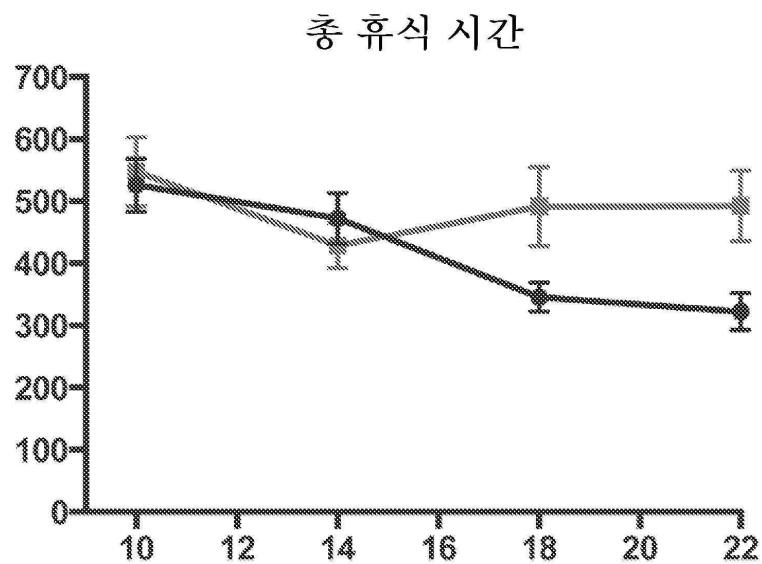
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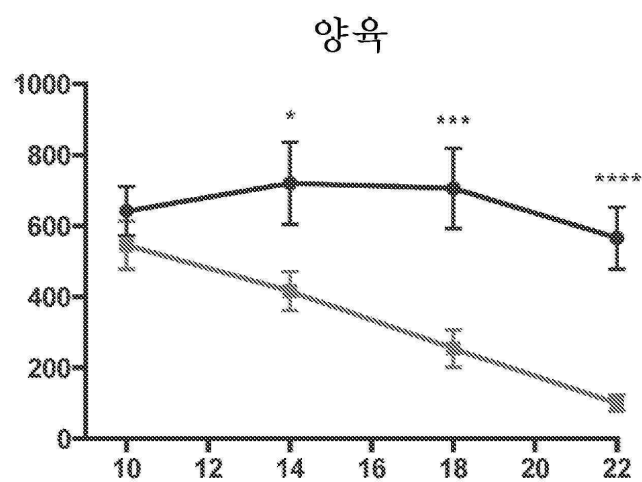
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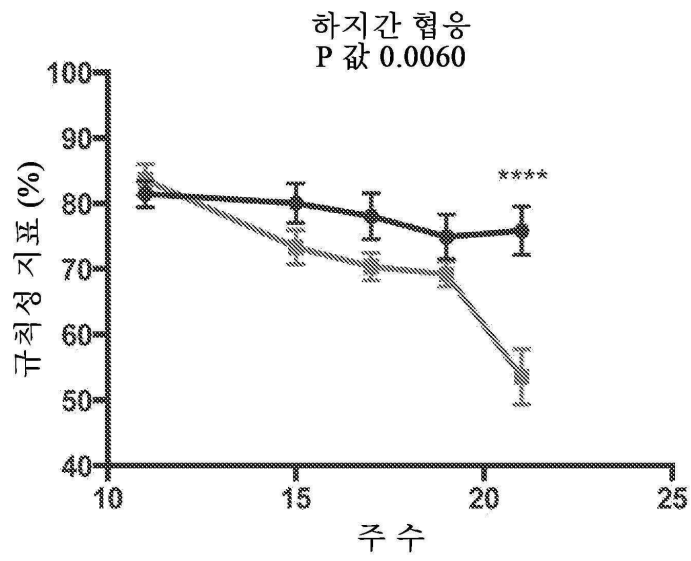
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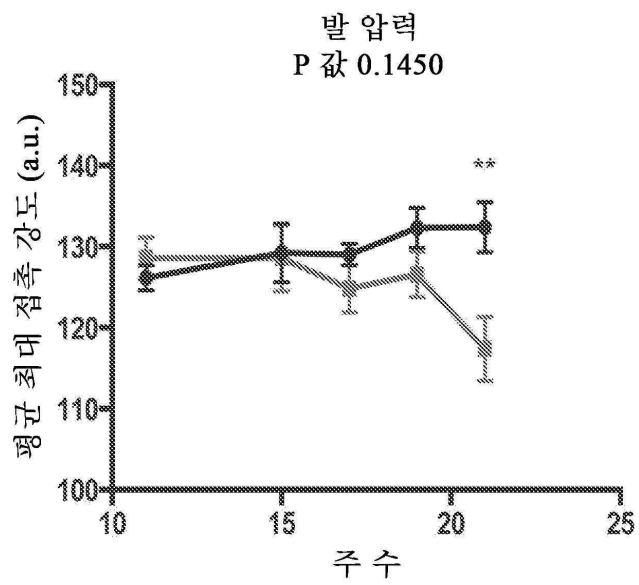
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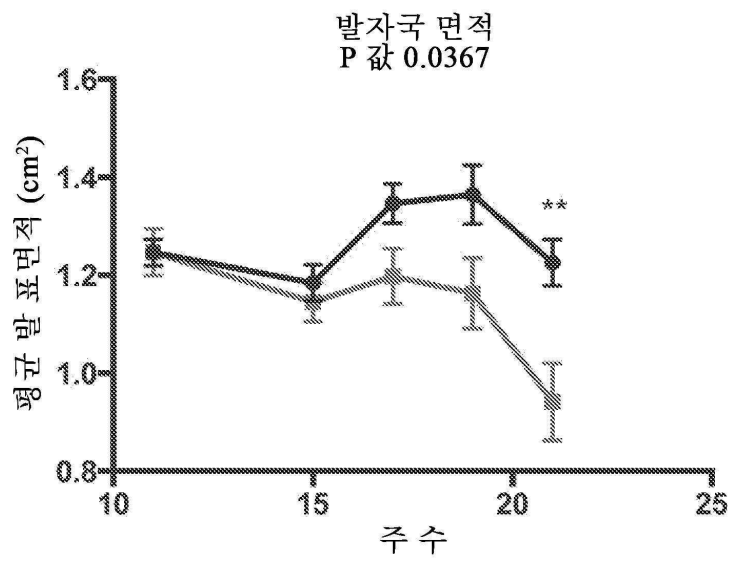
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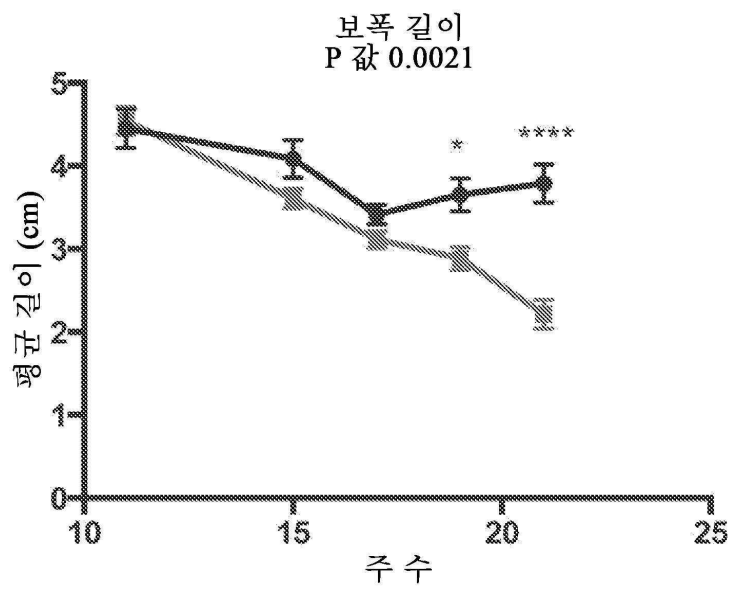
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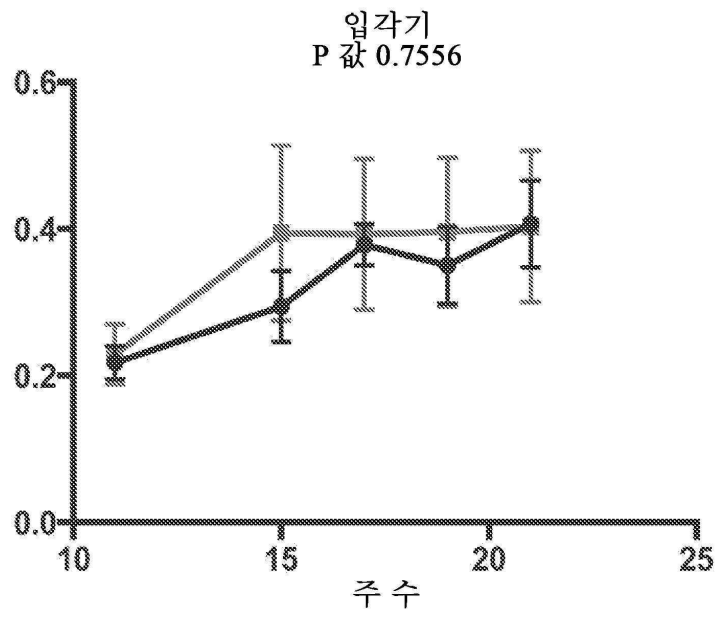
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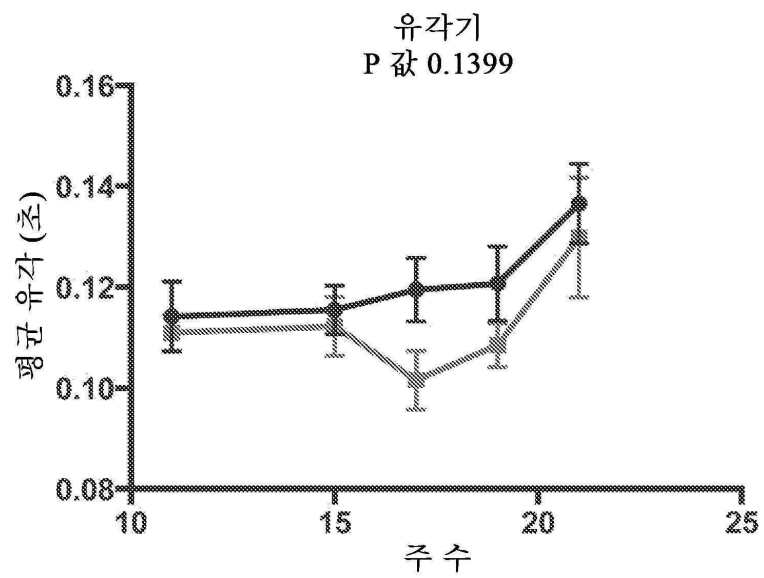
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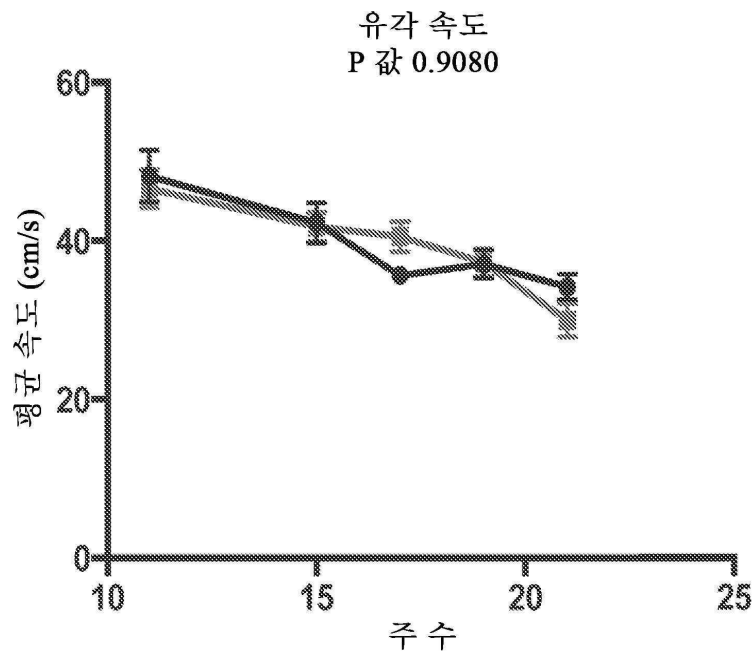
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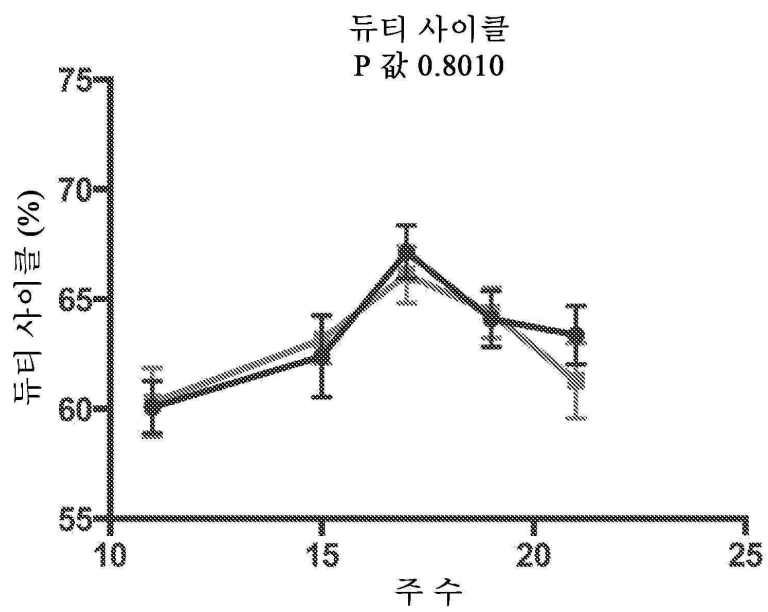
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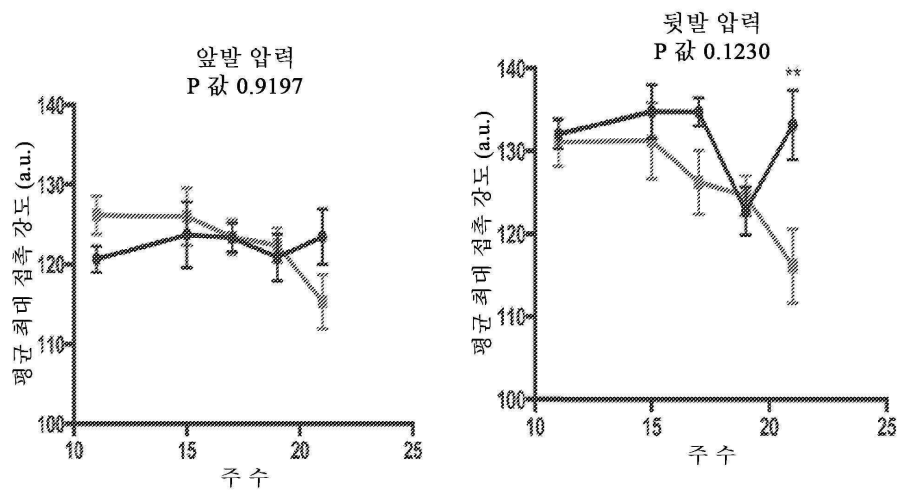
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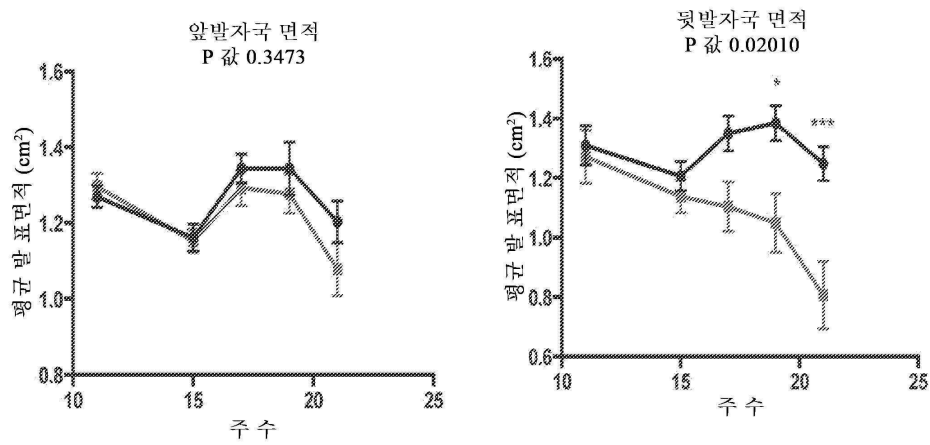
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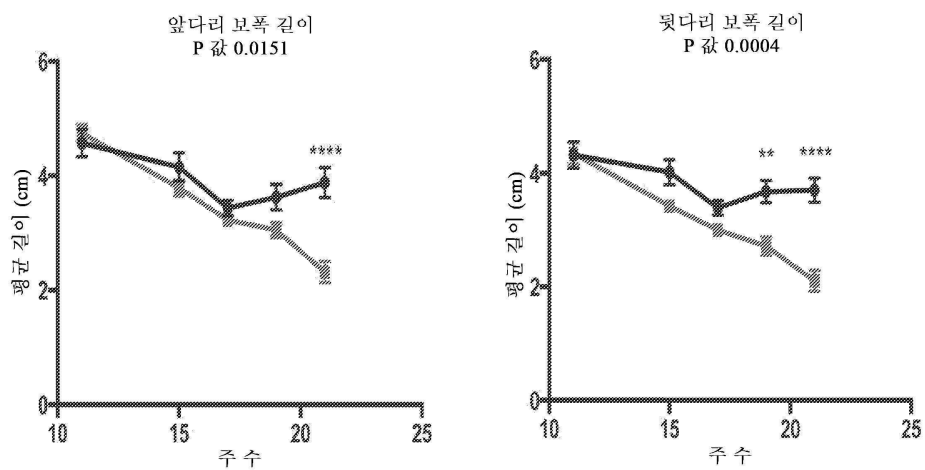
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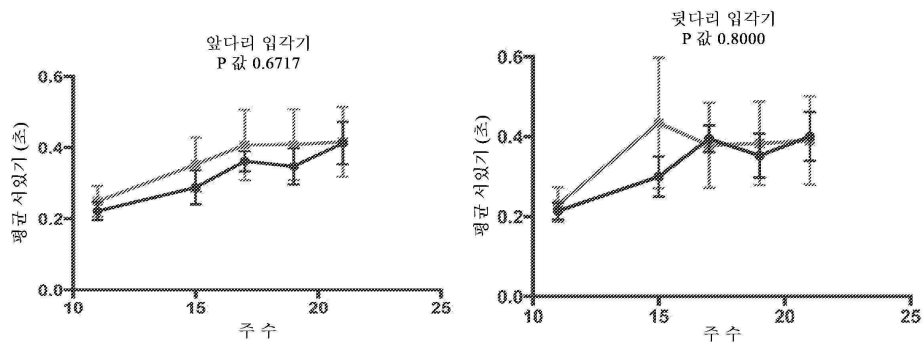
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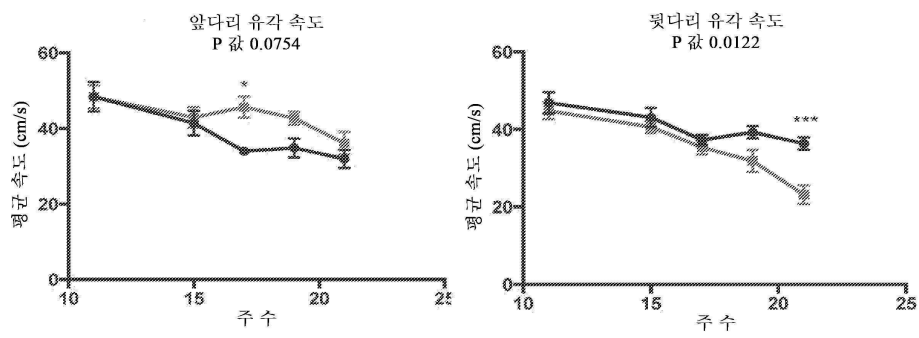
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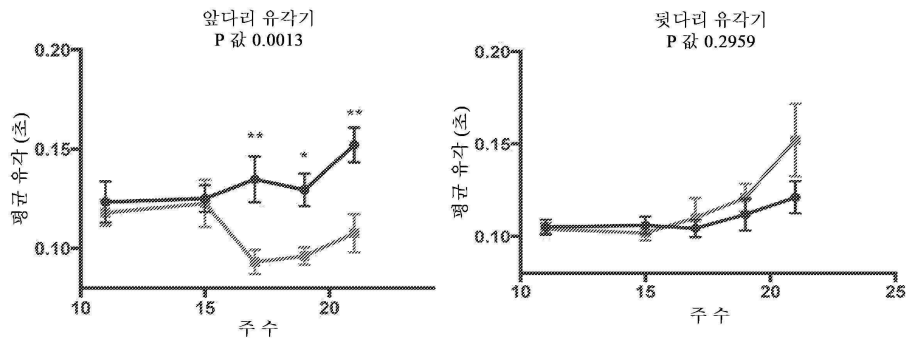
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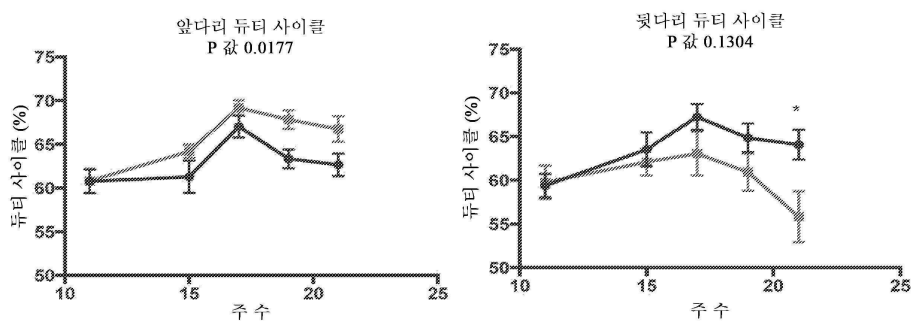
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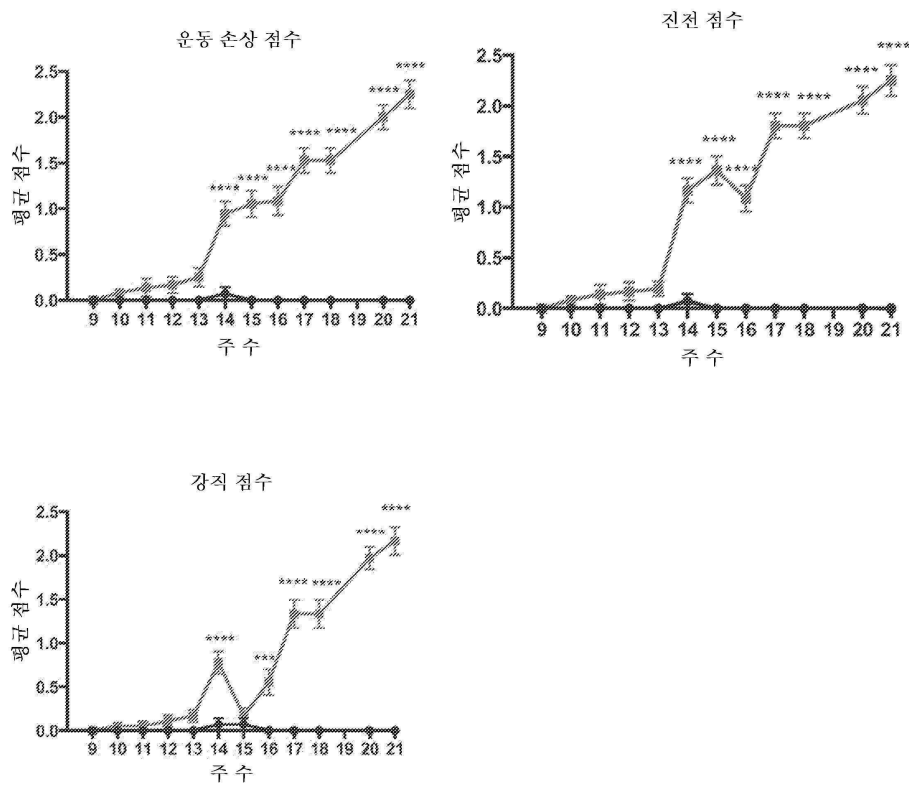
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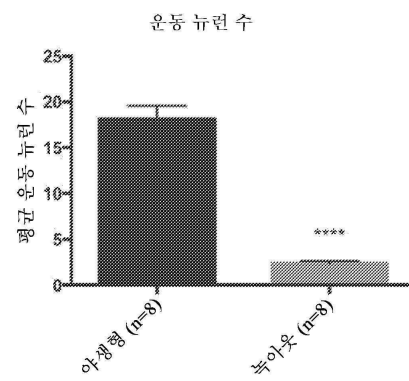
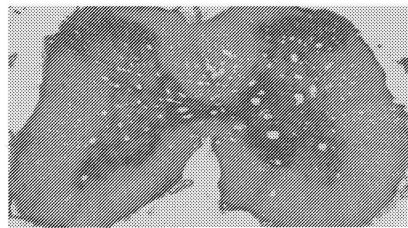
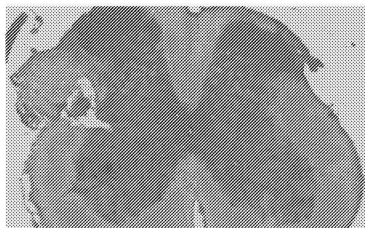
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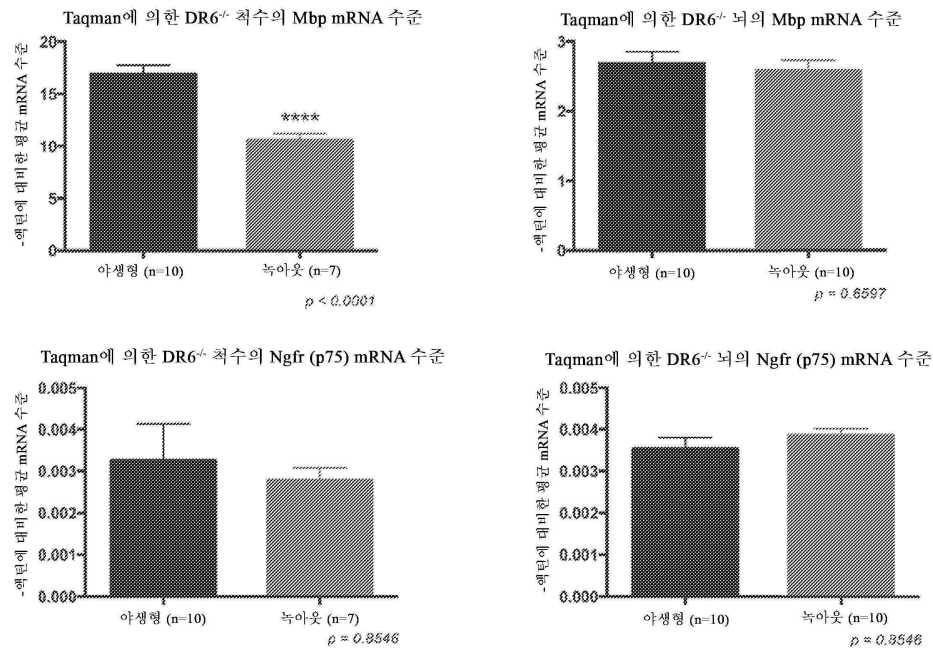
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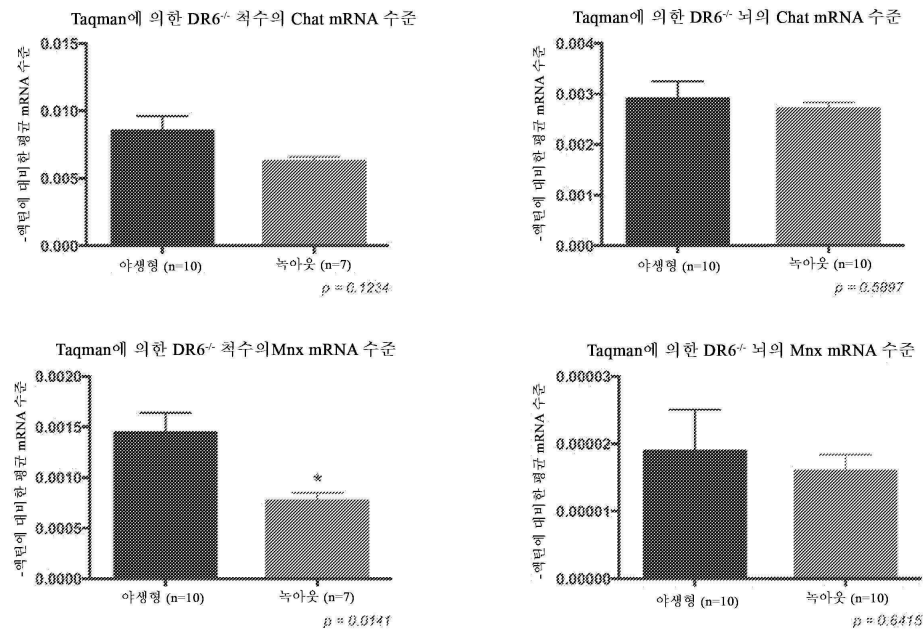
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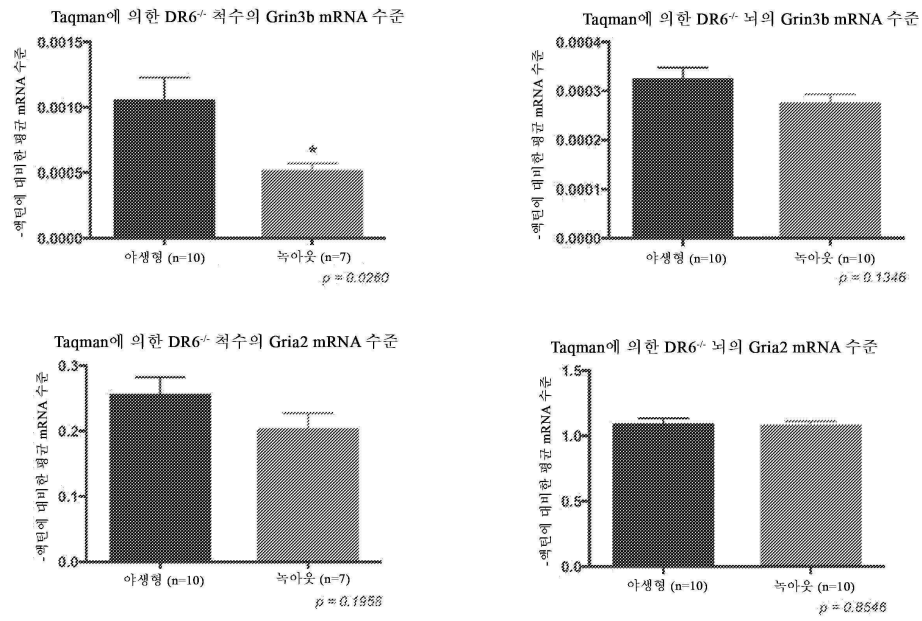
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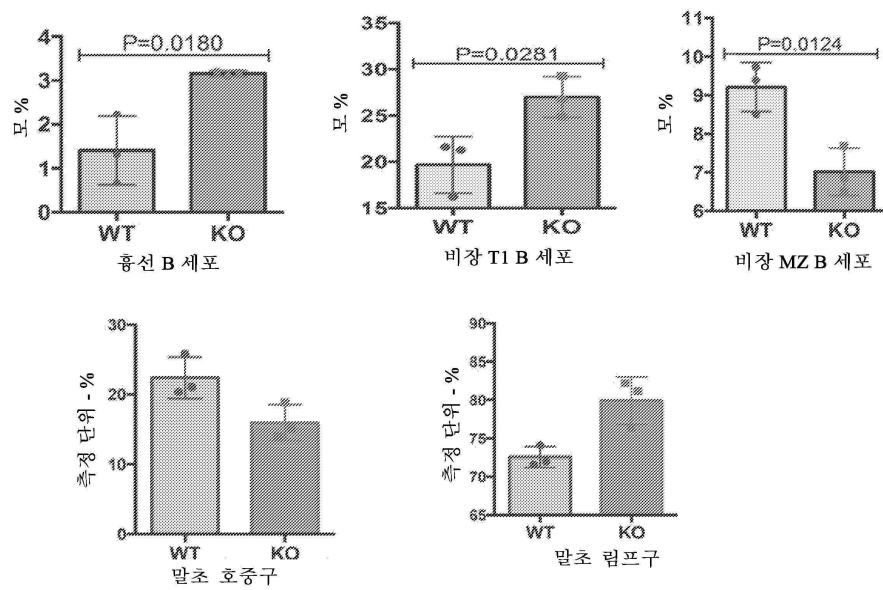
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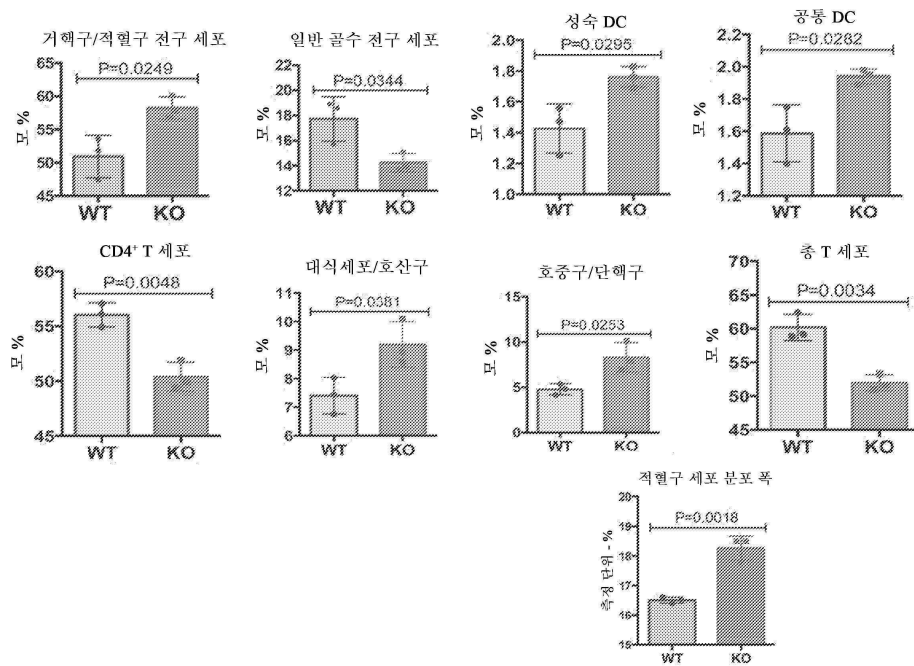
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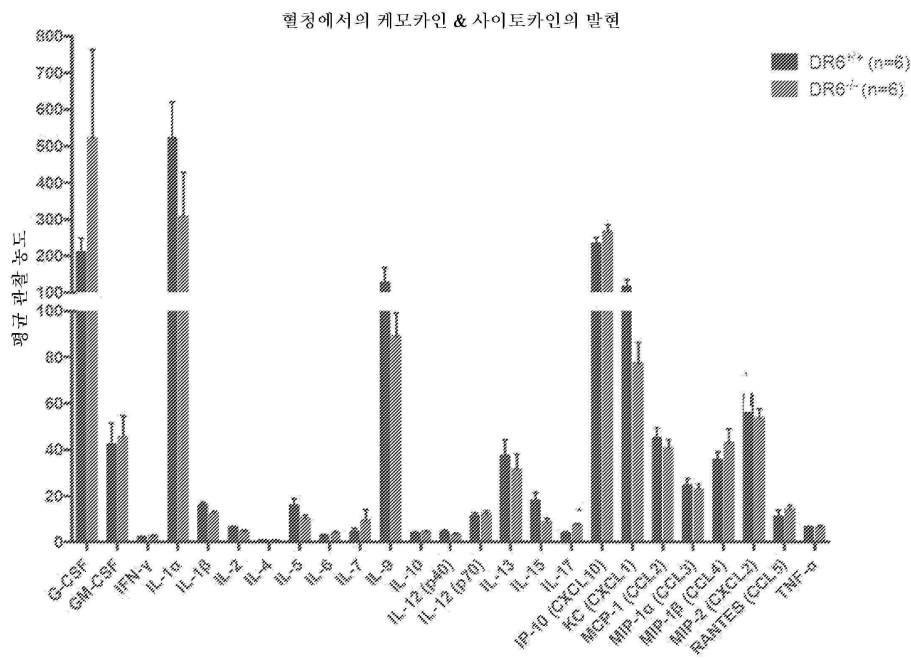
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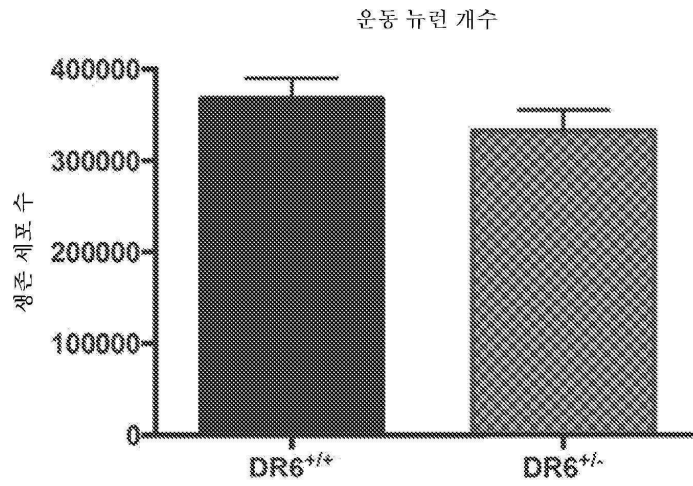
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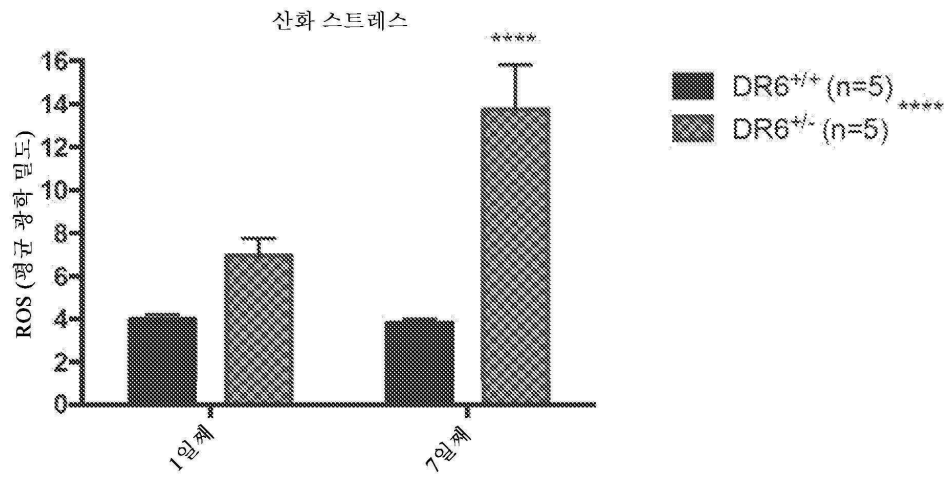
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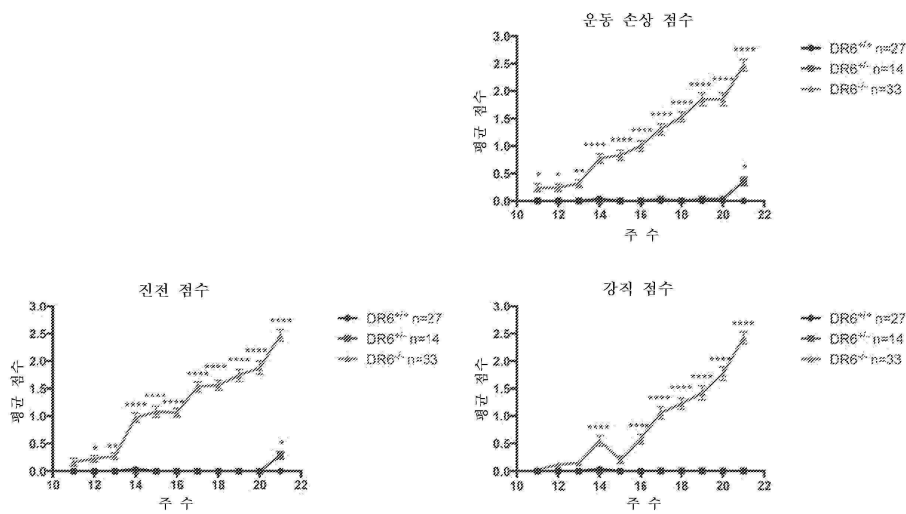
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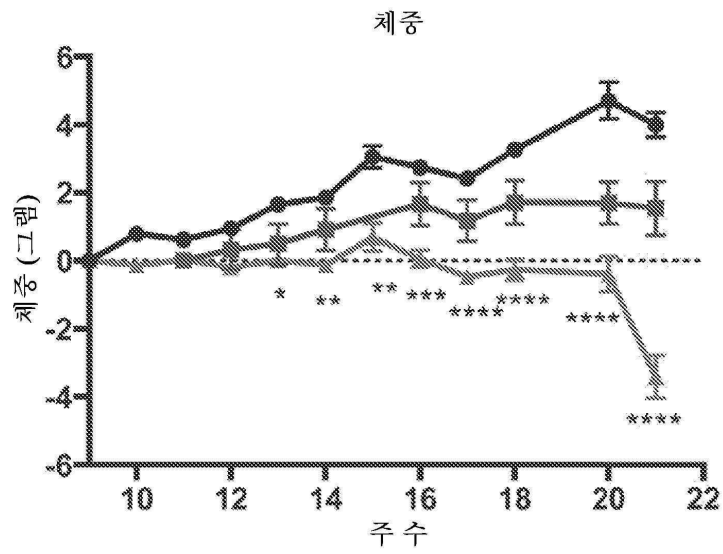
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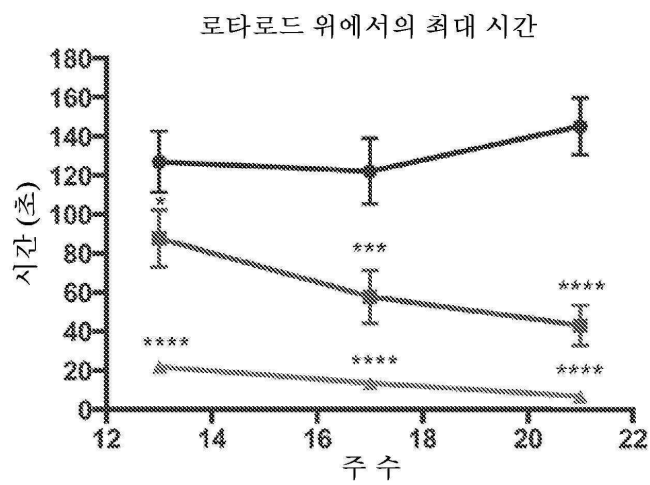
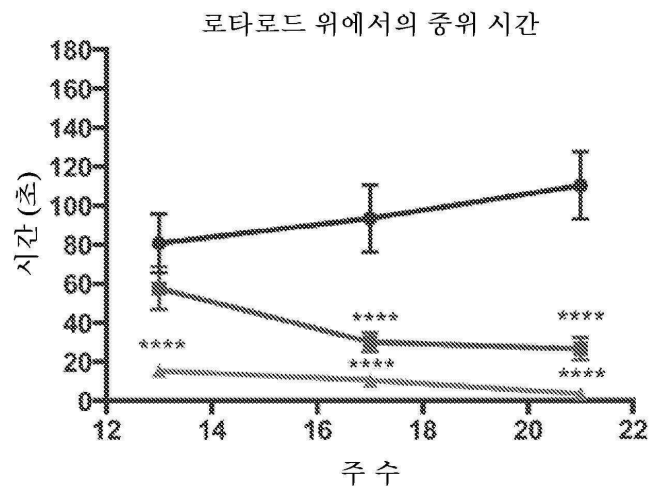
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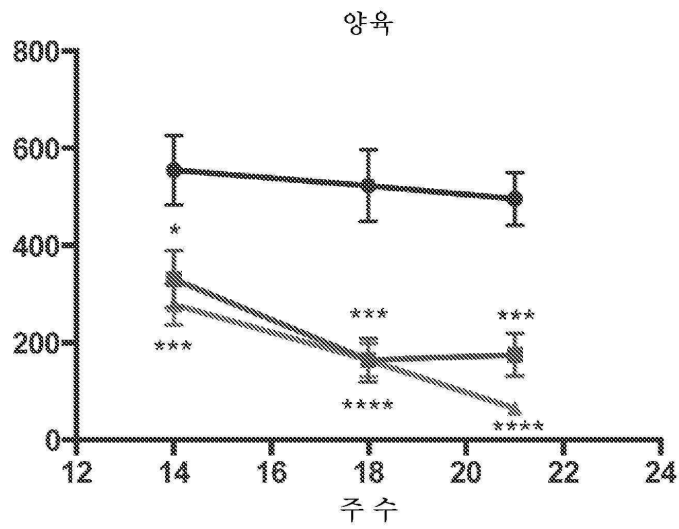
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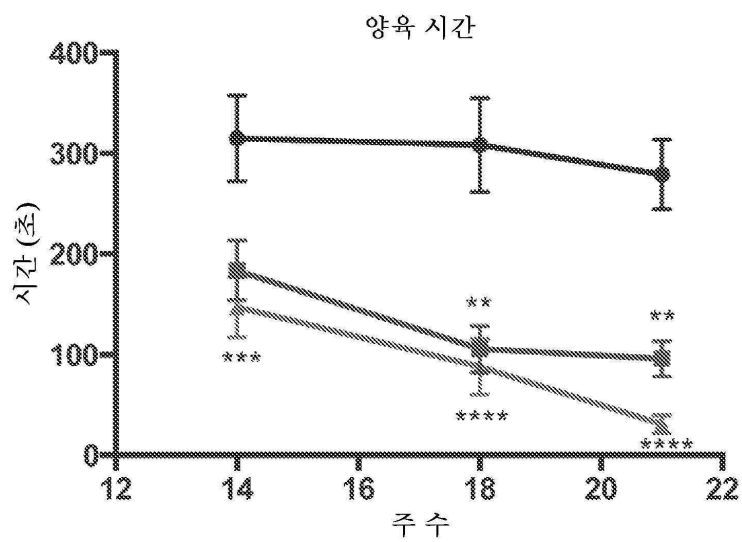
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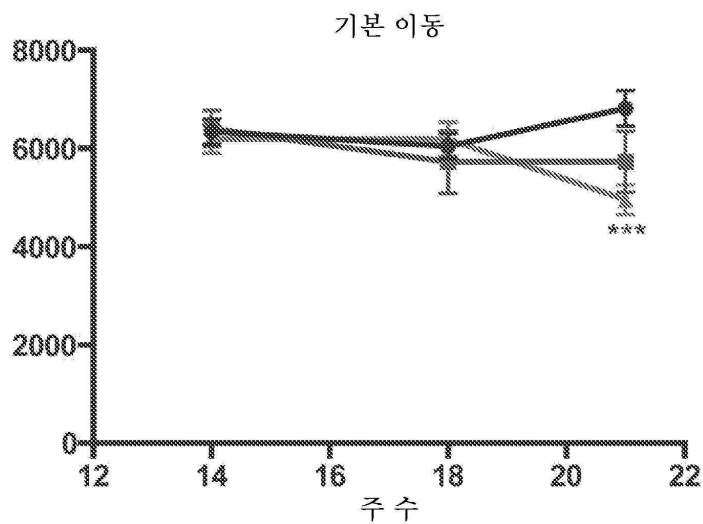
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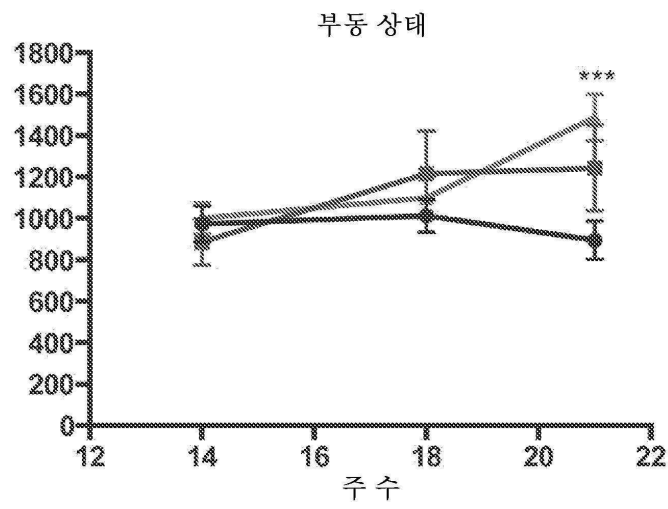
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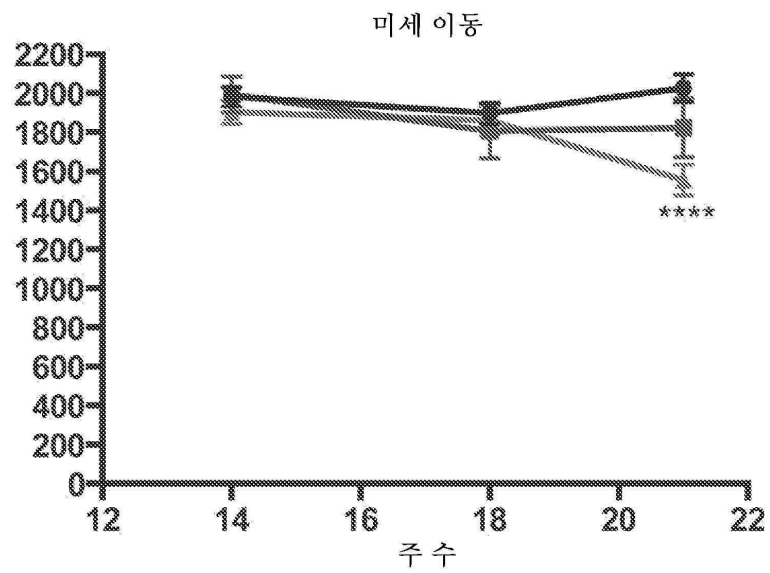
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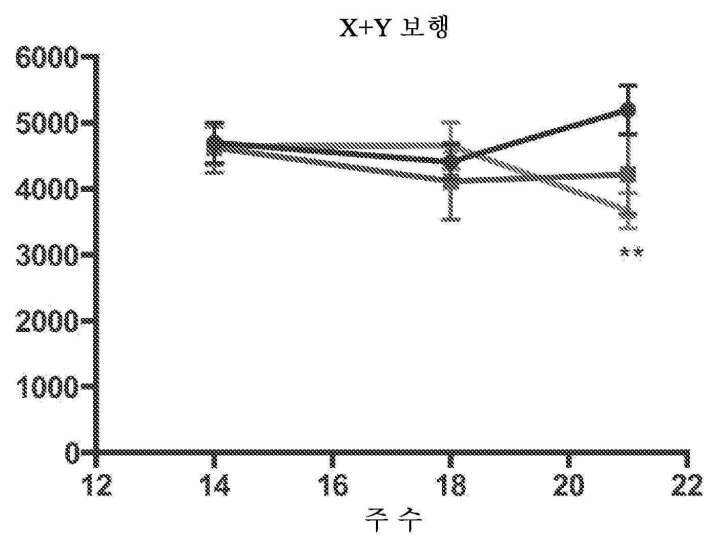
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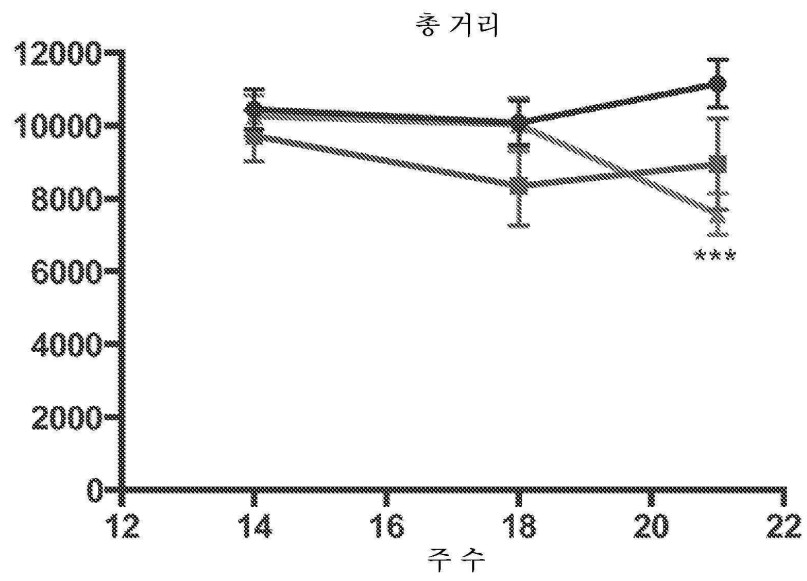
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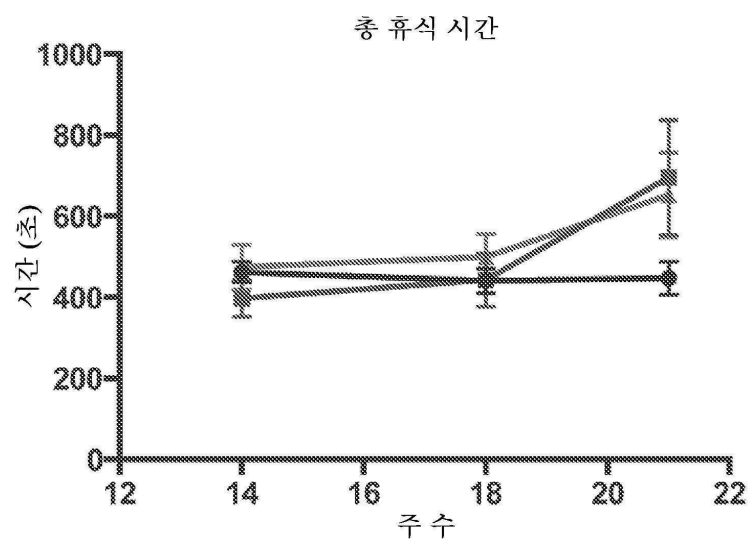
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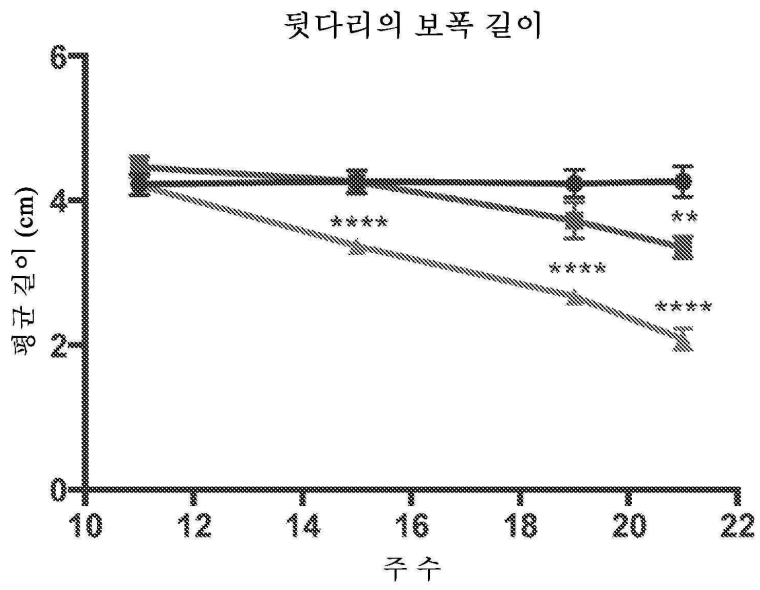
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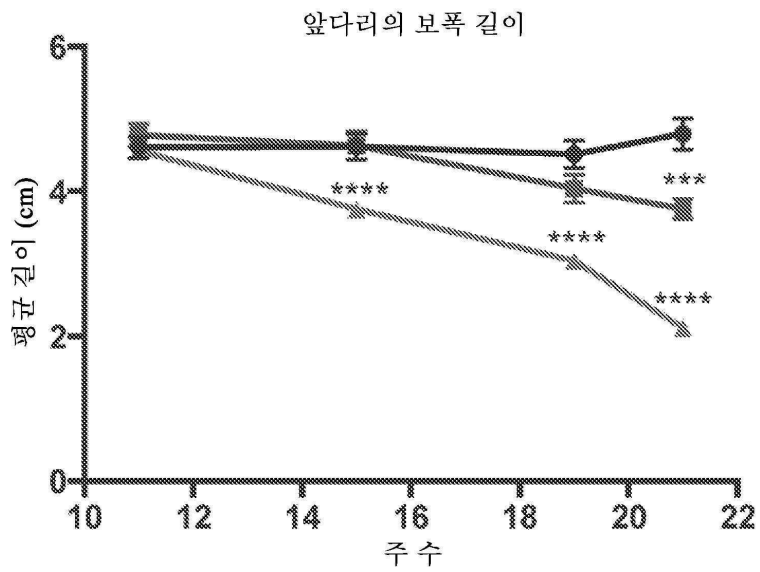
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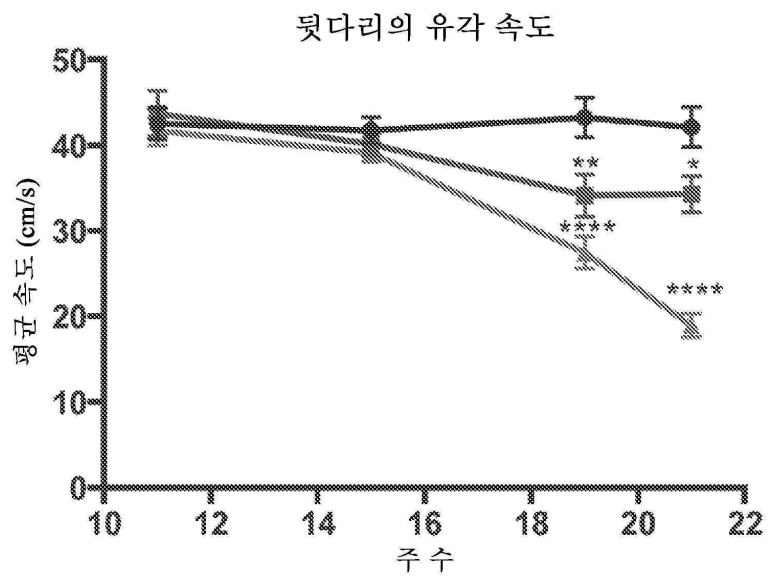
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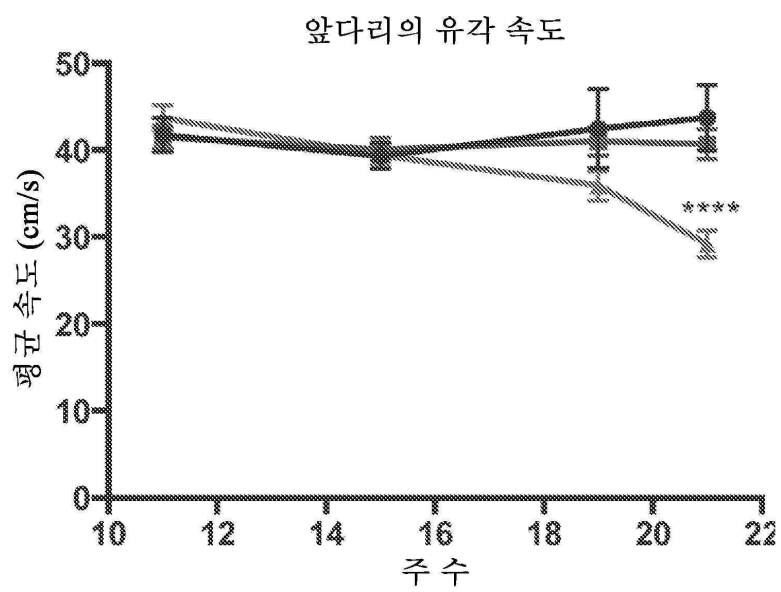
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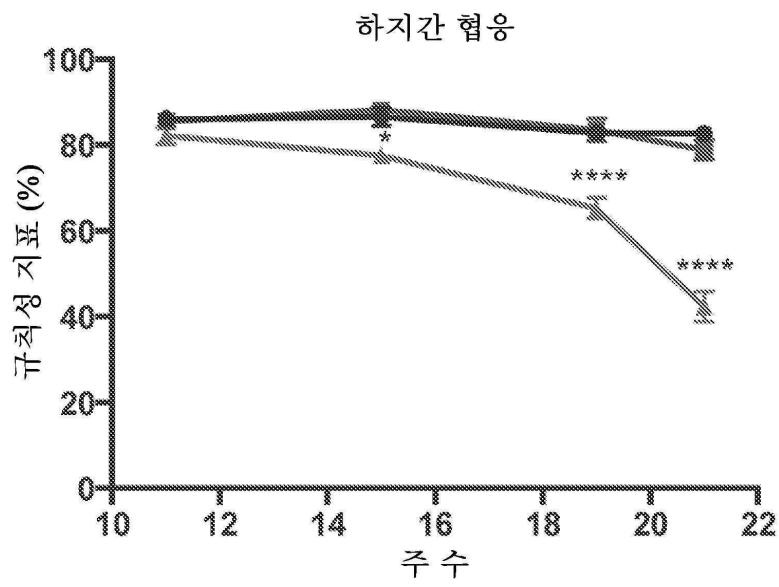
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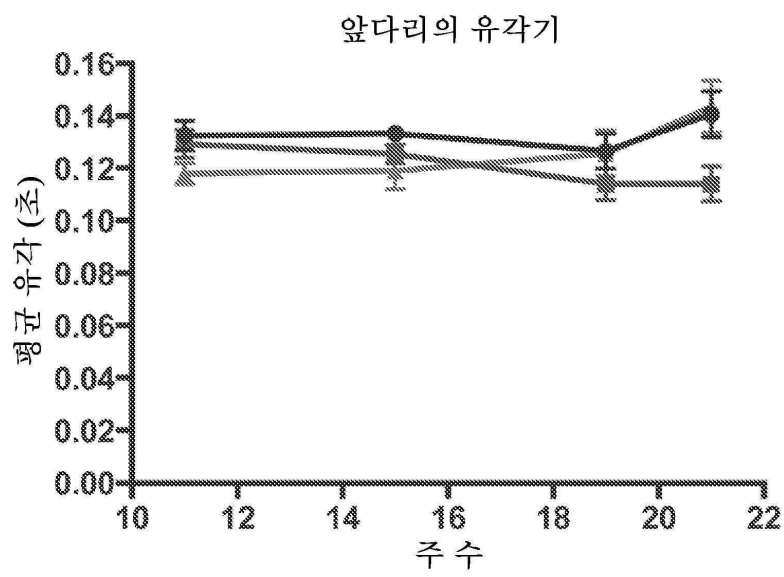
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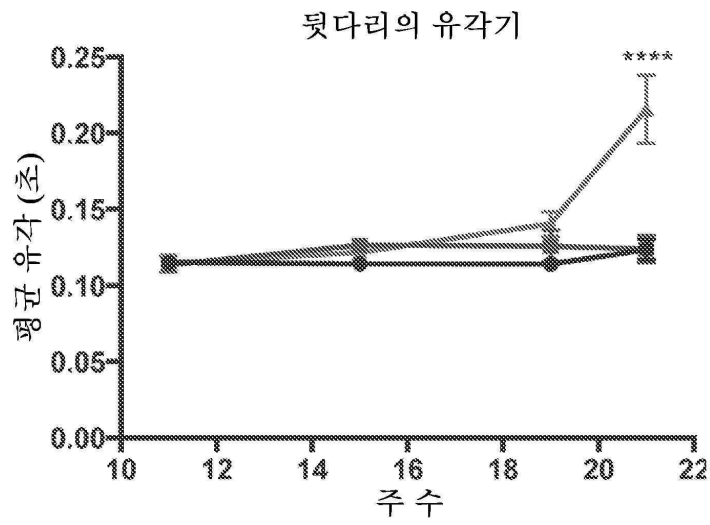
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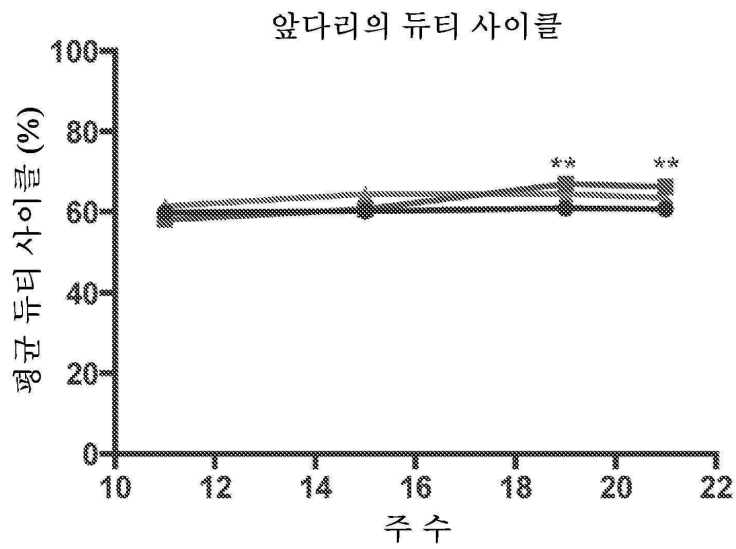
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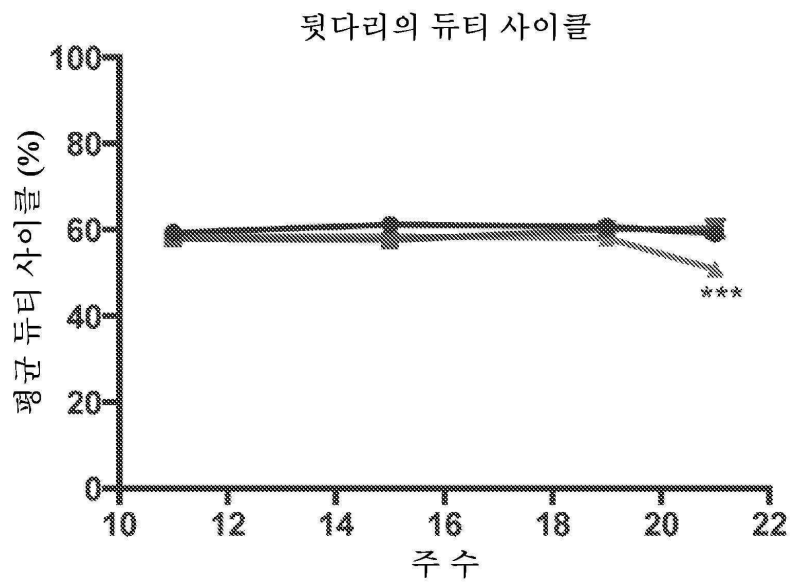
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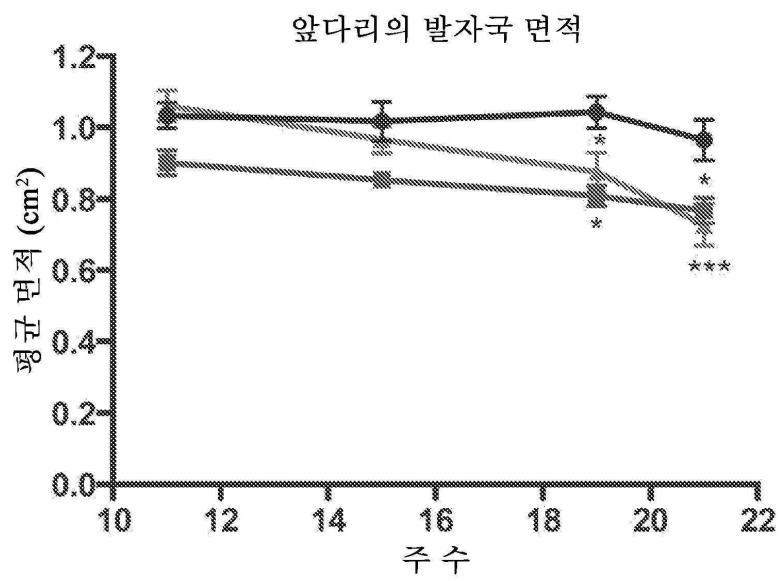
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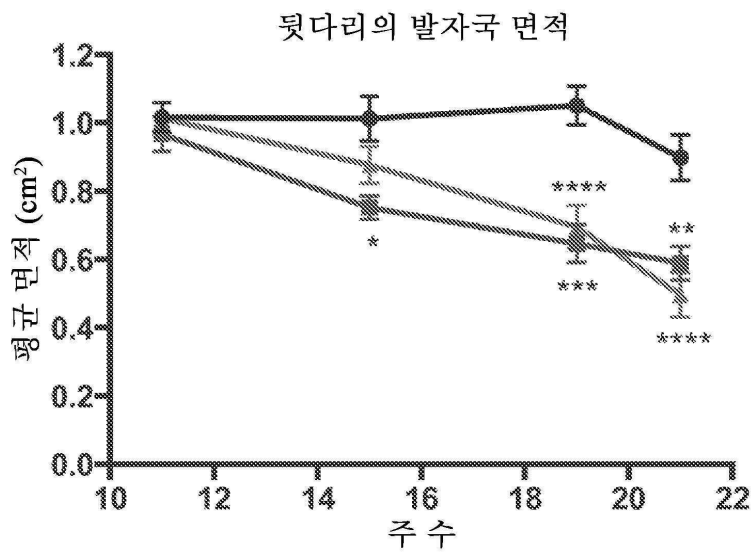
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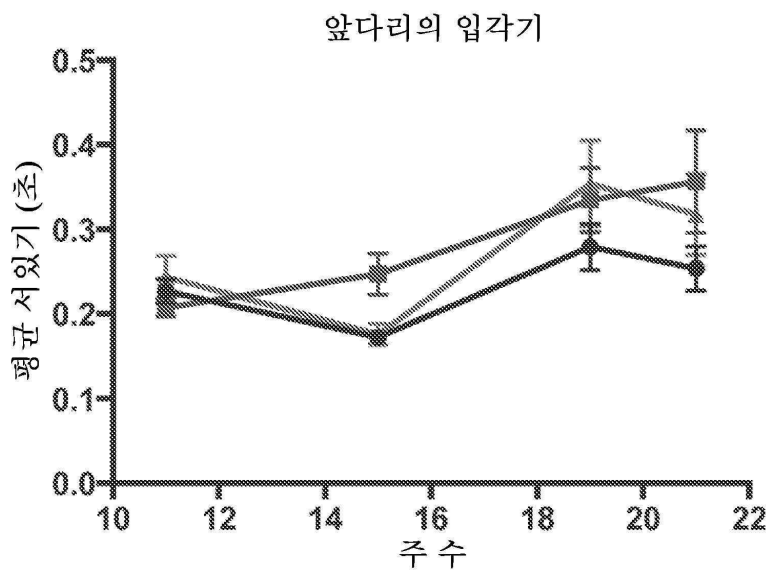
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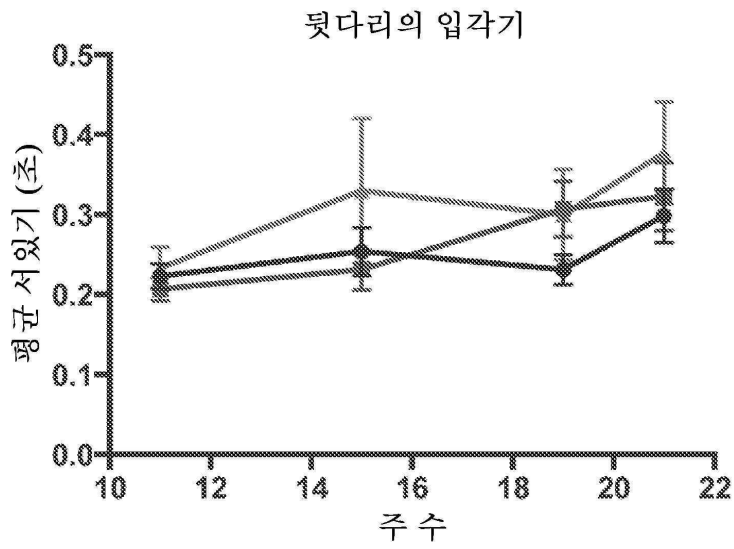
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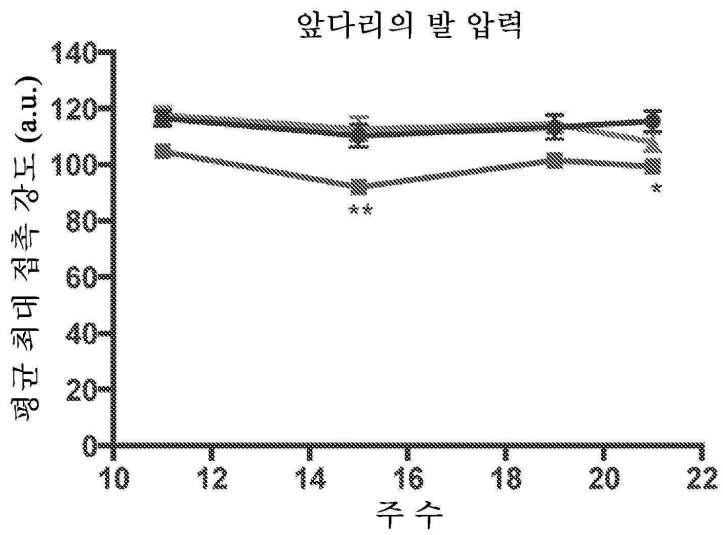
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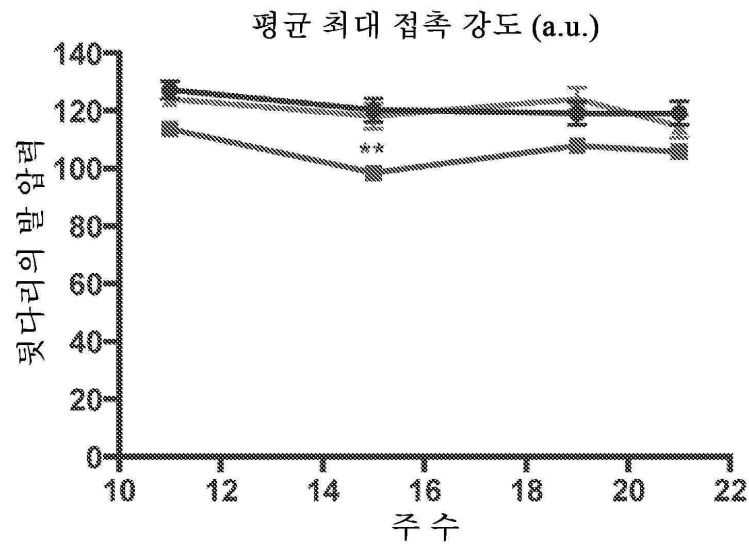
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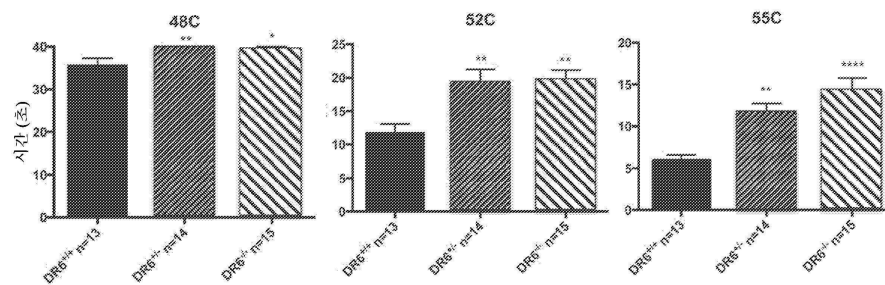
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도면17z



도면18



서열목록

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<110> Regeneron Pharmaceuticals, Inc.

Ikiz, Burcin

LaCroix-Fralish, Michael

Croll, Susan D.

<120> NON-HUMAN ANIMAL EXHIBITING DIMINISHED UPPER AND LOWER MOTOR
NEURON FUNCTION AND SENSORY PERCEPTION

<130> T0041US01

<150> 62/133,909

<151> 2015-03-16

<150> 62/250,229

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cag tct aat ggt acc tgc gct ccc cat aca gtg tgc ccc gtg ggc tgg	904
Gln Ser Asn Gly Thr Cys Ala Pro His Thr Val Cys Pro Val Gly Trp	
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Gly Val Arg Lys Lys Gly Thr Glu Asn Glu Asp Val Arg Cys Lys Gln	
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tgc gct cgg ggt acc ttc tct gac gtg cct tcc agt gtg atg aag tgt	1000
Cys Ala Arg Gly Thr Phe Ser Asp Val Pro Ser Ser Val Met Lys Cys	
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aaa gct cac acg gac tgt ctg ggt cag aac ctg gag gtg gtc aag cca	1048
Lys Ala His Thr Asp Cys Leu Gly Gln Asn Leu Glu Val Val Lys Pro	
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Gly Thr Lys Glu Thr Asp Asn Val Cys Gly Met Arg Leu Phe Phe Ser	

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Ser Thr Asn Pro Pro Ser Ser Gly Thr Val Thr Phe Ser His Pro Glu			
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cat atg gaa tcc cac gat gtc cct tcc tcc acc tat gag ccc caa ggc			1192
His Met Glu Ser His Asp Val Pro Ser Ser Thr Tyr Glu Pro Gln Gly			
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Met Asn Ser Thr Asp Ser Asn Ser Thr Ala Ser Val Arg Thr Lys Val			
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cca agt ggc atc gag gaa ggg aca gtg cct gac aat acg agc tca acc			1288
Pro Ser Gly Ile Glu Glu Gly Thr Val Pro Asp Asn Thr Ser Ser Thr			
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agt ggg aag gaa ggc act aat agg acc ctg cca aac cca cca caa gtt			1336
Ser Gly Lys Glu Gly Thr Asn Arg Thr Leu Pro Asn Pro Pro Gln Val			
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acc cac cag caa gcc ccc cac cac aga cac att ctg aag ctg ctg cca			1384
Thr His Gln Gln Ala Pro His His Arg His Ile Leu Lys Leu Leu Pro			
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tcg tcc atg gag gcc acg ggt gag aag tcc agc aca gcc atc aag gcc			1432
Ser Ser Met Glu Ala Thr Gly Glu Lys Ser Ser Thr Ala Ile Lys Ala			
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Pro Lys Arg Gly His Pro Arg Gln Asn Ala His Lys His Phe Asp Ile			
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aac gag cac ttg cct tgg atg atc gtc ctc ttc ctt ctg ctg gtc ctg			1528
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Lys Lys Ser Leu Thr Pro Thr Gln Asn Arg Glu Lys Trp Ile Tyr Tyr	
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Gly Ser Gln Trp Lys Asp Ile Tyr Gln Phe Leu Cys Asn Ala Ser Glu	
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Arg Glu Val Ala Ala Phe Ser Asn Gly Tyr Thr Ala Asp His Glu Arg	
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gcc tac gcg gct ctg cag cac tgg acc atc cgt ggc cct gag gcc agc	1864
Ala Tyr Ala Ala Leu Gln His Trp Thr Ile Arg Gly Pro Glu Ala Ser	
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Leu Ala Gln Leu Ile Ser Ala Leu Arg Gln His Arg Arg Asn Asp Val	
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Val Glu Lys Ile Arg Gly Leu Met Glu Asp Thr Thr Gln Leu Glu Thr	
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Asp Lys Leu Ala Leu Pro Met Ser Pro Ser Pro Leu Ser Pro Ser Pro	
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Ser Arg Asn Gly Ser Phe Ile Thr Lys Glu Lys Lys Asp Thr Val Leu			
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Leu Pro Asp Leu Leu			
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-5 -1 1 5

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His His Arg His Ile Leu Lys Leu Leu Pro Ser Ser Met Glu Ala Thr
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Thr Gln Asn Arg Glu Lys Trp Ile Tyr Tyr Arg Asn Gly His Gly Ile		
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Ile Tyr Gln Phe Leu Cys Asn Ala Ser Glu Arg Glu Val Ala Ala Phe		
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