



(51) International Patent Classification:

A61K 47/48 (2006.01) C07K 14/16 (2006.01)
C12N 5/0775 (2010.01)

(21) International Application Number:

PCT/GB2016/051881

(22) International Filing Date:

23 June 2016 (23.06.2016)

(25) Filing Language:

English

(26) Publication Language:

English

(30) Priority Data:

1511158.6 24 June 2015 (24.06.2015) GB

(71) Applicant: THE UNIVERSITY OF NOTTINGHAM

[GB/GB]; Tech Transfer Office, Sir Colin Campbell Bldg,
Innovation Park, Triumph Road, Nottingham Nottingham-
shire NG7 2TU (GB).

(72) Inventor: DIXON, James; Wolfson Center for Stem Cells,
Tissue Engineering and Modelling (STEM), Center for Bio-
molecular Sciences (CSB), B59, School of Pharmacy, Uni-
versity Park, University of Nottingham, Nottingham Not-
tinghamshire NG7 2RD (GB).(74) Agent: BARKER BRETTELL LLP; 100 Hagley Road,
Edgbaston, Birmingham West Midlands B16 8QQ (GB).

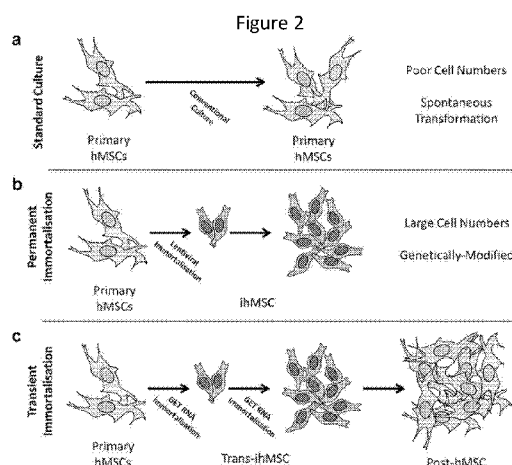
(81) Designated States (unless otherwise indicated, for every
kind of national protection available): AE, AG, AL, AM,
AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY,
BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM,
DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT,
HN, HR, HU, ID, IL, IN, IR, IS, JP, KE, KG, KN, KP, KR,
KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, ME, MG,
MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM,
PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC,
SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN,
TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States (unless otherwise indicated, for every
kind of regional protection available): ARIPO (BW, GH,
GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, ST, SZ,
TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU,
TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE,
DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU,
LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK,
SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ,
GW, KM, ML, MR, NE, SN, TD, TG).

Published:

- with international search report (Art. 21(3))
- with sequence listing part of description (Rule 5.2(a))

(54) Title: CELL MODIFICATION AND APPLICATION IN THERAPY



(57) Abstract: The invention relates to a method of producing a population of cells comprising: transiently transforming a cell with nucleic acid arranged to promote cell expansion, wherein the nucleic acid is linked to a delivery vehicle for cell attachment and internalization, wherein the delivery vehicle comprises: a glycosaminoglycan (GAG) binding element, which is capable of binding to GAG on the surface of the cell; and a protein transduction domain. The invention further relates to a population of cells, methods of manufacture of cells and articles, methods of transient modification of a cell, and use of a cell delivery vehicle.

CELL MODIFICATION AND APPLICATION IN THERAPY

The invention relates to methods of producing a population of cells and use of those cells in therapy, including bone and tissue repair.

5

A number of cell therapies are proposed where large numbers of patient-matched (autologous) cells are required, but there is presently no suitable method to produce them. For example, human adult stem cells such as mesenchymal stem cells (hMSCs; derived from tissues such as bone marrow, fat or isolated from circulating blood) have low proliferative capacity and a finite life-span¹. Mesenchymal stem cells (MSCs) are multipotent stromal cells that can differentiate into a variety of cell types, including: osteoblasts (bone cells), chondrocytes (cartilage cells), myocytes (muscle cells) and adipocytes (fat cells). Many regenerative medicine applications employ MSCs however their lack of expandability and senescence significantly inhibits their autologous clinical adoption². Other cell types such as pancreatic islets, hepatocytes, retinal pigment cells, and neurons are also difficult to expand to a large population for cell therapies.

In particular, many systems simply rely on conventional culture (Fig 2A), in which slow growing cells are pushed to proliferate by media conditions with progressive slowing of growth and eventual senescence. Most therapies cannot feasibly use patient-derived cells. Therefore most approaches employ heterologous donor cells which require patient immunosuppression and a reduction in efficacy and clinical outcome. Even if the desired numbers of patient-specific cells can be produced then they have been exhaustively manipulated over several weeks/months meaning that spontaneous transformation could result leading to considerable safety concerns². Therefore, there is an unmet clinical need for high numbers of patient-specific cells for cell therapies.

30 An aim of the present invention is to provide a method of producing higher numbers of patient-specific cells for cell therapies, which overcomes the above limitations.

According to a first aspect of the invention, there is provided a method of producing a population of cells comprising:

-transiently transforming a cell with nucleic acid arranged to promote cell expansion,

wherein the nucleic acid is linked to a delivery vehicle for cell attachment and internalization, wherein the delivery vehicle comprises:

- 5 - a glycosaminoglycan (GAG) binding element, which is capable of binding to GAG on the surface of the cell; and
- a protein transduction domain.

According to another aspect of the invention, there is provided a method of transient
10 modification of a cell comprising:

-exposing the cell to a transformation complex comprising a nucleic acid linked to a delivery vehicle for cell attachment and internalization, wherein the delivery vehicle comprises:

- a glycosaminoglycan (GAG) binding element, which is capable of binding to
15 GAG on the surface of the cell; and
- a protein transduction domain.

Advantageously transiently transforming a cell with nucleic acid, such as RNA, arranged to promote expansion can provide a rapid expansion of cells in one-to-
20 several weeks rather than months from small starting populations. Furthermore, the cells produced from the transiently transformed cells will be non-genetically modified as, for example, RNA will not be integrated into the chromosome, will not be replicated, and will be degraded within the cell. Such a technology greatly facilitates the possibilities of autologous cell therapies. There is also less likelihood of
25 spontaneous transformation, for example which can be observed in senescing hMSC cultures. Advantageously, the provision of the delivery molecule in the present invention can increase the efficiency of transduction of the nucleic acid into cells for transient transformation. The GAG binding element, such as HS-GAG binding element P21 (from a growth factor), in combination with a protein transduction domain, such
30 as 8mer arginine peptide, and a cargo, greatly facilitates the uptake of very large quantities of the nucleic acid into cells. Not only do the cells take up the delivery molecule (by macro-pinocytosis) but the delivery molecules have been shown to traverse the cellular matrix and be delivered to the nucleus.

The term “cell expansion” is understood to mean the reproduction of a cell into multiple copies of the cell, i.e. in order to form a population of cells derived from the cell. Expansion includes increasing the number of cells in a population. In one embodiment, expansion may comprise at least halving the doubling time of the cells.

5

The method may further comprise expanding the cell(s) to form an expanded cell population, for example by incubation following or during transient transformation. The incubation may be in any suitable cell growth media, which the skilled person can readily determine based on the cell type to be expanded. The incubation may be at a
10 suitable growth temperature and conditions, such as 37°C, 5% CO₂, humid atmosphere.

The term “transiently transforming” or “transient transformation” is understood that a genetic sequence, for example encoding a gene, is introduced into a cell, but it is not
15 integrated into the chromosome. The genetic sequence may function within the cell for a limited time, but it is not reproduced or an inherited genetic change. For example, the nucleic acid transiently transformed into the cell may be degraded after a period of time and is not passed onto subsequent generations of the cell during expansion of the cell population. RNA is transient as it is eventually degraded in the cell. The term
20 “transient immortalisation” may be used interchangeably with “transient transformation”.

The cell may be any cell type that can be promoted to increase expansion by nucleic acid capable of promoting expansion of the cell. The cell may comprise a
25 mesenchymal stem cell (MSC). In another embodiment, the cell may be a pancreatic islet cell. In another embodiment, the cell may be a hepatocyte. In another embodiment, the cell may be a retinal pigment cell. In another embodiment, the cell may be a neuron. The cell may be mammalian. In one embodiment, the cell is a human cell, such as a human mesenchymal stem cell.

30

A single cell may be expanded into a population of cells, or multiple cells may be expanded in a population of cells. For example, a population of cells may be collected from a patient and the population of cells may be expanded in number.

The expanded population of cells may not senesce. In an embodiment where the cells are MSCs, the expanded population of cells may retain multipotent differentiation into bone, fat and connective tissue. In one embodiment, the expanded population of cells (during or post-transient transformation) are not tumorigenic.

5

In one embodiment one or more of the following properties are exhibited from the cells, the properties selected from:

- at least 40% of the cells may exhibit protein expression from the transiently transformed nucleic acid;
- 10 - at least 80% increase in growth rate of the cells after transient transformation;
- gene expression may return to less than 10% variability in the expanded population of cells (post-immortalisation);
- retention of tri-lineage differentiation ability in embodiments providing
- 15 expanded population of MSCs;
- in embodiments where the cell is an MSC, the cells may provide at least 80% capacity for osteogenic, adipogenic and chondrogenic differentiation compared with primary hMSCs;
- the expanded cell population may display normal diploid karyotype (no
- 20 aneuploidy observed using clinical criteria); and
- at least about 20% of the cell attributes in the expanded population of cells may return to pre-transient transformation levels.

25 The cell attributes may comprise retention of the cell morphology and/or retention of the cells differentiation capacity.

In one embodiment, at least 50% of the cells may exhibit protein expression from the transiently transformed nucleic acid. In another embodiment, at least 60% of the cells may exhibit protein expression from the transiently transformed nucleic acid. In one

30 embodiment, at least 100% increase in growth rate of the cells is provided after transient transformation. In another embodiment, at least 120% increase in growth rate of the cells is provided after transient transformation.

The nucleic acid may comprise RNA, or analogues thereof. In one embodiment, the

35 nucleic acid is RNA. The nucleic acid may comprise siRNA, modified messenger

RNAs (mRNAs), or micro RNA. In one embodiment, the nucleic acid may not comprise DNA.

The nucleic acid, such as RNA, may be modified for translation and stability. The RNA may comprise poly-A tail for translation and stabilisation. Additionally, or alternatively the RNA may comprise a cap or cap-analogue for translation and stabilisation. A cap-analogue may comprise ARCA cap (anti-reverse cap analogue). T7 polymerase may be used to synthesise RNA *in vitro* incorporating a cap-analogue to improve translation and stability. The RNA may comprise modified nucleotides to prevent cell immune response to delivered RNA. For example the RNA may comprise nucleotide changes to prevent immune response. Modifications may comprise the provision of methy-cytosine and/or pseudo-uracil.

The nucleic acid, such as RNA, may be arranged to promote cell expansion by encoding genes capable of temporally immortalizing cells. For example, the nucleic acid may encode TERT (telomerase reverse transcriptase), HPV E6/7 or SV40T (simian virus 40 T antigen); or combinations thereof; or functional variants thereof. In one embodiment, the nucleic acid encodes TERT (telomerase reverse transcriptase) and HPV E6/7. The nucleic acid may encode any one of the genes selected from SV40T, TERT, HPV E6/7, BMI1, Cyclins (such as D1 or 2), Survivin (BIRC5), p53, and Ras; or combinations thereof.

In one embodiment, combinations of genes may be delivered separately to the cell using separate delivery vehicles.

The delivery vehicle system herein may be referred to as GET (GAG-binding enhanced transduction) or otherwise Heparan-sulfate enhanced transduction domain (HETD)-mediated delivery. These terms may be used interchangeably.

The GAG binding element may be a heparan sulphate glycosaminoglycan (HS-GAG) binding element, which is capable of binding to HS-GAG on the surface of the cell.

Heparan sulfate glycosaminoglycan (HS-GAG) is a proteoglycan in which two or three HS chains are attached in close proximity to cell surface or extracellular matrix proteins. It is in this form that HS binds to a variety of protein ligands and regulates a

wide variety of biological activities, including developmental processes, angiogenesis, blood coagulation and tumour metastasis. Heparan sulfate is a member of the glycosaminoglycan family of carbohydrates and is very closely related in structure to heparin. Both consist of a variably sulfated repeating disaccharide unit. The most common disaccharide unit within heparan sulfate is composed of a glucuronic acid (GlcA) linked to N-acetylglucosamine (GlcNAc) typically making up around 50% of the total disaccharide units.

The GAG binding element may have specific affinity for GAG. The HS-GAG binding element may have specific affinity for HS-GAG. The HS-GAG binding element may comprise a heparin binding domain (HBD), or a variant thereof. The heparin binding domain variant may comprise a truncated heparin binding domain, or an extended heparin binding domain. The GAG binding element may comprise any protein, peptide or molecule that specifically or preferentially binds to GAG. The HS-GAG binding element may comprise any protein, peptide or molecule that specifically or preferentially binds to HS-GAG.

The HS-GAG binding element may comprise at least part of the heparin binding domain of Heparin-Binding EGF-like Growth Factor (HB-EGF). The heparin binding domain may comprise P21 of HB-EGF. The heparin binding domain may comprise a truncated, extended, or functional variant of P21.

The HS-GAG binding element may comprise a heparin binding domain of a fibroblast growth factor, or a functional part or variant thereof.

The HS-GAG binding element may be selected from any of the group comprising FGF, antithrombin, such as ATIII, VEGF, BMPs, Wnts, Shh EGFs, and PDGF; or variants thereof. The HS-GAG binding element may comprise any of FGF2, FGF7, or PDGF. The HS-GAG binding element may comprise one or more of the heparan binding sulphate domains of any FGF protein (e.g. domains A, B or C). The HS-GAG binding element may comprise FGF4. The HS-GAG binding element may comprise FGF1 HBD A (heparan sulphate binding domain A (the first HBD domain of FGF1)), FGF2 HBD A (heparan sulphate binding domain A), FGF4 HBD A (heparan sulphate binding domain A), FGF1 HBD C (heparan sulphate binding domain C), FGF2 HBD B (heparan sulphate binding domain B), FGF2 HBD C (heparan sulphate binding

domain C), FGF4 HBD C (heparan sulphate binding domain C), FGF7 HBD B (heparan sulphate binding domain B), FGF7 HBD C (heparan sulphate binding domain C), antithrombin, such as ATIII, VEGF, or PDGF, or variants thereof.

- 5 The HS-GAG binding element may be selected from any of the group comprising Hepatocyte Growth Factor, Interleukin, morphogens, HS-GAG binding enzymes, Wnt/Wingless, Endostatin, viral protein, such as foot and mouth disease virus protein, annexin V, lipoprotein lipase; or HS-GAG binding fragments thereof. The HS-GAG binding element may comprise any protein, peptide or molecule capable of
10 specifically binding HS-GAG.

A “variant” may be understood by the skilled person to include a functional variant, wherein there may be some sequence differences from the known, reported, disclosed or claimed sequence, but the variant may still bind to HS-GAG. Conservative amino
15 acid substitutions are also envisaged within the meaning of “variant”.

The HS-GAG binding element may comprise the amino acid sequence KRKKKGKGLGKKRDPCLRKYK (P21) SEQ ID NO. 1). The HS-GAG binding element may comprise a sequence having at least 80% identity to SEQ ID NO. 1. The
20 HS-GAG binding element may comprise a sequence having at least 90% identity to SEQ ID NO. 1. The HS-GAG binding element may comprise a sequence having at least 95% identity to SEQ ID NO. 1. The HS-GAG binding element may comprise a sequence having at least 98% identity to SEQ ID NO. 1. The HS-GAG binding element may comprise a sequence having at least 99% identity to SEQ ID NO. 1.

25

The HS-GAG binding element may comprise the amino acid sequence G R P R E S G K K R K R K R L K P T (PDGF, SEQ ID NO. 3). The HS-GAG binding element may comprise a sequence having at least 80% identity to SEQ ID NO. 3. The HS-GAG binding element may comprise a sequence having at least 90% identity to SEQ ID
30 NO. 3. The HS-GAG binding element may comprise a sequence having at least 95% identity to SEQ ID NO. 3. The HS-GAG binding element may comprise a sequence having at least 98% identity to SEQ ID NO. 3. The HS-GAG binding element may comprise a sequence having at least 99% identity to SEQ ID NO. 3.

The HS-GAG binding element may comprise the amino acid sequence T Y A S A K W T H N G G E M F V A L N Q ((FGF7, HBD B) SEQ ID NO. 5). The HS-GAG binding element may comprise a sequence having at least 80% identity to SEQ ID NO. 5. The HS-GAG binding element may comprise a sequence having at least 90% identity to
 5 SEQ ID NO. 5. The HS-GAG binding element may comprise a sequence having at least 95% identity to SEQ ID NO. 5. The HS-GAG binding element may comprise a sequence having at least 98% identity to SEQ ID NO. 5. The HS-GAG binding element may comprise a sequence having at least 99% identity to SEQ ID NO. 5.

10 The HS-GAG binding element may comprise the amino acid sequence T Y R S R K Y T S W Y V A L K R (FGF2 HBD B SEQ ID NO. 7). The HS-GAG binding element may comprise a sequence having at least 80% identity to SEQ ID NO. 7. The HS-GAG binding element may comprise a sequence having at least 90% identity to SEQ ID NO. 7. The HS-GAG binding element may comprise a sequence having at least 95%
 15 identity to SEQ ID NO. 7. The HS-GAG binding element may comprise a sequence having at least 98% identity to SEQ ID NO. 7. The HS-GAG binding element may comprise a sequence having at least 99% identity to SEQ ID NO. 7.

Sequence identity may be determined by standard BLAST alignment parameters
 20 (provided by <http://www.ncbi.nlm.nih.gov/>).

The GAG binding element may comprise a GAG binding antibody, or a variant or fragment thereof. The HS-GAG binding element may comprise a HS-GAG binding antibody, or a variant or fragment thereof. The antibody fragment may be an antibody
 25 variable domain, an scFv, a diabody, a FAb, a Dab, a F(ab)'2, a heavy-light chain dimer, or a single chain structure. The antibody variant may comprise a protein scaffold comprising CDRs, an antibody mimetic, or a DARPin.

The GAG or HS-GAG binding element may comprise a nanobody (single-domain
 30 antigen-binding fragments derived from heavy-chain antibodies that are devoid of light chains and occur naturally in Camelidae).

The single-domain antibody may comprise a V_HH fragment comprising a CDR1, CDR2 and CDR3 wherein

CDR1 may comprise or consists of the amino acid sequence of GFTVSSNE (SEQ ID NO: 21) or GFAFSSYA (SEQ ID NO: 22);

CDR2 may comprise or consists of the amino acid sequence of ISGGST (SEQ ID NO: 23) or IGTGGDT (SEQ ID NO: 24); and

5 CDR3 may comprise or consists of the amino acid sequence of GRRLKD (SEQ ID NO: 25) or SLRMNGWRAHQ (SEQ ID NO: 26).

The single-domain antibody may comprise a V_HH fragment comprising a CDR1, CDR2 and CDR3 wherein

10 CDR1 may comprise or consists of the amino acid sequence of GFTVSSNE (SEQ ID NO: 21);

CDR2 may comprise or consists of the amino acid sequence of ISGGST (SEQ ID NO: 23); and

15 CDR3 may comprise or consists of the amino acid sequence of GRRLKD (SEQ ID NO: 25).

Alternatively, the CDR3 may comprise the amino acid sequence GMRPRL (SEQ ID NO: 27), HAPLRNTRTNT (SEQ ID NO: 28), GSRSSR (SEQ ID NO: 29), GRTVGRN (SEQ ID NO: 30), GKVKLPN (SEQ ID NO: 31), SGRKGRMR (SEQ ID NO: 32),
20 SLRMNGWRAHQ (SEQ ID NO: 26), or RRYALDY (SEQ ID NO: 33).

The single-domain antibody may comprise a V_HH fragment comprising a CDR1, CDR2 and CDR3 wherein

25 CDR1 may comprise or consists of the amino acid sequence of GFAFSSYA (SEQ ID NO: 22);

CDR2 may comprise or consists of the amino acid sequence of IGTGGDT (SEQ ID NO: 24); and

30 CDR3 may comprise or consists of the amino acid sequence of SLRMNGWRAHQ (SEQ ID NO: 26).

Alternatively, the CDR3 may comprise the amino acid sequence LKQQGIS (SEQ ID NO: 34), AMTQKKPRKLSL (SEQ ID NO: 35), HAPLRNTRTNT (SEQ ID NO: 28), GMRPRL (SEQ ID NO: 27), RRYALDY (SEQ ID NO: 33), or SGRKYFRARDMN (SEQ ID NO: 36).

35

The HS-GAG binding element may comprise anti-HS scFv antibodies AO4B08, AO4B05, AO4F12, RB4CB9, RB4CD12, RB4EA12, or RB4EG12 (as described in Jenniskens *et al* (2000. *The Journal of Neuroscience*, 20(11):4099–4111) and Smits, et al (2006. *METHODS IN ENZYMOLOGY*, VOL. 416, pp. 61-87) incorporated herein by reference); or fragments thereof. The HS-GAG binding element may comprise AO4B08. The HS-GAG binding element may comprise CDR1, CDR2 and CDR3 of AO4B08, AO4B05, AO4F12, RB4CB9, RB4CD12, RB4EA12, or RB4EG12. The HS-GAG binding element may comprise CDR1, CDR2 and CDR3 of AO4B08.

10 The HS-GAG binding element may comprise HS3A8, LKIV69, EW3D10, EW4G2, NS4F5, RB4EA12, HS4E4 or HS4C3 (as described in Wijnhoven et al (2008) *Glycoconj J* 25:177–185) and Smits, et al (2006. *METHODS IN ENZYMOLOGY*, VOL. 416, pp. 61-87) incorporated herein by reference). The HS-GAG binding element may comprise HS4E4 or HS4C3. The HS-GAG binding element may comprise CDR1, CDR2 and CDR3 of HS3A8, LKIV69, EW3D10, EW4G2, NS4F5, RB4EA12, HS4E4 or HS4C3. The HS-GAG binding element may comprise CDR1, CDR2 and CDR3 of HS4E4 or HS4C3.

The HS-GAG binding element may comprise SEQ ID NO: 15 or 17 (AO4B08). The HS-GAG binding element may comprise SEQ ID NO: 11 or 13 (HS4C3). The HS-GAG binding element may comprise an antibody, or antibody fragment, heavy chain and/or light chain. The HS-GAG binding element may comprise an antibody, or antibody fragment, heavy chain, comprising HCDR1, HCDR2 and HCDR3 chains and/or light chain, comprising LCDR1, LCDR2 and LCDR3.

25

In one embodiment, the GAG binding element may not comprise any of the protein transduction domains described herein. In one embodiment, the GAG binding element and the protein transduction domain are different. In one embodiment, the GAG binding element is not TAT.

30

The protein transduction domain may be hydrophilic or amphiphilic. The protein transduction domain may comprise a majority of hydrophilic amino acid residues. The protein transduction domain may comprise a majority of arginine and/or lysine amino acid residues. The protein transduction domain may comprise a periodic sequence, having a repeated amino acid sequence motif. The protein transduction domain may

35

comprise penetratin, TAT such as HIV derived TAT, MAP, or transportan, pVec, or pep-1.

Where reference is made to a “majority” of residue, this may be understood by the skilled person to include greater than 50% of the residues. A majority may be 55%, 60%, 70%, 80%, 90% or 95% of the residues.

The protein transduction domain may be selected from any of the group comprising:

- Penetratin or Antennapedia PTD RQIKWFAQNRMMKWKK (SEQ ID NO: 37);
- 10 TAT YGRKKRRQRRR (SEQ ID NO: 38);
- SynB1 RGGRLSYRRRFSTSTGR (SEQ ID NO: 39);
- SynB3 RRLSYRRRF (SEQ ID NO: 40);
- PTD-4 PIRRRKKLRRLK (SEQ ID NO: 41);
- PTD-5 RRQRRTSKLMKR (SEQ ID NO: 42);
- 15 FHV Coat-(35-49) RRRNRTRNRNRVR (SEQ ID NO: 43);
- BMV Gag-(7-25) KMTRAQRRAAARRNRWTAR (SEQ ID NO: 44);
- HTLV-II Rex-(4-16) TRRQRTRRARRNR (SEQ ID NO: 45);
- D-Tat GRKKRRQRRRPPQ (SEQ ID NO: 46);
- R9-Tat GRRRRRRRRRPPQ (SEQ ID NO: 47);
- 20 Transportan GWTLNSAGYLLGKINLKALAALAKKIL (SEQ ID NO: 48) chimera;
- MAP KLALKLALKLALALKLA (SEQ ID NO: 49);
- SBP MGLGLHLLVLAALQGAWSQPKKKRKV (SEQ ID NO: 50);
- 25 FBP GALFLGWLGAAGSTMGAWSQPKKKRKV (SEQ ID NO: 51);
- MPG ac-GALFLGFLGAAGSTMGAWSQPKKKRKV-cya (SEQ ID NO: 52);
- MPG(?NLS) ac- GALFLGFLGAAGSTMGAWSQPKSKRKV-cya (SEQ ID NO: 53);
- Pep-1 ac-KETWWETWWTEWSQPKKKRKV-cya (SEQ ID NO: 54); and
- 30 Pep-2 ac-KETWFETWFTEWSQPKKKRKV-cya (SEQ ID NO: 55).

The protein transduction domain may comprise polyarginines, such as RxN (4<N<17) chimera, polylysines, such as KxN (4<N<17) chimera, (RAca)6R, (RAbu)6R, (RG)6R, (RM)6R, (RT)6R, (RS)6R, R10, (RA)6R, R7, or R8.

The protein transduction domain may comprise polyarginine or polylysine. The protein transduction domain may comprise an arginine and lysine repeat sequence. The protein transduction domain may comprise arginine residues, such as consecutive arginine residues. The protein transduction domain may consist essentially of arginine residues. The protein transduction domain may comprise arginine repeats, such as 4-20 arginine residues. The protein transduction domain may comprise 8 arginine residues. The protein transduction domain may comprise between about 6 and about 12 arginine residues. The protein transduction domain may comprise between about 7 and about 9 arginine residues.

10

The protein transduction domain may comprise between about 4 and about 12 amino acid residues. The protein transduction domain may comprise between about 6 and about 12 amino acid residues. The protein transduction domain may comprise between about 7 and about 9 amino acid residues. The protein transduction domain may comprise at least about 4 amino acid residues. The protein transduction domain may comprise at least about 6 amino acid residues.

15

The protein transduction domain may comprise lysine residues, such as consecutive lysine residues. The protein transduction domain may consist essentially of lysine residues. The protein transduction domain may comprise lysine repeats, such as 4-20 lysine residues. The protein transduction domain may comprise 8 lysine residues. The protein transduction domain may comprise between about 4 and about 12 lysine residues. The protein transduction domain may comprise between about 6 and about 12 lysine residues. The protein transduction domain may comprise between about 7 and about 9 lysine residues.

20

25

The protein transduction domain may comprise Q and R residues, such as consecutive QR repeat residues. The protein transduction domain may consist essentially of Q and R residues. The protein transduction domain may comprise QR repeats, such as 4-20 QR repeat residues. The protein transduction domain may comprise 8 QR repeat residues. The protein transduction domain may comprise between about 6 and about 12 QR repeat residues. The protein transduction domain may comprise between about 7 and about 9 QR repeat residues.

30

The bond or interaction between the nucleic acid and delivery vehicle may be reversible, or degradable, for example in the intracellular environment.

The GAG binding element and protein transduction domain may be bound to the nucleic acid by direct chemical conjugation or through a linker molecule. Direct chemical conjugation may comprise a covalent bond. The GAG binding element, protein transduction domain may be a single fusion molecule (e.g. it may be encoded and transcribed as a single peptide molecule), with the nucleic acid conjugated thereto.

The nucleic acid, GAG binding element, and protein transduction domain may be linked together by one or more linker molecules. The GAG binding element, protein transduction domain and linker molecule may be a single fusion molecule (e.g. it may be encoded and transcribed as a single peptide molecule), with the nucleic acid conjugated thereto, or complexed therewith. The linker molecule may comprise a nucleic acid interacting peptide. The nucleic acid interacting peptide may comprise synthetic amphipathic peptide LK15. In another embodiment, the nucleic acid interacting peptide may comprise KALA peptide. In another embodiment, the nucleic acid is linked to the GAG binding element and protein transduction domain via a conjugated PEI (polyethylenimine) molecule.

The delivery vehicle may comprise a marker for identifying and/or tracking the location of the delivery molecule. The marker may comprise a fluorescence marker, or a radioisotope. The marker may comprise mRFP1 (monomeric red fluorescent protein). The marker may comprise mNectarine, such as pH-sensitive mNectarine. mNectarine, is appropriate to measure physiological pH changes in mammalian cells, because it has a pKa' of 6.9. The marker may comprise a red fluorescent protein (RFP) homologue of avGFP. The marker may comprise a fluorescent protein selected from the mFruit series RFPs, derived from tetrameric Discosoma RFP. The marker may comprise any of mTangerine, mOrange, mCherry, mStrawberry, yellow FP Citrine. mApple and TagRFP-T. The marker may be pH-sensitive. The marker may be used to confirm delivery of the delivery molecule into the cell or tissue. The marker may be cell-type specific, for example the marker may only be activated or fluoresce in specific cell types. The marker may be encoded on the nucleic acid.

The delivery vehicle may comprise a tag to aid in purification, isolation, detection and/or determination of location. The tag may be an affinity tag. The tag may be a peptide. The tag may be a FLAG-tag / FLAG octapeptide.

- 5 The transduction may be for at least about 1 second. The transduction may be for at least about 1 minute. The transduction may be for at least about 2 minutes. The transduction may be for at least about 10 minutes. The transduction may be for at least about 30 minutes. The transduction may be for at least about 1 hour. The transduction may be for 12 hours or less, such as 8 hours or less. The transduction may be for about 10 6 hours or less. The transduction may be for between about 1 hour and 6 hours. The transduction may be for less than about 1 hour. The transduction may be for less than about 30 minutes. The transduction may be for less than about 10 minutes. The transduction may be for less than about 1 minute.
- 15 According to another aspect of the present invention, there is provided a method of treatment or prevention of a disease comprising:
- providing a cell;
 - transiently transforming the cell with nucleic acid for causing expansion of the cell in accordance with the method of the invention;
 - 20 -expanding the cell to form an expanded population of cells;
 - administering the expanded population of cells to the patient.

According to another aspect of the present invention, there is provided a method of treatment for tissue damage or a bone defect comprising:

- 25 - providing a cell;
- transiently transforming the cell with nucleic acid for causing expansion of the cell in accordance with the method of the invention;
 - expanding the cell to form an expanded population of cells;
 - administering the expanded population of cells to the patient.

30

According to another aspect of the present invention, there is provided an expanded population of cells for use in a method of treatment or prevention of a disease or treatment for tissue damage or a bone defect, the method comprising:

- providing a cell;

- transiently transforming the cell with nucleic acid for causing expansion of the cell in accordance with the method of the invention;
- expanding the cell to form an expanded population of cells;
- administering the expanded population of cells to the patient.

5

The provided cell may be from the patient to be treated. The expanded population of cells may be autologous for the patient.

10 The expanded population of cells may be administered to the patient by implantation of a scaffold populated with the expanded population of cells. The expanded population of cells may be administered to the tissue damage or bone defect of the patient, for example by injection. The expanded population of cells may be administered topically for skin regeneration treatments.

15 According to another aspect of the present invention, there is provided a method of manufacturing an implant for treatment of tissue damage or a bone defect in a patient comprising:

- providing a cell;
- transiently transforming the cell with nucleic acid for causing expansion of
- 20 the cell in accordance with the method of the invention;
- expanding the cell to form an expanded population of cells;
- populating the expanded population of cells into the implant.

25 According to another aspect of the present invention, there is provided a method of manufacturing a scaffold-forming composition comprising cells for treatment of tissue damage or a bone defect in a patient comprising:

- providing a cell;
- transiently transforming the cell with nucleic acid for causing expansion of
- the cell in accordance with the method of the invention;
- 30 - expanding the cell to form an expanded population of cells;
- mixing the expanded population of cells with a scaffold-forming composition.

35 According to another aspect of the present invention, there is provided a method of manufacturing a wound dressing comprising cells for treatment of tissue damage in a patient comprising:

- providing a cell;
- transiently transforming the cell with nucleic acid for causing expansion of the cell in accordance with the method of the invention;
- expanding the cell to form an expanded population of cells;
- 5 -mixing the expanded population of cells with a wound dressing material.

According to another aspect of the invention, there is provided an agent for transiently transforming a population of cells, the agent comprising:

- nucleic acid arranged to promote cell expansion,
- 10 wherein the nucleic acid is linked to a delivery vehicle for cell attachment and internalization, wherein the delivery vehicle comprises:
 - a glycosaminoglycan (GAG) binding element, which is capable of binding to GAG on the surface of the cell; and
 - a protein transduction domain.

15

According to another aspect of the present invention, there is provided the use of the method of the invention or the use of the agent of the invention for providing a population of cells suitable for seeding onto a scaffold implant or wound dressing.

- 20 The scaffold or scaffold-forming composition may comprise any synthetic or natural polymer capable of solidifying to form a scaffold. The scaffold or scaffold-forming composition may comprise a polymer blend (for example a PLGA/PEG blend). The scaffold or scaffold-forming composition may comprise a synthetic bone alternative. The scaffold may comprise extracellular matrix. The scaffold may be a biocompatible scaffold. The skilled person will be familiar with a range of scaffold material and
- 25 compositions suitable for use with seeded cells. For example, Freed et al. (1994. Biodegradable polymer scaffolds for tissue engineering. *Nature Biotechnology*, 12, 689–693) describes suitable scaffold compositions that may be used with this invention, the contents of which are incorporated herein by reference.

30

According to another aspect of the present invention, there is provided a method of manufacturing a suspension of autologous cells for treatment of a patient in need thereof comprising:

- providing a cell;

- transiently transforming the cell with nucleic acid for causing expansion of the cell in accordance with the method of the invention;

- expanding the cell to form an expanded population of cells which are autologous to the patient.

5

In accordance with the methods of the invention herein, the cell may be from the patient to be treated.

The treatment may comprise autologous therapy selected from the group comprising
10 burns/skin grafting; diabetes therapy with pancreatic islet cell expansion; liver therapy with hepatocyte cell expansion; retinal pigment epithelial cell expansion for macular degeneration; and expansion of cardiac progenitors for transplant in ischemia.

According to another aspect of the invention, there is provided a population of cells
15 produced by the method of the invention.

The population of cells may be derived from a patient to be treated with the cells. In an embodiment wherein the population of cells is derived from a patient to be treated, the population of cells may be autologous to the patient.

20

According to another aspect of the invention, there is provided the use of delivery vehicle for transiently transforming a mesenchymal stem cell with nucleic acid, wherein the delivery vehicle comprises:

- a glycosaminoglycan (GAG) binding element, which is capable of binding to
25 GAG on the surface of the cell; and

- a protein transduction domain; and

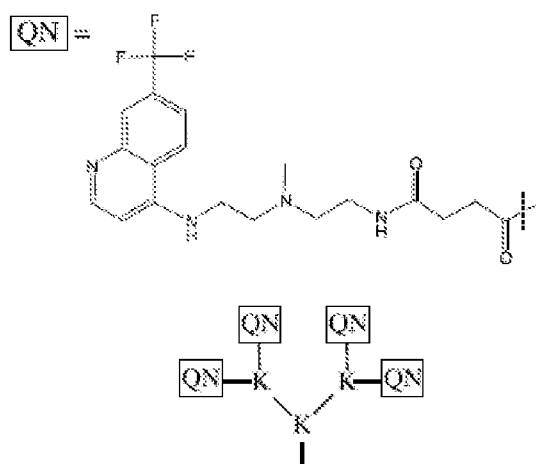
wherein the delivery vehicle is linked to the nucleic acid.

The transient transformation and/or expansion of the cell may be *in vitro*.

30

With reference to the methods of the invention, the delivery vehicle may be transduced in the presence of, or co-administered with, a vesicle/endosome release agent for promoting release of the delivery molecule from micropinocytic vesicles. The vesicle release agent may comprise chloroquine. The chloroquine concentration
35 may be between about 1 μ M and about 100 μ M.

The delivery vehicle may further comprise an endosome release agent. The endosome release agent may comprise one or more trifluoromethylquinolines, for example four trifluoromethylquinolines as described in Lindberg et al. (*International Journal of*
 5 *Pharmaceutics* 441 (2013) 242– 247), which is incorporated herein by reference. For example, the endosome release agent may be linked to the delivery vehicle and may comprise the following structure:

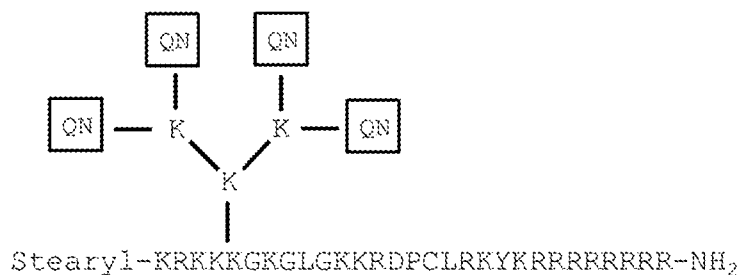


10 , which is arranged to be linked to the delivery vehicle, optionally the link is to a lysine residue of the delivery vehicle.

The endosome release agent linked to a delivery vehicle according to the invention may comprise or consist of the following structure (P21-8R-QN1):

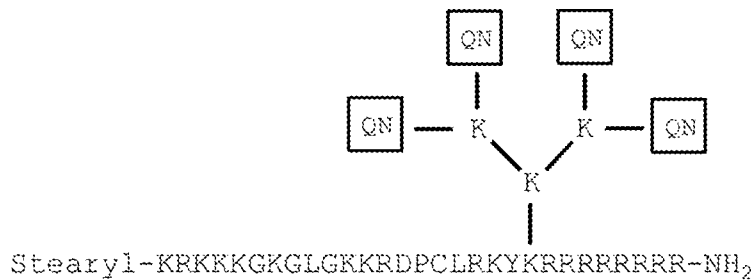
15

Stearyl-KRKKK1(QN-K3[QN]K2{QN-K3[QN]})GKGLGKKRDPCLRKYKRRRRRRRR, alternatively represented as:



The endosome release agent linked to a delivery vehicle according to the invention may comprise or consist of the following structure (P21-8R-QN2):

Stearyl-KRKKKGKGLGKKRDPCLRKYK1(QN-K3[QN]K2{QN-
5 K3[QN]})RRRRRRRR, alternatively represented as:



According to another aspect of the invention, there is provided a method of
10 genetically modifying a cell comprising:

- transiently transforming the cell according to the method of the invention, such that the cell is in an expansion phase to form a population of cells; and
- transforming the cells during the expansion phase with nucleic acid arranged to stably modify the chromosomal DNA of the cell.

15

Following the transient transformation and expansion into a population of cells, the nucleic acid, such as RNA, may be degraded and the cells may return to their original state, whilst retaining the chromosomal DNA modification.

20 According to another aspect of the invention, there is provided the use of the method of the invention to transiently transform a cell for achieving a cell expansion phase in order to facilitate stable genetic modification of the cell by transformation with nucleic acid arranged to modify the cell chromosomal DNA.

25 The nucleic acid for stable genetic modification may comprise DNA. The method of genetically modifying a cell may comprise modifying a mesenchymal stem cell. The method may comprise stable genetic modification of any slow growing cell type. In another embodiment, the method of genetically modifying a cell may comprise modifying an epithelial cell, keratinocyte, beta cell, or cardiac or skeletal myocyte.

Reference to “stably genetically modifying” or “stable genetic modification” is understood to mean stable integration of DNA, mutation, or removal of DNA sequence, for example from the chromosome of the cell. For example, transformed
5 DNA may be integrated into the chromosome, replicated and passed on to generations of the cell.

Advantageously, cells which are proliferating/expanding are more susceptible to stable genetic transfection. Therefore, genetic modifications can be made to cells such
10 as mesenchymal stem cells, which are typically difficult to transform and modify genetically.

The methods of the invention herein may further comprise a second or third transient transformation step (e.g. one or more serial transformations).
15

The skilled person will understand that optional features of one embodiment or aspect of the invention may be applicable, where appropriate, to other embodiments or aspects of the invention.

20 Embodiments of the invention will now be described in more detail, by way of example only, with reference to the accompanying drawings.

Figure 1: Genetic Immortalisation (via Lentivirus) of hMSCs using TERT, E6/7 and SV40T overexpression. A) Primary hMSCs were transduced with
25 TERT, E6/7 or SV40T-expressing lentiviruses which generated a rapid up-regulation in proliferation and population-doubling capacity. B) ihMSC-TERT E6/7 cells retain the ability to differentiate down osteogenic, chondrogenic and adipogenic lineages (Alizerin Red, Alcian Blue and Oil-O red, respectively).

Figure 2: Project Hypothesis: Transient Immortalisation of hMSCs can be achieved using GET delivery of TERT, E6/7 or SV40T RNA. A)
30 Conventional hMSC culture senesces and yields low cell numbers. Also this long term culture can promote a transformed phenotype. B) Permanent Immortalisation creating ihMSCs yields high cell numbers of genetically
35 modified cells. C) The transient immortalisation process proposed to expand

hMSCs through an immortalised intermediate (Trans-ihMSCs) driven by GET delivery of RNA.

Figure 3: Production of Modified RNAs to direct cell behaviour.

5 Initially the genes/factors to be expressed are selected. B) The open-reading frame (ORF) of the gene is isolated incorporating codon changes to optimise human expression. C) The ORF is cloned into an in-house produced vector pEXPRESS which allows the *in vitro* expression of RNA which contains the human β -actin untranslated regions to enhance translation of protein from the
10 RNA. D) PCR is used to insert a 90 adenosine poly-A tail required for translation and RNA stabilisation. E) T7 polymerase is used to synthesise RNA *in vitro* incorporating a cap-analogue to improve translation and RNA stability, and modified nucleotides to prevent cell immune response to delivered RNA. F) The synthesised RNA is checked for purity and intactness. G-H) RNA is
15 delivered to cells using the GET system, and the RNA produces the factor protein which affects the cell behaviour (programs the cell).

Figure 4: Transfection of human mesenchymal stem cells (ihMSCs) using GET.

Initially we assessed binding capacity of LK15 peptides for plasmid (p)DNA (SIN GFP, to express GFP on transfection), RNA (modRNA) (to
20 express GFP on transfection) and small-inhibitory (si)RNAs (labelled with FAM fluorophore to detect delivery). A) Schematic of the GET transfection peptide (P21-LK15-8R). B) Delivery of nucleic acid cargoes to hMSCs by GET transfection. After optimising ratios we transfected ihMSCs with P21-LK15-8R and pDNA (10 μ g), modRNA (10 μ g) or siRNA (1 μ g) and visualised
25 transfection by fluorescence microscopy (scale bar, 100 μ m). (C) Quantification of GET-LK15 transfection of ihMSCs by flow cytometry (% transfection efficiency or relative fluorescence for siRNA) compared to lipofectamine (LIPO)2000 as a commercial standard. Error bars indicate s.d.

30 **Figure 5: Analyses of Trans-ihMSCs (Ana)** A) Primary, Genetically, transiently, and post-transiently immortalised hMSCs will be compared. B) Gene expression analyses through Illumina mRNA-Seq, Tri-lineage differentiation, karyotype stability, cell growth and tumorigenicity of the cells
35 will be assessed.

Figure 6: Transfection of primary human mesenchymal stem cells (hMSCs), long-term passaged (P10) hMSCs and immortalised hMSCs (ihMSCs) using GET or LIPO2000. hMSCs were transfected, long-term hMSCs (P10) and ihMSCs with P21-LK15-8R (GET) and pDNA (10µg of SIN GFP expressing eGFP) and quantified transfection SCs by flow cytometry (% transfection efficiency) and compared to lipofectamine (LIPO)2000 as a commercial standard. Error bars indicate s.d. pDNA transfections are strongly influenced by proliferation of the cell line transfected. Younger, growing hMSCs (P1) were transfected at low levels, older senescing hMSCs (P10) have ~10-fold less transfection efficiency in comparison. If the same cells are immortalised and induced to grow this resulted in >8-fold increase in transfection efficiency.

Figure 7 P21 improves PTD-mediated transduction. (a) Schematic of the proteins created after screening domains which improve efficiency of protein delivery to cells. mR and mR-8R are described in Fig 1. P21-mR is mRFP with an N-terminal fusion of the P21 domain of heparin-binding EGF (HB-EGF). P21-mR-8R is mRFP with N-terminal fusion of P21 and C-terminal fusion of 8R. **(b)** Fusion of P21 to mR-8R significantly improves transduction into NIH3t3 cells. Fluorescence microscopy images of NIH3t3 cells treated with proteins (20µg/ml) for twelve hours in standard media conditions. Scale bar, 100µm. **(c)** P21-mR-8R transduces efficiently into human and mouse embryonic stem cells (HUES7 and CGR-8, respectively) and human induced pluripotent stem cells (IPS2) and mouse cardiomyocyte cell line HL1. Flow cytometry analyses of the mR-8R-inefficiently transduced cell lines treated with proteins mR-8R (20µg/ml) for twelve hours. **(d)** P21-mR-8R initially strongly interacts with cell membranes and progressively transduces be localised peri-nuclearly. Fluorescence (top) and confocal laser scanning microscopy (bottom) images of NIH3t3 cells treated with P21-mR-8R (20µg/ml) for either 1 hour, 1 hour with washes and a further 5 hours incubation (in serum-free media) or 6 hours treatment. Cells were pre-incubated for 1 hour in serum-free media, transduced for the desired time in serum-free media. Scale bars, 50µm (top) and 10µm (bottom). **(e)** Enhancement of transduction mediated by P21 and 8R are affected by Trypsin proteolysis. Flow cytometry analyses

NIH3t3 cells treated with proteins (20µg/ml) for 1 hour and a further 5 hour incubation (in serum-free media), with or without 10min pre-digestion with Trypsin or treatment with non-proteolytic cell dissociation solution (CDS). Cells were pre-incubated for 1 hour in serum-free media, treated with Trypsin and transduced for 1 hour in serum-free media. (f) Cell surface interaction of P21-containing proteins is disrupted by Tritonx100 treatment. Flow cytometry analyses NIH3t3 cells treated with proteins (20µg/ml) for 1 hour and a further 5 hour incubation (in serum-free media) with 10min pre-treatment of PBS or PBS containing 0.1% (v/v) Tritonx100 (Tx100). Cells were pre-incubated for 1 hour in serum-free media, treated with PBS or PBS with Tx100 and transduced for 1 hour in serum-free media. Error bars indicate s.d.

Figure 8 P21 binds directly to Heparin and cell surface HS-GAG. (a) Soluble Heparin in media during transduction inhibits cell membrane interaction and transduction of P21-containing proteins. Fluorescence microscopy images of CGR-8 cells treated with P21-mR-8R (20µg/ml) for 6 hours in serum-free media containing 0 or 50µg/ml Heparin. Scale bar, 100µm. (b) Flow cytometry analyses NIH3t3 cells treated with P21-mR-8R (20µg/ml) for 6 hours with or without a variety of GAGs (50µg/ml) in serum-free medium. CS is Chondroitin sulphate. Cells were pre-incubated for 1 hour in serum-free media and transduced for 6 hours in serum-free media with or without GAGs. (c-d) Only high-doses of Heparin inhibit 8R activity whereas P21 activity is inhibited dose-dependently by Heparin in (c) NIH3t3 cells and (d) CGR-8 cells. (e) and (f) Cell surface Heparan sulphate is required for efficient P21-mediated protein delivery. Heparan sulphates/Heparin-containing FCS inhibits P21-mediated transduction but can also replace cell surface GAGs and mediate P21-transduction in cells deficient for Heparan sulphate. Fluorescence microscopy images of CGR-8 cells and *EXT1*^{-/-} mESCs treated with P21-mR-8R (20µg/ml) for 6 hours in media containing 0 or 20% FCS. Scale bar, 100µm. Error bars indicate s.d.

30

Figure 9 GET / HETD-mediated nuclear delivery of Cre Recombinase. (a) Schematic of the construct created to mark Cre activity in cells. Cre-mediated excision of a transcriptional STOP region flanked by *loxP* sites induces the constitutive expression of eGFP. Pr, promoter; βGal, β-galactosidase; Neo, Neomycin phosphotransferase. The NIH3t3 LSL-eGFP cell line was created by

35

transfection and selection of NIH3t3 cells. **(b)** eGFP expression in untreated NIH3t3 LSP-eGFP cells or those transduced with SIN Cre lentivirus. Left shows fluorescence microscopy and right shows flow cytometry histogram of eGFP expression. Scale bar, 50µm **(c)** Scheme of testing transduction of Cre activity in NIH3t3 LSL-eGFP cells. Cells were transduced with Cre proteins for 1 hour, washed and cultured for 2 days before analyses. **(d-e)** P21-mR-Cre-8R is efficiently transduced and recombines DNA. **(d)** Fluorescence microscopy images Cre-transduced NIH3t3 LSL-eGFP with the variety of dosages. Scale bar, 50µm. **(e)** Flow cytometry analyses of NIH3t3 LSL-eGFP cells transduced for 1 hour with mR-Cre, mR-Cre-8R and P21-mR-Cre-8R at a variety of dosages (0, 1, 10, 100 and 500µg/ml), washed and cultured for 2 days. Graph shows % recombination (i.e. % of eGFP+ve from total cell population). Error bars indicate s.d.

Figure 10 GET / HETD-mediated delivery of domain position protein variants. **(a)** Schematic of the proteins created to test the effect of domain position on protein delivery to cells. **(b)** Fusion of P21 and 8R to mR in any orientation significantly improves transduction. Flow cytometry analyses of NIH3t3 and HUES7 cells incubated with the protein variants (20µg/ml) for twelve hours. Error bars indicate s.d.

Figure 11 GET / HETD-mediated delivery of PTD protein variants. **(a)** Schematic of the proteins created to test the enhancing effect of P21 on other PTDs for protein delivery to cells. 8R is RRRRRRRR (SEQ ID NO: 19), TAT is HIV-1 TAT protein RKKRRQRRR (SEQ ID NO: 20), 8K is KKKKKKKK (SEQ ID NO: 56), 8RQ is RQRQRQRQ (SEQ ID NO: 57) **(b)** Fusion of P21 and any PTD to mR significantly improves transduction. Flow cytometry analyses of NIH3t3 and HUES7 cells incubated with the protein variants (20µg/ml) for twelve hours. Error bars indicate s.d.

Figure 12 GET / HETDs can achieve higher intracellular levels of cargo delivery than transgenic systems. **(a)** Fluorometry of soluble extracts generated from NIH3t3 mR (transgenic NIH3t3 cells transduced with SIN mR) compared with those from NIH3t3 cells transduced for 6 hours with different doses of mR-8R or P21-mR-8R (0, 10, 20, 50, 100 or 200µg/ml in serum-free media) **(b)** Flow

cytometry of NIH3t3 mR (transgenic NIH3t3 cells transduced with SIN mR) compared with those from NIH3t3 cells transduced for 6 hours with different doses of mR-8R or P21-mR-8R (0, 10, 20, 50, 100 or 200µg/ml in serum-free media). Fluorescence is normalised to untreated NIH3t3 cells. Error bars indicate s.d.

Figure 13 HERD-mediated Cre Recombinase nuclear activity is promoted by vesicle escape but repressed by inhibitors of macropinocytosis or cholesterol depletion. NIH3t3: LSL-eGFP cells were pre-incubated in serum-free media (with or without drugs), transduced with Cre proteins (mR-Cre: 100µg/ml or P21-mR-8R: 10µg/ml) for 1 hour in serum-free media (with or without drugs), washed and cultured for 12 hours in full growth media (with or without drugs) and a further 36 hours in full growth media before analyses. **(a)** Methyl-β-cyclodextrin (used to deplete cholesterol) inhibits Cre transduction and recombination. (Methyl-β-cyclodextrin doses were 0, 1 2 and 5 mM). **(b)** Nystatin (a drug which sequesters cholesterol) inhibits Cre transduction and recombination. (Nystatin doses were 0, 10, 20 and 50µg/ml). **(c)** Amiloride (a specific inhibitor of Na⁺/H⁺ exchanged required for macropinocytosis) inhibits Cre transduction and recombination. (Amiloride doses were 0, 1, 5 or 10mM). **(d)** Cytochalasin D (an F-actin elongation inhibitor) inhibits Cre transduction and recombination. (Cytochalasin D doses were 0, 1, 5 or 10µM). **(e)** Chloroquine promotes the release of Cre from endosomal vesicles and increases recombination (Chloroquine doses were 0, 10 and 100µM). **(f)** Picogram per millilitre amounts is required to induce recombination with enhanced vesicle escape. The dose of transduced P21-mR-Cre-8R was varied in ten-fold dilutions (0-100µg/ml) with 1 hour incubation in the presence of Chloroquine. All data is presented as % of the maximal recombination. Error bars indicate s.d.

Figure 14 GET / HETD-mediated transduction increases general cellular macropinocytosis. Flow cytometry of cells incubated in 70kDa FITC-Dextran and transduced with recombinant proteins. NIH3t3 cells were pre-incubated in serum-free media for 1 hour and transduced with mR, P21-mR, mR-8R or P21-mR-8R (20µg/ml in serum-free media) containing 70kDa FITC-Dextran (neutral) for 1 hour. Error bars indicate s.d.

Figure 15 GET of non-protein Cargoes. **(a)** GET of biotinylated cargoes using monomeric streptavidin (mSA2). **(i)** Schematic of the mSA2 proteins engineered to bind to and transduce biotinylated cargoes. P21-8R was used as a non-interacting control, mSA2 as a non-transducing control, and P21-mSA2-8R as the test protein. **(ii)** Schematic of the antibody (Ab) complexes of a biotinylated primary (1°) antibody (Goat anti-rabbit; GtαRb) bound to an FITC-conjugated secondary (2°) antibody (Rabbit anti-mouse; Rb αMu) used to test activity. **(iii)** GET-delivery of Ab complexes were visible by fluorescence microscopy (scale bar, 50μm). With co-incubation of P21-mSA2-8R (10μg/ml, bottom image), Ab complexes were efficiently delivered to cells **(iv)** Flow cytometry demonstrating that 1°/2° Ab complexes (1μg/ml) are taken into NIH3t3 cells poorly by direct incubation or when co-incubated with mSA2 only. **(b)** GET of nucleic acids by employing LK15 peptide. **(i)** Schematic of the LK15 proteins engineered to bind to and transduce nucleic acids. **(ii)** Transfection of human mesenchymal stem cells (iHMSCs) using GET-LK15. Initially we assessed binding capacity of LK15 peptides for plasmid (p)DNA (SIN GFP, to express GFP on transfection), modified synthetic messenger RNA (modRNA) (Milenyi Biotech; to express GFP on transfection) and small-inhibitory (si)RNAs (labelled with FAM fluorophore to detect delivery). After optimising ratios we transfected iHMSCs with P21-LK15-8R and pDNA (10μg), modRNA (10μg) or siRNA (1μg) and visualised transfection by fluorescence microscopy (scale bar, 100μm). **(iii)** Quantification of GET-LK15 transfection of iHMSCs by flow cytometry (% transfection efficiency or relative fluorescence for siRNA) compared to lipofectamine (LIPO)2000 as a commercial standard. Error bars indicate s.d. **(c)** GET of Magnetic Nanoparticles. **(i)** Schematic of the P21-8R peptide synthesised and test magnetic nanoparticles (MNPs). We tested 250nm Nanomag-D dextran shell/iron oxide core MNPs and conjugated P21-8R peptide to surface COOH groups. **(ii)** MNPs are taken into NIH3t3 cells most efficiently in serum-free media (SFM; left panel). Light microscopy images of Prussian blue iron stained NIH3t3 cells treated with MNPs (50μg/ml) for twelve hours in standard media conditions (10% FCS) or SFM. Conjugation of P21-8R to MNPs significantly increases cellular uptake in both 10% FCS and SFM conditions (circular image is of entire well, scale bar, 100μm).

Figure 16 Ligand auto-labelling of intracellular and extracellular membrane-anchored HALO proteins. (a) Schematic of the HALO (intracellular) and LAMP2b-HALO (extracellular membrane-anchored) transgenic SIN lentivirus constructs. In LAMP2b-HALO the expressed protein is localised to the cell membrane by the signal peptide (SIG) which is cleaved and presented on the extracellular side of the cell membrane (b) NIH3t3 cells transgenic for intracellular HALO protein (NIH3t3-HALO) are only efficiently labelled by cell permeant ligands (HALO^{TAG} Oregon Green). NIH3t3 cells transgenic for membrane-anchored extracellular HALO protein (NIH3t3-LAMP2b) is efficiently labelled by both cell permeant and cell impermeant ligands (HALO^{TAG} Alexafluor⁴⁸⁸). Data shows flow cytometry of the NIH3t3 cell-lines incubated in ligand (1µM) for 15mins, followed by 3 media washes and a 15mins incubation to remove unbound ligand. Error bars indicate s.d. This provides an assay to assess intra- versus extracellular localisation of HALO proteins.

Figure 17 Ligand labelling of GET-HALO proteins demonstrates rapid cell binding and transduction. (a) Schematic of HALO proteins created (as described for mRFP in fig 1). (b) P21-HALO-8R and P21-HALO efficiently bind NIH3t3 cells but do not significantly internalise with a 1 hour incubation. (c) Bound P21-HALO-8R efficiently transduces into NIH3t3 cells with further incubation (1h-5h). Bound P21-HALO does not as efficiently enter cells and remains bound to cell membrane with further incubation. Data shows flow cytometry analyses of NIH3t3 cells treated with proteins (20µg/ml) for 1 hour followed direct ligand labelling (1h) or further incubation of 5 hours (1h-5h). Error bars indicate s.d.

Figure 18 pH-sensitivity of GET-mNectarine (mNect) proteins demonstrates rapid cell binding and transduction. (a) Schematic of HALO proteins created (as described for mRFP in fig 1). (b-c) GET-mNect or GET-mR proteins (20µg/ml) were transduced into NIH3t3 cells for 1h (to demonstrate membrane binding activity), 1h followed by a further 5h incubation without protein (1h-5h) (to demonstrate transduction activity) or 6h (to demonstrate sustained delivery). Flow cytometry was used to compare intensities of mNect and mR GET-proteins. Fluorescence signal from transducing mNect proteins (unlike mR versions) is rapidly lost after internalisation new to endosomal acidification and protein unfolding. Error bars indicate s.d. (d-f) GET-mNect proteins (20µg/ml) were

transduced into NIH3t3 cells for the same regimes but washed in DMEM at pH7.5 or pH5.5 before cytometry. Membrane localised mNect protein fluorescence is extinguished by pH5.5 but is retained at pH7.5 indicating at 1h incubations leave P21-mNect-8R external to cells, bound to membranes and not protected from pH-mediated unfolding. 1h-5h incubations demonstrate that P21-mNect-8R localisation is shifted and protected from pH-mediated unfolding demonstrating internalisation of the protein and protection by the cell membrane. Error bars indicate s.d.

Figure 19 GET protein must be delivered intracellularly to allow successful re-transduction (a) Scheme of testing the effect of re-transduction of GET proteins in NIH3t3 cells. Cells were pre-incubated in fresh media for 1 hour and transduced with P21-mR-8R (20µg/ml) for 1 hour. Cells were then either analysed for fluorescence or re-transduced with P21-mR-8R (20µg/ml) for a further 1 hour. This re-transduction was either immediate (0h) or with a 1-6 hour incubation between re-transduction before fluorescence analyses by flow cytometry. Immediate re-transduction is inhibited whereas >1 hour incubation between transductions allows the most efficient re-transduction of GET-protein. Error bars indicate s.d. * p<0.05.

Figure 20 GET is biocompatible in multiple clinically relevant cell types. Cell lines were transduced with P21-mR-8R at 20 or 200µg/ml over 24 hours and assessed by trypan blue for cell viability (cell lines were those described in Fig. 1 including rat aortic smooth muscle cells (rSMC) and neonatal cardiomyocytes (rCMs)). Viability remained high in all cell types for both concentrations tested. Error bars indicate s.d.

Figure 21 A: Prussian blue staining of Nanomag particles incubated with 3t3 cells for 24 hours, B: Iron assay results for the amount of iron per cell after the 24 hour incubation.

Figure 22 Graph showing NIH3T3, CGR-8 and HUES7 cells treated with mRFP conjugated peptides (20ug/ml) for 12h. Flow cytometry analysis was used to quantify fluorescence (relative fluorescence units (RFU)) of cells. Error bars indicate s.d, n=3.

Figure 23 Graph showing the increase in transduction of mRFP into cells by modified CPPs (HS-GAG binding domain mRFP 8R) over an unmodified CPP (mRFP 8R). NIH3T3, CGR-8 and HUES7 cells were treated with mRFP conjugated peptides (20ug/ml) for 12h. Error bars indicate s.d., n=3.

Figure 24 Efficient delivery of mRFP to cells via modified peptides shown to promote GET. Fluorescence microscopy images of NIH3T3, CGR8 and HUES-7 cells treated with P21 mRFP 8R, FGF2B mRFP 8R, FGF7B mRFP 8R and PDGF mRFP 8R peptides (20µg/ml) for twelve hours. Scale bar, 100µm.

Figure 25 Optimization of (+/-) charge ratio of P21 LK15 8R to pSIN GFP using YO-PRO-1 assay. Graph shows a decrease in % of fluorescence as P21 LK15 8R binds pSIN GFP. Error bars indicate s.d., n=3.

Figure 26 Examples of flow cytometry dot plots showing GFP expression of NIH3T3 cells following transfection with pSIN GFP for 6h. Following transfection, cells were fixed at a 48h time-point. Flow cytometry analysis was used to quantify % of GFP positive cells.

Figure 27 Transfection optimization of pSIN GFP into NIH3T3 cells by P21 LK15 8R peptide. Cells were treated with the optimum (+/-) charge ratio of P21 LK15 8R to pSIN GFP of 2:1, respectively. Optimization was carried out at varying transfection times (3, 6 and 24h) in (A) 10% serum transfection media (B) serum free transfection media. Following transfection, cells were fixed at a 48h time-point. Flow cytometry analysis was used to quantify % of GFP positive cells. Error bars indicate s.d., n=3.

Figure 28 Graph showing the optimization of the transfection of pSIN GFP into NIH3T3 cells by Lipofectamine2000, in serum free conditions. Flow cytometry analysis was used to quantify % of GFP positive cells. Error bars indicate s.d., n=3.

Figure 29 Transient immortalisation of human MSCs. A) The transient immortalisation process demonstrated to expand hMSCs through a proliferative

intermediate (Trans-ihMSCs) driven by GET or lipid-transfection delivery of RNA⁵. B) Proof-of-principle that the delivery promotes hMSC growth post-TERT E6/7 or SV40T RNA transfection. C) ihMSCs⁶ (immortalised by lentivirus expressing TERT & E6/7) remain karyotypically stable and euploid.

5

Figure 30 Optimisation of RNA transfection and cytotoxicity in hMSCs. A) The transient transfection of hMSCs is efficient but rapidly lost without serial transfection (bar = 10um). B) Flow cytometry showing a 3x serial transfections. 2x serial transfection is best to maintain expression and retain cell viability). Arrows show 'Yes' and 'No' for transfected and untransfected, respectively. C) Transfection remains high after 2 transfections (2x). D) Viability is rapidly lost beyond 2x serial transfections.

10

15

Figure 31 Transfection 'resets' cellular senescence and triggers cell cycle in aged human MSCs. A) SA- β galactosidase senescence assay shows P10 cells are mainly senescent, whereas Lentiviral-transduced ihMSCs are not senescent. Transfection of TE6/7 or SV40T RNA using the invention partially 'resets' the senescence of P10 hMSCs. (arrows show non-senescent cells. Bar = 20um) B) Quantification of senescence 'resetting' by the invention. C) Transfection partially triggers cells to enter cell-cycle (as a function of G2/M cell cycle analyses using PI).

20

Example 1

25 There is an unmet clinical need for high numbers of patient-specific cells for cell therapies. The solution is the use of transient immortalisation to expand the small cell population to numbers feasible for cell therapies.

Transgenic systems (like those used in genetic immortalisation for ihMSCs; Fig 2B) have been used for basic research and one example exists in cell therapies (CTX trial of immortalized neural cells for stroke treatment by ReNeuron, UK)⁷. Stable genetic modification has been used by several studies¹. However these cells are not clinically relevant. Transient immortalisation of clinically valuable cells to generate extensive cell numbers (without losing the primary cell phenotype) will be a step change in what is possible using hMSCs in cell therapies. There are many examples of other cell

35

therapies were large numbers of patient-matched (autologous) cells are required but there is presently no approach to produce them.

Many systems simply rely on conventional culture (Fig 2A), in which slow growing
5 cells are pushed to proliferate by media conditions with progressive slowing of growth
and eventual senescence. Most therapies cannot feasibly use patient-derived cells.
Therefore most approaches employ heterologous donor cells which require patient
immunosuppression and a reduction in efficacy and clinical outcome. Even if the
desired numbers of patient-specific cells can be produced then they have been
10 exhaustively manipulated over several weeks/months meaning that spontaneous
transformation could result leading to considerable safety concerns².

Immortalised hMSCs (ihMSCs) have been created by transgenically expressing human
telomerase (hTERT) and human papilloma virus E6/7 (HPV E6/7) genes³. These cells
15 grow rapidly, do not senesce and retain multipotent differentiation into bone, fat and
connective tissue (**Fig 1**). Importantly these should not form tumours and have normal
karyotype¹. These ihMSCs represent an unlimited source of material for transplant,
but will not be clinically adopted due to their permanent genetic modification with
hTERT/HPV oncogenes. With transient activity of these factors achieved without
20 delivery of DNA, these cells would have a transformative impact on hMSC therapeutic
use (**Fig 2**).

A peptide-mediated delivery system for RNA (**Fig 3 & 4**) can transiently deliver the
activity of any gene⁴. The delivery mechanism for this technology is termed
25 Glycosaminoglycan-binding Enhanced Transduction (GET) and is described and
demonstrated in international patent application No. PCT/GB2014/053764, the content
of which is incorporated by reference herein.

GET is used to deliver hTERT and HPV E6/7 activity without genetic modification to
30 hMSCs. This yields proliferative, karyotypically normal cells (trans-ihMSCs) which
can be expanded. Upon GET removal, cells return to their original phenotype but with
vastly amplified numbers (**Fig 2C**). This technology can therefore be directly applied
to clinical research allowing transformative patient-specific cell therapy approaches
that would not presently be achievable.

Example 2**GET intracellular delivery system**

Protein transduction domains (PTDs) are powerful non-genetic tools that allow intracellular delivery of conjugated cargoes to modify cell behaviour. Their use in biomedicine has been hampered by inefficient delivery to nuclear and cytoplasmic targets. This deficiency is overcome by a fusion protein that couples a membrane docking peptide to heparan sulfate glycosaminoglycans (GAGs) with a PTD (termed **GAG-binding Enhanced Transduction (GET)**).

This GET (**GAG-binding Enhanced Transduction**) system can deliver enzymes (Cre, neomycin phosphotransferase), transcription factors (NANOG, MYOD), antibodies, native proteins (Cytochrome-C), magnetic nanoparticles (MNPs) and nucleic acids (plasmid (p)DNA, modified (mod)RNA and siRNA) at efficiencies of up to two-orders of magnitude higher than previously reported in cell types considered hard to transduce, such as mouse embryonic stem cells (mESCs), human ESCs (hESCs), induced pluripotent stem cells (hiPSCs) and human mesenchymal stem cells (hMSCs).

Delivery of RNAs (**Fig 3 & 4**) using GET temporarily produces a proliferative phenotype in clinically-relevant cells which are hard and slow to expand to sufficient numbers for human cell therapies.

Immortalised hMSCs retain differentiation capacity, are non-tumorigenic and proliferate

Immortalised hMSCs (ihMSCs) were created by over-expressing oncogenes from lentiviruses in primary cells (TERT and HPV E6/7 or SV40T) (**Fig 1A**). These are robustly proliferating cell lines which also retain the differentiation of the primary hMSCs (**Fig 1B**). The technological advantages afforded by the GET system (which is non-toxic, can be used in serum-containing systems and does not affect cell proliferation) is used to immortalise primary hMSCs by delivering RNAs for the TERT, E6/7 or SV40T genes.

Modified Synthetic RNA production to regulate gene expression stoichiometrically

A major technological improvement to the approach of RNA transfection was detailed in a recent study⁴. RNA can be synthetically made using modified nucleotides and cap analogues which express well in human cells, are not cytotoxic and do not generate a cellular immune response (**Fig 3**). RNA is not stability inherited unlike integrated DNA and is more efficient at producing a translated protein product as it is translated in the cytosol rather than needing transcription in nuclei like delivered DNAs.

An important benefit of using RNA delivery verses DNA is that RNA does not integrate into the host cell genome and that it is rapidly turned-over meaning that protein-expression from it is transient. RNA can be delivered daily to create a situation of constant protein expression and simply ceasing the delivery results in no further protein-expression. Therefore the level and duration of the expressed protein's activity can tightly and stoichiometrically controlled.

15 **GET of immortalisation RNAs to transiently proliferate hMSCs:**

Creation of Tran-ihMSCs

In this example the efficient, non-toxic and serum-resistant GET transfection system was used to express immortalisation factors in primary hMSCs to promote their proliferation. This is temporary and once GET delivery is stopped, proliferation will return to normal levels but have yielded a significantly larger population of cells that could be used for therapies.

If this approach is indeed to be clinically useful then we must prove a number of attributes that cells have post-processing. They should return to normal primary cell behaviour and essentially be identical to the starting cell-type. They should not form tumours, they must still have the same differentiation capacity, they should not contain any proportion of genetically unstable or mutated cells. It is important that the process is an immortalisation which is reversed, and is not a transformative process. Given that ihMSCs genetically-expressing TERT E6/7 are not transformed, are euploid and do not form tumours in mice¹ this approach will yield similar attributes in the resulting cells.

35 References

1. Okamoto, T., Aoyama, T., Nakayama, T., Nakamata, T., Hosaka, T., Nishijo, K., Nakamura, T., Kiyono, T. & Toguchida, J. (2002). Clonal heterogeneity in differentiation potential of immortalised human mesenchymal stem cells. *Biochem. Biophys. Res. Com.* 295:354-361
- 5 2. Jin, H.J., Bae, Y.K., Kim, M., Kwon, S-J., Jeon, H.B., Choi, S.J., Kim, S.W., Yang, Y.S. Oh, W. & Chang, J. W. (2013). Comparative Analysis of Human Mesenchymal Stem Cells from Bone Marrow, Adipose Tissue, and Umbilical Cord Blood as sources of Cell Therapy. *IJMS*. 14:17986-18001.
- 10 3. Saeed, A., Francini, N., White, L., Dixon, J. E., Gould, T., Rashidi, H., Cheikh Al Ghanami, R., Hruschka, V., Redl, H., Saunders, B. R., Alexander, A. & Shakesheff, K. M. (2014). A Thermoresponsive and Magnetic Colloid for 3D Cell Expansion and Reconfiguration. *Advanced Materials*. 27(4):662-8
- 15 4. Warren, L., Manos, P. D., Ahfeldt, T., Loh, Y-H., Li, H., Lau, F., Ebina, W., Mandal, P. K., Smith, Z. D., Meissner, A., Daley, G. Q., Brack, A. S., Collins, J. J., Cowan, C., Schlaeger, T. M. & Rossi D. J. (2010). Highly efficient reprogramming to pluripotency and directed differentiation of human cells with synthetic mRNA. *Cell Stem Cell*. 7(5):618-630.
- 20 5. Dixon, J.E., Dick, E., Shakesheff, K.M. & Denning, C. (2011). Directed differentiation of human embryonic stem cells to interrogate the cardiac gene regulatory network. *Mol. Therapy*. 19(9):1695-703
- 25 6. Dixon, J.E., Shah, D.A., Rogers, C., Hall, S., Weston, N., McNally, D., Denning, C. & Shakesheff, K.M. (2014). Combined hydrogels that switch human pluripotent stem cells from self-renewal to differentiation. *PNAS*. 111(15):5580-5
- 30 7. CTX Neural Stem Cell Clinical trial. *ReNeuron*. <http://www.reneuron.com/clinical-trials>.

Example of the GET system

Results

Isolation of P21, a HBD that enhances PTD function through HS-GAG interaction

Several short peptides were screened which have been reported to interact with molecules known to be present on mESC, HESC or HiPSC membranes including integrins, CD markers and GAGs. Peptides were fused *N*-terminally to mRFP1, expressed, affinity purified and incubated with the three cell-types. Screening of 12 variants yielded one which clearly increased localisation of mRFP1-fluorescence (mR) to cells and their membranes, termed P21 (KRKKKGKGLGKKRDPCLRKYK (SEQ ID NO: 1)) (Fig 7). Interestingly this peptide also demonstrated transduction activity as evidenced by punctate intracellular fluorescence indicative of endosomal localisation (Fig 7b,c). P21 was derived from HB-EGF, which belongs to the EGF family of cytokines. HB-EGF shows a strong affinity to heparin and binds to the same receptor as EGF and TGF- α {Sakuma, 1997 #76}. The interaction of HB-EGF with cell surface HS-GAG is essential for its optimal binding to EGFR and for promoting its growth/migratory activity toward vascular smooth muscle cells {Higashiyama, 1993 #90}. Mutagenesis and protease digestion of recombinant HB-EGF, coupled with analyses using synthetic peptides and heparin, revealed the P21 sequence in the amino-terminal region of the soluble HB-EGF is responsible for its binding to heparin so is considered a typical HBD {Thompson, 1994 #77}. In addition, P21 also interacts with cell surface HS-GAG but not the EGFR which is mediated by other sequences in HB-EGF. Therefore, a short 21-residue peptide, P21, was isolated, which enhances the association of a fluorescent reporter to both mouse and human pluripotent stem cells.

P21-mediated binding of cell membranes was tested to determine if it could enhance PTD-mediated transduction of mR by combining both moieties in one molecule (Fig 7a). P21-mR-8R was cloned, expressed and purified, and its activity compared to P21-only (termed P21-mR) or 8R-only (mR-8R) proteins (Fig. 7a). Data demonstrated that the inclusion of both P21- and 8R- moieties synergized in the same protein to significantly enhance the fluorescence of all cell-lines tested. (Fig. 7b). Importantly mouse and human pluripotent stem cells (CGR-8, HUES7 and IPS2) and cardiomyocytes (HL1) only transduced efficiently with the inclusion of P21-tag, and both tags synergized to produce the high-levels of transduction similar to that seen in other cell lines (Fig. 7c). These motifs were also placed in tandem at *N*- and *C*-terminal of mRFP (P21 or 8R first; P21-8R-mR, 8R-P21-mR or mR-P21-8R, mR-8R-

P21) or switched their termini (i.e. 8R-mR-P21) with all variants demonstrating similar synergy and cell transducing behaviour (Fig 10) as seen for P21-mR-8R. Furthermore 8R was swapped for alternative well characterised PTDs (TAT, 8K and 8RQ; {El-Andaloussi, 2005 #36}) and showed that these also synergized with P21
5 (Fig 11).

By incubating cells with protein for different timings and including a post-culture period it could be efficiently distinguished between fluorescence signal produced at the cell surface with that internalised (Fig. 7d). Cells demonstrated membrane
10 localisation of fluorescence with short incubation times (1 hour), termed 1h. With this short incubation and a subsequent culture period (1 hour with a 5 hour post-culture), termed 1h-5h we observed near exclusive intracellular fluorescence indicating transduction. Using longer incubation times (6 hours), termed 6h, cells exhibited strong punctate peri-nuclear fluorescence indicative of endosomal-mediated
15 transduction. This synergistic delivery mechanism is described as GAG-binding enhanced transduction (GET) or otherwise Heparan-sulfate enhanced transduction domain (HETD)-mediated delivery.

20 **GET requires the presence of trypsin-sensitive and detergent-soluble cell membrane molecules**

To evaluate the mechanism of GET interaction and uptake by cells, a series of experiments were performed which were previously used to assess PTD. To assess which cell membrane components are required for both initial cell association and transduction by HETDs it was determined whether similar transduction would be
25 obtained by enzymatic depletion of cell membrane before transduction. Cells were pre-treated with proteolytic enzyme trypsin and tested cell transduction using the 1h-5h regime protocol. Enzymatic removal of cell-surface proteins potentially inhibited GET / HETD-mediated transduction (~8.4-fold; $p<0.05$) (Fig 7e). In contrast, non-enzymatic release of cells from the culture plastic using ionic cell
30 dissociation solution (CDS) did not alter HETD-mediated uptake.

Next it was tested if depletion of detergent-soluble cell-membrane molecules would also have a similar effect on transduction. Cells were pre-incubated in 0.1% (v/v) Triton X-100 and using the 1h-5h protocol a decrease (~2.2-fold; $p<0.05$) was
35 observed in GET (Fig 7f) without a decrease in viability. Therefore, it was

demonstrated that both protein and detergent soluble moieties on the cell membrane affect the efficacy of protein transduction through P21- and PTD- synergy in GET / HETD-transduction.

5 GET generates higher intracellular protein levels than Lentiviral transgenesis

Several studies have concluded that PTD-mediated transduction is sufficiently refined to allow the transport of biologically active cargos for clinical studies. These now include trials of cancer therapies {Gump, 2007 #39}, siRNAs {Meade, 2007 #37} and *in vivo* imaging technologies {Bullok, 2006 #79}. As well as the benefits of avoiding
 10 genomic modification, if PTD-mediated transduction is to be preferential to gene-therapy approaches it must achieve the delivery of high-levels of molecule, be amenable to control of protein levels over short time-frames and also allow cell-type specific delivery. The levels achieved in cells by PTD- or GET / HETD-delivery were compared to those achieved by efficient lentiviral transduction {Dick, 2011 #10} and
 15 exogenous expression of mRFP1 (with stable EF1 α -promoter driven) (Fig 12). To achieve this soluble protein was extracted from transduced cells and measured amounts by fluorometry (Fig 12a), or flow cytometry was used (Fig 12b). Using 6 hour incubations, mR-8R levels were several times lower ($\sim$ 3-fold; $p<0.05$) than that achieved by viral transgenesis even at the highest tested doses (200 μ g/ml). However
 20 P21-mR-8R levels under the same conditions were $\sim$ 16-fold higher ($p<0.001$) than transgenic cells. Importantly of the amount of P21-mR-8R protein incubated a significant proportion was recovered as soluble intracellular protein ($\sim 46 \pm 3.5 \mu$ g/200 μ g used; $\sim 23 \pm 1.7\%$ recovery). It is important to note that under these conditions transduced cells appear red/purple in colour under normal light,
 25 demonstrating the efficient enrichment of large quantities of HETD-tagged / GET proteins in cells.

The rate at which these proteins were concentrated in cells was investigated by measuring the depletion of fluorescence in media over the incubation period. Proteins
 30 were diluted (20 μ g/ml) and incubated with cells for 12 hours in serum-free conditions. 8R-tagged proteins were depleted by $\sim$ 12% in NIH3t3- and $\sim$ 3.5% in HUES7-incubations. This precisely mirrors flow cytometric data with 8R-proteins poorly transduced into HUES7 cells but at moderate levels in NIH3t3 cells (Fig 7). P21-tagged proteins were significantly depleted in both cell types ($\sim$ 37% and $\sim$ 25% in
 35 NIH3t3 and HUES7 cells, respectively; $p<0.05$) with HETD-proteins depleted from

media to the highest levels and majority of protein removed (~72 and ~66% in NIH3t3 and HUES7 cells, respectively; $p < 0.01$).

The time required to deplete half of the fluorescence ($T^{1/2}$) was determined, with P21-mR-8R requiring only ~9.4 hours, in comparison to mR-8R which required ~62 hours and untagged protein never achieving half-depletion even after 7 days. These data are corroborative with the cytometric data proving a rapid and efficient enrichment of exogenous GET-protein/HETD-protein into cells. Therefore, it was demonstrated that within a relatively short incubation period (6 hours) that a significant protein concentration can be achieved within cells. In less than a day, the majority of extracellular protein has been effectively internalised using GET delivery. This system will be amenable to precise regulation of protein stoichiometry, while avoiding the stochastic transgene expression variation and silencing of integrating vectors used in gene-therapy approaches.

15

GET enhances Cre-mediated genome modification

It was determined that HETDs bind rapidly to cell membranes through HS-GAGs and transduce efficiently into cells, but it was yet to be confirmed if the mode of uptake was through macropinocytosis as for PTDs. Also, what proportion of this protein escaped endosomes and may be considered successfully delivered was not assessed. Previous studies have avoided issues associated with direct measurement of fluorescent-tagged proteins (such as being unable to distinguish membrane, vesicle or functional cytosolic/nuclear protein) by assaying for the successful nuclear activity of Cre recombinase {Gump, 2010 #2}. This system was used to measure Cre-mediated recombination of a *loxP*-STOP-*loxP* (LSL) enhanced green fluorescent protein (eGFP) reporter gene in live NIH3t3 mouse fibroblast cells (NIH3t3: LSL-eGFP cells) as an indicator of cellular uptake (Fig 9a). This system is rigorous as activation of green fluorescence requires exogenous Cre protein to enter the cell, undergo nuclear-translocation and excise the LSL fragment of the transgene. This must occur in live cells and be non-toxic for the subsequent expression of eGFP. However, this process requires only one functional Cre recombinase molecule to be delivered to activate eGFP so does not allow the determination of the precise amount of cargo delivered. To overcome this issue Cre proteins were delivered at limiting dilutions for a short exposure time (1 hour) and the minimum dose required to activate green fluorescence after 48 hours (Fig 9c) was determined.

35

Transduction of NIH3t3: LSL-eGFP cells with SIN Cre lentiviruses to overexpress *Cre* transgenically led to near complete ($92\pm6\%$; $p<0.001$) activation of eGFP-expression in all cells confirming the utility of this system (Fig 9b). The benefits of the fluorescence system and delivered fluorescent-versions of Cre-recombinase protein were retained by purifying proteins with *mRFP1* cloned to the *N*-terminal of *Cre* cDNA. Treatment of NIH3t3: LSL-eGFP cells with mR-Cre (mRFP fused to Cre) resulted in recombination and eGFP activation ($22.1\pm6.7\%$; $p<0.05$) at the highest doses ($500\mu\text{g/ml}$) (Fig 9d). eGFP activation was inhibited at 4°C and negatively affected by serum concentration-dependently. mR-Cre-8R demonstrated that the 8R PTD enhanced functional delivery of Cre (~ 22 -fold; $p<0.01$).

The GET-protein / HETD-protein, P21-mR-Cre-8R, required as little as one minute incubation with cells at a low dose ($1\mu\text{g/ml}$) to elicit recombination ($4.3\pm2.5\%$; $p<0.05$) confirming that binding and internalization is an efficient and rapid process. For a moderate dose ($10\mu\text{g/ml}$) GET/ HETD-transduction achieved a functional delivery ~ 15 -fold ($p<0.01$) above PTD only levels and completely recombined all NIH3t3: LSL-eGFP cells (Fig 9d,e). Importantly this activity was ~ 340 -fold better than mR-Cre ($p<0.001$). Heparinase III, free-heparin and serum-free experiments were repeated using the Cre recombination system. It was confirmed that heparinase III pre-treatment reduced recombination to basal-levels and that media serum plays a role in replenishing cell membrane GAGs depleted by heparinase. Overall these data correlate well with the fluorescence delivery conclusions and show synergy between P21- and PTD- moieties to achieve significant increases in functional transduction of protein cargo.

GET protein enters cells by lipid raft macropinocytosis

Previously it has been shown that PTD-mediated internalization is via macropinocytosis rather than other endocytotic pathways {Wadia, 2004 #25}. It was next determined whether the cellular uptake of GET-proteins / HETD-proteins occurs through a specific endocytotic pathway employing the Cre assay system. Removal of cholesterol from the cell plasma membrane disrupts several lipid raft-mediated endocytotic pathways, including caveolae and macropinocytosis {Anderson, 1998 #29; Nichols, 2001 #30; Liu, 2002 #28}. NIH3t3: LSL-eGFP cells treated with methyl- β -cyclodextrin and nystatin were used to deplete or sequester cholesterol, respectively,

then transduced HETD-tagged proteins. Both methyl- β -cyclodextrin (Fig 13a) and nystatin (Fig 13b) disruption of lipid rafts resulted in a dose-dependent inhibition of functional delivery. These data demonstrates that GET / HETD-mediated transduction specifically requires lipid raft-mediated endocytosis.

5

Macropinocytosis is a rapid, lipid raft-dependent and receptor-independent form of endocytosis which requires actin membrane protrusions that envelope into vesicles termed macropinosomes {Nichols, 2001 #30; Liu, 2002 #28; Conner, 2003 #22}. To confirm macropinocytosis was indeed the endocytotic mechanism of HETD-mediated transduction cells were pre-treated with macropinocytosis-inhibiting compounds (Fig. 11a,b). Amiloride is a specific inhibitor of the Na^+/H^+ exchange required for macropinocytosis {West, 1989 #31}. Cytochalasin D is an inhibitor of F-actin elongation which is required for macropinosome-linked membrane protrusions {Sampath, 1991 #32}. Amiloride and Cytochalasin D did not disrupt cell binding of HETD-proteins but resulted in a dose-dependent reduction of functional transduction into cells (Fig 13c and 13d, respectively). These data confirm that P21 enhances the macropinocytotic pathway used by PTD to internalize cargo molecules.

10
15

GET-delivery promotes general macropinocytosis

The effects of GET-binding / HETD-binding on the induction of macropinocytosis was investigated. PTD-mediated transduction has previously been shown to promote the uptake of other proteins by an increase in the overall level of macropinocytosis {Wadia, 2004 #25}. Cells were incubated with a fluorescent fluid-phase macropinocytotic maker, FITC-labeled 70kDa neutral dextran, in combination with GET / HETD protein, P21-mR-8R (Fig. 14). Other studies {Oliver, 1984 #24; Araki, 1996 #23; Wadia, 2004 #25} have demonstrated that neutral dextrans are taken up by amiloride-sensitive macropinocytosis. P21-mR-8R induced a significant dose dependant increase in fluid-phase dextran uptake over steady-state control levels. PTD-tagged versus GET / HETD-tagged activity to stimulate this macropinocytosis was compared. P21-mR-8R enhanced FITC-dextran uptake ~ 2.5 -fold ($p < 0.05$) over the stimulation achieved by the same concentration of mR-8R demonstrating that engagement with HS-GAG through P21 and its subsequent effect on PTD-mediated transduction stimulates macropinocytotic uptake.

20
25
30

Significant amounts of GET-delivered protein is trapped in Endosomes which can be efficiently released with Chloroquine

The majority of PTD-delivered molecules remain trapped in macropinosomes even after further incubation indicating that release from these vesicles is inefficient. If
5 fine-tuned and graded amounts of delivery are to be controlled then it would be beneficial if the majority of internalized protein could be considered as functional. Cells were treated with chloroquine, an ion-transporting ATPase inhibitor that disrupts endosomes by preventing their acidification {Seglen, 1979 #33} (Fig. 13e). Similar doses have been demonstrated to significantly improve the functional delivery of
10 PTD-delivered proteins {Wadia, 2004 #25}. Sub-cytotoxic doses of Chloroquine (100 μ M) resulted in a marked increase (95.3 $\pm$ 4.8-fold; p <0.001) in functional HETD-tagged / GET protein delivery at a sub-threshold dose (0.1 μ g/ml) indicating that this is point in the pathway is still a major issue to resolve for GET/HETD medicinal application. Nevertheless the GET-delivery / HETD-delivery system was so efficient
15 that with chloroquine treatment we achieved significant and measureable levels of recombination (4.8 $\pm$ 2.9%; p <0.05) with short (1 hour) incubations of >10pg/ml (Fig 13f). Combination of GET / HETD-delivery efficiency with endosomal-escape technologies may therefore allow precise and temporally controlled amounts of cargo function in cells.

20

GET-mediated internalisation is efficient after cell membrane association

Even for incubations using low amounts of GET-protein functional quantities of protein activity were observed within cells. However, to categorically and stringently prove that most GET protein was indeed efficiently internalising a series of analyses
25 was conducted using reporters that are responsive to their cellular or extracellular localisation. HALO (Halo^{Tag}) was used, which is a self-labelling protein derived from DhaA²⁹. HALO rapidly forms a covalent attachment to synthetic chloroalkane-based ligands; with cell permeant and impermeant ligands available. Intra- versus extracellular labelling of HALO was confirmed using transgenic over-expression of
30 untagged HALO (for intracellular) and LAMP2b-HALO which is presented on the external cell membrane (for extracellular) and labeling with cell permeant (HALO^{TAG} Oregon Green) or impermeant (HALO^{TAG} Alexafluor⁴⁸⁸) ligands (Fig 16). GET-HALO proteins were constructed and recombinantly expressed (Fig 17a) and delivered them to cells testing the the internalisation by sensitivity to labelling with the cell
35 impermeant ligand. One hour incubation demonstrated that GET-proteins remained

mainly extracellularly localised and attached to the cell membrane (Fig 17b). However, with further incubation (1h exposure with 5h further incubation; 1h-5h) GET-protein (P21-HALO-8R) is effectively internalised (remaining cell permeant ligand-sensitive but impermeant ligand-insensitive) (Fig 17c). These experiments were repeated using the mNectarine (mNect) variant of RFP which is pH-sensitive and loses almost all fluorescence in environments $< \text{pH}6$ ³⁰ (Fig 18). In agreement with HALO transduction, mNect remained mostly membrane localised after 1 h and its fluorescence sensitive to acidic pH media incubation (pH5.5) (Fig 18 d). After further incubation post-delivery (1h-5h) GET-mNect fluorescence was no longer sensitive to extracellular pH (Fig 18e); however interestingly the absolute fluorescence levels were significantly decreased when compared to GET-mR, presumably due to the internal pH change the protein is experiencing during endosomal acidification¹. These data demonstrates that GET protein membranes association is rapid and transduction is efficient post-cell binding. It was hypothesised that membrane clearance of GET-protein could be a rate-limiting step in the delivery process and this was tested by undertaking multiple transductions (1h) of GET-mR varying the time between transductions (Fig 19a). Indeed re-transduction directly after the initial transduction decreases the effectiveness of the second transduction however as little as 1 h between new transductions is required to obtain a maximal efficiency of re-transduction (Fig 19b).

Experimental Procedures

Expression and Purification of Recombinant Proteins

cDNA was obtained for *mRFP1* (*mR*) as a kind gift from Prof. R. Y. Tsien (University of California, USA) {Campbell, 2002 #12}. *8R*, *TAT*, *8K*, *8RQ*, *P21*, *Cre*, *NANOG*, *MYOD* and *NEO* cDNAs were synthesized *de novo* (Eurofins MWG Operon). cDNAs were cloned into the pGEX6-P1 expression vector (Novagen) to create in-frame fusions and expressed proteins in BL21 (DE21) pLysS *Escherichia coli* (Novagen). Exponentially growing LB cultures ($\text{OD}_{600} = 0.4$) shaken at 220rpm at 37°C were induced using 1 mM IPTG for 24 hours at 25°C. Bacterial pellets were lysed and sonicated (7 amplitudes, 1 minute, 5 times) in 1X STE extraction buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 1 mM EDTA containing 1mM DTT, 0.2 mg/ml lysozyme, and 1X protease inhibitor cocktail). Insoluble protein was retrieved using the Rapid GST inclusion body solubilisation and renaturation kit (AKR-110; Cell Biolabs, Inc., San Diego, CA). Recombinant proteins were purified by affinity chromatography using

Glutathione-Sepharose resin (GE Healthcare). GST-tags were removed and eluted from resin by PreScissionTM Protease cleavage (GE healthcare) in 1X cleavage buffer (50 mM Tris-HCl pH 7.0, 150 mM NaCl, 1 mM EDTA and 1 mM DTT). Protein concentration was determined using a BCA-based protein assay (BioRad) with absorbance measured at 595nm using recombinant mR protein as a standard. Integrity and full-length protein expression was confirmed by SDS-PAGE. The fluorescence of recombinant proteins (excitation: 584 nm; emission: 607 nm) was determined with all preparations <10% intensity difference between samples (fluorescence/ μ g). Standards and samples were analysed using the TECAN infinite 200PRO multimode reader. Aliquots were stored at -80°C.

Cell Culture

NIH3T3 mouse fibroblast cells, HEK293T human embryonic kidney cells, C2C12 mouse myoblast cells, iHMSC immortalised human mesenchymal stem cells (created as described {Okamoto, 2002 #8}) and MEF murine embryonic fibroblasts (harvested as described {Anderson, 2007 #9}) were maintained in DMEM with 10% (v/v) fetal calf serum (FCS; Sigma) media supplemented with 2mM L-glutamine, 100units/ml penicillin and 100 μ g/ml streptomycin). CGR-8 mouse embryonic stem cells (mESCs) and *EXT1*^{-/-} mESCs (a kind gift from Dr. D. E. Wells, University of Houston, USA; {Lin, 2000 #78}) were maintained in DMEM, 20% (v/v) FCS, 1000units/ml leukaemia inhibitory factor (LIF), non-essential amino acids, 100 μ M β -mecaptoethanol (Sigma) 2mM L-glutamine, 100units/ml penicillin and 100 μ g/ml streptomycin). HL1 mouse cardiomyocyte cells were maintained as described {Claycomb, 1998 #7}. HUES7 human embryonic and IPS2 induced pluripotent stem cells were cultured as previously described {Dick, 2011 #10}. HUES7fib human fibroblasts derived from HUES7 cells were generated and cultured as previously described {Dick, 2011 #10}. All cells were cultured at 37°C under 5% CO₂.

Flow Cytometry and Microscopy

For flow cytometry, cells were trypsinized (unless otherwise stated), fixed in 4% (w/v) PFA, resuspended in PBS (pH7.5) and analysed on a MoFloTM DP (DAKO) Flow Cytometer using a 488nm green laser. (50,000 cells; gated on live cells by forward/side scatter). Median fluorescence was used for statistical analyses with background from unlabelled/transduced cells subtracted and values taken as ratios to the experimental control. Data shown are three experiments of triplicate samples. For

microscopy, cultures were rinsed twice with PBS and imaged with inverted fluorescence microscope (Nikon Eclipse TS100).

Fluorescence Delivery Assay

5 For testing multiple cell lines we plated 2×10^5 cells/well (in 12-well plates) onto the surface relevant to the tested cell line, attached cells for 2 hours and transduced with recombinant proteins in cell-type specific growth media. After transduction cells were washed with PBS, trypsinized and fixed in 4% PFA for flow cytometry. For membrane localization, intracellular localisation or both we plated cells as above, but cultures
10 pre-incubated in serum-free media for 1 hour before transduction. Membrane localization to assess cell interaction was achieved by a short transduction of 1 hour in serum-free media. Intracellular localization to assess transduction efficiency was achieved by a short transduction of 1 hour followed by 5 hour incubation in serum-free media only. Cell association (membrane and intracellular levels) were assessed by
15 transducing cells for 6 hours in serum-free medium. For flow cytometry cells were trypsinized, washed and fixed in 4% PFA and for microscopy cells were imaged live after washing in PBS. For trypsin depletion of cell-surface proteins, cells were treated with trypsin/EDTA (Invitrogen) or EDTA-based cell dissociation solution (CDS) (Sigma) for 15 minutes at 37 °C, followed by washes with PBS and 1X soybean
20 trypsin inhibitor (10 mg/ml in PBS; Sigma). Cells were then treated with proteins for 1 hour at 37 °C in serum-free medium. For detergent depletion of cell membranes, cells were treated with PBS (pH7.5) containing 0.1 % (v/v) Triton-X100 (Tx100) for 10 minutes at 37 °C, followed by washes with PBS. Cells were then treated with proteins for 1 hour at 37 °C in serum-free medium. For GAG-treatment cells were pre-
25 treated with GAGs in DMEM without serum before transduction and were included in the transduction media. This included heparin and chondroitin sulphate A, B and C (0-50 µg/ml).

Total delivered Protein Analyses

30 5×10^6 NIH3t3 cells were plated (in T25 flasks), pre-incubated cells in serum-free DMEM for 1 hour, and transduced them with mR-8R or P21-mR-8R (0-200µg/ml; 1 ml volume) in serum-free DMEM for 6 hours. NIH3t3 cells transduced with SIN-mR lentiviruses were used as a control for the levels achieved by transgenic systems {Dixon, 2011 #15; Dick, 2011 #10}. Cells were harvested by trypsinization, fixed
35 in 4% PFA for flow cytometry or washed several times in cold PBS with soluble

protein extracted in cold HKM buffer (20mM HEPES, pH 7.5, 5mM KCl, 0.5 mM MgCl₂ and 0.5 mM DTT with 1X complete EDTA-free protease inhibitor cocktail) for fluorometry {Medina, 2000 #14}. Extracts were sonicated, centrifuged and NaCl added to yield a final concentration of 100mM prior to analyses. Fluorometry was used to compare soluble extracts with purified mRFP protein diluted in HKM buffer with 100mM NaCl as standards. Flow cytometry was used to assess total delivered protein in intact cells.

Media depletion Assessment

2 x 10⁶ NIH3t3 cells or HUES7 HESCs were plated (in 6-well plates), pre-incubated cells in serum-free DMEM for 1 hour, and transduced with recombinant proteins (20µg/ml; 1 ml volume) in serum-free DMEM for 12 hours. Media was harvested and fluorometry was used to compare the remaining fluorescence in media verses that before cell-incubation. Fluorescence of media pre-incubation was assigned as 100% fluorescence units and background of serum-free media subtracted.

Heparin-binding assay, Heparinase treatment and depletion of P21-binding molecules from Serum

For Heparin binding activity we incubated 1ml of recombinant proteins (20µg/ml) in DMEM with 50µl of PBS-washed Heparin-sepharose beads (Sigma) for 1 hour at 37°C shaking at 100rpm. Media pre- and post-incubation was compared by fluorometry. For Heparinase treatment, we plated NIH3t3 cells at 2 x 10⁵/well (in 12-well plates) and were pre-incubated in serum-free media for 1 hour with Heparinase III (0-1 U/ml) or Heparin (0-50µg/ml). Cells were then washed and transduced with mR or P21-mR-8R (20µg/ml in serum-free media or media with different FCS concentrations) containing Heparinase III or Heparin for 12 hours. FCS was depleted of P21-binding material by affinity chromatography. This was achieved by incubating 50 ml FCS with 2 ml Glutathione-Sepharose resin (GE Healthcare) pre-absorbed with GST-P21 protein expressed in *Escherichia coli*.

Macropinocytosis Assessment

To measure the effects of protein transduction on general macropinocytosis, cells were incubated with 100µg/ml FITC-70kDa neutral dextran (Sigma), along with different recombinant proteins (0-10µg/ml) for 1 hour at 4°C or 37°C. Cells were trypsinized and washed in PBS before analyses by flow cytometry.

Cre Recombination Assay

To measure Cre Recombinase activity the NIH3t3: LSL-eGFP cell line was created using the pZ/EG plasmid transfection and G-418 selection {Novak, 2000 #6}. To confirm Cre activity efficiently led to recombination and eGFP activation cells were transduced with SIN-Cre lentiviruses (as described in Dixon et al. 2011) and >95% of cells were confirmed eGFP-positive 48 hours post-transduction. 2×10^5 cells/well were plated (in 12-well plates), pre-incubated them in DMEM without serum for 1 hour and treated with Cre proteins (0-500 $\mu\text{g/ml}$) in DMEM without serum. After the Cre incubation cells were trypsinized, replated into complete media and incubated for 2 days. Cells were pre-treated with drugs for the stated time-period in DMEM without serum, were included in Cre-transduction medium and were added after replating. Pre-treatments included: heparin (0-50 $\mu\text{g/ml}$), chondroitin sulphate A, B and C (0-50 $\mu\text{g/ml}$), chloroquine (0-100 μM), cytochalasin-D (0-10 μM), amiloride (0-5 mM), methyl- β -cyclodextrin (0-5mM), and nystatin (0-50 $\mu\text{g/ml}$). After incubations cell were trypsinized, washed, fixed in 4% PFA and % recombined cells was determined by flow cytometry. For mR-Cre-8R and P21-mR-Cre-8R comparisons concentrations of 100 $\mu\text{g/ml}$ and 10 $\mu\text{g/ml}$ were used, respectively and data was expressed as % maximum recombination (i.e. the % relative to the maximum recombination achieved at the stated dose of Cre).

Antibody, Nucleic acid and Nanoparticle delivery

Biotinylated-Goat anti-Rabbit and FITC-Rabbit anti-mouse antibodies (Sigma), pSIN-GFP (Dixon et al. 2014), modified nucleotide RNA (modRNA) for GFP (Milenyi Biotech) and FAM-labelled siRNA against GAPDH (Sigma), and nanomag-D (250nm) (MircoMod) were complexed with GET-proteins or -peptides and added to cells. For antibodies complexes were allowed to form in growth media for 20mins before cell addition. For nucleic acids a 2:1 peptide:nucleic acid charge ratio was used for complexation. GET- or LIPO2000 (lipofectamine 2000; Invitrogen) transfection used 10 μg or 1 μg nucleic acid per transfection of 100,000 hMSCs in 12 well plates. GET-peptide substituted LIPO2000 following the exact manufacturer's instructions. For MNPs, a final concentration of 25 μM peptide was used in an EDAC/NHS reaction using 2mg MNPs according to manufacturer's instructions. Prussian blue was carried out using potassium ferrocyanide (2.5% w/v) in 2.5% w/v HCl.

Statistical Analysis

Statistical comparisons were carried out using the GraphPad Prism software package. Comparisons were made using Tukey-Kramer analysis of variance (ANOVA). Results were considered significant if $p < 0.05$.

5

References:

1. Gump JM & Dowdy SF (2007) TAT transduction: the molecular mechanism and therapeutic prospects. *Trends in molecular medicine* 13(10):443-448.
2. El-Andaloussi S, Holm T, & Langel U (2005) Cell-penetrating peptides: Mechanisms and applications. *Curr Pharm Design* 11(28):3597-3611.
3. Goun EA, Pillow TH, Jones LR, Rothbard JB, & Wender PA (2006) Molecular transporters: Synthesis of oligoguanidinium transporters and their application to drug delivery and real-time imaging. *Chembiochem* 7(10):1497-1515.
4. Meade BR & Dowdy SF (2007) Exogenous siRNA delivery using peptide transduction domains/cell penetrating peptides. *Adv Drug Deliver Rev* 59(2-3):134-140.
5. Fischer R, Fotin-Mleczek M, Hufnagel H, & Brock R (2005) Break on through to the other side - Biophysics and cell biology shed light on cell-penetrating peptides. *Chembiochem* 6(12):2126-2142.
6. Nakase I, Takeuchi T, Tanaka G, & Futaki S (2008) Methodological and cellular aspects that govern the internalization mechanisms of arginine-rich cell-penetrating peptides. *Adv Drug Deliver Rev* 60(4-5):598-607.
7. Heitz F, Morris MC, & Divita G (2009) Twenty years of cell-penetrating peptides: from molecular mechanisms to therapeutics. *Brit J Pharmacol* 157(2):195-206.
8. Gump JM, June RK, & Dowdy SF (2010) Revised Role of Glycosaminoglycans in TAT Protein Transduction Domain-mediated Cellular Transduction. *J Biol Chem* 285(2):1500-1507.
9. Norbury CC, Hewlett LJ, Prescott AR, Shastri N, & Watts C (1995) Class I MHC presentation of exogenous soluble antigen via macropinocytosis in bone marrow macrophages. *Immunity* 3(6):783-791.

10

15

20

25

30

10. Meier O, *et al.* (2002) Adenovirus triggers macropinocytosis and endosomal leakage together with its clathrin-mediated uptake. *J Cell Biol* 158(6):1119-1131.
11. Conner SD & Schmid SL (2003) Regulated portals of entry into the cell. *Nature* 422(6927):37-44.
12. Wadia JS, Stan RV, & Dowdy SF (2004) Transducible TAT-HA fusogenic peptide enhances escape of TAT-fusion proteins after lipid raft macropinocytosis. *Nat Med* 10(3):310-315.
13. Skehel JJ, Cross K, Steinhauer D, & Wiley DC (2001) Influenza fusion peptides. *Biochem Soc T* 29:623-626.
14. Han X, Bushweller JH, Cafiso DS, & Tamm LK (2001) Membrane structure and fusion-triggering conformational change of the fusion domain from influenza hemagglutinin. *Nat Struct Biol* 8(8):715-720.
15. Sakuma T, Higashiyama S, Hosoe S, Hayashi S, & Taniguchi N (1997) CD9 antigen interacts with heparin-binding EGF-like growth factor through its heparin-binding domain. *Journal of biochemistry* 122(2):474-480.
16. Higashiyama S, Abraham JA, & Klagsbrun M (1993) Heparin-Binding Egf-Like Growth-Factor Stimulation of Smooth-Muscle Cell-Migration - Dependence on Interactions with Cell-Surface Heparan-Sulfate. *J Cell Biol* 122(4):933-940.
17. Thompson SA, *et al.* (1994) Characterization of Sequences within Heparin-Binding Egf-Like Growth-Factor That Mediate Interaction with Heparin. *J Biol Chem* 269(4):2541-2549.
18. Kaplan IM, Wadia JS, & Dowdy SF (2005) Cationic TAT peptide transduction domain enters cells by macropinocytosis (vol 102, pg 247, 2005). *J Control Release* 107(3):571-572.
19. Lawrence R, Lu H, Rosenberg RD, Esko JD, & Zhang LJ (2008) Disaccharide structure code for the easy representation of constituent oligosaccharides from glycosaminoglycans. *Nat Methods* 5(4):291-292.
20. Lin X, *et al.* (2000) Disruption of gastrulation and heparan sulfate biosynthesis in EXT1-deficient mice. *Dev Biol* 224(2):299-311.
21. Dick E, Matsa E, Young LE, Darling D, & Denning C (2011) Faster generation of hiPSCs by coupling high-titer lentivirus and column-based positive selection. *Nat Protoc* 6(6):701-714.

22. Anderson RGW (1998) The caveolae membrane system. *Annu Rev Biochem* 67:199-225.
23. Nichols BJ & Lippincott-Schwartz J (2001) Endocytosis without clathrin coats. *Trends Cell Biol* 11(10):406-412.
- 5 24. Liu NQ, *et al.* (2002) Human immunodeficiency virus type 1 enters brain microvascular endothelia by macropinocytosis dependent on lipid rafts and the mitogen-activated protein kinase signaling pathway. *J Virol* 76(13):6689-6700.
25. West MA, Bretscher MS, & Watts C (1989) Distinct Endocytotic Pathways in
10 Epidermal Growth Factor-Stimulated Human Carcinoma A431 Cells. *J Cell Biol* 109(6):2731-2739.
26. Sampath P & Pollard TD (1991) Effects of Cytochalasin, Phalloidin, and Ph on the Elongation of Actin-Filaments. *Biochemistry-Us* 30(7):1973-1980.
27. Oliver JM, Berlin RD, & Davis BH (1984) Use of Horseradish-Peroxidase and
15 Fluorescent Dextran to Study Fluid Pinocytosis in Leukocytes. *Method Enzymol* 108:336-347.
28. Araki N, Johnson MT, & Swanson JA (1996) A role for phosphoinositide 3-kinase in the completion of macropinocytosis and phagocytosis by macrophages. *J Cell Biol* 135(5):1249-1260.
- 20 29. Seglen PO, Grinde B, & Solheim AE (1979) Inhibition of the Lysosomal Pathway of Protein-Degradation in Isolated Rat Hepatocytes by Ammonia, Methylamine, Chloroquine and Leupeptin. *Eur J Biochem* 95(2):215-225.
30. Eustice DC & Wilhelm JM (1984) Mechanisms of Action of Aminoglycoside Antibiotics in Eukaryotic Protein-Synthesis. *Antimicrob Agents Ch* 26(1):53-60.
- 25 31. Yu JY, Chau KF, Vodyanik MA, Jiang JL, & Jiang Y (2011) Efficient Feeder-Free Episomal Reprogramming with Small Molecules. *Plos One* 6(3).
32. Warren L, *et al.* (2010) Highly Efficient Reprogramming to Pluripotency and Directed Differentiation of Human Cells with Synthetic Modified mRNA. *Cell Stem Cell* 7(5):618-630.
- 30 33. Kim D, *et al.* (2009) Generation of human induced pluripotent stem cells by direct delivery of reprogramming proteins. *Cell Stem Cell* 4(6):472-476.
34. Zhou HY, *et al.* (2009) Generation of Induced Pluripotent Stem Cells Using Recombinant Proteins (vol 4, pg 381, 2009). *Cell Stem Cell* 4(6):581-581.

35. Takahashi K, *et al.* (2007) Induction of pluripotent stem cells from adult human fibroblasts by defined factors. *Cell* 131(5):861-872.
36. Dixon JE, *et al.* (2010) Axolotl Nanog activity in mouse embryonic stem cells demonstrates that ground state pluripotency is conserved from urodele
5 amphibians to mammals. *Development* 137(18):2973-2980.
37. Chambers I, *et al.* (2003) Functional expression cloning of Nanog, a pluripotency sustaining factor in embryonic stem cells. *Cell* 113(5):643-655.
38. Robinton DA & Daley GQ (2012) The promise of induced pluripotent stem cells in research and therapy. *Nature* 481(7381):295-305.
- 10 39. Do Kwon Y, *et al.* (2005) Cellular manipulation of human embryonic stem cells by TAT-PDX1 protein transduction. *Mol Ther* 12(1):28-32.
40. Hidema S, Tonomura Y, Date S, & Nishimori K (2012) Effects of protein transduction with intact myogenic transcription factors tagged with HIV-1 Tat-PTD (T-PTD) on myogenic differentiation of mouse primary cells. *J Biosci Bioeng* 113(1):5-11.
15
41. Liang QL, Mo ZY, Li XF, Wang XX, & Li RM (2013) Pdx1 protein induces human embryonic stem cells into the pancreatic endocrine lineage. *Cell Biol Int* 37(1):2-10.
42. Bichsel C, *et al.* (2013) Direct Reprogramming of Fibroblasts to Myocytes via Bacterial Injection of MyoD Protein. *Cell Reprogram* 15(2):117-125.
20
43. Chan EM, *et al.* (2009) Live cell imaging distinguishes bona fide human iPS cells from partially reprogrammed cells. *Nat Biotechnol* 27(11):1033-U1100.
44. Smith KP, Luong MX, & Stein GS (2009) Pluripotency: Toward a Gold Standard for Human ES and iPS Cells. *J Cell Physiol* 220(1):21-29.
- 25 45. Burridge PW, *et al.* (2011) A Universal System for Highly Efficient Cardiac Differentiation of Human Induced Pluripotent Stem Cells That Eliminates Interline Variability. *Plos One* 6(4).
46. Campbell RE, *et al.* (2002) A monomeric red fluorescent protein. *Proceedings of the National Academy of Sciences of the United States of America* 99(12):7877-7882.
30
47. Okamoto T, *et al.* (2002) Clonal heterogeneity in differentiation potential of immortalized human mesenchymal stem cells. *Biochem Bioph Res Co* 295(2):354-361.

48. Anderson D, *et al.* (2007) Transgenic enrichment of cardiomyocytes from human embryonic stem cells. *Mol Ther* 15(11):2027-2036.
49. Claycomb WC, *et al.* (1998) HL-1 cells: A cardiac muscle cell line that contracts and retains phenotypic characteristics of the adult cardiomyocyte. *Proceedings of the National Academy of Sciences of the United States of America* 95(6):2979-2984.
50. Dixon JE, Dick E, Rajamohan D, Shakesheff KM, & Denning C (2011) Directed differentiation of human embryonic stem cells to interrogate the cardiac gene regulatory network. *Mol Ther* 19(9):1695-1703.
51. Medina D, Moskowitz N, Khan S, Christopher S, & Germino J (2000) Rapid purification of protein complexes from mammalian cells. *Nucleic Acids Res* 28(12).
52. Novak A, Guo CY, Yang WY, Nagy A, & Lobe CG (2000) Z/EG, a double reporter mouse line that expresses enhanced green fluorescent protein upon Cre-mediated excision. *Genesis* 28(3-4):147-155.
53. Bayoussef Z, Dixon JE, Stolnik S, & Shakesheff KM (2012) Aggregation promotes cell viability, proliferation, and differentiation in an in vitro model of injection cell therapy. *J Tissue Eng Regen M* 6(10):e61-e73.

20 **Improving the Efficiency of Iron Oxide Nanoparticle Uptake Using Cell Penetrating Peptides**

Background

Superparamagnetic Iron Oxide nanoparticles (SPIONS) are small highly magnetised particles consisting of an iron oxide core and surface coating. SPIONS have been clinically approved for use in MRI contrast agents¹, and are currently being researched for use in targeted drug delivery², hyperthermia treatment and cell labelling³. SPIONS have been approved for uses in MRI contrast agents and commercially available products include Lumiren, Resivist and Feridex.¹

Applications of SPIONS require an adequate concentration being internalised into cells and without the required targeting of nanoparticles it can lead to an inefficient outcome. The efficiency of cell internalisation can depend on the size, coating and additional ligands to name a few⁴. Literature shows that without the attachment of

internalisation agents researchers are achieving a range of 15-30 pg of iron per cell^{5,6}. The functional groups on nanoparticle coatings can be exploited to target increase cell internalisation by attaching monoclonal antibodies, cell penetrating peptides and small molecules as internalisation agents.⁷

5

A currently researched cell penetrating peptide is Arg-Gly-Asp (RGD). RGD was designed to target the $\alpha v \beta 3$ integrin⁸. The integrin can be found predominantly on cancer cells, so can also be used as a targeting peptide. Research found that the RGD peptide increased nanoparticle uptake by 50%.⁹

10

The following study focuses on a cell penetrating peptide of the invention herein, in particular P218R. The peptide has two domains, P21 binds to the heparan sulphate (HS) on the cell membrane and the 8R aids in the transduction. The aim of the study was to identify the efficiency of the P218R and to investigate its mechanism.

15

Materials and Methods

Nanoparticle labelling

A 31 mM EDAC with 0.1 M NHS dissolved in 0.5M MES buffer was added to Nanomag-D (250 nm) particles in a 1:5 ratio respectively and mixed for 1 hour. The particles are then washed in a 0.1M MES buffer and 0.2 $\mu\text{g}/\mu\text{l}$ of the required labelling agent dissolved in the same buffer was added to give a 1:1 ratio of labelling solution and nanoparticles, an aliquot of the labelling solution was kept for testing labelling efficiency. The solution is then continuously mixed at room temperature for 3 hours. Once the particles are labelled a 25 mM glycine solution is added to the particles then further incubated for 30 minutes. An aliquot of the labelling solution is kept for comparison with the earlier aliquot and the particles washed in 0.1% BSA in PBS. Particles are finally diluted in 0.1% BSA in PBS to give a 1mg/ml solution. Both aliquots of labelling solution and some of the labelled nanoparticles were assessed for fluorescence.

30

Cell Culture

NIH 3T3 fibroblast cells were cultured in Dulbecco's modified Eagle's media (DMEM; Gibco), supplemented with 10% (v/v) Fetal Calf Serum (FCS, Sigma), 2mM L-glutamine and (PS) at 37°C and 5% CO₂. The cells were then cultured until confluent.

35

Cell Labelling

Confluent cells were split into 12 well plates at 200,000 cells/well and incubated for 24 hours at 37°C. After 24 hours 50 µg of Nanomag-D iron oxide nanoparticles (250 nm) and either 0, 0.01, 0.05, 0.1, 0.5, 2, 1, 5 and 10 µM of cell penetrating peptide were added to the cells with either 10% FCS DMEM or serum free DMEM media and left for 24 hours for iron nanoparticles to be internalised. After incubation cells were washed in PBS to remove excess nanoparticles then harvested for qualitative Prussian blue staining, quantitative colorimetric iron assay or fluorescence activated flow cytometry.

10

Prussian Blue Staining

Cells were labelled then fixed in 4% (w/v) PFA for 15-20 minutes at 4°C. A staining solution of 2.5% potassium ferrocyanide in 2.5% HCL was added to cells and incubated for an hour at room temperature. If nanoparticles were present a blue stain appeared which is proportional to the concentration of iron.

15

Quantitative Colorimetric Iron Assay Cells were labelled, trypsinised and pelleted then all media removed. 40µl of 37% HCL was added to the cells and heated at 70°C until dissolved, then neutralised with 50µl of NaOH. Those samples containing a high concentration of iron were diluted 1:10 then 40 µl of Quantichrom working reagent was added and the instructions in the Quantichrom iron assay followed.

20

Flow Cytometry

Cells were labelled with 50 µg of Nanomag-D particles, 1 µM P218R and either 0, 0.1, 1 and 5 µg/ml of FitC-BSA. Cells were then fixed cells and run through a Coulter Altra flow cytometer to assess the green fluorescence. Findings were then statistically analysed by Wesal software.

25

Results

30 Nanoparticle and Cell labelling

Nanomag-D (250 nm) particles were successfully labelled with mR, P21mR, 8RmR and P21mR8R.

Quantitative Assessment of Nanoparticle Uptake

35 Optimisation of Protein Concentration

As shown in figure 21, Prussian blue staining proves that the P218R is the most effective peptide for particle uptake compared to P21 and 8R alone. The iron assay results showed that when cells are incubated for 24 hours with 1 μ M of P218R and 50 μ g of Nanomag-D particles 100% of the particles become associated with the cells and 63 pg/cell. Therefore it was concluded that only 1 μ M of P218R is needed for 100% uptake of particles.

Discussion

The results show that the addition of a small amount of P218R leads to 100% uptake of iron oxide nanoparticles. Microscopy and the trypsinisation of the cells indicate that the particles are being internalised. Experiments were also conducted using mesenchymal stem cells showing a 90% association of particles. The mechanism behind the uptake is dependent on the symbiotic action of the two domains of the peptide. The hypothesis is that the P21 can bind to both the HS on the cell membrane and the dextran in the coating of the nanoparticles, the peptide either has multiple binding points by which both nanoparticle and cell can both be attached to the same P21. Therefore the pre bound protein to the particle can also bind to the membrane keeping the particle in close proximity to the cell. The 8R can then aid in the transduction of the nanoparticle by endocytosis. Or the other mechanism could involve the peptide pre binding to the nanoparticle then when in close proximity to a cell membrane the HS has a higher binding efficiency so the P21 then binds to the cell. This may then lead to the particle being internalised. The advantages of using the P218R peptide is its efficiency in serum media which is more relatable to the in vivo environment and that the system does not require the use of the functional group on the nanoparticles surface coating. The free functional group means that targeting molecules or drugs can be covalently attached to the particle.

Conclusion

The peptide P218R has been found to cause 100% cell association of nanoparticles. This has been found to be due to a dextran binding mechanism which can be utilised for many applications for example targeting of nanoparticles for specific tissues by attaching antibodies, or drug delivery.

References

1. Singh, A & Sahoo, S, (2013), Magnetic Nanoparticles: a novel platform for theranostics, *Drug Disc Today*,
2. Arruebo, M et al, (2007) Magnetic Nanoparticles for drug delivery, *Nanotoday*, 3, 22
- 5 3. Wang, Z and Cuschieri A, (2013) Tumor cell labelling by magnetic nanoparticles with determination of intracellular iron content and spatial distribution of the intracellular iron, *Int. J. Mol. Sci*, 14, 9111
4. Sun, C, Lee, J and Zhang, M, (2008), Magnetic Nanoparticles in MR Imaging and drug delivery, *Adv Drug Deli Rev*, 60, 1253
- 10 5. Schlorf, T et al, (2011), Biological properties of iron oxide nanoparticles for cellular and molecular magnetic resonance imaging, *Int. J. Mol. Sci.* 12, 12
6. Markides, H et al, (2013) Whole body tracking of superparamagnetic iron oxide nanoparticle labelled cells – a rheumatoid arthritis mouse model, *Stem cell Res & ther*, 4, 126
- 15 7. Peng et al, (2008), targeted magnetic iron oxide nanoparticles for tumour imaging and therapy, *Int. J. of Nanomed.* 3, 311
8. Zhang, C et al, (2007), Specific Targeting of Tumor Angiogenesis by RGD-Conjugated Ultrasmall Superparamagnetic Iron Oxide Particles Using a Clinical 1.5-T Magnetic Resonance Scanner, *Can. Res.* 67, 1555
- 20 9. Ji, S et al, (2012), RGD-conjugated albumin nanoparticles as a novel delivery vehicle in pancreatic cancer therapy, *Cancer Biology & Therapy* 13:4, 206.

Modified CPPs for Efficient Cell Type Specific Delivery of Therapeutic Molecules via GET (Glycosaminoglycan (GAG)-binding Enhanced Transduction)

25 **Introduction**

The first aim of this study was to investigate whether the GET-mediated synergistic increase in delivery of mRFP into cells with P21 8R could be observed when P21 was replaced by growth factor derived HS-GAG binding domains. The second aim of this study was to show cell type specific delivery in a heterogeneous population of cells by

30 targeting a specific cell surface HS-epitope. Merry et al have demonstrated the utility of a HS-epitope binding antibody in targeting a subpopulation of cells during mesodermal differentiation [13]. The variable region of this antibody was conjugated to 8R to show an example of cell type specific delivery. The third aim of this study

was to demonstrate the GET-mediated delivery of therapeutic biomolecules. The transfection of reporter gene (pSIN GFP) was optimized with P21 LK15 8R peptide and compared to a 'gold standard' commercial lipid based transfection reagent Lipofectamine2000.

5 Cell Type Specific Delivery via GET

Experimental Procedures

Preparation of Peptides

Peptides, mRFP, mRFP 8R, P21 mRFP 8R, FGF1A mRFP, FGF1A mRFP 8R, FGF2A mRFP, FGF2A mRFP 8R, FGF4A mRFP, FGF4A mRFP 8R, FGF7A mRFP, FGF7A mRFP 8R, FGF1B mRFP, FGF1B mRFP 8R, FGF2B mRFP, FGF2B mRFP 8R, FGF4B mRFP, FGF4B mRFP 8R, FGF7B mRFP, FGF7B mRFP 8R, FGF1C mRFP, FGF1C mRFP 8R, FGF2C mRFP, FGF2C mRFP 8R, FGF4C mRFP, FGF4C mRFP 8R, FGF7C mRFP, FGF7C mRFP 8R, ATIII mRFP, ATIII mRFP 8R, PDGF mRFP, PDGF mRFP 8R, VEGF mRFP, VEGF mRFP 8R, HS4C3 mRFP and HS4C3 mRFP 8R, were cloned as cDNAs into pGEX6-PI vector (Novagen), expressed in BL21 (DE21) pLysS *Escherichia coli* (Novagen) and purified as previously described [12]. Integrity and full length peptide expression was confirmed by SDS-PAGE. Fluorescence of the recombinant peptides was confirmed using the TECAN infinite 200PRO multimode reader, the difference in fluorescence intensity measurements between samples was <10%.

Peptide Assay

Bradford assay was used to quantify protein concentration [14]. Absorbance was measured at 595nm using recombinant mRFP protein as a standard [12]. Samples were analysed using the TECAN infinite 200PRO multimode reader.

25 Cell Culture of NIH3T3, CGR-8 and HUES7 Cells

NIH3T3, CGR-8 and HUES7 cells were grown and maintained as previously described [12]. NIH3T3 mouse fibroblast cells were maintained in *D*ulbecco's *m*odified *E*agle's *m*edium (DMEM) with 10% (v/v) *f*etal *c*alf *s*erum (FCS) supplemented with 2mM L-glutamine and 100 ug/ml streptomycin. CGR-8 mouse embryonic stem cells were maintained in DMEM with 20% (v/v) FCS supplemented with 1000units/ml *l*eukaemia

inhibitory factor (LIF), 100 μ M β -mecaptoethanol, 2mM L-glutamine and 100ug/ml streptomycin. HUES7 human embryonic stem cells were cultured on gelatin coated tissue culture flask. The cells were maintained in DMEM with 20% (v/v) FCS supplemented with 1000units/ml LIF, 100 μ M β -mecaptoethanol, 2mM L-glutamine and 100ug/ml streptomycin. All cells were incubated at 37°C under humidified 5% CO₂ conditions.

Peptide Delivery to Cells

Cells were seeded at 2×10^5 cells/well in 12-well plates and incubated for 2h in 1mL of relevant growth media (GM) at 37°C in a 5% CO₂ humidified incubator. The peptide was diluted to 20ug/ml in 500ul of GM. Each well of cells was aspirated, washed with phosphate buffered saline (PBS) and replaced with 500ul of peptide solution. The cells were incubated with the peptide at 37°C in a 5% CO₂ humidified atmosphere for 20h. Following incubation, each well of cells was washed with PBS, trypsinized and fixed with 3.7% paraformaldehyde (PFA) in preparation for flow cytometry. Each experiment was done in duplicate and repeated 3 times, n=3.

Maintenance and Differentiation of Bry-GFP ES Cells, Generation of EBs

Bry-GFP murine embryonic stem cell line was maintained and differentiated as previously described [13]. Bry-GFP cells were maintained on feeders in DMEM-ES (DMEM with 15% FCS supplemented with 1.5×10^{-5} M monothioglycerol (MTG), 10ng/ml LIF and 2mM L-glutamine).

Bry-GFP cells were differentiated as EBs. Prior to differentiation the cells were passaged twice, first onto a gelatin coated flask in DMEM-ES and second into a flask in Iscove's modified Dulbecco's medium (IMDM)-ES (IMDM with 15% FCS supplemented with 1.5×10^{-4} M monothioglycerol (MTG), 10ng/ml LIF and 2mM L-glutamine). Cells were then differentiated as EBs for 2.8 days in IMDM with 15% FCS supplemented with 4×10^{-4} M MTG, 300ug/ml transferrin, 25ug/ml ascorbic acid and 2mM L-glutamine in Petri-grade dishes. 3h before dissociation, EBs were treated with 50ug/ml of HS4C3 mRFP or HS4C3 mRFP 8R. Following differentiation EBs were separated into single cells by 10min incubation and agitation in cell dissociation buffer and fixed in PFA.

Flow Cytometry Analysis

Cells were analysed on a MoFlo™ DP (DAKO) Flow Cytometer using a 488nm green laser and/or 633nm red laser. (40,000 cells; gated on live cells by forward/side scatter). Median fluorescence was used for statistical analyses.

5 *Results and Discussion*

CPPs Modified to Include GET

In this study the HS-GAG binding domains of fibroblast growth factor (FGF)-1, FGF-2, FGF-4, FGF-7, platelet derived growth factor (PDGF) and antithrombin-III (ATIII) were coupled to 8R. These growth factors play important biological roles in embryonic development and angiogenesis. They have also been shown to interact with cell surface HS-GAGs, similarly to P21. NIH 3T3 murine fibroblasts, CGR8 murine embryonic stem cells and HUES-7 human embryonic stem cells were treated with these modified CPPs to investigate whether any of the peptides demonstrated a GET-mediated increase in delivery of mRFP (Figure 22). It was also important to explore whether any of the modified peptides would preferentially target HS epitopes that were more abundantly expressed in any of the different cell types.

A panel of four modified CPPs that showed GET-mediated enhanced transduction into cells have been identified. P21 8R, FGF2B 8R, FGF7B 8R and PDGF 8R have demonstrated 30-100 fold increase in transduction of mRFP into cells over using an 8R alone (Figure 23 and 24). P21 8R, FGF7B 8R and PDGF 8R showed preferential transduction into HUES-7 embryonic stem cells. This demonstrates preferential transduction of CPPs into a cell-type that is considered difficult to transduce in to. FGF2B 8R showed pluripotency specific transduction into CGR8 and HUES7 embryonic stem cells. The diverse delivery profiles of the modified CPPs into the three cell types suggests the HS-GAG binding domains of different growth factors target and bind different cell surface HS-epitopes. Targeting HS-epitopes that are expressed more abundantly by specific cell types can be utilized for the selection of CPPs that are more suitable for their application.

30

GET Mediated Delivery of Plasmid DNA

Experimental Procedures

Preparation of Peptides

P21-LK15-8R peptide was synthesised using solid phase t-Boc chemistry (Novabiochem (Beeston, Nottinghamshire, UK)).

5 Cell culture

NIH3T3 mouse fibroblast cells were maintained in DMEM with 10% (v/v) fetal calf serum (FCS) media supplemented with 2mM L-glutamine and 100ug/ml streptomycin. The cells were incubated at 37°C under humidified 5% CO₂ conditions.

Preparation of Plasmid DNA

- 10 DNA (pSIN GFP) was amplified in *E. coli*. The DNA was extracted and purified using a QIAGEN Plasmid Maxi kit (Qiagen). DNA was precipitated in 100% ethanol and rehydrated in dH₂O. Plasmid purity was confirmed using the nanodrop.

Peptide-DNA Complexation Assay

- 10ug DNA was diluted in 60ul 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)-buffered saline (10mM HEPES, 150mM sodium chloride (NaCl) solution, pH 7.4). 1mM YO-PRO-1 stock solution was diluted to 0.1mM in dimethyl sulfoxide (DMSO). 2.7ul of the diluted YO-PRO-1 solution was made up to 60ul in HEPES-buffered saline and added dropwise to the diluted DNA. The DNA/YO-PRO-1 solution was mixed, wrapped in foil and incubated for 5h at room temperature. After 5h, the
- 20 DNA/YO-PRO-1 solution was made up to 1ml in HEPES-buffered saline and 100ul aliquots were pipetted into eppendorf tubes per treatment condition. Peptide amounts corresponding to the desired (+/-) charge ratios were added to each eppendorf (Appendix 1). Peptide/DNA/YO-PRO-1 solutions were mixed and incubated at room temperature for 10min. Fluorescence measurements were then analysed using the
- 25 TECAN infinite 200PRO multimode reader. Similarly, a no DNA control was made by diluting 2.7ul of the diluted YO-PRO-1 solution in 120ul HEPES-buffered saline and following the procedure above.

Design and Optimization of Transfection Experiment

- Cells were seeded at 80,000 cells per well on 12-well plates and incubated overnight
- 30 in 1mL of 10% GM at 37°C in a 5% CO₂ humidified incubator. For each well of cells to be transfected, DNA was diluted in 100ul Opti-MEM® and mixed. The peptide was

- added directly to the diluted DNA at the optimal (+/-) charge ratio. The solution was then mixed and incubated for 25min at room temperature. The cells were aspirated, washed with PBS and replaced with 400ul Opti-MEM®. Each well of cells was treated with 100ul of Peptide/DNA complex and incubated 37°C in a 5% CO₂ humidified atmosphere. Appendix 2 shows the different treatment conditions used for each well. Following incubation, the cells were washed with PBS and replaced with 1ml GM. After 48h, each well of cells was washed with PBS, trypsinized and fixed with 3.7% PFA. Experiments were repeated 3 times.
- 10 Lipofectamine2000 transfection optimization was carried out as described in the manufacturers guide (Invitrogen). Cells were seeded at 80,000 cells per well on 12-well plates and incubated overnight in 1mL of GM at 37°C in a 5% CO₂ humidified incubator. For each well of cells to be transfected, DNA was diluted in 100ul Opti-MEM® and mixed. 1.5ul of Lipofectamine2000 was added directly to the diluted
- 15 DNA. The mixture was then mixed and incubated for 25mins at room temperature. The cells were aspirated, washed with PBS and replaced with 400ul Opti-MEM®. Each well of cells was treated with 100ul of Lipofectamine2000/DNA complex and incubated 37°C in a 5% CO₂ humidified atmosphere. After 6h, the cells was washed with PBS, trypsinized and fixed with 3.7% PFA. This was repeated with varying
- 20 volumes of Lipofectamine2000 (3ul and 4.5ul) to find the optimal ratio of Lipofectamine2000 to DNA. Experiments were repeated 3 times.

Flow Cytometry Analysis

- Cells were analysed on a MoFlo™ DP (DAKO) Flow Cytometer using a 633nm red laser. (40,000 cells; gated on live cells by forward/side scatter). Median fluorescence
- 25 was used for statistical analyses.

Results and Discussion

Peptide to DNA Binding

- YO-PRO-1 assay can be used to investigate the DNA condensation ability of a DNA binding peptide. YO-PRO-1 is a cyanine dye that binds DNA to form a fluorescent
- 30 DNA/dye complex. Different (+/-) charge ratios of peptide can be added to the fluorescent DNA/dye complex. As the peptide out competes the dye by binding the DNA a reduction in fluorescence intensity is observed. In this study, LK15 was fused

to P21 8R transduction protein to improve the DNA binding ability of the modified cell penetrating peptide. Fusion of LK15 peptide to TAT has been shown to significantly improve transfection of pDNA into HT29 and HT1080 cultured cells [19]. Enhanced transduction efficiency of Tat-LK15 over Tat is thought to be due to
5 the improved condensation ability of the peptide and DNA, and better transduction of the DNA across the cell membrane [20].

A graph of (+/-) charge ratio was plotted against % fluorescence to investigate the optimum ratio of P21 LK15 8R to pSIN GFP (Figure 25). Results showed that the
10 optimal (+/-) charge ratio of P21 LK15 8R to pSIN GFP was 2:1, respectively. This ratio was used in the transfection experiments.

Transfection of pDNA Reporter Gene via P21 LK15 8R

The phospholipid bilayer of the cell membrane acts as an impenetrable barrier to nucleic acids and thus pDNA will be conjugated to a modified CPP to facilitate its
15 transport into the cell [2]. In this study the GET-mediated transfection of the reporter gene pSIN GFP into NIH 3T3 murine fibroblast cells was optimized in terms of transfection time (3, 6 or 24h), transfection media (with or without serum) and amount of DNA (1, 4 or 10ug). The reporter gene pSIN GFP was transfected into cells using
20 P21 LK15 8R where P21 targets and binds cell surface HS-GAGs, LK15 complexes pSIN GFP and 8R transduces pSIN GFP across the cell membrane. The transfection efficiency of pSIN GFP with P21 LK15 8R was compared to the transfection efficiency of commercially used lipid-based transfection reagent lipofectamine2000. Cells were fixed at 48h following transfection to allow time for the transient
25 expression of GFP to be captured and transfection efficiencies were quantified by flow cytometry (Figure 26).

Gene carrier systems must be serum resistant for efficacious in-vivo applications, however most gene carries, including lipofectamine2000, have demonstrated steep decreases in transfection efficiency in serum containing media [21]. This is believed
30 to be because serum molecules competitively bind the gene carrier, therefore decreasing free gene carriers available to bind the DNA [22]. The transfection efficiency of P21 LK15 8R was characterised in serum and serum free transfection media. The optimal transfection conditions were when cells were transfected with 10ug DNA for 24h in serum conditions where transfection efficiency reached $17.9 \pm$

4.8%. (Figure 27). This is 3 fold lower than the optimized transfection efficiency observed for lipofectamine2000 in serum free conditions ($54.7 \pm 10.3\%$, Appendix 3) however, the serum-resistance of P21 LK15 8R transfection is advantageous for any sort of clinical/ in-vivo delivery of therapeutic biomolecules. In addition, it is well documented that endosomal escape strategies greatly increase the efficiencies of CPP-mediated transfections.

Conclusions

A panel of CPPs that have been modified to include growth factor derived cell surface HS-GAG binding domains have shown 30-100 fold increase in transduction into cells, compared to unmodified CPPs. The hypothesis is that the GET-mediated delivery of these peptides is due to the dual functionality of the peptide in i) increasing interaction with the cell membrane via the HS-GAG binding domain, and ii) transducing protein across cell membrane via 8R. The modified CPPs showed preferential delivery profiles of mRFP into different cell types, this is due the HS-GAG binding domains targeting specific HS-epitopes that are more abundantly expressed in different cell types. Future work is to modify CPPs to include specific antibody-derived HS-epitope binding domains. HS-epitope binding libraries of antibodies can be utilized for the cell type specific delivery of therapeutic molecules via GET.

20

To demonstrate the utility of these peptides for the delivery of therapeutic molecules P21 LK15 8R was used to deliver the reporter gene pSIN GFP into cells. Results showed GET-mediated transfection efficiencies of up to $17.9 \pm 4.8\%$ in serum conditions, without any endosomal escape strategy. CPPs modified to include HS-GAG binding domains show great promise as alternatives to using viral and lipid based delivery vehicles for the in-vivo delivery of therapeutic biomolecules.

References

- [1] Bechara C, Sagan S. Cell-penetrating peptides: 20 years later, where do we stand? *Febs Letters* 2013;587:1693-702.
- [2] Tanaka K, Kanazawa T, Ogawa T, Suda Y, Takashima Y, Fukuda T, et al. A Novel, Bio-Reducible Gene Vector Containing Arginine and Histidine Enhances Gene

30

- Transfection and Expression of Plasmid DNA. Chemical & Pharmaceutical Bulletin 2011;59:202-7.
- [3] Mitchell DJ, Kim DT, Steinman L, Fathman CG, Rothbard JB. Polyarginine enters cells more efficiently than other polycationic homopolymers. Journal of Peptide Research 2000;56:318-25.
- [4] Nakase I, Niwa M, Takeuchi T, Sonomura K, Kawabata N, Koike Y, et al. Cellular uptake of arginine-rich peptides: Roles for macropinocytosis and actin rearrangement. Molecular Therapy 2004;10:1011-22.
- [5] Ma DX, Shi NQ, Qi XR. Distinct transduction modes of arginine-rich cell-penetrating peptides for cargo delivery into tumor cells. International Journal of Pharmaceutics 2011;419:200-8.
- [6] El-Sayed A, Futaki S, Harashima H. Delivery of Macromolecules Using Arginine-Rich Cell-Penetrating Peptides: Ways to Overcome Endosomal Entrapment. Aaps Journal 2009;11:13-22.
- [7] Shiraishi T, Nielsen PE. Enhanced delivery of cell-penetrating peptide-peptide nucleic acid conjugates by endosomal disruption. Nature Protocols 2006;1:633-6.
- [8] Matsubara Y, Chiba T, Kashimada K, Morio T, Takada S, Mizutani S, et al. Transcription activator-like effector nuclease-mediated transduction of exogenous gene into IL2RG locus. Scientific Reports 2014;4.
- [9] Parelkar SS, Letteri R, Chan-Seng D, Zolochovska O, Ellis J, Figueiredo M, et al. Polymer-Peptide Delivery Platforms: Effect of Oligopeptide Orientation on Polymer-Based DNA Delivery. Biomacromolecules 2014;15:1328-36.
- [10] Yang HY, Vonk LA, Licht R, van Bortel AMG, Bekkers JEJ, Kragten AHM, et al. Cell type and transfection reagent-dependent effects on viability, cell content, cell cycle and inflammation of RNAi in human primary mesenchymal cells. European Journal of Pharmaceutical Sciences 2014;53:35-44.
- [11] Ma Y, Gong C, Ma YL, Fan FK, Luo MJ, Yang F, et al. Direct cytosolic delivery of cargoes in vivo by a chimera consisting of D- and L-arginine residues. Journal of Controlled Release 2012;162:286-94.
- [12] James E. Dixon GM, Nina Lane, Chris Denning and Kevin M. Shakesheff Highly Efficient Delivery of Functional Proteins by the Synergistic Effect of GAG Binding Motifs and Cell-Penetrating Peptides. Unpublished 2014.
- [13] Baldwin RJ, ten Dam GB, van Kuppevelt TH, Lacaud G, Gallagher JT, Kouskoff V, et al. A Developmentally Regulated Heparan Sulfate Epitope Defines a

Subpopulation with Increased Blood Potential During Mesodermal Differentiation. Stem Cells 2008;26:3108-18.

- [14] Bradford MM. RAPID AND SENSITIVE METHOD FOR QUANTITATION OF MICROGRAM QUANTITIES OF PROTEIN UTILIZING PRINCIPLE OF PROTEIN-DYE BINDING. Analytical Biochemistry 1976;72:248-54.
- [15] Schamhart DHJ, Kurth KH. Role of proteoglycans in cell adhesion of prostate cancer cells: From review to experiment. Urological Research 1997;25:S89-S96.
- [16] Delehedde M, Deudon E, Boilly B, Hondemarck H. Proteoglycans in breast cancer. Pathologie Biologie 1997;45:305-11.
- [17] Shao C, Shi XF, Phillips JJ, Zaia J. Mass Spectral Profiling of Glycosaminoglycans from Histological Tissue Surfaces. Analytical Chemistry 2013;85:10984-91.
- [18] Thompson KE, Bashor CJ, Lim WA, Keating AE. SYNZIP Protein Interaction Toolbox: in Vitro and in Vivo Specifications of Heterospecific Coiled-Coil Interaction Domains. Acs Synthetic Biology 2012;1:118-29.
- [19] Saleh AF, Aojula H, Arthanari Y, Offerman S, Alkotaji M, Pluen A. Improved Tat-mediated plasmid DNA transfer by fusion to LK15 peptide. Journal of Controlled Release 2010;143:233-42.
- [20] Dufourcq J, Neri W, Henry-Toulme N. Molecular assembling of DNA with amphipathic peptides. Febs Letters 1998;421:7-11.
- [21] Zhang X, Hu HM, Liu TB, Yang YY, Peng YF, Cai QQ, et al. Multi-armed poly(L-glutamic acid)-graft-polypropyleneimine as effective and serum resistant gene delivery vectors. International Journal of Pharmaceutics 2014;465:444-54.
- [22] Wu HM, Pan SR, Chen MW, Wu Y, Wang C, Wen YT, et al. A serum-resistant polyamidoamine-based polypeptide dendrimer for gene transfection. Biomaterials 2011;32:1619-34.

Example 3

- There is an unmet clinical need for high numbers of patient-specific cells for cell therapies. In this example RNAs have been delivered (Figures 29-31) that temporarily produce a proliferative phenotype in clinically-relevant cells which are hard and slow to expand to sufficient numbers for human cell therapies.

Modified Synthetic RNA production to regulate gene expression stoichiometrically

RNA was synthetically made using modified nucleotides and cap analogues which express well in human cells, are not cytotoxic and do not generate a cellular immune response, specifically in primary cells using the interferon B18R inhibitor (Fig 29).
5 Clearly RNA is not stability inherited unlike integrated DNA and is more efficient at producing a translated protein product as it is translated in the cytosol rather than needing transcription in nuclei like delivered DNAs.

10 RNA expression was demonstrated to be short lived (to background levels by 72h) meaning that RNA can be serially delivered (daily to every 3 days) to create a situation of constant protein expression and simply ceasing the delivery results in no further protein-expression (Fig. 29). Therefore, it has been proven that the level and duration of the expressed protein's activity can tightly and stoichiometrically
15 controlled in the target cell type.

Transient immortalisation of clinically valuable cells to generate extensive cell numbers (without losing the primary cell phenotype) is a step change in what is possible using hMSCs in cell therapies. We found that aged cells (by over passaging
20 them; Fig 31. P4-10) benefited the most from this technology. It is well known that stem cell derived from the elderly or diabetic patients are the most difficult to expand.

Transformative benefits of the invention are:

- 1) A rapid expansion of cells in one-to-several weeks rather than months from small
25 starting populations. We have demonstrated a doubling of proliferation after a single dose of delivered RNA
- 2) Non-genetically modified cells will be produced. No modification to the genome has been detected.
- 3) This is a technology to facilitate autologous cell therapies.
- 30 4) No spontaneous transformation has been seen using this technology in hMSC cultures.
- 5) There is application to other therapeutic cell types (Pancreatic islets, hepatocytes, retinal pigment, neurons).

Example Sequences

35

Example HS-GAG binding sequences

P21 amino acid sequence

(SEQ ID NO. 1)

KRKKKGKGLGKKRDPCLRKYK

5

P21 nucleotide sequence (with a methionine/ATG):

(SEQ ID NO: 2)

aagcgcaagaagaaggcgaaggcctgggcaagaagcgcgatccgtgcctgcgcaagtataag

PDGF (194-211) amino acid sequence:

10 (SEQ ID NO. 3)

G R P R E S G K K R K R K R L K P T

PDGF (194-211) nucleotide sequence:

(SEQ ID NO: 4)

15 ggccgcccgcgcgaaagcggcaaaaaacgcaaacgcaaacgcctgaaaccgacc

FGF7B amino acid sequence:

(SEQ ID NO. 5).

T Y A S A K W T H N G G E M F V A L N Q

20

FGF7B nucleotide sequence:

(SEQ ID NO: 6)

acctatgcgagcgcgaaatggaccataacggcgggcgaaatgtttgtggcgctgaaccag

25 FGF2 HBD B(247-262) amino acid sequence:

(SEQ ID NO. 7).

T Y R S R K Y T S W Y V A L K R

FGF2 HBD B(247-262) nucleotide sequence:

30 (SEQ ID NO: 8)

acctatcgagccgcaaatataaccagctggtatgtggcgctgaaacgc

Nucleotides encoding 8R protein transduction domain sequence:

(SEQ ID NO: 9)

35 CGA AGA CGC AGG AGA CGT CGA AGG

Example delivery molecule nucleotide sequence (P21-cargo-8R):

(SEQ ID NO: 10)

aagcgcaagaagaagggcaaaggcctgggcaagaagcgcgatccgtgcctgcgcaagtataagNcgaagacgcagga
 5 gacgtcgaagg

N=cargo nucleic acid sequence of various length (i.e. the number of nucleotide residues may vary), or another molecular entity.

Two versions of each of the nanobody variants of the ScFv antibodies were made; one
 10 with identical sequence to the ScFv vHH domain (Frame domain1-CDR1-Frame domain 2-CDR2-Frame domain 3-CDR3-IgA Hinge domain/Frame domain 4) and one in which the CDR1, 2 and 3 domains were grafted into a generic vHH domain sequence. Both versions have comparable activity and the grafting version was created to prove that simply grafting the CDR domains onto a generic antibody also works.

15

Below are the sequences of the HS4C3, and AO4BO8 ScFv vHH and grafted vHH:

HS4C3 ScFv vHH

(SEQ ID NO: 11)

20 EVQLVESGGGLVQPRGSLRLSCAASGFTVSSNEMSWIRQAPGKGLEWVSSISGG
STYYADSRKGRFTISRDNSKNTLYLQMNNLRAEGTAAYYCGRRLKDPSTPPTPS
 PSTPPTSPS

CDR1 GFTVSSNE

25 CDR2 ISGGST

CDR3 GRRLKD

HS4C3 ScFv vHH nucleotide sequence

(SEQ ID NO: 12)

30 gaagtgcagctggtgaaagcggcgggcctggtgcagccgcgcggcagcctgcgcctgagctgcgcggcgagcgg
 cttaccgtgagcagcaacgaaatgagctggattcgccaggcgccgggcaaaggcctggaatgggtgagcagcattagcg
 gcggcagcacctattatgcggatagccgaaaggccgctttaccattagccgcgataacagcaaaaacacctgtatctgca
 gatgaacaacctgcgcgcggaaggcaccgcggcggtattattgcggccgcccgcctgaaagatccgagcaccgccgacc
 ccgagcccagcaccgccgaccccgagcccagc

35

HS4C3 grafted vHH

(SEQ ID NO: 13)

QVQLVESGGGSVQAGGSLRLSCTASGFTVSSNELGWFRQAPGQERWAVAAISG
GSTYYADSVKGRFTISRDNAKNTVTLQMNNLKPEDTAIYYCGRRLKDWGQGTQ
5 VTVSSPSTPPTSPSTPPTSPS

CDR1 GFTVSSNE

CDR2 ISGGST

CDR3 GRRLKD

10

HS4C3 grafted vHH nucleotide

(SEQ ID NO: 14)

caggtgcagctggtgaaagcggcgggcggcagcgtgcaggcggcgaggcagcctgcgcctgagctgcaccgcgagcgg
ctttaccgtgagcagcaacgaactgggctggtttcgccaggcgccgggcccaggaacgtgggcggtggcgggcgattagc
15 ggcggcagcacctattatgcggatagcgtgaaaggcgcgtttaccattagccgcgataacgcgaaaaacacgtgaccctg
cagatgaacaacctgaaaccggaagataccgcgattattattgcggccgcgcctgaaagattggggccagggcacca
ggtgaccgtgagcagcccgagcaccgcgcgaccccgagcccgagcaccgcgcgaccccgagcccgagc

AO4B08 ScFv vHH

20 (SEQ ID NO: 15)

EDQLVESGGGLVQPGGSLRPSCAASGFAFSSYALHWVRRAPGKGLEWVSAIGT
GGDTYYADSVMGRFTISRDNAKKSLYLHMNSLIAEDMAVYYCSLRMNGWRAH
QPSTPPTSPSTPPTSPS

25 CDR1 GFAFSSYA

CDR2 IGTGGDT

CDR3 SLRMNGWRAHQ

AO4B08 ScFv vHH nucleotide sequence

30 (SEQ ID NO: 16)

gaagatcagctggtgaaagcggcgggcggcctggtgcagccggcgaggcagcctgcgcccagctgcgcggcgagcgg
ctttcggttagcagctatgcgctgcattgggtgcgcgcggcgccgggcaaaggcctggaatgggtgagcgcgattggcac
cggcgggcgatacctattatgcggatagcgtgatgggcccgtttaccattagccgcgataacgcgaaaaaagcctgtatctg
catatgaacagcctgattgcggaagatatggcggtgtattattgcagcctgcgcatgaacggctggcgcgcatcagccg
35 agcaccccgccgaccccgagcccgagcaccgcgcgaccccgagcccgagc

AO4B08 grafted vHH

(SEQ ID NO: 17)

QVQLVESGGGSVQAGGSLRLSCTASGFAFSSYALGWFRQAPGQERWAVAAIGT
5 GGDTYYADSVKGRFTISRDNAKNTVTLQMNNLKPEDTAIYYCSLRMNGWRAH
QWGQGTQVTVSSPSTPPTPSPSTPPTSPS

CDR1 GFAFSSYA

CDR2 IGTGGDT

10 CDR3 SLRMNGWRAHQ

AO4B08 grafted vHH nucleotide sequence

(SEQ ID NO: 18)

caggtgcagctggtgaaagcggcggcggcagcgtgcaggcggcggcagcctgcgcctgagctgcaccgcgagcgg
15 ctttgcgttttagcagctatgcgctgggctggtttcgccaggcgccgggcccaggaacgctgggcggtggcggcgattggca
ccggcggcgatacctattatgcggatagcgtgaaaggccgctttaccattagccgcgataacgcgaaaaacaccgtgaccc
tgcagatgaacaacctgaaaccggaagataccgcgattattattgcagcctgcgcctgaacggctggcgcgcgcgcacgt
ggggccagggcacccaggtgaccgtgagcagcccagcaccgccgaccccgagcccgagcaccgccgaccc
gagcccgagc

20

CLAIMS

1. A method of producing a population of cells comprising:
-transiently transforming a cell with nucleic acid arranged to promote cell
5 expansion,
wherein the nucleic acid is linked to a delivery vehicle for cell attachment and
internalization, wherein the delivery vehicle comprises:
- a glycosaminoglycan (GAG) binding element, which is capable of binding to
GAG on the surface of the cell; and
10 - a protein transduction domain.
2. A method of transient modification of a cell comprising:
-exposing the cell to a transformation complex comprising a nucleic acid linked to a
delivery vehicle for cell attachment and internalization, wherein the delivery vehicle
15 comprises:
- a glycosaminoglycan (GAG) binding element, which is capable of binding to
GAG on the surface of the cell; and
- a protein transduction domain.
- 20 3. The method according to claim 1 or claim 2, wherein the cell comprises a
mesenchymal stem cell (MSC).
4. The method according to any preceding claim, wherein the resulting expanded
population of cells retain multipotent differentiation into bone, fat and connective
25 tissue.
5. The method according to claim 1 or claim 2, wherein the cell comprises a
pancreatic islet cell, a hepatocyte, a retinal pigment cell, or a neuron.
- 30 6. The method according to any preceding claim, wherein the nucleic acid comprises
RNA.
7. The method according to claim 6, wherein the RNA is modified for translation and
stability.

8. The method according to any preceding claim, wherein the nucleic acid is arranged to promote cell expansion by encoding genes capable of temporally immortalizing cells
- 5 9. The method according to any preceding claim, wherein the nucleic acid encodes any one of the genes selected from SV40T, TERT, HPV E6/7, BMI1, Cyclins, Survivin (BIRC5), p53, and Ras; or combinations thereof.
- 10 10. The method according to any preceding claim, wherein the GAG binding element is a heparan sulphate glycosaminoglycan (HS-GAG) binding element, which is capable of binding to HS-GAG on the surface of the cell.
11. The method according to any preceding claim, wherein the protein transduction domain is hydrophilic or amphiphilic.
- 15 12. The method according to any preceding claim, wherein the protein transduction domain comprises a majority of arginine and/or lysine amino acid residues.
- 20 13. A method of treatment or prevention of a disease, or treatment for tissue damage or a bone defect comprising:
- providing a cell;
 - transiently transforming the cell with nucleic acid for causing expansion of the cell in accordance with the method of any preceding claim;
 - expanding the cell to form an expanded population of cells;
 - 25 -administering the expanded population of cells to the patient.
14. The method according to claim 13, wherein the expanded population of cells is administered to the patient by implantation of a scaffold populated with the expanded population of cells; or administered by injection; or administered topically for skin regeneration treatments.
- 30 15. A method of manufacturing an implant for treatment of tissue damage or a bone defect in a patient comprising:
- providing a cell;

- transiently transforming the cell with nucleic acid for causing expansion of the cell in accordance with the method of any of claims 1 to 12;
- expanding the cell to form an expanded population of cells;
- populating the expanded population of cells into the implant.

5

16. A method of manufacturing a scaffold-forming composition comprising cells for treatment of tissue damage or a bone defect in a patient comprising:

- providing a cell;
 - transiently transforming the cell with nucleic acid for causing expansion of
- 10 the cell in accordance with the method of any of claims 1 to 12;
- expanding the cell to form an expanded population of cells;
 - mixing the expanded population of cells with a scaffold-forming composition.

17. A method of manufacturing a suspension of autologous cells for treatment of a

15 patient in need thereof comprising:

- providing a cell;
 - transiently transforming the cell with nucleic acid for causing expansion of the cell in accordance with the method of the invention;
 - expanding the cell to form an expanded population of cells which are
- 20 autologous to the patient.

18. The method according to any of claims 13 to 17, wherein the cell is from the patient to be treated.

25 19. A population of cells produced by the method of any preceding claim.

20. Use of delivery vehicle for transiently transforming a mesenchymal stem cell with nucleic acid, wherein the delivery vehicle comprises:

- a glycosaminoglycan (GAG) binding element, which is capable of binding to
- 30 GAG on the surface of the cell; and
- a protein transduction domain; and
- wherein the delivery vehicle is linked to, or complexed with, the nucleic acid.

21. The use of claim 20, wherein the transient transformation and/or expansion of the

35 cell is *in vitro*.

22. A method of stably genetically modifying a cell comprising:

- transiently modifying the cell according to the method of any of claims 1 to 12, such that the cell is in an expansion phase to form a population of cells;

5 - stably transforming the cells during the expansion phase with nucleic acid arranged to stably modify the chromosomal DNA of the cell.

23. Use of the method according to any of claims 1 to 12 to transiently transform a cell for achieving a cell expansion phase in order to genetically modify the cell by
10 transformation with nucleic acid.

24. A method of manufacturing a wound dressing comprising cells for treatment of tissue damage in a patient comprising:

- providing a cell;

15 - transiently transforming the cell with nucleic acid for causing expansion of the cell in accordance with the method of any of claims 1 to 12;

- expanding the cell to form an expanded population of cells;

-mixing the expanded population of cells with a wound dressing material.

20 25. An agent for transiently transforming a population of cells, the agent comprising:

- nucleic acid arranged to promote cell expansion,

wherein the nucleic acid is linked to a delivery vehicle for cell attachment and internalization, wherein the delivery vehicle comprises:

- a glycosaminoglycan (GAG) binding element, which is capable of binding to

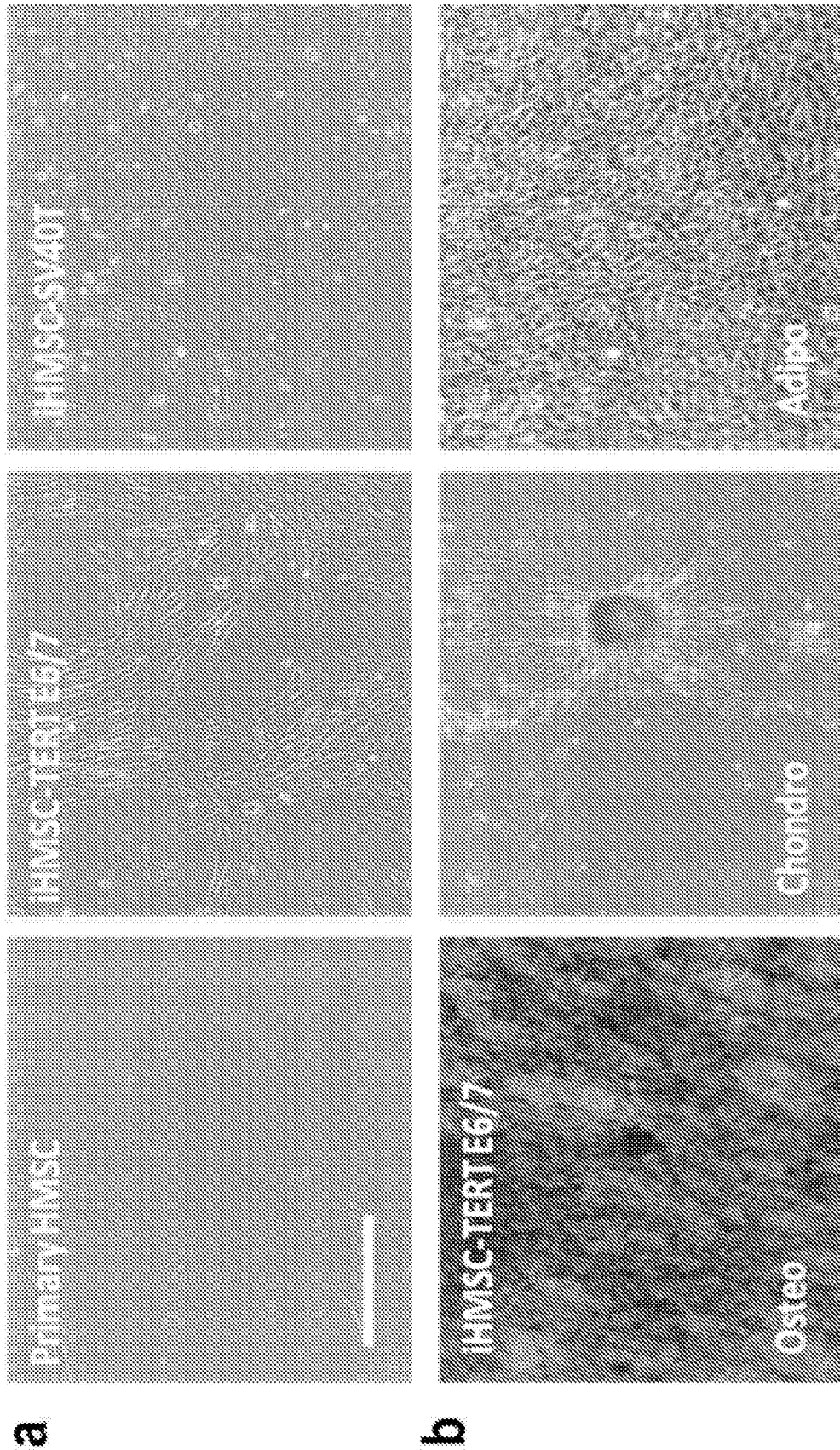
25 GAG on the surface of the cell; and

- a protein transduction domain.

26. Use of the method of any of claims 1 to 12 or the use of the agent of claim 25 for providing a population of cells suitable for seeding onto a scaffold implant or wound
30 dressing.

1/43

Figure 1



2/43

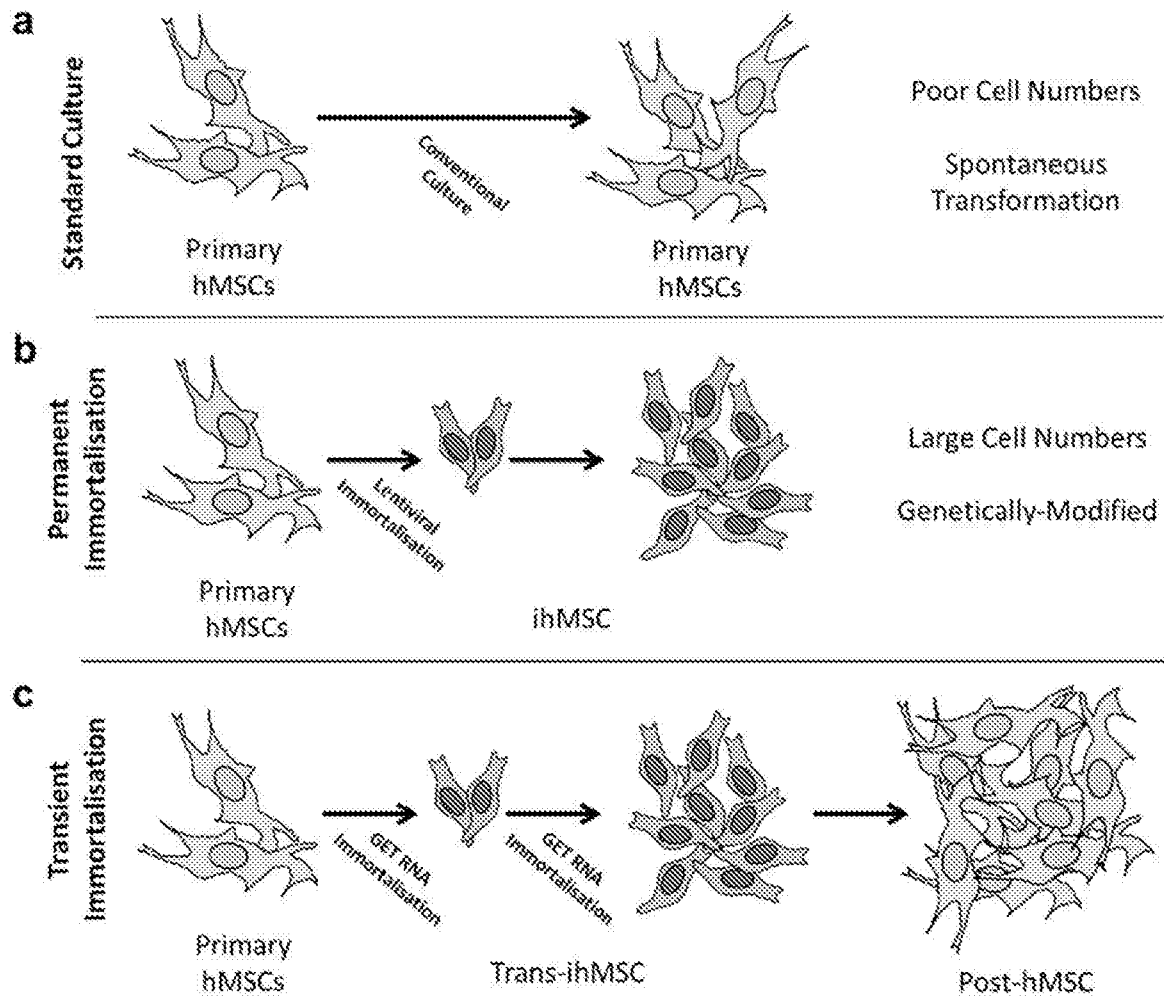


Figure 2

3/43

Figure 3

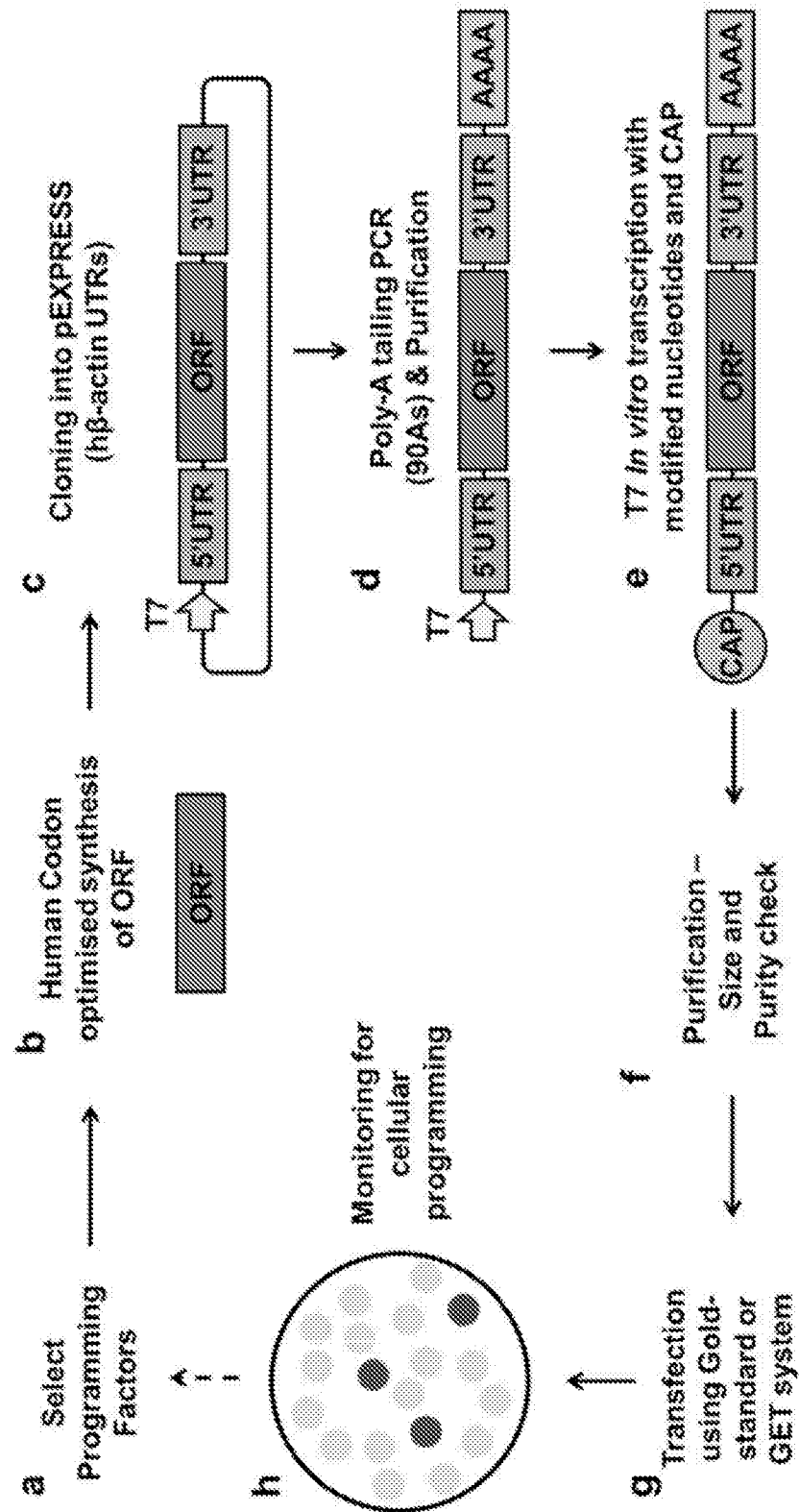


Figure 4

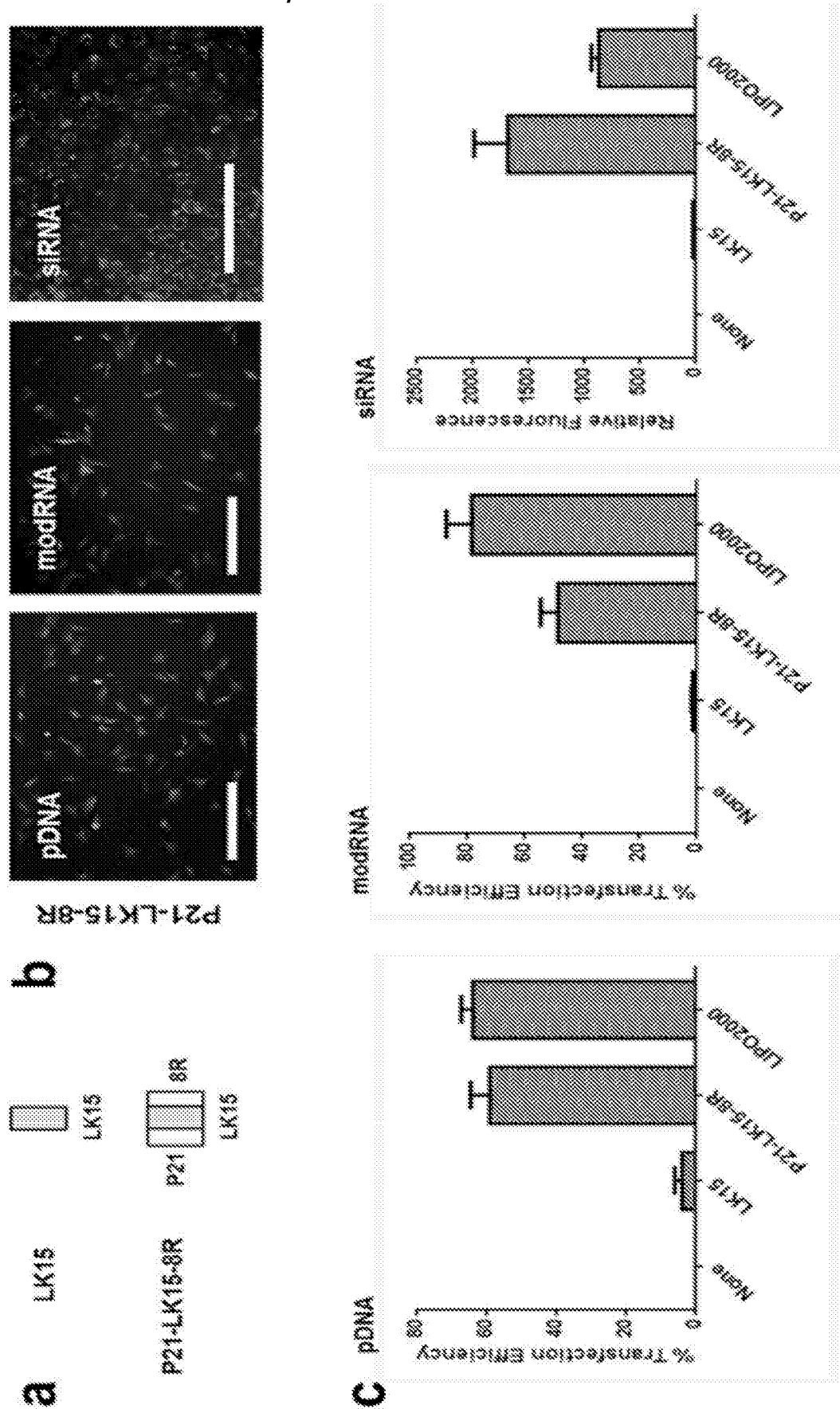
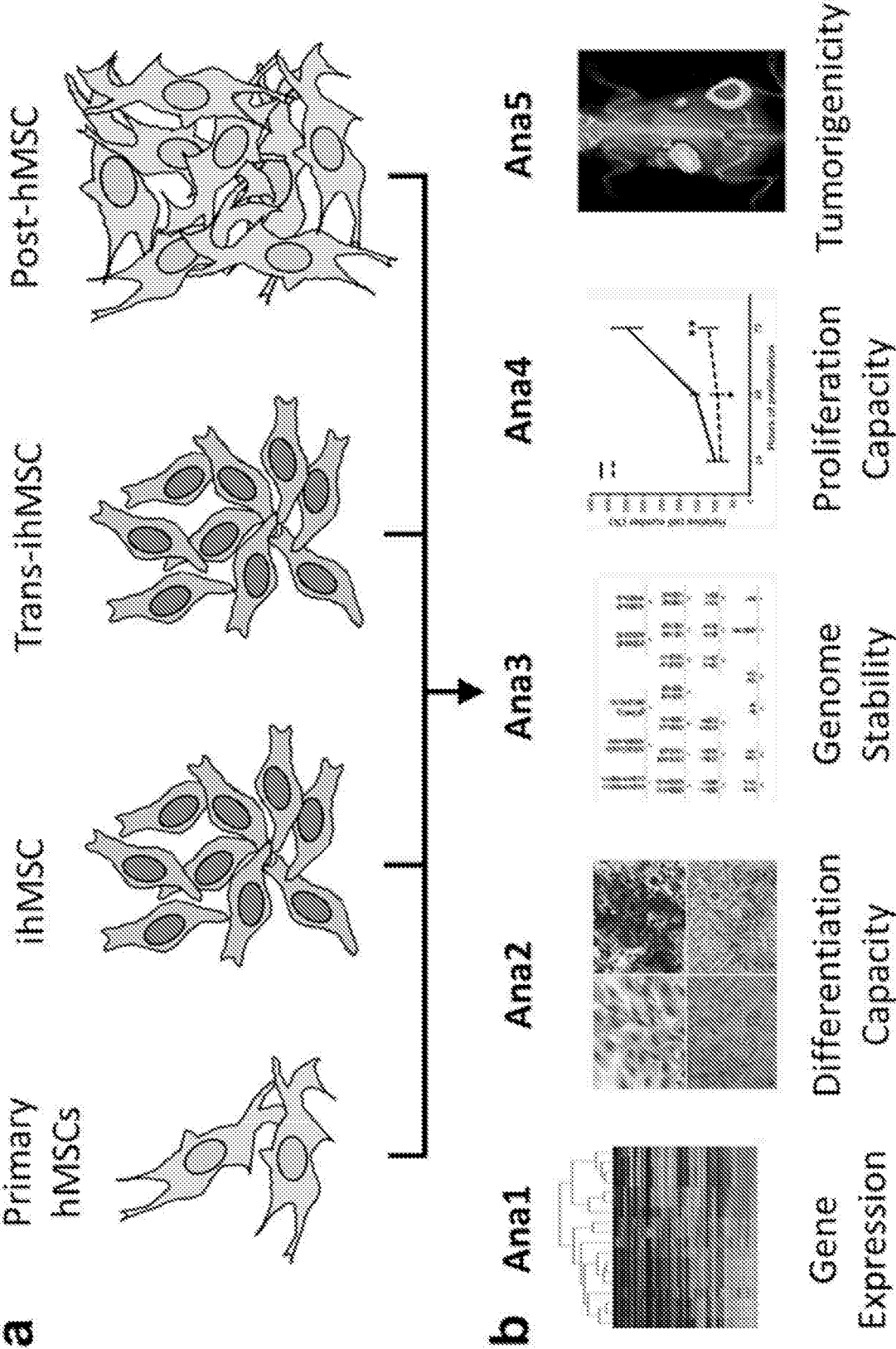


Figure 5



6/43

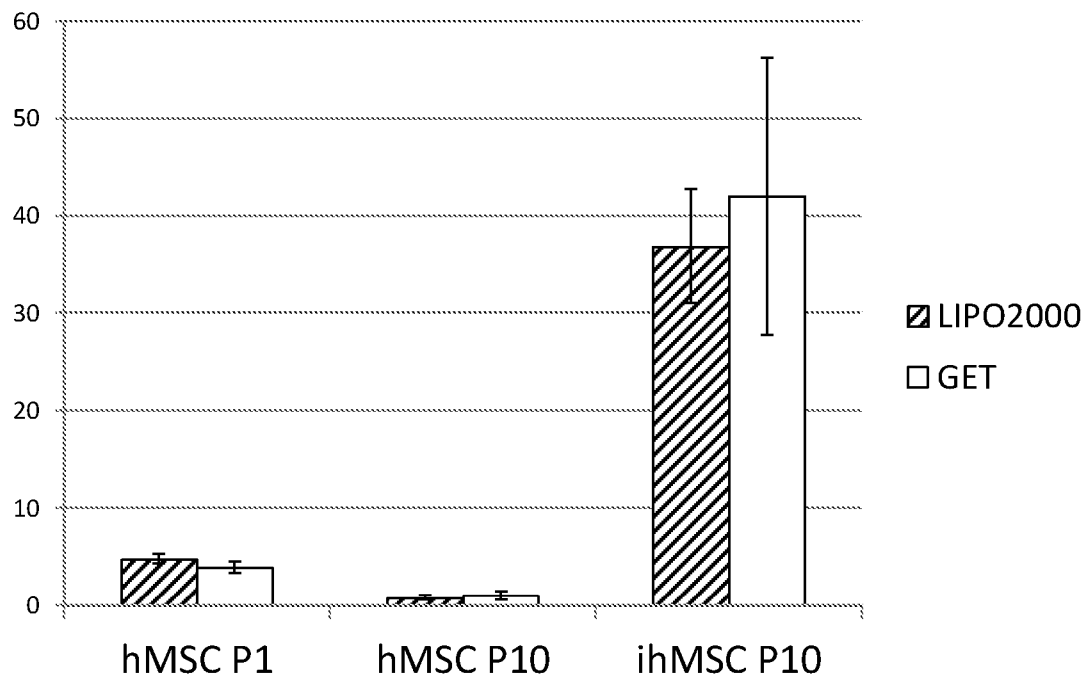


Figure 6

7/43

Figure 7

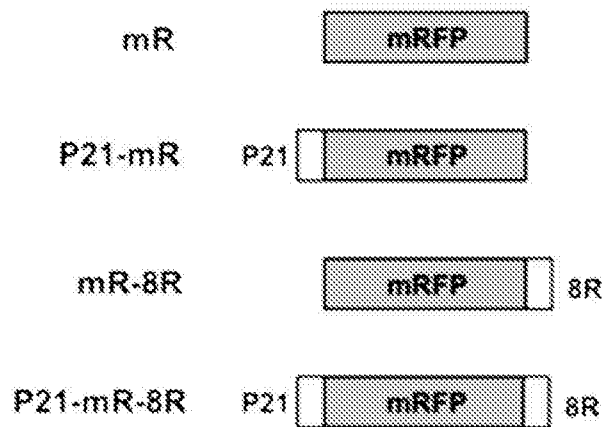
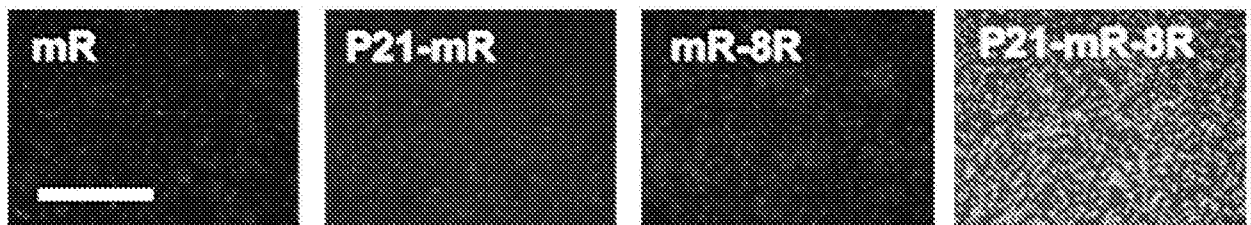
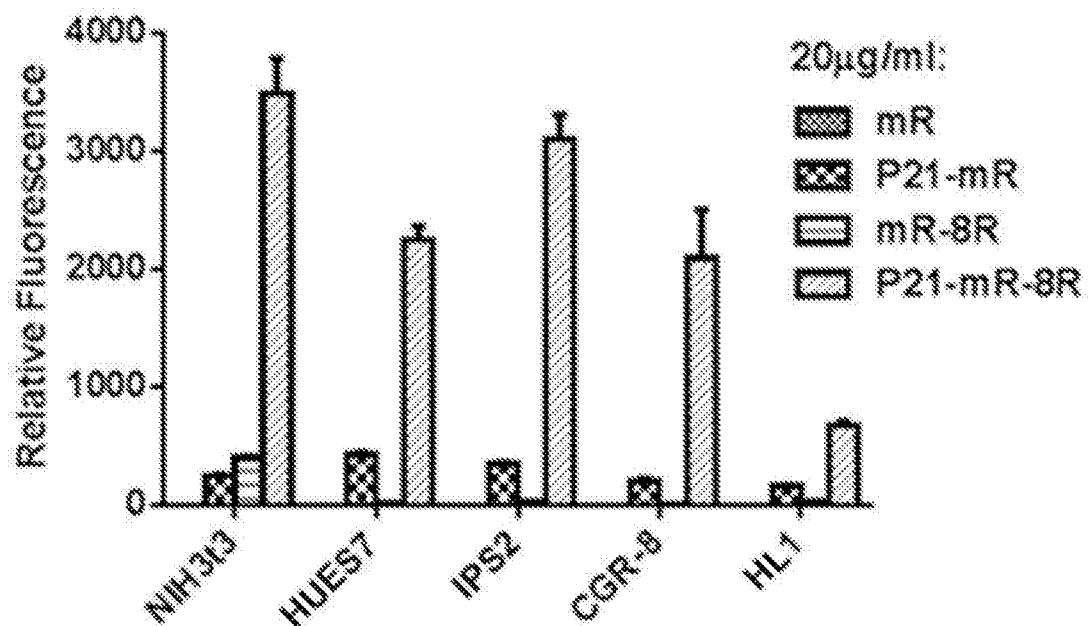
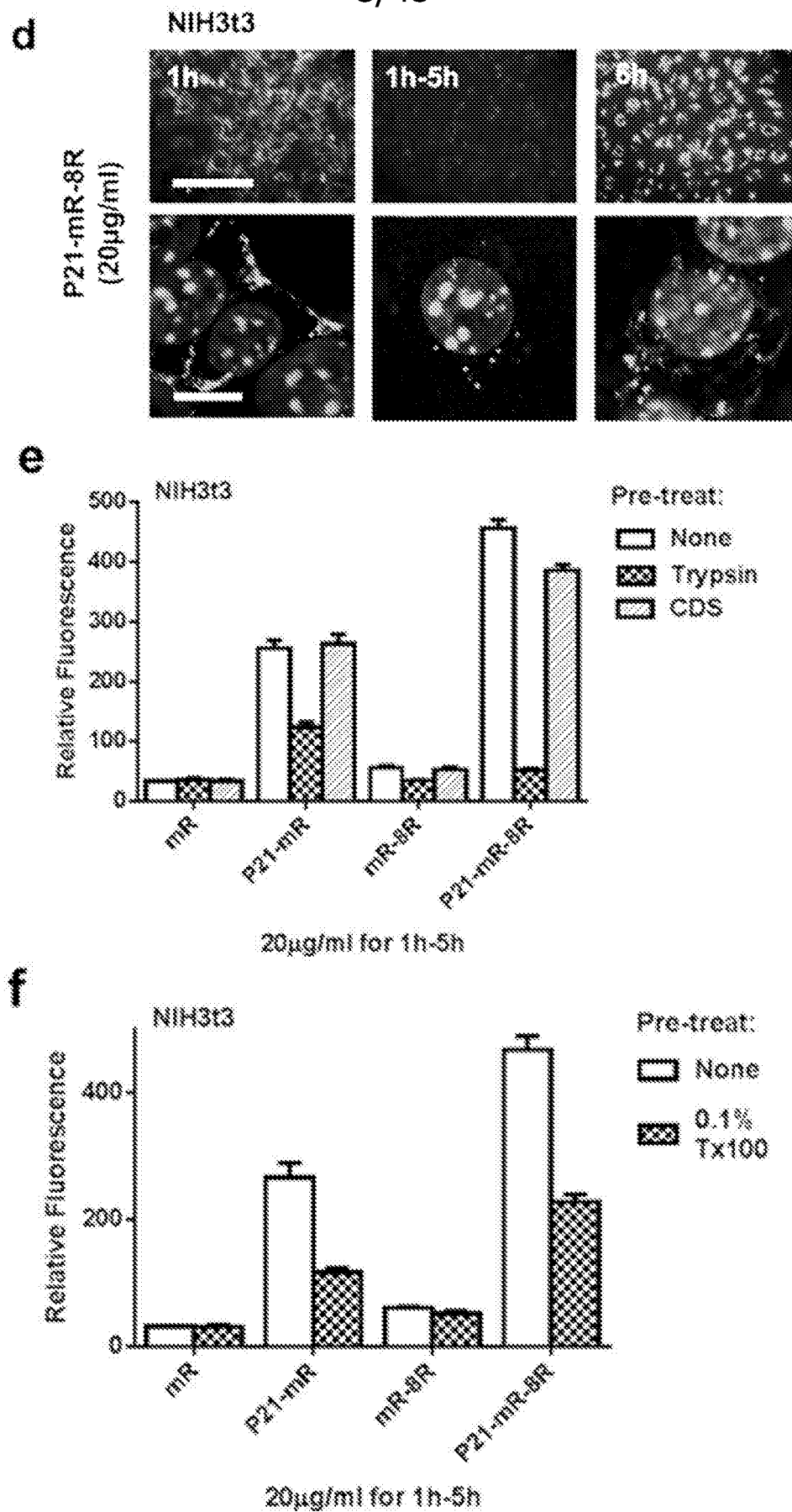
a**b**NIH3t3 (20 μ g/ml, 12h)**c**

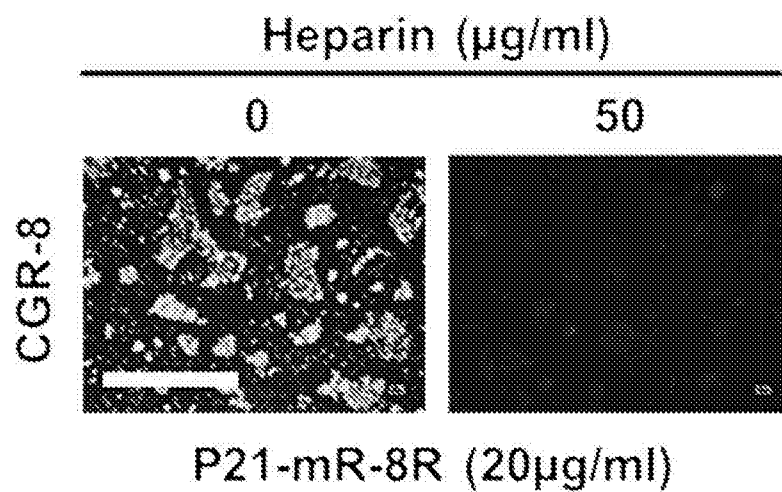
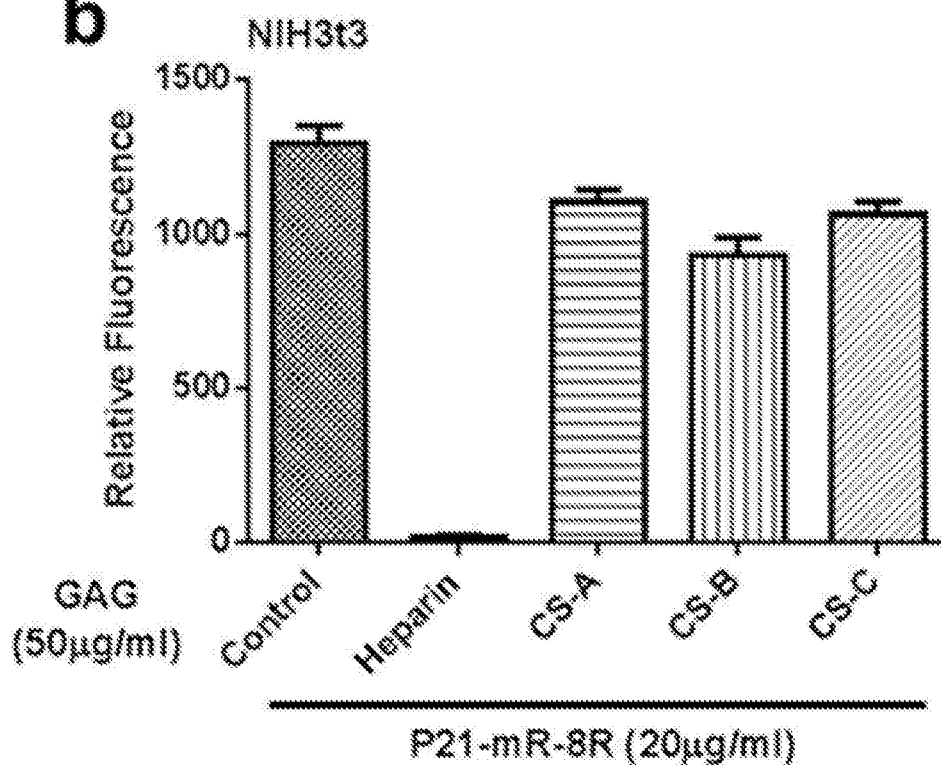
Figure 7 continued

8/43



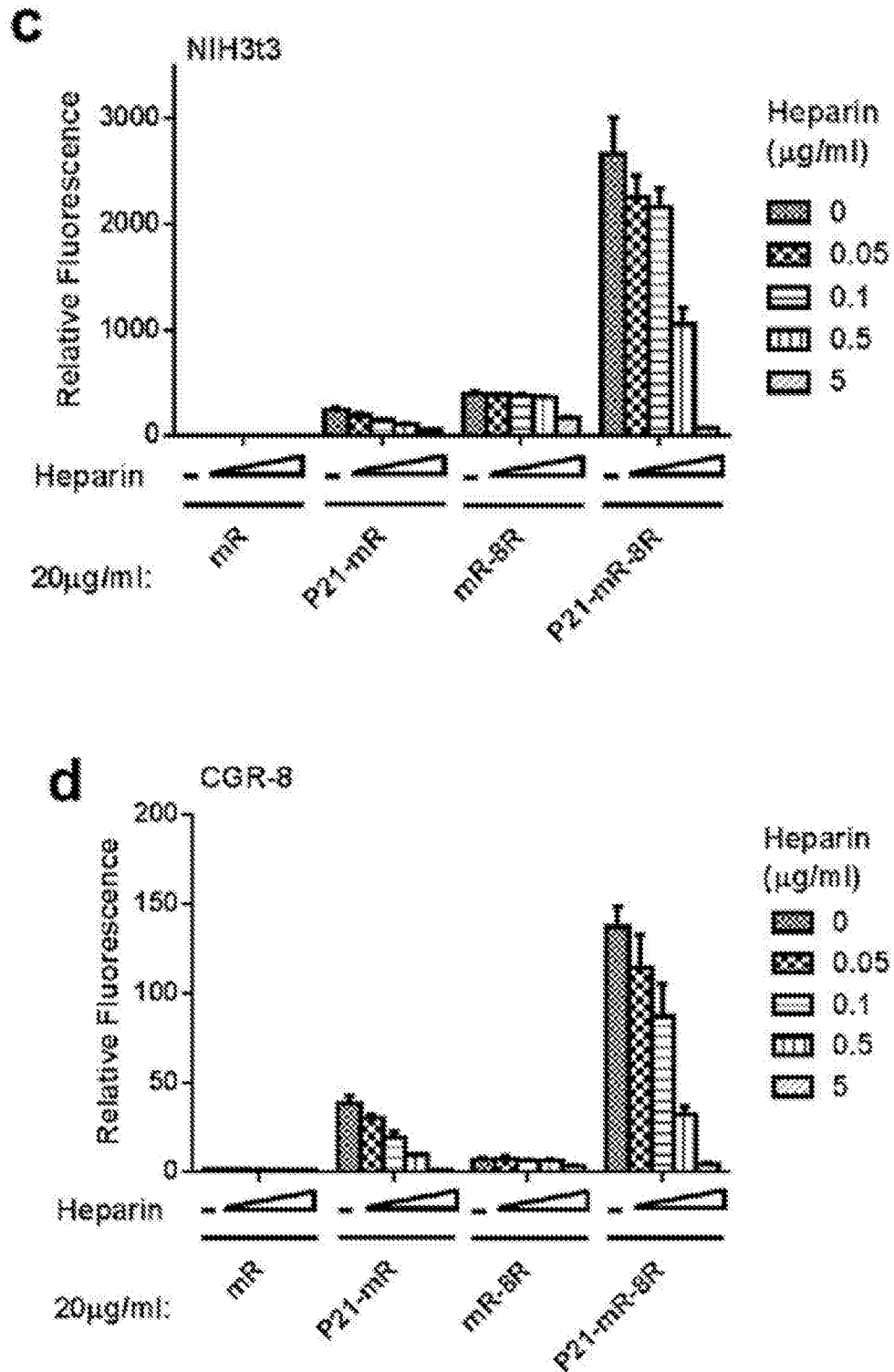
9/43

Figure 8

a**b**

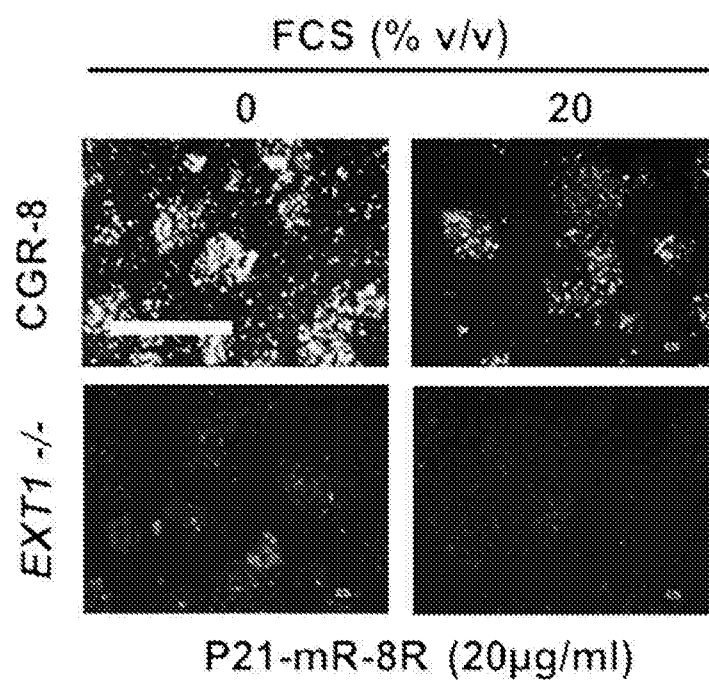
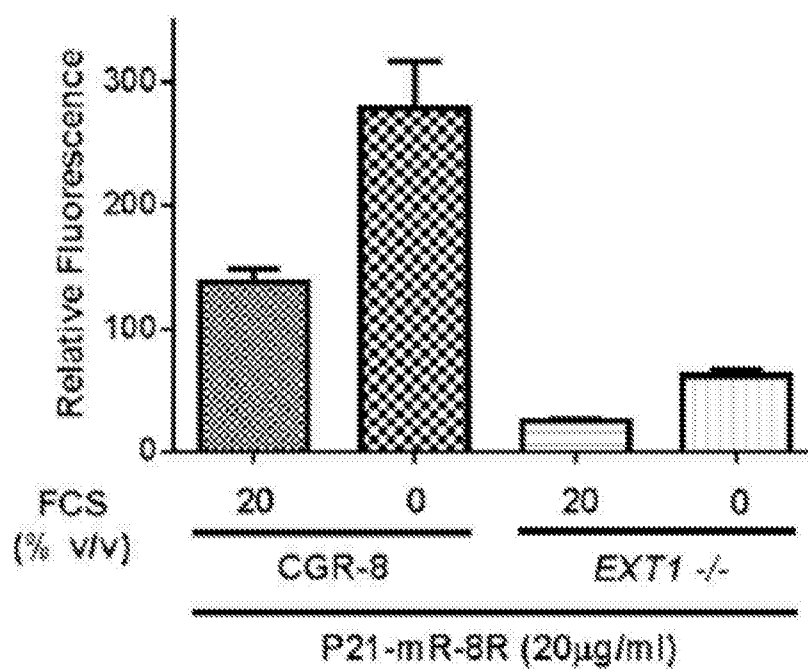
10/43

Figure 8 continued



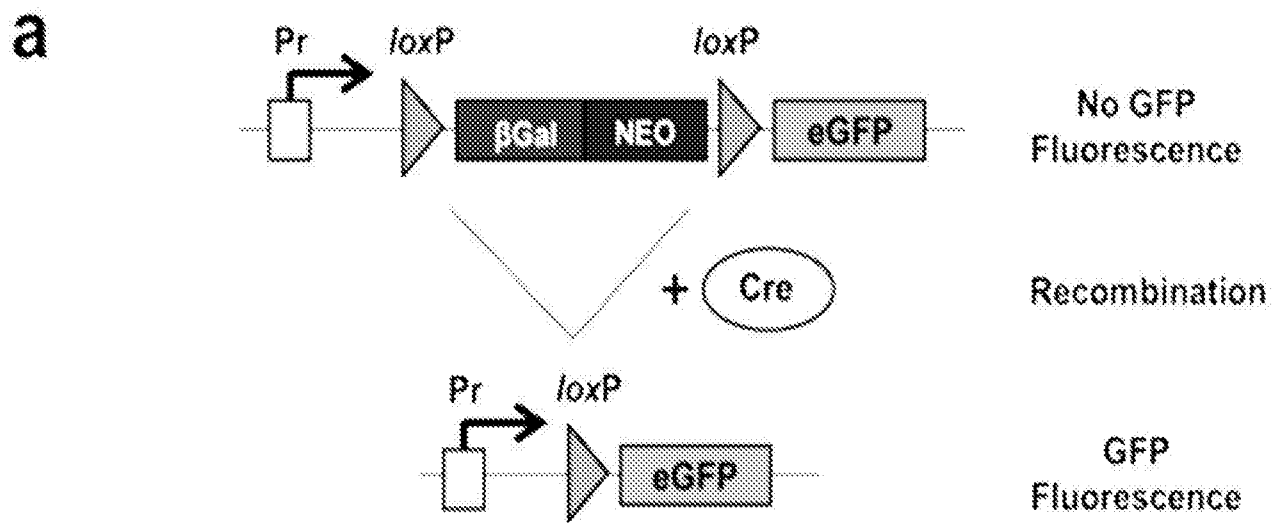
11/43

Figure 8 continued

e**f**

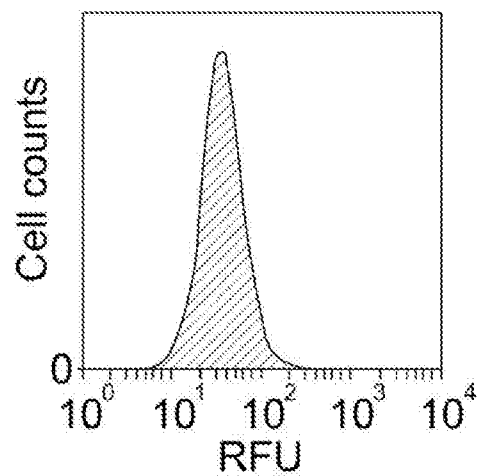
12/43

Figure 9

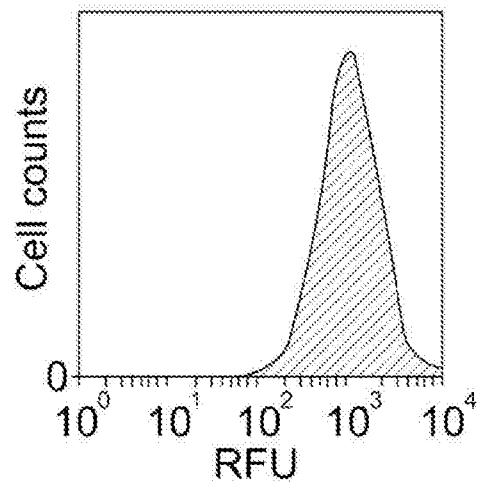
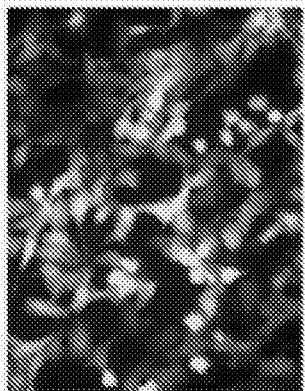
**b**

NIH3t3 LSL-eGFP

None

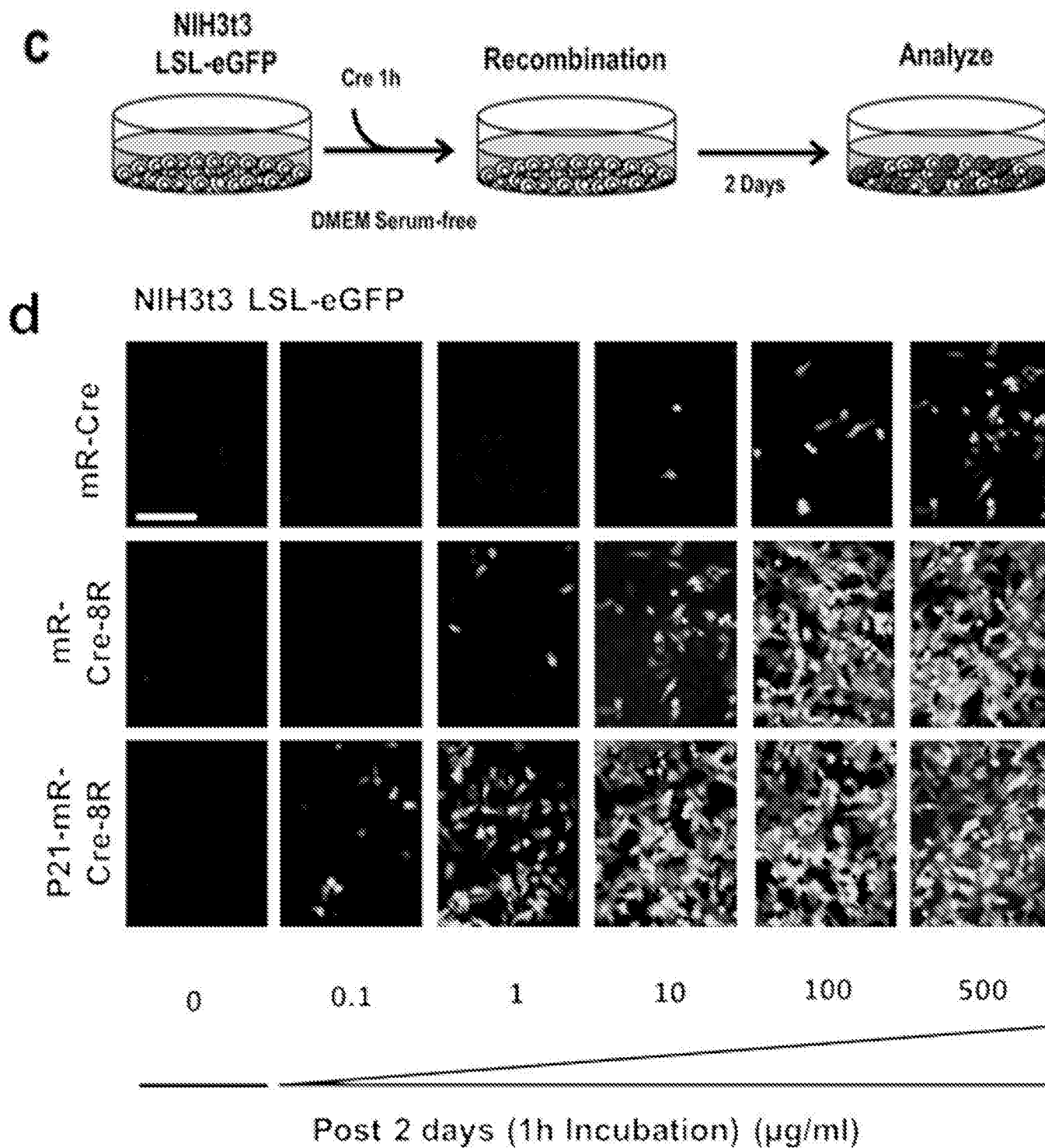


SIN Cre



13/43

Figure 9 continued



14/43

Figure 9 continued

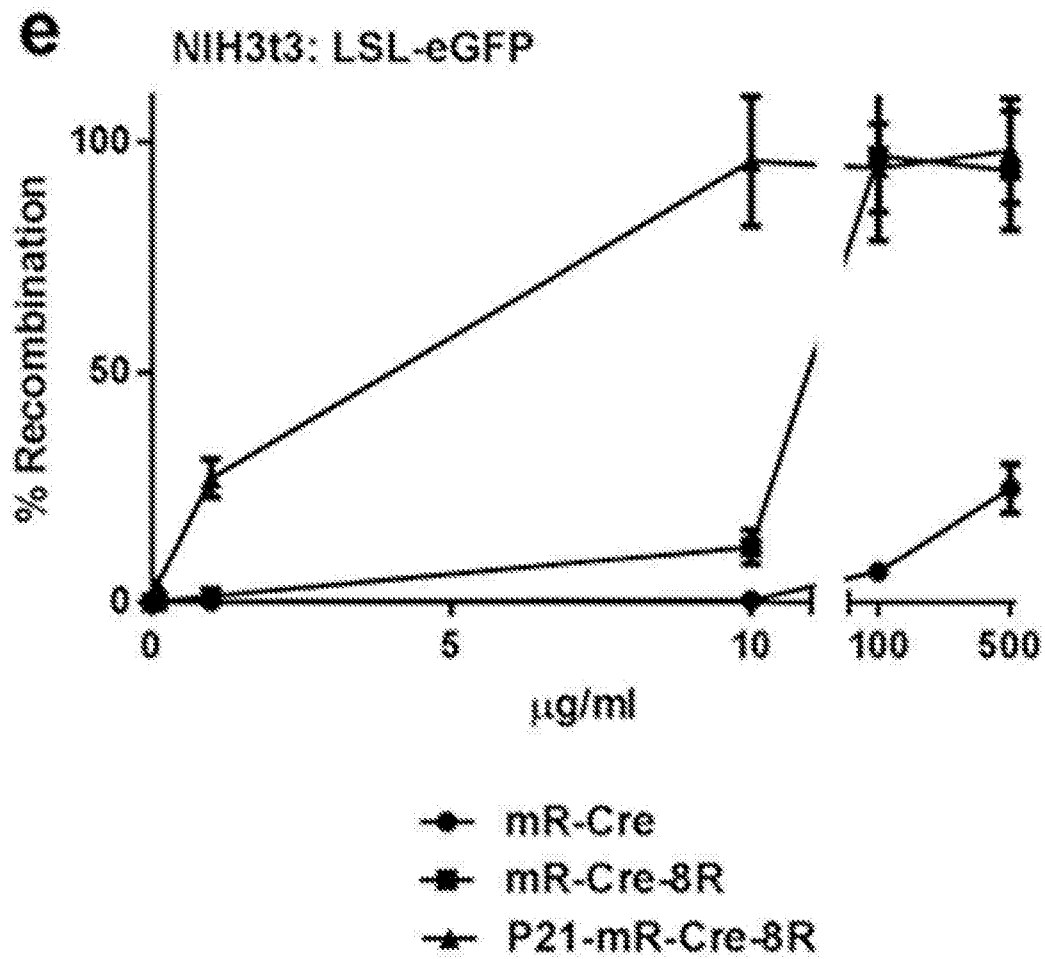
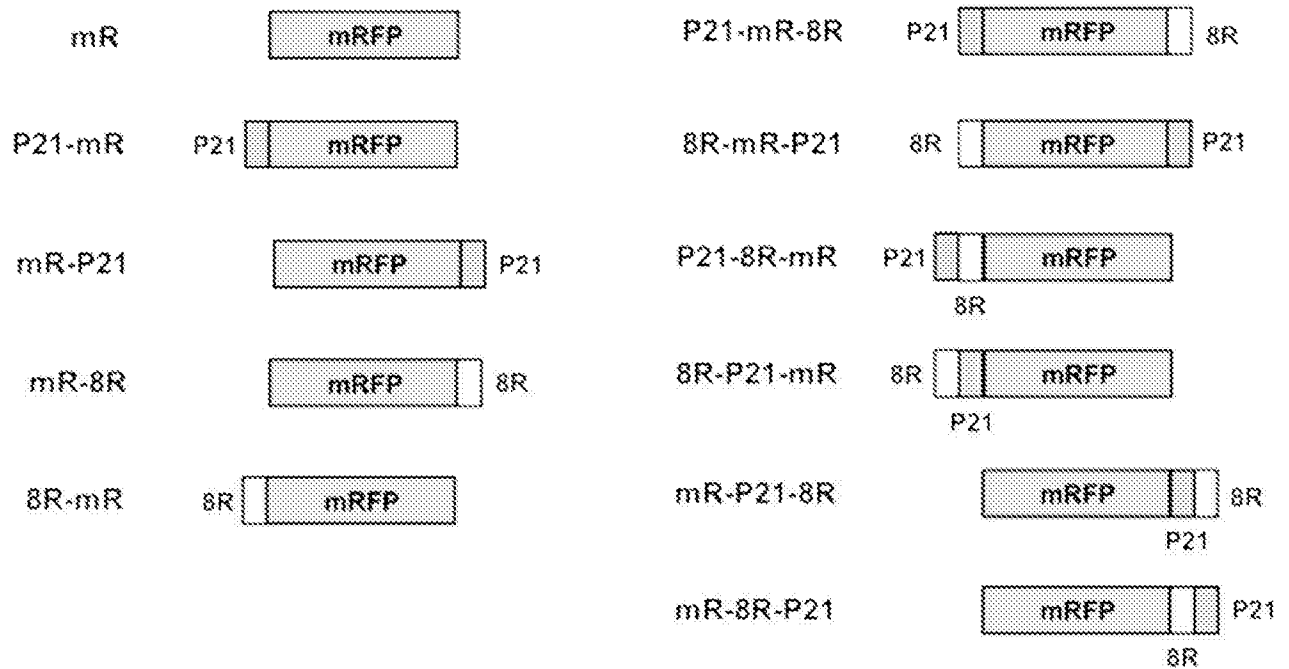
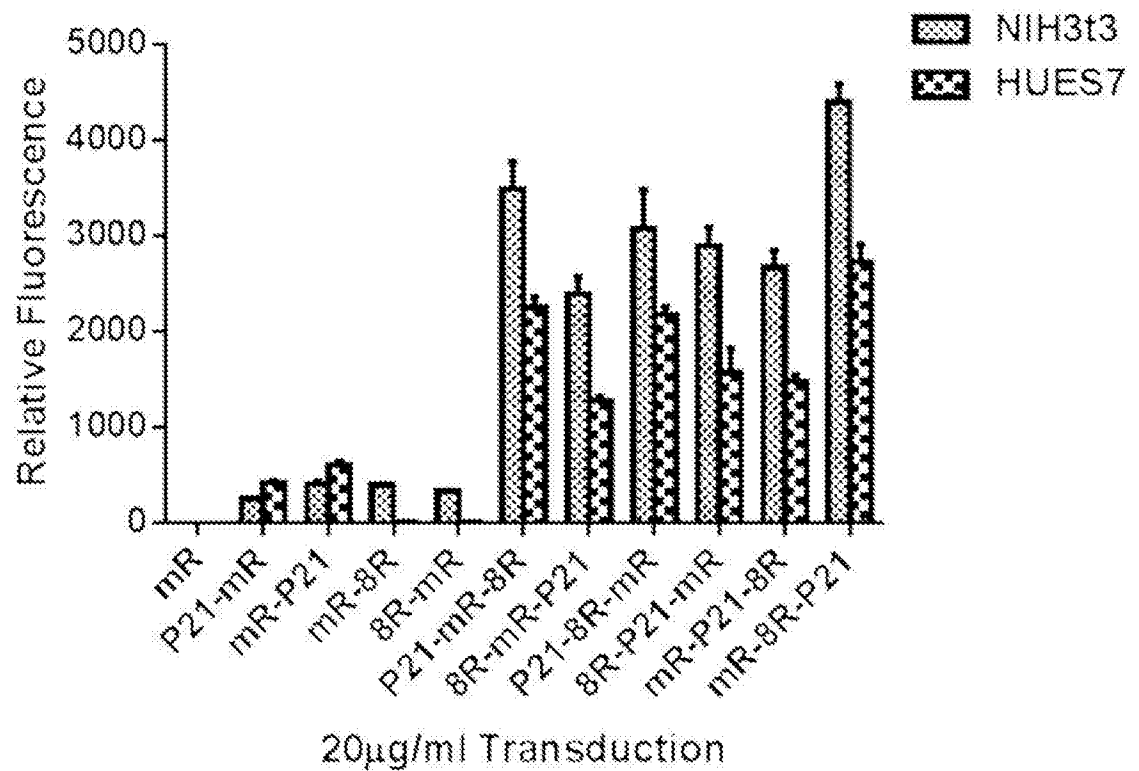


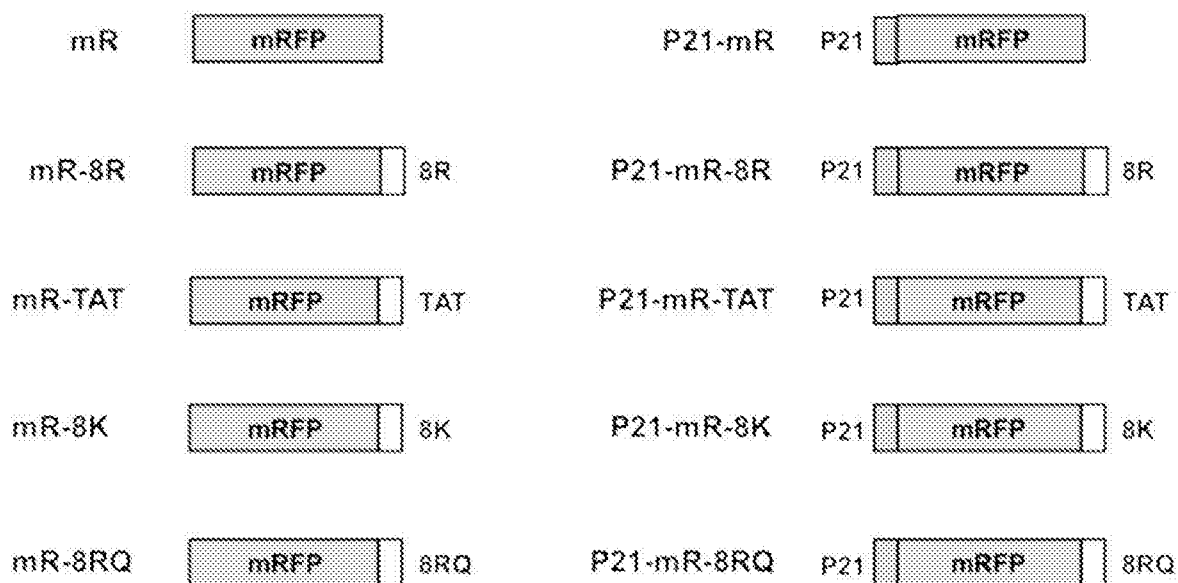
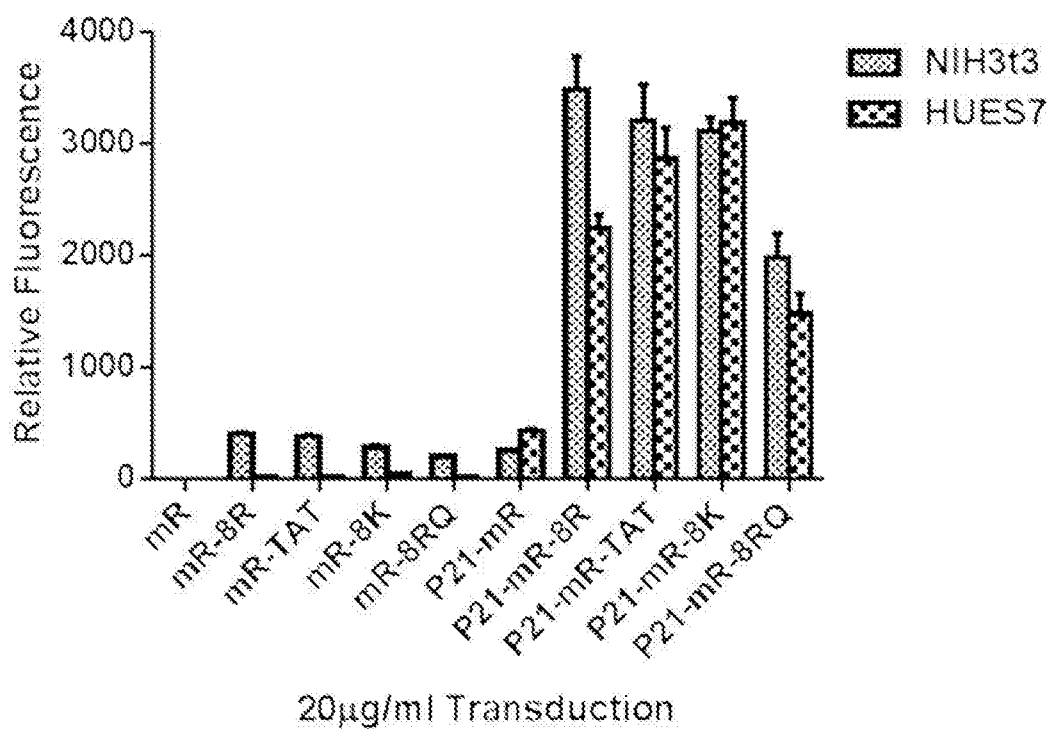
Figure 10

15/43

a**b**

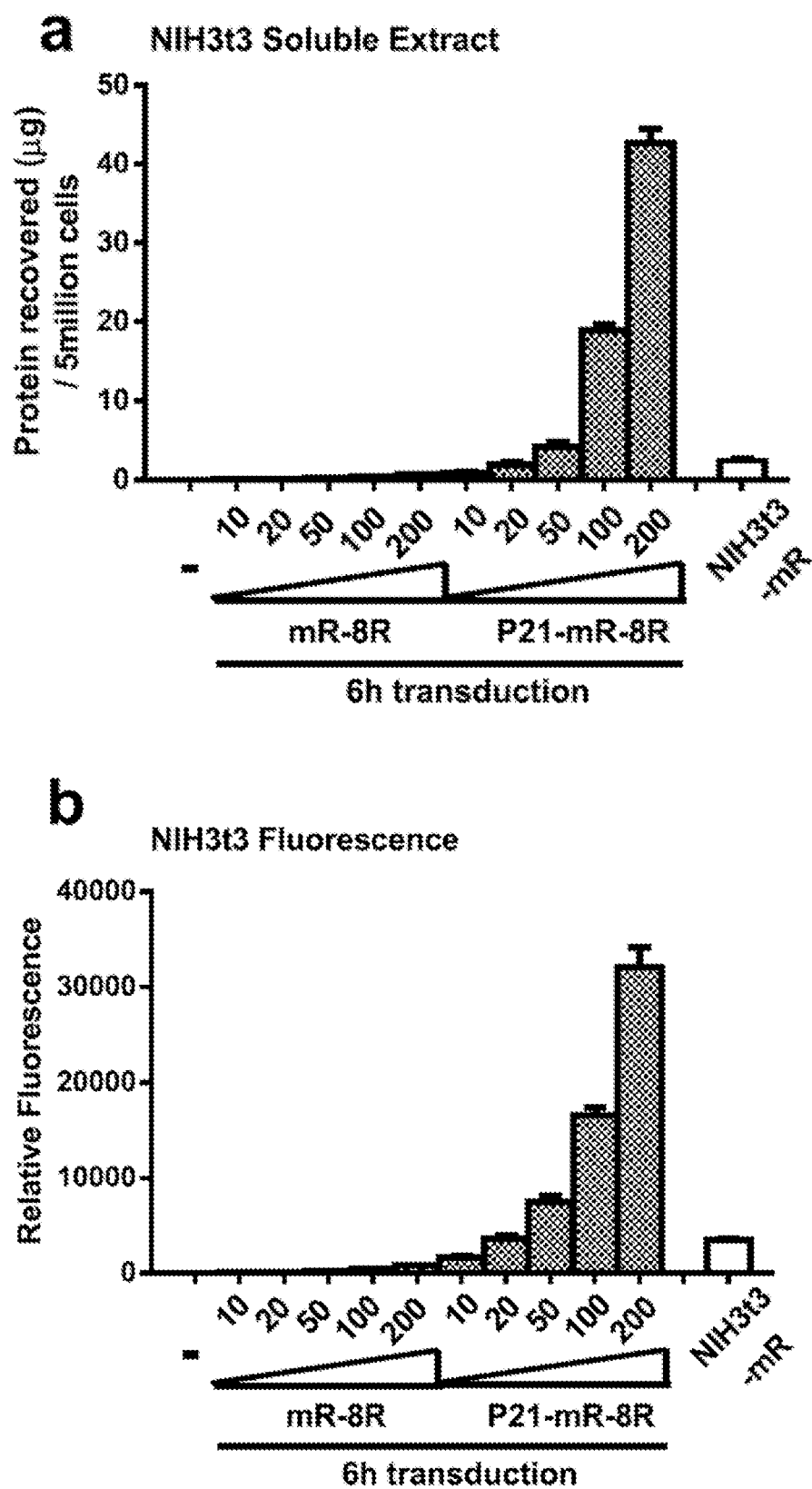
16/43

Figure 11

a**b**

17/43

Figure 12



18/43

Figure 13

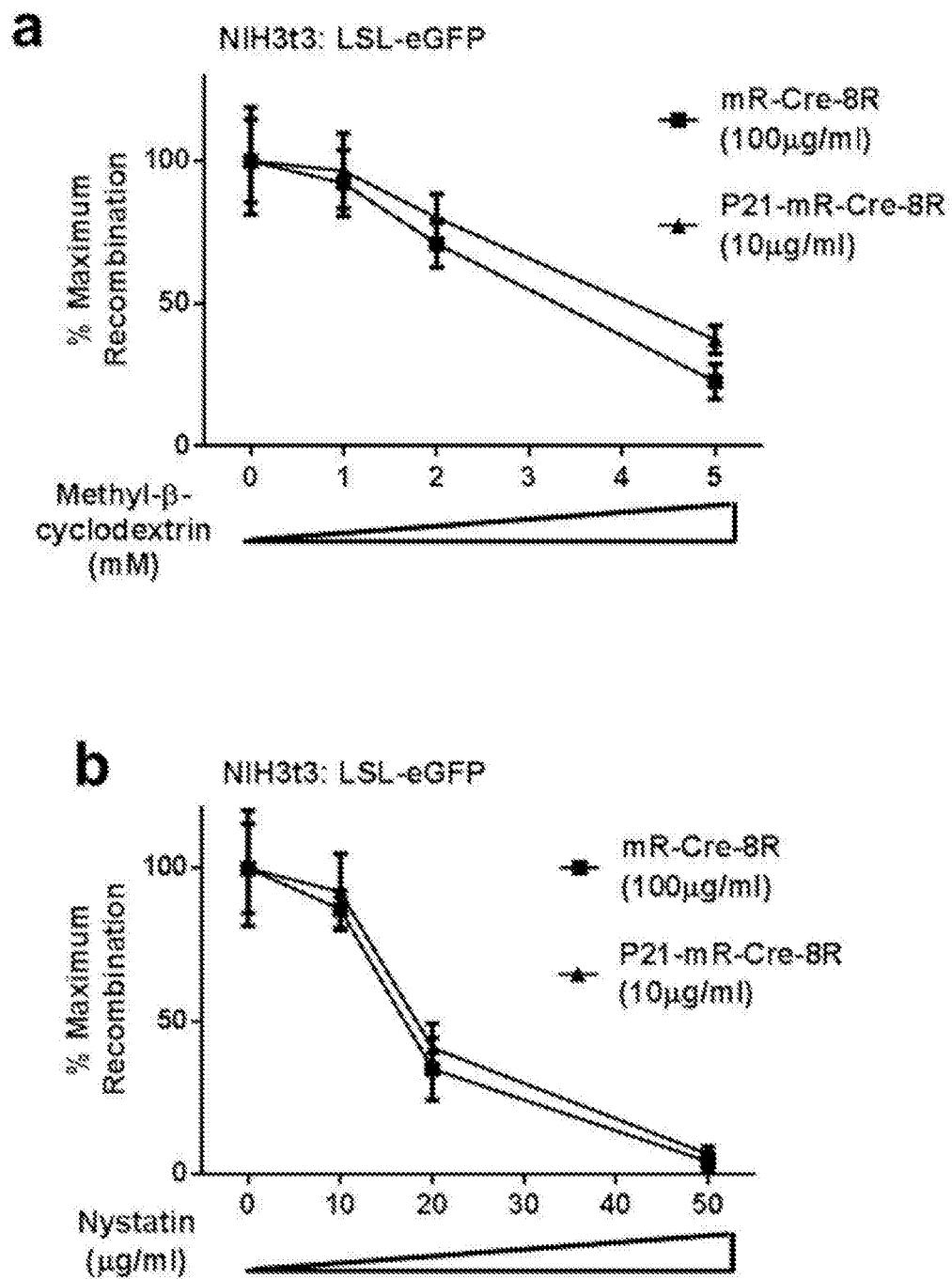
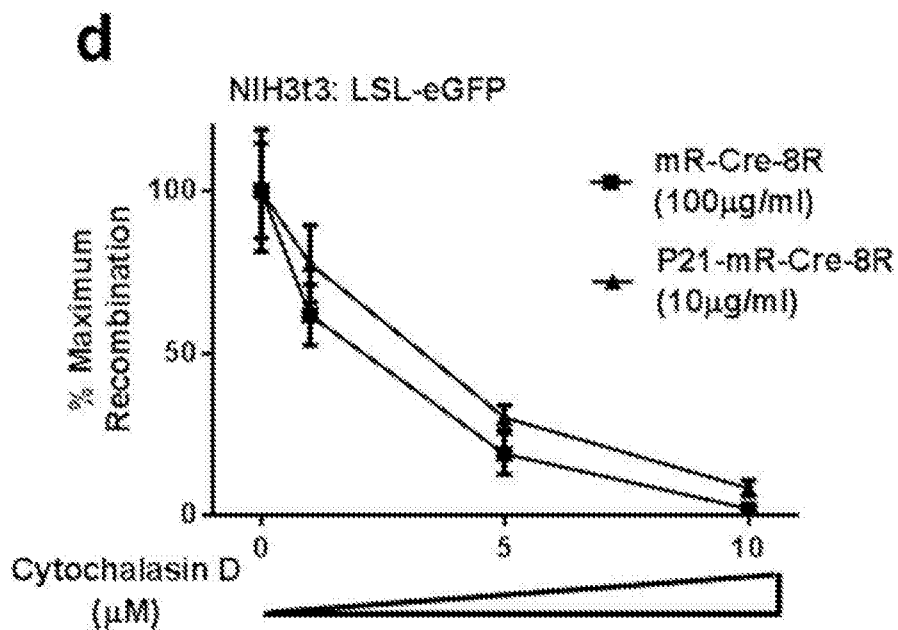
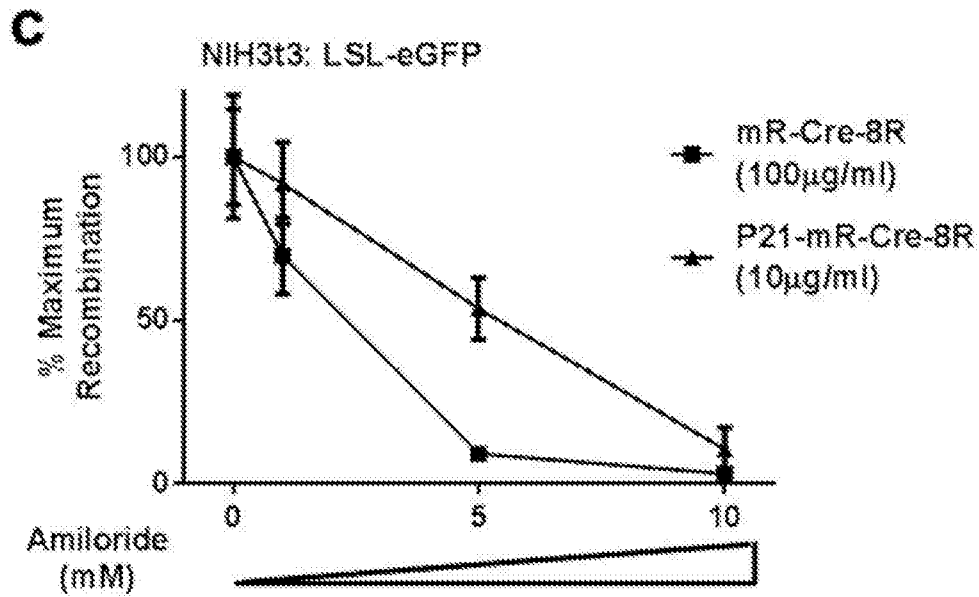


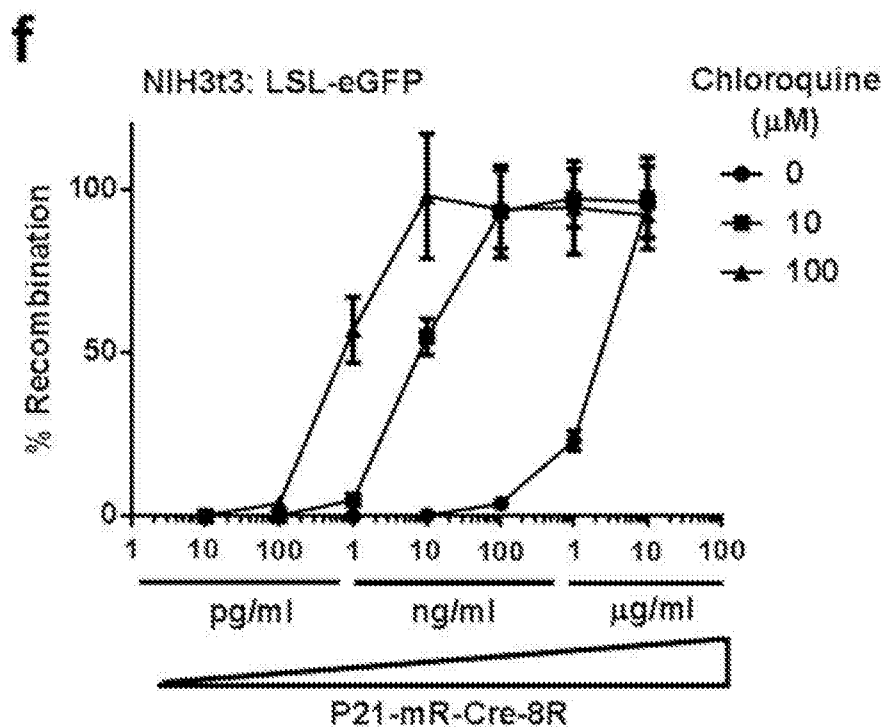
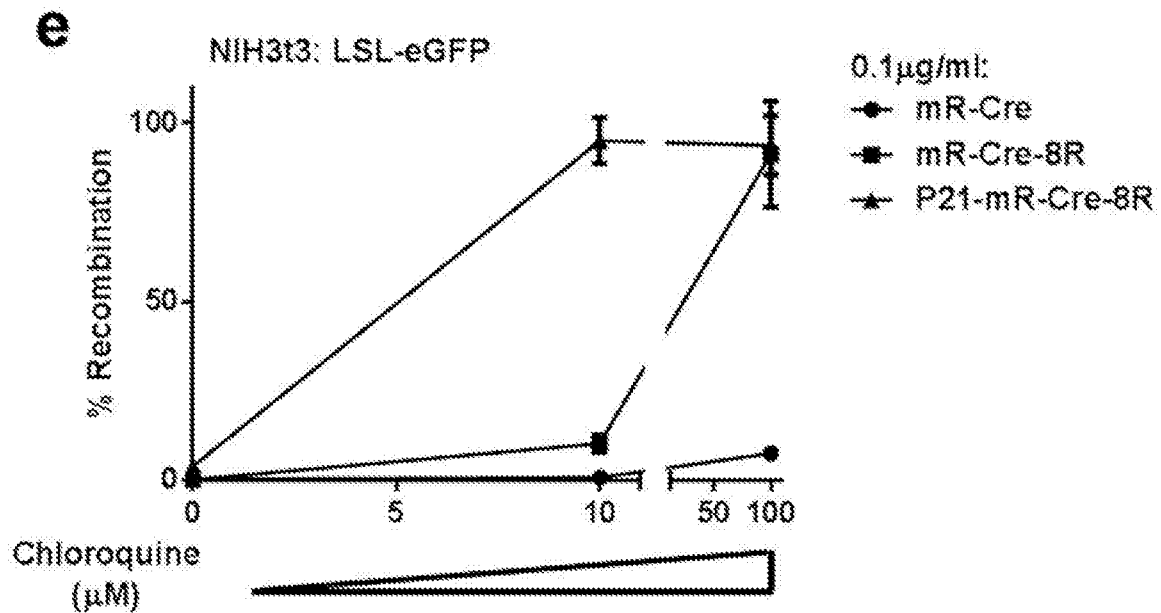
Figure 13 continued

19/43



20/43

Figure 13 continued



21/43

Figure 14

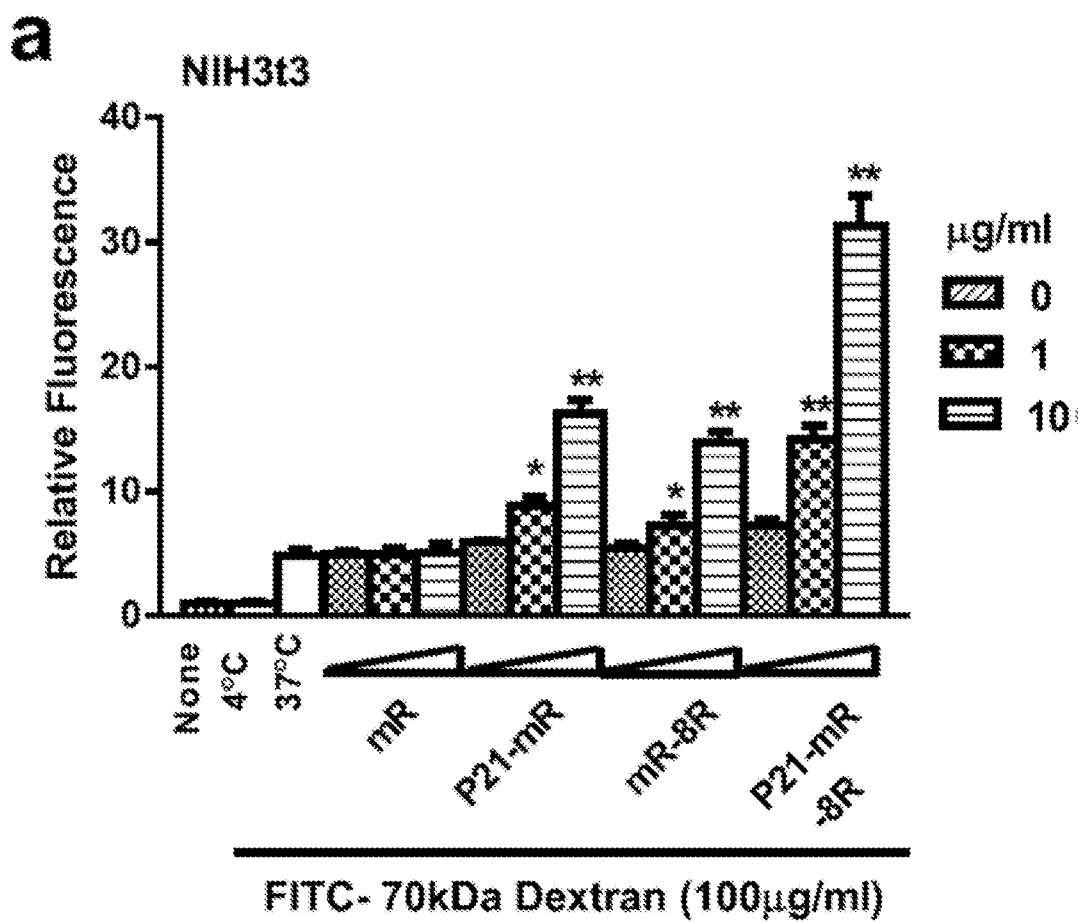


Figure 15

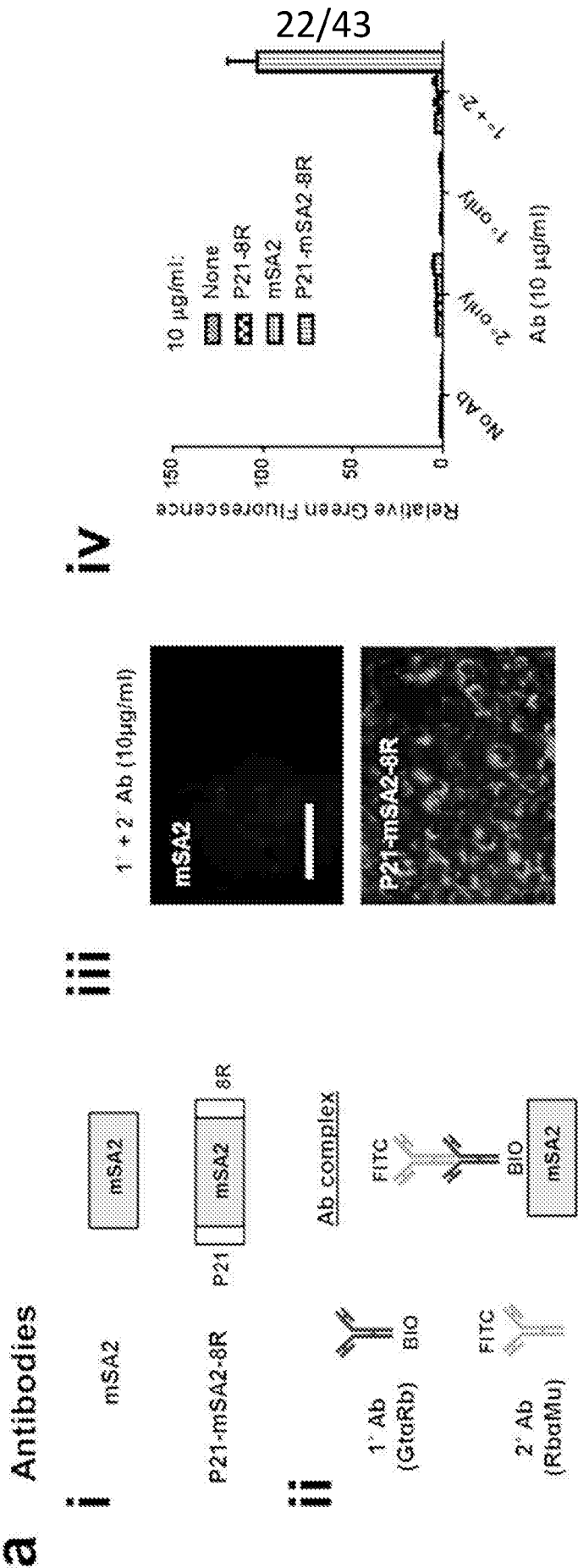


Figure 15 continued

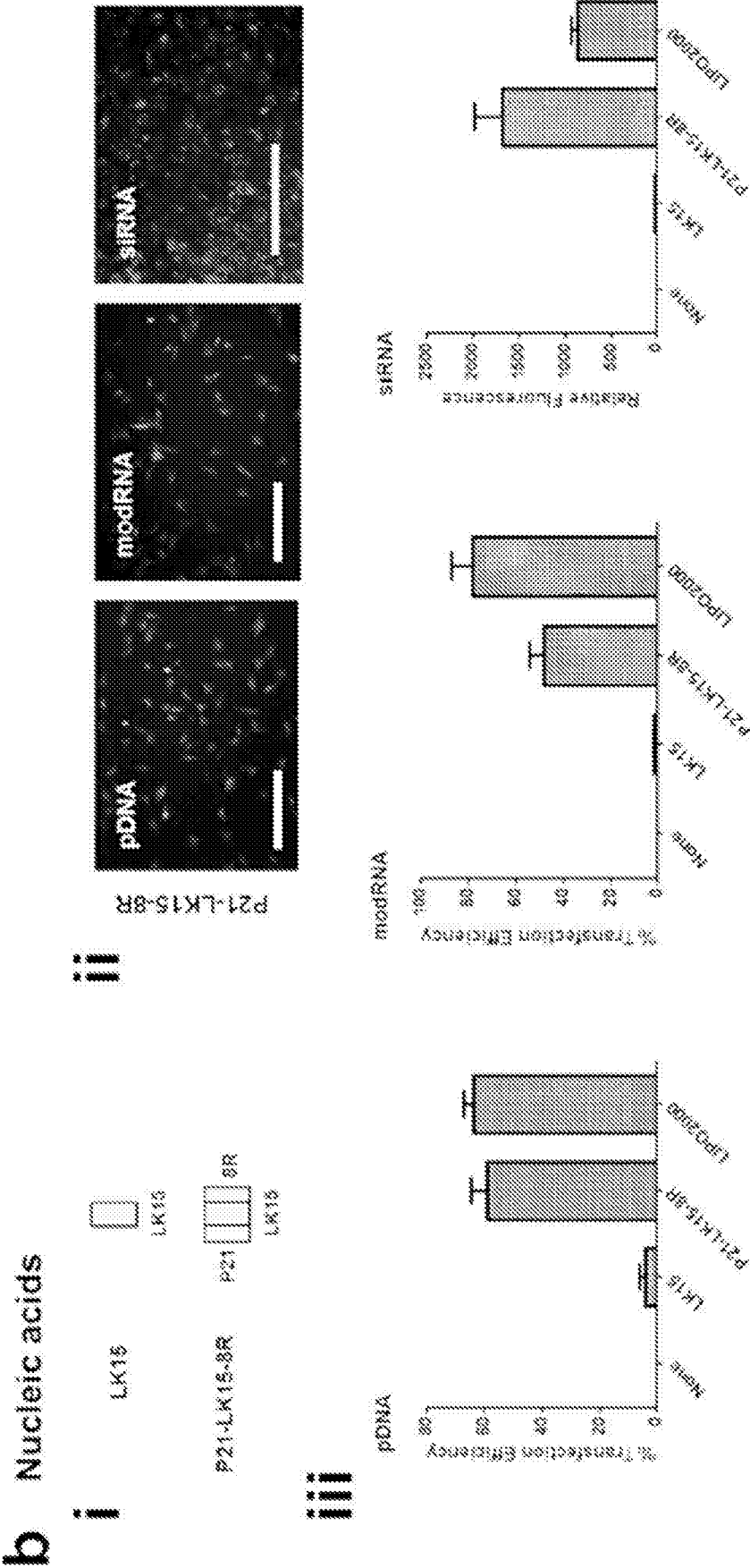


Figure15 continued

24/43

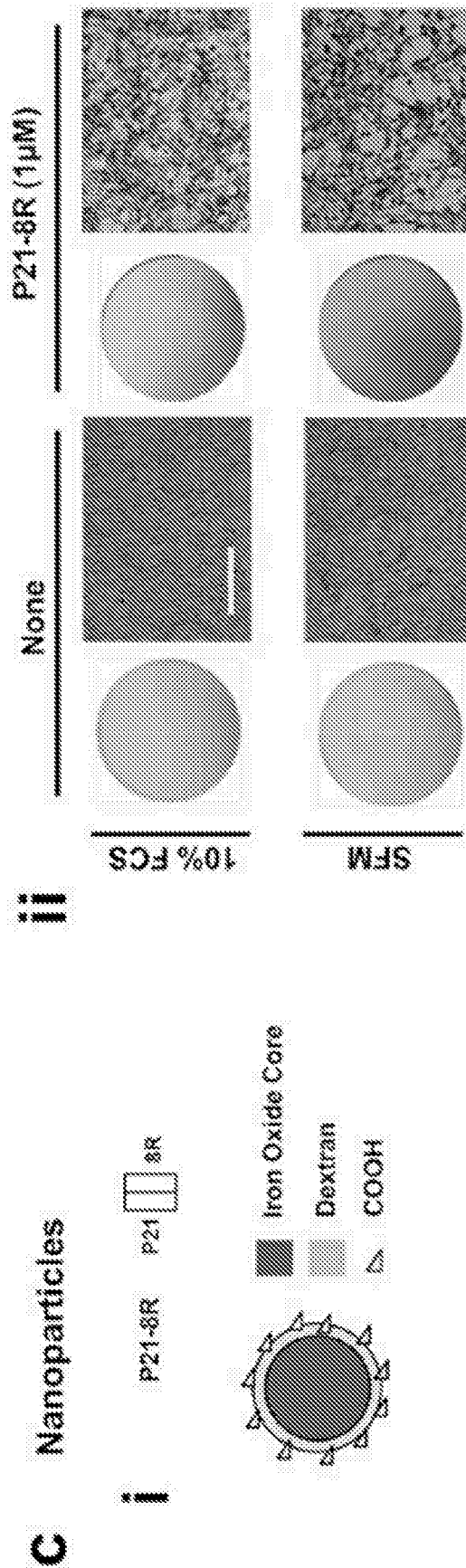
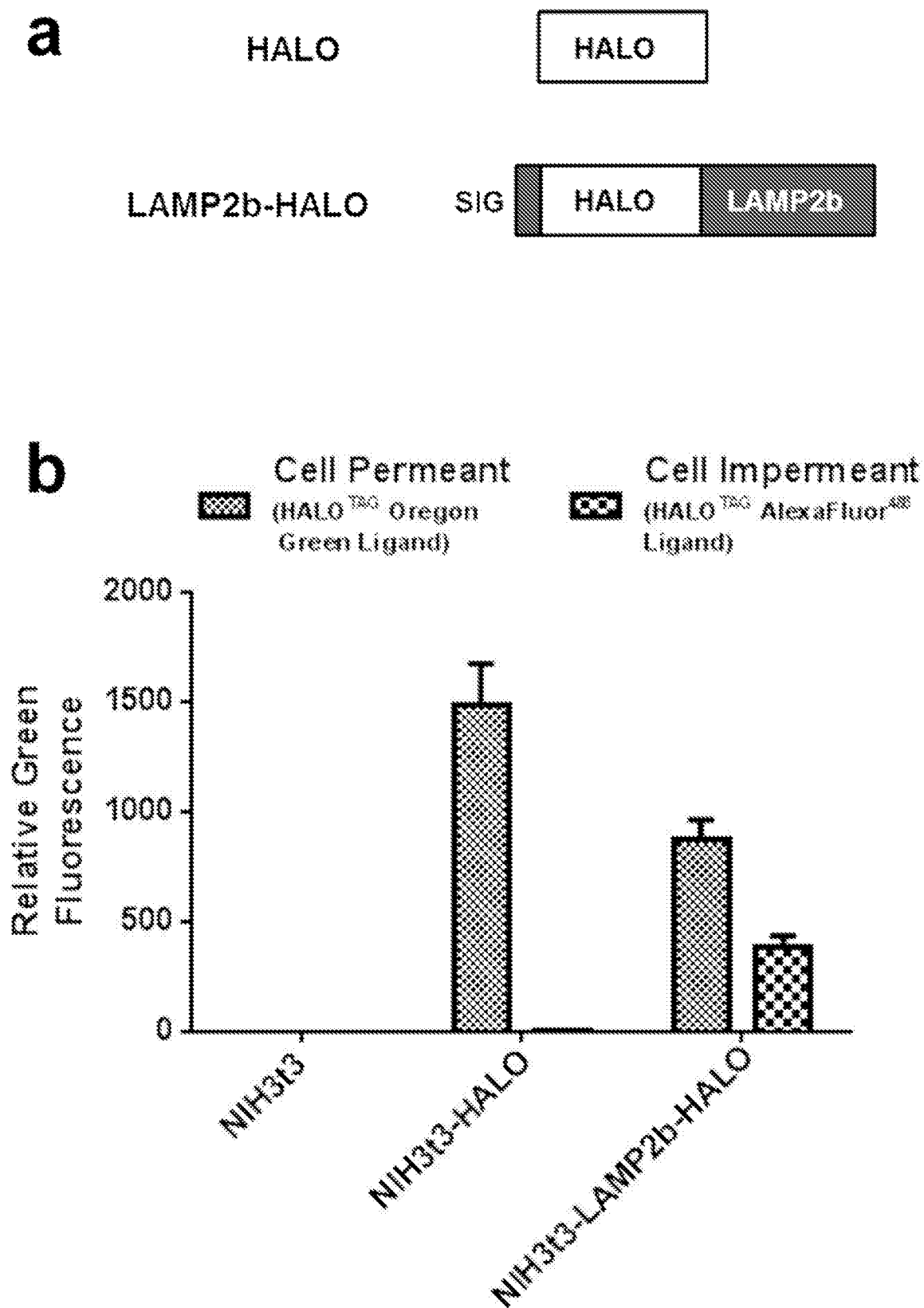


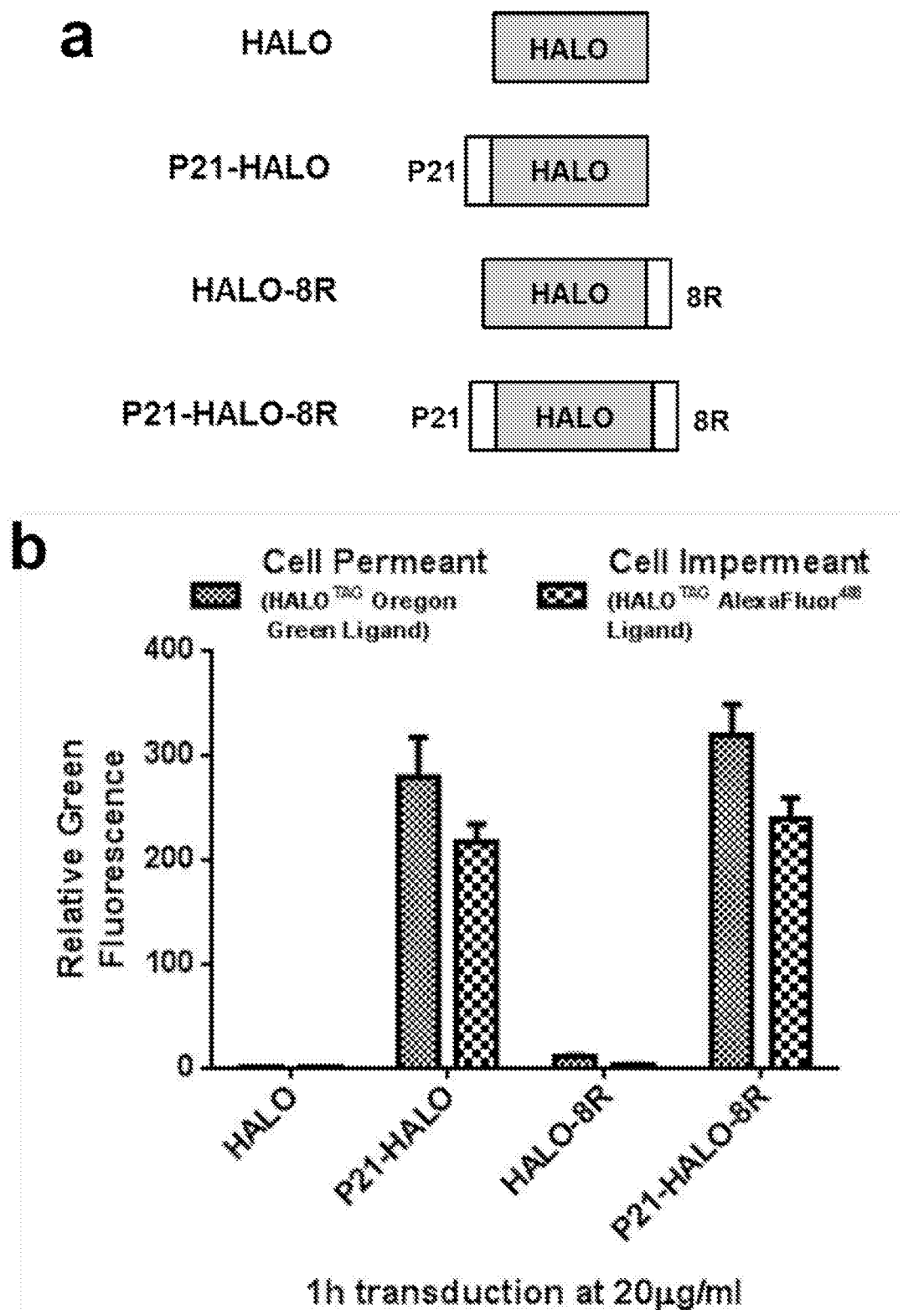
Figure 16

25/43



26/43

Figure 17



27/43

Figure 17 continued

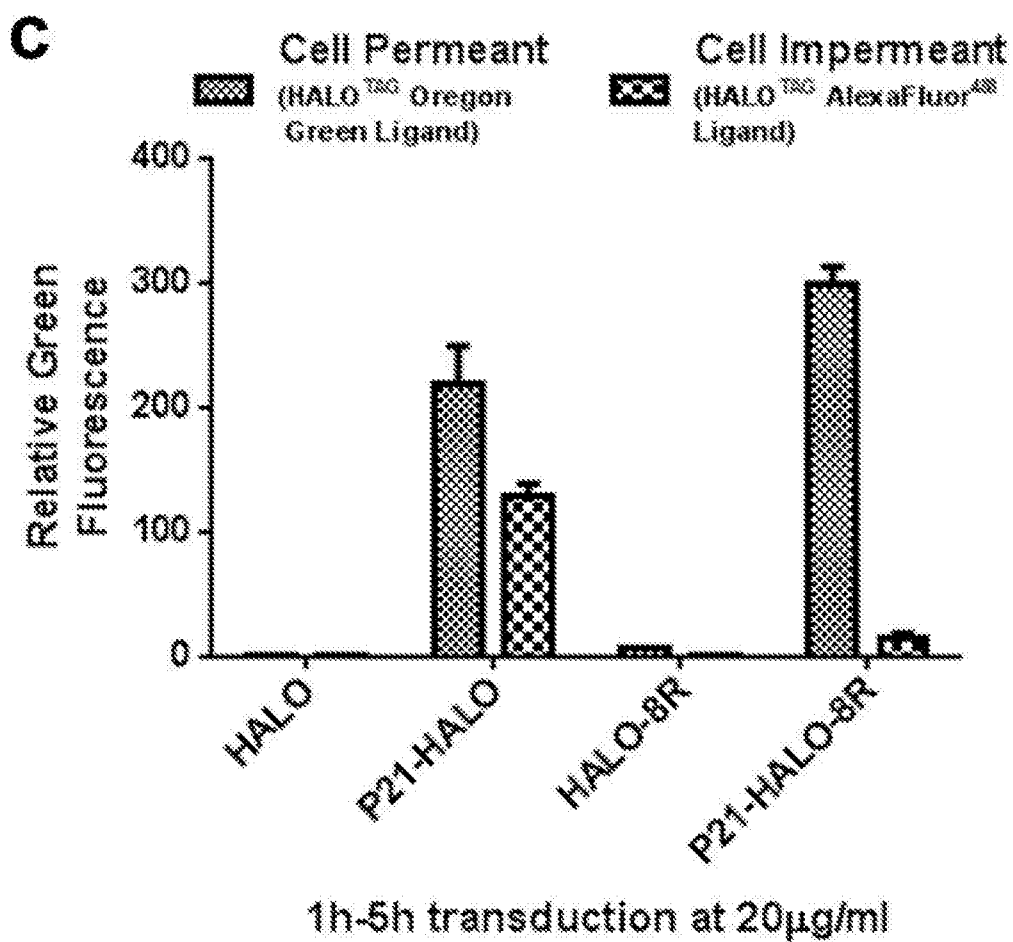


Figure 18

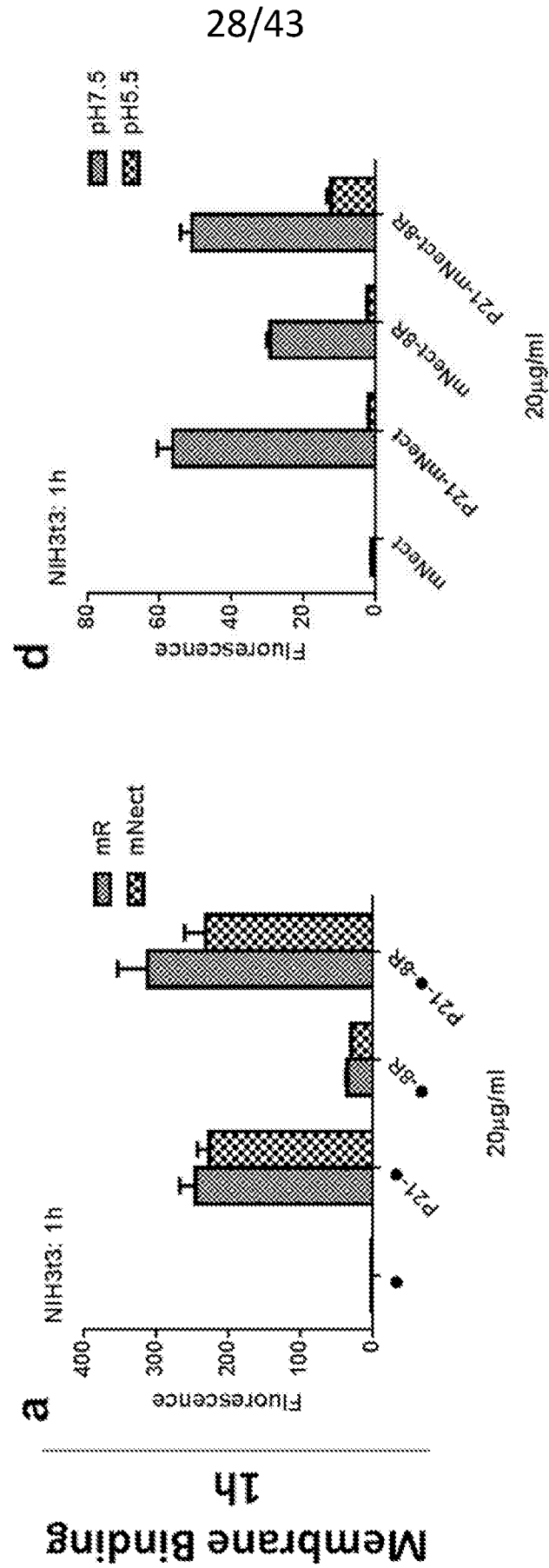


Figure 18 continued

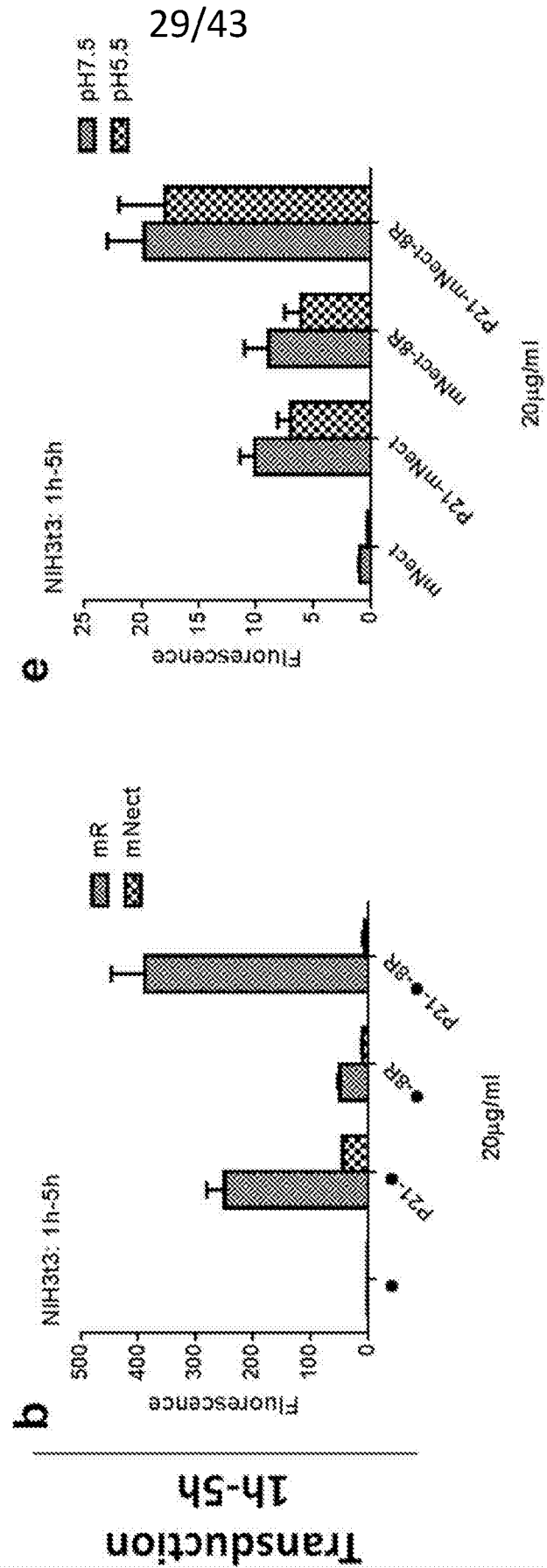
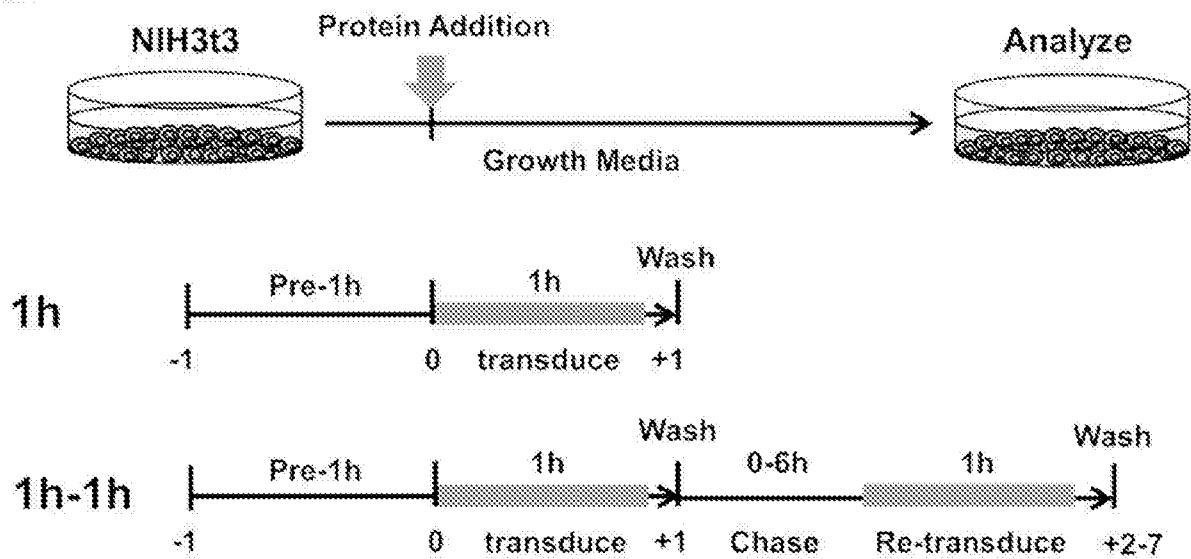


Figure 18 continued

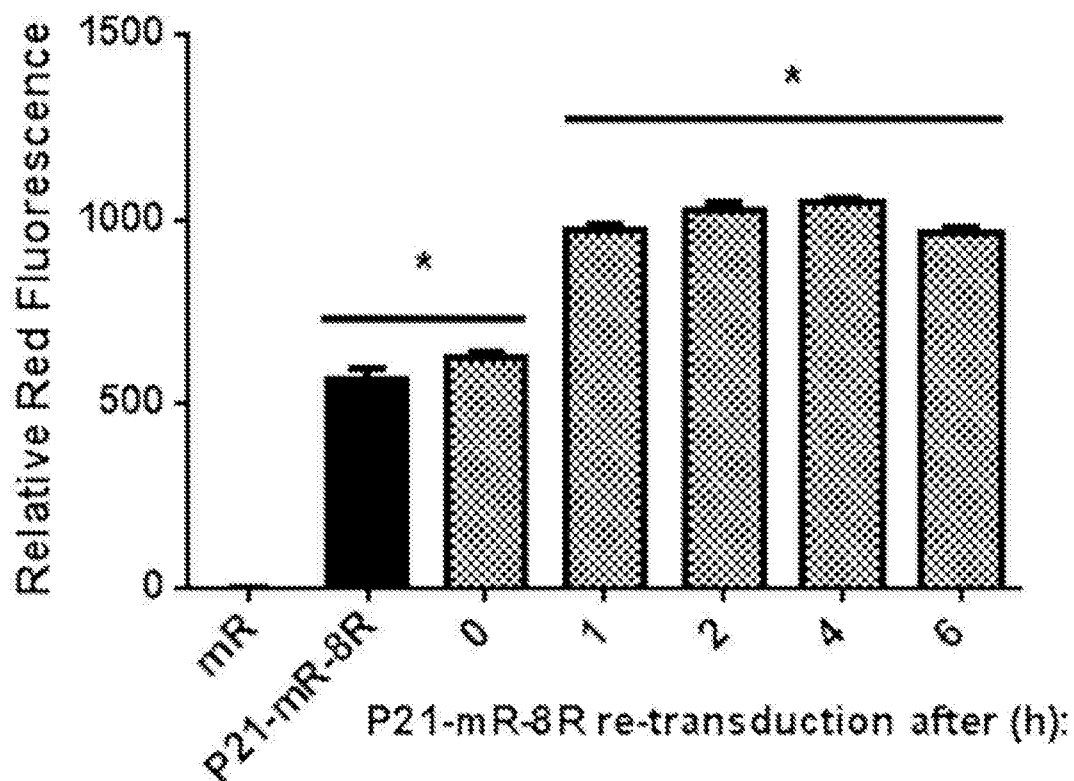


31/43

Figure 19

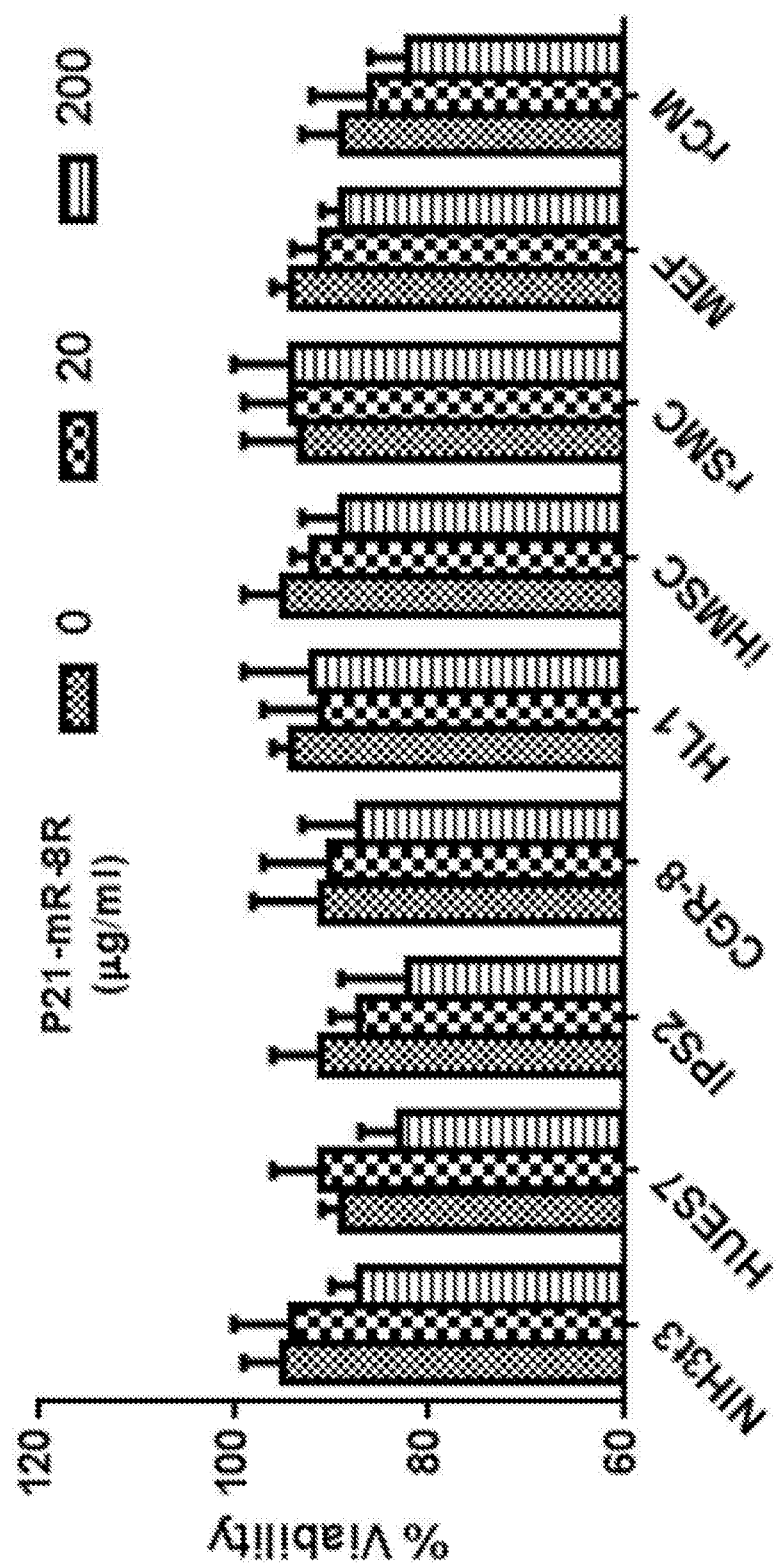
a**b**

NIH3T3: Re-transduction



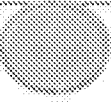
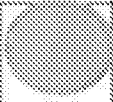
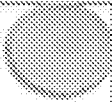
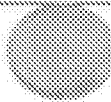
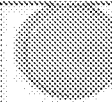
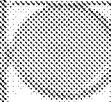
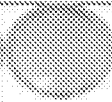
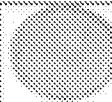
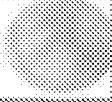
32/43

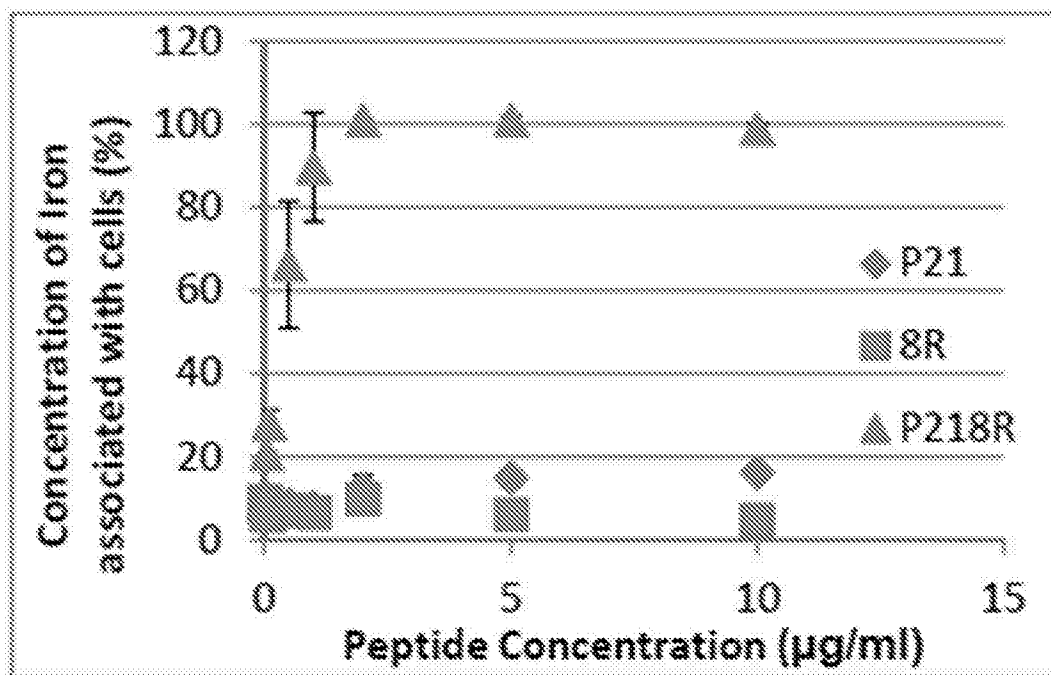
Figure 20



33/43

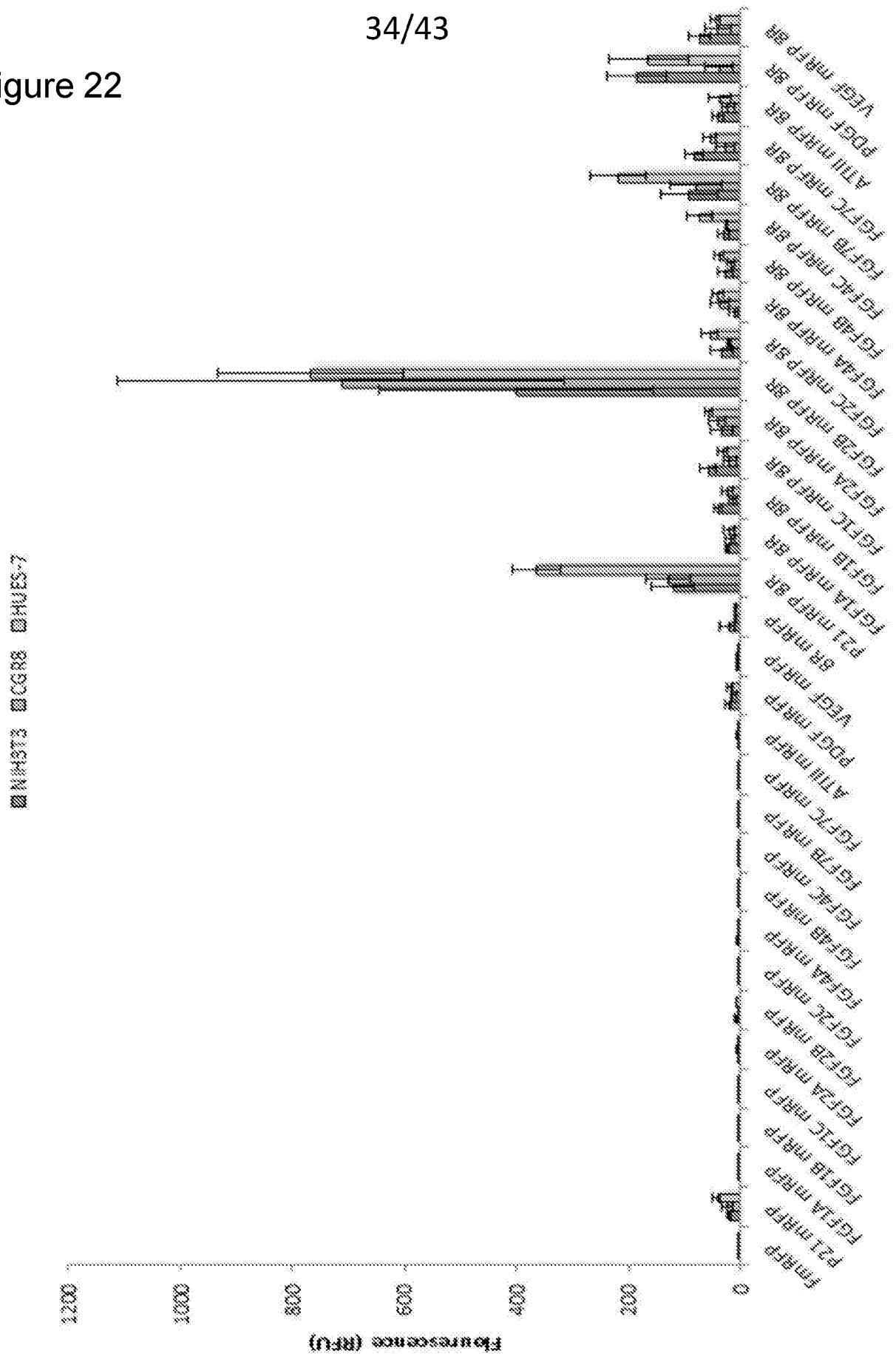
Figure 21

NIH 3t3: Incubated for 24 hours with 50 μ g Nanomag-D (250 nm)									
Peptide Conc (μ g/ml)	0	0.01	0.05	0.1	0.5	1	2	5	10
P21									
8R									
P218R									



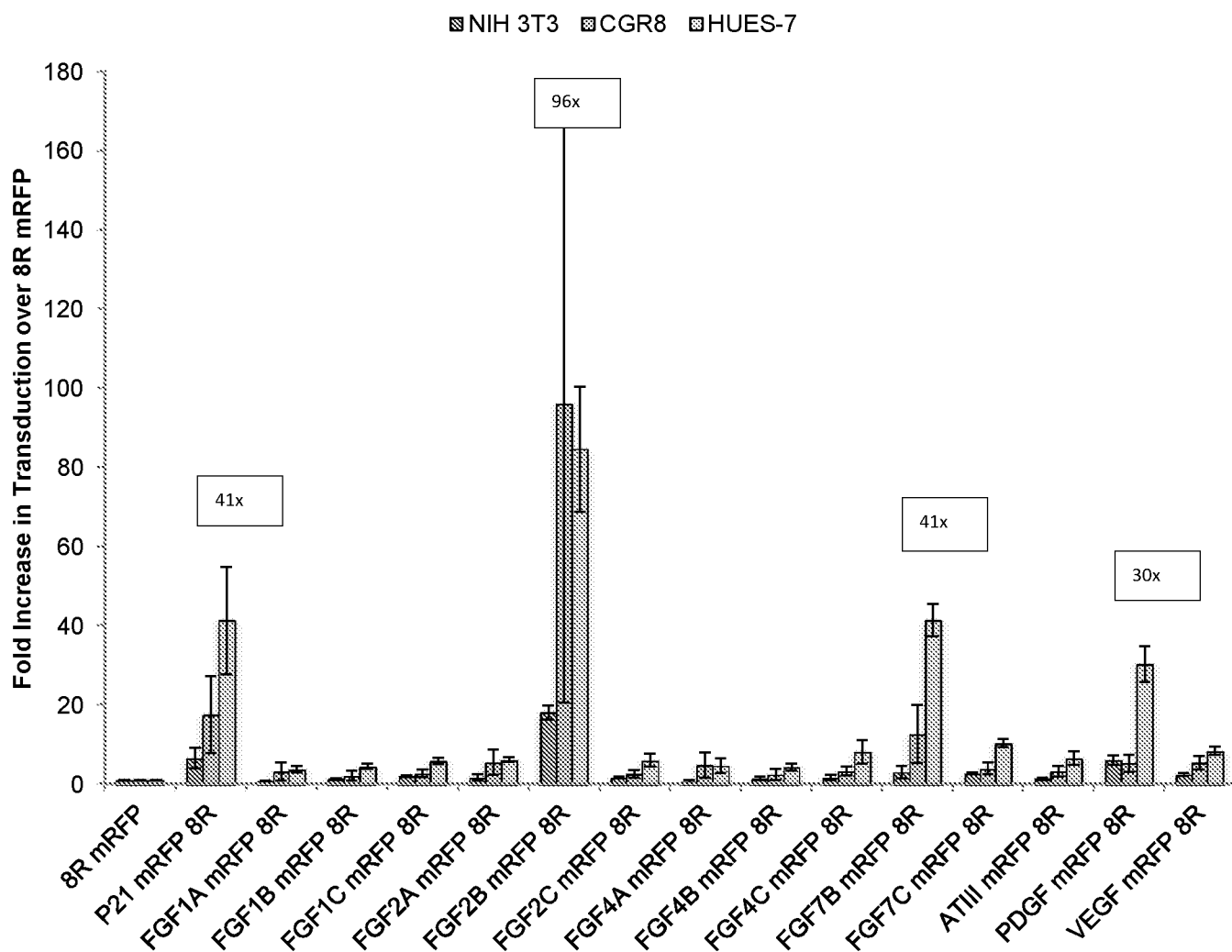
34/43

Figure 22



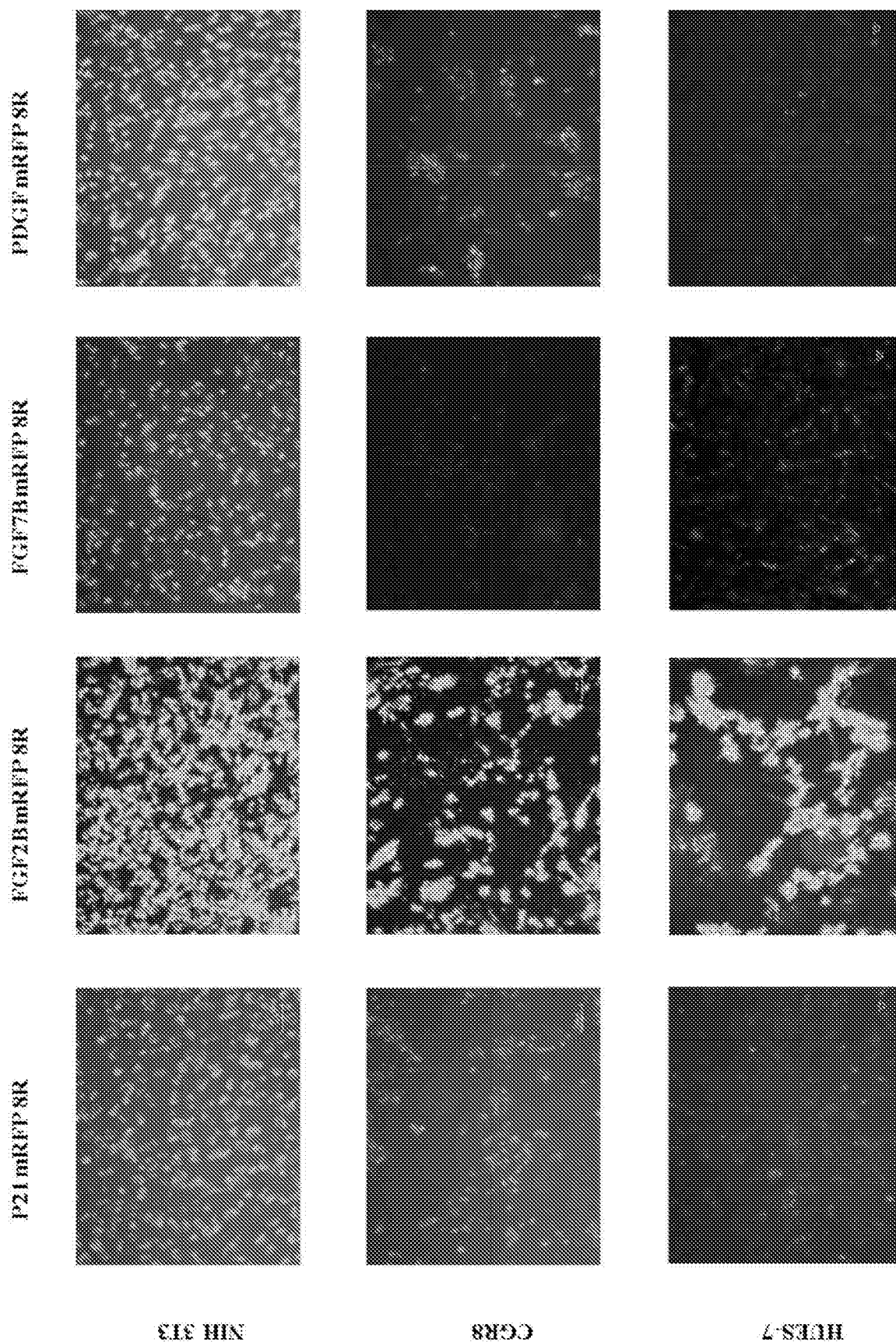
35/43

Figure 23



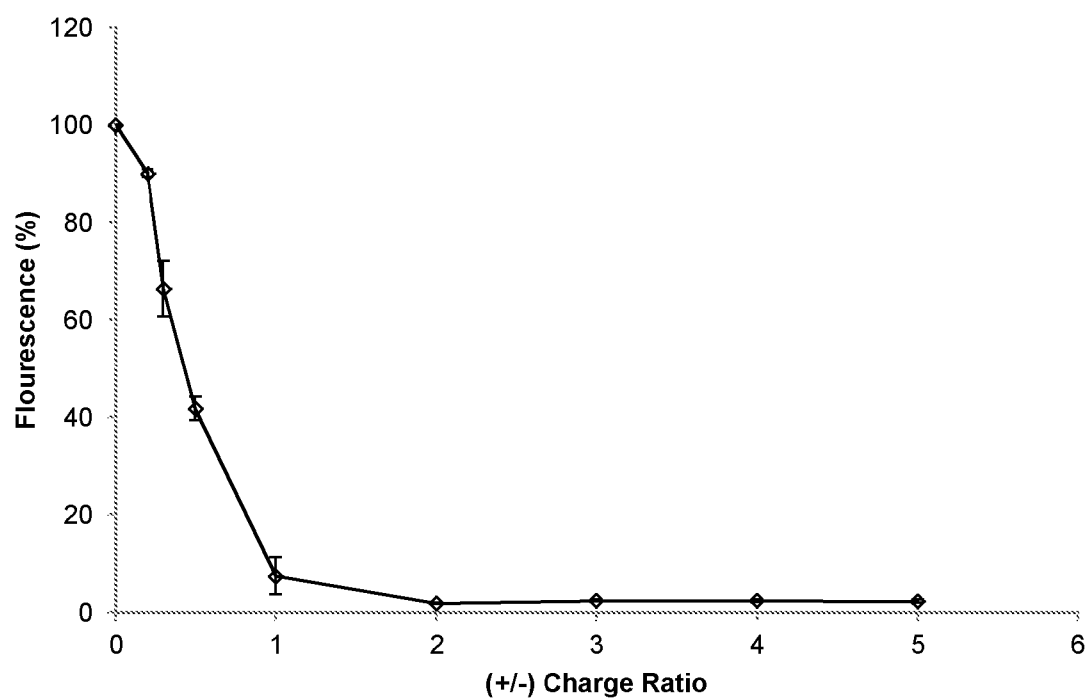
36/43

Figure 24



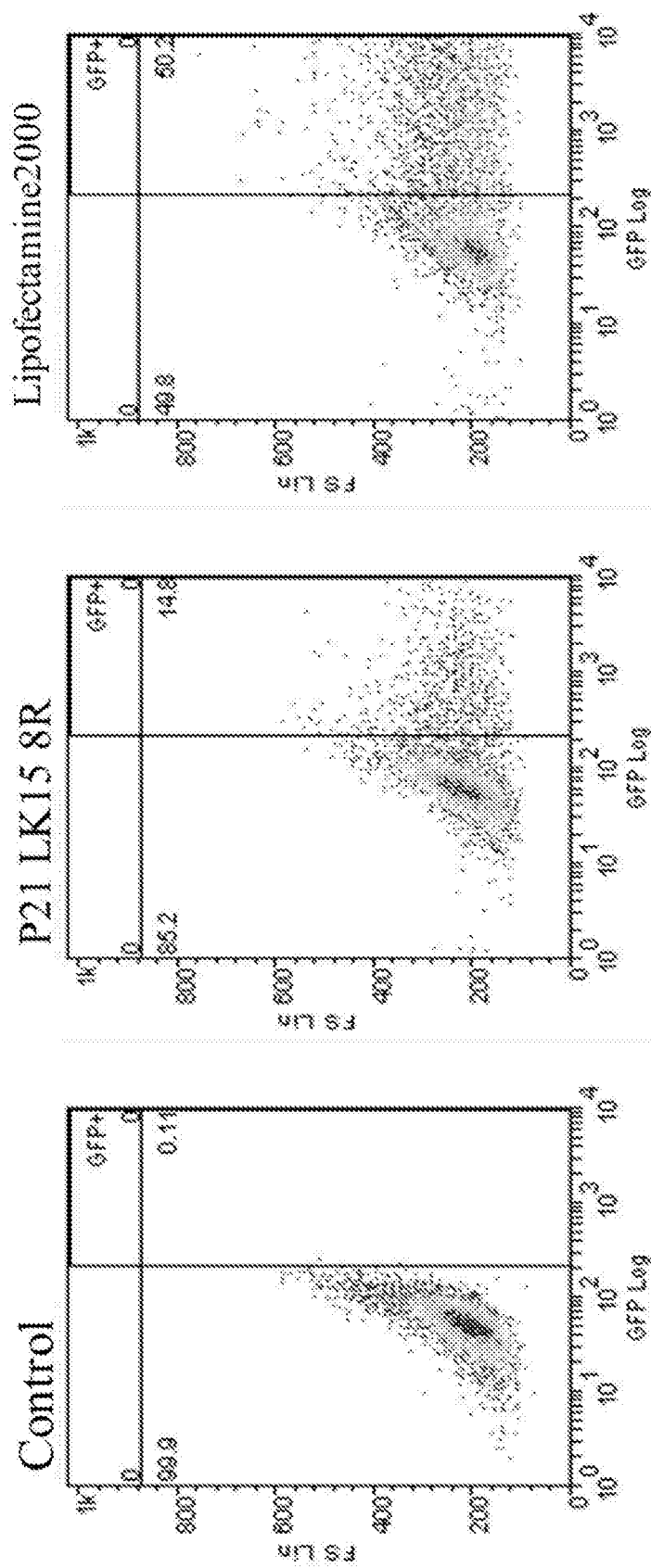
37/43

Figure 25



38/43

Figure 26



39/43

Figure 27

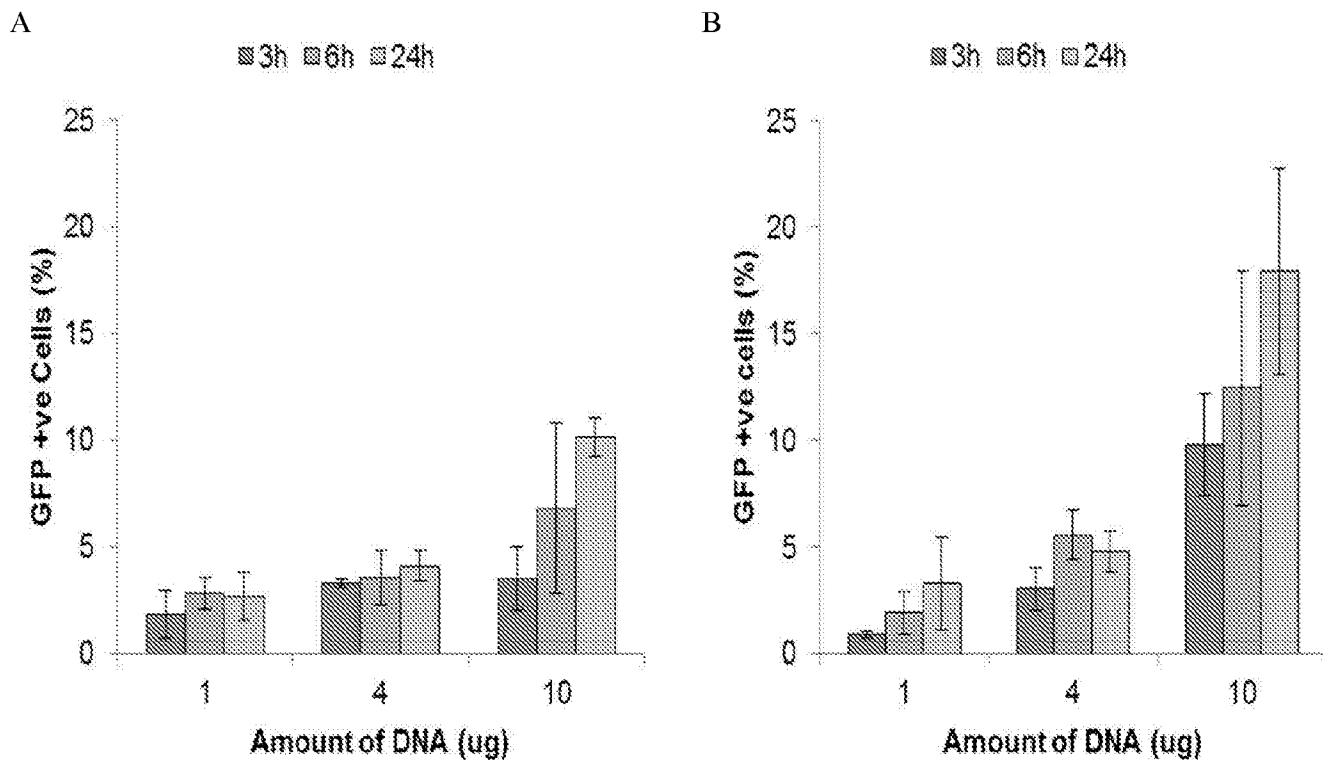
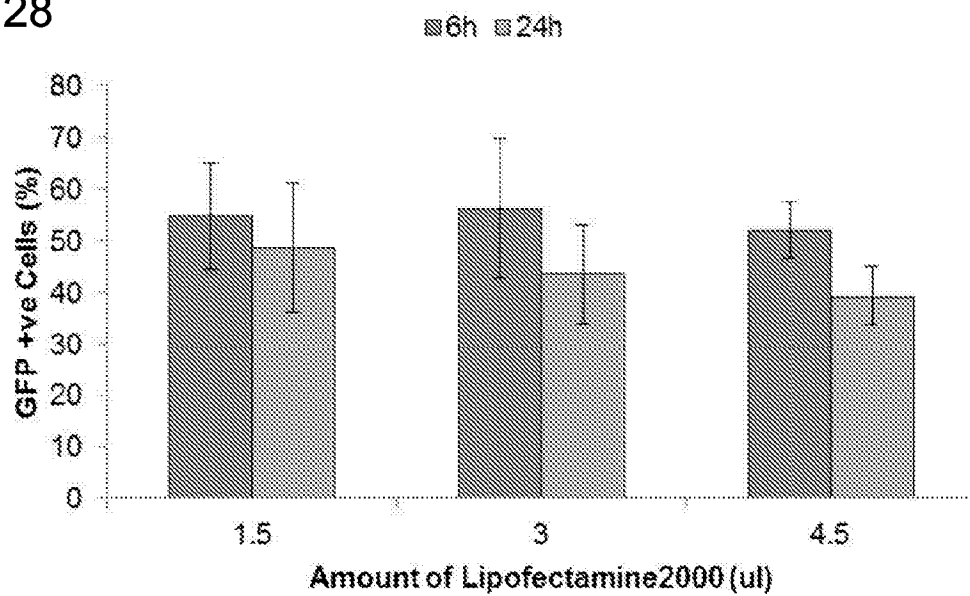
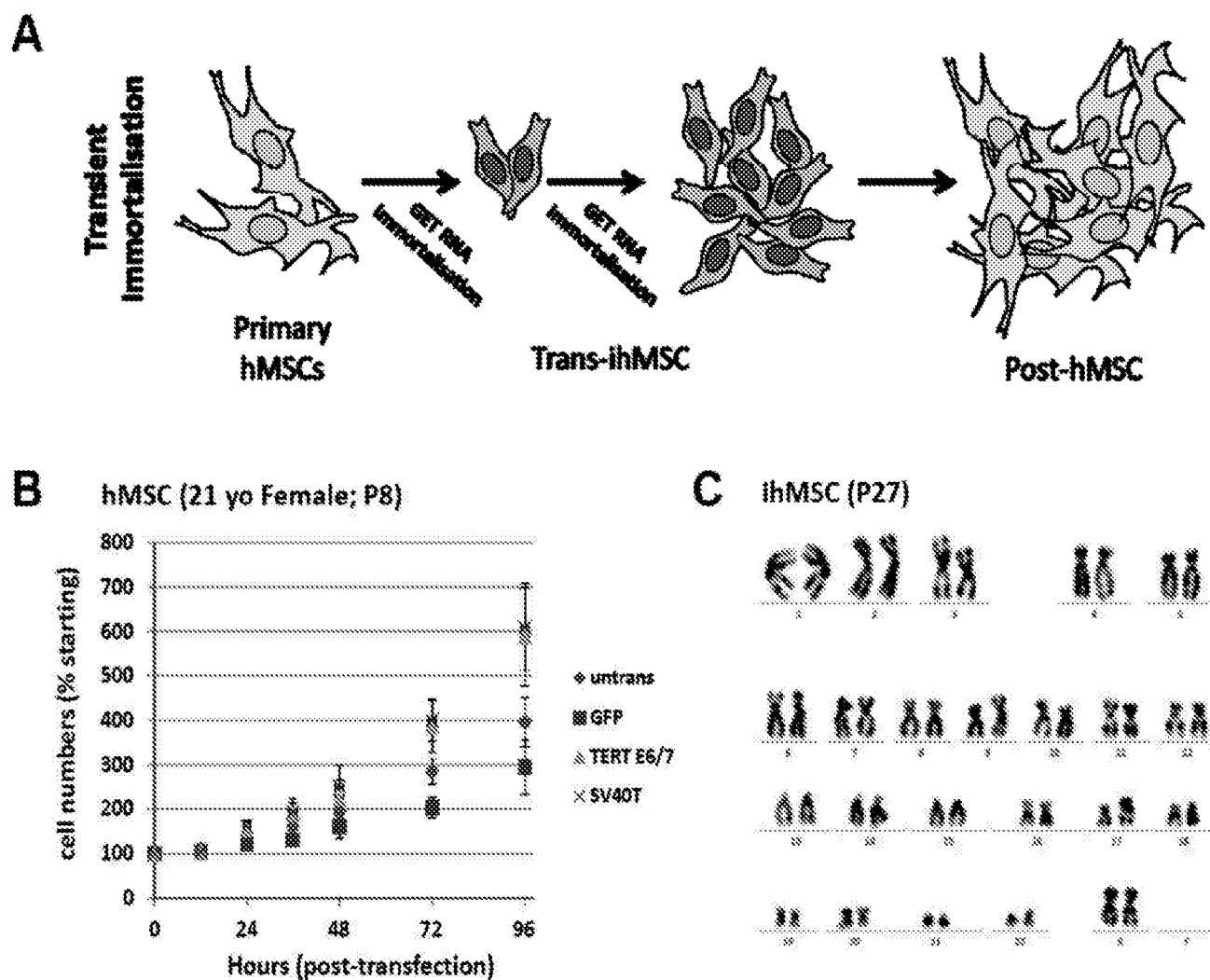


Figure 28



40/43

Figure 29



41/43

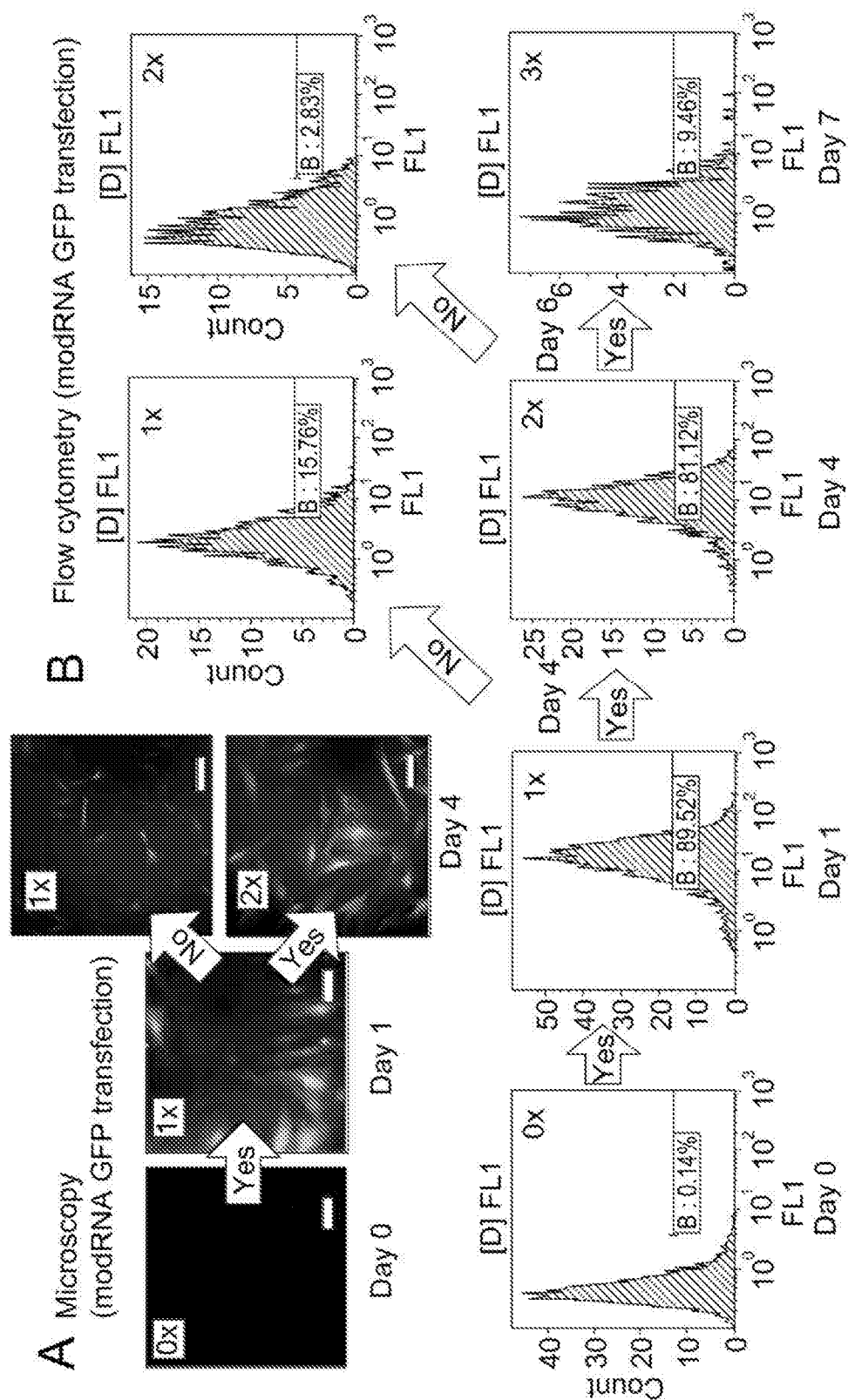
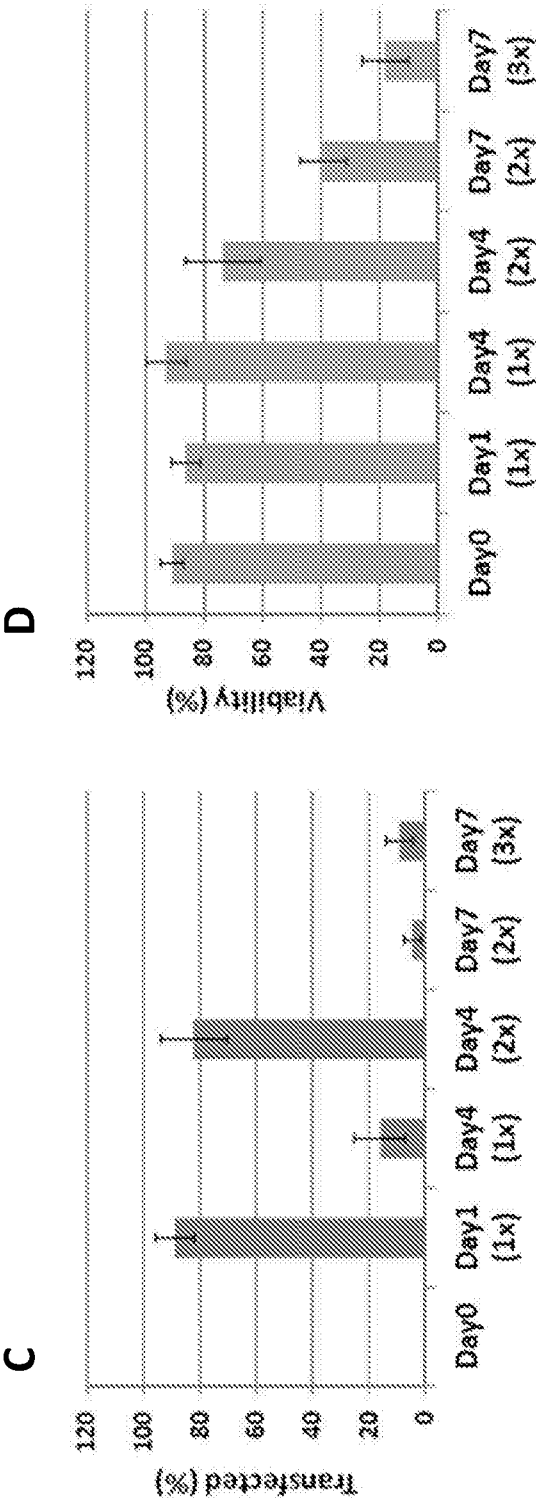


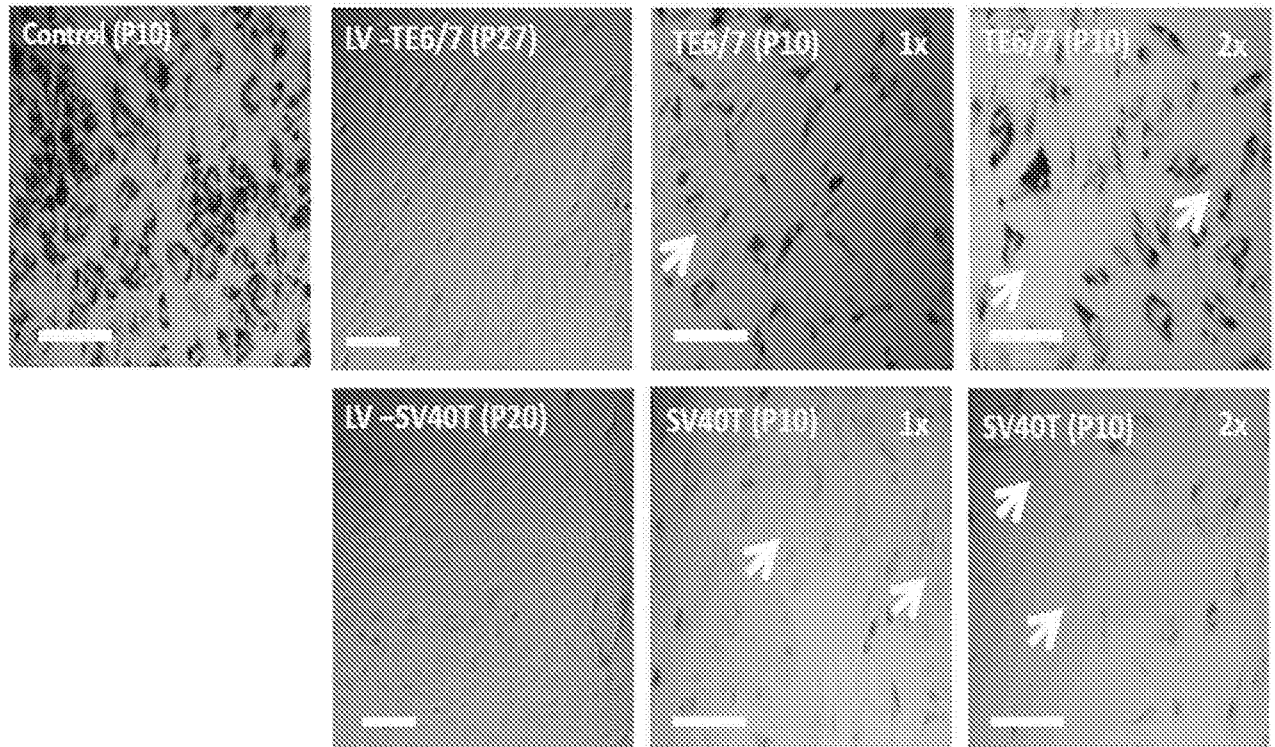
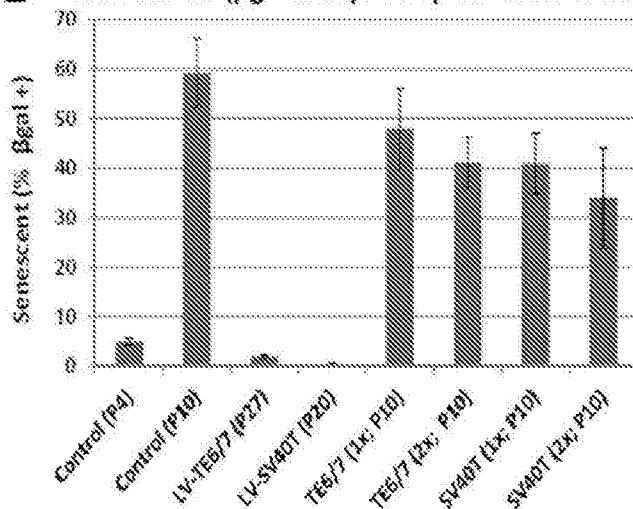
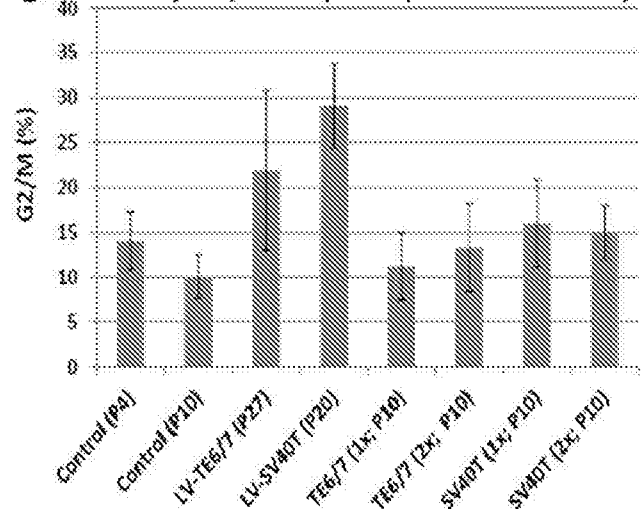
Figure 30

Figure 30 continued



43/43

Figure 31

A Senescence (β gal assay: 24h post-transfection)**B** Senescence (β gal assay: 24h post-transfection)**C** Cell-cycle (PI assay: 24h post-transfection)

INTERNATIONAL SEARCH REPORT

International application No

PCT/GB2016/051881

A. CLASSIFICATION OF SUBJECT MATTER

INV. A61K47/48 C12N5/0775 C07K14/16
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

A61K C07K C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EPO-Internal, BIOSIS, EMBASE, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	James E Dixon ET AL: "Get: glycosaminoglycan (gag)-binding enhanced transduction of functional proteins", European Cells and Materials, 1 January 2014 (2014-01-01), page 63, XP055177030, Retrieved from the Internet: URL: http://www.ecmjournal.org/journal/supplements/vol028supp04/pdf/Vol028Supp04a063.pdf [retrieved on 2015-03-17]	19
Y	the whole document ----- -/--	1-18, 20-26



Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

27 September 2016

Date of mailing of the international search report

07/10/2016

Name and mailing address of the ISA/

European Patent Office, P.B. 5818 Patentlaan 2
NL - 2280 HV Rijswijk
Tel. (+31-70) 340-2040,
Fax: (+31-70) 340-3016

Authorized officer

Offermann, Stefanie

INTERNATIONAL SEARCH REPORT

International application No

PCT/GB2016/051881

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Hoda M Eltaher ET AL: "Intracellular transduction of proteins in three dimensional gradients", European Cells and Materials, 1 January 2014 (2014-01-01), page 84, XP055177029, Retrieved from the Internet: URL: http://www.researchgate.net/profile/Hoda_Eltaher/publication/271511473_Intracellular_transduction_of_proteins_in_three_dimensional_gradients/links/54ca11490cf2002b93ca9f86.pdf [retrieved on 2015-03-17]	19
Y	the whole document	1-18, 20-26

X	WO 2007/095152 A2 (UNIV CALIFORNIA [US]; DOWDY STEVEN F [US]; WADIA JEHANGIR S [US]; MEAD) 23 August 2007 (2007-08-23) paragraphs [0021], [0041] - [0043]	2,6,7, 11,12,19
Y		1,3-5, 8-10, 13-18, 20-26

X	HEITZ FREDERIC ET AL: "Twenty years of cell-penetrating peptides: from molecular mechanisms to therapeutics", BRITISH JOURNAL OF PHARMACOLOGY,, vol. 157, no. 2, 1 January 2009 (2009-01-01), pages 195-206, XP002566600, ISSN: 0007-1188, DOI: 10.1111/J.1476-5381.2008.00057.X [retrieved on 2009-03-20]	2,6,7, 11,12,19
Y	the whole document	1,3-5, 8-10, 13-18, 20-26

X	CHEN CHIEN-JUNG ET AL: "In Vitro Characterization and in Vivo Application of a Dual Functional Peptide", 2013 SEVENTH INTERNATIONAL CONFERENCE ON COMPLEX, INTELLIGENT, AND SOFTWARE INTENSIVE SYSTEMS, IEEE, 3 July 2013 (2013-07-03), pages 576-581, XP032485643, DOI: 10.1109/CISIS.2013.104 [retrieved on 2013-09-18]	19
Y	the whole document	1-18, 20-26

-/--		

INTERNATIONAL SEARCH REPORT

International application No

PCT/GB2016/051881

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	J. R. AGUILA ET AL: "SALL4 is a robust stimulator for the expansion of hematopoietic stem cells", BLOOD, vol. 118, no. 3, 20 May 2011 (2011-05-20), pages 576-585, XP055092565, ISSN: 0006-4971, DOI: 10.1182/blood-2011-01-333641	19
Y	the whole document	1-18, 20-26

X	GREGORY A. BIRD ET AL: "Expansion of Human and Murine Hematopoietic Stem and Progenitor Cells Ex Vivo without Genetic Modification Using MYC and Bcl-2 Fusion Proteins", PLOS ONE, vol. 9, no. 8, 29 August 2014 (2014-08-29), page e105525, XP055284106, DOI: 10.1371/journal.pone.0105525	19
Y	the whole document	1-18, 20-26

T	JAMES E. DIXON ET AL: "Highly efficient delivery of functional cargoes by the synergistic effect of GAG binding motifs and cell-penetrating peptides", PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES, vol. 113, no. 3, 5 January 2016 (2016-01-05), pages E291-E299, XP055290762, US ISSN: 0027-8424, DOI: 10.1073/pnas.1518634113	

T	ELTAHER HODA M ET AL: "Highly efficient intracellular transduction in three-dimensional gradients for programming cell fate", ACTA BIOMATERIALIA, ELSEVIER, AMSTERDAM, NL, vol. 41, 3 June 2016 (2016-06-03), pages 181-192, XP029666133, ISSN: 1742-7061, DOI: 10.1016/J.ACTBIO.2016.06.004	

X,P	WO 2015/092417 A1 (UNIV NOTTINGHAM [GB]) 25 June 2015 (2015-06-25) the whole document claims 1-40	1-26

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

PCT/GB2016/051881

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2007095152 A2	23-08-2007	AU 2007215275 A1	23-08-2007
		CA 2638915 A1	23-08-2007
		EP 1984399 A2	29-10-2008
		JP 2009532017 A	10-09-2009
		US 2009093026 A1	09-04-2009
		WO 2007095152 A2	23-08-2007

WO 2015092417 A1	25-06-2015	AU 2014369443 A1	14-07-2016
		EP 3082871 A1	26-10-2016
		KR 20160101083 A	24-08-2016
		WO 2015092417 A1	25-06-2015
