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Declarations under Rule 4.17:

— *of inventorship (Rule 4.17(iv))*

Published:

— *with international search report (Art. 21(3))*

— *with sequence listing part of description (Rule 5.2(a))*

(54) Title: PROTEIN DELIVERY SYSTEMS

(57) Abstract: Disclosed are compositions and method for delivering a target protein into a cells. The compositions, which are used in the methods of the invention, include fusion proteins comprising a repeat sequence of DSS.



WO 2014/164684 A1

PROTEIN DELIVERY SYSTEMS

CROSS-REFERENCE TO RELATED APPLICATIONS

This application claims the benefit of U.S. Provisional Application No.
5 61/776,227, filed March 11, 2013, which is incorporated by reference in its entirety.

STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH OR DEVELOPMENT

10 This invention was made with government support under DE 19633 awarded
by the National Institutes of Health. The government has certain rights in the
invention.

SEQUENCE LISTING STATEMENT

The text file containing a Sequence Listing created March 7, 2014 and filed
concurrently with the filing of this application is incorporated herein by reference.

FIELD OF THE INVENTION

20 The present invention relates generally to compositions and methods for
delivering proteins to cells. More specifically, the present invention relates to
compositions and methods for delivering to cells target proteins expressed as fusion
proteins with a cell penetrating polypeptide having multiple aspartate-serine-serine
(DSS) repeats.

BACKGROUND OF THE INVENTION

25 There is considerable interest in delivering proteins into cells. Introduction of
exogenous proteins into cells has applications, for example, in treating disease,
studying cellular functions, and affecting cell differentiation. One of the major
challenges to the delivery of proteins therapeutic agents into target cells is the
30 selective permeability of the plasma membranes, in that most proteins are unable to
cross the plasma membrane.

One approach to facilitating delivery of proteins into cells is to attach the protein of interest to an antigen, ligand, or antibody specific for a cell receptor e.g., a hormone receptor or a T cell receptor. T cell receptors are considered to be an attractive therapeutic target; however, applicability of this approach is limited to T cells. Another approach is to use one of a class of polypeptides or proteins known as cell penetrating peptides (CPPs), which can function as carriers for therapeutic targets. One member of this class that has been extensively studied is the HIV TAT peptide, which has been shown to be capable of delivering biologically active target proteins *in vivo*. However, with TAT and other known CPPs, efficiency of delivery is reduced by lysosomal degradation.

Therefore, there is a demand in the art for compositions and methods for delivery of polypeptides and proteins across the plasma membrane that bypass the lysosomal degradation pathway. The present invention satisfies this demand.

SUMMARY OF THE INVENTION

The present invention provides compositions and methods for the delivery of target polypeptides, i.e. polypeptides of interest, into cells.

In certain embodiments, the invention provides a fusion polypeptide that includes a cell penetrating polypeptide having an amino acid sequence that includes at least six DSS repeats and a target polypeptide.

In certain embodiments, the cell penetrating polypeptide of the fusion polypeptide includes at least six DSS repeats. In certain embodiments, the cell penetrating polypeptide of the fusion polypeptide may include from six to as many as 100, 200, 300, or more DSS repeats. The DSS repeats may be contiguous or substantially contiguous, i.e., one or more DSS repeats may be separated by one or more amino acid residues that is not a member of a DSS repeat.

The target polypeptide may include any polypeptide of interest, including, without limitation, polypeptides having therapeutic value, polypeptides that promote differentiation or dedifferentiation of cells, and polypeptides of interest to scientific researchers.

In certain embodiments, the fusion protein comprises an organelle-specific localization signal, i.e., a sequence that preferentially causes localization of the fusion protein to a specific organelle. In certain embodiments, the localization signal localizes the fusion protein to the nucleus, mitochondria, or endoplasmic reticulum.

The localization signal may be within the target polypeptide or separate from the target polypeptide.

In certain embodiments, target polypeptides having therapeutic include, without limitation, antibodies, transcription factors, and enzymes.

5 In certain embodiments, transcription factors may include transcription factors that affect the degree of differentiation of the cell into which the transcription factor is delivered. In certain embodiments, the transcription factor may promote expression of genes that result in the cell becoming more differentiated. In certain
10 embodiments, the transcription factor may promote expression of genes that result in the cell becoming less differentiated, e.g., the cell becomes pluripotent or totipotent.

In other embodiments, the target peptide may include one or more or pluripotency-associated gene products, or "reprogramming factors", to effect dedifferentiation of differentiated cells.

The target polypeptide may be directly or indirectly linked to the DSS cell
15 penetrating polypeptide at the N- or C-terminus of the cell penetrating polypeptide. In certain embodiments, the target polypeptide is directly linked in-frame to the DSS cell penetrating peptide. In certain embodiments, the target polypeptide and the DSS cell penetrating peptide may be separated by a hydrolysable linker.

In certain embodiments, the fusion polypeptide may include an affinity tag to
20 facilitate isolation of the fusion polypeptide. The affinity tag may be any tag suitable for facilitating isolation of the fusion protein, including, but not limited to, any of numerous affinity tags known in the art. Non-limiting examples of affinity tags include His tags, biotin, FLAG, glutathione S-transferase (GST), and the like. It is an advantage that the DSS cell penetrating peptide of the fusion proteins of the present
25 invention promotes rapid endocytosis by any cell type, i.e., the delivery system is universally applicable to all cell types. It is a further advantage that the mechanism by which the DSS cell penetrating peptide promotes uptake of the fusion proteins is efficient, in that once the fusion proteins are endocytosed, they are not substantially degraded by the lysozomal pathway.

30 The present invention and its attributes and advantages will be further understood and appreciated with reference to the detailed description below of presently contemplated embodiments, taken in conjunction with the accompanying drawings.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 is a dose response curve for DSS endocytosis by MC3T3-E1 cells showing a linear response.

Fig. 2 is a dose response curve for Runx2-DSS fusion protein endocytosis by MC3T3-E1 cells.

Fig. 3 is a graph showing the fold change in expression levels of three Runx2 downstream target genes in human marrow stromal cells (HMSCs) upon treatment with Runx2-DSS fusion protein for 24 and 48 hours.

DETAILED DESCRIPTION OF EMBODIMENTS OF THE INVENTION

Dentin phosphophoryn (DPP) is a highly acidic protein found predominantly in the dentin extracellular matrix. It contains numerous Asp-Ser (DS) rich repeats, making it highly anionic, with an isoelectric point (pI) of 1.1. Therefore DPP tends to be negatively charged at physiological pH. DPP is normally associated with matrix mineralization as it can bind avidly to Ca^{2+} .

In the examples below, it is shown that DPP can be internalized by several cell types via a non-conventional endocytic process. Additionally, using the 244-amino acid carboxy terminal domain of DPP (SEQ ID NO:1), which is rich in DSS repeats, it was demonstrated that it is the DSS repeat rich domain of DPP that facilitates endocytosis.

Further, it was shown that DSS repeat rich polypeptides can be used as a protein delivery vehicle by delivering the osteoblast transcription factor Runx2 to the nucleus of mesenchymal cells. The functionality of the endocytosed Runx2 protein was demonstrated by performing gene expression analysis of Runx2 target genes. Nuclear localization was also demonstrated with the fusion protein DSS-Runx2 conjugated to quantum dots in 2D and 3D culture models *in vitro* and *in vivo*. A DSS polypeptide containing six repeats and designated $(\text{DSS})_6$ was shown to be sufficient to allow delivery of an antibody into cells. The results illustrate that the DSS domain of DPP and polypeptides having as few as six DSS repeats function as novel cell penetrating peptides. These findings present new opportunities for intracellular delivery of therapeutic proteins and cell tracking *in vivo*.

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Cell culture:

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Recombinant dentin phosphophoryn (DPP) was expressed as a GST fusion protein in *Escherichia coli* and purified. The 244-residue carboxyl-terminal domain of rat DPP, which contains aspartic acid-serine repeats (DSS) (SEQ ID NO:1) was cloned into PGEX4T-3 GST vector and expressed as a GST-DSS fusion protein.

5 The fusion protein was purified using standard methods. For experiments with GST-DSS, the GST was not cleaved and the fusion protein was eluted using glutathione. Runx2-DSS fusion protein (SEQ ID NO:2) was obtained by cloning the Runx2 coding sequence in PGEX4T-1 GST fusion vector) upstream of the DSS coding sequence in the PGEX4T-3 vector. The fusion protein was then purified

10 using standard methods. The purity of all recombinant proteins were verified by SDS-PAGE analysis.

FITC labeling of recombinant proteins:

FITC labeling of DSS was performed as published previously. The FITC

15 labeled protein was imaged after UV illumination following separation on an SDS gel.

Quantum dot (QD) labeling of recombinant proteins:

QD conjugation of the recombinant proteins were performed according to standard protocols.

Binding and Endocytosis:

20 Binding of biotinylated DSS to T4-4 cells at 4°C was performed according to standard protocols. Endocytosis experiments were performed using FITC labeled proteins or quantum dot labeled proteins. For the FITC labeled proteins, 10µg/ml of the protein (rDPP, DSS, rR2DSS, GST-DSS or BSA) were incubated with the cells grown on cover slips for various time points. The cells were then fixed with 4%

25 paraformaldehyde, mounted on slides and imaged using a Zeiss LSM 510 Meta or LSM 710 confocal microscope. When required, the cells were immunostained with the appropriate primary and secondary antibodies. The following primary antibodies were used in this study: Mouse monoclonal anti- tubulin antibody (Sigma, 1/1000), rabbit polyclonal anti-caveolin – 1 antibody (Santa Cruz biotechnology 1/75), rabbit

30 polyclonal anti-clathrin antibody (Santa Cruz Biotechnology 1/100), rabbit polyclonal anti EEA-1 FITC labeled antibody (BD Biosciences 1/50).

Dose response curves for DSS, GST-DSS and R2DSS were obtained by incubating MC3T3-E1 cells (50,000cells/well) seeded in 24 well plates with increasing amounts of the FITC labeled proteins. The cells were incubated with the

proteins at 37°C for 2 hours. They were then lysed using 100µl of MPER (Thermo Scientific) protein extraction reagent per well and green fluorescence was measured using a BioTek micro titer plate reader using appropriate filter sets. All experiments were performed in triplicate.

5 For endocytosis blocking experiments, T4-4 cells were pretreated for 60 minutes with different inhibitors and then treated for 15 minutes with 10µg/ml of FITC DSS. Wherever time points are not mentioned, the cells were incubated with the fluorescently labeled proteins for 15 minutes. The following inhibitors were used in this study: Hyper-osmotic sucrose (HOS) (0.45M), ikarugamycin (Ikg) (2µM), methyl
10 β cyclodextrin (MBCD) (1%), colchicine (20µM) and cytochalasin D (1µM). The experiments were also performed under acidic (pH 4.0) and basic (pH 10.0) conditions, using concentrated HCl or NaOH to adjust media pH. T4-4 cells were pretreated for 30 minutes in acidic or alkaline media and the experiment was performed for 15 minutes under the same conditions at 37°C. Endocytosis
15 experiments were also performed at 4°C and at room temperature. MC3T3-E1 were incubated in a refrigerator or at room temperature for 30 minutes followed by treatment with FITC-labeled proteins (DSS, GST-DSS) for 1 hour. After incubation, the cells were fixed in 4% paraformaldehyde, permeabilized and immunostained as required. The cells were then imaged using a Zeiss LSM 510 META confocal
20 microscope using appropriate lasers and emission filters. All imaging conditions for comparative experiments were maintained constant. To quantify the fluorescence, the green intensities in cells were measured using the Zeiss Axiovision Software and expressed as mean intensity per condition +/- S.E.M.

Endocytosis experiments with proteins conjugated with quantum dots (QDs)
25 were performed by incubating cells cultured on coverslips or embedded in collagen scaffolds(30) with 25µl of the conjugates (estimation of protein concentration was not possible). However, prior to conjugation, the protein concentration was 1mg/ml and the concentration of the protein used for conjugation was maintained the same each time and the suitable amount of conjugate required to get a good signal for the
30 indicated time points was empirically determined. The cells were fixed in 4% paraformaldehyde, mounted on to slides and imaged using the Zeiss LSM 510 Meta confocal microscope (The cells were excited using a UV laser and emission was observed in the red spectrum).

Gene expression analysis:

Quantitative real-time PCR was used to analyze gene expression levels of Runx2 targets upon treatment with Runx2- DSS fusion protein. HMSCs were treated with either 2µg of the DSS-Runx2 fusion protein or 2µg of DSS peptide alone for 24 and 48 hours. At the end of these time points, the RNA from the cells was extracted using the Qiagen kit as per the manufacturer's protocol. cDNA synthesis was then performed and the expression levels of three Runx2 target genes namely, alkaline phosphatase (ALP), SMAD 4 and DMP1, were analyzed using gene specific primers. The data was normalized to GAPDH and changes in expression levels were calculated using the comparative $\Delta\Delta CT$ method.

In vivo implantation:

DPSCs were seeded onto biomimetic scaffolds as described previously. 24 hours post seeding, the scaffolds were incubated with 25µl of DSS-Runx2 QD conjugates for 24 hours. Scaffolds were prepared in triplicates and then implanted subcutaneously into 1-month-old athymic nude male mice (Charles River Labs) for a period of 2 weeks. Scaffolds without the QD conjugates served as controls. All experiments were performed using approved UIC animal care protocols (Assurance No: A3460-01). After 1 and 2-week post-implantation, the animals were euthanized and imaged using an IVIS Spectrum animal imager. The samples were excited using UV light and emission was observed in the red spectrum. The scaffolds were then removed, fixed in 4% formalin, embedded in paraffin and 5µm thick sections were obtained and stained with DAPI nuclear stain and imaged using a Zeiss LSM 710 confocal microscope. The slides were excited using UV laser and emission was observed in the blue spectrum for DAPI nuclear fluorescence and in the red spectrum to observe the QDs.

Endocytosis of (DSS)₆ peptide:

In order to identify the minimum number of DSS repeats responsible for endocytosis, a short peptide containing six DSS amino acid repeats (DSS)₆ (SEQ ID NO:3) followed by a FLAG tag (DSSDSSDSSDSSDSSDSSDYKDDDDK) (SEQ ID NO:4) was synthesized. The peptide (50µg) was incubated with affinity purified rabbit polyclonal FLAG antibody (20µg) (Sigma) for 1 hour at room temperature. The

mixture was then added to MC3T3-E1 cells seeded on a cover glass (25mm) and incubated at 37°C for 1 hour. 20µg of FLAG antibody added to cells served as control. After incubation, the cells were washed, fixed, permeabilized and fluorescently immunostained with anti-rabbit secondary antibody (FITC) to visualize the FLAG antibody. The cells were also immunostained with mouse monoclonal tubulin antibody (Sigma) followed by a TRITC conjugated anti-mouse secondary antibody. The cells were imaged using a Zeiss LSM 710 confocal microscope.

RESULTS

10 Internalization of rDPP:

Confocal microscopy of three different mesenchymal cell types (T4-4 pre-odontoblasts, C3H10T1/2 embryonic mesenchymal cells) and DPSCs treated with FITC labeled rDPP showed that all of these cell types are able to internalize FITC-rDPP.

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Internalization of the DSS domain lacking RGD:

DPP homologs from several species contain a conserved RGD domain, which may interact with integrin receptors to mediate internalization. Confocal microscopy of FITC labeled DSS polypeptide, which lacks the RGD domain, endocytosed in T4-4 and primary pulp cells revealed extensive uptake of the DSS polypeptide within two minutes. Additionally, the polypeptide was also found to be endocytosed by epithelial cells (HAT 7), endothelial cells (HUVECs) and osteoblast cells (MC3T3). These results confirm that DPP internalization is not mediated through integrin receptors.

25

Endocytosis does not occur via the clathrin or caveolae mediated endocytic pathways:

The involvement of classical endocytic mechanisms for the internalization of DSS was evaluated using inhibitors of clathrin- and caveolae-mediated endocytosis. Inhibitors of the clathrin endocytic pathway (ikarugamycin or 0.45M) and caveolar endocytosis (MBCD) had no effect on DSS uptake. Immunofluorescence colocalization experiments showed that there was no colocalization of the endocytic vesicles with either caveolin-1 (caveolae marker, or clathrin. Thus, neither clathrin-

coated pits nor caveolae/lipid rafts were involved in the endocytic process. Additionally, a dose response endocytosis experiment showed that endocytosis is not saturable, but showed a linear increase with increase in dosage indicating absence of specific cell surface binding. Binding experiments performed with biotinylated DSS to determine whether DSS binds to the plasma membrane prior to endocytosis. No specific binding of DSS to T4-4 and MC3T3-E1 cells were observed.

Disruption of microtubules and microfilaments does not affect endocytosis of DSS:

Endocytic vesicles are transported from the plasma membrane through microtubules and microfilaments. Experiments were performed to determine whether disruption of the intracellular transportation mechanisms affect the endocytic process. Results show that disruption of the microtubules with colchicine or microfilaments with cytochalasin D did not prevent the endocytosis of the DSS polypeptide.

Effect of pH on the endocytosis of DSS:

Perturbations in the cytoplasmic and extracellular pH are known to inhibit the endocytosis of cell surface proteins. Results indicate that neither acidic pH nor alkaline pH affect the endocytic process by which DSS is internalized.

Temperature dependence of DSS endocytosis:

Results from endocytosis experiments performed at 4°C and at room temperature indicate that the endocytosis of DSS is reduced but not abrogated by changes in temperature. Results also show that the vesicles containing DSS were not observed on the microtubules, suggesting random diffusion of the protein within the cytoplasm. This indicates that the DSS polypeptide requires ATP for regulated intracellular transportation.

Use of the DSS polypeptide as a vehicle for protein delivery:

Because the DSS peptide is able to cross the plasma membrane of several cell types via a non-classical, non-receptor mediated pathway, the possibility of using

this peptide as a protein delivery agent was evaluated. To visualize the delivery process, uptake of an FITC-labeled GST-DSS fusion protein was examined in T4-4 and MC3T3-E1 cell. GST protein alone was used as control and was not endocytosed by either of the cell types. A dose response for the endocytosis of this fusion protein by MC3T3-E1 cells indicated a linear profile similar to the endocytosis of the DSS polypeptide (FIG. 1).

Targeted nuclear delivery of the osteoblast transcription factor Runx2 in various mesenchymal cell types:

A FITC-labeled fusion protein containing a DSS polypeptide and Runx2 was used to assess the ability of DSS polypeptide to deliver Runx2, which contains an NLS, to the nucleus of eukaryotic cells. Results demonstrated that the FITC labeled DSS-Runx2 fusion protein was internalized and localized to the nucleus in T4-4 cells, MC3T3-E1 cells, and DPSCs. Nuclear localization was confirmed by performing a z-stack confocal imaging. Additionally, similar to the DSS and the GST-DSS proteins, a linear dose response curve was obtained for the endocytosis of the FITC labeled DSS-Runx2 fusion protein, indicating that the addition of a cargo protein does not alter the endocytic mechanism (FIG. 2).

The DSS tagged Runx2 retains its intracellular functionality:

The endocytosed transcription factor Runx2 was found to be functionally active, as measured by its ability to increase expression levels of DMP1, ALP and SMAD4, three of the many downstream target genes for Runx2. Results show that there was no change in gene expression at the 24 hr time point, but, expression levels of DMP1, ALP and SMAD4 increased at 48 hrs, (FIG. 3), indicating the effectiveness of using DSS to introduce functional Runx2 into cells, which may be used to differentiate stem cells into bone forming osteoblast-like cells.

Endocytosis of Runx2-DSS fusion protein conjugated to nanocrystals/ QDs:

QDs are resistant to photobleaching and can serve as tools for tracking the internalized proteins or as marker for implanted cells. Runx2-DSS fusion protein conjugated to QDs was internalized. Quantum dots are frequently being trapped in endosomes after endocytosis. Internalized Runx2-DSS-QD conjugates did not co-localize with the early endosome antigen 1 (EEA-1), an endosomal marker after 4 or

24 hours. In fact, QD-tagged Runx2-DSS fusion protein was found to localize in the nucleus of MC3T3-E1 cells. BSA tagged QDs and QDs alone were used as negative controls and did not show any internalization.

5 **Visualization of the DSS-Runx2-QD conjugates *in vivo*:**

The ability to track cells treated with the DSS-Runx2-QD conjugates *in vivo* was evaluated. Biomimetic scaffolds containing DPSCs pretreated for 24 hours with the QD conjugates were implanted subcutaneously into nude mice. The mice were euthanized at 1 week and 2-weeks post implantation and imaged. The results
10 indicate that it is possible to observe the QD tagged cells *in vivo* in the animal and *in vitro* after removal of the implant.

Endocytosis of (DSS)₆-Flag peptide:

A polypeptide containing six DSS repeats was evaluated for the ability to be
15 taken up by cells by endocytosis. Results from endocytosis experiments with the (DSS)₆-FLAG peptide indicate that the FLAG antibody, when pre-bound to the peptide can be endocytosed. However, unbound antibody is not endocytosed and instead binds to the cell surface and the cover glass.

While the disclosure is susceptible to various modifications and alternative
20 forms, specific exemplary embodiments of the present invention have been shown by way of example in the drawings and have been described in detail. It should be understood, however, that there is no intent to limit the disclosure to the particular embodiments disclosed. Rather, all modifications, equivalents, and alternatives falling within the scope of the disclosure are included, as defined by the appended
25 claims.

CLAIMS

It is claimed:

5

1. A fusion polypeptide comprising a cell penetrating peptide sequence having at least six DSS repeats and a target polypeptide sequence.

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2. The fusion polypeptide of claim 1, wherein the target polypeptide sequence is a therapeutic polypeptide.

3. The fusion polypeptide of claim 1 or claim 2, wherein the target polypeptide is an antibody, transcription factor, or an enzyme.

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4. The fusion polypeptide of claim 3, wherein the target polypeptide is a transcription factor.

5. The fusion polypeptide of claim any one of claims 1-4, wherein the fusion polypeptide comprises a cellular organelle localization signal.

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6. The fusion polypeptide of claim 5, wherein the cellular organelle localization signal promotes localization of the fusion polypeptide to nucleus, mitochondria, or endoplasmic reticulum of the cell..

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7. The fusion polypeptide of claim 6, wherein the cellular organelle localization signal is comprised within the target polypeptide.

8. The fusion polypeptide of any one of claims 1-7, further comprising a detectable label.

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9. The fusion polypeptide of 8, wherein the detectable label is a fluorescent protein sequence within the fusion polypeptide.

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10. The fusion polypeptide of claim 8, wherein the detectable label is a fluorescent label attached to the fusion polypeptide.

11. The fusion polypeptide of claim 8, wherein the detectable label is a quantum dot.

5 12. A nucleic acid encoding the fusion polypeptide of any one of claims 1-9.

13. A cloning vector comprising the nucleic acid of claim 12.

10 14. A method of introducing a target polypeptide into a cell comprising contacting the cell with the fusion polypeptide of any one of claims 1-11 under conditions suitable to allow uptake of the fusion protein by the cell.

15 15. The method of claim 12, wherein the cell is contacted with the fusion polypeptide *in vivo*, *in vitro*, or *ex vivo*.

16. The method of claim 14 or claim 15, wherein the cell is a mammalian cell.

17. The method of claim 16, wherein the cell is an epithelial cell, mesenchymal cell, or an endothelial cell.

20 18. The method of any one of claims 14-17, wherein expression of one or more genes in the cell is altered following uptake of the fusion protein by the cell.

25 19. The method of claim 18, wherein the cell undergoes differentiation or dedifferentiation.

20. The method of any one of claims 14-19, further comprising detecting localization of the fusion polypeptide after contacting the cell with the fusion polypeptide.

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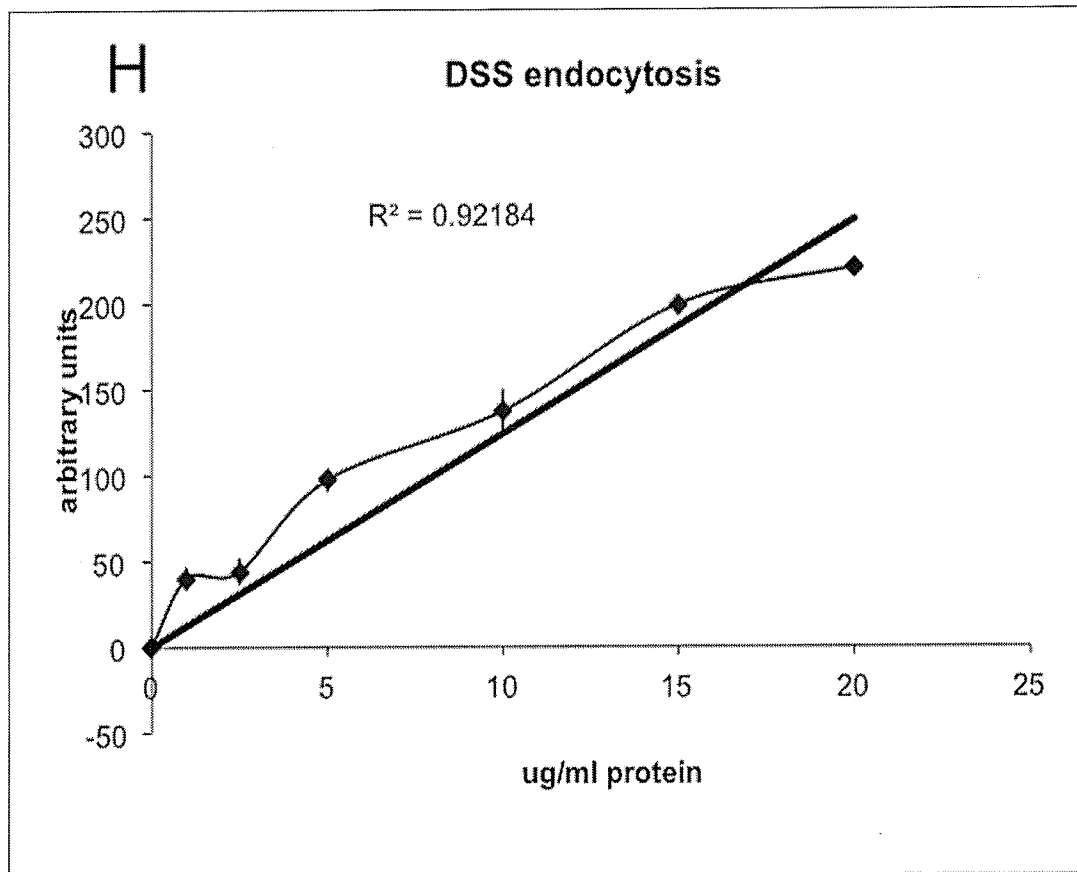


FIG. 1

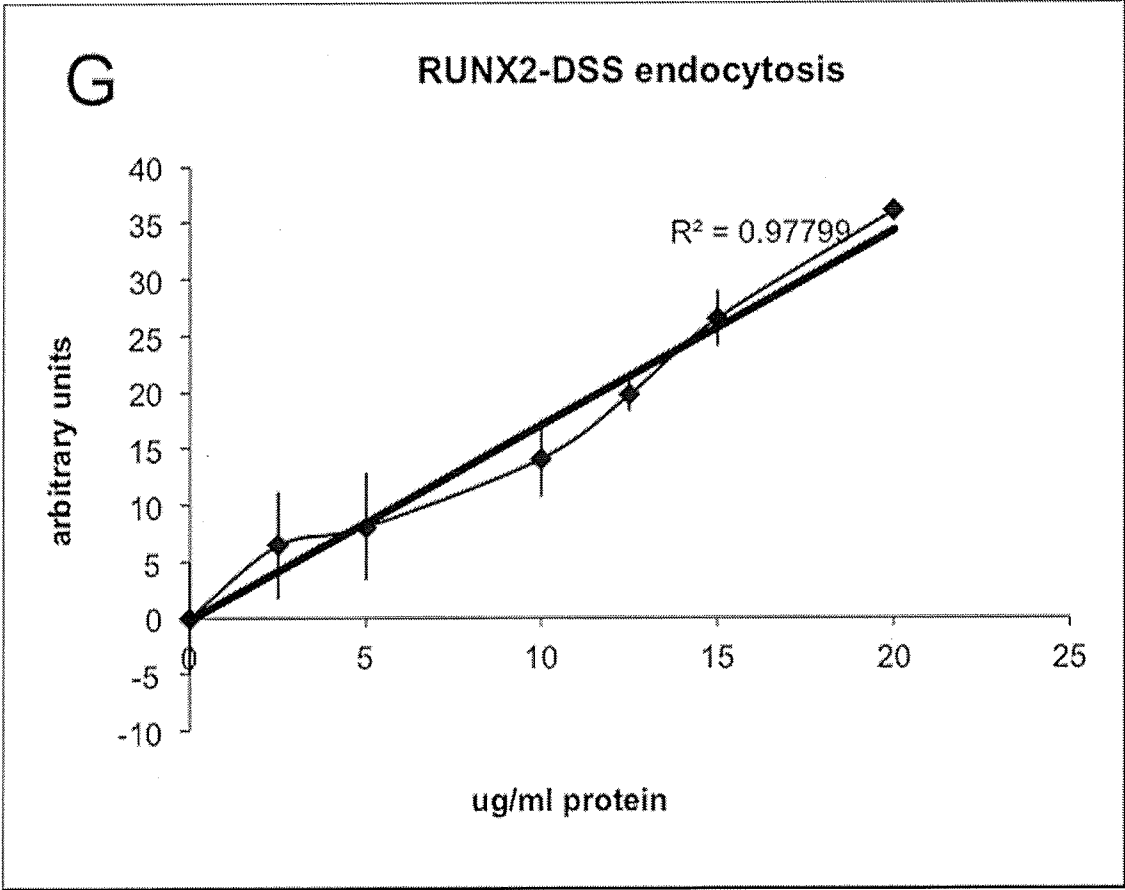


FIG. 2

3/3

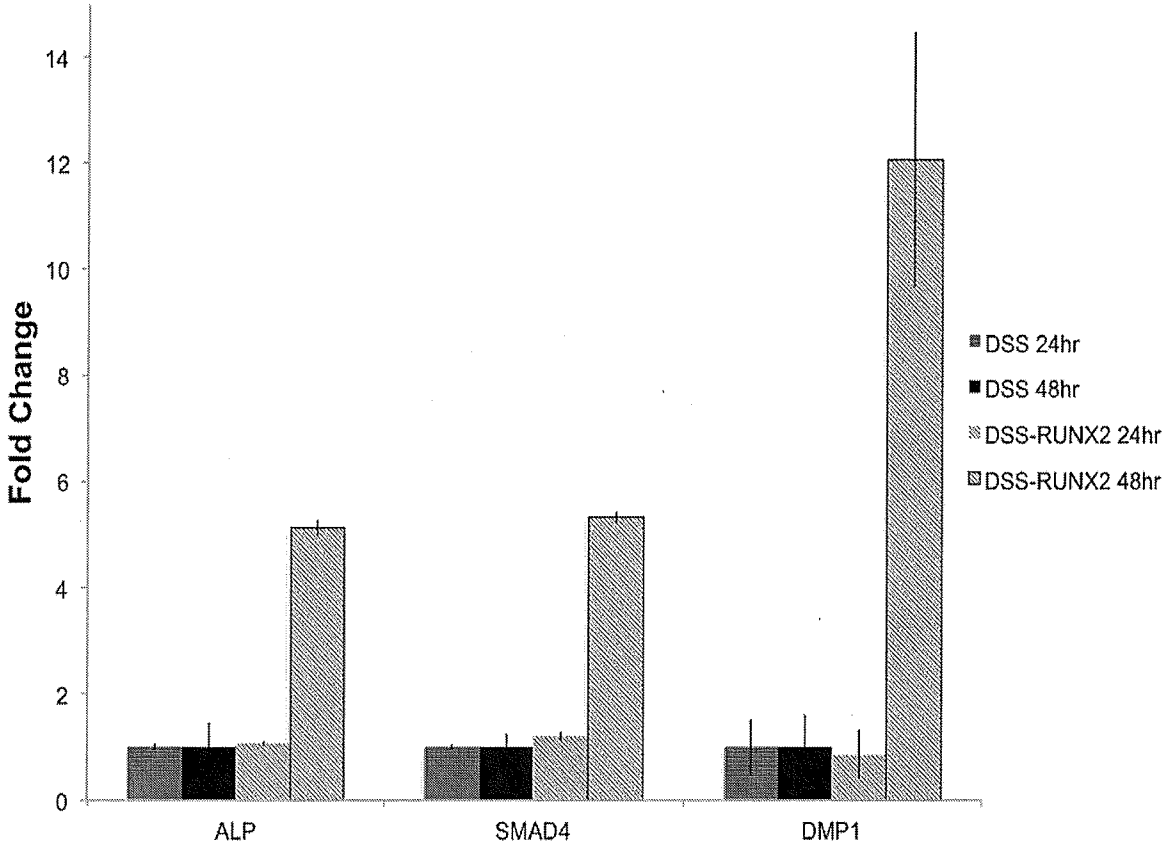


FIG. 3

INTERNATIONAL SEARCH REPORT

2014/023213 13.06.2014

International application No.

PCT/US 14/23213

A. CLASSIFICATION OF SUBJECT MATTER

IPC(8) - C07K 1/00, C07K 14/00, C07K 17/00, A61K 38/00 (2014.01)

USPC - 530/350, 530/324

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)
USPC- 530/350, 530/324Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
USPC- 14/1.1; 514/19; 514/16.7; 435/69.7; 536/23.4, 530/325; 530/326; 435/377Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
PubWEST(PGPB,USPT,USOC,EPAB,JPAB); PatBase, Google/Scholar: DSS repeats, fusion, cell penetrating peptide, Internalizing, protein transduction domain, transcription factor, DSSDSSDSSDSSDSSDSS, (DSS)6; Asp Ser Ser; George, Anne; transcription factor, Runx2.
GenCore 6.4.1: SEQ ID NO:3

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X -- A	2009/0098050 A1 (Yarbrough, et al.) 16 April 2009 (16.04.2009) para [0062], [0063], SEQ ID NO:29, SEQ ID NO:31	1-2 --- 3-4
X,P	Ravindran, et al. Acidic domain in dentin phosphophoryn facilitates cellular uptake: implications in targeted protein delivery. J Biol Chem. Epub 15 April 2013, 288(22):16098-109; Abstract, pg 16099, co 1	1-4



Further documents are listed in the continuation of Box C.



* 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

28 May 2014 (28.05.2014)

Date of mailing of the international search report

13 JUN 2014

Name and mailing address of the ISA/US

Mail Stop PCT, Attn: ISA/US, Commissioner for Patents
P.O. Box 1450, Alexandria, Virginia 22313-1450

Facsimile No. 571-273-3201

Authorized officer:

Lee W. Young

PCT Helpdesk: 571-272-4300

PCT OSP: 571-272-7774

INTERNATIONAL SEARCH REPORT

2014/023213 13.06.2014

International application No.

PCT/US 14/23213

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☒ Claims Nos.: 5-20
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- ☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- ☐ No protest accompanied the payment of additional search fees.