BITERIALS Co., Ltd

Technology Overview

1. Background of Technology

1. Definition of gene therapy

Gene therapy is the insertion of genes into an individual's cells and tissues to treat a diseases such as a hereditary disease in which a deleterious mutant allele is replaced with a functional one.

There are two types of gene therapy as shown in Figure 1. Somatic gene therapy is directly introducing therapeutic genes into human cells. Germ line gene therapy is first removing a cancer cells from patients and then inserting the missing genes or functional ones into the cancer cells and finally infusing genetically modified ones back into the patient.

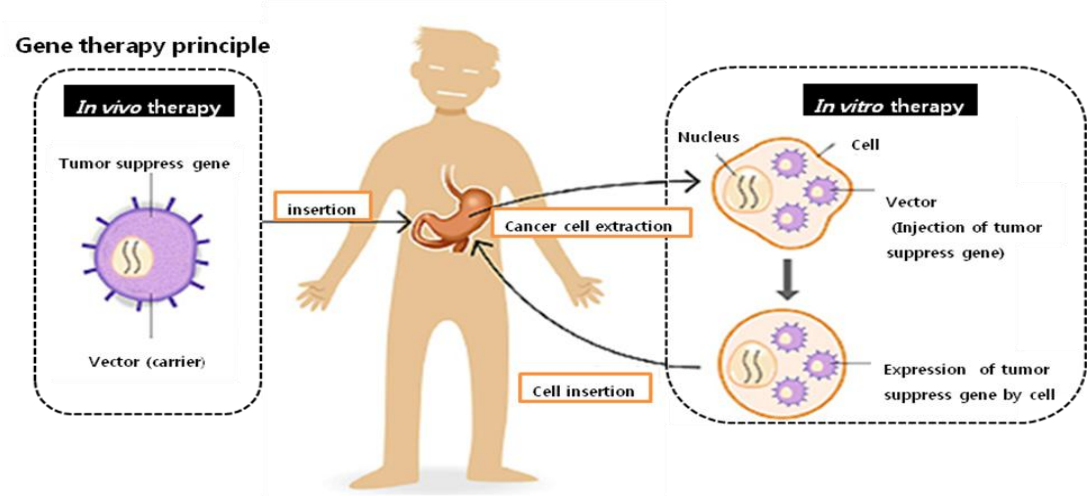

Figure 1. Principle of gene therapy

2. Characteristics of gene therapy

The most significant benefit of gene therapy is a therapy which is basically repairing abnormal and nonfunctional gene. The others are possibilities to be able to use engineered materials selectively for therapy and to remain the effectiveness of materials as one introduction. For the case of cancer therapy gene therapy is much more specific than chemical one and starts earlier therapy like in the beginning of cancer than other treatment. Also, there are fewer side effects than others because of partial treatment. There are not commercially available gene therapeutic materials presently and they are in the state for clinical trials. If the relationship between functionality of gene and illnesses is identified by the success of human genome project (HGP), the target gene for therapy will increase from about five hundred to a million. So, it can be estimated that the market of gene therapy is very positive. The expense and time to develop gene therapeutic materials are relatively less than those by chemical medicines. The period for experiments is estimated as 3~5 years.

Therefore, gene therapy has tremendous potential for ability of replacement of chemical medicines developed in advance.

3. Methods of gene therapy

Gene-delivery vehicles can be divided into two categories: recombinant viruses called as virus vector and synthetic vectors called as non-virus vectors. The majority of synthetic vectors, furthermore, can be divided into polymers (including polypeptides) and lipids. Viruses such as retrovirus, lentivirus (for example, HIV), adenovirus, adeno-associated virus, herpes simplex virus and pox virus can be transformed into gene-delivery vehicles by replacing part of the genome of a virus with a therapeutic gene. Because viruses evolved essentially as sophisticated gene-delivery vehicles, such recombinant viral vectors are typically very efficient. However, safety concerns have been the primary barrier to the clinical application of viral gene delivery. Although recombinant viral vectors are rendered non-replicative, and therefore non-pathogenic, there still exists the possibility that the virus will revert to a wild-type virion or co-purify with replication-competent virions. Furthermore, viruses are inherently immunogenic, leading to difficulty with repeat administrations and the possibility of dangerous immune reactions. On other hands, synthetic vectors provide opportunities for improved safety, greater flexibility for applications and more facile manufacturing although the effectiveness of gene delivery is much less than virus vectors. In current the method to use biodegradable polymer as a vehicle for gene delivery is representative among the non-virus vectors. For example, it is reported that platelet-derived growth factor (PDGF) in poly (lactide-co-glycolid, PLGA) was released gradually, which improved the generation of blood tubes.[1] In addition, a gelatin charged by positive ions was used for aggregating genes which have negative charges. The releasing of gene-gel aggregates were regulated by the degree of interaction of charges. [2] These methods were thought to be very useful because the high concentration of DNA can be provided during the balancing between growth of cell and decomposition of polymer. However, it is difficult to regulate the decomposition rate of cell membrane, endosomal escape and nucleus membrane, which is important, factors to determine the efficiency of non-virus vectors. Therefore, it is recently popular to change lipids or polymers to biodegradable polymers. Induction of gene using electric pulse into cell is also useful for rapid introduction and high efficiency. But, cell can be damaged and the electric field is not stable enough.

4. Properties of vectors for gene deliver, Table 1.

Vectors		Advantages	Disadvantages
Viral	Retrovirus (MLV)	- Biology well understood - Efficient entry - No viral genes in vector	- Low titer - Infection limited to dividing cells - Expression difficult to control and stabilize - Expensive and complex to prepare
	Adenovirus	- High titers - Efficient entry into most cell types - High level of expression - Infection of nondividing cells	- Vectors contain viral genes - Immunogenic, stimulating T- and B-cell responses - Generation of replication competent virus - Factors controlling tropism not well understood
	Lenti-virus (HIV)	- Efficient entry into most cell types - Gene delivery at liver cell	- Low level of safety - Hard to prepare and store
	Adeno-associated virus	- Integration at specific sites	- Requires replacing adenovirus to grow - No helper cell line - Limited insert size
Non-Viral	Naked DNA	- Easy to prepare - No size constraints - High level of safety - No viral genes - Lack of integration	- Inefficient entry and uptake - Limited persistence and lack of stability
	Positive lipid	- Easy to prepare and store - Efficient entry into most cell types - Lack of integration - High level of safety	- Low level of expression *in vivo* - Limited persistence and lack of stability
	Polymer	- Easy to prepare and store - High level of expression ex vivo - Lack of integration - High level of safety	- Low level of expression *in vivo* - Limited persistence and lack of stability
	Electroporation	- High level of expression	- Low viability by electric shock

5. Market of gene therapy

In the world the percentages of distribution of research about gene therapy are 64 % for cancer, 13 % for genetically gene disorder, 8 % for HIV infection. Gene therapy can be divided into three categories which are monogenic disease, cancer and cardiovascular disease. Market plane transition of these diseases is shown in the Table 2.

Table 2. Gene-therapy market plane transition (unit : Million Dollar, $)

Year	Monogenic	Cancer	Cardiovascular Disease	The others	Total
2004	-	125	-	-	125
2005	20	255	126	-	431
2006	189	499	344	10	1,042
2007	410	1,723	630	38	2,800
2008	739	2,783	1,050	979	5,551
2009	1,813	6,044	2,128	9,686	19,671
2010	4,449	13,127	4,319	95,835	117,730

Frost& Sullivan, US Gene Therapy Market, 2002.

The most of diseases about 4,000 are related to monogenic disease as seen in table 3. These cannot be cured completely in present. There are 86 therapeutic cases which are in the clinical experiments.

Table 3. Representative monogenic diseases

No.	Disease gene	No.	Disease gene
1	Prostate cancer, glaucoma, dementia	13	Breast cancer, Retinoblastoma
2	Parkinson's disease, colon cancer	14	Dementia
3	Lung cancer	15	Marfan syndrome
4	Huntington's disease	16	Crohn’s disease
5	Alopecia	17	Breast cancer
6	Diabetes, epilepsy	18	Pancreatic cancer
7	Obesity	19	Arteriosclerosis
8	Progeria	20	Immune deficiency, Creutzfeldt-Jakob disease
9	Skin cancer, leukemia	21	Muscular atrophy, Down syndrome epilepsy, dementia, leukemia
10	Progressive retinal atrophy	22	Leukemia, Cat-eye syndrome
11	Heart attack	23	Colorblind, Muscular Dystrophy
12	Phenyl ketonuria	24	Infertility

The most popular cancer therapy is a surgery in present. When the cancer is metastasis to whole body, radioactive therapy and chemical therapy are applied. However, these methods can destroy the normal cell and thus gene therapy which is non-toxic is being important. The

most important targets of gene therapy are prostate cancer, melanoma and laryngeal cancer, etc.

The death related to cardiovascular disease is reported as about 500 thousands in every year in the USA. Cardiovascular disease can be divided into arteriosclerosis and angiogenesis. The gene therapies of these diseases are in the clinical experiments by Collateral Therapeutic, Inc. and Genvec, Inc.

2. Description on Technology Applied

In the present study, we have constructed a non-viral peptide vector and applied it in the treatment of experimentally induced systemic lupus erythematosus (SLE) like disease in dogs. For therapeutic gene construction, the extracellular domain of canine CTLA-4, and the CH2–CH3 domains of canine immunoglobulin alpha constant region were inserted between the cytomegalovirus promoter and poly-adenylation sequence of bovine growth hormone. The constructed therapeutic gene was ligated to the non-viral synthetic peptide vector and was applied to systemic lupus erythematosus-like disease induced dogs. After gene therapy, clinical signs of systemic lupus erythematosus were reduced dramatically: the anti-nuclear antibody titers and urine protein/creatinine ratios were recovered to normal values, and the skin regained its normal histological features. The peptide vector did not show either tissue specific tropism or host induced immune response.

1. Peptide vector construction

The delivery vector includes leader peptide and linker DNA as shown in Figure 2. Leader peptide has 16 amino acids, which are designed to have functions for membrane fusion and penetration. The linker DNA bridges the leader peptide and a therapeutic gene. The sequences of leader peptide, linker-C, and linker-2 were as follows:

- Leader peptide: Ac-Gly-Leu- Gly-Ile-Ser-Tyr-Gly-Arg-Lys-Lys-Arg-Arg-Gly-Arg-Arg-Cys
- Linker-C: 5’-Cys–OO–CTAATACGACTCACTAT-3’ (–OO–: ester bond)
- Linker-2: 3’-GATTATGCTGAGTGAT-5

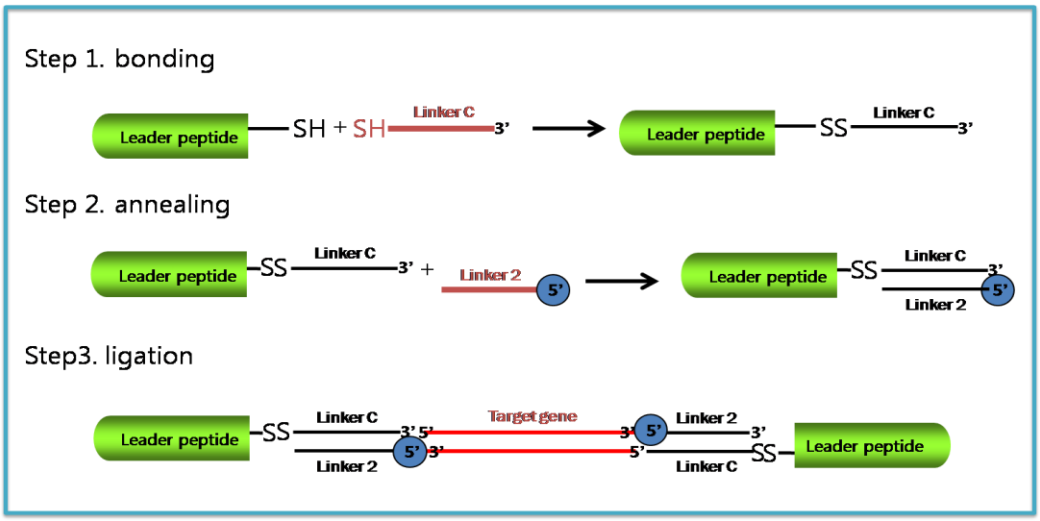

Figure 2. Construction of therapeutic gene

The N-terminal amino group was replaced with an acetyl group to remove molecular activity. Leader peptide and linker-C were conjugated with a disulfide bond by incubating in an S–S bond buffer (50 mM, Tris, 0.1 mM EDTA, 10 mM DTT, pH 10.5) at 37 °C for 1 h as shown in Step 1. After this procedure, linker-2 was added and incubated at 60 °C for 30 min for the hybridization between linker-C and linker-2 as shown in Step 2. The peptide vector was aliquoted in 100 pmol (20 pmol/mL). The therapeutic gene was amplified by PCR and the product was purified by silica based gel extraction. The purified PCR product (3–5 μg) was ligated into peptide vector (20 pmol) using T4 ligase as shown in Step 3. The final product ligated with the peptide vector was purified by ethanol precipitation and it was rehydrated in phosphate buffered saline and then injected intravenously in two dogs with SLE-like disease.

2. Construction of the therapeutic gene

A therapeutic gene is composed of the extracellular domain of canine CTLA-4 to inhibit the B7:CD28 co-stimulatory pathway and the CH2–CH3 domains of the canine immunoglobulin alpha constant (IGHAC) region to prolong the half-life of therapeutic protein *in vivo*. The fusion sequence of oncostatin M, CTLA-4 extracellular domain and the CH2–CH3 domains of the IGHAC region was ligated to *Hin*dIII and *Xba*I sites in pcDNA3.1(+) (Invitrogen, USA). Primer pairs were prepared: CMV-F 5'-GCCAGATATACGCGTTGACAT-3' and BGH-R 5'-GCTTAATGCGCCGCTACA-3'. With these primers, approximately 2213 bp fragments were amplified using pcDNA 3.1(+)/CTLA4Ig as templates.

3. Induction of an SLE-like disease in dogs

We utilized heparan sulfate (HS) to induce an SLE-like disease in eight male dogs. HS is the major glycosaminoglycan of glomerular basement membrane. Autoimmunity to HS has been suggested to be responsible for the induction of tissue damage and kidney dysfunction in

SLE in both *in vitro* and *in vivo*. All eight dogs developed SLE-like disease. Before the therapeutic gene construction, four of eight SLE induced dogs died. Two of four surviving dogs were treated by peptide vector encoding the therapeutic gene and the other two dogs were used as control.

4. Gene therapy by using Peptide Vector

1) Detection of therapeutic gene

Therapeutic gene was injected intravenously or intraperitoneally into Sprague–Dawley rats (5 weeks old, female). Control rats were injected an equal volume of PBS intravenously. Total RNAs were prepared from the sacrificed rat tissues, which were obtained 3 days after injection by using of Trizol reagent.

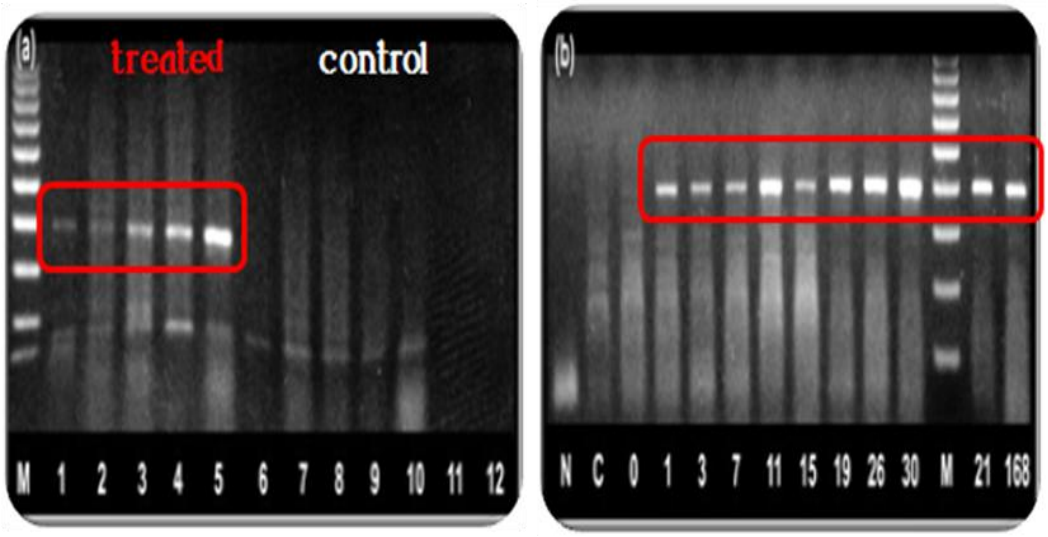

Figure 3. RT-PCR to confirm the transcription level of the therapeutic gene in rat tissues and dog PBMCs. (a) Expected size of 394 bp band size is shown. (M: 100 bp ladder, 1, 6: liver, 2, 7: kidney, 3, 8: spleen, 4, 9: lung, 5, 10: muscle, 11, 12: distilled water negative control, 1–5: gene injected rat, 6–10: PBS injected control rat.). (b) RT-PCR was carried out from PBMCs. A single band of 394 bp is shown (N: negative control, distilled water, C: negative control from non-treated dog; 0, 1, 3, 7, 11, 15, 19, 26, and 30 days after gene therapy, respectively, from treated dog 2. M: 100 bp, ladder, 21 and 168 days after gene therapy, respectively, from treated dog.

2) Gross morphologic change in gene therapy

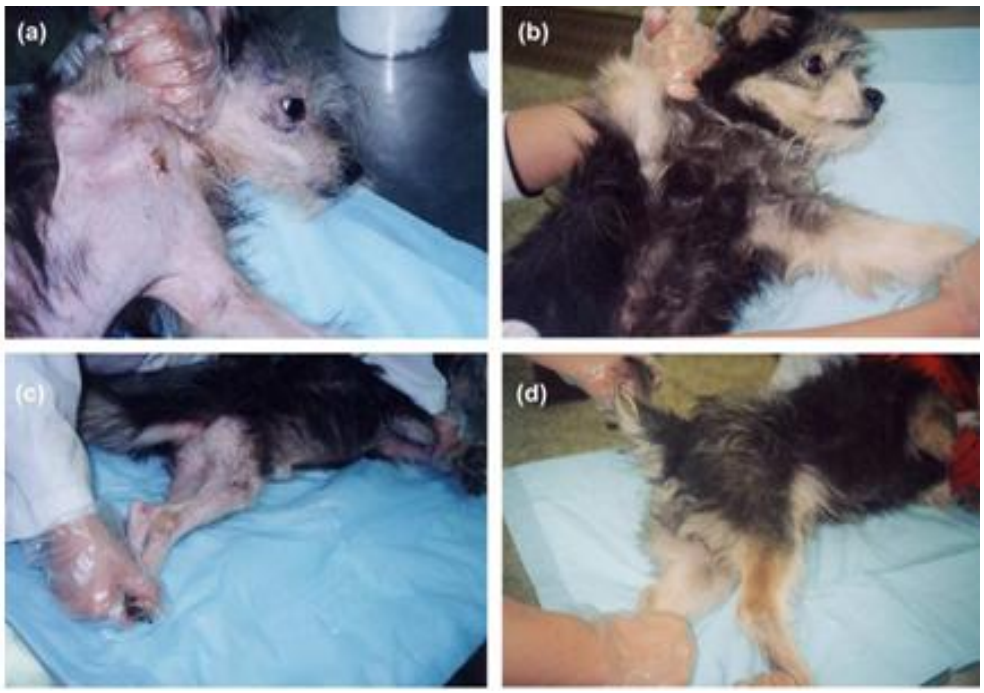

Figure 4. In experimentally-induced SLE dogs, severe alopecia was shown in the chest and head (a) and the hind limbs (c). After gene therapy, the skin was regained and its normal gross morphologic features are shown in the (b) and (d). Alopecia was not shown any more after gene therapy.

3) Microscopic observation in gene therapy.

In haematoxylin and eosin (H&E) staining, the superficial dermal infiltration of lymphocytes and plasma cells was remarkably reduced as shown in the Fig. 5a (before gene therapy) and 5b (after gene therapy). Hair follicles showed telogen phase before gene therapy (Fig. 5c) and after gene therapy it was recovered to normal anagen phase (Fig. 5d). In immunohistological examination of skin from dogs treated with the therapeutic gene, the deposition of immunoglobulin M (IgM) and C3 (data not shown) along the dermal–epidermal junction of the skins were negligible (Fig. 5f).

Finally, after our gene therapy, clinical signs of systemic lupus erythematosus were reduced dramatically: the anti-nuclear antibody titers and urine protein/creatinine ratios were recovered to normal values, and the skin regained its normal histological features. The peptide vector did not show either tissue specific tropism or host induced immune response.

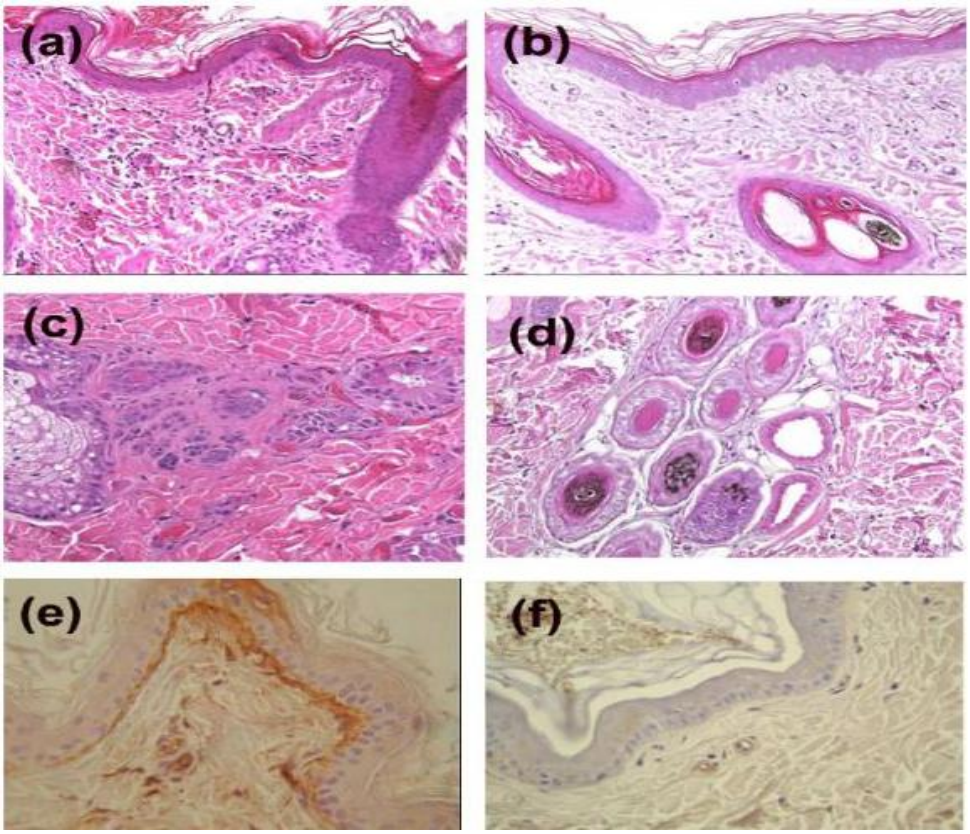

Figure 5. Skin biopsy from the HS-immunized dog 12 weeks after the final immunization (a, c, and e) and after CTLA4Ig gene therapy (b, d, and f). (a) Orthokeratotic hyperkeratosis in the epidermis and peri-vascular lymphoplasmacytic infiltration in the superficial dermis (x400, H&E). (b) Note slight (negligible) superficial dermal infiltration of lymphocytes and plasma cells compared to that of non-treated skin (x400, H&E). (c) All of the hair follicles were severely atrophied showing telogen phase (x400, H&E). (d) The hair follicles recovered to normal anagen phase (x400, H&E). (e) Deposition of IgM along the dermal–epidermal junction is prominent (often known as a positive ‘‘lupus band’’, x400, ABC). (f) Deposition of IgM in the dermal–epidermal junction is negligible (x400, ABC).

5. Safety of Peptide Vector

1) ELISA for detection of anti-peptide vector

IgG antibodies to peptide vector was measured in the sera of control and treated dogs collected on 0, 3, 7, 15, and 30 days after gene therapy as shown in Fig 4. Compared to control, absorbance of anti-peptide vector antibodies in treated dogs was not significantly different. Therefore, it is clear that this method is much safer than virus vector.

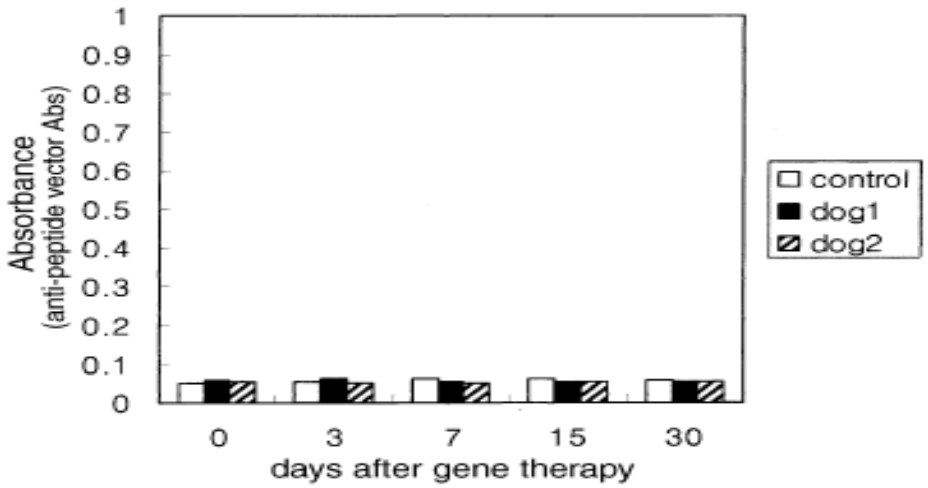

Figure 6. Assay of anti-peptide vector antibodies. Antibody against the peptide vector was determined in dog sera on 0, 3, 7, 15, and 30 days after gene therapy.

3. Differential Point, Superiority or Characteristics of Technology Applied

Our peptide vector is a non-virus vector and so there is no induction of cancer or host induced immune response. Also, it does not have any toxicity like polymers or lipids in the cells. In addition, because it can deliver any genes by high yield, it will be useful for a large scale production of induced Pluripotent Stem Cell and for an increase of productive efficiency. Therefore, it is expected that induction of Pluripotent Stem Cell, its clinical applications and its commercialization are possible.

1. There is no possibility of induction of cancer and host induced immune response because our peptide vector is a non-virus vector method,.
2. There is not any toxicity because polymers or lipids were not used in synthesis of our vector.
3. Our synthetic procedures are relatively simple compared to those of other non-virus vectors.
4. The our synthetic procedures are same for all different genes
5. Our peptide vector can be applied to any genes regardless of size and kind.
6. There is no concern about easy introduction inside of the cell because peptide used is designed to have functions for membrane fusion and penetration.
7. In our vector, there is only one disulfide bond for a gene to be free in the cell. So, it is faster to start gene transfection than that of biodegradable polymers in the cell because they have much more bonds to release genes.

8. After development of our peptide vector, there is no need to obtain the approval of safety for clinical experiments. So, it needs short time to develop our peptide vector as a medicine.

Table 4. Superiority and Characteristics of Technology Applied

Design criteria		Peptide Vector
Preparation	Synthesis	Easy
	Economy	Inexpensive
	Application	Easy administration
	Size of gene therapy	No limitation
Stability	Immunogenicity	ND
	Toxicity	ND
	Integration	ND
Durability	Long term expression	Above 7 months
	Reinjection	In experiment

ND: not detected

Reference

1. Murphy, W. L., Mooney, D. J. *J. Periodontal Res*. **1999**, *34*, 413.
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3. Faaber, P., Rijke, T.P., Van De Putte, L.B., Capel, P.J., Berden, J.H., *J. Clin. Invest*. **1986**, *77*, 1824
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Specific Patent

NO.	Name of Patent	Application No.	Date of application /approval	Country	Status (Applied/approval)	Cost for patent (KRW)
1	PEPTIDE VECTOR	10/2001/0006587	2001.02.10	KR	Approval	
2	PEPTIDE VECTOR	10/071,476	2002.02.08	US	Approval	
3	PEPTIDE VECTOR	02104729.4	2002.02.09	CN	Approval	
4	PEPTIDE VECTOR	02 002 623.3	2002.02.05	EU	Applied	

※ Please provide accurate information for Application No and Date of application/approval. It will be used for patent search.

※ In case of Cost for patent, please consider administrative cost for patent application only.

※ In case of PCT or overseas patent (application) except domestic patent, please attach a certificate of application/approval (or patent abstract) as a separate file.