

Construction of Hypoxia-Responsive VEGF Gene-Expression System Using Synthetic Biological Approach

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Abstract Three-dimensional (3D) tissue construction from individual cells is an important process in regenerative medicine to enhance cell functions. In transplantation of tissue-engineered constructs, a limited oxygen/nutrient supply due to insufficient vascular network formation causes cell death. Thus, it is necessary to develop a system for inducing vascular networks into 3D tissue constructs under hypoxic conditions. In our previous study (Ono, A., *et al.*, 2017), we developed a hypoxia-inducible transgene expression system in which a target gene can be expressed in response to hypoxic stress using hypoxia-responsive promoter RTP801 as a trigger, tTA transactivator as an amplifier, and oxygen-dependent degradation sequence as a noise canceler. In this study, to improve oxygen and nutritional limitation within engineered 3D tissue constructs, a hypoxia-responsive gene expression system for vascular endothelial growth factor (VEGF) was introduced into cells. We demonstrated that genetically engineered cells could regulate VEGF expression autonomously in an oxygen concentration-dependent manner. Using the genetically engineered cells, 3D tissue constructs were fabricated using a magnetic force-based tissue engineering technique (Ito, A., *et al.*, 2005). The tissue constructs were transplanted into mice to evaluate the feasibility of the hypoxia-responsive VEGF gene expression system *in vivo*. The results indicated that the VEGF gene expression system is promising for the induction of vascular networks into 3D tissue constructs.

1 Introduction

In recent years, regenerative medicine has attracted attention for the purpose of functional recovery using functionalized cells as a solution for defects and dysfunction of tissues and organs. Three-dimensional (3D) tissue construction from individual cells is an important process in regenerative medicine to enhance cell functions. However, in transplantation of tissue-engineered constructs, lack of oxygen/nutrition supply within the constructs due to “hypovascularity” causes a decrease in cell viability of the constructs. To address this problem, approaches such as artificial blood vessels and co-culture with vascular endothelial cells have been examined (Arai *et al.*, 2018; Okudaira, *et al.*, 2016). We previously succeeded in inducing angiogenesis of tissue constructs using VEGF expressing cells (Akiyama *et al.*, 2010). VEGF binds to vascular endothelial growth factor receptor (VEGFR) on the surface of vascular endothelial cells and promotes cell division and migration. On the other hand, excessive expression of VEGF increases vascular membrane permeability (Lioret *et al.*, 2013), and hence regulation of VEGF expression is an important issue. Accordingly, we focused on hypoxia in 3D

constructs and developed a hypoxia-responsive VEGF gene-expression system using synthetic biological methods (Figure 1). Hypoxia responsive promoter RTP801 regulates gene-expression in a hypoxic environment (Kim *et al.*, 2009; Lee *et al.*, 2004; Shoshani, *et al.*, 2002). The RTP801 promoter activity is controlled by a hypoxia inducible factor (HIF) consisting of unstable HIF-1 α in the presence of oxygen and constitutively stable HIF-1 β . The stability of HIF-1 α

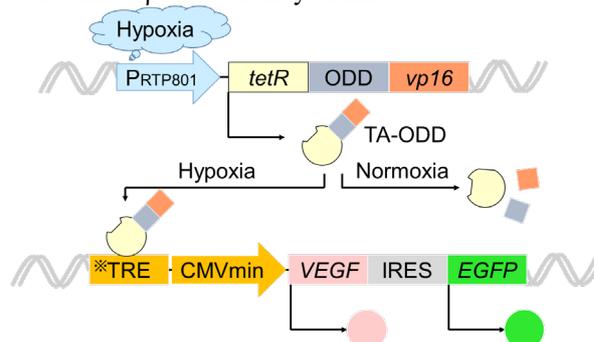


Figure 1. Gene construction of hypoxia-responsive VEGF gene expression system. (* TetO region that recognizes TA-ODD protein contained in TRE sequence is repeated seven times)

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is regulated by the ubiquitin-proteasome system, and central rule of the system is the oxygen-dependent degradation (ODD) domain (Ivan *et al.*, 2001; Jaakkola, *et al.*, 2001). The RTP801 promoter has a problem that the expression strength of downstream genes is low and it weakly works under normoxic conditions. In the hypoxia-responsive VEGF gene-expression system, an artificial transcription activator TA-ODD comprising tetracycline-responsive transactivator incorporated ODD domain is expressed under control of RTP801 promoter, and the expression of VEGF and EGFP genes is driven by TA-ODD. This system allows cells to control VEGF gene expression autonomously in response to hypoxia.

In this study, mouse myoblast C2C12 cells equipped with the hypoxia-responsive VEGF gene expression system were prepared, and transgene expression of the cells was evaluated. Furthermore, 3D tissue constructs were fabricated using the genetically engineered C2C12 cells. The tissue constructs were transplanted into mice to evaluate the feasibility of the hypoxia-responsive VEGF gene expression system *in vivo*.

2 Experimental

2.1 Materials and method

2.1.1 Cells and cultures

Mouse myoblast C2C12 cells were grown in low-glucose Dulbecco's modified Eagle medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 0.1 mg/ml streptomycin sulfate, 100 U/ml potassium penicillin G, 3.7 mg/ml sodium hydrogen carbonate (NaHCO₃) and 20 mM 2-[4-(2-hydroxyethyl)-1-piperazinyl] ethanesulfonic acid (HEPES). 293FT cells were maintained on collagen coated dish in high-glucose DMEM supplemented with 10% FBS, 0.1 mg/ml streptomycin sulfate, 100 U/ml potassium penicillin G, 3.7 mg/ml NaHCO₃ and 0.1 mM non-essential amino acid (NEAA). For lentivirus vector production, DMEM supplemented with 20 mM HEPES, 3.7 mg/ml NaHCO₃ and 77.6 mg/ml caffeine was mixed with normal medium for 293FT cells at ratio of 1:9. These cells were cultured at 37°C in a 5% CO₂ incubator.

2.1.2 Production of lentiviral vector

For lentiviral vector production, 293FT cells were seeded at 1.0×10^6 cells in 10 cm dish. At reaching 80% confluence, the cells were transfected with 3000 ng pLP1, 7200 ng pLP2, 4800 ng pLP-VSV-G and 9000 ng viral vector plasmids using Lipofectamine2000 reagent. At 6 h post-transfection, medium was changed to caffeine containing medium. After 48 h, the culture supernatant was filtered using a 0.45 µm cellulose acetate filter, and virus vector solution was concentrated by ultracentrifugation at $60,000 \times g$ for 2 h.

2.1.3 Measurement of viral titer

The virus RNA titer was measured using Retrovirus titer set and One Step SYBR PrimeScript RT-PCR Kit according to the manufacturer's protocol.

One day after C2C12 cells were seeded at 1.0×10^4 cells/well in a 6-well plate, 20 µl of virus vector solution was added. After 20 h, the medium was changed, and the cells were cultured for 2 d. Then, green-fluorescent cell number was counted using a FACS device. Viral infectious titer was calculated from the number of infected cells, GFP positive rate, dilution ratio of virus, and liquid volume at the time of infection.

2.1.3 Gene expression analysis for EGFP

In gene expression analysis for screening and cloning, EGFP gene expression of cells was monitored. C2C12 cells infected with viral vector were cultured for 2 d and analysed using FACS. The fluorescent intensity threshold was set on wild type (WT) C2C12 cells. The gene expression profiles of cells were evaluated by the fluorescent percentage of cells and median of the fluorescence intensity.

2.1.4 Quantification of VEGF expression

To quantify VEGF expression in cell culture supernatants, enzyme-linked immunosorbent assay (ELISA) was performed using Mouse VEGF-A platinum ELISA BMS619/2 according to manufacturer's protocol.

2.1.5 Preparation of MCLs

The magnetite (Fe₃O₄; average particle size is 10 nm) was purchased from Daiichikousyuha. The magnetic nanoparticles were dispersed by ultrasonic treatment for 1 h. For the preparation of magnetite cationic liposome (MCL), N-(α-trimethylammonioacetyl)-didodecyl-D-glutamate chloride (TMAG), dilauroylphosphatidylcholine (DLPC) and dioleoylphosphatidyl-ethanol-amine (DOPE) in a molecular ratio of 1 : 2 : 2 were dissolved in chloroform in a flask, and the solvent was evaporated to form a lipid film layer. After evaporation of the solvent, 10 mg/ml dispersed magnetic nanoparticles were added, and the flask was vortexed for 15 min. Finally, sonication was applied for 1 h for dispersion and sterilization of the magnetic particles.

2.1.6 Fabrication of cell sheets using Mag-TE

For fabrication of cell sheets using a magnetic force-based tissue engineering (Mag-TE), C2C12 cells were cultured to harvest sufficient numbers of cells for magnetic labeling. Then 100 pg/cell MCLs were added to the cells, and cells were cultured for 4 h. Subsequently, the cells were harvested and seeded at 1.2×10^6 cells in 500 µl medium inside a silicone rubber tube, which was placed on the center of a 35 mm culture dish. Immediately thereafter, a cylindrical neodymium magnet (diameter; 30 mm, height; 15 mm) was placed under the dish to accumulate MCL-labeled cells on the dish surface and

form a cell sheet. After 1 h culture, the silicone tube was removed and 1.5 ml medium was added.

2.1.7 Subcutaneous transplantation of cell sheets

For transplantation experiments, female 4-5 weeks old KSN/Slc (Japan SLC) nude mice were used. Mice were anesthetized using isoflurane and a small incision was created in the dorsal skin. Cell sheets cultured for 1 d were rinsed 3 times with PBS and moved on thin films as a physical support using a magnet. Subsequently, the cell sheet-film was inserted subcutaneously into the dorsal skin and the film was removed, leaving the cell sheet. Thereafter, incision was closed using silk sutures. At 14 d post-implantation, mice were sacrificed and the tissues were harvested.

All animal experiments in this study were approved by the Ethics Committee for Animal Experiments of the Faculty of Engineering, Kyushu University (A30-247-0).

2.1.8 Statistical analysis

All data are expressed as means \pm SD. Statistical comparisons were evaluated using one-way analysis of variance (ANOVA), and any values of $P < 0.05$ were considered significantly different.

2.2 Results

The hypoxia-responsive VEGF gene expression system (Figure 1) was transduced into C2C12 cells by lentiviral vector. To generate variation in gene expression range, cells were infected using two different multiplicity of infection (MOI), MOI2 and MOI30. Using the limiting dilution method, 48 cell clones were obtained from MOI2 and MOI30 cells. In order to select clones that responded strictly to the hypoxic environment from transduced cells, the gene expression of EGFP reporter was analyzed. Clone MOI30 #5 was selected because the cells exhibited the most difference in EGFP fluorescent percentage of cells between hypoxic culture (O_2 : 1%) and normoxic culture (O_2 : 19%) (Figure 2). Clone MOI30 #8 was also selected because the cells exhibited the most difference in EGFP mean fluorescent intensity (Figure 3).

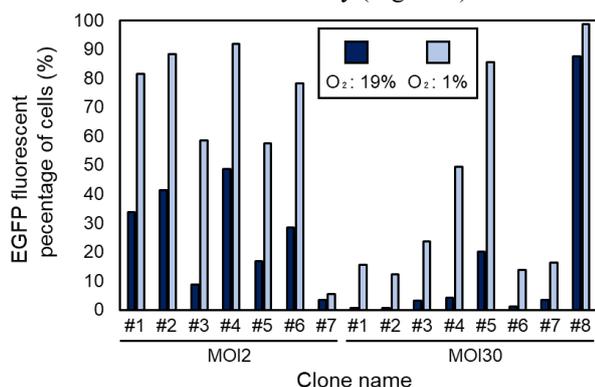


Figure 2. EGFP fluorescent percentage of clones

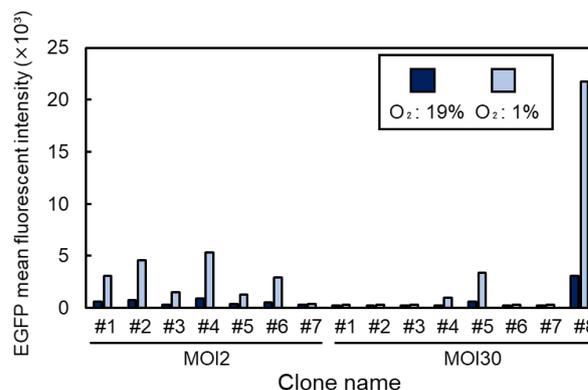


Figure 3. EGFP mean fluorescent intensity of clones

VEGF production in the culture supernatants for the selected two clones was quantified using ELISA (Table 1). VEGF production of both clones was significantly enhanced under hypoxic conditions. Especially, the level of VEGF production for MOI30 #5 cultured under normoxic conditions was almost equivalent to C2C12 WT cells, indicating strict hypoxia responsiveness. Therefore, clone MOI30 #5 was used for subsequent cell sheet experiments and *in vivo* assay.

Table 1. VEGF gene quantification of clones

Cell	VEGF expression (ng/ml)	
	O_2 : 19%	O_2 : 1%
C2C12 WT cells	3.31 \pm 0.05	3.88 \pm 0.46
MOI30 #5	3.77 \pm 0.02	14.9 \pm 2.06
MOI30 #8	26.2 \pm 0.19	39.8 \pm 0.54

Cell sheets using C2C12 WT, MOI30 #5 and constitutively VEGF gene expressing clone CMV #2 were prepared using Mag-TE method, and VEGF production was quantified by ELISA. As the result, VEGF production was observed for cell sheets using MOI30 #5 and CMV #2, suggesting that VEGF production was induced in response to hypoxic environment inside the tissue construct for MOI30 #5.

Table 2. VEGF gene quantification of cell sheets

Cell sheet (1.2×10^6 cells/sheet)	VEGF expression (ng/ml)
C2C12 WT cells	0.481 \pm 0.533
MOI30 #5	95.5 \pm 8.49
CMV #2	187 \pm 18.4

Cell sheets (C2C12 WT, MOI30 #5 and CMV #2) were implanted subcutaneously in mice and incubated for 14 d. The tissues were removed from the mice using biopsy punch and observed by hematoxylin-Eosin (HE) staining (Fig. 4A, 4B and 4C). The cell sheets containing magnetic nanoparticles as detected in black dots. Furthermore, microvessel-like structures were observed around MOI30 #5 and CMV #2 cell sheets (Fig. 4B and 4C).

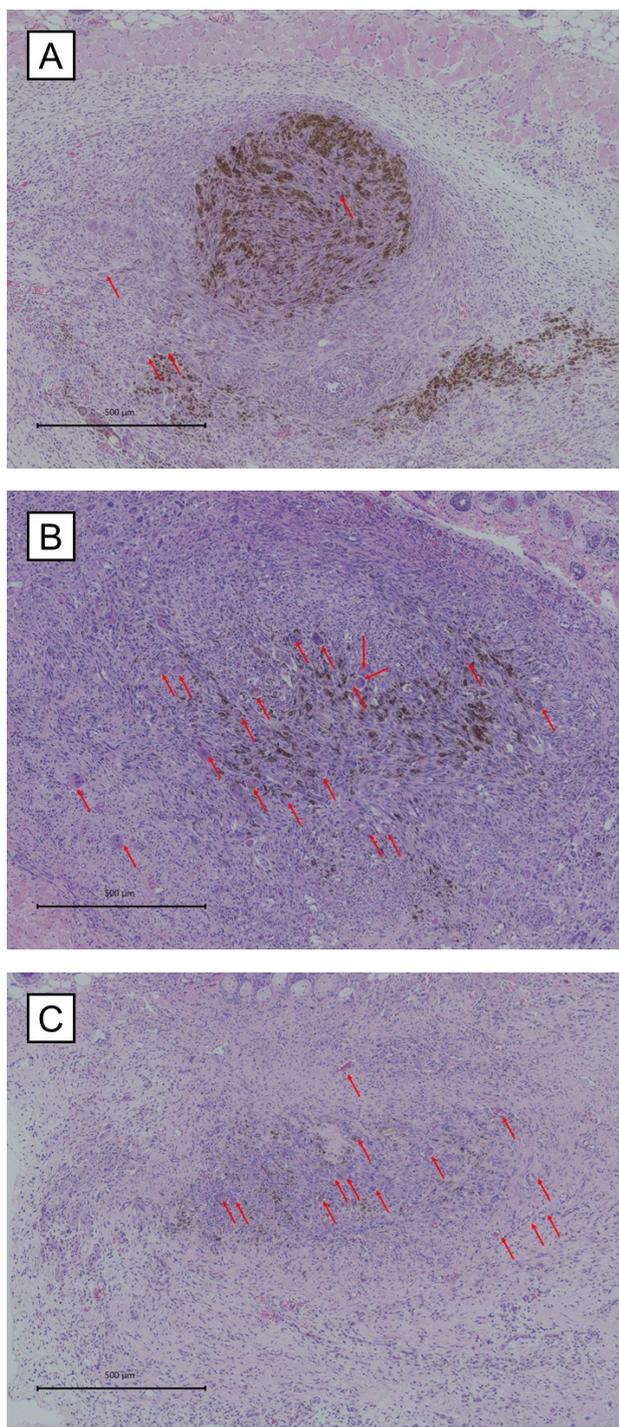


Figure 4. HE staining of C2C12 WT cell sheets (A), MOI30 #5 cell sheets (B) and CMV #2 cell sheets (C)

3 Discussion

In this study, we created genetically engineered cells equipped with hypoxia-responsive gene expression of angiogenesis factor VEGF. Microvessel-like structures were induced in mouse body by transplanting cell sheet comprising the genetically engineered cells. Angiogenesis in response to hypoxia is often observed for tumor tissues. In general, local hypoxic environment is caused inside tumor tissues because angiogenesis cannot keep up with the growth rate of cancer cells. Further, HIF

is often activated and promote angiogenesis in tumor tissues, causing cancer metastasis/invasion, and treatment resistance (Semenza, 2012; Ji *et al.*, 2010; Gardner *et al.*, 2001; Harada, 2011). By applying the VEGF gene expression system developed in this study to transplanted tissues, various advantages can be obtained. Autonomous angiogenesis allows the creation of microvascular networks that cannot be produced using artificial devices. In addition, by expressing an angiogenesis factor specifically under hypoxia, an ideal vascular network for transplanted tissue will be created. On the other hand, there are some issues to be considered. One of the problems is the risk of tumorigenesis of transplanted tissues due to genetic modification. The use of human artificial chromosomes (HACs) has been proposed as a method for stably introducing gene expression systems into cells while reducing the risk of tumorigenesis (Kuroiwa *et al.*, 2000). Secondly, the original angiogenic system is more complex, and expression of factors such as angiopoietin1, may be essential for the creation of a robust vascular network (Thurston, 2002; Brudno *et al.*, 2013).

This VEGF gene expression system provides the first platform for the creation of an autonomous vascular network by the tissue itself. Furthermore, we will analyze the expression behavior of VEGF gene in the cell sheets and the presence of endothelial cells to confirm the usefulness of this hypoxia-responsive VEGF gene expression system in regenerative medicine.

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