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权利要求书2页 说明书14页
序列表35页 附图5页

(54)发明名称

一种可视化标记基因组位点的方法

(57)摘要

本发明公开了一种可视化标记基因组位点的方法。本发明通过在传统的转录激活样效应元件(TALE)蛋白的C端融合硫氧还蛋白TRX,创建了新型的基因组可视化标记工具TTALE。通过实验证明:TTALE可用于在肿瘤细胞系、胚胎干细胞、成体干细胞、终末分化细胞等不同类型的人类细胞中精确标记端粒、着丝粒和核糖体RNA编码序列(Ribosomal DNA,rDNA)等基因组重复序列,以及编码基因座(MUC4)。TTALE技术能够弥补目前科研和临床市场缺乏精确性、简便性和长效性的活细胞基因组可视化标记技术的空白,从而推动人类衰老和重要疾病的基础研究和临床诊疗技术的发展。

CN 106800602 A

1.一种用于可视化标记基因组位点的试剂盒,为如下1)-8)中任一种:

1)包括融合蛋白,所述融合蛋白包括用于识别或结合基因组靶序列的转录激活样效应元件蛋白TALE和硫氧还蛋白TRX;

2)包括融合蛋白,所述融合蛋白包括用于识别或结合基因组靶序列的转录激活样效应元件蛋白TALE、荧光蛋白和硫氧还蛋白TRX;

3)包括蛋白组合物,所述蛋白组合物包括用于识别或结合基因组靶序列的转录激活样效应元件蛋白TALE和硫氧还蛋白TRX;

4)包括蛋白组合物,所述蛋白组合物包括用于识别或结合基因组靶序列的转录激活样效应元件蛋白TALE、荧光蛋白和硫氧还蛋白TRX;

5)编码所述融合蛋白的DNA分子;

6)编码所述蛋白组合物的DNA分子;

7)包括表达1)或2)所述融合蛋白的载体;

8)包括表达3)或4)所述蛋白组合物的载体。

2.根据权利要求1所述的试剂盒,其特征在于:

所述靶序列为端粒DNA、着丝粒DNA、核仁组织区核糖体RNA编码基因或MUC4蛋白编码基因;

或所述端粒DNA的靶序列为序列14;

或所述着丝粒DNA的靶序列为序列15;

或所述核仁组织区核糖体RNA编码基因的靶序列为序列16;

或所述MUC4蛋白编码基因的靶序列为序列17。

3.根据权利要求2所述的试剂盒,其特征在于:

用于识别或结合基因组中所述端粒DNA的靶序列的融合蛋白为a1)或a2);

a1)由核定位序列、用于识别或结合基因组中端粒DNA的靶序列的转录激活样效应元件蛋白TALE和硫氧还蛋白TRX融合得到的蛋白质;

a2)由核定位序列、用于识别或结合基因组中端粒DNA的靶序列的转录激活样效应元件蛋白TALE、荧光蛋白和硫氧还蛋白TRX融合得到的蛋白质;

或用于识别或结合基因组中所述着丝粒DNA的靶序列的融合蛋白为a3)或a4);

a3)由核定位序列、用于识别或结合基因组中着丝粒DNA的靶序列的转录激活样效应元件蛋白TALE和硫氧还蛋白TRX融合得到的蛋白质;

a4)由核定位序列、用于识别或结合基因组中着丝粒DNA的靶序列的转录激活样效应元件蛋白TALE、荧光蛋白和硫氧还蛋白TRX融合得到的蛋白质;

或用于识别或结合基因组中所述核仁组织区核糖体RNA编码基因的靶序列的融合蛋白由核定位序列、用于识别或结合基因组中核仁组织区核糖体RNA编码基因的靶序列的转录激活样效应元件蛋白TALE、荧光蛋白和硫氧还蛋白TRX融合得到的蛋白质;

或用于识别或结合基因组中MUC4蛋白编码基因的靶序列的融合蛋白由核定位序列、用于识别或结合基因组中MUC4蛋白编码基因的靶序列的转录激活样效应元件蛋白TALE、荧光蛋白和硫氧还蛋白TRX融合得到的蛋白质。

4.根据权利要求1-3中任一所述的试剂盒,其特征在于:

所述荧光蛋白为EGFP蛋白或mCherry蛋白;

a1)中所述融合蛋白的氨基酸序列为序列4；

a2)中所述融合蛋白的氨基酸序列为序列7；

a3)中所述融合蛋白的氨基酸序列为序列5；

a4)中所述融合蛋白的氨基酸序列为序列8；

用于识别或结合基因组中所述核仁组织区核糖体RNA编码基因的靶序列的融合蛋白的氨基酸序列为序列10；

用于识别或结合基因组中所述MUC4蛋白编码基因的靶序列的融合蛋白的氨基酸序列为序列12。

5.权利要求1-4中任一所述的试剂盒或权利要求1-4中任一所述的试剂盒中的融合蛋白或权利要求1-4中任一所述的试剂盒中的蛋白组合物在基因组可视化中的应用；

或权利要求1-4中任一所述的试剂盒或权利要求1-4中任一所述的试剂盒中的融合蛋白或权利要求1-4中任一所述的试剂盒中的蛋白组合物在制备基因组可视化的产品中的应用。

6.权利要求1-4中任一所述的试剂盒或权利要求1-4中任一所述的试剂盒中的融合蛋白或权利要求1-4中任一所述的试剂盒中的蛋白组合物在如下b1)-b14)中任一种中的应用：

b1)可视化标记基因组位点；

b2)制备可视化标记基因组位点的产品；

b3)可视化标记细胞基因组中的端粒；

b4)制备可视化标记细胞基因组中的端粒的产品；

b5)可视化标记细胞基因组中的着丝粒；

b6)制备可视化标记细胞基因组中的着丝粒的产品；

b7)可视化标记细胞基因组中的核仁组织区核糖体RNA；

b8)制备可视化标记细胞基因组中的核仁组织区核糖体RNA的产品；

b9)可视化标记细胞基因组中的MUC4编码基因位点；

b10)制备可视化标记细胞基因组中的MUC4编码基因位点的产品；

b11)可视化检测端粒或着丝粒在细胞分裂不同时期的动态变化；

b12)制备可视化检测端粒或着丝粒在细胞分裂不同时期的动态变化的产品；

b13)可视化检测端粒在不同细胞衰老模型的动态变化；

b14)制备可视化检测端粒在不同细胞衰老模型的动态变化的产品。

7.权利要求1-4中任一所述的试剂盒中的融合蛋白或权利要求1-4中任一所述的试剂盒中的蛋白组合物在作为基因组可视化工具中的应用。

8.编码权利要求1-4中任一所述的试剂盒中的融合蛋白或权利要求1-4中任一所述的试剂盒中的蛋白组合物的DNA分子在制备基因组可视化工具中的应用。

9.一种可视化标记基因组位点的方法，包括将权利要求1-4中任一所述的试剂盒中的融合蛋白或权利要求1-4中任一所述的试剂盒中的表达所述融合蛋白的载体导入细胞中，实现细胞基因组位点的可视化。

10.根据权利要求6所述的应用或权利要求9所述的方法，其特征在于：

所述细胞为人或动物细胞。

一种可视化标记基因组位点的方法

技术领域

[0001] 本发明属于生物技术领域，具体涉及一种可视化标记基因组位点的方法。

背景技术

[0002] 在人类细胞的细胞核中，大约32亿对碱基组合成庞大的人类基因组，并且进一步凝缩形成不同大小的23对染色体。尽管人类基因组计划的完成帮助我们获得了人类基因组的全部序列信息，但是这仅仅是我们认识人类基因组的结构，以及了解由基因组组成的染色质三维结构与人类发育、衰老和疾病等重要生物学过程之间关系的第一步。事实上，已有多项研究证明基因组结构失序是导致衰老和若干严重疾病的重要因素(Zhang,W.,etal.,Aging stem cells.A Werner syndrome stem cell model unveils heterochromatin alterations as a driver of human aging.Science,2015.348(6239):p.1160-3.2.Liu,G.H.,et al.,Progressive degeneration of human neural stem cells caused by pathogenic LRRK2.Nature,2012.491(7425):p.603-7.3.Misteli,T.,Higher-order genome organization in human disease.Cold Spring Harb Perspect Biol,2010.2(8):p.a000794.)。因此，明确人类基因组和由其构成的染色质的空间结构，及其与蛋白质和RNA调节子直接的相互作用，将非常有助于我们理解导致衰老和人类疾病的细胞生物学进程(Lopez-Otin,C.,etal.,The hallmarks of aging.Cell,2013.153(6):p.1194-217.2.Misteli,T.,Beyond the sequence:cellular organization of genome function.Cell,2007.128(4):p.787-800.3.Misteli,T.,The cell biology of genomes:bringing the double helix to life.Cell,2013.152(6):p.1209-12.)。

[0003] 可视化的基因组标记技术可以大大提升对于基因组和染色质结构和功能研究的效率。尽管目前已有多种基因组可视化标记技术应用于科研，但是荧光标记Lac或Tet系统需要将约10kb的外源大片段整合进入基因组目的位点，因此存在低标记效率和潜在的基因组损伤等问题(Robinett,C.C.,etal.,In vivo localization of DNA sequences and visualization of large-scale chromatin organization using lac operator/repressor recognition.J Cell Biol,1996.135(6Pt 2):p.1685-700.2.Heun,P.,etal.,Chromosome dynamics in the yeast interphase nucleus.Science,2001.294(5549):p.2181-6.)；荧光原位杂交(FISH)是目前研究特定序列在基因组中定位的金标准，但是该方法需要在化学固定的细胞中进行操作，因此无法实现活细胞中的基因组可视化标记(Levsky,J.M.and R.H.Singer,Fluorescence in situ hybridization:past,present and future.J Cell Sci,2003.116(Pt 14):p.2833-8.)；尽管近年来兴起的CRISPR/Cas9技术可以实现端粒等基因组特殊位点的活细胞精确标记，但是细胞核内的高背景导致的低信噪比以及复杂的系统等缺点导致其无法在肿瘤细胞系之外的其它人类细胞(如多能干细胞和终末分化细胞等)中实现基因组位点的精确标记(Levsky,J.M.and R.H.Singer,Fluorescence in situ hybridization:past,present and future.J Cell Sci,2003.116(Pt 14):p.2833-8.)；另一种被用做基因编辑技术的转录激活因子样效应元件

(TALE)同样可以被用于基因组位点的可视化标记,然而现有的报道中既没有用FISH金标准验证TALE介导的基因组标记的正确性,同时显示其在不同细胞类型中标记的强异质性(Ma,H.,P.Reyes-Gutierrez,and T.Pederson,Visualization of repetitive DNA sequences in human chromosomes with transcription activator-like effectors.Proc Natl Acad Sci USA,2013.110(52):p.21048-53.)。

[0004] 综上所述,现有的基因组可视化标记技术都存在缺陷,无法满足精确标记人类细胞基因组元件和特殊位点的需要。因此,目前无论是科研还是临床市场,都急需具备精确性、简便性和长效性的活细胞基因组可视化标记技术来弥补空白,从而推动人类衰老和重要疾病的基础研究和临床诊疗技术的发展。

发明内容

[0005] 本发明的一个目的是提供一种用于可视化标记基因组位点的试剂盒。

[0006] 本发明提供的用于可视化标记基因组位点的试剂盒为如下1)-8)中任一种:

[0007] 1)包括融合蛋白,所述融合蛋白包括用于识别或结合基因组靶序列的转录激活样效应元件蛋白TALE和硫氧还蛋白TRX;

[0008] 2)包括融合蛋白,所述融合蛋白包括用于识别或结合基因组靶序列的转录激活样效应元件蛋白TALE、荧光蛋白和硫氧还蛋白TRX;

[0009] 3)包括蛋白组合物,所述蛋白组合物包括用于识别或结合基因组靶序列的转录激活样效应元件蛋白TALE和硫氧还蛋白TRX;

[0010] 4)包括蛋白组合物,所述蛋白组合物包括用于识别或结合基因组靶序列的转录激活样效应元件蛋白TALE、荧光蛋白和硫氧还蛋白TRX;

[0011] 5)编码所述融合蛋白的DNA分子;

[0012] 6)编码所述蛋白组合物的DNA分子;

[0013] 7)包括表达1)或2)所述融合蛋白的载体;

[0014] 8)包括表达3)或4)所述蛋白组合物的载体。

[0015] 上述试剂盒中,所述靶序列为端粒DNA、着丝粒DNA、核仁组织区核糖体RNA编码基因或MUC4蛋白编码基因。

[0016] 上述试剂盒中,所述靶序列可以根据本领域公知常识,选择它们的部分序列作为靶序列。优选的,本发明选择了如下序列作为靶序列:

[0017] 所述端粒DNA的靶序列为序列14;

[0018] 所述着丝粒DNA的靶序列为序列15;

[0019] 所述核仁组织区核糖体RNA编码基因的靶序列为序列16;

[0020] 所述MUC4蛋白编码基因的靶序列为序列17。

[0021] 上述试剂盒中,

[0022] 用于识别或结合基因组中所述端粒DNA的靶序列的融合蛋白为a1)或a2);

[0023] a1)由核定位序列、用于识别或结合基因组中端粒DNA的靶序列的转录激活样效应元件蛋白TALE和硫氧还蛋白TRX融合得到的蛋白质;所述融合蛋白自N端至C端依次包括核定位序列、用于识别或结合基因组中端粒DNA的靶序列的转录激活样效应元件蛋白TALE和硫氧还蛋白TRX;

[0024] a2)由核定位序列、用于识别或结合基因组中端粒DNA的靶序列的转录激活样效应元件蛋白TALE、荧光蛋白和硫氧还蛋白TRX融合得到的蛋白质;所述融合蛋白自N端至C端依次包括核定位序列、用于识别或结合基因组中端粒DNA的靶序列的转录激活样效应元件蛋白TALE、荧光蛋白和硫氧还蛋白TRX;

[0025] 或用于识别或结合基因组中所述着丝粒DNA的靶序列的融合蛋白为a3)或a4);

[0026] a3)由核定位序列、用于识别或结合基因组中着丝粒DNA的靶序列的转录激活样效应元件蛋白TALE和硫氧还蛋白TRX融合得到的蛋白质;所述融合蛋白自N端至C端依次包括核定位序列、用于识别或结合基因组中着丝粒DNA的靶序列的转录激活样效应元件蛋白TALE和硫氧还蛋白TRX;

[0027] a4)由核定位序列、用于识别或结合基因组中着丝粒DNA的靶序列的转录激活样效应元件蛋白TALE、荧光蛋白和硫氧还蛋白TRX融合得到的蛋白质;所述融合蛋白自N端至C端依次包括核定位序列、用于识别或结合基因组中着丝粒DNA的靶序列的转录激活样效应元件蛋白TALE、荧光蛋白和硫氧还蛋白TRX;

[0028] 或用于识别或结合基因组中所述核仁组织区核糖体RNA编码基因的靶序列的融合蛋白由核定位序列、用于识别或结合基因组中核仁组织区核糖体RNA编码基因的靶序列的转录激活样效应元件蛋白TALE、荧光蛋白和硫氧还蛋白TRX融合得到的蛋白质;所述融合蛋白自N端至C端依次包括核定位序列、用于识别或结合基因组中核仁组织区核糖体RNA编码基因的靶序列的转录激活样效应元件蛋白TALE、荧光蛋白和硫氧还蛋白TRX;

[0029] 或用于识别或结合基因组中MUC4蛋白编码基因的靶序列的融合蛋白由核定位序列、用于识别或结合基因组中MUC4蛋白编码基因的靶序列的转录激活样效应元件蛋白TALE、荧光蛋白和硫氧还蛋白TRX融合得到的蛋白质;所述融合蛋白自N端至C端依次包括核定位序列、用于识别或结合基因组中MUC4蛋白编码基因的靶序列的转录激活样效应元件蛋白TALE、荧光蛋白和硫氧还蛋白TRX。

[0030] 上述试剂盒中,

[0031] 所述荧光蛋白为EGFP蛋白或mCherry蛋白;

[0032] a1)中所述融合蛋白的氨基酸序列为序列4;

[0033] a2)中所述融合蛋白的氨基酸序列为序列7;

[0034] a3)中所述融合蛋白的氨基酸序列为序列5;

[0035] a4)中所述融合蛋白的氨基酸序列为序列8;

[0036] 用于识别或结合基因组中所述核仁组织区核糖体RNA编码基因的靶序列的融合蛋白的氨基酸序列为序列10;

[0037] 用于识别或结合基因组中所述MUC4蛋白编码基因的靶序列的融合蛋白的氨基酸序列为序列12。

[0038] 上述试剂盒中,

[0039] 所述融合蛋白的N端还带有Flag标签序列;所述FLAG标签序列具体为3xFlag标签序列。在实际应用中,本领域技术人员还可以利用N端的Flag标签序列通过免疫荧光染色实验的方法实现细胞基因组位点的可视化检测。

[0040] 上述试剂盒中,

[0041] 所述EGFP蛋白的编码基因为序列13第7382-8098位所示的DNA分子;

[0042] 所述mCherry蛋白的编码基因为序列6所示的DNA分子；

[0043] 所述硫氧还蛋白TRX的编码基因为序列3所示的DNA分子；

[0044] 所述3xFlag标签序列的编码基因为序列1第2113-2181位所示的DNA分子；

[0045] 所述核定位序列的编码基因为序列1第2182-2232位所示的DNA分子；

[0046] 所述用于识别或结合基因组中端粒DNA的靶序列的转录激活样效应元件蛋白TALE的编码基因为序列1第2233-4725位所示的DNA分子；

[0047] 所述用于识别或结合基因组中着丝粒DNA的靶序列的转录激活样效应元件蛋白TALE的编码基因为序列2第121-2613位所示的DNA分子；

[0048] 所述用于识别或结合基因组中核仁组织区核糖体RNA编码基因的靶序列的转录激活样效应元件蛋白TALE的编码基因为序列9第121-2613位所示的DNA分子；

[0049] 所述用于识别或结合基因组中MUC4蛋白编码基因的靶序列的转录激活样效应元件蛋白TALE的编码基因为序列11第121-2613位所示的DNA分子。

[0050] 上述试剂盒中，

[0051] 表达a1)中所述融合蛋白的载体是以用于识别或结合端粒DNA的靶序列的TALE载体为骨架载体，将序列3所示的人硫氧还蛋白TRX的编码基因序列插入骨架载体的KpnI和XhoI位点间得到的载体；其中，所述用于识别或结合端粒DNA的靶序列的TALE载体的核苷酸序列为序列1；

[0052] 表达a2)中所述融合蛋白的载体是以用于识别或结合端粒DNA的靶序列的TTALE载体为骨架载体，将序列6所示的mCherry编码基因序列插入骨架载体的HpaI和KpnI位点间得到的载体；其中，所述用于识别或结合端粒DNA的靶序列的TTALE载体是以所述用于识别或结合端粒DNA的靶序列的TALE载体为骨架载体，将序列3所示的人硫氧还蛋白TRX的编码基因序列插入骨架载体的KpnI和XhoI位点间得到的载体；

[0053] 表达a3)中所述融合蛋白的载体是以用于识别或结合着丝粒DNA的靶序列的TALE载体为骨架载体，将序列3所示的人硫氧还蛋白的编码基因序列插入骨架载体的KpnI和XhoI位点间得到载体；其中，所述用于识别或结合着丝粒DNA的靶序列的TALE载体的核苷酸序列为将序列1中第2113-4725位所示的DNA片段替换为序列2所示的DNA片段后得到的序列；

[0054] 表达a4)中所述融合蛋白的载体是以用于识别或结合着丝粒DNA的靶序列的TTALE载体为骨架载体，将序列13第7382-8098位所示的EGFP编码基因序列插入骨架载体的HpaI和KpnI位点间得到的载体；其中，所述用于识别或结合着丝粒DNA的靶序列的TTALE载体是以所述用于识别或结合着丝粒DNA的靶序列的TALE载体为骨架载体，将序列3所示的人硫氧还蛋白TRX的编码基因序列插入骨架载体的KpnI和XhoI位点间得到的载体；

[0055] 表达用于识别或结合基因组中所述核仁组织区核糖体RNA编码基因的靶序列的融合蛋白的载体是以用于识别或结合核仁组织区核糖体RNA编码基因的靶序列的TALE载体为骨架载体，将序列3所示的人硫氧还蛋白TRX的编码基因序列插入骨架载体的AscI和XhoI位点间得到的载体；其中，所述用于识别或结合核仁组织区核糖体RNA编码基因的靶序列的TALE载体的核苷酸序列为将序列1第2113-4725位所示的DNA片段替换为序列9所示的DNA片段，且将序列13第7382-8098位所示的EGFP编码基因序列插入序列1的HpaI和KpnI位点间后得到的序列；

[0056] 表达用于识别或结合基因组中MUC4蛋白编码基因的靶序列的融合蛋白的载体是以用于识别或结合MUC4蛋白编码基因的靶序列的TALE载体为骨架载体，将序列3所示的人硫氧还蛋白TRX的编码基因序列插入骨架载体的AscI和XhoI位点间得到的载体；其中，所述用于识别或结合MUC4蛋白编码基因的靶序列的TALE载体的核苷酸序列为将序列1第2113-4725位所示的DNA片段替换为序列11所示的DNA片段，且将序列13第7382-8098位所示的EGFP编码基因序列插入序列1的HpaI和KpnI位点间后得到的序列。

[0057] 本发明的第二个目的是提供上述试剂盒或上述试剂盒中的融合蛋白或上述试剂盒中的蛋白组合物的新用途。

[0058] 本发明提供了上述试剂盒或上述试剂盒中的融合蛋白或上述试剂盒中的蛋白组合物在基因组可视化中的应用。

[0059] 本发明还提供了上述试剂盒或上述试剂盒中的融合蛋白或上述试剂盒中的蛋白组合物在制备基因组可视化的产品中的应用。

[0060] 本发明还提供了上述试剂盒或上述试剂盒中的融合蛋白或上述试剂盒中的蛋白组合物在如下b1)-b14)中任一种中的应用：

[0061] b1)可视化标记基因组位点；

[0062] b2)制备可视化标记基因组位点的产品；

[0063] b3)可视化标记细胞基因组中的端粒；

[0064] b4)制备可视化标记细胞基因组中的端粒的产品；

[0065] b5)可视化标记细胞基因组中的着丝粒；

[0066] b6)制备可视化标记细胞基因组中的着丝粒的产品；

[0067] b7)可视化标记细胞基因组中的核仁组织区核糖体RNA；

[0068] b8)制备可视化标记细胞基因组中的核仁组织区核糖体RNA的产品；

[0069] b9)可视化标记细胞基因组中的MUC4编码基因位点；

[0070] b10)制备可视化标记细胞基因组中的MUC4编码基因位点的产品；

[0071] b11)可视化检测端粒或着丝粒在细胞分裂不同时期的动态变化；

[0072] b12)制备可视化检测端粒或着丝粒在细胞分裂不同时期的动态变化的产品；

[0073] b13)可视化检测端粒在不同细胞衰老模型的动态变化；

[0074] b14)制备可视化检测端粒在不同细胞衰老模型的动态变化的产品。

[0075] 本发明还有一个目的是提供上述试剂盒中的融合蛋白或上述试剂盒中的蛋白组合物的新用途。

[0076] 本发明提供了上述试剂盒中的融合蛋白或上述试剂盒中的蛋白组合物在作为基因组可视化工具中的应用。

[0077] 编码上述试剂盒中的融合蛋白或上述试剂盒中的蛋白组合物的DNA分子在制备基因组可视化工具中的应用也属于本发明的保护范围。

[0078] 本发明的最后一个目的是提供一种可视化标记基因组位点的方法。

[0079] 本发明提供的可视化标记基因组位点的方法包括将上述试剂盒中的融合蛋白或表达所述融合蛋白的载体导入细胞中，实现细胞基因组位点的可视化。

[0080] 上述应用或上述方法中，

[0081] 所述细胞为人或动物细胞；所述人或动物细胞为人肿瘤细胞、人胚胎肾细胞、人多

能干细胞、人成体干细胞、人终末分化细胞或小鼠OP9细胞;所述人或动物细胞具体为人肿瘤细胞系(U2OS、HeLa、MCF7和HepG2)、人胚胎肾细胞系(HEK293)、人多能干细胞(胚胎干细胞hESC)、人多能干细胞(诱导多能干细胞iPSC)、成体干细胞(间充质干细胞)、成体干细胞(神经干细胞hNSC)、终末分化细胞(血管平滑肌细胞hVSMC)或小鼠OP9细胞。

[0082] 本发明通过在传统的转录激活样效应元件(TALE)蛋白的C端融合硫氧还蛋白TRX,创建了新型的基因组可视化标记工具TTALE。通过实验证明:TTALE可用于在肿瘤细胞系、胚胎干细胞、成体干细胞、终末分化细胞等不同类型的人类细胞中精确标记端粒、着丝粒和核糖体RNA编码序列(Ribosomal DNA,rDNA)等基因组重复序列,以及编码基因座(MUC4)。TTALE技术能够弥补目前科研和临床市场缺乏精确性、简便性和长效性的活细胞基因组可视化标记技术的空白,从而推动人类衰老和重要疾病的基础研究和临床诊疗技术的发展。

附图说明

[0083] 图1为本发明通过将硫氧还蛋白TRX融合在TALE的C端建立了能够精确标记细胞基因组中的端粒(Telomere)和着丝粒(Centromere)的基因组可视化标记技术(TTALE)。其中,A为TTALE标记基因组位点的模式图;B为识别端粒(Telomere)的TTALE在细胞核内的标记结果,从左到右依次为:FLAG标签标记TTALE标记端粒的结果;FISH探针标记端粒的结果;前两张图片叠加的结果,说明TTALE与FISH信号很好的共定位效果;叠加图片的局部放大结果;C为识别端粒(Telomere)的TTALE在细胞核内标记出的荧光点的分布图;D为特异性标记端粒(Telomere)的FISH探针在细胞核内标记出的荧光点的分布图;E为识别着丝粒(Centromere)的TTALE在细胞核内的标记结果;F为识别着丝粒(Centromere)的TTALE在细胞核内标记出的荧光点的分布图;G为特异性标记着丝粒(Centromere)的FISH探针在细胞核内标记出的荧光点的分布图。

[0084] 图2为利用TTALE精确标记人肿瘤细胞系中的端粒(Telomere)和着丝粒(Centromere)。其中,A为识别端粒(Telomere)的TTALE在处于细胞间期的人肿瘤细胞系中的标记结果;B为识别着丝粒(Centromere)的TTALE在处于细胞间期的人肿瘤细胞系中的标记结果;C为用荧光蛋白(EGFP或mCherry)融合表达的识别端粒(Telomere)或着丝粒(Centromere)的TTALE同时标记处于细胞分裂周期不同时期的HeLa细胞基因组的结果。

[0085] 图3为利用TTALE精确标记人多能干细胞(胚胎干细胞hESC或诱导多能干细胞iPSC)及其衍生的成体干细胞(间充质干细胞hMSC和神经干细胞hNSC)和终末分化细胞(神经细胞hNeuron和血管平滑肌细胞hVSMC)中的端粒(Telomere)和着丝粒(Centromere)。其中,A为识别着丝粒(Centromere)的TTALE的标记结果;B为识别端粒(Telomere)的TTALE的标记结果。

[0086] 图4为利用TTALE精确标记人类细胞中的核仁组织区核糖体RNA编码序列(NOR-rDNA)。其中,A为识别核仁组织区核糖体RNA编码序列的TALE载体的标记结果;B为在hMSC中证明识别NOR-rDNA的TTALE的标记结果能够与NOR-rDNA的FISH杂交信号共定位,并且其标记信号围绕核仁组织区的分子标志物Nucleolin和Fibrillarin;C为利用TTALE精确标记人肿瘤细胞(HeLa和U2OS)以及人多能干细胞(hESC,hMSC和hNSC)中的NOR-rDNA。

[0087] 图5为利用TTALE精确标记人类细胞中的基因编码序列(MUC4)。A为与识别编码基因位点(MUC4)的TALE载体的标记结果;B为利用TTALE精确标记人肿瘤细胞(HeLa)以及人多

能干细胞(hMSC)中的MUC4基因位点;C为用荧光蛋白(mCherry)融合表达的识别MUC4基因位点的TTALE标记处于细胞分裂周期不同时期的HeLa细胞基因组的结果。

[0088] 图6为利用识别端粒(Telomere)的TTALE在不同的细胞衰老模型中可视化标记基因组中端粒(Telomere)随细胞衰老进程的动态变化。A为不同细胞衰老模型的模式图;B为利用识别端粒(Telomere)的TTALE在不同的细胞衰老模型中可视化标记基因组中端粒(Telomere)的结果;C为WRN基因缺失的间充质干细胞的验证结果(WRN基因缺失验证实验);D为HGPS患者来源的间充质干细胞的验证结果(Progerin表达验证实验);E为三种细胞衰老模型中端粒缩短的qPCR验证;F为利用识别端粒(Telomere)的TTALE在不同的细胞衰老模型中可视化标记基因组中端粒(Telomere)的荧光强度统计结果。

[0089] 图7为利用识别端粒(Telomere)的TTALE在动物体内可视化标记基因组中端粒(Telomere)的结果。A为构建表达EGFP融合的TTALE的慢病毒载体(Lentivirus)的模式图;B为利用表达EGFP融合的TTALE的慢病毒载体感染人肿瘤细胞U2OS的结果;C为利用表达EGFP融合的TTALE的慢病毒载体感染小鼠细胞OP9的结果;D为利用表达EGFP融合的TTALE的慢病毒载体(Lentivirus)在小鼠体内可视化标记基因组中端粒(Telomere)的模式图;E为利用表达EGFP融合的TTALE的慢病毒载体(Lentivirus)在小鼠肌肉、肝脏和脑组织中可视化标记基因组中端粒(Telomere)的结果。

具体实施方式

[0090] 下述实施例中所使用的实验方法如无特殊说明,均为常规方法。

[0091] 下述实施例中所用的材料、试剂等,如无特殊说明,均可从商业途径得到。

[0092] 下述实施例中的定量试验,均设置三次重复实验,结果取平均值。

[0093] 下述实施例中的HGPS病人来源的iPSC(简称HGPS-iPSCs):记载于“Liu,G.H.,Barkho,B.Z.,Ruiz,S.,Diep,D.,Qu,J.,Yang,S.L.,Panopoulos,A.D.,Suzuki,K.,Kurian,L.,Walsh,C.,et al.(2011a).Recapitulation of premature ageing with iPSCs from Hutchinson-Gilford progeria syndrome.Nature 472,221-225.”一文,公众可从申请人处获得,仅用于重复本发明实验使用。HGPS病人来源的iPSC(HGPS-iPSCs)中的LMNA基因发生了突变(LMNA基因组序列为GenBank:NG_008692.2;cDNA序列为GenBank:NM_170707.3),突变类型为C1824T(GGCGG突变为GGTGG),该位点参比序列为LMNAcDNA序列GenBank:NM_170707.3中的CDS区域。

[0094] 下述实施例中的HGPS-GC-iPSCs为与HGPS-iPSC具有相同遗传背景的经过基因矫正的对照细胞系,记载于“Liu,G.H.,Suzuki,K.,Qu,J.,Sancho-Martinez,I.,Yi,F.,Li,M.,Kumar,S.,Nivet,E.,Kim,J.,Soligalla,R.D.,et al.(2011).Targeted gene correction of laminopathy-associated LMNA mutations in patient-specific iPSCs.Cell Stem Cell 8,688-694.”一文,公众可从申请人处获得,仅用于重复本发明实验使用。

[0095] 下述实施例中的WRN基因缺失的人胚胎干细胞系(WS-ESC)由第一发明人创建,记载于“Zhang,W.et al.Aging stem cells.A Werner syndrome stem cell model unveils heterochromatin alterations as a driver of human aging.Science 348,1160-1163”一文,公众可从申请人处获得,仅用于重复本发明实验使用。WRN蛋白对于细胞核内组成型

异染色质的结构维持至关重要，与细胞衰老密切相关，其功能缺失是成年早衰症发生的直接原因，可引起多组织器官衰老和衰老相关疾病。

[0096] 下述实施例中的pLE4载体记载于“Huize Pan,Di Guan,Xiaomeng Liu,Jingyi Li,Lixia Wang,Jun Wu,Junzhi Zhou,Weizhou Zhang,Ruotong Ren,Weiqi Zhang,Ying Li,Jiping Yang,Ying Hao,Tingting Yuan,Guohong Yuan,Hu Wang,Zhenyu Ju,Zhiyong Mao,Jian Li,Jing Qu,Fuchou Tang,Guang-Hui Liu(2016).SIRT6 safeguards human mesenchymal stem cells from oxidative stress by coactivating NRF2.Cell Research 26,190-205.”一文，公众可从申请人处获得，仅用于重复本发明实验使用。

[0097] 下述实施例中的人胚胎干细胞H9细胞系(WT-ESC)是WiCell公司的产品，货号：WA09(H9)-DL-7。

[0098] 实施例1、精确标记细胞基因组中的端粒(Telomere)和着丝粒(Centromere)的基因组可视化标记技术(TTALE)的建立

[0099] 一、融合表达TALE和TRX的表达载体TTALE的构建

[0100] 1、识别端粒和着丝粒的TALE载体

[0101] 参照文献“Zhang,F.,et al.,Efficient construction of sequence-specific TAL effector for modulating mammalian transcription.Nat Biotechnology,2011.29(2):p.149-53，通过Golden Gate Assembly”中的方法利用TALE Toolbox Kit(美国Addgene，货号为1000000019)分别构建识别端粒的TALE载体和识别着丝粒的TALE载体。

[0102] (1)识别端粒的TALE载体

[0103] 识别端粒的TALE载体的核苷酸序列如序列1所示。识别端粒的TALE载体表达融合蛋白$TALE^{telo}$，融合蛋白$TALE^{tel}$识别的端粒区DNA序列的靶序列如下：5’-AACCCTAACCCTAACCCT-3’(序列14)。

[0104] (2)识别着丝粒的TALE载体

[0105] 识别着丝粒的TALE载体的核苷酸序列为将序列1中第2113-4725位所示的DNA片段替换为序列2所示的DNA片段后得到的序列。识别着丝粒的TALE载体表达融合蛋白$TALE^{centro}$，该融合蛋白N端带有FLAG标签序列和核定位序列NLS。融合蛋白$TALE^{centro}$识别的着丝粒区DNA序列的靶序列如下：5’-CCATTCCATTCCATTCCA-3’(序列15)。

[0106] 2、识别端粒和着丝粒的TTALE载体

[0107] (1)识别端粒的TTALE载体

[0108] 以步骤1中的识别端粒的TALE为骨架载体，将序列3所示的人硫氧还蛋白TRX的编码基因序列插入骨架载体的KpnI和XhoI位点间，得到重组载体，将其记为识别端粒的TTALE载体(图1A)。识别端粒的TTALE载体表达融合蛋白$TALE^{telo}$-TRX，该融合蛋白N端带有FLAG标签序列和核定位序列NLS。融合蛋白$TALE^{telo}$-TRX的氨基酸序列如序列4所示。

[0109] (2)识别着丝粒的TTALE载体

[0110] 以步骤1中的识别着丝粒的TALE为骨架载体，将序列3所示的人硫氧还蛋白的编码基因序列插入骨架载体的KpnI和XhoI位点间，得到重组载体，将其记为识别着丝粒的TTALE载体(图1A)。识别着丝粒的TTALE载体表达融合蛋白$TALE^{centro}$-TRX，该融合蛋白N端带有FLAG标签序列和核定位序列NLS。融合蛋白$TALE^{centro}$-TRX的氨基酸序列如序列5所示。

[0111] 二、细胞基因组中的端粒和着丝粒分布位点检测

[0112] 1、转染

[0113] 将步骤一中制备的识别端粒的TTALE载体和识别着丝粒的TTALE载体分别转染U2OS细胞(美国ATCC,货号:HTB-96),同时以识别端粒的TALE载体和识别着丝粒的TALE载体为对照载体(NoTRX),转染24-48小时后,分别得到转染后细胞。

[0114] 2、免疫荧光染色实验检测细胞基因组中的端粒和着丝粒分布位点

[0115] 利用识别端粒的TTALE载体、识别着丝粒的TTALE载体和对照载体N端的FLAG标签序列进行免疫荧光染色实验,检测转染后细胞基因组中的端粒和着丝粒分布位点。具体步骤如下:用4%多聚甲醛(北京鼎国昌盛生物技术有限责任公司,货号:AR-0211)固定转染后细胞,然后用含0.4%TritonX100(美国Sigma公司,货号为T9284)的PBS室通透15分钟,用一抗稀释液(含有10%驴血清的PBS)室温封闭30分钟,用一抗稀释液配制的小鼠抗FLAG抗体4度孵育过夜,用PBS室温清洗3次,每次10分钟,用ALEXA-488标记的驴抗小鼠IgG室温孵育1小时,用PBS室温清洗3次,每次10分钟,用1:2000的Hoechst标记细胞核,最后利用荧光显微镜进行观察,并利用ImageJ软件进行荧光强度计算和统计分析。如需与FISH探针进行共定位分析,则在孵育小鼠抗FLAG抗体之后,进行FISH探针孵育,再用Biotin标记的抗小鼠IgG室温孵育1小时,最后用ALEXA-488标记的Streptavidin(Vectorlabs,货号为SA-5488)孵育1小时。

[0116] 3、荧光原位杂交实验检测细胞基因组中的端粒和着丝粒分布位点

[0117] 利用特异性识别端粒和着丝粒的FISH探针完成荧光原位杂交实验。具体步骤为:用4%多聚甲醛固定转染后细胞,然后用含0.4%TritonX100(美国Sigma公司,货号为T9284)的PBS室通透15分钟,再用100微克/毫升的RNAase A(美国Sigma公司,货号为83831)于37度孵育30分钟,用90度5分钟变性过的浓度为50nM FISH探针(韩国PANAGENE公司,端粒FISH探针货号为F1002;着丝粒FISH探针货号为F3003)85度孵育10分钟,之后于室温避光孵育过夜,用PBS室温清洗3次,每次10分钟,用1:2000的Hoechst标记细胞核,最后利用荧光显微镜进行观察,并利用ImageJ软件进行荧光强度计算和统计分析。

[0118] 结果如图1所示。从图1中可以看出:与识别端粒或着丝粒的TALE(图1B中的No TRX,图1E中的No TRX)相比,识别端粒或着丝粒的TTALE在细胞核中标记的荧光点与FISH探针标记的荧光点几乎完全重合(图1B和图1E),并且其荧光点分布与FISH探针标记的荧光点的分布非常相似(图1C,1D,1F,1G),说明本发明的TTALE能够精确标记人类细胞基因组中的端粒或着丝粒位点。

[0119] 实施例2、TTALE在精确标记不同类型人类细胞中的端粒、着丝粒、核仁组织区核糖体RNA编码序列(NOR-rDNA)以及编码基因位点(MUC4)中的应用

[0120] 一、TTALE在精确标记不同类型人类细胞中的端粒和着丝粒中的应用

[0121] 将实施例1中的U2OS细胞分别替换为如下细胞:人肿瘤细胞系(MCF7和HepG2,美国ATCC公司,货号分别为HTB-22和HB-8065)、人胚胎肾细胞系(HEK293,美国ATCC公司,货号为CRL-1573)、人多能干细胞(胚胎干细胞hESC,Wicell公司,货号为WA-09)、人多能干细胞(诱导多能干细胞iPSC,Wicell公司,货号为IISH6i-CML17)、成体干细胞(间充质干细胞,Lonza公司,货号为PT-2501)、成体干细胞(神经干细胞hNSC,Wicell公司,货号为NSC-H9)、终末分化细胞(血管平滑肌细胞hVSMC,Lonza公司,货号为CC-2571),且保持其他步骤不变,然后利用荧光显微镜进行观察,并利用ImageJ软件进行荧光强度计算和统计分析。

[0122] 结果表明:TTALE可精确标记不同类型人类细胞基因组中的端粒和着丝粒,实现上述各个细胞中端粒和着丝粒的可视化(图2A-B,图3)。

[0123] 二、荧光蛋白融合表达的识别端粒或着丝粒的TTALE在精确标记人类细胞中的端粒和着丝粒中的应用

[0124] 1、mCherry荧光蛋白融合表达的识别端粒的TTALE的制备

[0125] 以实施例1中的识别端粒的TTALE为骨架载体,将序列6所示的mCherry编码基因序列插入骨架载体的HpaI和KpnI位点间,得到重组载体,将其记作mCherry融合表达的识别端粒的TTALE载体。mCherry融合表达的识别端粒的TTALE载体(mCherry-TTALE^{telo})表达融合蛋白TTALE^{telo}-mCherry-TRX,该融合蛋白N端带有FLAG标签序列和核定位序列NLS。融合蛋白TTALE^{telo}-mCherry-TRX的氨基酸序列如序列7所示。

[0126] 2、EGFP荧光蛋白融合表达的识别着丝粒的TTALE的制备

[0127] 以识别着丝粒的TTALE为骨架载体,将序列13第7382-8098位所示的EGFP编码基因序列插入骨架载体的HpaI和KpnI位点间,得到EGFP融合表达的识别着丝粒的TTALE。EGFP融合表达的识别着丝粒的TTALE(EGFP-TTALE^{centro})表达融合蛋白TTALE^{centro}-EGFP-TRX,该融合蛋白N端带有FLAG标签序列和核定位序列NLS。融合蛋白TTALE^{centro}-EGFP-TRX的氨基酸序列如序列8所示。

[0128] 3、荧光蛋白融合表达的识别端粒或着丝粒的TTALE标记HeLa细胞

[0129] 分别将mCherry融合表达的识别端粒的TTALE(mCherry-TTALE^{telo})和EGFP融合表达的识别着丝粒的TTALE(EGFP-TTALE^{centro})转染处于细胞分裂周期不同时期的HeLa细胞中,转染24-48小时后,用4%多聚甲醛固定细胞,然后利用荧光显微镜进行观察,并利用ImageJ软件进行荧光强度计算和统计分析。

[0130] 结果表明:荧光蛋白融合表达的识别端粒或着丝粒的TTALE可精确标记人类细胞中的端粒和着丝粒,实现处于细胞分裂周期不同时期的细胞中端粒和着丝粒的可视化(图2C)。

[0131] 三、TTALE在精确标记不同类型人类细胞中的核仁组织区核糖体RNA编码序列(NOR-rDNA)中的应用

[0132] 1、EGFP融合表达的识别核仁组织区核糖体RNA编码基因的TALE的制备

[0133] 参照文献"Zhang,F.,et al.,Efficient construction of sequence-specific TAL effector for modulating mammalian transcription.Nat Biotechnology,2011.29(2):p.149-53,通过Golden Gate Assembly"中的方法利用TALE Toolbox Kit(美国Addgene,货号为1000000019)构建识别核仁组织区核糖体RNA编码序列的TALE载体。

[0134] 识别核仁组织区核糖体RNA编码基因的TALE载体的核苷酸序列为将序列1第2113-4725位所示的DNA片段替换为序列9所示的DNA片段,且将序列13第7382-8098位所示的EGFP编码基因序列插入序列1的HpaI和KpnI位点间后得到的序列。识别核仁组织区核糖体RNA编码基因的TALE载体表达融合蛋白TALE^{rDNA}-EGFP,该融合蛋白N端带有FLAG标签序列和核定位序列NLS。融合蛋白TALE^{rDNA}-EGFP识别的核仁组织区核糖体RNA编码基因的靶序列如下:5'-ACCCTACTGATGATGTGT-3'(序列16)。

[0135] 2、荧光蛋白融合表达的识别核仁组织区核糖体RNA编码基因的TTALE的制备

[0136] 以步骤1中的识别核仁组织区核糖体RNA编码基因的TALE为骨架载体,将序列3所

示的人硫氧还蛋白TRX的编码基因序列插入骨架载体的AscI和XhoI位点间，得到重组载体，将其记为识别核仁组织区核糖体RNA编码基因的TTALE载体(EGFP-TTALE^{rDNA})。识别核仁组织区核糖体RNA编码基因的TTALE载体(EGFP-TTALE^{rDNA})表达融合蛋白TALE^{rDNA}-EGFP-TRX。融合蛋白TALE^{rDNA}-EGFP-TRX的氨基酸序列如序列10所示。

[0137] 将识别核仁组织区核糖体RNA编码序列的TTALE载体(EGFP-TTALE^{rDNA})中的EGFP编码基因替换为序列6所示的mCherry编码基因，得到识别核仁组织区核糖体RNA编码基因的TTALE载体(mCherry-TTALE^{rDNA})。识别核仁组织区核糖体RNA编码基因的TTALE载体(mCherry-TTALE^{rDNA})表达融合蛋白TALE^{rDNA}-mCherry-TRX。

[0138] 3、识别核仁组织区核糖体RNA编码基因的TTALE标记细胞

[0139] 分别将识别核仁组织区核糖体RNA编码基因的TALE载体和识别核仁组织区核糖体RNA编码基因的TTALE转染人肿瘤细胞(HeLa和U2OS)以及人多能干细胞(hESC，hMSC和hNSC)中，转染24-48小时后，用4％多聚甲醛固定细胞，然后利用荧光显微镜进行观察，并利用ImageJ软件进行荧光强度计算和统计分析，分别检测不同细胞中的核仁组织区核糖体RNA编码基因的荧光分布情况。

[0140] 结果表明：与识别核仁组织区核糖体RNA编码基因的TALE载体相比(图4A)，识别核仁组织区核糖体RNA编码基因的TTALE可精确标记人肿瘤细胞(HeLa和U2OS)以及人多能干细胞(hESC，hMSC和hNSC)中的核糖体RNA，实现核仁组织区核糖体RNA编码基因的可视化(图4B，4C)。

[0141] 四、TTALE在精确标记不同类型人类细胞中的编码基因位点(MUC4)中的应用

[0142] 1、EGFP融合表达的识别编码基因位点(MUC4)的TALE的制备

[0143] 参照文献“Zhang，F.，et al.，Efficient construction of sequence-specific TAL effector for modulating mammalian transcription.Nat Biotechnology，2011.29(2)：p.149-53，通过Golden Gate Assembly”中的方法利用TALE Toolbox Kit(美国Addgene，货号为1000000019)构建识别编码基因位点(MUC4)的TALE载体。

[0144] 识别编码基因位点(MUC4)的TALE载体的核苷酸序列为将序列1第2113-4725位所示的DNA片段替换为序列11所示的DNA片段，且将序列13第7382-8098位所示的EGFP编码基因序列插入序列1的HpaI和KpnI位点间后得到的序列。识别编码基因位点(MUC4)TALE载体表达融合蛋白TALE^{MUC4}-EGFP，该融合蛋白N端带有FLAG标签序列和核定位序列NLS。融合蛋白TALE^{MUC4}-EGFP识别的编码基因位点(MUC4)的靶序列如下：5’-CCTGTCACCGACACTTCC-3’(序列17)。

[0145] 2、荧光蛋白融合表达的识别编码基因位点(MUC4)的TTALE的制备

[0146] 以步骤1中的识别编码基因位点(MUC4)的TALE为骨架载体，将序列3所示的人硫氧还蛋白TRX的编码基因序列插入骨架载体的AscI和XhoI位点间，得到重组载体，将其记为识别编码基因位点(MUC4)的TTALE载体(EGFP-TTALE^{MUC4})。识别编码基因位点(MUC4)的TTALE载体表达融合蛋白TTALE^{MUC4}-EGFP-TRX。融合蛋白TTALE^{MUC4}-EGFP-TRX的氨基酸序列如序列12所示。

[0147] 将识别编码基因位点(MUC4)的TTALE载体(EGFP-TTALE^{MUC4})中的EGFP编码基因替换为序列6所示的mCherry编码基因，得到识别核仁组织区核糖体RNA编码序列的TTALE载体(mCherry-TTALE^{MUC4})。识别核仁组织区核糖体RNA编码序列的TTALE载体(mCherry-TTALE

^{MUC4})表达融合蛋白TALE^{MUC4}-mCherry-TRX。

[0148] 3、识别编码基因位点(MUC4)的TTALE标记细胞

[0149] 分别将识别编码基因位点(MUC4)的TALE和识别编码基因位点(MUC4)的TTALE分别转染处于细胞间期和细胞分裂周期的hMSC和HeLa细胞中，转染24-48小时后，用4％多聚甲醛固定细胞，然后利用荧光显微镜进行观察，并利用ImageJ软件进行荧光强度计算和统计分析。

[0150] 结果表明：与识别编码基因位点(MUC4)的TALE载体相比(图5A)，识别编码基因位点(MUC4)的TTALE可精确标记细胞间期和细胞分裂周期的hMSC和HeLa细胞中的编码基因位点(MUC4)，实现编码基因位点(MUC4)的可视化(图5B-C)。

[0151] 实施例3、TTALE标记不同细胞衰老模型中端粒在可视化细胞衰老进程的动态变化中的应用

[0152] 1、EGFP融合表达的识别端粒的TTALE载体的制备

[0153] 将序列13第7382-8098位所示的EGFP编码基因序列替换实施例2步骤二的1中mCherry荧光蛋白融合表达的识别端粒的TTALE载体(mCherry-TTALE^{telo})中的mCherry编码基因序列，得到EGFP融合表达的识别端粒的TTALE载体(EGFP-TTALE^{telo})，该融合蛋白N端带有FLAG标签序列和核定位序列NLS。

[0154] 2、EGFP融合表达的识别端粒的TTALE载体标记不同衰老模型的人类间充质干细胞

[0155] (1)不同衰老模型的人类间充质干细胞的建立

[0156] 1)野生型人间充质干细胞(WT-MSC)和WRN基因缺失的人间充质干细胞(WS-MSC)的制备

[0157] 本发明将野生型人胚胎干细胞H9细胞系(WT-ESC)和WRN基因缺失的人胚胎干细胞系(WS-ESC)，进一步体外定向分化为间充质干细胞(WT-MSC)和WRN基因缺失的人间充质干细胞(WS-MSC)，具体方法如下：

[0158] A、将野生型人胚胎干细胞H9细胞系(WT-ESC)和WRN基因缺失的人胚胎干细胞系(WS-ESC)分别进行拟胚体(EB)分化，获得拟胚体(EB)。拟胚体(EB)分化具体步骤如下：准备含有300-500个细胞、大小均一的ESC克隆，用室温PBS(Gibco，10010023)清洗一次，用Dispase(Invitrogen公司，货号为17105041)37℃消化20-30min。待ESC克隆形成球体后，用CDF12培养基重悬后，加到低粘附培养板(Corning公司，货号3471)中，37℃，5％CO_2条件培养1-3天后即形成拟胚体。

[0159] B、将步骤A获得的拟胚体(EB)接种于基质胶(matrigel)包被的6孔板中进行培养，继续培养2周至纤维状细胞出现。再经过一次传代后，利用流式细胞术分选其中的CD73、CD90和CD105均为阳性的细胞类群(图1)，即为野生型间充质干细胞(记为WT-MSC)和WRN基因缺失的人间充质干细胞系(记为WS-MSC)。

[0160] 2)HGPS病人来源的(HGPS-MSC)和经过基因矫正的人间充质干细胞(HGPS-GC-MSC)的制备

[0161] 本实施例将HGPS-iPSCs和HGPS-GC-iPSCs进一步体外定向分化为间充质干细胞HGPS-MSC和HGPS-GC-MSC。具体方法如下：

[0162] 分别将HGPS-iPSCs和HGPS-GC-iPSCs进行拟胚体(EB)分化，分化14天，将EB接种于基质胶(matrigel)包被的6孔板中进行培养，继续培养2周至纤维状细胞出现。再经过一次

传代后，利用流式细胞术分选其中CD73、CD90和CD105均为阳性的细胞类群，即为HGPS病人来源的间充质干细胞（记为HGPS-MSC）和经过基因矫正的人间充质干细胞（记为HGPS-GC-MSC）。

[0163] 3）野生型间充质干细胞的早代细胞（EP-WT-MSC）与晚代细胞（LP-WT-MSC）的制备

[0164] 将步骤（1）中的野生型间充质干细胞（WT-MSC）连续传代培养至12代（记为P12代），将P1-P6代细胞记为早代间充质干细胞（记为EP-WT-MSC），将P10-P12代细胞记晚代间充质干细胞（记为LP-WT-MSC）。选取P6和P12代WT-MSC细胞作为EP-WT-MSC和LP-WT-MSC的代表进行下述相关实验。

[0165] （2）EGFP融合表达的识别端粒的TTALE载体标记不同衰老模型的人类间充质干细胞

[0166] 将EGFP融合表达的识别端粒的TTALE载体利用化学转染方法导入不同衰老模型的人类间充质干细胞中（图6A，6C和6D），转染24-48小时后，用4％多聚甲醛固定细胞，然后利用荧光显微镜进行观察，并利用ImageJ软件进行荧光强度计算和统计分析。

[0167] 3、qPCR方法检测不同衰老模型的人类间充质干细胞中端粒长度

[0168] 以步骤2中不同衰老模型的人类间充质干细胞为供试细胞。分别从供试细胞中提取基因组DNA，通过实时定量PCR的方法检测供试细胞中端粒长度。其中，以36B4为端粒长度检测的对照基因。引物序列如下：

[0169] Tel-F：5’-GGTTTTTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGT-3’；

[0170] Tel-R：5’-TCCCGACTATCCCTATCCCTATCCCTATCCCTATCCCTA-3’；

[0171] 36B4u：5’-CAGCAAGTGGGAAGGTGTAATCC-3’；

[0172] 36B4d：5’-CCCATTCTATCATCAACGGGTACAA-3’。

[0173] 结果表明，利用识别端粒的TTALE能够在不同的细胞衰老模型中可视化标记基因组中端粒，并能够反映出衰老细胞中标记的端粒的荧光强度显著降低（图6B，6F），该结果与利用qPCR方法检测的衰老细胞中端粒长度缩短的结果一致（图6E），说明TTALE技术能够精确地可视化标记基因组中端粒随细胞衰老进程的动态变化。

[0174] 实施例4、识别端粒的TTALE在体外和动物体内可视化标记基因组中端粒中的应用

[0175] 一、识别端粒的TTALE在体外可视化标记基因组中端粒中的应用

[0176] 1、EGFP融合表达的识别端粒的TTALE慢病毒载体质粒的制备及包装

[0177] 将EGFP融合表达的识别端粒的TTALE的编码基因序列插入pLE4载体的限制性内切酶酶切位点MluI和SalI之间，且保持pLE4载体的其他序列不变，得到慢病毒载体质粒pLE4-EGFP-TTALE（其核苷酸序列如序列13所示）。然后将慢病毒载体质粒pLE4-EGFP-TTALE在HEK293T（美国ATCC，货号：CRL-3216）细胞中进行慢病毒的病毒包装，慢病毒包装质粒购自Addgene，货号如下：psPAX（12260），pMD2.G（12259）。

[0178] 将慢病毒载体质粒pLE4-EGFP-TTALE与包装质粒psPAX和pMD.2G共转染至HEK293T细胞中，与转染后48小时收集培养上清，并通过超高速离心纯化慢病毒颗粒。

[0179] 2、EGFP融合表达的识别端粒的慢病毒载体质粒标记人或小鼠细胞

[0180] 利用产生表达EGFP融合的识别端粒的TTALE的慢病毒感染人U2OS细胞（美国ATCC，货号：HTB-96）和小鼠OP9细胞（美国ATCC，货号：CRL-2749），感染24-72小时后，用4％多聚甲醛固定细胞，然后利用荧光显微镜进行观察。

[0181] 结果表明，利用表达EGFP融合的识别端粒的TTALE的慢病毒载体能够在体外可视化标记人和小鼠细胞基因组中端粒(图7B，7C)。

[0182] 二、EGFP融合表达的识别端粒的TTALE在动物体内可视化标记基因组中端粒中的应用

[0183] 用Opti-MEM(ThermoFisher公司，货号：51985042)将步骤一中制备的表达EGFP融合的识别端粒的TTALE的慢病毒载体稀释为10^8病毒/微升剂量，分别在小鼠的胫骨前肌、肝脏和大脑海马区注射5微升病毒液(图7D)，注射7-10天后，分离上述小鼠组织，用4％多聚甲醛固定后进行冰冻切片，分别用WGA抗体(肌肉)(ThermoFisher公司，货号：W32464)、ALB抗体(肝脏)(Abcam公司，货号：ab8940)和NeuN抗体(大脑海马区)(Abcam公司，货号：ab177487)进行免疫荧光染色，最后用荧光显微镜观察。免疫荧光染色的具体步骤如下：用4％多聚甲醛(北京鼎国昌盛生物技术有限责任公司，货号：AR-0211)室温固定切片10分钟，然后用含0.4％TritonX100(美国Sigma公司，货号为T9284)的PBS室通透30分钟，用一抗稀释液(含有10％驴血清的PBS)室温封闭45分钟，用一抗稀释液配制的WGA抗体(肌肉)(ThermoFisher公司，货号：W32464)、ALB抗体(肝脏)(Abcam公司，货号：ab8940)和NeuN抗体(大脑海马区)(Abcam公司，货号：ab177487)分别4度孵育过夜，用PBS室温清洗3次，每次10分钟，分别用ALEXA-488标记的IgG室温孵育1小时，用PBS室温清洗3次，每次10分钟，用1：2000的Hoechst标记细胞核，最后利用荧光显微镜进行观察，并利用ImageJ软件进行荧光强度计算和统计分析。

[0184] 结果表明：利用表达EGFP融合的识别端粒的TTALE的慢病毒载体能够在小鼠不同组织内实现可视化标记基因组中端粒(图7E)。

[0001] 序列表

[0002] <110>中国科学院生物物理研究所

[0003] <120>一种可视化标记基因组位点的方法

[0004] <160>17

[0005] <210>1

[0006] <211>7746bp

[0007] <212>DNA

[0008] <213>人工序列

[0009] <220>

[0010] <223>

[0011] <400>1

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[0014] tgcaaacaaa aaaaccaccg ctaccagcgg tggtttgttt gccggatcaa gagctaccaa 180

[0015] ctcttttcc gaaggtaact ggcttcagca gagcgcagat accaaatact gttcttctag 240

[0016] tgtagccgta gttagcccac cacttcaaga actctgtagc accgcctaca tacctcgctc 300

[0017] tgctaatcct gttaccagtg gctgctgcca gtggcgataa gtcgtgtctt accgggttgg 360

[0018] actcaagacg atagttaccg gataaggcgc agcggtcggg ctgaacgggg ggttcgtgca 420

[0019] cacagcccag cttggagcga acgacctaca ccgaactgag atacctacag cgtgagctat 480

[0020] gagaaagcgc cacgcttccc gaagggagaa aggcggacag gtatccggta agcggcaggg 540

[0021] tcggaacagg agagcgcacg agggagcttc cagggggaaa cgcctggtat ctttatagtc 600

[0022] ctgtcgggtt tcgccacctc tgacttgagc gtcgattttt gtgatgctcg tcaggggggc 660

[0023] ggagcctatg gaaaaacgcc agcaacgcgg cctttttacg gttcctggcc ttttgctggc 720

[0024] cttttgctca catgttcttt cctgcgttat cccctgattc tgtggataac cgtattaccg 780

[0025] cctttgagtg agctgatacc gctcgccgca gccgaacgac cgagcgcagc gagtcagtga 840

[0026] gcgaggaagc ggaaggcgag agtagggaac tgccaggcat caaactaagc agaaggcccc 900

[0027] tgacggatgg cctttttgcg tttctacaaa ctctttctgt gttgtaaaac gacggccagt 960

[0028] cttaagctcg ggccccctgg gcggttctga taacgagtaa tcgttaatcc gcaaataacg 1020

[0029] taaaaacccg cttcggcggg ttttttttatg gggggagttt agggaaagag catttgtcag 1080

[0030] aatatttaag ggcgcctgtc actttgcttg atatatgaga attatttaac cttataaatg 1140

[0031] agaaaaaagc aacgcacttt aaataagata cgttgctttt tcgattgatg aacacctata 1200

[0032] attaaactat tcatctatta tttatgattt tttgtatata caatatttct agtttgttaa 1260

[0033] agagaattaa gaaaataaat ctcgaaaata ataaagggaa aatcagtttt tgatatcaaa 1320

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[0042] ggcagtacat ctacgtatta gtcatcgcta ttaccatggt gatgcggttt tggcagtaca 1860

[0043] tcaatgggcg tggatagcgg tttgactcac ggggatttcc aagtctccac cccattgacg 1920

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[0141] cccttg 7746

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[0143] <211>2613bp

[0144] <212>DNA

[0145] <213>人工序列

[0146] <220>

[0147] <223>

[0148] <400>2

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[0151] gtagatttga gaactttggg atattcacag cagcagcagg aaaagatcaa gcccaaagtg 180

[0152] aggtcgacag tcgcgcagca tcacgaagcg ctggtgggtc atgggtttac acatgcccac 240

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[0154] atgattgcgg cgttgccgga agccacacat gaggcgatcg tcggtgtggg gaaacagtgg 360

[0155] agcggagccc gagcgcttga ggccctgttg acggtcgcgg gagagctgag agggcctccc 420

[0156] cttcagctgg acacgggcca gttgctgaag atcgcgaagc ggggaggagt cacggcggtc 480

[0157] gaggcggtgc acgcgtggcg caatgcgctc acgggagcac ccctcaacct gaccccagag 540

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[1342] <211>18bp

[1343] <212>DNA

[1344] <213>人工序列

[1345] <220>

[1346] <223>

[1347] <400>16

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[1351] <212>DNA

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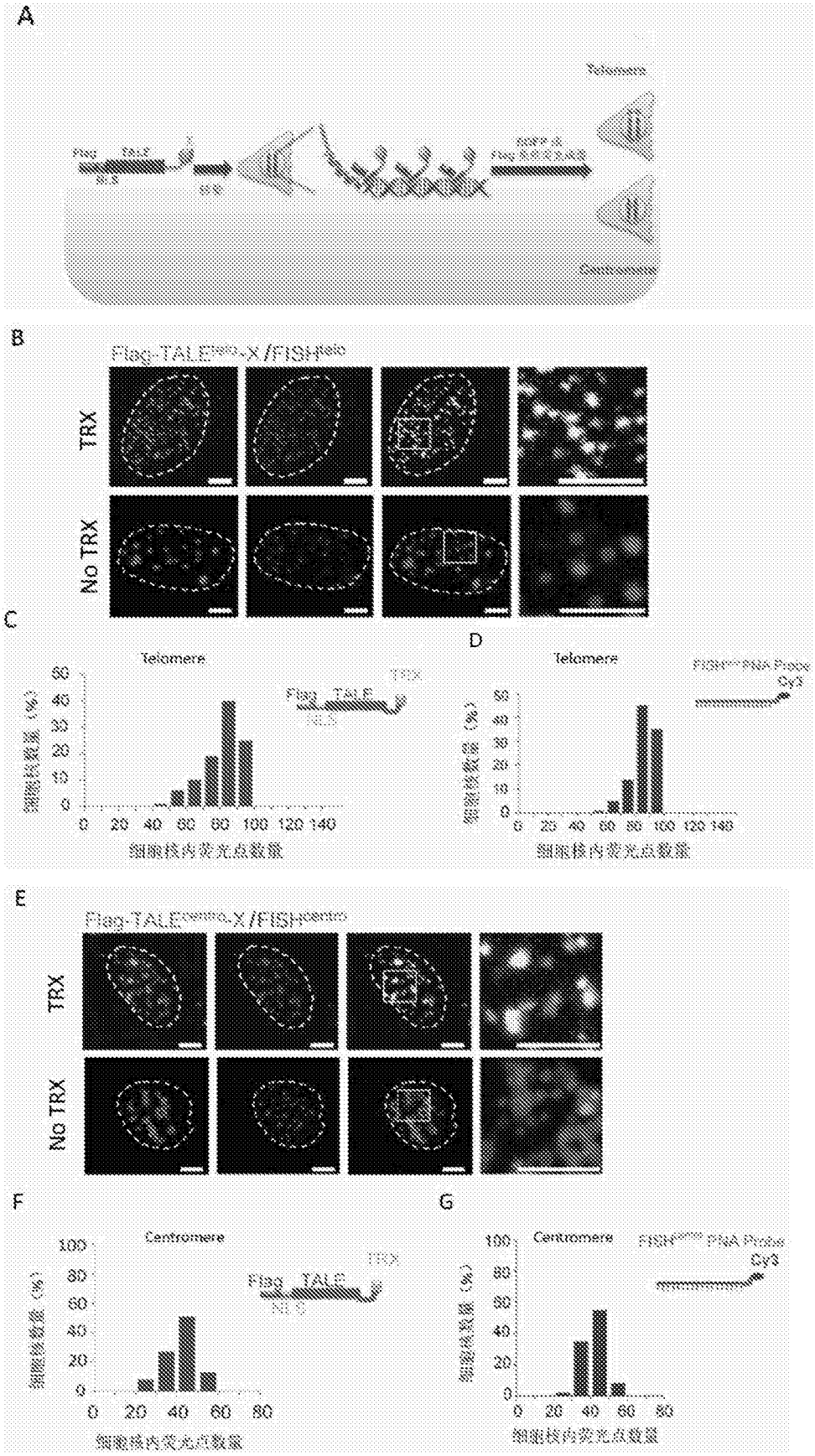
A
Telomere
Centromere
B
TRX
No TRX
C
Telomere
Flag TALE
D
Telomere
细胞核数量（%）
细胞核内荧光点数量
E
TRX
No TRX
F
Centromere
Flag TALE
G
Centromere

图1

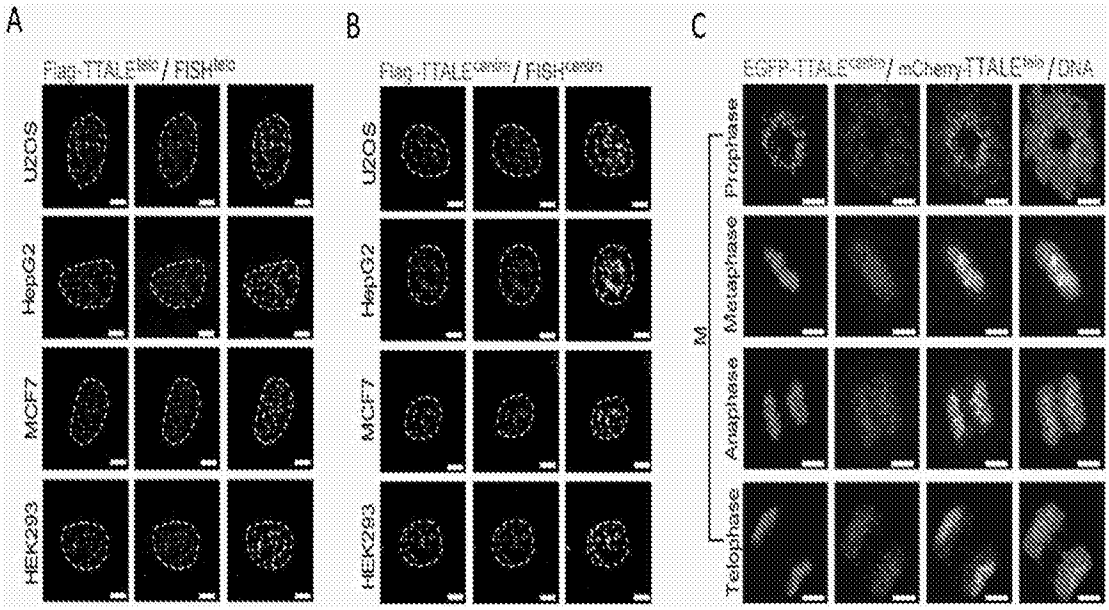
A
B
C
U2OS
HepG2
MCF7
HEK293
M
Prophase
Metaphase
Anaphase
Telophase

图2

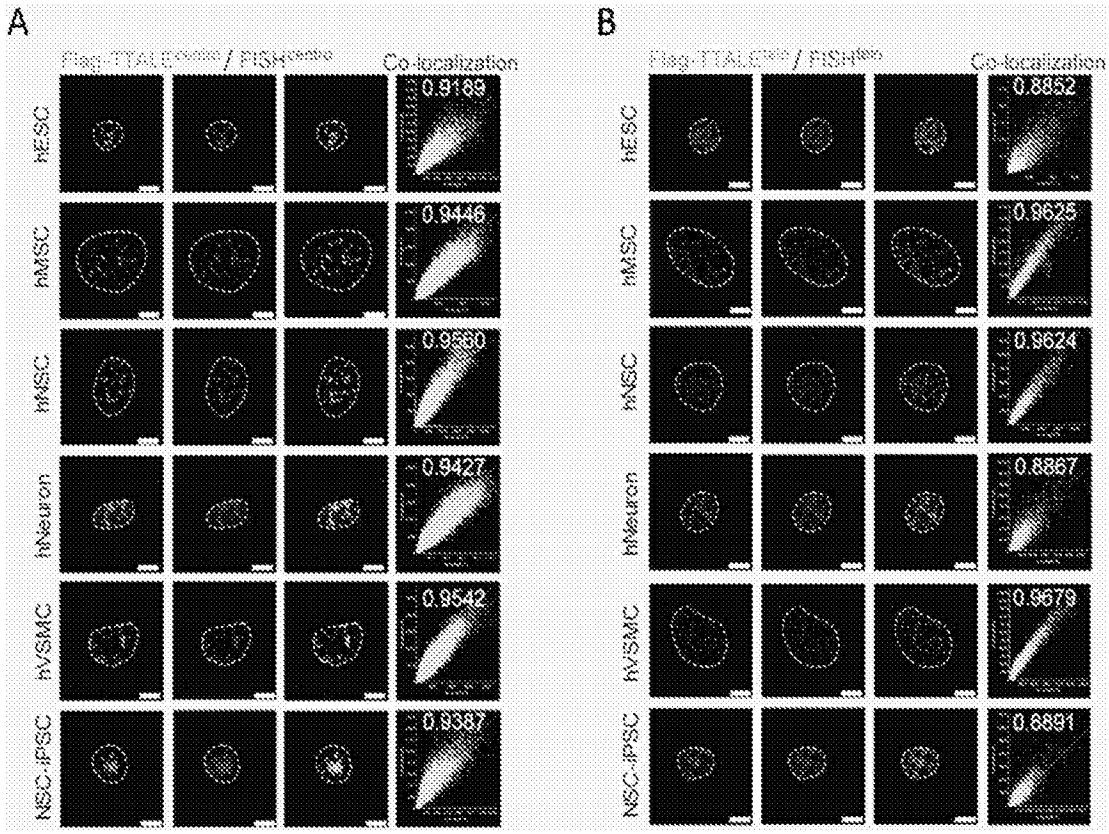
A
B
Co-localization
hESC
hMSC
hMSC
hNeuron
hVSMC
NSC-iPSC
0.9189
0.9446
0.9560
0.9427
0.9542
0.9387
0.8852
0.9625
0.9624
0.8867
0.9679
0.8891

图3

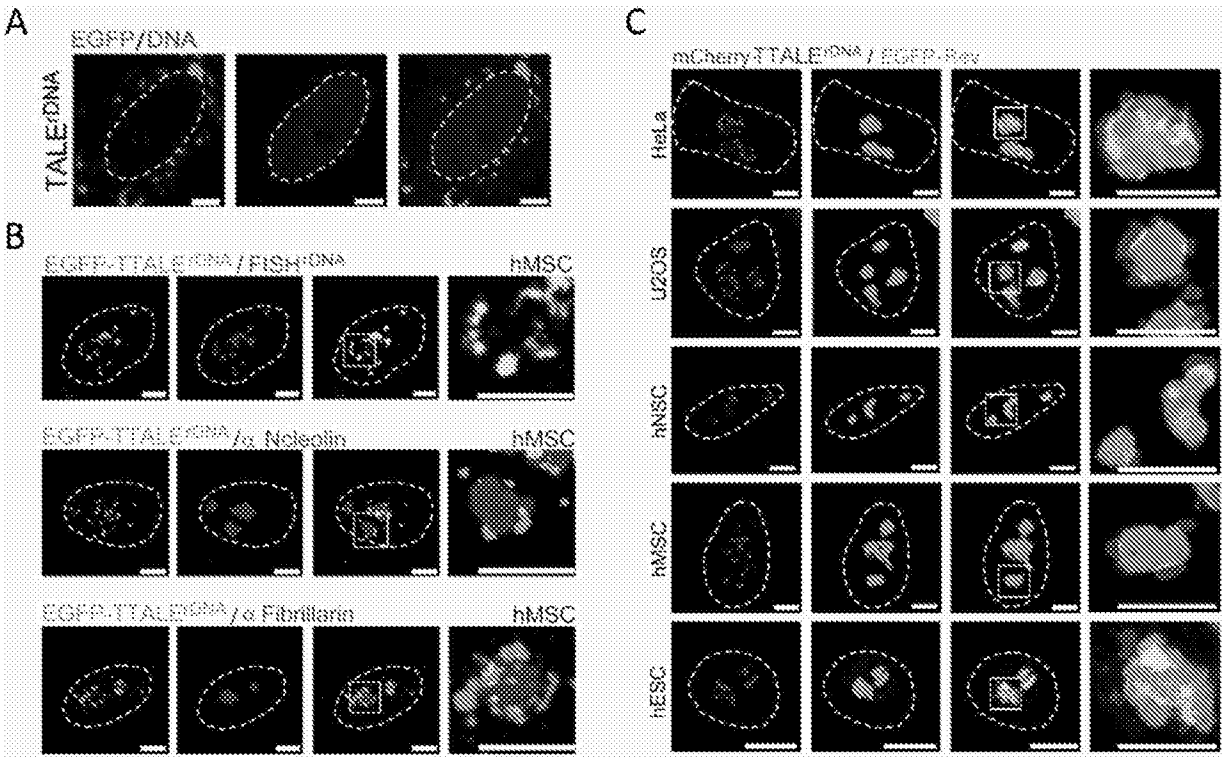
A
EGFP/DNA
TALE-DNA
B
EGFP-TTALE-DNA / FISH-DNA
hMSC
EGFP-TTALE-DNA / α Nucleolin
hMSC
EGFP-TTALE-DNA / α Fibrillarin
hMSC
C
mCherry-TTALE-DNA / EGFP-Poly
HeLa
U2OS
hNSC
hMSC
hESC

图4

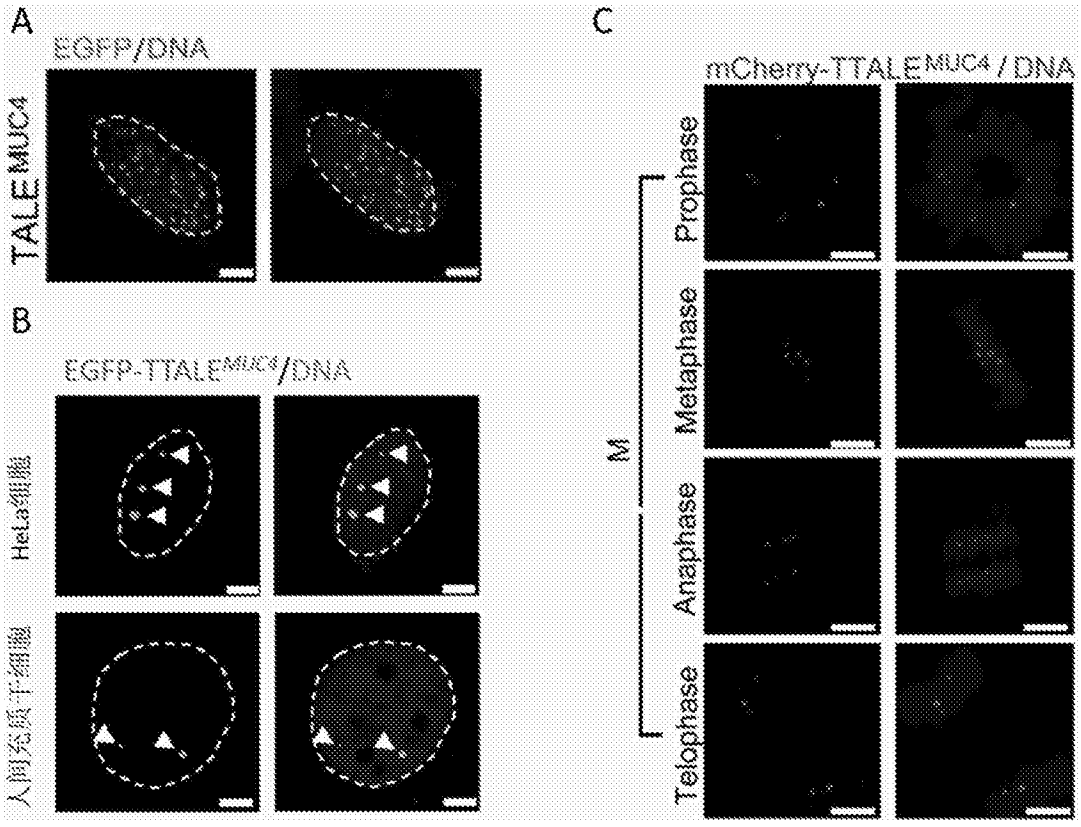
A
EGFP/DNA
TALE MUC4
B
EGFP-TTALE MUC4/DNA
HeLa细胞
人间充质干细胞
C
mCherry-TTALE MUC4 / DNA
M
Prophase
Metaphase
Anaphase
Telophase

图5

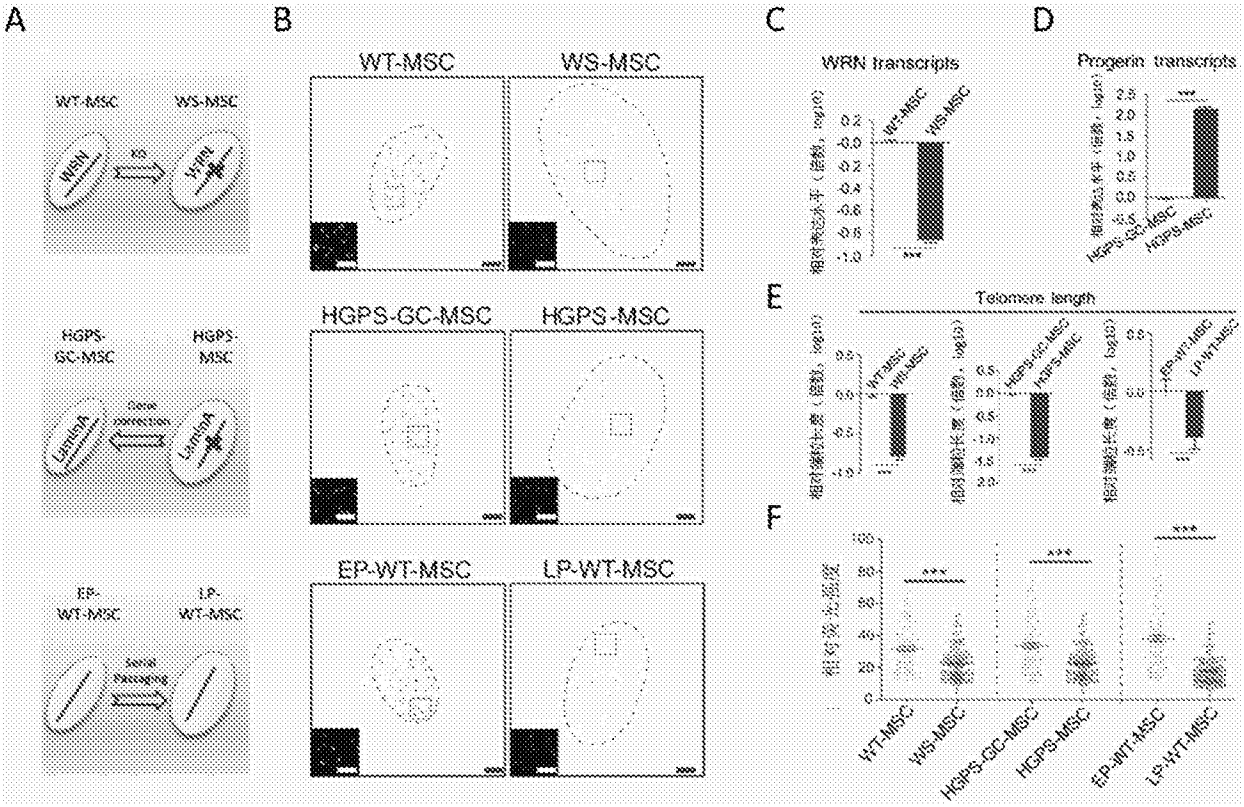
A
WT-MSC
WS-MSC
WRN
KO
WRN
HGPS-GC-MSC
HGPS-MSC
Lamin A
Gene correction
Lamin A
EP-WT-MSC
LP-WT-MSC
Nuclear Reshaping
B
WT-MSC
WS-MSC
HGPS-GC-MSC
HGPS-MSC
EP-WT-MSC
LP-WT-MSC
C
WRN transcripts
D
Progerin transcripts
E
Telomere length
F

图6

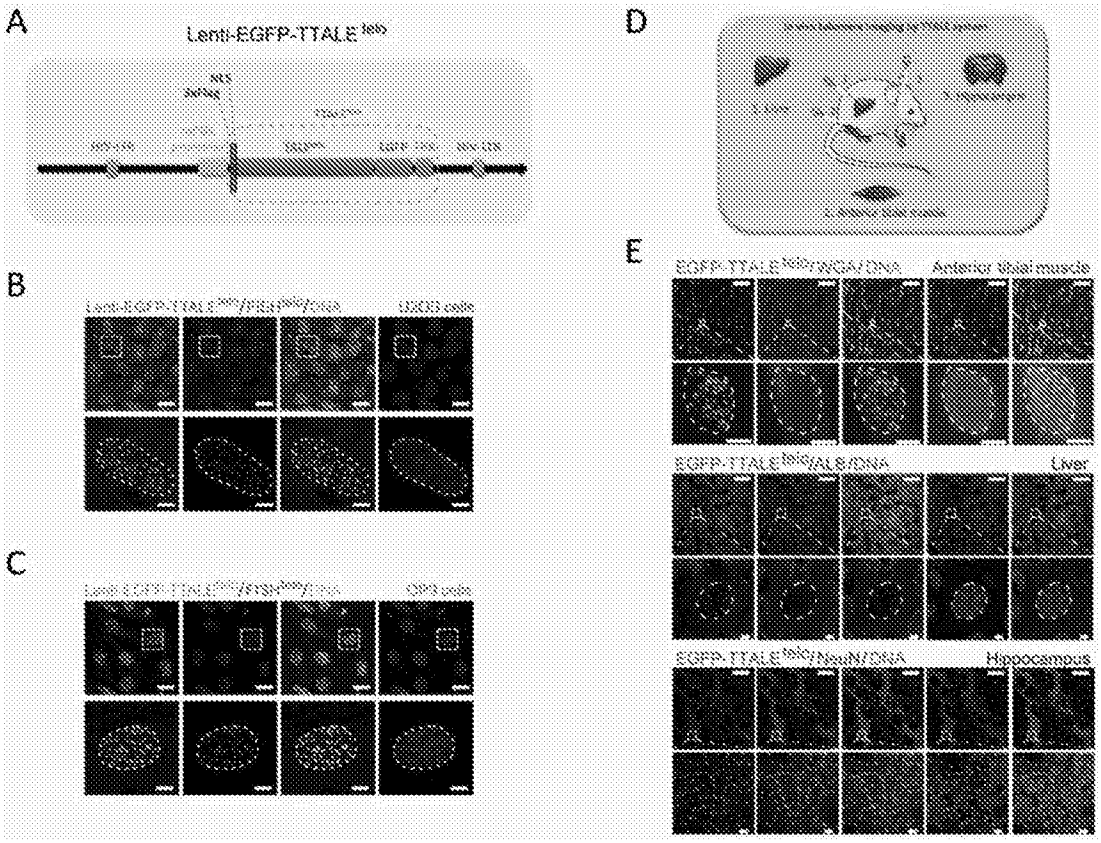
A
Lenti-EGFP-TTALE
B
C
D
E
Anterior tibial muscle
Liver
Hippocampus

图7