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석사학위논문

IN VIVO BIOLUMINESCENCE IMAGING OF RAT CARDIOMYOBLAST TRANSPLANTATION

LONG-TERM MONITORING CELL SURVIVAL IN MOUSE SKELETAL MUSCLES WITH IMMUNOSUPPRESSIVE REGIMENS

조선대학교 대학원

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LE NGUYEN UYEN CHI

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지도교수 송 창 훈

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조 선 대 학 교 대 학 원

의학과

LE NGUYEN UYEN CHI

LE NGUYEN UYEN CHI 의
석사학위논문을 인준함

위원장 조선대학교 교수 김 종 중 

위원 조선대학교 교수 이 미 자 

위원 조선대학교 교수 송 창 훈 

2006 년 5 월

조 선 대 학 교 대 학 원

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LIST OF ABBREVIATIONS

Adv : Adenoviral vector

BLI : bioluminescence

CCD camera : charged coupled detector camera

CsA : Cyclosporine A

Dex : Dexamethasone

FL : Firefly luciferase protein

Fluc : Firefly Luciferase gene

IL : interleukine

IM : intramyocadium

IVIS : In vivo Imaging System

MOI : multiplicity of infection

MR : magnetic resonance

MSCs : mesenchymal stem cells

NK : natural killer

RLU : Relative light unit

ROI : region of interest

SPECT: single photon emission computed tomograghy

PET : positron emission tomography

Tac : Tacrolimus

TCR : T cell receptor

ABSTRACT

IN VIVO BIOLUMINESCENCE IMAGING OF CARDIOMYOBLAST TRANSPLANTATION

LONG-TERM MONITORING CELL SURVIVAL IN MURINE SKELETAL MUSCLES WITH IMMUNOSUPPRESSIVE REGIMENS

Le Nguyen Uyen Chi

Advisor: Prof. Chang-Hoon Song Ph.D.

Department of Medicine,

Graduate School of Chosun University

The rapid donor cell death and rejection are basic limitations encountered in normal and stem cell transplantation. Nowadays, the survival of transplanted cells can be visualized using a novel optical imaging technique. Our study aimed to use the bioluminescence imaging technique to monitor the cardiomyoblasts transplanted into murine skeletal thigh muscles, together with the applications of some immunosuppressive agents to prevent the rejection of host immune system.

Embryonic rat cardiomyoblasts were transfected with Fluc reporter gene-containing adenovirus in different multiplicity of infection (1, 10, 100) and cell doses (1×10^4 - 5×10^6), followed by the intramuscular injection into murine skeletal muscle (n=9 per group). In immunosuppressive therapy, mice and rats underwent transient immunosuppression with either Cyclosporine (5mg/kg/day) or Tacrolimus (1mg/kg/day) or Dexamethasone (4mg/kg/day), beginning on day -3 of implantation. Bioluminescence imaging

was then daily carried out using a cooled CCD camera (Xenogen). Luciferase activity was measured by luciferase assay and normalized to the protein amount determined by protein assay.

Viral transduction at MOI 100 and the 5×10^6 cell-dose implantation resulted in optimal transfection efficiency. Nude mice received immunosuppressive agents displayed the long-term Fluc expression for 30 days, in which Tacrolimus and Cyclosporine successfully suppressed the cell loss (day 4: 2.71E+07 (Tac), 9.47E+07 (CsA), 5.25E+07 (Dex), and 1.25E+07 p/s/cm^2/sr (control); day 30: 4.31E+05 (Tac), 1.24E+05 (CsA) and 1.09E+04 p/s/cm^2/sr (Dex)). In Balb/c mice, Tacrolimus and Dexamethasone also kept cells alive until day 8 as compared with the signal reduction up to day 6 of control mice. Bioluminescence expressed for 25 days in rat with immunosuppressants and 6 days in rat without drugs.

From this study, the induction of immune tolerance using pharmaceutical agents during cardiomyoblast transplantation improved the donor cell survival in murine skeletal muscles. Utility of bioluminescence imaging resulted in a potential to noninvasively and repetitively monitor implanted cardiac myoblasts over time.

초록

쥐의 심근모세포이식에 대한 분자 영상법 연구

면역억제요법을 사용하여 murine 의 골격근육에 주입한 세포생존에 대한 장기간 모니터링

래 누엔 웬지
지도교수 : 송창훈 교수님
의과대학
조선대학교 대학원

증여세포의 사멸 및 재주입은 정상세포와 이식한 줄기세포의 결합이 기본적으로 제한되어져 있다. 현재 이식되어진 세포의 생존은 새로운 광학영상기법을 이용하여 볼 수 있다. 본 실험은 murine 의 넓접골격 근육에 심근세포를 이식한 후 세포생존 모니터를 생물발광 영상 기법을 이용하는데 목적이 있으며, 또한 숙주 면역세포의 거부반응을 막기 위해 여러 개의 면역 억제재를 적용하였다.

Rat 배아심근세포를 표지 유전자인 firefly luciferase 가 포함되어 있는 아데노바이러스를 이용하여 감염시켰으며, 1, 10, 100 의 MOI 를 가진 아데노바이러스를 사용하여 murine (n=9 per group)의 근육세포내에 intramuscular injection 방법으로 바이러스가 감염된 세포를 $1x10^4$ 부터 $5x10^6$ 주입하였다. 면역억제 치료로서 배아심근세포를 이식한 후 3 일째부터 mice 와 rat 에 Cyclosporine (5mg/kg/day) or Tacrolimus (1mg/kg/day) or Dexamethasone (4mg/kg/day) 같은 면역억제제를 가지고 일시적으로 면역억제를 수행하였다. 생물발광영상은 Xenogen company 에서 구입한 in vivo imaging system 를 이용하여 매일 영상을 획득하였다. BSA protein assay 를 통하여 단백질을 정량화 한 후 luciferase assay kit 를 이용하여 luciferase 의 활성도를 측정하였다.

감염다중도가 100 인 바이러스를 형질 도입한 배아심근세포를 $5x10^6$ 골격근육에 이식한 그룹에서 최적에 감염효율을 야기하였다. 면역억제제를 투여한 nude mice 에서 Tacrolimus 와 Cyclosporine 이 이와 같이 (day 4: 2.71E+07 (Tac), 9.47E+07 (CsA), 5.25E+07 (Dex), and 1.25E+07

p/s/cm2/sr (control); day 30: 4.31E+05 (Tac), 1.24E+05 (CsA) and 1.09E+04 p/s/cm2/sr (Dex) 성공적으로 세포손실을 성공적으로 억제함을 확인하였으며 30 일 동안이나 생물발광영상을 통하여 firefly luciferase 의 발현을 보여주었다. Balb/c 에서도 역시 Tacrolimus 와 Cyclosporine 같은 면역억제제를 이용한 그룹이 정상그룹보다 오랫동안 세포가 생존해 있음을 영상을 통하여 8 일 동안 확인하였다. rat 에서는 면역억제제를 사용한 그룹에서는 25 일 동안, 약물을 사용하지 않은 정상그룹에서는 6 일까지만 세포가 생존해 있음을 영상으로 확인하였다.

이러한 실험을 통하여 심근세포를 이식하는 동안 약물을 이용하여 면역내성을 유도하여 murine 골격근육에 이식되어진 증여세포의 생존율을 향상 시킬 수 있음을 알 수 있었다. 또한 이러한 세포생존을 in vivo imaging system 과 같은 영상기기 장비를 이용한 생물발광 영상을 통하여 이식된 심근세포의 생존기간을 비침습적, 반복적으로 모니터 할 수 있는 잠재성을 보여주었다.

I. INTRODUCTION

A. Cell transplantation for treating heart diseases

Cell transplantation has emerged as a potential therapeutic intervention to enhance angiogenesis, provide structural support, and perhaps even to restore lost myocardial mass in diseased hearts.

Several approaches in cell transplantation and cardiac tissue engineering have been investigated as potential treatments to enhance cardiac function after myocardial injury, including the recruitment of endogenous myocyte precursors, the injection of skeletal myoblasts, the infusion of endothelial progenitor cells, the ex vivo cultivation of myocardium-like tissue, and fetal or embryonic stem cell-derived cardiomyocyte replacement [1-2]. Each strategy is full of promise but also encounters to some extraordinary challenges. Replacement of the scar tissue by cells that have the same or similar properties as the cardiomyocytes – the cardiac muscle cells – is a novel approach for MI treatment.

B. Problems with survival of transplanted cells and the host immune response

1. Cell death is a hurdle in transplantation efficacy

For stem or other cell transplantation therapies, the introduced donor cells must be able to survive in their host environment. However, intramuscular (IM) injection of cultured isolated myoblasts in classical myoblast transfer therapy shows that there is a massive and rapid necrosis of donor myoblasts, with over 90% dead within the first hour after injection [3-6]. This rapid myoblast death appears to be due to exposure to tissue culture condition altering the myoblasts so that when transferred in vivo, they provoke an

acute adverse host immune response. A very important note is when equivalent intact donor muscles are implanted directly, without exposure to cell isolation or tissue culture conditions, there is no adverse immune response and grafts show excellent survival for up to a year.

The extent to which such immune problems also occur with IM transplantation of donor stem cells or with delivery of donor (stem or other) cells via the circulation is unclear [7], but this aspect should be carefully considered. The report that mesenchymal stem cells (MSCs) from adult human bone marrow are exempt from rejection after xenotransplantation into sheep (in contrast to other stem cells) is intriguing [8]. These donor MSCs were detected in many tissues (although their survival was not quantified), and such expected immune tolerance has major implications for clinical cell therapies and requires further investigation.

Massive death of injected donor cells is also recognized as a major problem with transplanted cardiomyocytes, especially in the inflammatory conditions that follow infarction [9]. Many cardiomyocytes die by apoptosis and exposure of cultured cardiomyocytes to heat shock before transplantation strikingly increased cell survival at 1 day. Host natural killer (NK) cells appear to play a particularly important role in this rapid death of cultured donor myoblasts [3]. As for transplanted cultured skeletal myoblasts, a central role for host NK cells is also implicated in cardiac allograft rejection (Maier et al. 2001), and it appears likely that the immune problems encountered with myoblast transfer therapy will also apply to cultured cells transferred into the heart.

Moreover, the donor cell death and rejection caused by humoral and cellular immune reactions are a basic limitation encountered in stem cell therapy for cardiovascular disease treatment.

2. Preventing the host immune response

In order to achieve successful allotransplantation, immunosuppressive drugs are necessary to prevent graft rejection. A number of immunosuppressants, including steroids, cyclosporine (CsA), and tacrolimus, has remarkably improved cell survival rates in solid organ transplantation

a. Cyclosporine

The cyclosporine is fungal metabolite comprising a class of cyclic oligopeptides that act as immunosuppressants. Unlike other medications of immunosuppression that act by killing cells of immune system, Cyclosporine, sometimes referred as "cyclosporine A" acts by interfering with Helper T-lymphocyte interleukin production. Cyclosporine A binds and forms a complex with the intracellular receptor cyclophilin. The cyclosporine/cyclophilin complex binds to and inhibits calcineurin, a Ca.sup.2+ -calmodulin-dependent serine-threonine-specific protein phosphatase. Calcineurin mediates signal transduction events required for T-cell activation. Cyclosporines and their functional and structural analogs suppress the T-cell-dependent immune response by inhibiting antigen-triggered signal transduction. This inhibition decreases the expression of proinflammatory cytokines, such as interleukin-2 (IL-2). Without interleukins, T lymphocytes cannot communicate with one another and ultimately Killer T-cells armed and sent out to destroy the donated organ.

b. Tacrolimus

Tacrolimus, also known as FK506 or Prograf, is an immunosuppressant that inhibits signal transduction pathways involved in T-cell activation [10-12]. It is used clinically for allograft rejection, graft-versus-host disease, and autoimmune diseases [13]. Tacrolimus binds to an intracellular protein

FK506 binding protein (FKBP-12) that is not structurally related to cyclophilin (Harding et al. 1989; Siekienka et al. 1989; and Soltoffet et al. 1992). The FKBP/FK506 complex binds to calcineurin and inhibits calcineurin's phosphatase activity. This inhibition prevents the dephosphorylation and nuclear translocation of NFAT, a nuclear component that initiates gene transcription required for lymphokine (e.g., IL-2, gamma interferon) production and T-cell activation. Thus, tacrolimus inhibits T-cell activation.

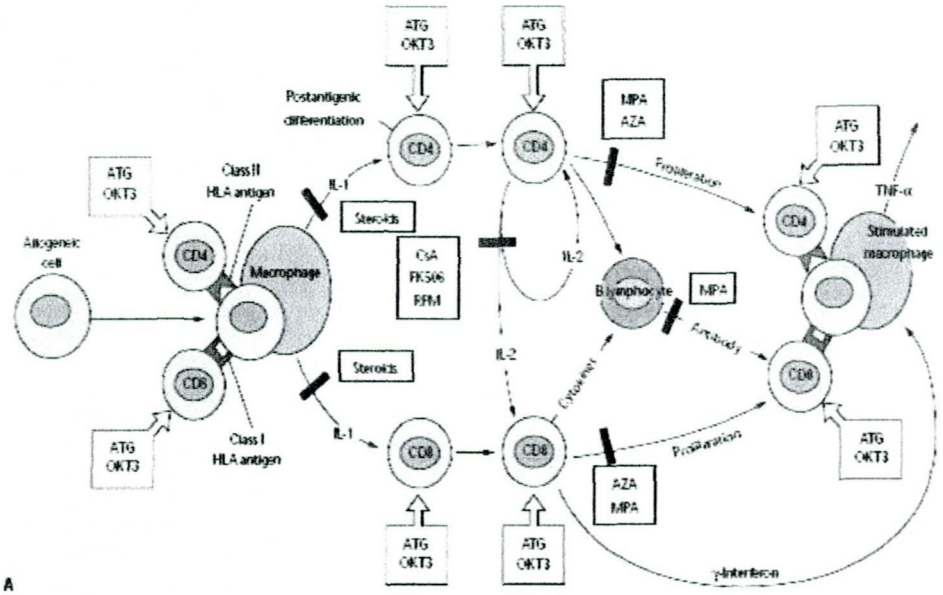


Figure 1: The site of action of the commonly used immunosuppressive drugs. Immunosuppressive drugs interfere with allograft rejection at various sites in the rejection pathways. Glucocorticoids block the release of IL-1 by macrophages, CsA and Tacrolimus interfere with IL-2 production from activated T cells.

c. Dexamethasone

Molecular formula: $C_{22}H_{29}FO_5$

Chemical name: *9-Fluoro-11β,17,21-trihydroxy-16α-methylpregna-1,4-diene-3,20-dione.*

As a glucocorticoid (GC), Dexamethasone binds to cytoplasmic GC receptor (GR), a member of the steroid hormone receptor superfamily. Several studies have confirmed that GCs induce and regulate T-cell apoptosis [14-15]. Recent studies have demonstrated that dexamethasone induces apoptosis not only in immature thymocytes but also in mature peripheral T cells [16-17].

C. How to detect cell survival in vivo?

1. Novel techniques for cell monitoring

For the study of stem cell transplantation in animal models, most of recent methods demand histological analysis in order to determine the fate and migration of cells. At each period of time, the animals need to be sacrificed, that appears to be a disadvantage when the necessity of tracking the cell activities inside an intact whole-body is required, which helps us attain a far-reaching knowledge underlying biological and physiological properties of cells. Furthermore, recent developments in stem cell and gene therapy also require methods to monitor stem cell survival and integration repeatedly and non-invasively with a high temporal and spatial resolution in vivo. Novel techniques that allow noninvasive and repetitive imaging of cell transplants have recently been proposed and evaluated in a limited number of cell delivery models. One of them involves the use of reporter gene technology for bioluminescence, fluorescence, MR, SPECT, or PET imaging [18-19]. With this approach, cell survival of genetically modified cells can

be monitored more accurately because only viable cells will express the reporter genes. Moreover, since the reporter genes are passed on to daughter cells, cell proliferation can also be reliably quantified [20].

2. In vivo bioluminescence imaging

Imaging strategies that use genetically tagged cells that express bioluminescent reporter proteins offer the advantages of fine temporal analyses, labeling versatility, accessible instrumentation, and a signal-to-noise ratio that provides high sensitivity of detection. Weak internal sources of biological light, bioluminescence, can be externally detected using sensitive charge-coupled device (CCD) cameras as low-light detection systems. This imaging modality, known as bioluminescent imaging (BLI), employs light-emitting proteins for real-time in vivo detection of tagged cells. Given that there are very few sources of light in mammalian cells and bioluminescent proteins do not require an excitation source, the background in BLI is nearly nonexistent.

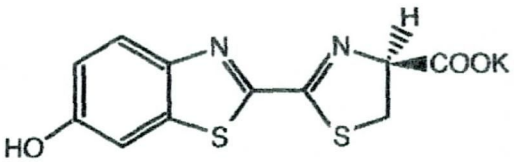
BLI is based on the in vivo expression of luciferase, the light-emitting enzyme produced by the firefly Photinus pyralis, for instance. This type of imaging has been successfully used in many areas of research including tumor tracking [21-24], infectious diseases [25-27], and drug efficacy studies [21, 28-29]. First described by Contag et al. in 1995, this technique relies on an adenosine triphosphate- (ATP) and oxygen-dependent photochemical reaction between luciferin and luciferase, resulting in the release of photons by living cells. Because of the low background luminescence of normal tissue and the rapid turnover of the luciferase enzyme, this technique is well suited for studying the temporal expression of genes by living cells [24].

a. Luciferase enzyme

Luciferase, a small, single polypeptide with molecular weight of 62 KDa, is a family of proteins produced by luminescent organisms like several insects, marine species, and some prokaryotes. It is encoded by luciferase gene that has been isolated and widely used as a high effective reporter gene. As compared to the bacterial CAT reporter gene, the assay of luciferase activity is more than 100-fold more sensitive. Luciferase catalizes for a reaction requiring high-energy molecules such as ATP, and thus light production is coupled to metabolic activity of the cells expressing the reporter gene. Other advantages of using the luciferase gene as a reporter gene are that mammalian cells do not have endogenous luciferase activity, and that luciferase can produce chemiluminescent light with very high efficiency, which can be readily detected. Genes coding for luciferases are useful tools for molecular and cellular biologists and, due to the penetration of bioluminescent light through mammalian tissues, have more recently been shown to be valuable for in vivo analyses.

b. Luciferin

The substrate for catalyzing reaction of luciferase is luciferin, a small, water-soluble molecule that quickly penetrates the membranes of cells in tissues following intraperitoneal or intravenous injection, even crossing the blood-brain and placental barriers. Luciferin is easily administered intraperitoneally together with anesthetic for imaging.



$C_{11}H_7N_2O_3S_2K = 318.42$

Figure 2: D-Luciferin (*4,5-Dihydro-2-(6-hydroxy-2-benzothiazolyl)-4-thiazolecarboxylic acid*)

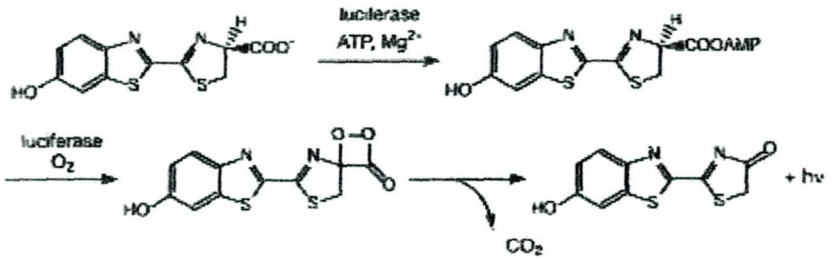


Figure 3: Bioluminescence reaction between substrate Luciferin and its Luciferase enzyme.

The most commonly used Luciferase is derived from the Firefly *Photinus*, called Fluc. This enzyme and its substrate have several characteristics that make them useful reporter proteins [30]. Firefly luciferase does not need external light excitation and self-emits light from yellow to green wavelengths (550-575nm) in the presence of D-luciferin, ATP, Mg^{2+}, and O_2. Secondly, the fast rate of enzyme turnover ($T_{1/2}$ = 3h) in the presence of substrate D-luciferin allows for the real-time measurements because the enzyme doesn't accumulate intracellularly to the extent of other reporters. Thirdly, the relationship between the enzyme concentration and the peak height of emitted light in vitro is linear up to 7-8 orders of magnitude. These properties allow for the sensitive noninvasive imaging of Fluc reporter gene expression in living subjects.

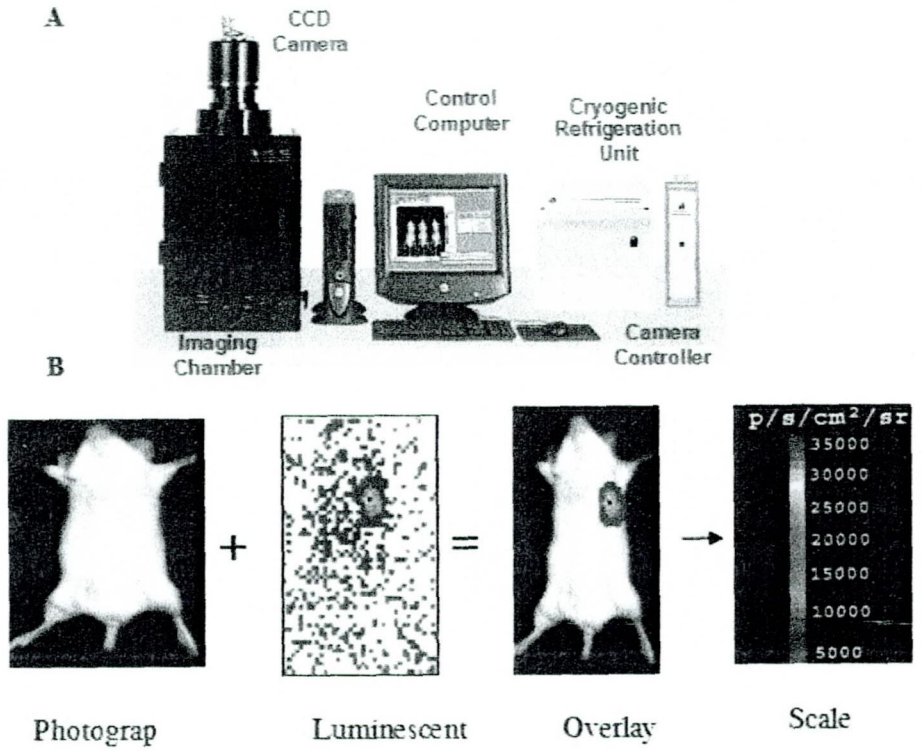


Figure 4: The Xenogen In Vivo Imaging System (IVIS) consists of a cooled charged-coupled device camera mounted on a light-tight chamber, a cryogenic refrigerator, and a computer system for data analysis (A). B-Pseudocolor luminescent image is overlaid on a grayscale photographic image.

c. Bioluminescence imaging system

The instrumentation for low-light imaging is called as the sensitive CCD detectors that are required to capture transmitted photons have been widely used in biological imaging (Fig. 4). Cooling the CCD detector to temperatures of −100 to −120°C is an effective means of reducing thermal

noise and results in extremely sensitive CCD cameras that can detect light over a broad spectral range, including the wavelengths that are more readily transmitted through living tissue (650-1100 nm)

For BLI the animal is anesthetized and the substrate, luciferin, is injected. After a fixed time, usually 15 minutes, the animal is placed in a light-tight chamber upon which a cooled CCD camera has been mounted. A second image is then acquired, in the absence of an external light source, collecting the light that is generated internally and transmitted through the animal's tissues.

D. Purpose of study

As a result of this, our study aimed to determine the utility of bioluminescence in vivo imaging system to monitor Fluc gene expression in cardiomyoblasts in culture and in animal skeletal muscles adenovirus expressing FL. We then evaluate the potential of some immunosuppressive drugs that induce the tolerance of these donor cells against the host immune responses in intact whole body animal.

II. MATERIALS AND METHODS

A. Materials

1. Culture media and solutions:

- Medium: Dulbecco's modified Eagle's medium (Gibco, Invitrogen).
- Serum: Fetal Bovine Serum (TerraCell International S.A., Canada).
- Antibiotic - Antimycotic (100X): prepared with 10,000 units/ml penicillin G sodium, 10,000 ug/ml streptomycine sulfate and 25 ug/ml amphotericin B as fungizone in 0.85% saline (Gibco).
- Dulbecco's Phosphate - buffered saline (PBS) (Gibco).
- Trypsin-EDTA (10X): includes 0.5% Trypsin with EDTA-4Na (Gibco).

2. Mammalian cell lines:

H9c2 cardiomyoblasts were obtained from the American Type Culture Collection, Rockville, MD.

3. Immunosuppressive agents:

- Tacrolimus (Prograf) from Jujisawa, Ireland
- Cyclosporine (Sandimmun) from Novartis
- Dexamethasone from Choongwae Pharma Corporation, Korea

4. Anaesthetics

- Isoflurane
- Ketamin
- Rompun (Xylazine)

5. Imaging:

D-Luciferin Firefly K+ salt (synthetic) or 4,5-Dihydro-2-[6-hydroxy-2-benzothiazolyl]-4-thiazolecarboxylic acid potassium salt, obtained from Xenogen

6. Luciferase Assay System from Promega, Madison, WI

- Reporter Lysis 5X Buffer

- Passive Lysis Buffer
- Luciferase Assay Reagent

B. Animals

Three female murine models were used, including immunodeficient nude mouse (20-25g, 4-6week old), immunocompromised Balb/c mouse (20-25g) and Sprague Dawley rat (200-250g) achieved from the Japan SLC, Inc. Haruno breeding branch.

C. Cell culture

H9c2 cardiomyoblasts were cultured in Dulbecco's Modified Eagle's Medium supplemented with 3.7 g/L sodium bicarbonate, 1% penicillin and streptomycin, 0.2mM L-glutamine, and 10% fetal bovine serum (FBS), at 37°C in a 5% CO2 humidified atmosphere. Medium is renewed every 2 to 3 days. Cell subcultivation is recommended (ratio of 1:2 to 1:4) as reaching 70% of confluence. The culture method follows the protocol supplied by manufacturer.

D. Recombinant adenovirus vector and Transient transfection to cardiomyoblasts

1. Construction of recombinant adenovirus

Replication-defective recombinant adenovirus carrying cytomegalovirus promoter driving firefly luciferase reporter gene (Ad-CMV-Fluc) was constructed and amplificated. The firefly luciferase gene and enzyme are subsequently referred to as Fluc and FL.

2. Adenovirus-mediated transient transfection

One day before transfection, H9c2 cells were seeded in 6-well plate (1×10^5/well) or T_{150} Flask (2×10^6/flask) in such a way that they would obtain 70% confluence on the next day. Ad-CMV-Fluc vector was incubated overnight with cells at multiplicity of infection from 1 to 100 in a certain

volume of serum free DMEM covering cells. After 6 hours, the virus supernatant was removed and cells were consequently incubated in DMEM containing FBS and antibiotics overnight. One day after transfection, cells were washed 3 times in 1X phosphate-buffer saline (PBS, pH 7.0), detached by 1X Trypsin-EDTA and implanted into animals.

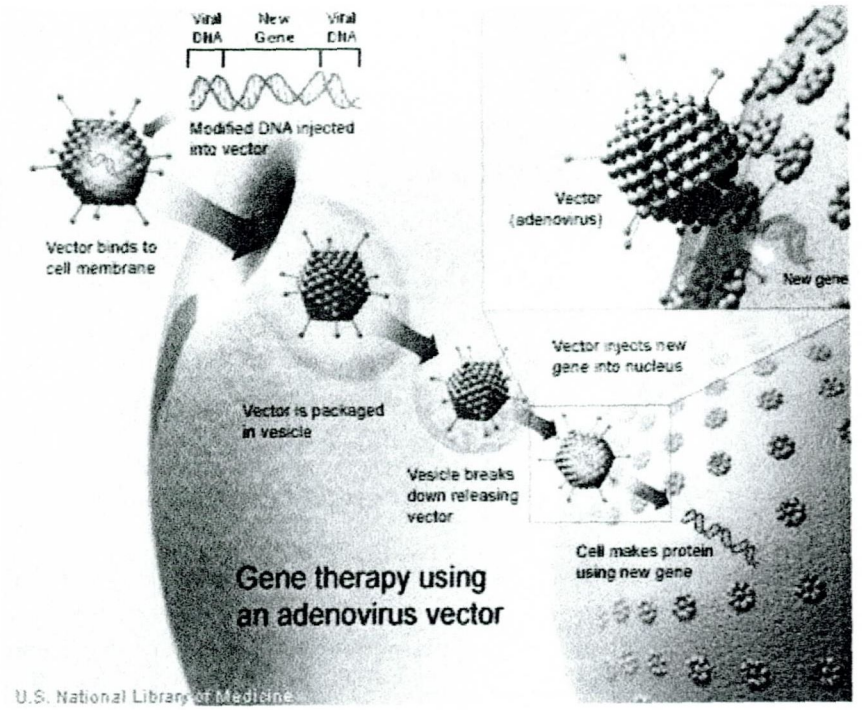


Figure 5: Application of adenoviral vector for gene transferring into mammalian cells. Fluc reporter gene was constructed in adenovirus so that it can be introduced into H9c2 cells.

E. Intramuscular Implantation of cardiomyoblasts

Murine animals (n=9/group) received a 4:1 mixture of Ketamin (80mg/kg) and Xylazine (10mg/kg) for anesthesia were implanted with FL-expressing H9c2 cardiomyoblasts at the right thigh skeletal muscles. In *in vivo* MOI test,

H9c2 cells were injected at $1x10^6$ per mouse; and $5x10^6$ cells were injected in each mouse for pharmaceutical therapy.

F. Pharmaceutical effects for the prolongation of cell survival

From three days before cell transplantation mice (n=3 for each group) were daily pre-treated with Cyclosporine (5mg/kg/day), or Tacrolimus (1mg/kg/day), or Dexamethasone (4mg/kg/day). Mice were then implanted with H9c2 cells expressing the Fluc gene. The control animals were received saline injections (1ml/kg/day, n=3).

Cell survival was daily assessed after cell transplantation by using optical bioluminescence imaging technique. All the experiments were performed in triplicate.

G. Imaging of cardiomyoblast implantation

Optical bioluminescence imaging was performed with the Xenogen In Vivo Imaging System (IVIS) (Figure 5) consisting of a cooled charged couple device (CCD) camera mounted on a light-tight specimen chamber, a cryogenic refrigeration unit, a camera controller, and a computer system for data analysis. Both the IVIS and its imaging analysis software are commercially available (Xenogen Corp.)

After intraperitoneal injection of D-Luciferin at a dose of 375mg/kg body weight, mice were serially imaged for 30 minutes using 30 x 1-minute acquisition scan everyday until the bioluminescent signals had completely faded away. The bioluminescence was quantified in units of photons · $second^{-1}$ · $centimeter^{2-1}$ · $steridian^{-1}$.

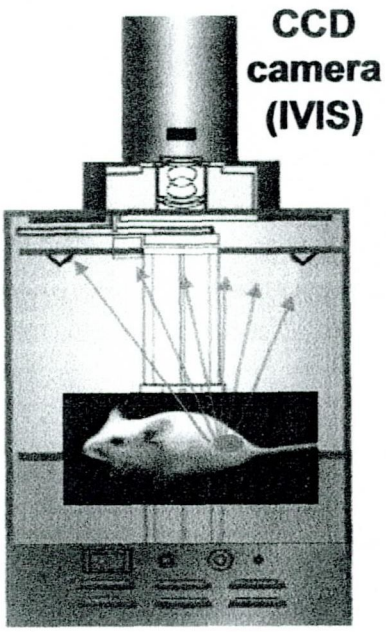


Figure 6: IVIS imaging system monitor H9c2 cells at mouse thigh skeletal muscle.

H. Luciferase assay

Luciferase expression in transfected cells was monitored according to the instructions given by the supplier using the Luciferase Assay System from Promega. For in vitro study, cells were washed extensively and lysed by adding 4 volumes double-distilled H_2O and 1 volume Reporter Lysis 5X Buffer. The lysate was centrifuged at 12,000 rpm for 2 minute at 4°C to remove cell debris. A volume of 20 ul of this lysate was dispensed into wells of 96-well plate and loaded into the luminometer (Centro LB 960, Berthold) followed by the autoinjection of 100 ul of the Luciferase assay reagent per well controlled by a MicroWin software. Light intensity was shown as relative light unit (RLU) and normalized to the protein as measured by Bio-

Rad Protein Assay System (Bio-Rad, Hercules, CA). Transfection were performed in triplicates with <10% variation.

For in vivo study, after imaging, the mouse thigh muscles were excised and homogenized in Passive Lysis Buffer at 4ml/g tissue. After freezing and thawing three times at -80oC for 15 min each, the homogenate was centrifuged at 14,000 rpm for 15 min. Fluc activity was assessed using Luciferase assay reagent as described.

I. Protein assay

As following the Bio-Rad protein assay, the standard curve was set up with BSA dilution in PBS. 800ul of each sample was added with 200ul of Bio-Rad protein assay reagent, vortexed and loaded into cuvette. Light absorbance was read at 595nm, compared to the standard curve to determine the protein concentration of experimental sample.

J. Data Analysis

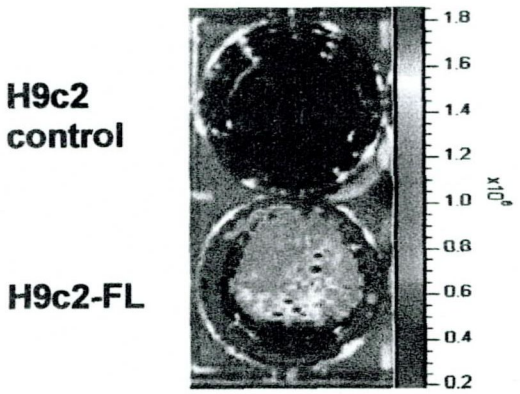
Data are given as mean ± standard deviation. For statistical analysis, the 2-tailed Student's t test was used. Differences were considered significant at $P<0.05$. Luciferase activities from in vivo CCD imaging were correlated by least-squares linear regression.

III. RESULTS

1. Validation of the presence of Fluc gene and protein in transfected H9c2 cells

After transfection with adenoviral vector on the 6-well plate, $1x10^5$ H9c2 cells was incubated for 24 hours and checked for the luciferase-mediated signals by imaging and luciferase assay. For imaging, Fluc expression was detected in H9c2-Fluc cells only (Fig. 7A). In contrast, control H9c2 cells produced no Fluc expression. Moreover, the high RLU obtained in H9c2-Fluc samples by luciferase assay as compared to the background levels of H9c2 samples and the control confirmed for the expression of Fluc protein in adenovirus-mediated injected H9c2 cells (Fig. 7B).

A



B

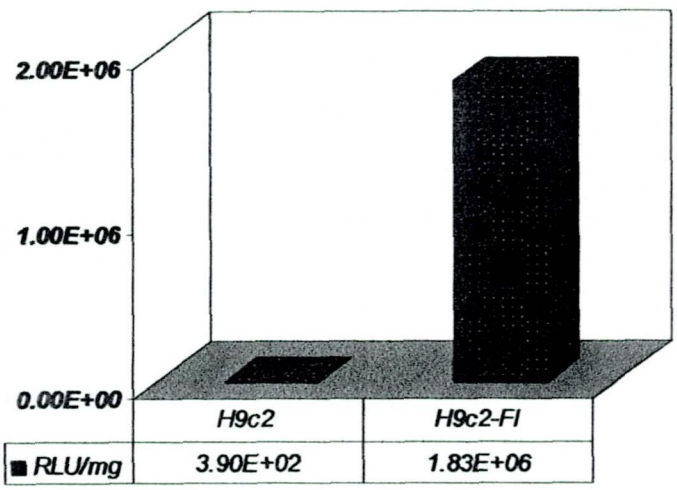


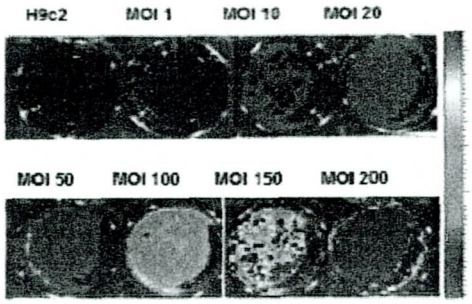
Fig. 7: Validation of firefly luciferase expression in H9c2 cells. (A) *In vitro* bioluminescence imaging reveals firefly luciferase-mediated signals only in H9c2-Fluc cells and not in control H9c2 cells. (B) Fluc protein presenting in transfected cells was quantified as RLU/ mg protein.

2. Fluc gene transferring efficiency of adenoviral vector.

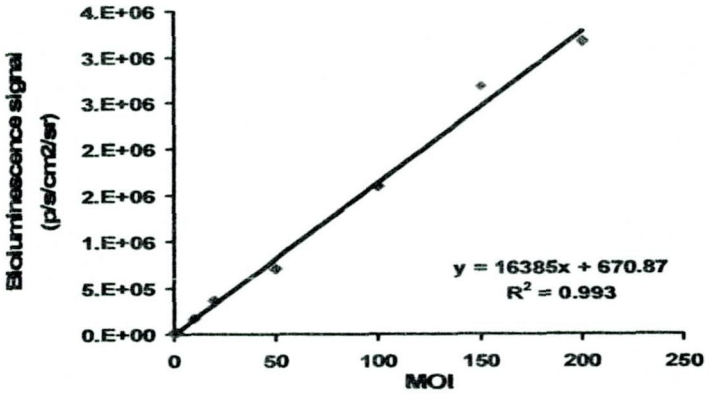
Using adenovirus for gene transfer to mammalian cells depends on the multiplicity of infection (MOI) of virus. We performed the viral transfection in both in vivo and in vitro studies to estimate the potential of adenovirus for gene transferring into cardiomyoblasts and determined the optimal amount of adenovirus for transfection.

To evaluate the efficacy of adenovirus-mediated transfection, we incubate Fluc-adenovirus with cardiomyoblasts at MOI from 1 to 200 overnight in 6-well plate or T125 flasks. The plate was then imaged by IVIS or cell-harvested for in vivo injection. The bioluminescence emission was shown as in figure 8A with the correlation between bioluminescence signal detected by in vitro imaging and viral MOI at r^2= 0.993 (Fig. 8B), demonstrating for the adenoviral transfection efficacy on embryonic cardiomyoblasts. These results are in familiar with those of in vivo study when injecting H9c2-Fl cells with different MOI into nude mice (Fig. 8C). The virus titer at MOI 100 offers the best transfection as Fluc activity always prevailed over the other MOI (Fig. 8D), thus, the use of MOI 100 was chosen for all next experiments because it offer the high efficiency of viral transduction (day 1 - MOI 100: 2.79E+07 ±3.8%, MOI 10: 1.24E+07 ±24%, MOI 1: 2.01E+06 ±10.2% , the longest time for cell detecting (5 days) as well as the nontoxic effect for cell physiology.

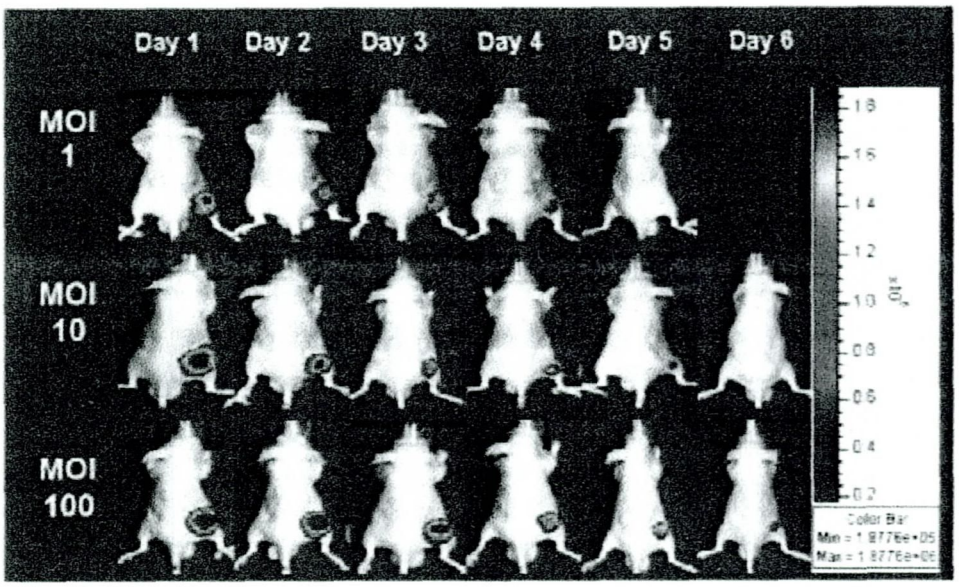
A


H9c2
MOI 1
MOI 10
MOI 20
MOI 50
MOI 100
MOI 150
MOI 200

B


Bioluminescence signal (p/s/cm2/sr)
4.E+06
3.E+06
3.E+06
2.E+06
2.E+06
1.E+06
5.E+05
0.E+00
0
50
100
150
200
250
MOI
y = 16385x + 670.87
R2 = 0.993

C


Day 1
Day 2
Day 3
Day 4
Day 5
Day 6
MOI 1
MOI 10
MOI 100
Color Bar

D

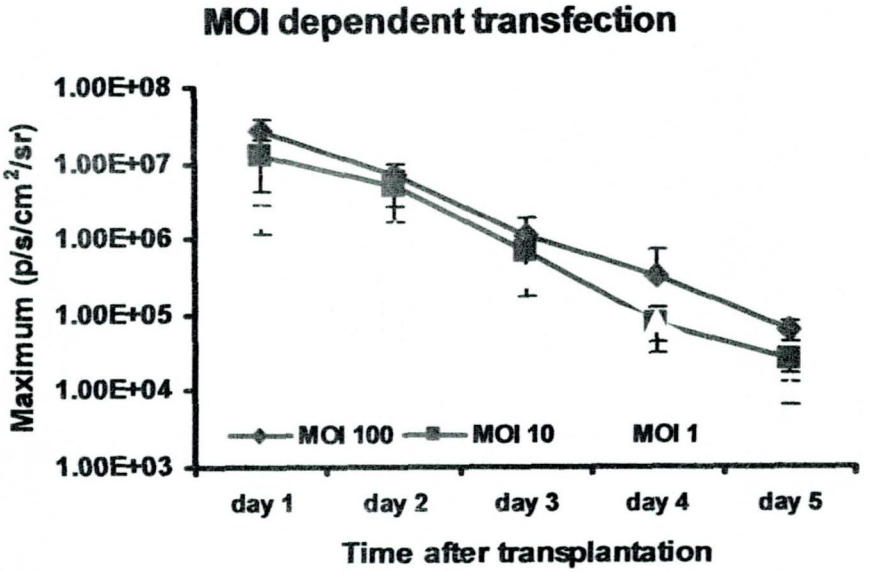


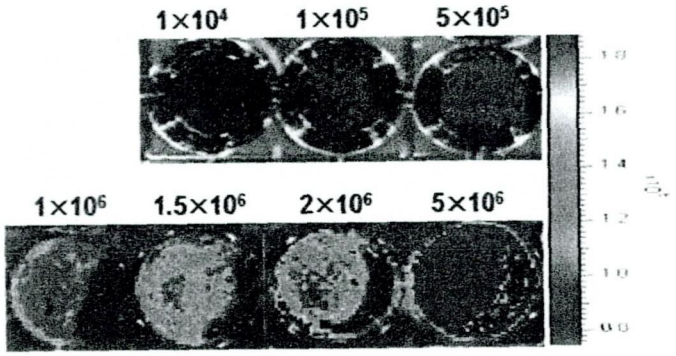
Figure 8: Dependence of Adenoviral transfection on the multiplicity of infection. In vitro results showed the potential of adenoviral transfection in gene transfer belongs to the MOI in the direct ratio (A, B). The in vivo imaging confirmed for that correlation of MOI and bioluminescence signal performed by IVIS (C), and MOI is the optimal choice for adenoviral transfection next (D).

3. Sensitivity of detection and the correlation of bioluminescence signal implanted cell dose.

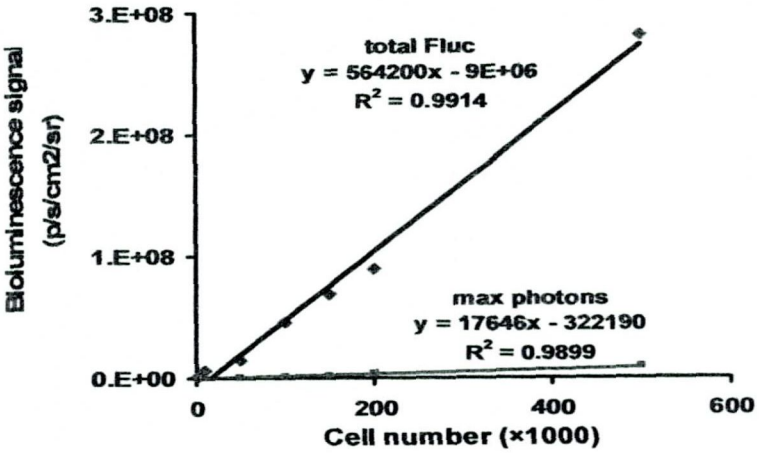
For studying the kinetic of Fluc expression on cell density dependence, Fluc-transferred H9c2 were plated at to 6-well plate in different cell dose from $1x10^3$ to $5x10^5$ per well and measure either the bioluminescence using IVIS cooled CCD camera or the Luciferase activity using luminometer. The results showed an increase in cell number brought about a highly correlative increase of bioluminescence (Fig. 9A). The cell densities created linearly proportional and highly correlated BL signals in imaging (r^2=0.99) (Fig. 9B) and even correlated with RLU of Fluc activity in cell lysates by Luciferase assay (r^2=0.99) (Fig. 9C).

For estimating the sensitive detection threshold of cooled CCD camera in detecting number of implanted cells in vivo, serially amount of Fluc-transferred H9c2-cells ($1x10^5$, $5x10^5$, $1x10^6$, and $5x10^6$ cells) were injected into thigh skeletal muscles of nude mice. The highest signal detected in mouse imaging was induced as injecting $5x10^6$ cells that lasted until 7 days post-transplantation (Fig. 9D). The Fluc expression showed in bioluminescence images was in cell-dependent manner (Fig. 9E). Remarkably, a strong correlation exists between the BL signals obtained in mouse imaging and the in vitro Fluc activity of implanted cell lysates determined from the Luciferase assay (Fig. 9F). These results revealed the potential use of bioluminescence imaging technique in tracking transplanted cells deep inside animal tissue and the ability of introducing 5 million cells to mouse muscle so that the longest bioluminescent emission can be detected from these cardiomyoblasts.

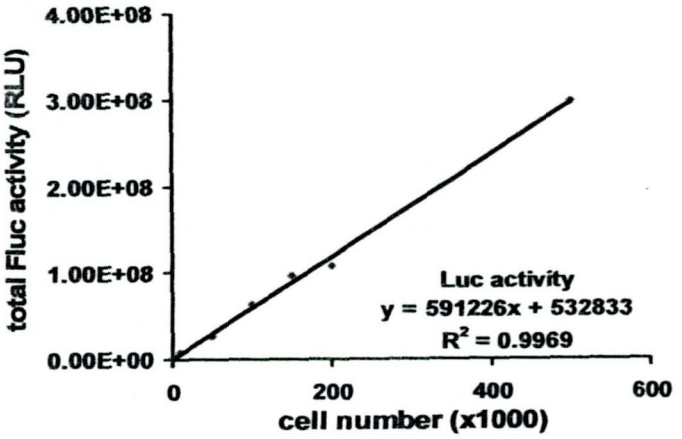
A


1×10⁴
1×10⁵
5×10⁵
1×10⁶
1.5×10⁶
2×10⁶
5×10⁶

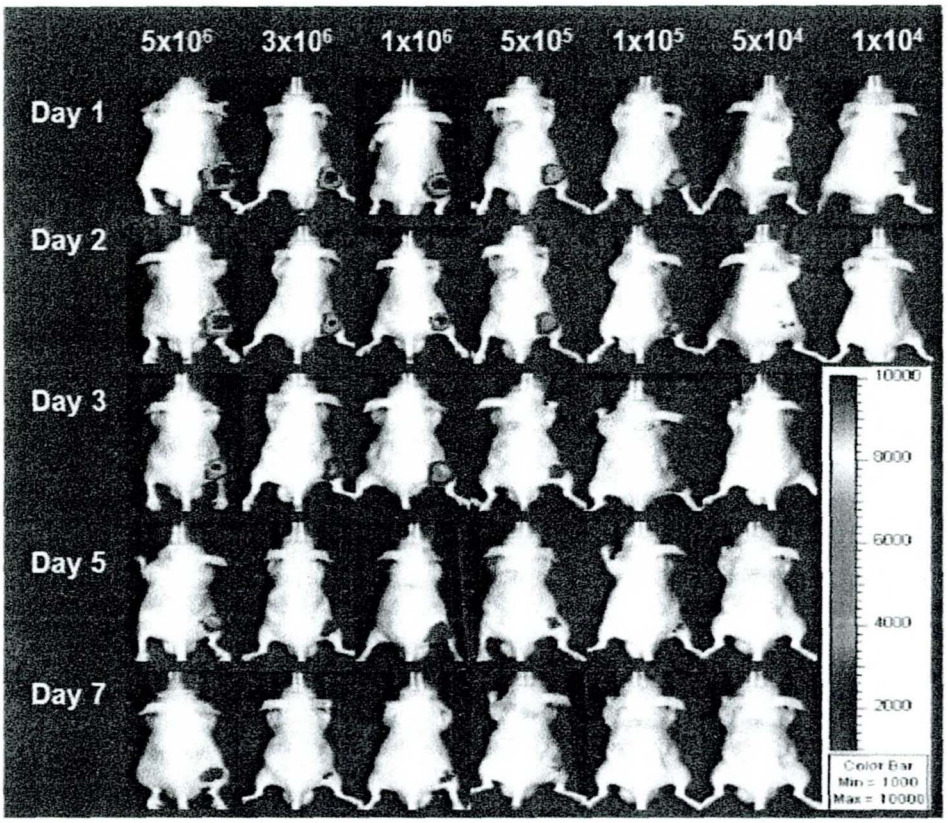
B


Bioluminescence signal (p/s/cm2/sr)
3.E+08
2.E+08
1.E+08
0.E+00
total Fluc
y = 564200x - 9E+06
R² = 0.9914
max photons
y = 17646x - 322190
R² = 0.9899
0
200
400
600
Cell number (×1000)

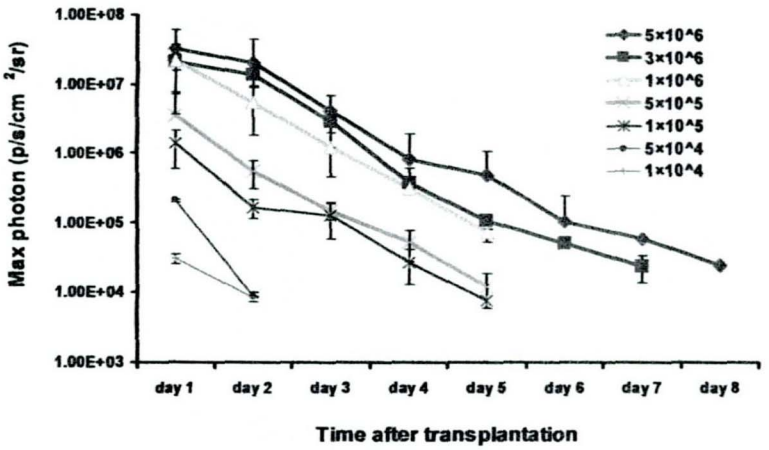
C


total Fluc activity (RLU)
4.00E+08
3.00E+08
2.00E+08
1.00E+08
0.00E+00
Luc activity
y = 591226x + 532833
R² = 0.9969
0
200
400
600
cell number (x1000)

D


5x10^6
3x10^6
1x10^6
5x10^5
1x10^5
5x10^4
1x10^4
Day 1
Day 2
Day 3
Day 5
Day 7
10000
8000
6000
4000
2000
Color Bar
Min = 1000
Max = 10000

E


1.00E+08
1.00E+07
1.00E+06
1.00E+05
1.00E+04
1.00E+03
Max photon (p/s/cm ²/sr)
5×10^6
3×10^6
1×10^6
5×10^5
1×10^5
5×10^4
1×10^4
day 1
day 2
day 3
day 4
day 5
day 6
day 7
day 8
Time after transplantation

F

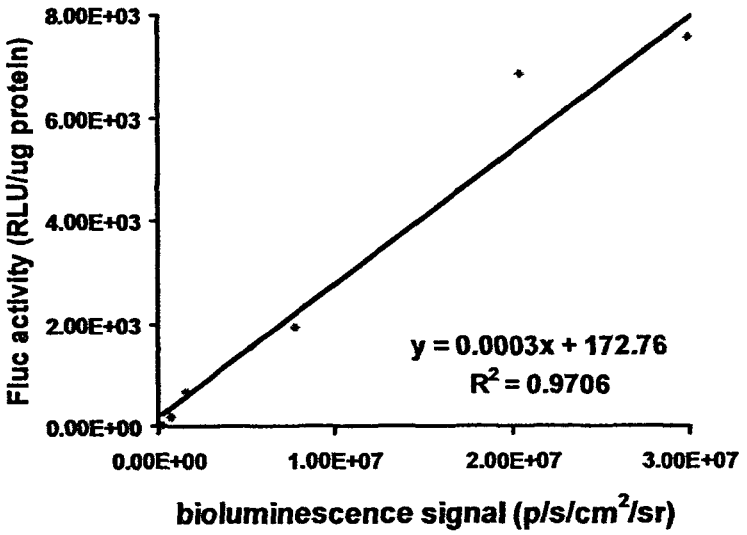


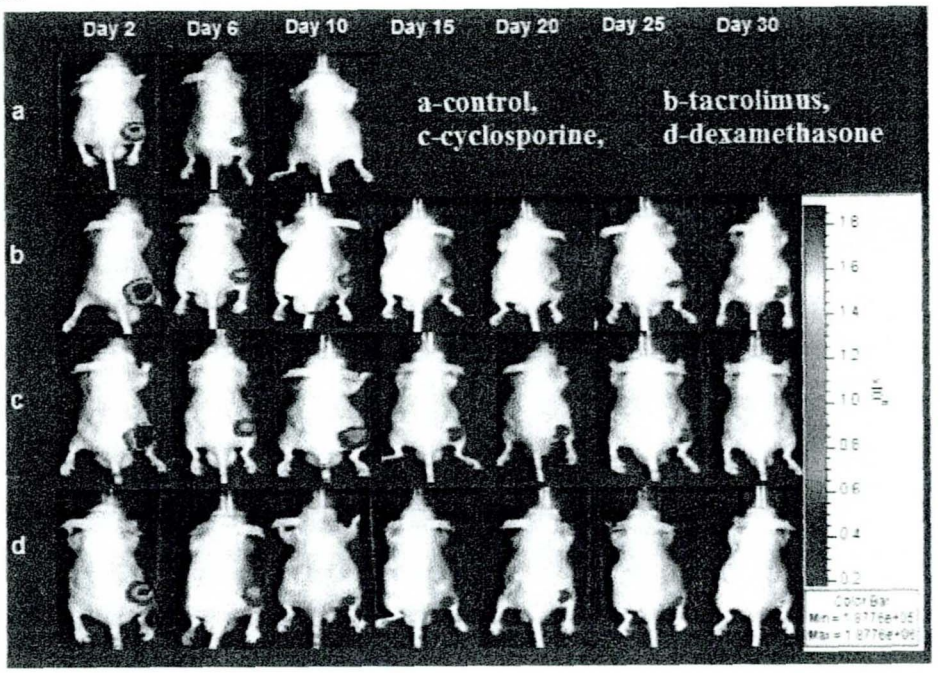
Figure 9: Correlation of cell number to bioluminescence signals and Fluc activity. (A) Increasing implanted cell dose brought about a correlative increase of bioluminescence. (B) The cell densities created linearly proportional and highly correlated BL signals as imaging (r2=0.99) and even correlated with RLU of Fluc activity in Luciferase assay (r2=0.99) (C). (D) Nude mice injected with series of cell number showed a cell dose-dependent manner in vivo with the optimal used cell number can be reach to 5 million cells to have the optimal Fluc expression in H9c2 (E). Finally, a strong correlation exists between the BL signals obtained in mouse imaging and the in vitro Fluc activity of implanted cell lysates determined from the Luciferase assay (F).

4. Effects of Immunosuppressive agents on cell survival

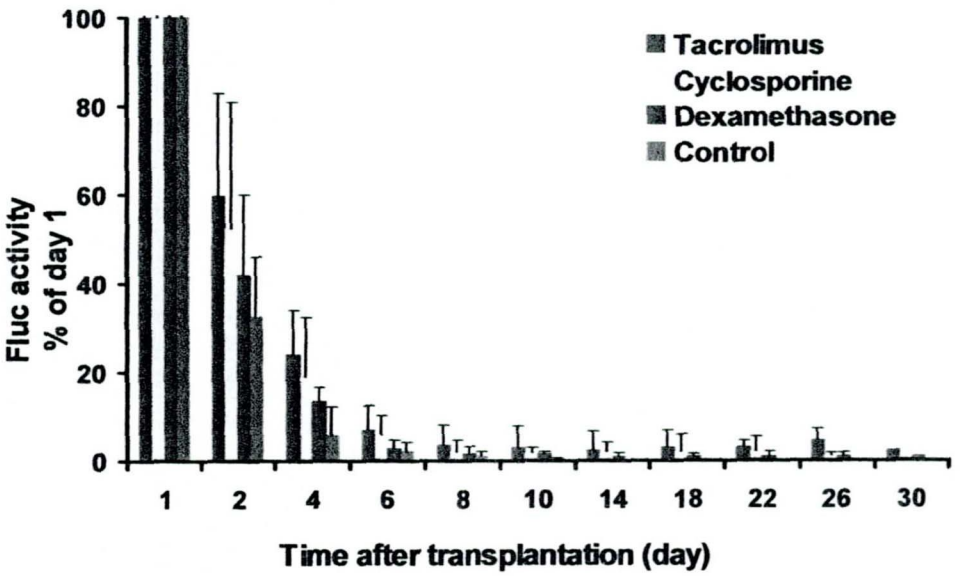
4.1 Cell implantation into nude mice

To determine if different immunosuppressive drugs can help prolong cell survival, $5x10^6$ H9c2-Fluc cells were transplanted into the skeletal muscles of both nude and Balb/c mice. Animals were treated with Cyclosporine (5 mg kg^{-1} d^{-1}), dexamethasone (4 mg kg^{-1} d^{-1}), tacrolimus (Prograf) (1 mg kg^{-1} d^{-1}), or saline (1 ml kg^{-1} d^{-1}) as control. Here, we show the longitudinal bioluminescent emission expressed in agent-treated groups (Fig. 10A). Animals treated with immunosuppressants showed significantly stronger Fluc activities from one day after transplantation compared to the control ($P < 0.05$). Tacrolimus-treated nude mice had longer visible signal until 30 days compared to other treated groups (Cyclosporine and Dexamethasone: around 25 days, Control: 8 days). The quantification of bioluminescence signal counted on the ROI (region of interest) in each case is followed in Table 1. Cell survival in all drug-treated groups shows in the same manner of a drastic decline for early 8 days post-transplantation and stably sustains by one month (Tacrolimus: 59.8% [day 2], 3.68% [day 8]; Cyclosporine: 52.4% [day 2], 2.0% [day 8]; Dexamethasone: 41.7% [day 2], 1.6% [day 8] as the percentage of cell survival on day 1) when compared to control mice (day 2: 32.4% and day 8: 1%) (Fig.10B). Especially, Fluc activity in cell lysate revealed the existence of H9c2 cell implanted in mouse muscles was improved by administration of immunosuppressive agents in early 10 days compared with control mice (Fig. 10C).

A


Day 2
Day 6
Day 10
Day 15
Day 20
Day 25
Day 30
a
b
c
d
a-control,
b-tacrolimus,
c-cyclosporine,
d-dexamethasone

B


100
80
60
40
20
0
Fluc activity
% of day 1
Tacrolimus
Cyclosporine
Dexamethasone
Control
1
2
4
6
8
10
14
18
22
26
30
Time after transplantation (day)

C

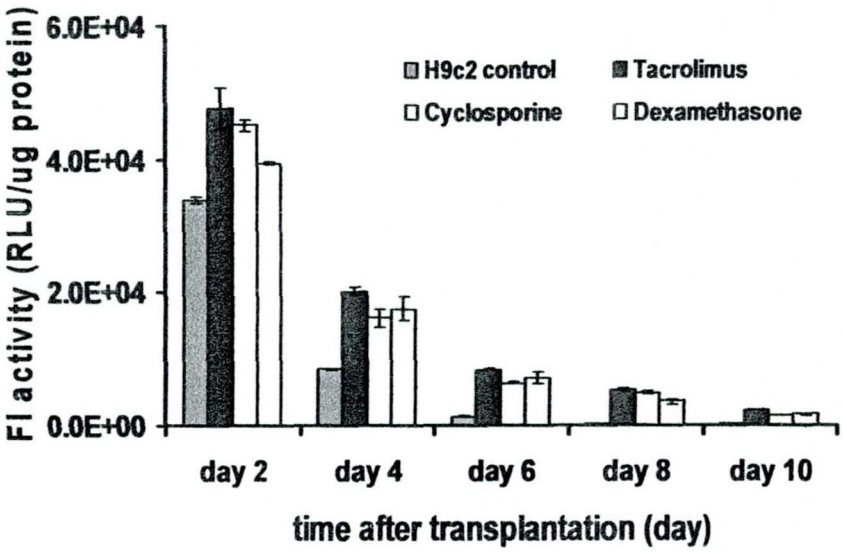


Figure 10: Effect of immunosuppressive agents on post-transplanted cell survival on nude mice.

(A) Longitudinal bioluminescent emission expressed in agent-treated groups. Tacrolimus-treated nude mice have longer visible signal until 30 days compared to other treated groups.

(B) Cell survival in all drug-treated groups shows in the same manner of a drastic decline for early 6 days post-transplantation and stably sustains by one month .

(C) Fluc activity in cell lysate revealed the existence of H9c2 cell implanted in mouse muscles was improved by administration of immunosuppressive agents in early 10 days compared with control mice.

Agents	Day 1	Day 2	Day 6	Day 10	Day 30
Tacrolimus	1.87E+07 ± 5254562	1.12E+07 ± 2317659	1.28E+06 ± 534062	5.59E+05 ± 245007	4.31E+05 ± 147714
Cyclosporine	1.93E+07 ± 5286847	1.01E+07 ± 1401725	1.17E+06 ± 694366	3.64E+05 ± 154324	1.24E+05 ± 23726
Dexamethasone	1.44E+07 ± 5456494	5.99E+06 ± 2171549	3.74E+05 ± 189578	2.03E+05 ± 91988	1.09E+05 ± 46669
Fl-H9c2 control	1.33E+07 ± 2598559	4.31E+0 ± 1087027	2.93E+05 ± 163187	9.46E+04 ± 25575	0

* Bioluminescence unit: photon/sec/cm^2/sr

Table 1: Quantification of bioluminescence signals in the in vivo imaging of nude mice with or without injection of immunosuppressive agents.

4.2 Cell implantation into Balb/c mice

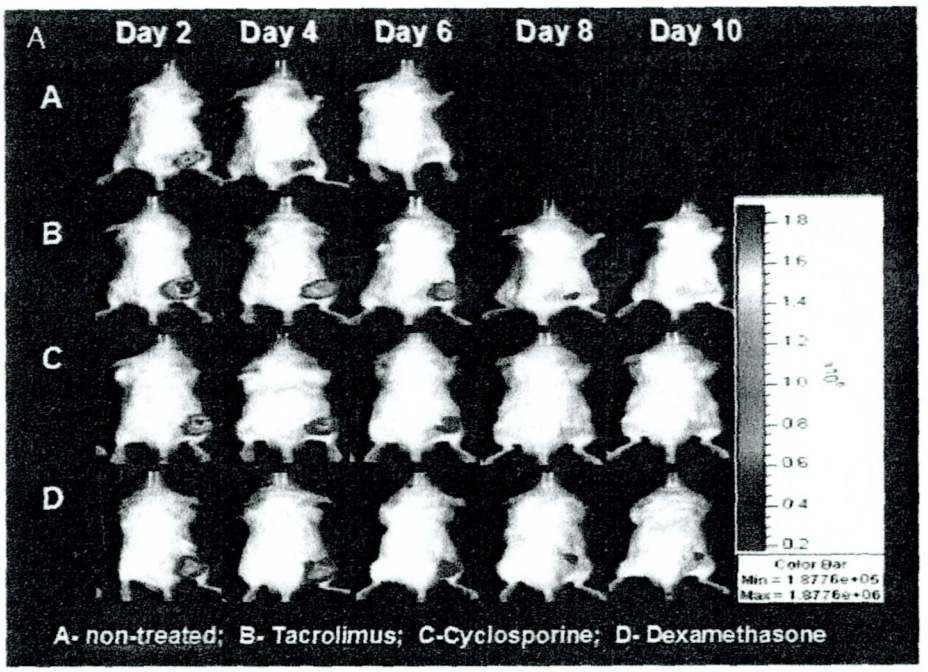
To survey the monitoring capability of bioluminescence imaging technology on cell survival supported with the immunosuppression on several murine models, we compare the effects of all previous immunosuppressants on the immunocompromised Balb/c mice. Thirty-six Balb/c mice were divided into 4 groups like the protocol of nude mouse experiments. Figure 11A showed the detection of CCD camera on cell survival in all four groups of animals, in which the bioluminescence signals were prominently expressed on day 1 and 2 after transplantation, followed by the gradual decline of signal, first on the control at day 6 and on other groups during 4 days later. The chart (Fig. 11B) which illustrates the reduction of Fluc signal, in another meaning is the effect of H9c2 cell survival's prolongation induced by immunosuppressants, demonstrated that the relative cell survival of control mice was detected for 5 days and expressed the Fluc activities were 24.5% on day 2, 5.1% on day

3, and 1.1% on day 5, reduced to the same denominator of the signals on day 1. Cardiomyoblast survival was prolonged by immunosuppressants, such as Tacrolimus for 8 days (1.2%) and Dexamethasone for 10 days (0.4%). The percentage of cell survival is equivalent with bioluminescence max photons noted in Table 2. Although the effect of Cyclosporine was helpful for cell survival around early 5 days after transplantation as compared to the control [Cyclosporine: 4.13E+05 p/s/cm2/sr (3.0%) and control group: 1.64E+05 p/s/cm2/sr (1.1%)], but it was no as effective as other two drugs (1.32E+05 p/s/cm2/sr on day 8).

Agents	Day 1	Day 2	Day 3	Day 5	Day 10
Tacrolimus	2.46E+07 ± 154353567	1.28E+07 ± 7776252	3.28E+06 ± 3882203	2.22E+06 ± 1420036	8.68E+04 ± 625
Cyclosporine	1.40E+07 ± 9981223.9	7.28E+06 ± 2366768	1.66E+06 ± 1414227	4.13E+05 ± 263359.9	3.91E+04 ± 5256
Dexamethasone	2.49E+07 ± 21688105	1.03E+07 ± 2753118	2.74E+06 ± 1943989	1.65E+06 ± 689828	1.07E+05 ± 62555
Fl-H9c2 control	1.55E+07 ± 5572862	3.80E+06 ± 824875	7.99E+05 ± 800527	1.64E+05 ± 89130.15	7.40E+03 ± 176

* Bioluminescence unit: photon/sec/cm^2/sr

Table 2: Quantification of bioluminescence signals in the in vivo imaging of Balb/c mice with or without injection of immunosuppressive agents.



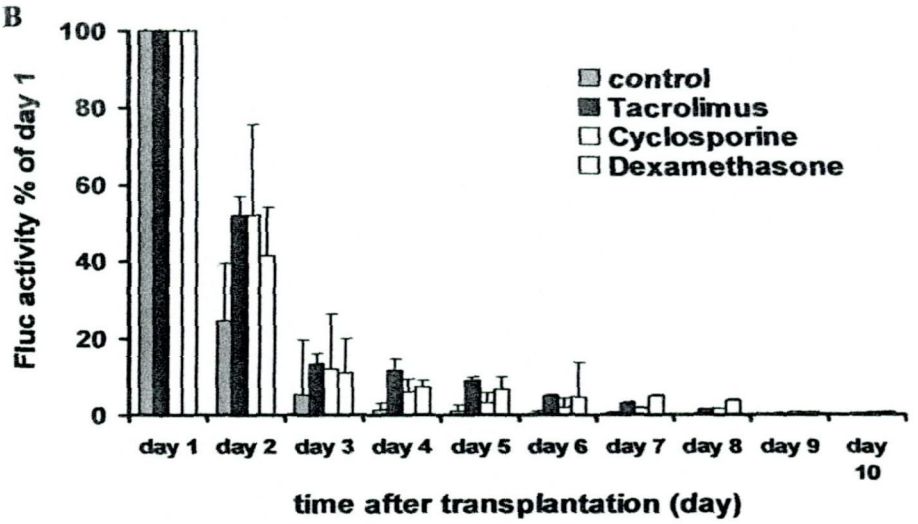


Figure 11: Effect of immunosuppressive agents on Balb/c mice.

(A) Mice injected with agents remain the longitudinal imaging for 10 days. (B) Tacrolimus and Dexamethasone were more effective than CsA. The decline of cell survival was clearly seen in treated groups for early 8 days.

4.3 Cell implantation into rat

Experimental SD rats (n=8) were implanted with Fluc-expressing H9c2 cells ($5x10^6$), together with the daily injection of immunosuppressants or saline, as described before. Fluc gene expressing density is in correspondence with the amount of living H9c2 cells in the host environment. In figure 12A, control rats without immunosuppressants could express the photonic light until the days 6 and wholly vanished afterward, whereas those at rats applied with agents were detected for long time, for example, 10 days with CsA and 25 days with Tacrolimus and Cyclosporine agents (imaging could not be performed after that time since the animals were sacrificed because of habitat contamination). A comparison among 4 groups about daily Fluc activities on the percentage of the activity of day 1 could be related briefly: on day 2 : control 18.4%, Tacrolimus 29.8%, CsA 71.5%, Dexamethasone 27%; on day 6: control 1.9%, Tacrolimus 16.2%, CsA 4.4%, Dexamethasone 3.5%; on day 10: Tacrolimus 4.6%, CsA 2.9%, Dexamethasone 6.5% (Fig. 12B). The percentage of cell survival by day is equivalent with bioluminescence max photons noted in Table 3.

A remarkable point in this series of experiment, as well as in the Balb/c experiments, is the limitation of CsA in suppressive the recipient's immune system, in other words, in improving donor cell's survival, as compared to the immune-tolerating effects of Tacrolimus and Dexamethasone, although CsA effectively prevent cell rejection at the early stage (4 days) after transplantation. Furthermore, Fluc expression particularly increased again after 10 days in Dexamethasone treated rats

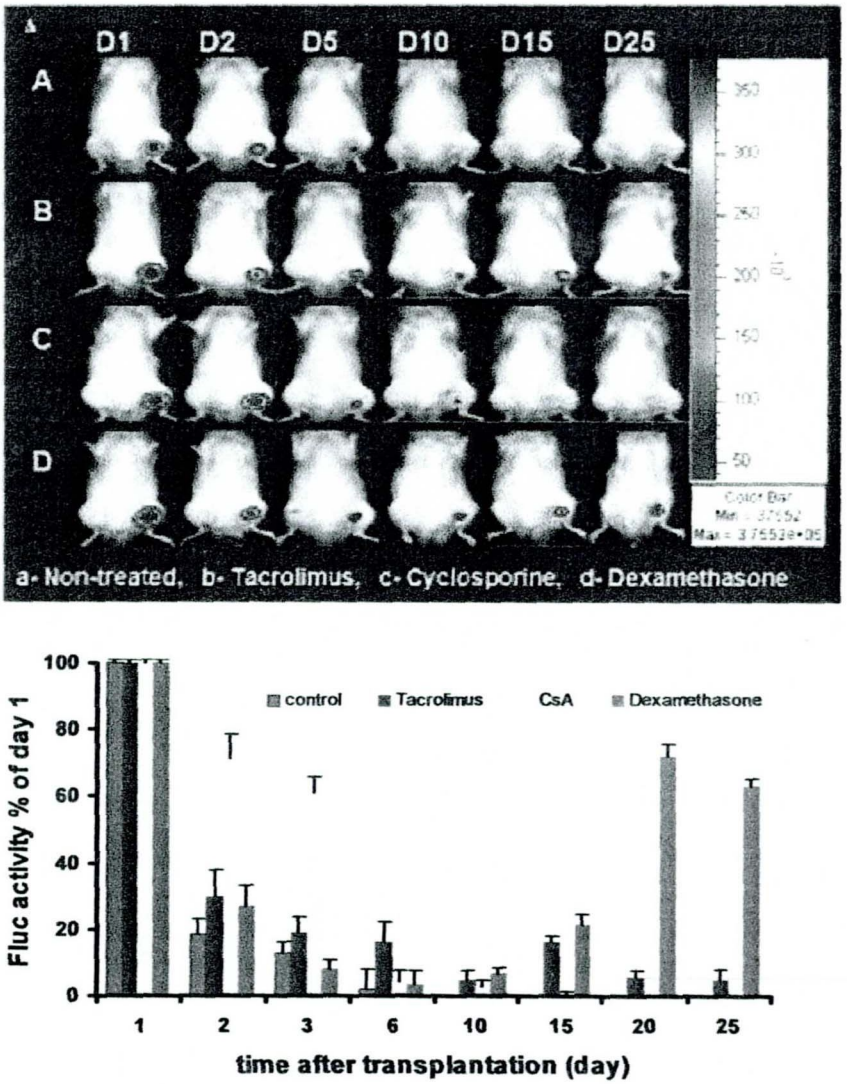


Figure 12: Effect of immunosuppressive agents on SD rats.

(A) Rats injected with agents remain the longitudinal imaging for 25 days. (B) CsA prevented cell rejection much more effective than Tacrolimus and Dexamethasone at 3 previous day but early stop its function later. An increase of signal was detected in Dexamethasone treated group from day 10 to day 25.

Agents	Day 1	Day 2	Day 5	Day 15	Day 25
Tacrolimus	1.94E+06 ± 263687	5.76E+06 ± 153731	3.14E+05 ± 73525	3.09E+05 ± 23668	9.58E+04 ± 4567
Cyclosporine	2.61E+06 ± 236622	1.87E+06 ± 626852	1.16E+05 ± 25787	1.39E+04 ± 2575	7.40E+03 ± 652
Dexamethasone	2.57E+06 ± 156385	6.96E+05 ± 836686	8.99E+04 ± 4729	5.54E+05 ± 3736	1.63E+06 ± 9636
Fl-H9c2 control	9.85E+05 ± 63667	1.81E+05 ± 16267	1.82E+04 ± 8368		

* Bioluminescence unit: photon/sec/cm^2/sr

Table 3: Quantification of bioluminescence signals in the in vivo imaging of SD rats with or without injection of immunosuppressive agents.

IV. DISCUSSION

Before the discovery of molecular imaging techniques, researches on animals for cellular or subcellular mechanisms belonged to postmortem analysis (histological staining, DNA extraction, TUNEL apoptosis) in which the animals were required to be sacrificed at every certain time of experiment, that seemed to be a limitation for continuously observing the processes taking place in animal bodies. Using a new developed technique of molecular imaging, our study was able to address the cardiomyoblast survival and especially, combine with the immunosuppressive assistance in an allogeneic cell transplant model. Our major findings are: first, bioluminescence imaging is useful for monitoring cell graft in living animals from the early to the late phases after transplantation; the second is the immunosuppressive agents can enhance cell survival against host immune response for the early 10 days and continuously sustain graft survival for one month on immunodificient nude mouse and 10 days on immunocompromised mouse.

Although studying on the embryonic rat cardiomyoblasts, we performed all of cardiomyoblast transplantation into skeletal muscle site instead of myocardium for the researching convenience. Although the H9c2 cell line was derived originally from embryonic rat myocardium, its morphologic, biochemical, and electrophysiologic properties more closely resemble those of a skeletal muscle cell line [31]. Therefore H9c2 can survive and fuse with the thigh muscle cells.

To transfer the Fluc reporter gene into embryonic rat cardiomyoblast H9c2, we used adenovirus as a transgene vector at an optimal multiplicity of

infection to penetrate into cardiomyoblast. The MOI 100 was determined after testing on series of MOI in both in vitro and in vivo (Fig. 8), showing that Fluc-adenovirus has high transduction efficiency [32-33]. To date, replication-deficient Ad vectors are proved as the most efficient gene transfer vehicles for the gene therapy strategies, and E1-deleted, so-called first generation vectors are widely used in both animal and human trials (Crystal et al., 1995), because of their ability to efficiently infect target cells and translocate the expression cassette to the nuclei (Brenner et al., 1995, Crystal et al., 1995).

In cell transplantation therapy, the significant limitation for grafting success is the rapid cell death and rejection. Humoral and cellular immune reactions are responsible for the poor outcome of myoblast transplantations, especially allo- and xenografts [34-35]. In Balb/c mice and SD rats implanted with myoblast without immune suppression, cells survival decreased more rapidly than from day 2 (20.5%) to day 6 (0.9%) (Fig. 11, 12), that could be caused by immune cells as macrophages, natural killer cells and T lymphocytes [36]. On the other hand, it seems that the cell death was caused anti-viral defense of the NK cells. The active NK cells are detected within two days of a virus infection and they have been identified as a major effecter cells against cytomegalovirus (CMV), the promoter of adenovirus in this study. Here we use a replication-deficient adenoviral vector which carries the single reporter gene encoding for Firefly luciferase under the control of the cytomegalovirus (CMV) promoter, and is deleted the E1 region. Despite deletion of the E1 region, leaky expression of immunologic adenoviral protein occurred, which would lead to the host immune response against H9c2 cells expressing the reporter gene and this response may be

responsible for the loss of transgene expression. Therefore, as injected into mouse muscles, H9c2 secreted the adenoviral proteins which excited the immunological cells in mouse to recognize and destroy H9c2 cells in the acute rejection phase, results in the swift decrement of cell survival right after transplantation in Balb/c mice and SD rats.

Notably, although experimental nude mouse is immunodeficient animal that does not have the immune responses caused by T lymphocyte activity; therefore cell rejection does not entirely take place and implanted cells could exist for long time, graft survival can sustain for approximately 6-8 days, for example, in the control group without drug administration (Figure 10), cell survival was detected until 8 days with gradually weakened signal and entirely lose tract afterward (32.4% on day 2, 5.7% on day 4 and 1% on day 8). It can be explained by the activities of other immune cells except T lymphocytes. According to the Animal Lab information, these nude mice in spite of absence of T cell function but have high functioning activity of macrophages and NK cells, which induce the acute rejection that usually appears 6 hours after transplantation and increases on the following days afterward. Furthermore, small numbers of cells carrying T-cell markers have been found in athymic nude mice and there is also accumulating evidence to suggest that bone-marrow precursors can home to mucosal epithelia and mature to form functional T-cells with γδ TCRs, and probably also T cells with αβ TCRs, without the need for a thymus [36]. Once the T-lymphocyte present, it can activate for the cytokine production and consequently stimulate the activities of other immune cells to reject graft. In addition, according to other findings of graft rejection after myoblast

transplant performed in the muscles of histocompatible or severe combined immunodeficient (SCID) mice or immunosuppressed animals in some studies highlight the importance of other unknown innate factors that are involved in graft rejection [37-38].

Our efforts in preventing cell death and rejection using some immunosuppressive agents (Tacrolimus, Cyclosporine and Dexamethasone) introduced a prolongation of cardiomyoblasts in animal body and a longitudinal imaging in murine animals. The use of Cyclosporine and Dexamethasone during the crucial early phase after myoblast transplantation can result in a long-term survival of cells. However, Cyclosporine did not result in the high potential maintenance for H9c2 in Balb/c mice (Fig. 11) and SD rats (Fig. 12), probably because it is just effective when administered before the transplantation but is ineffective in suppressing ongoing rejection [39]. In generally, these pharmaceutical regimens were responsible for the longitudinal survival of cardiomyoblast in Balb/c mice until days 10, whereas their prominent effects showed in nude mice for over 30 days, as well as more than 25 days in SD rats.

In this primary research on imaging grafted cell survival's prolongation, we encountered on some hurdles that limited our results. First, we hypothesized that if the bioluminescence signal decline in athymic animal model showed in figure 10 was not caused by the activities of host immune system, whether the cell were still survive in mouse muscle? Here we used adenoviral vector for the transient transfection of Fluc gene; thus, the in vivo reporter gene activity declines after few days, which may have further contributed to signal reduction. It was possible

that H9c2 cells liberated Fluc gene at that time but still maintain in muscles. We need more evidence for the presence of these cells by other analysis modalities such as histology or immunohistochemistry staining. Though adenoviral vector has a high transduction efficacy, but it can not preserve the long-time gene expression because the reporter gene is not integrated into the chromatin of mother or daughter cells. This can be the severe limitation as the desired goal of noninvasive imaging is to tract cell survival and proliferation longitudinally. Because of this drawback, we have now switched our delivery system from adenovirus- to lentivirus-based vectors. Lentivirus vectors have some advantages such as they can stably integrate into cell genome with minimal cytotoxicity, infect both dividing and nondividing cells and are not subjected to gene silencing [40].

From this study, we conclude that induction of immune tolerance by using separating immunosuppressive agents like Tacrolimus, Dexamethasone and Cyclosporine in initial phase during myoblast transplantation improve long-term cell survival and may help to reduce the adverse effects associated with sustained immunosuppressive treatment. However, in nowadays, there are strategies for combining immunosuppressive agents. Currently, monotherapy is not commonly used any longer; instead of it, dual therapy, usually revolving Cyclosporine or Tacrolimus is more and more popular [41]. Combinations of immunosuppressive agents have proved to be an effective strategy to inhibit diverse pathways of the multifaceted immune system, allowing the reduction of both dosage and adverse effects of each individual drug.

Besides the effect of host immune response which causes the rapidly reduce of cell survival, grafted cell can die due to apoptosis, the programmed cell death. The dead cardiomyocytes implanted into ischemic heart had features of apoptosis [9], which can be limited by activating the Akt pathways or even more effectively by heat shock prior to transplantation. Since the potential of Akt (protein kinase B), a serine/threonine kinase with antiapoptotic and oncogenic activities, acts as a critical enzyme in several signal transduction pathways involved in cell proliferation, apoptosis, angiogenesis, and diabetes, application of Akt gene therapy against cell apoptosis is our on-going goal in cardiac cell survival prolongation.

V. CONCLUSION

In conclusion, our study revealed that the use of a molecular imaging technique is excessively potential in tracking grafted cell survival in living animals. It brings about the benefit of analyzing time-consumption, of effectiveness, noninvasiveness and repetitiveness for imaging on animals, with either a little or a great number of implanted cells.

We prolonged cell survival by using immunosuppressive medications of which the efficacies were applied not only in clinical treatments but also in other studies. It demonstrated that immunosuppressive intervention on cell implantation resulted in an immune tolerance in a certain time duration after transplantation in murine animals

Our focusing work now is challenging the firefly luciferase reporter gene transfection by another virus vehicle, the lentivirus, required for the stable maintenance of Fluc expression, together with using the Akt gene therapy for anti-apoptosis. We definitely believe in this potential to improve the graft survival, supporting to the monitoring studies of gene expression, cell survival, especially cardiac stem cell proliferation and differentiation for cell therapy in heart failure treatment.

REFERENCES

1. Dowell, J.D. Rubart, M. Pasumarthi, K.B. Soonpaa, M.H. Field, L.J.. "Myocyte and myogenic stem cell transplantation in the heart." *Cardiovasc. Res.* 58:336-350, 2003.

2. Hassink, R. Dowell, J. Brutel de la Riviere, A. Doevedans, P. Field, L.. "Stem cell therapy for ischemic heart disease." *Trends Mol. Med.* 10:436-441, 2003.

3. Hodgetts, S.I. Beilharz, M.W. Scalzo, T. Grounds, M.D.. "Why do cultured transplanted myoblasts die in vivo? DNA quantification shows enhanced survival of donor male myoblasts in host mice depleted of CD4+ and CD8+ or NK1.1+ cells." *Cell Transplant* 9:489-502, 2000.

4. Skuk, D. Tremblay, J.P.. "Progress in myoblast transplantation: a potential treatment of dystrophies." *Microsc Res Tech* 48:213-222, 2000.

5. Smythe, G.M. Hodgetts, S.I. Grounds, M.D.. "Immunobiology and the future of myoblast transfer therapy." *Mol Ther* 1:303-313, 2000.

6. Smythe, G.M. Hodgetts, S.I. Grounds, M.D.. "Problems and solutions in myoblast transfer therapy." *J Cell Mol Med* 5:33-47, 2001.

7. Grounds, M.D.. "Muscle regeneration: molecular aspects and therapeutic implications." *Curr Opin Neurol* 12:535-543, 1999.

8. Liechty, K.W. MacKenzie, T.C. Shaaban, A.F. Radu, A. Moseley, A.B. Deans, R. Marshak, D.R. Flake, A.W.. "Human mesenchymal stem cells engraft and demonstrate site-specific differentiation after in utero transplantation in sheep." *Nature Med* 6:1282-1286, 2000.

9. Zhang, M. Methot, D. Poppa, V. Fujio, Y. Walsh, K. Murry, C.E.. "Cardiomyocyte grafting for cardiac repair: graft cell death and anti-death strategies." *J Mol Cell Cardiol* **33**:907-921, 2001.

10. Thomson, A.W.. "FK506: profile of an important new immunosuppressant." *Transplant Rev* **4**:1, 1990.

11. Tocci, M.J. Matkovich, D.A. Kwoek, D.. "The immunosuppressant FK506 selectively inhibits expression of early T cell activation gene." *J Immunol* **143**:718, 1989.

12. Yoshimura, N. Matsui, S. Hamashima, T. Oka, T.. "The effect of a new immunosuppressive agent, FK506, on human lymphocyte response in vitro: inhibition of the production of IL-2 and Î³-IFN, but not B cell-stimulating factor 2." *Transplantation* **47**: 356, 1989.

13. Tzakis, A.G. Abu-Elmagd, K. Fung, J.J.. "FK506 rescue in chronic graft-versus-host disease after bone marrow transplantation." *Transplant Proc* **23**:3225, 1991.

14. Gottschalk, A.R. Boise, L.H. Thompson, C.B, Quintas, J.. "Identification of immunosuppressant-induced apoptosis in a murine B-cell line and its prevention by bcl-x but not bcl-2." *Proc Natl Acad Sci USA* **91**:7450, 1994.

15. McCarthy, S.A. Cacchione, R.N. Mainuaring, M.S. Cairns, J.S.. "The effects of immunosuppressive drugs on the regulation of activation-induced apoptotic cell death in thymocytes." *Transplantation* **54**:543, 1992.

16. Perandones, C.E. Illera, V.A. Peckham, D. Stunz, L. Ashman, R.. "Regulation of apoptosis in vitro in mature murine spleen T cells." *J Immunol* **151**:3521, 1993.

17. Tuosto, L. Cundari, E. Montani, M.S.G. Piccolella, E.. "Analysis of susceptibility of human T lymphocytes to dexamethasone-induced apoptosis." *Eur J Immunol* **24**:1061, 1994.

18. Weissleder, R. Moore, A. Mahmood, U. Bhorade, R. Benveniste, H. Chiocca, E.A. Basilion, J.P.. "In vivo magnetic resonance imaging of transgene expression." *Nat Med.* **6**:351-5, 2000.

19. Wu, J.C. Chen, I.Y. Sundaresan, G. Min, J.J. De, A. Qiao, J.H. Fishbein, M.C. Gambhir, S.S.. "Molecular imaging of cardiac cell transplantation in living animals using optical bioluminescence and positron emission tomography." *Circulation* **108**(11):1302-5, 2003.

20. Wu, J.C. Tseng, J.R. Gambhir, S.S.. "Molecular imaging of cardiovascular gene products." *J Nucl Cardiol* **11**(4):491-505, 2004.

21. Contag, P.R.. "Whole-animal cellular and molecular imaging to accelerate drug development." *Drug Discov Today.* **7**:555-562, 2002.

22. Edinger, M. Sweeney, T.J. Tucker, A.A.. "Noninvasive assessment of tumor cell proliferation in animal models." *Neoplasia.* **1**:303-310, 1999.

23. Sweeney, T.J. Mailander, V. Tucker, A.A.. "Visualizing the kinetics of tumor-cell clearance in living animals." *Proc Natl Acad Sci USA.* **96**:12044 - 12049, 1999.

24. Vooijs, M. Jonkers, J. Lyons, S.. "Noninvasive imaging of spontaneous retinoblastoma pathway-dependent tumors in mice." *Cancer Res.* **62**:1862-1867, 2002.

25. Burns, S.M.. Revealing the spatiotemporal patterns of bacterial infectious diseases using bioluminescent pathogens and whole body imaging. *Contrib Microbiol.* 71-88, 2001.

26. Contag, C.H. Contag, P.R. Mullins, J.I.. "Photonic detection of bacterial pathogens in living hosts." *Mol Microbiol.* **18**:593-603, 1995.

27. Francis, K.P. Joh, D. Bellinger-Kawahara, C.. "Monitoring bioluminescent Staphylococcus aureus infections in living mice using a novel luxABCDE construct." *Infect Immun.* **68**:3594 -3600, 2000.

28. Luker, G.D. Bardill, J.P. Prior, J.L.. "Noninvasive bioluminescence imaging of herpes simplex virus type 1 infection and therapy in living mice." *J Virol.* **76**:12149 -12161, 2002.

29. Rehemtulla, A. Stegman, L.D. Cardozo, S.J.. "Rapid and quantitative assessment of cancer treatment response using in vivo bioluminescence imaging." *Neoplasia.* **2**:491-495, 2000.

30. Wu, J.C. Sundaresan, G. Iyer, M. Gambhir, S.S.. "Noninvasive Optical Imaging of Firefly Luciferase Reporter Gene Expression in Skeletal Muscles of Living Mice." *Molecular Therapy* **4**(4):297-306, 2001.

31. Kimes, B.W. Brandt, B.L.. "Properties of a clonal muscle cell line from rat heart." *Exp Cell Res.* **98**:367-81, 1976.

32. Wu, J.C. Inubushi, M. Sundaresan, G. Schelbert, H. Gambhir, S.S.. "Optical Imaging of Cardiac Reporter Gene Expression in Living Rats." *Circulation* **105**(14):1631-4, 2002.

33. Wu, J.C. Spin, J.M. Cao, F. Lin, S. Xie, X. Gheysens, O. Chen, I.Y. Sheikh, A.Y. Robbins, R.C. Tsalenko, A. Gambhir, S.S. Quertermous, T.. "Transcriptional profiling of reporter genes used for molecular imaging of embryonic stem cell transplantation." *Physiol Genomics* **25**:29-38, 2006.

34. Irintchev, A. Zweyer, M. Wernig, A.. "Cellular and molecular reactions in mouse muscles after myoblast implantation." *J Neurocytol* **24**:319, 1995.

35. Skuk, D. Caro, N. Goulet, M.. "Dynamics or early immune cellular reactions after myogenic cell transplantation." *Cell Transplant* 11:671, 2002.

36. Roitt, I. Brostoff, J. Male, D.. *Immunology* fifth edition, London: Mosby International Ltd, 1998, pp.161-162.

37. Huard, J. Roy, R. Guerette, B. Verreault, S. Tremblay, G. Tremblay, J.P.. "Human myoblast transplantation in immunodeficient and immunosuppressed mice: evidence of rejection." *Muscle Nerve* 17:224-34, 1994.

38. Sammels, L.M. Bosio, E. Fragall C.T. Grounds, M.D. van Rooijen, N. Beilharz, M.W.. "Innate inflammatory cells are not responsible for early death of donor myoblasts after myoblast transfer therapy." *Transplantation* 77:1790-7, 2004.

39. Coico, R. Sunshine, G. Benjamini, E. *Immunology a short course*, USA: John Wiley and Sons, Inc., 2003, pp.265-267.

40. Pfeifer, A. Ikawa, M. Dayn, Y. Verma, I.M.. " Transgenesis by lentiviral vectors: lack of gene silencing in mammalian embryonic stem cells and preimplantation embryos." Proc *Natl Acad Sci USA* 99:214, 2002.

41. Migita, K. Eguchi, K. Kawabe, Y. Origuchi, T. Tominaga, M. Nagataki, S.. "FK506 potentiaes steroid-induced T-cell apoptosis." *Transplantation* 64(9):136-141, 1997.

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