Membrane Transducing Activity of Recombinant Hoxc8 and Its Application as a Delivery Vector

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Membrane Transducing Activity of Recombinant Hoxc8 and Its Application as a Delivery Vector

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This certifies that the Master's Thesis
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<u>시편 139편</u> 여호와여 주께서 나를 감찰하시고 아셨나이다 주께서 나의 앉고 일어섬을 아시며 멀리서도 나의 생각을 통촉하시오며 나의 길과 눕는 것을 감찰하시며 나의 모든 행위를 익히 아시오니 여호와여 내 혀의 말을 알지 못하시는 것이 하나도 없으시니이다 주께서 나의 전후를 두르시며 내게 안수하셨나이다 이 지식이 내게 너무 기이하니 높아서 내가 능히 미치지 못하나이다 내가 주의 신을 떠나 어디로 가며 주의 앞에서 어디로 피하리이까 내가 하늘에 올라갈찌라도 거기 계시며 음부에 내 자리를 펼찌라도 거기 계시니이다 내가 새벽 날개를 치며 바다 끝에 가서 거할찌라도 곧 거기서도 주의 손이 나를 인도하시며 주의 오른손이 나를 붙드시리이다 내가 혹시 말하기를 흑암이 정녕 나를 덮고 나를 두른 빛은 밤이 되리라 할찌라도 주에게서는 흑암이 숨기지 못하며 밤이 낮과 같이 비취나니 주에게는 흑암과 빛이 일반이니이다 주께서 내 장부를 지으시며 나의 모태에서 나를 조직하셨나이다 내가 주께 감사하옴은 나를 지으심이 신묘막측하심이라 주의 행사가 기이함을 내 영혼이 잘 아나이다 내가 은밀한데서 지음을 받고 땅의 깊은 곳에서 기이하게 지음을 받은 때에 나의 형체가 주의 앞에 숨기우지 못하였나이다 내 형질이 이루기 전에 주의 눈이 보셨으며 나를 위하여 정한 날이 하나도 되기 전에 주의 책에 다 기록이 되었나이다 하나님이여 주의 생각이 내게 어찌 그리 보배로우신지요 그 수가 어찌 그리 많은지요 내가 세려고 할찌라도 그 수가 모래보다 많도소이다 내가 깰 때에도 오히려 주와 함께 있나이다 하나님이여 주께서 정녕히 악인을 죽이시리이다 피 흘리기를 즐기는 자들아 나를 떠날찌어다 저희가 주를 대하여 악하게 말하며 주의 원수들이 헛되이 주의 이름을 칭하나이다 여호와여 내가 주를 미워하는 자를 미워하지 아니하오며 주를 치러 일어나는 자를 한하지 아니하나이까 내가 저희를 심히 미워하니 저희는 나의 원수니이다 하나님이여 나를 살피사 내 마음을 아시며 나를 시험하사 내 뜻을 아옵소서 내게 무슨 악한 행위가 있나 보시고 나를 영원한 길로 인도하소서

스물아홉의 인생 중에 짧다고 하면 짧다고 할 수 있는 지난 5학기 하지만 가장 긴 시간으로만 여겨진 이 2년 반을 잊을 수가 없을 것입니다. 건강의 악화(?)로 어쩔 수 없이 지도 교수님이 바뀌버린 사건! 모든 사람들이 졸업할 수 없을 것이라는 부정적인 결과를 던지는 가운데 3학기 후반부에 세부전공을 바꿀 수밖에 없었던 웃지 못 할 해프닝을 비롯하여 세상에서 가장 존경하고 정말 다시는 세상 어디에서도 이렇게 훌륭한 사람을 만나지는 못할 아버지를 잃은 슬픈 일들! 그 모든 일들을 이 짧은 감사 글에 남길 수 없다는 생각이 듭니다. 이렇게 힘들고 어려운 시간에 이 시간들을 이길 수 있도록 믿고 인내해줬으며 정신적으로 뿐만 아니라 재정적으로 도와준 우리 가족, 엄마를 비롯하여 희전 언니와 은지, 은영 동생들에게, 특별히 천국에서 가장

기뻐하실 아버지께 먼저 깊은 감사를 글을 올립니다.

그리고 과학의 기초! 알레르기만 아니었으면 정말 파고들어 연구하고 싶은 가치가 있는 또한 관심이 있는 신경 과학의 참 맛을 알게 해주신 임상의학연구센터 전기 생리학 교실 가족들 이경희 선생님, 김은정 선생님, 정세정 선생님께 감사를 드리며 특별히 연구원으로 일 년 반 그리고 석사 생으로 일 년 반 이상 일하다가 훌쩍 떠나 민폐만 끼친 격이 되어버린 이배환 교수님께 깊은 감사의 뜻을 표하기를 원합니다.

남들은 석사 생활 중 일 년은 그 학문을 이해하는데 시간을 들이고 나머지 일 년은 배운 지식으로 프로젝트를 수행시키는데도 많은 부족함을 느끼는데 3학기 때 정신없이 들어온 저를 게다가 건강까지 좋지 않은 상황이었음에도 저를 받아주시고 또 분자생물과 발생학의 참 맛을 알게 해주시고 참 과학도로서 아직은 많이도 부족하지만 추리하고 생각하는 머리를 갖도록 지도해준 김명희 교수님과 박형우 교수님 이하 인내심을 한껏 발휘하여 옆에서 도와준 발생학 실험실 식구들 유병기 선생님, 양혜원 선생님, 정현주 선생님, 나의 첫 사수 친절한 권윤정 선생님, 박성도 선생님, 정명섭 선생님, 공경아 선생님, 강진주 선생님, 김혜선 선생님, 이은영 선생님, 이미희 선생님, 이승리 선생님, 김승혜 선생님에게도 깊은 감사를 드립니다.

특별히 이 기간동안에 참 신앙으로 지도해주시고 옳은 길로 가도록 도와주신 우선동 목사님, 신호섭 목사님, 석기신 목사님, 은정수 목사님, 심경숙 전도사님 그리고 임민철 목사님께도 깊은 감사를 드리며 참 힘들 때 옆에서 친구가 되어주고 말동무가 되어준 15청년회 친구들에게도 깊은 감사를 표합니다. 그리고 무엇보다 뒤에 가장 큰 백으로 계신 하나님께 이 모든 일이 있기까지 여기까지 이렇게 인도하셨음에 깊은 영광을 드립니다. 앞으로 과학도로서 귀하게 쓰임 받을 삶을 살기로 다짐하며 또 서원하며 이 감사의 글을 마칩니다.

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Abstract

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All macromolecules are not actively taken up by living cells as its impermeability. The observation of protein transduction domain (PTD) has triggered its excitement. Since then, the most actively studied PTDs are those derived from the human immunodeficiency virus (HIV-1) transcriptional regulator Tat and the Drosophila transcription factor Antennapedia (Antp), that shown a rapid cellular delivery of proteins and peptides. Like Antp, Hoxc8 is one of the homeodomains that encodes protein domain of

60 amino acids, which is composed of three α-helices, with one β turn between helix 2 and 3 and that helix 3 is known to have translocation properties. Therefore, this is to study the transducing activity of recombinant Hoxc8 and its application as a delivery vector in membrane.

Hoxc8 full gene (pGEX:Hoxc8$_{1\text{-}242}$) was obtained from mouse RNA and prepared as a cDNA. By cloning technique, homeobox of *Hoxc8* with acidic portion could be isolated and named as pGEX:Hoxc8$_{152\text{-}242}$ with homeobox only of pGEX:Hoxc8$_{149\text{-}208}$. Recent paper have reported that PTD that contains purely basic amino acids is more efficient. Therefore, the third clone consisted with homeobox only which showed to contain basic amine groups. The proteins were labeled Oregon 488 and labeled proteins were treated to cells.

Similar efficiency in protein transduction was seen in all those three recombinants. In the DNA-protein complex formation experiment, the construct with basic amine group was more efficient and also the negative charge portion at the C-terminus suppressed from the DNA-protein complex binding. It has been shown that the protein indeed enters cells with macromolecules and the macromolecules were able to translate and work inside the cell.

This opens a great promise as a tool for a delivery vector with some modifications it may become alternatives for the replacement of liposomes or viral vectors that are toxic or may produce immune reaction from the targeted cell.

Key words: PTD, Hoxc8, homeodomains, macromolecules

Membrane Transducing Activity of Recombinant Hoxc8 and Its Application as a Delivery Vector

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I. Introduction

Practically all macromolecules are not actively imported by living cells as the cell membrane acts as an impermeable barrier and has shown to have poor permeability for large molecules[1]. One of the main difficulties in the manufacturing of new line of drugs whose targets are located within host cells is this membrane impermeability which is reflected in the fact that greater than 95% of all new therapeutics possess poor pharmacokinetics. Typically,

delivery of macromolecules are undertaken using lipids, electroporation or through viral vectors, however, these exhibit several limitations, including: (1) the inability to deliver to primary, non-dividing cells, (2) the requirement for optimization with each cell type, (3) low transduction level, (4) cellular toxicity[2,3,4,5].

For these reasons, considerable excitement was generated following the observation of protein transduction domain (PTD), in particular, those derived from the human immunodeficiency virus (HIV-1) transcriptional regulator Tat[6,7] and the Drosophila transcription factor Antennapedia (Antp). These PTDs have shown to mediate the rapid cellular delivery of proteins[8] and peptides[9] that have observed to possess the ability to traverse the lipid bilayer of cells in a concentration dependent manner[10], which of the mechanism is generally known to undergo endocytosis but the exact mechanism is not fully understood yet[11,12,13]. The importance of these proteins that traverse membrane lies in their ability to uniformly transport large, biologically active molecules to a population of mammalian cells growing under standard culture conditions[14].

Hoxc8 is one of the homeobox containing genes which encode a set of master transcription factors[15] and that is similar to the transduction domain of Antp[16]. Homeobox encodes protein domain of 60 amino acids and that is known to play an important role in defining the body plan of vertebrates[17]. Homeodomain proteins belong to a class of transcription factors involved in multiple morphological processes. The homeodomain, composed of three α

helices, with one β turn between helix 2 and 3. Third helix is known to possess translocation property comparable to that of the entire homeodomain[18] (refer to the illustration of Hoxc8 in Fig. 1)

Whist studying the structure of Hoxc8, it has been found that the C-terminus of pGEX:Hoxc8$_{1\text{-}242}$ is full of negative charged cations (acidic portion) such as glutamic acid or aspartic acid. As DNA itself is highly negatively charged when DNA and a construct that contains homeobox of *Hoxc8* with acidic portion at the C-terminus is mixed together, DNA-protein complex would not formed as they repel each other[19]. Although the entire protein can be used for this purpose, as it has been reported by Noguchi et al.[20], that smaller and poly-arginine (polyR) and poly-lysine protein have shown to exhibit greater efficiency in terms of delivery of several peptides and proteins.

Therefore, it can be hypothesized that having homeodomain would be more efficient than the ones with Hoxc8 full sequence. Therefore, three different constructs have been designed and examined on their membrane transducing activity as well as their application as the possible delivery vectors for nucleic acids; GST:Hoxc8$_{1\text{-}242}$, GST:Hoxc8$_{152\text{-}242}$, GST:Hoxc8$_{149\text{-}208}$. GST:Hoxc8$_{1\text{-}242}$ is full length of Hoxc8 protein fused to GST (glutathione S-transferase) gene. GST:Hoxc8$_{152\text{-}242}$ contains homeodomain, of which the N-terminus of homeodomain was partially deleted and still containing the acidic portion at the C-terminus fused to the homeodomain. GST:Hoxc8$_{149\text{-}208}$ contains homeodomain only.

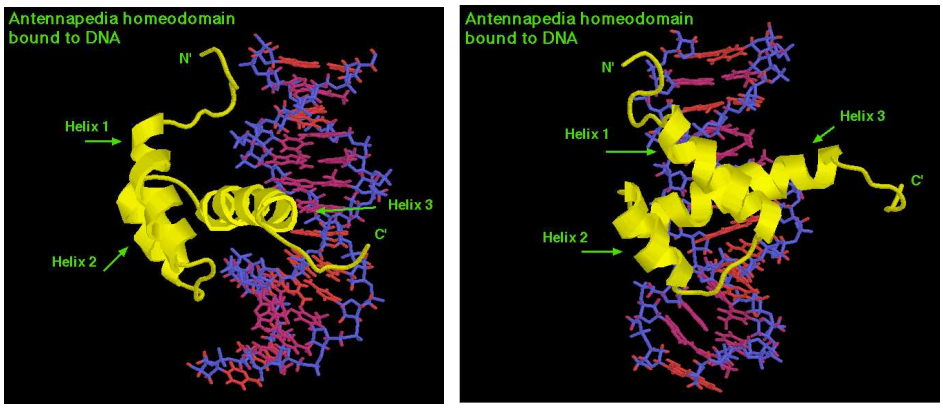

Fig. 1. 3D Structure Antennapedia homeodomain bound to DNA

(Source originated from http://www.biosci.ki.se/groups/tbu/homeo/)

II. MATERIALS AND METHODS

1. Plasmid Construction. The plasmid pGEX:Hoxc8$_{1-242}$ harboring a 780 bp Hoxc8 fragment was cloned into the *Bam*HI/*Xho*I site of the expression vector, pGEX4T-1 (Amersham) was kindly provided by Hyun-Joo Chung in our lab. pGEX:Hoxc8$_{1-242}$ was digested with *Sal*I to produce 306 bp fragment and subcloned it into the *Sal*I site of pGEX4T-1 to produce pGEX:Hoxc8$_{152-242}$. To isolate DNA fragment containing homeobox only of *Hoxc8*, both forward (16 mer; 5' GGA TCC CGG CGC AGC GGT CG 3') and reverse (14 mer; 5' CT CGA GTT CTC CTT TTT CCA C 3') primers were synthesized (Bioneer, Tae Jeon, Republic of Korea) and designed to contain *Bam*HI and *Xho*I site, repectively (Takara). PCR was done in the following conditions; first denaturation, 94 °C for 5 min; second denaturation, 94 °C for 30 sec; annealing temperature, 56 °C for 30 sec; extension, 72 °C for 60 sec; the last extension step, 72 °C for 5 min. The amplified fragment of homeobox was cloned into the pGEM-T Easy vector (Promega). The clone was then double digested with *Bam*HI and *Xho*I endonucleases, and the 180 bp fragment was isolated and inserted into the *Bam*HI and *Xho*I site of the expression vector, pGEX4T-1, to produce pGEX:Hoxc8$_{149-208}$. The schematic illustration of the *Hoxc8* deletion constructs of GST:Hoxc8$_{1-242}$, GST:Hoxc8$_{152-242}$, GST:Hoxc8$_{149-208}$ were shown in Fig. 2. Also the Detailed sequence of the negatively charged portion is shown in Fig. 3.

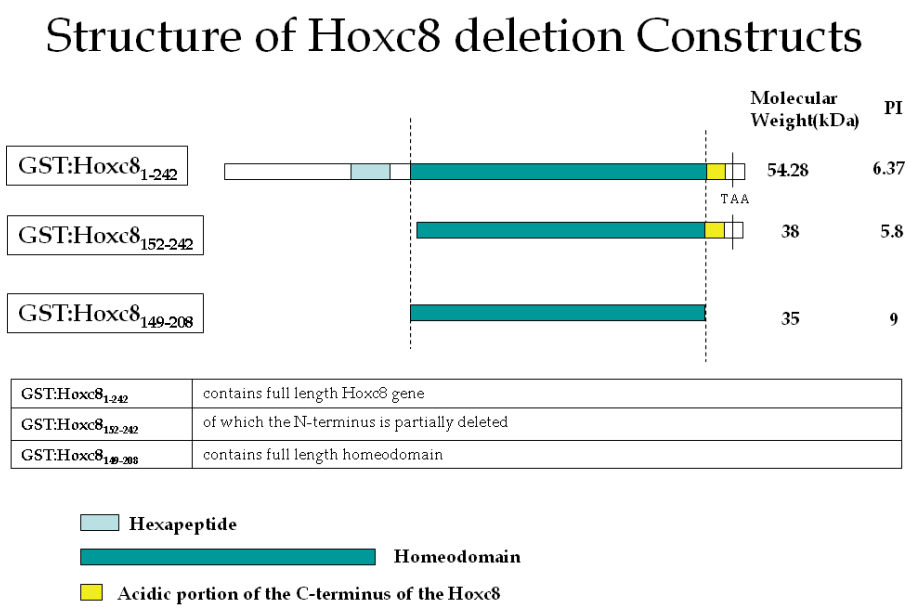

Fig. 2. Structure of Hoxc8 deletion constructs of GST:Hoxc8$_{1-242}$, GST:Hoxc8$_{152-242}$, GST:Hoxc8$_{149-208}$. The molecular weight of each GST:Hoxc8 fusion protein was shown on the right in kilodalton (kDa) along with its PI value. The hexapeptide, homeodomain, and the acidic portion of Hoxc8 protein were indicated in colors.

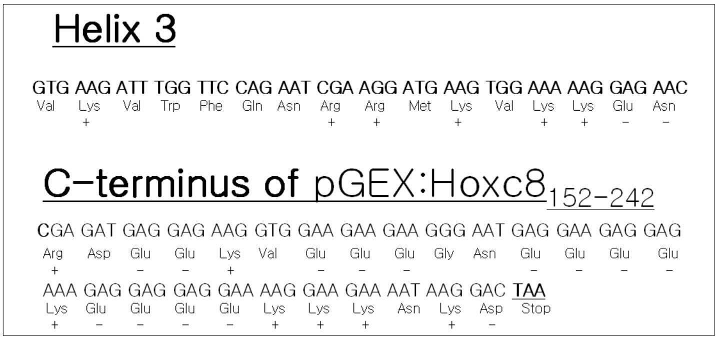

Fig. 3. The Detailed sequence of the negatively charged portion. As it can be seen from the sequence, many glutamic acid and aspartic acid were present at the c-terminus of the sequence.

2. Gel Extraction using Jetsorb Gel Extraction Kit (Genomed GmbH, LÖhne) Agarose was solubilized using 300 ㎕ of buffer A1 and 10 ㎕ of JETSORB suspension for each 100 ㎎ of Gel slice. Then DNA binding was allowed by incubating the assay at 50 °C for 15 min. The assay was mixed every 3 min during incubation. After this, the assay was centrifuged for 30 sec at 10000 g then the supernatant was removed completely using a pipette. The pellet was washed with A1 buffer and centrifuged as before to recover JETSORB and the supernatant was removed using pipette. Then the JETSORB was washed the 300 ㎕ of buffer A2 and centrifuged, then is repeated. JETSORB pellet was dried by air and under vacuum and eluted in TE buffer.

3. Transformation. Competent cell (DH5α) was melted in ice. 30 ㎕ of the competent cell was added to 1.5 ㎖ micro tube and 100 nM of desired DNA was added. Kept in the ice for 20 min with occasional tapping. Heat shock were given for 90 sec at 42 °C and 120 ㎕ of LB was added at room temperature (RT). The tube was incubated for 45 min at 37 °C and spread in a fresh LB plate with selective antibiotics, ampicillin (100 ㎍/㎕) in this clone.

4. Plasmid miniprep. Cells were grown in 3 ㎖ of LB broth (10 g of trytone, 5 g of yeast extract and 10 g of NaCl in 950 ㎖ of deionized H_2O then pH was adjusted to 7.0 with 5 N NaOH. The total volume was finally adjusted to 1 liter with deionized H_2O)

overnight in 14 ㎖ polystyrene Round-Bottom Tube (Falcon, NJ, USA). Cells were collected by the centrifugation at a speed of 12k rpm for 10 min at 4 °C in a 1.5 ㎖ micro tube (Sarstedt, Germany). After discarding the supernatant, the cell pallet was dissociated in 100 ㎕ of SolI (50 mM of glucose, 25 mM of Tris-Cl in pH 8.0 and 10 mM EDTA in pH 8.0) and vortexed well. The cells were then lysed by adding 200 ㎕ of SolII (0.2 N of NaOH and 1% (w/v) of SDS) and left for 5 min at RT then 150 ㎕ of SolIII (60 ㎖ of 5 M potassium acetate, 11.5 ㎖ of glacial acetic acid and 28.5 ㎖ of H2O) was added and shook to mix well (vortex three to five times in inverted position) then centrifuged at 12k rpm for 5 min at 4 °C. Then supernatant was transferred and the pellet was discarded. 450 ㎕ of Chloroform:Isoamylalcohol=24:1 (C/I) was added to the supernatant and shaken vigorously for at least 30 sec to 1 min then centrifuged at 12k rpm for 5 min. The supernatant was transferred into a new micro tube and then 900 ㎕ of absolute ethanol was added and vortexed vigorously. After leaving at RT for 2 min, it was centrifuged at 12k rpm for 5 min at 4 °C and the supernatant was discarded. The pellet was washed with ice cold 70% ethanol and centrifuged at 12k rpm for 5 min at 4 °C. Then the pellet was air dried at 55 °C for 10 min. 50 ㎕ of TE containing RNase (10 ㎍/㎕) was added to dissolve the DNA pellet.

5. Plasmid mediprep. Cells were grown in 25 ㎖ of LB broth for overnight in 50 ㎖ conical tube (SPL, Seoul, Republic of Korea).

The tube was centrifuged at a speed of 3.5k rpm for 10 min at 4 °C and the supernatant was discarded. The pellet was dissociated by adding 1 ㎖ of SolI and vortexed well. The cells were then lysed by adding 2 ㎖ of SolII and inverted for several times and left for 5 min at RT then 1.5 ㎖ of SolIII was added and inverted to mix well then centrifuged at 4k rpm for 10 min at 4 °C. 15 ㎖ tube with gauze covered up was prepared to isolate the pallet and the supernatant successfully. The same volume of 2-propanol (Duksan, Seoul, Korea) was added to the supernatant, shook vigorously, left for 2 min at RT, and then centrifuged for 10 min at 3.5k rpm at 4 °C. The pellet was washed with 3 ㎖ of ice cold 70% ethanol and centrifuged at 3.5k rpm for 10 min at 4 °C and air dried at 55 °C for 10 min. 400 ㎕ of deionized DW with 10 ㎕ of RNase (10 ㎍/㎕) was added and incubated at 37 °C for at least an hour. 200 ㎕ of phenol and 200 ㎕ of C/I were added and vortexed. After centrifugation at 12k rpm for 5 min at RT, the supernatant was transferred. 400 ㎕ of C/I were added, vortexed and centrifuged, and the supernatant was transferred. This step was repeated until the supernatant was purely isolated. To the supernatant, 1/10 volume of 3M Sodium Acetate (pH5.2) and 2.5 volume of absolute ethanol was added and kept at -20 °C for overnight. On the next day, DNA was collected by the centrifugation at 3.5k rpm for 10 min at 4 °C. The DNA pallet was washed with 1 ㎖ of 70% of ethanol and air dried. Then the pellet was dissolved in 50 ㎕ of TE.

6. Protein purification. In order to purify the fusion proteins, 100 ㎍ of each plasmid DNA of pGEX:Hoxc8$_{1\text{-}242}$, pGEX:Hoxc8$_{152\text{-}242}$, pGEX:Hoxc8$_{149\text{-}208}$ was transformed into the *E. coli* strain BL21 (DE3). BL21 (DE3) cells containing the expression plasmids were cultured at 37 °C to an OD_{600} of 0.8. Isopropyl-b-D-thiogalactopyranoside (IPTG) was added to a final concentration of 500 nM, and the cells were further incubated for 3 hr at 28 °C. After collecting cells through centrifugation, they were cracked with French Pressor (SLMOAMINCO, SLM instrument Inc.), and the supernatant was recovered after centrifuging it at 12k rpm for 60 min. The supernatant was incubation with glutathione sepharose (GST) beads (Sigma) overnight and the beads were recovered after centrifugation at 2k rpm for 10 min and washed three times with ice cold PBS. To separate GST bead with the proteins, They were incubated with reduced glutathione elution buffer {50 mM Tris in pH 8.0 and 10 mM reduced glutathione (sigma)}. After measuring the concentration of the proteins using Eppendorf Biophotometer (Eppendorf), and the protein sample was concentrated with Amicon Bioseparations Centricon (Millipore). For the better yield, optimal IPTG concentration and incubation temperature were determined.

7. Western blot. Purified recombinant proteins were resolved on a 10% polyacrylamide gel, transferred to nitrocellulose membrane, blocked with 5% (w/v) milk powder in TBST (20 mM Tris.HCl,

pH 7.5, 150 mM NaCl, and 0.05% (v/v) Tween 20), and incubated with primary antbody to GST (Santa Cruz Biotechnology) at a 1:10000 dilution. After washing the membrane three times in TBST, it was incubated with horseradish peroxidase-conjugated secondary antibody at a 1:20000 dilution. Finally, the bands were detected using chemiluminescence (ECL; Amersham Biosciences).

8. Transfection. The plasmid pDsRed1-C1 (red fluorescence) and pEGFP-C1 (green fluorescence) were chosen as reporter plasmids for their red and green fluorescent protein, respectively. 1 x 10^5 cells were seeded the day before transfection on to 6-well plate. One hr before the transfection cell was washed with PBS and incubated in DMEM without serum. For the transfection, DNA was pre-complexed with the PLUS Reagent by adding DNA with PLUS Reagent and left at RT for fifteen min. Then in a separate tube, LIPOFECTAMINE was diluted and the diluted Lipofectamine was brought to the pre-complexed DNA, mixed and left at RT for another fifteen min. The mixture was then added to each well containing fresh medium on cells. Cells were incubated at thirty seven °C at 5% CO_2 for three hr. And then, the media containing the mixture was suck out and fresh media containing 20% FBS was added and incubated further 24 hr. Then the cell was brought to the fluorescent microscopy for the analysis.

9. Concentration of proteins. Since it is advised to used the

concentration of higher than 2 ㎍/㎕ for the effective labeling with Oregon 488, purified proteins were concentrated to 4~10 ㎍/㎕ using centrifugal filter device, Centricon (Milipore, USA) as the initial protein concentrations were far less than 1.0 ㎍/㎕. Purified protein was loaded on to the column of the centrifugal filter device and centrifuged for 2000 g until the desired concentration of protein in obtained.

10. Labeling of protein with Oregon 488. About 5 ㎎ of the protein were dissolved in 500 ㎕ of 0.1 M sodium bicarbonate buffer. 5 ㎎ of the amine-reactive protein compound and the Oregon 488 dye were dissolved in 0.5 ㎖ of DMSO (10 ㎎/㎖). The the dye was freshly dissolved just before starting the reaction. Since reactive compounds are not very stable in solution. While vortexing protein solution prepared earlier, 50~100 ㎕ of the reactive dye solution (10 ㎎/㎖) was added slowly and incubated for an hour at RT with continuous stirring.

11. Nuclear (DNA) staining with Hoescht Dye. Media was aspirated from culture plate and cells were washed twice with PBS. Cells were then fixed with 4% formaldehyde at RT for 20 min using just enough solution to cover the dish thoroughly. After aspiration of the fix solution, cold absolute methanol was added. After leaving at RT for 20 min, the methanol was aspirated and rinsed thoroughly three times with PBS. 1:10,000 dilution of

Hoescht stock (1.2 ㎎/㎖ in 0.9% NaCl or water) in PBS was added and incubated for 15 min at RT. After rinsing five times with PBS, cells were analyzed under the fluorescence microscope.

12. Cell culture and protein internalization. Cell used for the experiment was PPFF (Pig Primary Fetal Fibroblast Cell) and T98G (Myeloma cells obtained from brain) cells. Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM, Welgene, Taegu, Korea) containing 10% Fetal Bovine Serum (FBS) and 5% Penicillin-streptomycine. All cells were cultured at 37 ℃ in a 5% CO_2 atmosphere. Cells were seeded on a 24-well plate at a density of 1 x 10^5 cells/well. After 24 hr incubation, the medium was changed with fresh medium containing 1 M of each fusion proteins purified proteins and incubated for 0, 5 min, 10 min, 15 min, 30 min, 1 hr, 2 hr and 3 hr, and then the optimal incubation time was decided. Then, series of proteins (10 nM, 100 nM, 500 nM and 1 mM) were treated to cells seeded on a 24-well plate at the same density described above. Cells were trypsinized to rule out electrostatic binding of protein to the cell surface and analyzed under the fluorescent microscope (Olympus IX70, Olympus, Melille, NY).

13. Formation of DNA-protein complex. The binding of Hoxc8 fusion proteins to the reporter plasmid DNA was investigated through a gel retardation experiment. Increasing amount of protein

was incubated with 0.1 μg plasmid pEGFP-C1 (green fluorescence) or pDsRed-C1 (red fluorescence), not exceeding 20 $\mu \ell$ in total volume and then glycerol was mixed to 30% of total volume. The mixture was left in RT for an hour. Then the electrophoretic mobility of the resulting protein-DNA complexes was analyzed in 1% agarose gel through electrophoresis.

14. Transduction of DNA-protein complex. To evaluate the transduction of protein-DNA complexes, cells were incubated in the presence of the medium harboring the DNA-protein complex for 3 hr, and then incubated overnight after adding fresh medium. The vitality of the cells were checked with light microscopy and the cells were kept another 24 hr for the protein expression. After washing three times with PBS, cells were fixed in 4% paraformaldehyde (PFA), stained with Hoescht, and analyzed under the fluorescence microscope.

III. Results

1. Construction of plasmids pGEX:Hoxc8$_{1\text{-}242}$, pGEX:Hoxc8$_{152\text{-}242}$ and pGEX:Hoxc8$_{149\text{-}242}$.

In order to examine the Hoxc8 transduction activity along with the nucleic acid complex, several Hoxc8-derived fusion proteins were cloned to be synthesized (Fig. 4.). Since the plasmid pGEX:Hoxc8$_{1\text{-}242}$ expresses the full length Hoxc8 protein, several deletion derivatives were constructed using this plasmid. The plasmid pGEX:Hoxc8$_{1\text{-}242}$ was digested with *Sal*I and created 306 bp of DNA fragment (Fig. 5.) containing homeobox along with the acidic portion at the C-terminus of Hoxc8 was isolated and cloned into the *Sal*I site of pGEX4T-1 vector. Of 45 colonies selected on the ampicillin plate, 10 were chosen to be checked. Among these four had the DNA fragment inserted in the right direction (pGEX:Hoxc8$_{152\text{-}242}$) and the rest of them had the DNA fragment inserted in the opposite direction.

To produce pGEX:Hoxc8$_{149\text{-}208}$, homeobox (180 bp) only of Hoxc8 was amplified using PCR technique as described in the materials and methods and the product was cloned into the pGEM-T Easy vector. Since pGEM-T Easy vector has a α-complementation region of *LacZ*, blue / white selection is possible after cloning. Among 14 colonies (12 white and 2 blue colonies) analyzed, all 12 white colonies turned out to contain about 800 bp

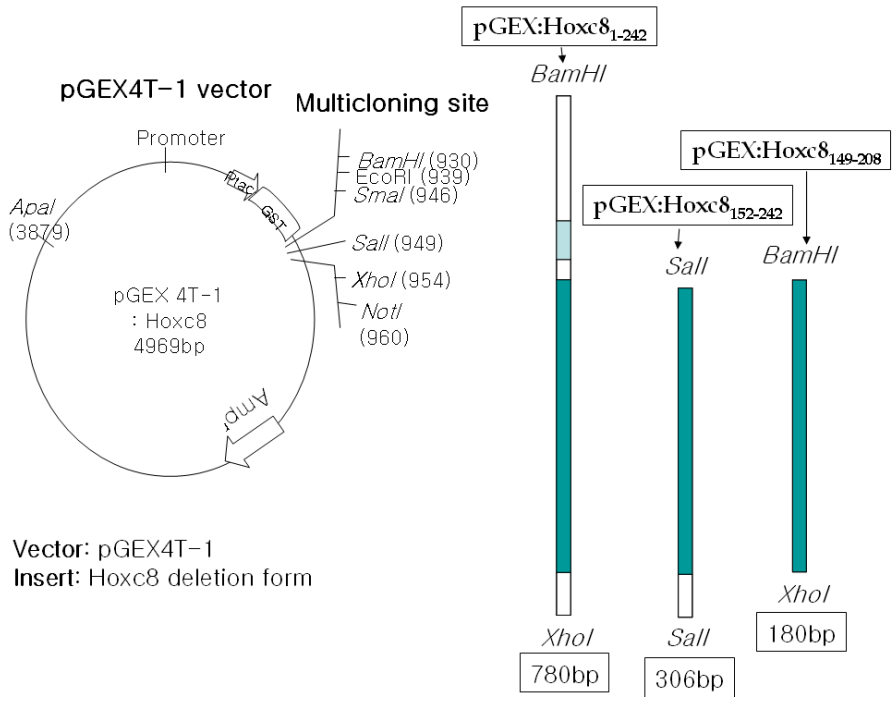

Fig. 4. **Schematic illustration of different clones.** pGEX:Hoxc8$_{152-242}$ was obtained by digesting pGEX:Hoxc8$_{1-242}$ with *Sal*I endonucleases and ligase into pGEX4T-1 vector. pGEX:Hox8$_{149-208}$ was cloned by amplification of homeobox using PCR with the template of pGEX:Hoxc8$_{1-242}$. The amplified homeobox was then cloned into pGEX4T-1 vector.

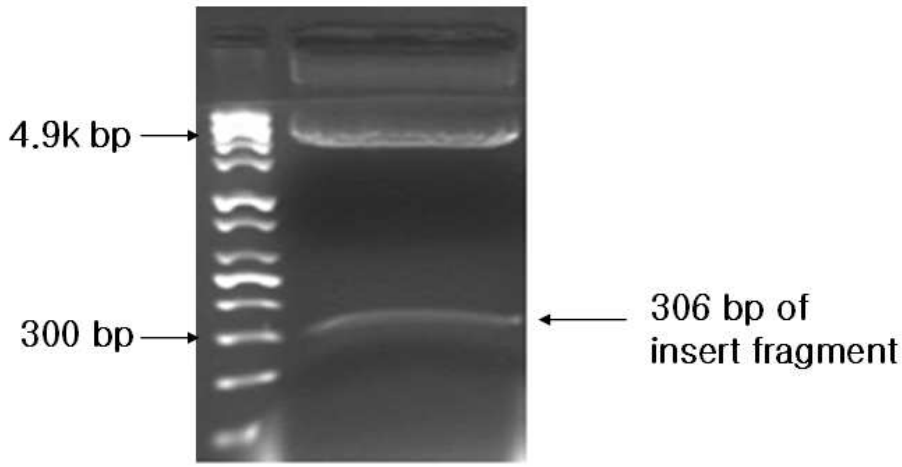

Fig. 5. 306 bp fragment harboring the C-terminus of Hoxc8 was confirmed. pGEX:Hoxc8$_{1-242}$ was digested with *Sal*I restriction endonucleases, and the 306 bp fragment harboring the C-terminus of Hoxc8 was confirmed in a 1.0% agarose gel.

*Bam*HI-*Xho*I fragment instead of 180 bp PCR fragment (Fig. 6.) Unexpectedly however, two blue colonies (lane 14 and 15) have revealed to contain about 200 bp *Bam*HI-*Xho*I fragment containing the amplified homeobox region of *Hoxc8*. For further analysis, # 14 colony was selected and used.

With the DNA gained from the mediprep, DNA was digested with *Xho*I/*Bam*HI and the 180 bp of homeobox was isolated, at the same time pGEX4T-1 was digested with the endonucleases. Ligation of the vector and the fragment were performed, for the result 1 colony was obtained. For the confirmation of the clones, all the different clones were digested with *Sal*I and *Bam*HI/*Xho*I. As it can be observed from the Fig. 7. all the clones obtained were confirmed.

2. Protein purification of GST:Hoxc8$_{1\text{-}242}$, GST:Hoxc8$_{152\text{-}242}$ and GST:Hoxc8$_{149\text{-}208}$ fusion proteins.

In order to get the highest yield of fusion proteins each plasmid (GST:Hoxc8$_{1\text{-}242}$, GST:Hoxc8$_{152\text{-}242}$ and GST:Hoxc8$_{149\text{-}208}$) was tested for the optimal IPTG concentration and induction temperature, since higher than 2 ㎎/㎖ was advised to use for the effective labeling with Oregon 488. As shown in Fig. 8., small amount of proteins were induced even in the absence of inducer IPTG (lanes 2 and 6). As the concentration of IPTG was increased from 0.1 mM to 1 mM (lanes 3 to 5 and 7 to 9), however, it seemed to reach its plateau at 0.5 mM of IPTG. Therefore, 0.5 mM of IPTG concentration was used to induce each fusion protein.

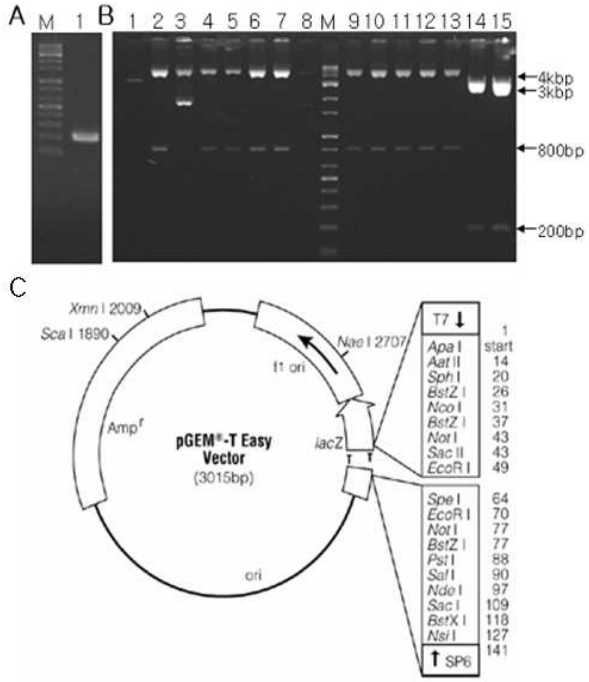

Fig. 6. PCR product (A), 15 clones obtained from the cloning (B), cloned PCR product into pGEM-T Easy vector (C). 0.5% agarose gel showing PCR product (A). 180 bp of PCR product was subcloned into pGEM-T Easy vector (C). (B) Showed the 15 clones obtained from the cloning of PCR product into the pGEM-T Easy Vector (1 - 13; white colony and 14 - 15; blue colony). As pGEM-T Easy vector contains LacZ, the clones were transformed into *E. coli* and then spread to a LB plate contains X-gal. The colonies were selected according to its color of the colones; white (lane 2 to 13) and blue (lane 14 and 15) colonies. Miniprep of the colony was performed and the DNA were cut with *Xho*I and *Bam*HI enzyme. Lane 1 shows single cut of pGEM-T Easy vector as a negative control.

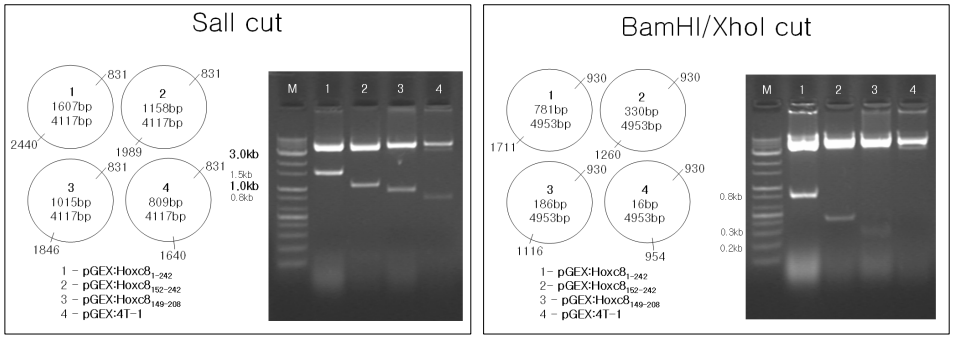

Fig. 7. **Different clones achieved after the cloning.** The colonies were confirmed using different restriction enzymes. Numbers on the plasmids show the cutting site of the each restriction endonucleases and the numbers in the illustrated plasmids shows the digesting points.

We tested the optimal temperature for the Hoxc8 production. Since some proteins were reported to be extracted best in low temperature of 28 °C and some in normal temperature of 37 °C. As shown in Fig, 9, 28 °C gave better yield than 37 °C. As Hoxc8 protein is one of transcription factor that are not expressed much normally, the optimal condition of 28 °C was chosen for the Hoxc8 full length protein after purifying and concentrating fusion proteins of GST:Hoxc8$_{1\text{-}242}$, GST:Hoxc8$_{152\text{-}242}$ and GST:Hoxc8$_{149\text{-}208}$.

3. Western blot.

To confirm whether fusion proteins, GST:Hoxc8$_{1\text{-}242}$, GST:Hoxc8$_{152\text{-}242}$ and GST:Hoxc8$_{149\text{-}208}$ were the ones which we have designed, each protein was partially purified using GST-agarose bead and analyzed on a polyacrylamide gel containing SDS. Using two identical SDS gel with the same proteins were loaded and ran, one was used to confirm the size of each protein, GST:Hoxc8$_{1\text{-}242}$, GST:Hoxc8$_{152\text{-}242}$ and GST:Hoxc8$_{149\text{-}208}$ through Commassie Blue staining (Fig. 10. A.), and the other gel was used to transfer the proteins into the nitrocellulose membrane and western analysis was performed using GST antibody (Fig. 10B).

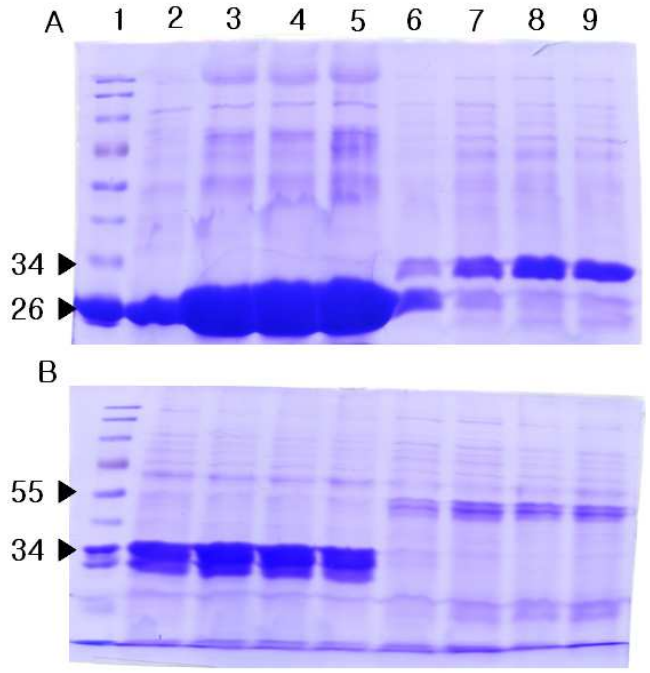

Fig. 8. Test for the optimal IPTG concentration. Test of optimal IPTG concentration of each constructs; A. Lane 1 indicate the protein maker, 2 to 5 were the overexpression of GST, 6 to 9 are the overexpression of GST:Hoxc8$_{149\text{-}208}$, B. Lane 1 indicate the protein maker, 2 to 5 show the overexpression of GST:Hoxc8$_{152\text{-}242}$, Lane 6 to 9 are the overexpression of GST:Hoxc8$_{1\text{-}242}$. The IPTG concentration was increased from 0 mM (lane 2 and 6) to 0.1 mM (lane 3 and 7), 0.5 mM (lane 4 and 8) and 1 mM (lane 4 and 9).

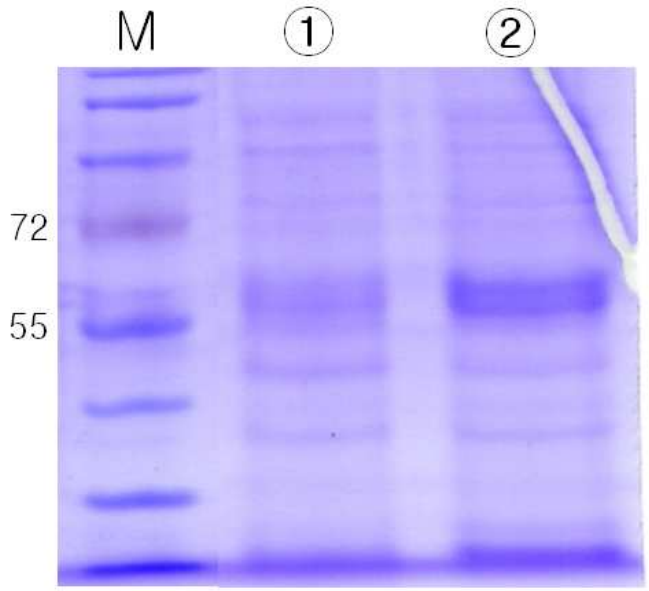

Fig. 9. Test of optimal temperature for the induction of GST:Hoxc8$_{1\text{-}242}$ protein. M lane indicates protein marker, ① lane indicates protein overexpressed in 37 ℃ and ② lane indicates protein overexpressed in 28 ℃.

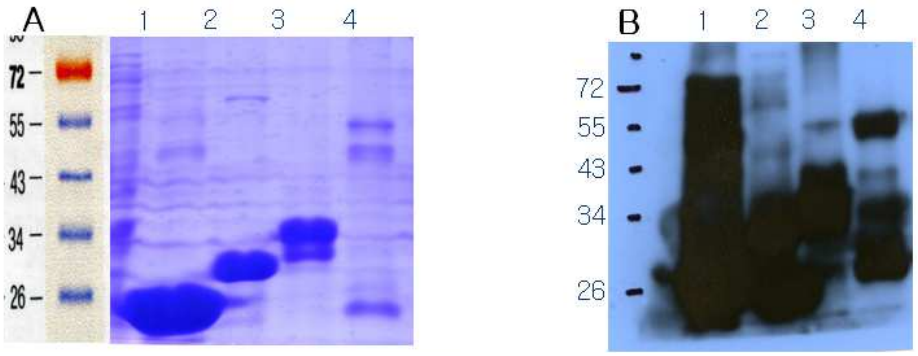

Fig. 10. Commassie staining of SDS-PAGE gel (A) and the western blot analysis using GST antibody (B) for the activities of proteins purified. Using two identical SDS gel with the same proteins were loaded and ran, one was used to confirm the size of each protein, GST:Hoxc8$_{1\text{-}242}$, GST:Hoxc8$_{152\text{-}242}$ and GST:Hoxc8$_{149\text{-}208}$ through Commassie Blue staining (A), and the other gel was used to transfer the proteins into the nitrocellulose membrane and western analysis was performed using GST antibody (B). Lane 1 indicates GST alone, Lane 2 indicates GST:Hoxc8$_{149\text{-}208}$, Lane 3 indicates GST:Hoxc8$_{152\text{-}242}$ and Lane 4 indicates GST:Hoxc8$_{1\text{-}242}$.

4. Cellular translocation of GST-fused Hoxc8 derivatives; GST:Hoxc8$_{1-242}$, GST:Hoxc8$_{152-242}$ and GST:Hoxc8$_{149-208}$ protein.

In order to see the translocation activity of GST-fused Hoxc8 derivatives, each protein was purified and labeled with Oregon 488 as described in the Materials and Methods. When the labeled GST:Hoxc8$_{1-242}$ (3 ㎎/㎖) was added into the culture media, green fluorescence was detected inside of PPFF cells as shown in Fig. 11, indicating that the labeled protein entered into the cell through the lipid bilayer. The figure still revealed that the protein entered the cell not only the in cytoplasm but also in the nucleus of the cell.

In the Fig. 12., the possibility of artifacts were tested by adding the dye only in the well containing cells (A) and GST:Hoxc8$_{1-242}$ was added as positive control. According to the data shown in Fig. 12., There was green fluorescence detected in the well with dye only (A), whereas green fluorescence detected in almost 100% of cells with the GST:Hoxc8$_{1-242}$ containing cells (B).

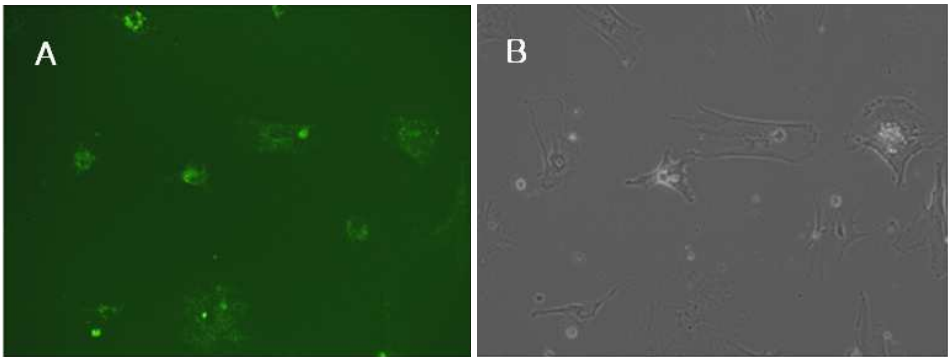

Fig. 11. Cellular transduction of the Oregon 488 labeled GST:Hoxc8$_{1\text{-}242}$ in PPFF cells. Picture above shows that cellular transduction of the Oregon 488 labeled GST:Hoxc8$_{1\text{-}242}$ in fluorescent (A) and light (B) microscopy shows that some of the proteins were retained in the nucleus of the cell and some are retained in the cytoplasm of the cell.

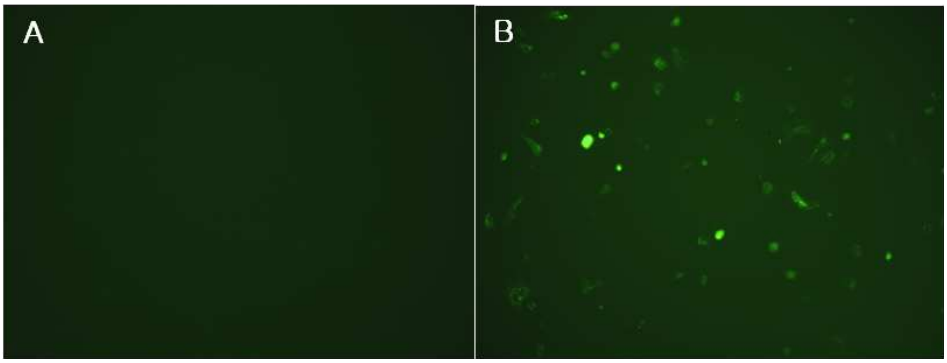

Fig. 12. To test the artifacts; dye (A) and 0.5 mM of GST:Hoxc8$_{1\text{-}242}$ (B) was treated. To confirm that the Oregon 488 dye itself is not staining the cells, dye only was treated (A) which was used as a negative control and 0.5 mM of GST:Hoxc8$_{1\text{-}242}$ (B) was treated to the cell as a positive control.

To compare the cellular translocation activity of each proteins, three different proteins of GST:Hoxc8$_{1\text{-}242}$ (Fig. 10. A), GST:Hoxc8$_{152\text{-}242}$ (Fig. 10. B) and 0.5mM of GST:Hoxc8$_{149\text{-}208}$ (Fig. 10. C) were added into the culture media at a concentration of 0.5mM each. After 1 hr incubation, cellular uptake of each proteins was analyzed under the fluorescence microscope. As shown in Fig. 13., all three proteins have a similar translocation activity, entering nearly 100% of the cells.

The time course for the cellular internalization of protein was tested using 0.5 mM of Oregon 488 labeled GST:Hoxc8$_{1\text{-}242}$ protein following different incubation time (5 min, 10 min, 15 min, 30 min, 1 hr, 2 hr and 3 hr). The intensity of the green fluorescence in the cell was gradually increased with the time until they reach its plateau at 1 hr of incubation (Fig. 14.).

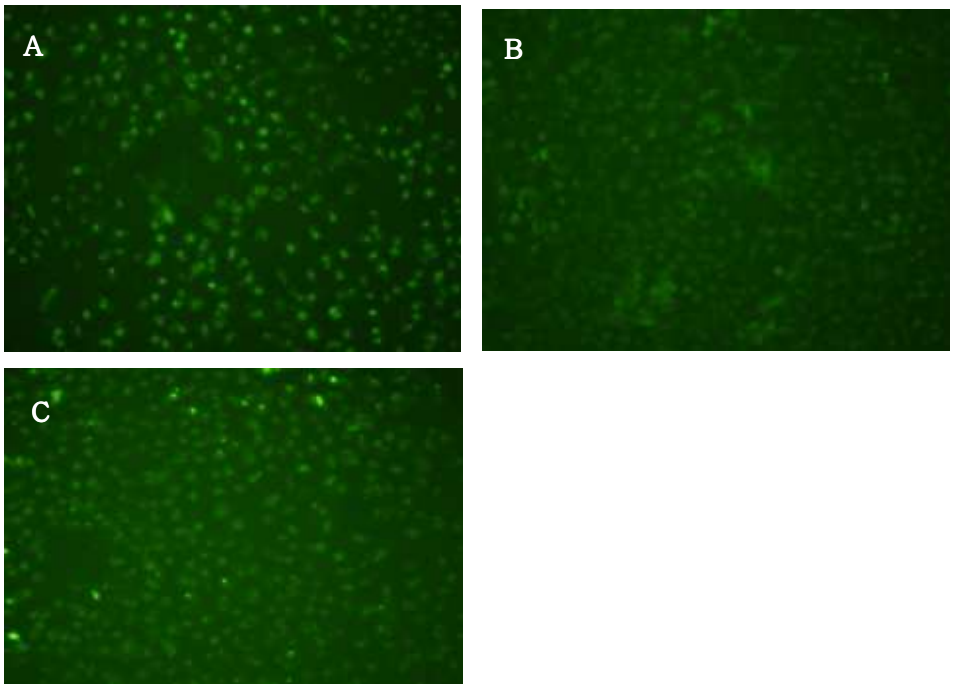

Fig. 13. The transduction of Oregon 488 labeled Hoxc8 fusion recombinants. The transduction of Oregon 488 labeled GST:Hoxc8$_{1\text{-}242}$ (A), GST:Hoxc8$_{152\text{-}242}$ (B) and GST:Hoxc8$_{149\text{-}208}$ (C) proteins in T98G cells displayed similar patterns with the ones observed from PPFF cells.

By the same method, optimum concentration was also tested using 10 nM, 100 nM, 500 nM and 1 mM of Oregon 488 labeled full length Hoxc8 fusion protein, GST:Hoxc8$_{1-242}$. As shown in Fig. 15., the intensity seemed to reach its peak at 500 nM of proteins.

In order to verify whether the GST itself has a cellular translocation activity, transduction efficiency was compared between GST and GST:Hoxc8$_{1-242}$ fusion protein. Although small amount of GST was found to enter the cell the efficiency of the GST:Hoxc8$_{1-242}$ was far greater than GST alone (Fig. 16.)

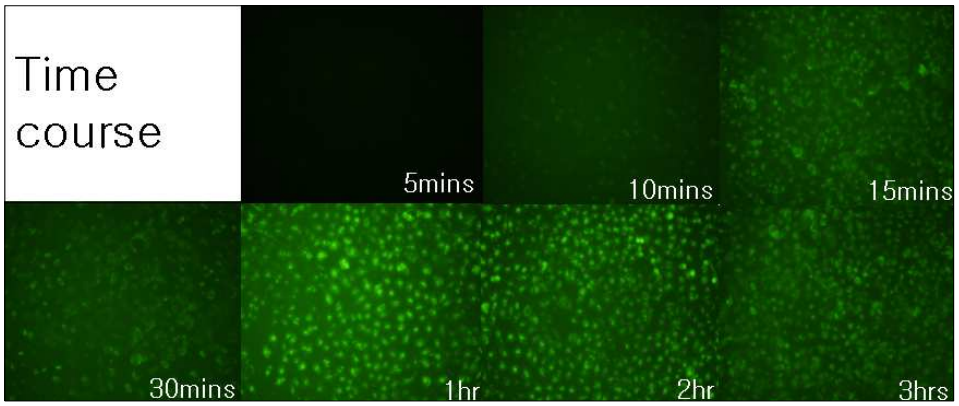

Fig. 14. The time course for the optimal protein transduction. The time course for the optimal transduction of proteins is tested only for GST:Hoxc8$_{1\text{-}242}$ as all of the proteins displayed similar patterns in the internalization of the protein. After the treatment of GST:Hoxc8$_{1\text{-}242}$ proteins for 5 min, 10 min, 15 min, 30 min, 1 hr, 2 hr, and 3 hr. 1 hr of the incubation time seems to be the most suitable for the internalization of the protein.

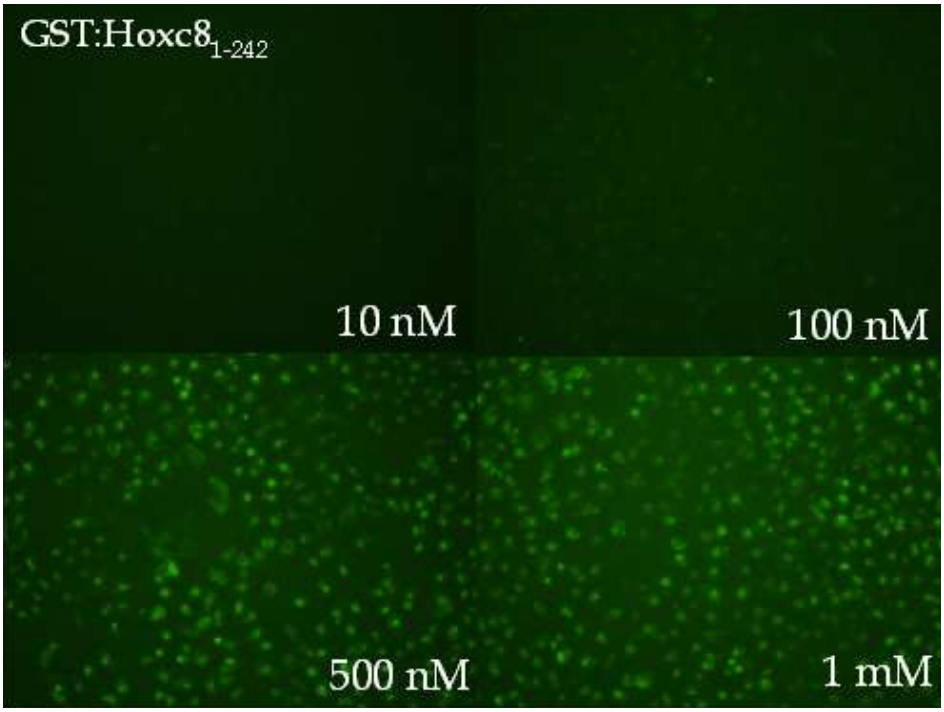

Fig. 15. The optimal concentration of the protein for the transduction. Same protein was tested to T98G cells for the optimal concentration of the protein to internalize the best. 500 nM seems to be the optimal concentration to treat with.

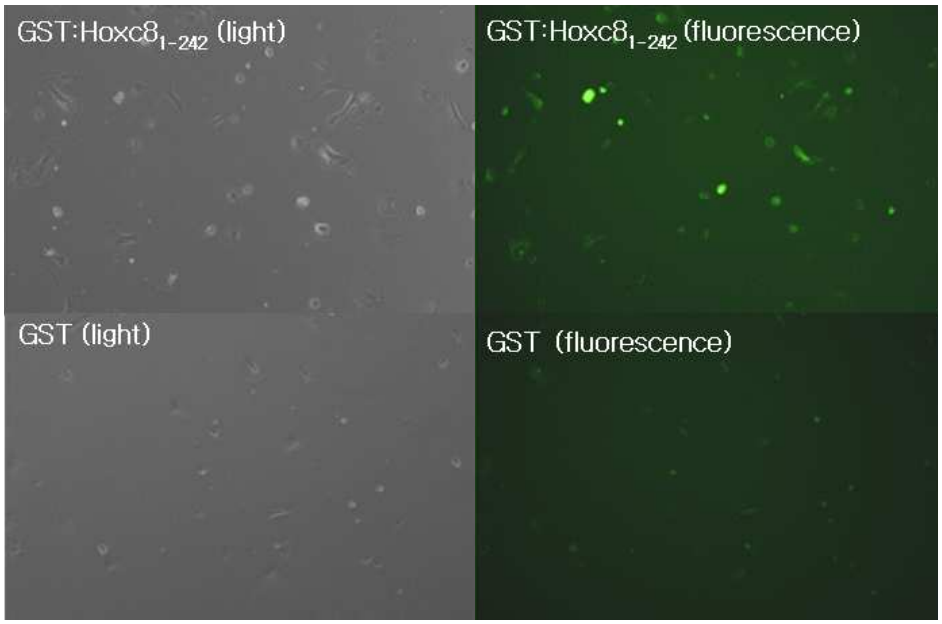

Fig. 16. The comparison of differences in transduction efficacy between GST protein to GST:Hoxc8$_{1-242}$. Although GST protein itself was able to transduce, it has clearly shown that the efficiency of GST:Hoxc8$_{1-242}$ was higher than GST protein only.

5. Formation of DNA-protein complex.

In order to test whether Hoxc8 protein could be used as a delivery vector for nucleic acid, the protein and DNA interaction was investigated using a gel retardation experiment. As described in the Materials and Methods, different concentrations of GST:Hoxc8 fusion proteins were incubated with the same amount of DNA. As shown in Fig. 17., GST:Hoxc8$_{1\text{-}242}$ seemed to bind well with DNA; most of DNA made complex with the protein at the ration of 1:15 (DNA:protein in mass). In the case of GST:Hoxc8$_{152\text{-}242}$ that has a negative charge at the C-terminus of the protein, the formation of DNA-protein complex seemed to be inhibited by repulsion negative charges between DNA and C-terminus of the protein. On the other hand, GST:Hoxc8$_{149\text{-}208}$ having the negative charges removed, seemed to retain the ability to form DNA-protein complex and making itself even more efficient than the ones with full length Hoxc8 protein: even with small amount of GST:Hoxc8$_{140\text{-}208}$ protein, high amount of protein-DNA complex was formed (Fig. 17. lane 2).

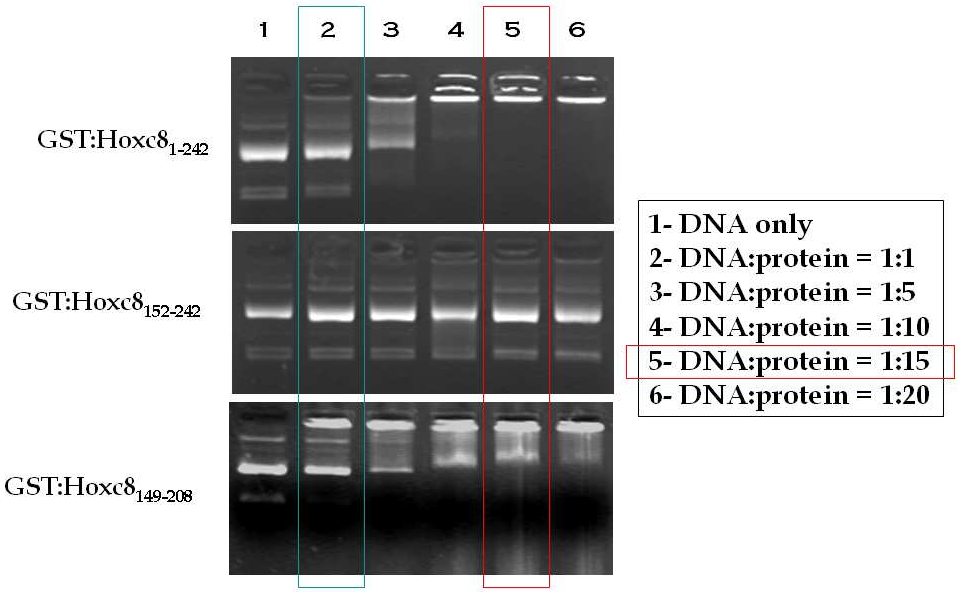

Fig. 17. Gel retardation experiment for DNA-protein complex. Gel retardation experiment was conducted to test the ability for the proteins to bind with macromolecules. The figure clearly showed that the binding affinity of PTD with negatively charged portion (GST:Hoxc8$_{152-242}$) has greatly suppressed whereas when the negatively charged portion has removed the binding affinity seems to increase greatly.

6. Transfection of DNA using Hoxc8 protein as a delivery vector.

In order to test the possibility of Hoxc8 protein as a delivery vector, the protein-DNA complex was applied into a fresh standard culture medium containing 20% FBS. The amount of the DNA added was decided by the amount of DNA used for the transfection using lipofectamine (0.7 μg in cell seeded onto 6 plate well). Transfection using 0.7 μg of DNA using lipofectamine were shown in Fig. 18. as a preliminary data.

Total amount of 0.7, 1.4, 2.8, 4.2 and 5.6 μg of pDsRed1-C1 DNA formed complex with each GST:Hoxc8$_{149\text{-}208}$ and those complex (1:15 mass ratio of DNA:protein) was applied into each well (6-well plate). After incubation for 3 hr, red fluorescence was analyzed under the fluorescence microscope. According to the fluorescence microscope, it the transfection rate was observed to be higher with the wells containing 5.6 μg of pDsRed1-C1 DNA complex with GST:Hoxc8 fusion protein, which of the data is not shown. The DNA-protein complex seemed to be transfected in similar efficiency for both GST:Hoxc8$_{149\text{-}208}$ and GST:Hoxc8$_{1\text{-}242}$, which of the data was shown in Fig. 19 that 5.6 μg of pDsRed1-C1 DNA formed complex with GST:Hoxc8$_{149\text{-}208}$ (A: 840 μg in a DNA:protein=1:15 ratio) and GST:Hoxc8$_{1\text{-}242}$ (B: 840 μg in a DNA:protein=1:15 ratio) was transfected.

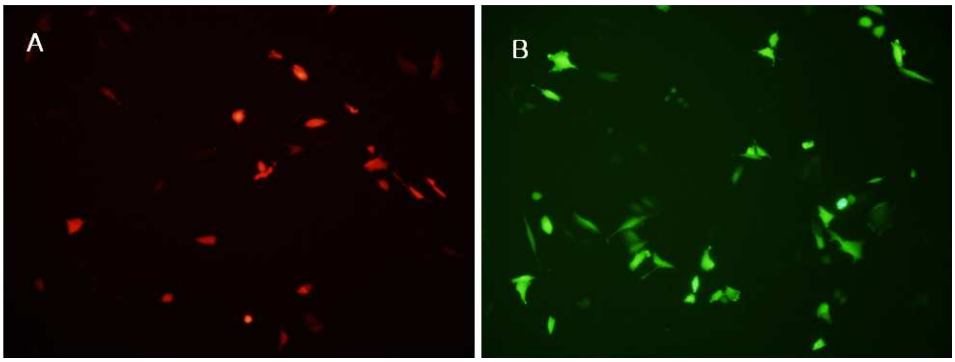

Fig. 18. DNA transfection using lipofectamine. DNA transfection was performed as a preliminary data to use in the transfection of the DNA-protein complex transfection. A showed transfection of pDsRed1-C1 (translates red fluorescence protein) and B shows transfection of pEGFP-C1 (translates green fluorescence protein).

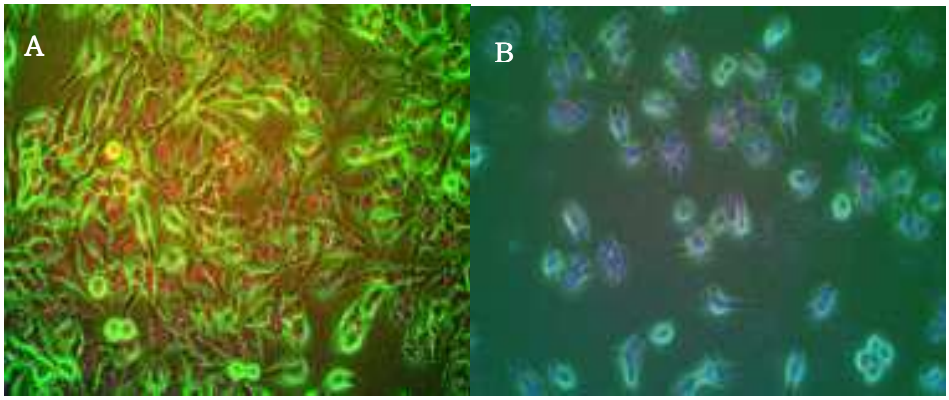

Fig. 19. DNA-protein complex transfection (A: GST:Hoxc8$_{149\text{-}208}$, B: GST:Hoxc8$_{1\text{-}242}$). Red color resulted from the translation of pDsRed C-1 vector, Blue color resulted from Hoescht, a staining of nucleus. The pictures above have merged pictures of Red fluorescence, Hoescht staining taken up by fluorescence microscopy and the cell taken by light microscopy.

IV. Discussion

Protein Transduction Domain (PTD) is on excitement and getting much attention as they are known to traverse biological membranes. What is more is that this protein binds with macromolecules and has the ability to deliver to cells as a delivery vector. The delightedness may come with their therapeutic and research use in the place of liposome and viral vectors[21].

Over the past years, the term "homeodomain" has evolved to define a class of protein domains that have recognizable similarity to a 60 amino acid motif (encoded by 180 bp homeobox sequences) originally identified in three Drosophila homeotic and segmentation proteins. Those homeodomains that have been tested contain sequence-specific DNA binding activities and are part of large protein family that function as transcriptional regulators[22]. It has been already known that a homeodomain of Hoxc8 has helix-turn-helix structure like other homeodomain proteins including Antp.

The homeodomain of Antennapedia is well studied and well known as they act as PTD. According to the study of Derossi et al[23], third helix of Antennapedia homeodomain is essential for internalization of Antennapedia. When they analyzed one mutant having two hydrophobic residues (tryptophan$_{48}$ and a phenylalanine$_{49}$) deleted in the third helix of the homeodomain, its translocating ability has been lost. Using synthesized peptides, they

found that a peptide of 16 amino acid residues, corresponding to amino acids 43 - 58 of the homeodomain (penetratin-1 or 'third helix'), possesses translocation properties comparable to that of the entire homeodomain[24].

Since Hoxc8 has homeodomain as the one from Antennapedia, and this domain is well known for its conserved structure, we hypothesized that the homeodomain would work in a similar pattern to that of Antp. It has been studied already that the Hoxc8 with its full lengths can be transduced into cells[25]. From the homeodomain sequence of Hoxc8, we have found that at the C-terminus many glutamic acid and aspartic acid residues, which make the protein negatively charged. As PTDs are known to be more effective when they are constituted with basic amino acids, three constructions were generated here: the protein having full length Hoxc8 (GST:Hoxc8$_{1\text{-}242}$), the protein having homeodomain along with the negatively charged C-term (GST:Hoxc8$_{152\text{-}242}$), and the protein having homeodomain only (GST:Hoxc8$_{149\text{-}208}$). In the case of GST:Hoxc8$_{152\text{-}242}$, 3 amino acids are missing at the beginning of the homeodomain. Roy, et al[26] has suggested that the first few amino acids of α-helix are not important although the last helix is crucial. Therefore, 3 amino acids missing were not seriously considered here.

In this study, three different GST:Hoxc8 fusion protein constructs were produced. In the cloning of PCR product to the pGEM-T Easy vector (Fig. 6. B.), it has not been expected to have fragment

of 800 bp where the inserted PCR product was 180 bp. Also in the comparison of the size of vectors, which lane 1 was showing empty pGEM-T Easy vector (3k bp) lane 2 to 13 showed somewhat bigger vector size as well. Therefore it can be interpreted that the PCR template might have been transformed and shown as white colony and as the PCR fragment was small that *lacI$_q$ZΔM15* region was expressed and shown blue colony in LB plate in the presence of X-gal.

According to the protein transduction study, all three different constructs (GST:Hoxc8$_{1\text{-}242}$, GST:Hoxc8$_{152\text{-}242}$ and GST:Hoxc8$_{149\text{-}208}$) was shown to transduce to almost 100% of the cells showing similar efficiency not only in the cytoplasm but also to the nucleus of the cells. It had been interpretate that the transducing efficiency of GST:Hoxc8$_{152\text{-}242}$ would be less efficient than GST:Hoxc8$_{1\text{-}242}$ and GST:Hoxc8$_{149\text{-}208}$. As those PTDs are known to electrostatically bind to the cell surface in order to endocytosis occur. However, in the transduction of GST Hoxc8 fusion protein, all three construct was turn out to be very efficient even in the low concentration (10 nM).

In order to eliminate the possibility of artifacts, free dye, GST protein and the GST:Hoxc8 fusion protein was added to cells on a standard culture media, respectively and the green fluorescence was detected. A high density of positive charges leads to a strong binding of the peptides to the overall negatively charged plasma membrane as was evidenced by mild fixations was performed[27].

According to the data obtained, we can understand that non of the detected green fluorescence was artifacts.

What excites us even more in the present study is that we have found the protein not only traversed to the cells but also GST:Hoxc8 fusion protein actually delivered macromolecules into the cells (refer to the Fig. 19.). There are not many studies on protein carrying macromolecules which to be delivered into cells and show the expression pattern. In the comparison of the transfection experiment, the transfection rate were not shown to be as effective as to lipofectamine or viral vectors. However, according to the data obtained from the transfection study of GST:Hoxc8 fusion protein combined with pDsRed1-C1 (Fig. 19.), red fluorescence protein was observed not only in the nucleus of the cells but also in the cytoplasm of the cells. To enhance the transfection rate, the use of common co-lipids in synergy may come useful as suggested by Mukherjee K, et al.[28], the use of common co-lipids in synergy may turn out to be rewarding in future design of novel liposomal transfection kits for use in non-viral gene therapy.

According to Namiki et al.[29], GST itself can act as PTDs. In the comparison of transduction of GST to GST:Hoxc8 fusion protein, our data revealed that GST:Hoxc8 fusion protein is more effective compared to GST alone. However, more experiment is needed to be designed in order to find out, if the Hoxc8 protein itself is effective as it is separated from GST. As the GST:Hoxc8 fusion

protein might have become effective protein as two proteins that are able to transduce to the cells were fused together.

In conclusions, the homeodomain of Hoxc8 has high translocating ability into cells without any sort of toxicity occurs in translocation of different cells. As far as gene therapy concerns, mostly viral vectors are used although they have reported to cause high toxicity and immune reaction, there are no alternatives available yet. With PTDs are now arising as a promising source of delivering oligonucleotide or macromolecules vector to cells. They might be considered to use clinically or for the research purposes.

V. Conclusion

Protein Transduction domain is one of the excitement as they translocate into cells without harming the target cell but also without causing immune response as they are reported to do so in the use of the viral vectors. Positively charged PTDs are known to electrostatically bind to negatively charged plasma membrane cell surface and then taken up by endocytosis of the cell, that makes energy or receptor independent pathway process possible.

In our studies, we have conducted three different constructs containing homeodomain of Hoxc8. One contained full lengths of Hoxc8 (GST:Hoxc8$_{1\text{-}242}$), second contained homeodomain with negatively charged C-terminus (GST:Hoxc8$_{152\text{-}242}$) and last contained homeodomain only (GST:Hoxc8$_{149\text{-}208}$). To sum up the results obtained from the study, we can conclude that containing of basic amino acid not particularly crucial in the development as a protein carrier in transduceness of the cells but is crucial in the development of a carrier of macromolecules in the transfection of the cells.

It can be concluded from our study is that Hoxc8 can be a promising source as PTD as they act not only as a good source as a carrier of other proteins but also a source as a carrier of macromolecules with it. It will come useful with some modifications, especially, in the gene therapy as viral vectors or liposomes have toxic effect on the target cell.

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ABSTRACT (IN KOREAN)

Hoxc8 단백질의 자가 전달 기능과 벡터로서의 응용에 관한 연구

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세포막이 투과성을 낮추는 장벽 역할을 하기 때문에 대부분의 고분자는 살아 있는 세포에 의해 활발히 이입되지는 않는다. 그러나 최근 단백질 transduction 도메인(PTD)이 지질층으로 이루어진 세포막을 통과하여 세포 내부로 도입되어질 수 있다는 것이 보고되었다. 가장 활발히 연구된 PTDs로는 인체면역결핍 바이러스(HIV-1)에서 얻어진 전사 조절 단백질 Tat와 초파리 전사 인자인 Antennapedia(Antp)가 있다. Antennapedia와 같이 Hoxc8은 호메오도메인을 포함하는 단백질이며 이것은 60개의 아미노산으로 구성되어 있다. 호메오도메인은 세 개의 α-helix를 갖고 있으며 2번째와 3번째 helix 사이에 β turn이 존재하며, 이 세 번째 α-helix가 PTD의 속성을 갖고 있음이 알려져 있다. 본 연구에서는 여러 종류의 재조합 Hoxc8 단백질을 제작하고 이들 각각의 PTD로서의 기능 및 핵산과 같은 고분자 물질의 세포내 도입 등을 분석하였다.

세 종류의 Hoxc8 단백질($Hoxc8_{1-242}$, $Hoxc81_{52-242}$, $Hoxc8_{149-208}$)을 glutathione S-transferase (GST)와 융합되어 대장균 내에서 발현하도록 제작하였으며 pGEX 4T-1에 클론하여 IPTG로 발현을 유도하고 또 glutathione agarose bead를 이용하여 이 단백질 분리 정제가 용이하도록 했다: GST:$Hoxc8_{1-242}$는 Hoxc8 단백질 전체를 포함하고 있으며, GST:$Hox8_{152-242}$는 Hoxc8의 N-term을 제거하여 호메오도메인과 산성 아미노산이 많이 존재하는 C-term을 포함하고 있으며, GST:$Hoxc8_{149-208}$은

호메오 도메인만을 포함한다. 단백질들은 Oregon 488을 이용하여 형광 표지한 후 PPFF와 T98G 세포에 처리 한 후 형광 현미경에서 단백질의 세포내 도입을 분석하였다.

결과 세 종류의 재조합 단백질 모두 비슷한 효율을 가지고 세포 안에 들어가는 것이 관찰이 되었다. 그리고 DNA-단백질 복합체 형성 실험에서는 음전하를 띄는 부분을 가지고 있는 단백질인 GST:Hoxc8$_{152-242}$ 에서는 매우 낮은 효율로 DNA-단백질 복합체를 형성했고, Hoxc8 단백질 전체를 다 갖고 있는 단백질보다는 호메오도메인만을 갖고 있는 GST:Hoxc8$_{149-208}$ 단백질이 매우 효율적으로 복합체를 형성하였다. 그 다음 이 DNA-단백질 복합체를 세포에 처리하여 Hox 단백질과 함께 도입된 DNA(pDsRed1-C1)의 세포 내 전달을 확인하였다.

이상의 결과는 재조합 Hoxc8 단백질을 이용하여 단백질이나 핵산 같은 생체 고분자 물질의 세포내 도입이 가능함을 제시해 주었으며 이는 더 나아가 치료용 물질의 전달 벡터로서의 개발 가능성을 보여 주었다.

핵심 되는 말: PTD, Hoxc8, homeodomains, macromolecules, 딜리버리 벡터