A Control (No treatment)   **B** 1 min treatment   **C** 24 h treatment

Fig. 6 **Electron micrographs of skin samples from hairless mice treated with a depilatory cream.** Dorsal skin samples from 5-week-old hairless mice; control (non-depilatory-treated) (A); treated with the depilatory cream for 1 min (B); and treated with depilatory cream for 24 h during the study (C). Expansion of the intercellular gaps in the basal and prickle-cell layers caused by depilatory processing was evident (B and C, arrows). Bar = 5 μm.

and exerted a direct penetration-enhancing effect on the transdermal route. However, these may be based on the difference in hairless mouse and normal hairy mouse. The hairless mice have been widely and frequently used to predict the effects of penetration enhancers in human skin. Nevertheless, the information on the suitability of hairless mice in percutaneous penetration is not uniform and sometimes contradictory (*35*). The skin of hairless mice is obviously thin in comparison with normal mice and has many folds. Moreover, because the skin penetration is a complex process, a judgement about this penetration-enhancing effect on hairless mouse skin must be cautious. In addition, we can conclude that differences in the delivery vehicles affect the penetration and bioavailability of the drug. Specifically, the effectiveness of gentamicin in an emulsion base (i.e. a cream) was higher than in oils and fats as determined by the readthrough activity and the LC-MS/MS analysis (data not shown).

Depilatory agent-treatment causes ultrastructural changes in the skin

To gain further insight into the mechanisms by which depilatory agents affect skin permeability, we evaluated ultrastructural changes in the skin caused by a thioglycolate-based depilatory agent (Fig. 6). As compared to the non-treated control, both the 1 min- and 24 h-treatments disrupted the structural integrity of the basal and prickle-cell layers. Jin-Ning Lee and colleagues (*34*) have shown that depilatory agents enhance transepidermal drug delivery by reducing the resistance of both the transcellular and intercellular routes of the stratum corneum. Our present results indicate that the alteration and expansion of the intracellular spaces in the basal and prickle-cell layers could

be due to the shrinkage of cells in those layers, which in turn leads to reduced resistance.

Readthrough for rescuing muscular dystrophy

In this study, we selected gentamicin to evaluate the efficacy of topical drug delivery. Aminoglycosides have emerged as vanguard pharmacogenetic agents for the treatment of human genetic disorders due to their ability to suppress translation termination caused by nonsense mutations. Gentamicin injections into *mdx* mice, a model for DMD, restored dystrophin in up to 20% of the muscle fibres and ameliorated the clinical symptoms of diseases (*15*). As gentamicin can cause serious side effects, such as inner ear and kidney toxicities, and may generate resistant bacteria, non-aminoglycoside, readthrough-inducing molecules are being actively sought. Previously, we reported that the dipeptide antibiotic negamycin has the ability to suppress nonsense mutations with lower toxicity than gentamicin (*36–38*). Thus, negamycin and its derivatives represent therapeutic candidates for genetic disorders, and are a topic for future studies. The small-molecular agent PTC124 (also known as Ataluren) has also been reported as a promising readthrough drug for DMD patients with nonsense mutations (*39*). Our transgenic mouse would be applicable to investigate the potential *in vivo* readthrough activity of these and other new molecules.

In the present study, we demonstrated that a transdermally delivered readthrough drug promotes the bypassing of a premature termination codon, which represents a novel approach for the treatment of genetic diseases caused by nonsense mutations. The transdermal delivery of drugs involves the continuous administration of therapeutic molecules through the skin, and has many advantages including the maintenance of low plasma drug levels and improving patient

compliance. We also showed the effectiveness of a thioglycolate-based depilatory agent to enhance the topical delivery of gentamicin. This finding may also be applicable for the transdermal delivery of other pharmacologically active molecules.

Acknowledgements

Conflict of interest
None declared.

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Novel chemotherapeutic agents for readthrough of nonsense mutations in muscular dystrophy

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Abstract

Readthrough of disease-causing premature termination codons might alleviate the pathologies of genetic disease caused by nonsense mutations. We previously reported that negamycin restored dystrophin expression with less toxicity than gentamicin in *mdx* mice. In order to explore more potent readthrough inducer, we have established transgenic mouse for readthrough-specific detection and tested several sterically related negamycin-like molecules searched by *in silico* screenings. Therefore, we found two compounds, which have a beneficial effect on readthrough action and confirmed that these readthrough-inducing compounds promote the dystrophin accumulation by immunohistochemistry and reduction of serum creatine kinase activity in *mdx* mice. We conclude that these compounds are promising new therapeutic candidates for nonsense mutation-mediated disorders including *Duchenne* muscular dystrophy.

This paper is to dedicate to Dr. Hideo Sugita for his 50th anniversary of the discovery of serum creatine kinase activity as a diagnostic marker of muscular dystrophy.

Key words : muscular dystrophy, Evans blue dye, creatine kinase, readthrough drug, nonsense mutation suppression

1. Introduction

Creatine kinase (CK), an abundant soluble enzyme in striated muscle became a strong diagnostic marker in serum of muscular dystrophy patients 50 years ago by the report of Sugita and colleagues[1]. The leakage of this enzyme into blood stream reflects the elevated permeability or damage of muscle membrane, leading "membrane damage hypothesis" of dystrophic muscle more than 30 years before the discovery of dystrophin which resides underneath the membrane. We have demonstrated that vital staining dye, Evans blue dye (EBD), can be used as a marker of degenerating fibers with elevated membrane permeability in *mdx* mouse when the dye injected via intravenously[2]. This method facilitates quantitative analyses of degenerating muscle fibers in *mdx* mouse, which does not develop severe symptom as compared with the case of human *Duchenne* muscular dystrophy (DMD). Since then many therapeutic analyses of gene transfer, myoblast transfer and drug administration were reported by using this method. It is easy to imagine that using the same leakiness of degenerating muscle membrane, CK comes out from and EBD comes into the degenerating muscle fiber (**Fig. 1**).
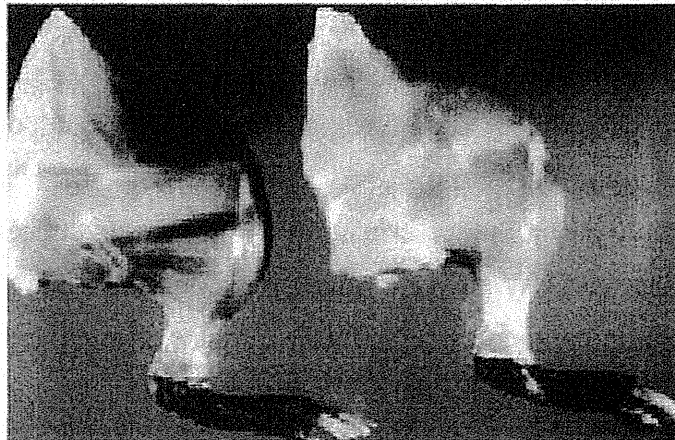

Fig. 1   Vital staining of Evans blue dye in dystrophic muscle
Permeable muscle fibers that have become damaged as a result
of muscular dystrophy, in the skeletal muscle of *mdx* mouse were
visualized by vital staining with Evans blue dye (A). In
comparison with B10 normal mouse has no staining (B).

To rescue DMD, we are currently working on the development of readthrough drugs with less toxicity than known readthrough drug, gentamicin.

Specific single genes that carry nonsense mutations cause more than 2,400 distinctly inherited human diseases. Nonsense mutations are alterations in the genetic code that prematurely stop the translation process and lead to the production of incomplete non-functional proteins. These premature termination codons are depending on the disorder, account for 5-70% of the individual cases of genetic diseases including cystic fibrosis, hemophilia, methylmalonic acidemia, Rett syndrome and numerous types of cancer. DMD is also due to alteration of the *dystrophin* gene. These are premature termination codons account for up to 20% of patients. Despite advances in gene therapy, it is still far from achieving clinical success. An alternative, pharmacologic approach is to induce translational readthrough by suppressing the nonsense mutations using antibiotics. It has been reported that aminoglycoside antibiotics can decrease translational fidelity and cause readthrough of the in-frame premature termination signals. The fact that aminoglycosides could suppress nonsense mutations in cultured mammalian cells was first demonstrated by Burke and Mogg in 1985[3]. Gentamicin has been shown to suppress nonsense mutations and partially restore protein expression in *mdx* mouse, which carries a premature termination codon in the *dystrophin* gene[4]. However, aminoglycoside antibiotics are associated with numerous side effects such as auditory and renal malfunctions, and excessive use can lead to the emergence of drug-resistant bacteria.

Previously we reported that a dipeptide antibiotic negamycin that binds to the ribosomal decoding site and alters translational accuracy, successfully restored dystrophin expression with less toxicity than gentamicin in *mdx* mice[5,6]. Unfortunately, negamycin is unapproved drug for human use and it is difficult to synthesize in large quantities. In order to investigate more potent readthrough inducer with less toxicity and move readthrough efficiency with quantitative accuracy, we have established three transgenic mouse strain, named READ (Readthrough Evaluation and Assessment by Dual reporter), which expressed a dual-reporter gene[7]. In the present study, we tested several sterically related negamycin-like molecules searched by *in silico* screenings from over one million compounds and found two compounds, which have a beneficial effect on readthrough action. In addition, we demonstrated that these readthrough-inducing compounds promote the dystrophin re-expression by immunohistochemistry and reduction of serum CK activity in *mdx* mice.

2. Materials & methods

1) Chemicals

#3 ($C_{10}H_{14}N_2O_7$; (E)-4-(2,2-bis (2-methoxy-2-oxoethyl) hydrazinyl)-4-oxobut-2-enoic acid) and #4 ($C_8H_{15}N_3O_4$; 6-(2-hydrazinyl-2-oxoethanamido) hexanoic acid) were purchased from Hodogaya Contract Laboratory Co. Ltd. (Tsukuba, Japan). Gentamicin solution for injection was obtained from Schering-Plough K. K. (Tokyo, Japan) under the trade name Gentacin. Negamycin was a gift from Microbial Chemistry Research Foundation, Tokyo, Japan. All other chemicals were purchased from Sigma Aldrich Co. Ltd. (St. Louis, MO, USA) and Wako Pure Chemical Industries (Osaka, Japan).

2) *In silico* screening

Compounds structurally similar to negamycin were identified via chemical similarity searching. In similarity searching, each compound is assigned a "fingerprint" based on the types of atoms in the compound and the connectivity between those atoms (*e. g.* Atoms bonded to each other, atoms bonded to one of the atoms in the first bonded pair, and so on)[8-10]. The fingerprints of different compounds are then used to score the structural similarity between compounds based on the Tanimoto Similarity Index[11]. This procedure was performed on a database of over one million commercially available low-molecular weight compounds[12,13]. For the search, the Tanimoto Similarity Index was adjusted to approximately 50 compounds. Compounds were then purchased from the appropriate vendor. All calculations were performed using the Molecular Operating Environment (MOE, Chemical Computing Group).

3) Animals

READ mouse strain on a C57/BL6 background expressed a dual-reporter gene, which was composed of the *lacZ* and *luc* genes connected with a premature termination codon region derived from exon 23 of the *mdx* mouse *dystrophin* gene. Although the premature termination codon was originally TAA, we used TGA in the present study. For further details of this mouse, see Shiozuka *et al.*[7]. Male *mdx* and normal (C57/BL10) mice (5 weeks old; approximately 20 g body weight) were obtained from Japan SLC, Inc. The mice were housed individually under controlled conditions of temperature and humidity and had free access to water and food. All experiments using mice were conducted under the approval of

the University of Tokyo Animal Ethics Committee.

4) Readthrough analysis on READ mice

READ mice (8 weeks old ; approximately 20 g body weight) were injected subcutaneously with 0.2 ml of compound in saline daily for 7 days. At the completion of the administration, the mice were euthanized with an overdose of ether. Tissue samples were collected from the rectus femoris, gastrocnemius and soleus. Dissected tissues were minced with scissors and homogenized in three volumes of the reporter lysis buffer (Promega, Madison, WI, USA) using a tissue grinder (Physcotron ; Niti-on, Japan). Tissue homogenates were subjected to one round of freeze-thawing. The lysate supernatants were collected after centrifugation at $17,710 \times g$ for 10 min, and then analyzed using the Beta-Glo and Bright-Glo luciferase assay systems (Promega). The ß-galactosidase and luciferase activities were measured according to the manufacturers' instructions using a luminometer (Luminescencer-JNRII AB-2300 ; Atto, Japan). The readthrough efficiency was determined as the ratio of luciferase activity to ß-galactosidase activity.

5) Immunohistochemical and Biochemical analysis on *mdx* mice

Mdx mice were injected subcutaneously with compound (1 mg/day) in saline (0.2 ml) daily for 21 days. Staining of dystrophin in muscle tissues was carried out on 8 μm transverse cryosections as described Welch *et al.*[14]. The sections were stained with a rabbit polyclonal antibody against C-terminus of dystrophin (ab-15277 ; Abcam Inc., Cambridge, MA, USA) diluted 1 : 1000 in 10% bovine serum albumin and detected with Alexa Fluor 488 secondary antibody (Molecular Probes Inc., Eugene, OR, USA) diluted 1 : 750 in 10% bovine serum albumin. Specimens were examined under a fluorescence microscope (Axioplan, Carl Zeiss GmbH, Oberkochen, Germany). The images were optimized for contrast and brightness using Photoshop CS3 software (Adobe Inc., San Jose, CA, USA).

Blood samples were collected by direct heart puncture under anesthesia and allowed to flow into tubes without anticoagulant for 30 min. Samples were then centrifuged at $2,500 \times g$, 4°C, for 10 min ; and the supernatants were assayed using an automated blood sample analyzer (Fujifilm DRI-CHEM 3500s ; Fujifilm Medical Co. Ltd, Japan).

3 . Results & discussion

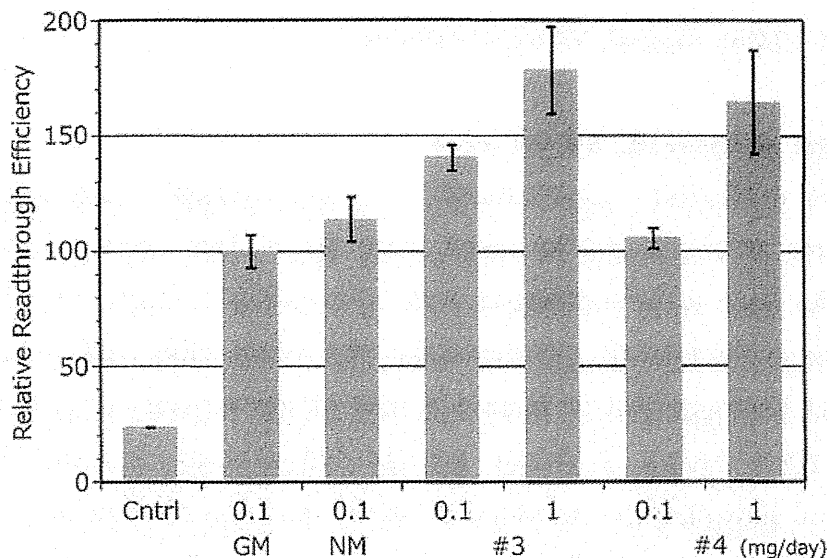We screened computationally to searching novel readthrough-inducing molecules from a

Fig. 2 Effects of readthrough-inducing molecules on READ mice

The readthrough activities following single, daily subcutaneous injections were compared with GM (gentamicin) and NM (negamycin). Cntrl (control) means the mice administrated with saline. Error bars indicate SDs.

data bank with 1,053,207 compounds, and picked up 29 compounds. Among them, we examined 19 compounds by injecting into READ mice to assess readthrough activity and found some newly identified compounds, which could induce readthrough. The dual reporter construct of READ mouse was composed of the *lacZ* and *luc* genes connected with a premature termination codon region. Although the premature termination codon of the *mdx* mouse was originally TAA, we adopted TGA-centered sequences because previous study indicated that aminoglycoside antibiotics tend to exhibit the highest readthrough activity for TGA[15]. When a test substance with no readthrough activity was administered to READ mice, only ß-galactosidase was translated. The readthrough efficiency was determined as the ratio of luciferase activity to ß-galactosidase activity.

1) Effects of readthrough-inducing molecules on READ mice

The readthrough activities of compound #3 and #4 were compared with gentamicin and negamycin that were treated by daily subcutaneous administration for 7 days (**Fig. 2**). Both compound #3 and #4 exhibited readthrough activity which is equal to gentamicin or negamycin known as readthrough-inducing molecules. As for compound #4, there was no decrease in body weight, no pathological changes by autopsy, and no abnormal value on
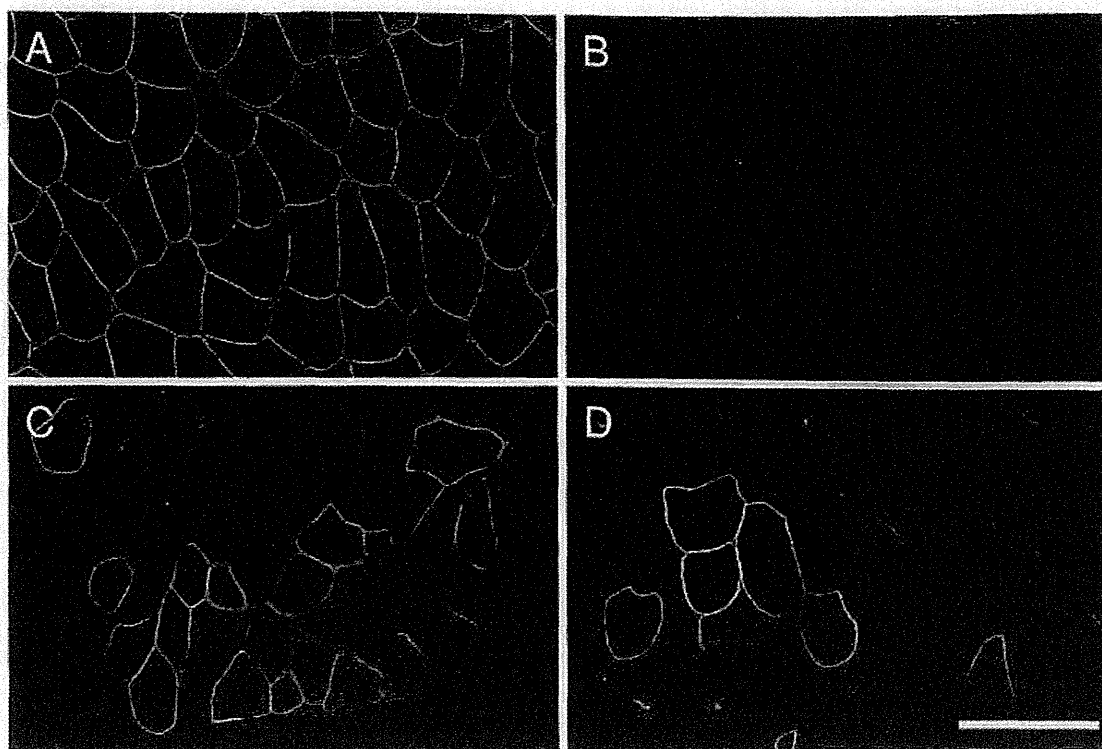
Fig. 3　Effects of #3 and #4 treatment on dystrophin expression
Immunohistochemistry of B10 (A) and *mdx* (B-D) skeletal muscle. The presence of dystrophin was detected by pAb to the COOH-terminus of dystrophin. Dystrophin was present in B10 muscle (A) and in *mdx* muscle treated with #3 (C) or #4 (D) in 3 weeks treated with 1 mg/body/day. No dystrophin was detected in untreated *mdx* muscle (B). Bar = 100 um

serum biochemistry test (22 items ; Albumin, Alanine aminotransferase, Alkaline phosphatase, Amylase, Aspartate aminotransferase, Blood urea nitrogen, Creatine kinase, Creatinin, Gamma-glutamyl transpeptidase, Glucose, High density lipoprotein-cholesterol, Lactose dehydrogenase, Na^+, K^+, Cl^-, Ca^{2+}, P^+, Phospholipid, Total Bilirubin, Total cholesterol, Total protein, Triglyceride) in ICR mice reared in a specific pathogen-free animal facility treated with intraperitoneal injection daily for 14 days up to 250 mg/kg/day (data not shown). Moreover, we confirmed that compound #4 promoted readthrough activity by oral administration as well in READ mice (data not shown).

2) Effects of #3 and #4 treatment on dystrophin expression

To determine whether both compounds could lead to suppression of the premature termination codon in dystrophin of *mdx* mice, compound #3 or #4 were treated by daily subcutaneous injection (50 mg/kg/day) for 21 days. Immunohistochemistry of muscle cross-sections to view dystrophin showed in **Fig. 3**. Administered compounds resulted in

synthesis of full-length dystrophin and its proper localization only in treated *mdx* mice. The dystrophin positive fibers in *mdx* mice treated with compound #3 or #4 had approximately 16.2% (84/523) or 18.8% (65/345) respectively. Hoffman *et al.* reported that in several cases of mild Becker muscular dystrophy, the phenotype was improved by dystrophin expressed at a level greater than or equal to 20% of normal[16]. Although we have no direct evidence as to whether normal dystrophin level observed in this study would be sufficient to improve muscle performance *in vivo*, it is likely to be far better than a complete lack of dystrophin.

Previous studies have shown that aminoglycosides suppress various stop codons with dramatically different efficiencies (UGA>UAG>UAA) and that the effectiveness of suppression is further dependent upon the identity of the first nucleotide immediately downstream from the stop codon (C>U>A/G) and the local sequence context around the stop codon[15]. The premature termination codon "UAA A" is found in the *dystrophin* gene of the *mdx* mouse and is lowest readthrough efficiency.

3）Effects of #3 and #4 treatment on CK activity

In blood samples, a high level of activity of the enzyme CK is another index of sarcolemmal fragility widely used as a diagnostic biochemical marker for muscular dystrophy[1]. To obtain a further indication of the degree of protection resulting from treatment of both compounds, we examined serum CK accumulation on *mdx* mice treated for 21 days. As shown in **Fig. 4**, Compounds #3 and #4 exhibit significantly reduced CK activities and its value was almost half. Thus, the level of protection afforded by the chemotherapy was functionally significant.

In conclusion, our results demonstrate that both compounds induce readthrough of the premature termination codon, resulting in the partial restoration of dystrophin protein and in the reduction of creatine kinase activity in *mdx* mice. These results demonstrate the feasibility of these investigational drugs to DMD and suggest that both compounds represents an important chemical entity for the potential treatment of genetic disorders caused by nonsense mutations. These novel chemotherapeutic agents that overrides premature termination codons may provide significant therapeutic value in treating or preventing genetic diseases associated with nonsense mutations. Readthrough-induced molecules have therapeutic potential for nearly one-third of all genetic disorders. These molecules may achieve even better rescue from DMD with lower toxicity and form the basis of the effective therapy for other inherited diseases involving nonsense mutations.
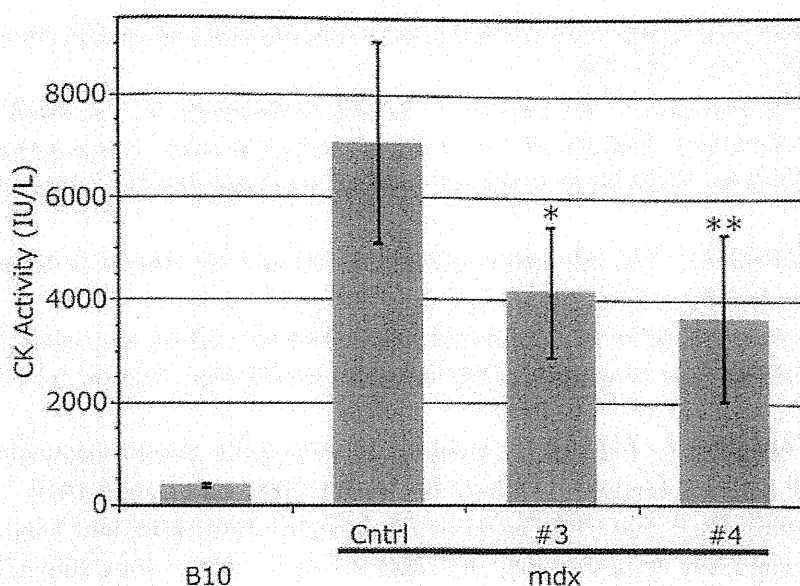
Fig. 4 Effects of #3 and #4 treatment on CK activity
Serum CK activities were dropped significantly in *mdx* mice
treated for 3 weeks. $^*p=0.056$ (vs. Cntrl), $^{**}p=0.039$ (vs.
Cntrl).

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Chapter 12
Fabrication of Growth Factor Array Using an Inkjet Printer

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Abstract Although multiple growth factors influence the fate of cells in vivo, it is technically difficult to reproduce similar condition in vitro. To overcome this problem, we have developed growth factor array, a system to study compound effects of multiple growth factors fabricated with a commercial color inkjet printer. By replacing color inks to 2–4 growth factors and printing them on the tissue culture substratum, we prepared growth factor arrays. Culturing cells on the array, we studied the compound effects of growth factors during myogenic and/or osteogenic differentiation of C2C12 myoblast and mesenchymal stem cells in a single culture dish. The cells grown on the array exhibited various levels of differentiation depending on the dose and the combination of growth factors. Since inkjet printer is capable to manipulate several colors simultaneously, this method is suitable for multivariate analyses of growth factors. This method may provide a powerful tool for regenerative medicine, especially for stem cell research on the control of cell-fate determination and differentiation.

12.1 Introduction

In living organisms, cells are constantly under the influence of extrinsic environmental factors such as growth factors, hormones, and extracellular matrices. Proliferation, differentiation, migration, and cell death are controlled by a complicated combination of stimuli from these factors. The mechanisms of these factors in the developmental processes of multi-cellular organisms have not yet been clarified completely, potentially due to the enormous number of factors that have to be examined in order to construct the in vivo conditions in vitro. Conventional studies

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with simple experimental systems considering one or two factors give insufficient analytical results.

Researches are actively pursuing methods to convert these stem cells into specific cell types to supplement damaged tissues. For example, it has been reported that marrow stromal cells can differentiate to adipocytes, chondroblasts, osteoblasts, cardiac muscle cells, hepatocytes, and neurons under certain culture conditions [1, 2, 3, 4]. Furthermore, muscle satellite cells, which are usually in quiescent state and activated for muscle regeneration process, can also differentiate to adipocytes, osteocytes, and chondrocytes by treating them with certain growth factors. Undifferentiated mouse embryonic stem (ES) cells have been found to express FGF receptor FGFR1, -R2, and -R3 [5]. The expression of IGF-1 receptors in C3H10T1/2 fibroblast is stimulated by BMP-2 [47]. As exemplified above, multiple receptors are expressed in one cell, suggesting that the stimulations from multiple factors may lead to novel effects which were not yet identified. Avila et al. [6] reported that neural differentiation of PC12 pheochromocytoma cell line was promoted by the presence of nicotine and nerve growth factor (NGF). Zebboudj et al. [7] reported that coexistence of matrix GLA protein and bone morphogenetic protein-2 (BMP-2) promotes calcification of blood vessels. Myogenic differentiation of C2C12 myoblast cell line was enhanced by IGF-I but inhibited by FGF-2 [8]. Furthermore, C2C12 muscle cells exhibit osteogenic differentiation by BMP-2 and IGF-I [9, 10]. All these reports suggest that experiments using a combination of multiple factors, not a single factor, at a wide range of concentrations is necessary.

It has been shown that no single growth factor is sufficient for deciding the differentiation fate of ES cells. Schuldiner et al. [11] examined eight different growth factors for human ES cells and reported that none of these directed differentiation to only one cell type, but, rather, altered the relative populations of a specific cell type. Loeser et al. [12] reported that coexistence of insulin-like growth factor-I (IGF-I) and bone morphogenetic protein 7 (BMP-7) stimulates the growth of chondrocytes. Although activin A induces various cell types in amphibian embryonic cells, it is not sufficient to induce phenotypes in murine ES cells [13, 14]. Therefore, the tools for analyzing the compound effect of multiple factors will make it possible to find the optimum conditions for cell differentiation efficiently.

In recent years, computer-aided tissue engineering technology called 'bioprinting' has emerged [15]. Inkjet technology has drawn attention as a powerful tool for biological research [16]. Inkjet technology is capable of ejecting solutions evenly in any place or area, enabling easy preparation of computer-generated patterns on the substratum. Since inkjet printers can eject tiny droplets at the picoliter scale, it is possible to easily control the amount of deposited substance by changing the number of ejections at any one place. Exploiting this characteristic, studies in DNA microarray preparation [17, 18, 19], protein handling [20, 21], and patterning of cells [22–24] have been reported. Furthermore, inkjet technology can be also used for analyzing the compound effects of multiple factors. We have previously proposed the concept of 'Growth Factor Array', a novel cell-based analysis tool. Growth factor arrays are fabricated with a conventional color inkjet

printer by replacing color inks to solutions containing growth factors and printing them on the tissue culture substratum [25]. Since growth factor arrays make it possible to analyze compound effects of multiple growth factors on single culture dish, it can be used to analyze the optimal conditions for differentiation of stem cells. This idea has been adopted by other research groups [26, 27].

In this chapter, we present our work on growth factor array fabrication, giving several experimental examples to prove the concept of the growth factor array. Growth factor arrays composed of 2–4 growth factors were fabricated with several approaches for growth factor immobilization onto the substratum.

12.2 Materials and Methods

12.2.1 Materials

Fibroblast Growth Factor-2 (FGF-2), Insulin-like Growth Factor-I (IGF-I), Bone Morphogenetic Protein-2 (BMP-2), Epidermal Growth Factor (EGF), Platelet-derived growth factor-BB (PDGF-BB) were purchased from R&D Systems Inc. (Minnesota, USA). [^{125}I] IGF-I and horseradish peroxidase conjugated anti-mouse IgG antibody were purchased from Amersham Biosciences Corp (New Jersey, USA). Alexa Fluoro 488 conjugated anti-rabbit IgG antibody, Alexa Fluoro 680 conjugated anti-mouse IgG antibody, and TOTO-3 were purchased from Molecular Probes, Inc. (Oregon, USA). Anti-myogenin polyclonal antibody was purchased from Santa Cruz Biotechnology. MF20 hybridoma was purchased from Developmental Studies Hybridoma Bank (Univ. Glowa), and its culture supernatant was used as the anti-mouse striated muscle type myocin heavy chain (MyHC) antibody. Anti-alkaline phosphatase monoclonal antibody was purchased from Biogenesis Ltd. (Poole, UK). IRDye 800 conjugated anti-mouse IgG antibody was purchased from Rockland Immunochemicals, Inc. (Pennsylvania, USA).

Dulbecco's Modified Eagle's Medium (DMEM) -high glucose type, fetal bovine serum (FBS), Antibiotic-Antimicotic, trypsin-EDTA, bovine insulin, and holo-transferrin were purchased from Invitrogen Corp. (California, USA). Bovine fibronectin was purchased from Itoham Foods Inc. (Hyogo, Japan). Other reagents were purchased from SIGMA-Aldrich, unless otherwise noted.

Inkjet printers used in this research were BJ F850, PIXUS 950i, Pixus iP8600 (all from Canon, Tokyo Japan). They were used with some modification, as described in the following sections.

Mouse myoblast cell line C2C12 was kindly provided from Dr. Yoichi Nabeshima of National Center of Neurology and Psychiatry, Japan. For C2C12 cell culture, DMEM containing 20% FBS was used as growth medium. As the differentiation medium, DMEM containing 10 µg/mL insulin, 5 µg/mL transferrin, 5 nM sodium selenite, 1 mg/mL bovine serum albumin (ITS medium) or DMEM containing 2% FBS was used.

12.2.2 Growth Factor Array Using Photoreactive Growth Factors

Growth factor arrays using photoreactive growth factors were prepared as described below. Photoreactive IGF-I, FGF-2, [^{125}I] IGF-I were prepared by introducing a phenyl azide group to growth factors, according to the methods described in previous works [28, 29]. The cover of the inkjet printer BJ F850 was removed, and the print head was washed with distilled water. Polystyrene substratum was placed 1 mm beneath the print head. Photoreactive growth factors were injected into the print head, and the growth factors were printed onto the substratum. To increase the density of the growth factor in a certain area, the number of printed droplets in that area was multiplied. After the printing, substratum was UV-irradiated at a distance of 10 cm for 20 s using a mercury lamp (Zeiss HBO 50, 50 W) to immobilize the growth factor on the substratum. Residual growth factors were removed by washing with PBS. With this method, a bifactor growth factor array with 16 areas representing combinations of IGF-I and FGF-2 at various concentrations was prepared. To examine the fidelity of printing and the efficiency of growth factor immobilization at each stage of this process, photoreactive [^{125}I] IGF-I was prepared and evaluated by measuring the γ-ray intensity.

For growth factor array analysis, C2C12 myoblasts were inoculated at 50 cells/mm^2 in growth medium, and after 24 h the medium was replaced with ITS-medium as differentiation medium. After a further 24 h of culture, cells were fixed with methanol, and immunostained with anti-myogenin antibody as a marker of myogenic differentiation. Anti-rabbit IgG-Alexa Fluor 488-labeled antibody was used as the secondary antibody. Nuclear DNA was also stained with Hoechst 33258. For comparison with the effect of growth factors in soluble state, combinations of IGF-I and FGF-2 in soluble state were examined at concentrations of 0, 2, 8 and 20 ng/mL, resulting in 16 combinations.

12.2.3 Growth Factor Array with Surface Activated Substratum

Substrata with activated surface to immobilize growth factors were prepared by treating the surface of the substrata with tresyl activated dextran according to the previous method [30]. Growth factors dissolved in carbonate buffer (pH 7.2) containing 0.3% glycerin were printed with inkjet printer Pixus 950i on the surface activated substratum. The substratum was incubated at 4°C for 16 h in moist conditions. Residual growth factors were removed by washing with PBS, and the remaining active groups were blocked with 0.1% gelatin or fibronectin solution. By this method, growth factor arrays composed of 3 growth factors (IGF-I, FGF-2, BMP-2) (Fig. 12.1) and 4 growth factors (IGF-I, FGF-2, BMP-2, PDGF) were prepared. The efficiency of growth factor immobilization on the surface activated substratum was extrapolated from the measured efficiency of immobilization of BSA.

For growth factor array analysis, C2C12 cells were cultured in growth medium for 24 h followed by the medium replacement with the differentiation medium containing 2% FBS. After 4 days cells were fixed with 10% formalin, blocked for
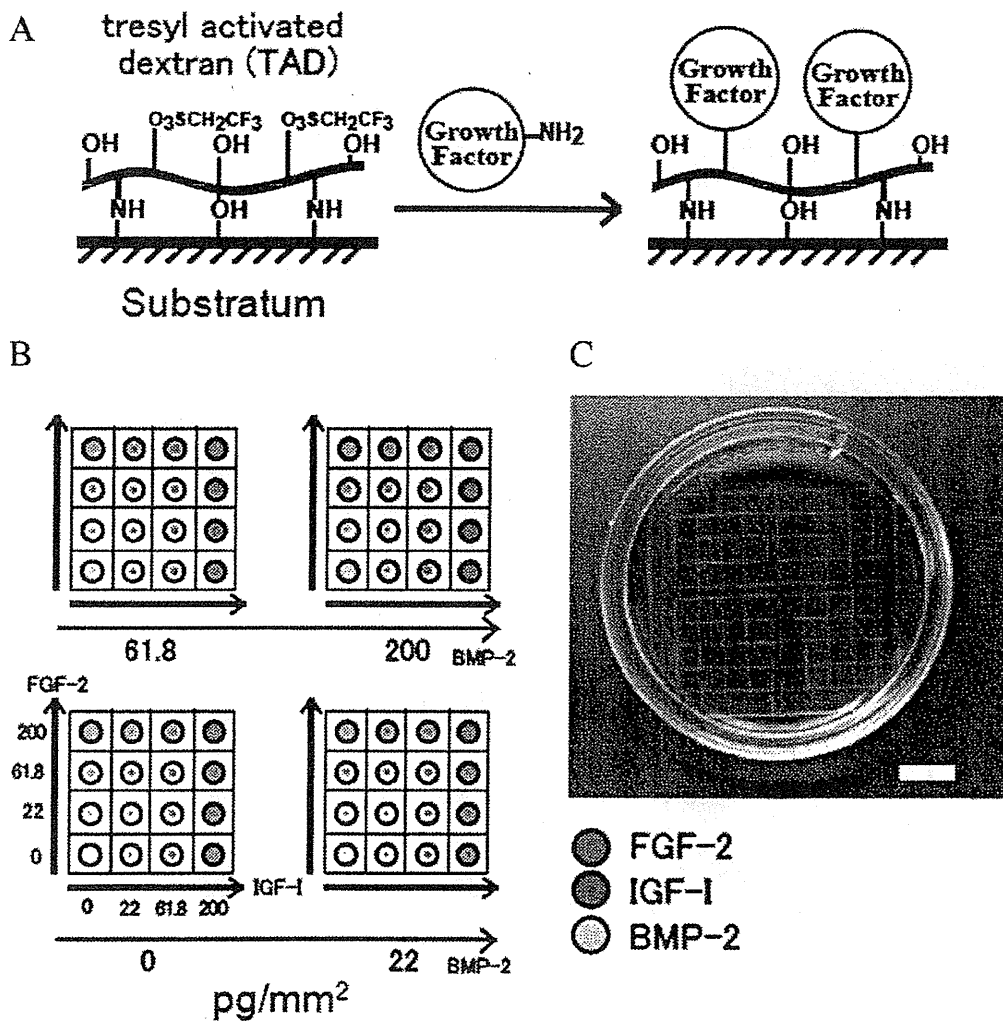
Fig. 12.1 Preparation of growth factor array using surface activated substratum. (a) Immobilization of growth factors by TAD-treated substratum. (b) Printed patterns of 3 growth factors on the surface activated substratum (pg/mm²). (c) Growth factor array with surface activated substratum placed in a culture dish (scale bar: 1 cm)

1 h, and immunostained with either monoclonal anti-ALP antibody as osteogenic marker or anti-MyHC monoclonal antibody as myogenic marker, and further stained with Anti-mouse IgG IRDye 800-labeled antibody. Cells were also stained with TOTO-3 as the indicator of the cell number. The fluorescent intensity from TOTO-3 and differentiation markers were measured with the fluorescent image analyzer Odyssey (LI-COR).

12.2.4 Growth Factor Array in Liquid System

In order to compare the effects of growth factors on the surface activated substratum with those in the soluble state, growth factor arrays in liquid system were prepared as described below. Solutions of growth factors were dissolved in distilled water containing 5% glycerin. The solutions were printed with Pixus 950i in the pattern that matches the wells of 96-well plate. After printing, 200 μL of differentiation
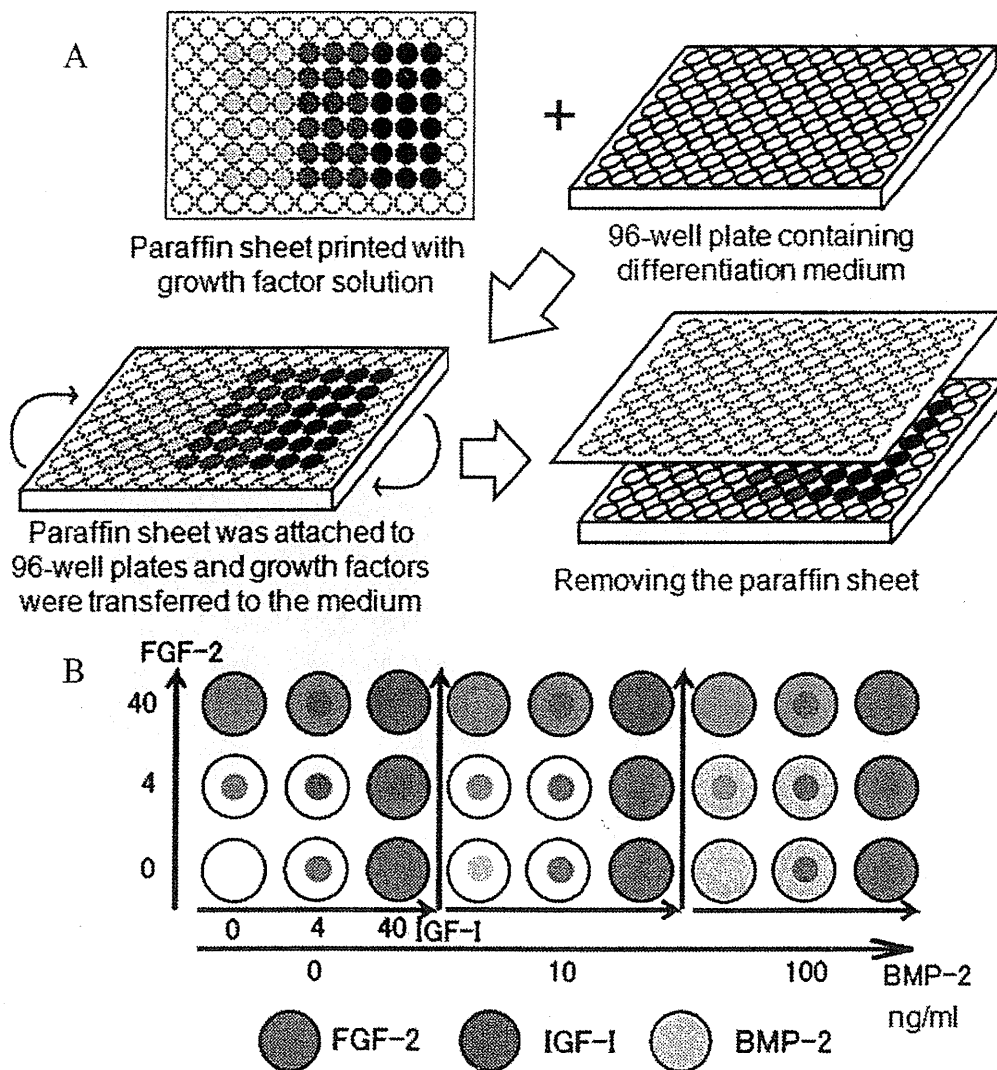
Fig. 12.2 Preparation of growth factor array in liquid system. (a) Schematic diagram of preparation of growth factor array in liquid system using paraffin sheets and 96-well plate. (b) Printed pattern of 3 growth factors on the paraffin sheets (ng/mL)

medium was injected in the wells of 96-well plates and the sheets were touched tightly to the 96-well plate, so that the printed areas corresponded to the wells. The plate was inverted to dissolve the growth factors in the medium, according to the previous method [31] (Fig. 12.2).

12.2.5 Growth Factor Array with Slow-Release System

Growth factor arrays with slow-release system were prepared as follows. Photoreactive gelatin was prepared by introducing a phenyl azide group to gelatin molecule in the same manner with the photoreactive growth factors. Photoreactive gelatin solution with and without growth factors dissolved in PBS were infused in the print head of inkjet printer iP8600. First, photoreactive gelatin without growth factors was printed on the substratum and air-dried. Then, solution containing growth factors were printed and air-dried. After printing, substratum was

UV-irradiated at an intensity of 20 mJ/cm^2 using a UV-crosslinker, and washed with distilled water 3 times. The total amount of photoreactive gelatin printed in each area was controlled such that the same amount was deposited in all areas. In order to avoid the attachment of cells outside of the printed areas, substrata were coated with graft copolymer of poly-L lysine and polyethylene glycol (PLL-g-PEG) synthesized according to the previous work [32].

To examine the slow-releasing effect of growth factors from the substratum, 96-well plate was incubated with the mixture of photoreactive gelatin and EGF then UV-irradiated at 0.2, 2, 20 and 200 mJ/cm^2. After the UV-irradiation, the wells were incubated with 200 μL of PBS containing 0.1% BSA at 37°C. The supernatant was sampled and the amount of EGF in the supernatant was determined by ELISA.

To examine the feasibility of growth factor array with slow-release system, C2C12 cells in growth medium were suspended in differentiation medium and cultured at 2×10^4 cells/cm^2. After 4 days of culture, the differentiation analysis was performed in the same manner with that of growth factor array with surface activated substratum.

MSCs were also cultured with growth factor arrays in a slow-releasing system as follows. MSCs isolated from the femur of a Wister rat (3 weeks, male) were cultured on the growth factor array with slow-release system at 1×10^4 cells/cm^2 in alphaMEM containing 15% FBS, 10^{-8} M dexamethasone, 10 mM β-glycerophosphate, 50 μg/mL ascorbic acid for 14 days [33]. Medium was changed every 4 days. After 14 days, cells were fixed with 10% formaldehyde and evaluated the bone differentiation by immunostaining with anti-ALP antibody.

12.3 Results

12.3.1 Growth Factor Array with Photoreactive Growth Factors

In order to examine the accuracy of growth factor printing with the inkjet printer and the immobilization rate of photoreactive growth factors, photoreactive [^{125}I] IGF-I was prepared. From the measured intensity of γ-ray from the printed [^{125}I] IGF-I as well as that remaining on the substratum after washing, it turned out that the printing of solutions is accurate and stable, but the immobilization efficiency decreased as the amount of protein increased.

Growth factor arrays consisted of 16 combinations of IGF-I and FGF-2 were prepared. The quantity of immobilized IGF-I and FGF-2 was calculated from the immobilization rate obtained from the previous result are 0, 21, 64, and 149 pg/mm^2 and 0, 41, 79, 175 pg/mm^2, respectively. C2C12 cells were cultured on the growth factor array for 48 hr in growth medium, followed by another 24 hr in differentiation medium. In order to compare the effect of growth factors immobilized on the array with those in a soluble state, C2C12 cells were cultured under 16 different conditions corresponding to combinations of four different concentrations (0, 2, 8, 20 ng/mL) of IGF-I and FGF-2. For both experiments, the onset of myogenin