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Construction of Protein Chip to Detect Binding of Mitf Protein (Microphthalmia Transcription Factor) and E-box DNA

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Abstract A protein chip was constructed to detect the binding of microphthalmia-associated transcription factor (Mitf) and E-box DNA. Mitf, a key regulatory transcriptional factor of pigmentation-related genes such as tyrosinase, binds to specific sequence (CATGTG) in E-box DNA within the promoter of tyrosinase in the melanocytes. We produced Mitf as a maltose-binding protein (MBP) fusion protein in *Escherichia coli*, purified it using an affinity column, and immobilized it on β-cyclodextrin-coated glass plate. Binding of Mitf to its target DNA, E-box oligomer, was monitored by surface plasmon resonance (SPR), SPR imaging (SPRi), and fluorescence-based system. Among these detection methods, fluorescence method was the most reliable. In this method, fluorescent intensity was proportional to the DNA concentration (up to 20 μM) and Mitf (up to 500 μg/ml). Kinetics of DNA binding with Mitf showed Langmuir isotherm, and its kinetic constants were determined. It is expected that Mitf-E-box DNA chip can be used as a screening tool for depigmenting agents in the cosmetic industry.

Keywords Mitf · E-box · Protein chip · HTS

Abbreviations

Mitf	Microphthalmia-associated transcription factor
β-CD	beta-cyclodextrin
MBP	Maltose Binding Protein
HTS	High Throughput Screening

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Introduction

Excessive exposure of skin to sunlight results in skin hyperpigmentation. Some skin disorders include melasma, post-inflammatory hyperpigmentation and lentigo senilis (LS) [1]. These hyper-pigmentation disorders can be medically treated with skin-lightening agents and/or treated with cosmeceuticals, defined as the cosmetic ingredients which have a curative potential. Although many ingredients have been developed and are currently used in dermatology and in the cosmetic industry, safer, more effective, and less irritating lightening agents are still required [1].

Pigmenting process occurs in the skin cells by melanocytes located in the bottom layer of the epidermis. Melanin, a brown-black biopolymer pigment is produced in the melanosome by a series of reactions converting tyrosine to melanin. Two screening methods for depigmenting agents are currently available. The first one is in vitro mushroom tyrosinase assay, using mushroom tyrosinase. As plant tyrosinase is different from mammalian tyrosinase in substrate specificity and requirements of cofactors, this method provides less satisfactory results [2, 3]. Furthermore, several papers indicated that many plant extracts showing inhibitory activity to mushroom tyrosinase in vitro did not reduce pigmentation activity in cells. Also, some compounds tested on mammalian tyrosinase did not give comparable results with mushroom tyrosinase [4]. The second method is melanin assay based on melanocyte cell culture assay. In addition to mushroom tyrosinase assay, this method is not perfect because it takes a long time, approximately 3~4 days, which is time-consuming. So, a novel screening method is required to provide a fast and compact screening tool for depigmenting agents and to overcome the disadvantages of these two methods.

Recently, it has been reported that microphthalmia transcription factor (Mitf) is a key regulator of melanogenesis in the melanocytes [5–7]. Mitf is a transcription factor having an essential basic helix–loop–helix–leucine zipper (bHLH-Lz) structure. This transcription factor plays an important role in regulating the program of gene expression in melanocyte [5–9]. Mitf target genes include enzymes which regulate the synthesis of melanin such as tyrosinase [5], tyrosinase-related protein-1 (TRP-1) [10], and tyrosinase-related protein-2 (TRP-2). This transcription factor binds to the E-box of the promoter and initiates the transcription of the genes including tyrosinase, TRP1, TRP2, and MC1R. This inhibition of Mitf binding to E-box was reported to block the transcription of the pigmenting genes resulting in depigmentation [11]. Therefore, targeting of this Mitf-DNA (E-box) interaction has potential for development of depigmenting agents.

In this study, we constructed a protein chip by immobilizing a Mitf fusion protein onto a glass plate. Direct binding of E-box DNA oligomer was also monitored, and the potential of utilizing a Mitf-protein chip as a screening tool was investigated. These results suggest that this protein chip can be used as a screening tool for depigmenting agents from numerous natural compounds and from the chemical libraries.

Materials and Methods

Production of MBP Fusion Mitf Protein

The glutathione-*S*-transferase (GST)–Mitf fusion protein expression vector, pGEX-Mitf, was kindly provided by Kitamura [12], and we constructed MBP–Mitf vector as shown in Fig. 2. To construct this vector, a polymerase chain reaction (PCR) was performed by each primer set as follows: primer NMBP-*Nde* I: 5′-CAA AAA CAT ATG AAA ATC GAA

GAA GGT AAA CTG GTA, CMBP-*Sma* I: 5′-GTG CAC GAA TTC AGT CTG CGC GTC TTT CAG GGC TT, Mitf-EcoR I (F): 5′-GGC GAA TTC ATG CTG GAA ATG CTA GAG TAC AGT C, Mitf-Sal I (R): 5′-GGC GTC GAC CTA ACA TGC ATG CTC CGT TTC TTC TG. After the PCR reaction, the MBP gene fragment and Mitf gene fragment were digested by EcoR1 and Sal 1. The gene fragments cloned to a pET vector, yielded pETMBP–Mitf. To produce purified MBP fusion proteins, MBP–Mitf was expressed in *Escherichia coli* BL21 (DE3) and cultured in an incubator at 37 °C. The expression of the MBP–Mitf fusion protein was induced by the addition of 1 mM IPTG (isopropyl-beta-D-thiogalactopyranoside). Cells were harvested by centrifugation at 6,000 rpm for 20 min at 4 °C. The resultant pellet was resuspended in 50 mM Tris–HCl, 10 mM ethylenediaminetetraacetic acid (EDTA), pH 8.0 cold buffer, and repeated twice. The supernatant was clarified by centrifugation, the cells broke up by sonication in 50 mM Tris–HCl, 0.5 M NaCl, pH 7.4 for 1.5 h, 6 min for crushing cells, and 5 min for resting at 4 °C. After sonication, cell lysates were centrifuged at 12,000 rpm for 30 min. Supernatant and sediments were then separated. MBP–Mitf was purified by MBP affinity exocellulose column (Bioprogen, Korea) using elution buffer (10 mM maltose, 20 mM Tris–HCl, 0.2 M NaCl, 1 mM EDTA, pH 7.4).

Design of Oligonucleotide (E-Box: CATGTG)

Mitf-binding oligonucleotide, probe oligo-DNA derived from tyrosinase promoter and melanocortin 1 receptor (MC1R) contains a CATGTG motif. It was manufactured as forward; 5′-AAG TTA GTC ATG TGC TTT GCA G-3′ and reverse; 5′-CTG CAA AGC ACA TGA CTA ACT T-3′ sequences (Bioneer, Korea). On Forward 5′of the sequence, Cy-3 dye was labeled for fluorescent imaging. Manufactured as a ssDNA, mixed pairs of ssDNAs were dimerized by heating up to 90 °C.

Construction of Protein Chip and Detection of Binding

A modified glass slide plate was used for immobilization of recombinant fusion protein, MBP–Mitf, as a principle of the protein chip Epoxy-treated glass slides were immersed in β-CD (70 g/L) in 0.1 M NaOH solution and gently agitated. Epoxy residues were blocked by immersion in 1% bovine serum albumin (BSA) solution and rinsed with ddH_2O and dried. The β-CD-treated glass slides were stored at 4 °C for 1 week. The β-CD displayed a binding affinity to maltose-binding protein (MBP) that can specifically fix MBP fusion protein.

Purified MBP–Mitf fusion protein was diluted in spotting buffer [50 mM Tris, 20% (*v*/*v*) tetraethylene glycol, pH 8.0]. MBP–Mitf was spotted on the β-CD-treated glass slide chip, covered with the solution to promote parallel spreading, and reacted for 1 h at a humidity of 50~60% at room temperature. The slides were then rinsed with a step of phosphate-buffered saline/Tween (PBST)-1 (tween20 in PBS), PBST-2 (triton X-100 in PBS), PBS, and ddH_2O and dried. On the Mitf immobilized chip, oligo-DNA (including E-box) was printed using a microarrayer CM-1000 (Proteogen, Korea) and carried out in an atmospherically controlled chamber with a relative humidity of 70~80% at room temperature. A Stealth pin (SMP10, Telechem) was used for arraying spots of 320 μm in diameter. The reaction solution included DNA, poly dIdC (0.25 mg/ml), binding buffer (10 mM HEPES pH 7.9, 50 mM KCl, 2.5 mM DTT, 0.1 mM EDTA, 0.05% NP-40, 10% glycerol, 5% BSA), and 25% (*v*/*v*) tetraethylene glycol. After 1 h Mitf-DNA binding time, the Mitf-DNA chip was rinsed with PBST-1, PBST-2, PBS, and ddH_2O, and dried.

Protein–DNA binding was detected by fluorescence and analyzed by a GenePix 4100A scanner (Axon) and GenePix 4.1 software program. On Forward 5′ of the nucleotide sequence, Cy-3 dye was labeled for fluorescent signaling. Evaluated results were displayed by graph using SigmaPlot 2001.

Results

Construction of the Protein Chip

The protein chip containing MBP–Mitf has been developed as illustrated in Fig. 1. The protein chip allows serial attachment of β-CD, Mitf fusion protein and target DNA (unlabeled or Cy3-labeled) on a glass plate. For this protein chip, we constructed MBP–Mitf fusion protein gene subcloned into pET vector (Fig. 2). The MBP domain is useful for purifying the fusion protein by affinity chromatography [14] as well as being immobilized on β-CD-coated solid surface. Fast protein liquid chromatography column chromatography (Fig. 3a) and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis (Fig. 3b) were performed to confirm the fusion protein after partial purification using an affinity column. As shown in Fig. 3b, MBP–Mitf protein marks 125 kDa molecular size.

Detection of Mitf–DNA Binding

To detect the MBP-tagged recombinant protein, surface plasmon resonance (SPR) and surface plasmon resonance imaging (SPRi) were attempted initially. SPR and SPRi are optical techniques to detect the specific binding of unlabeled biomolecules onto molecules attached to chemically modified gold thin films by measuring changes in the index of refraction upon adsorption [13, 15, 16]. In this work, however, binding of E-box DNA on

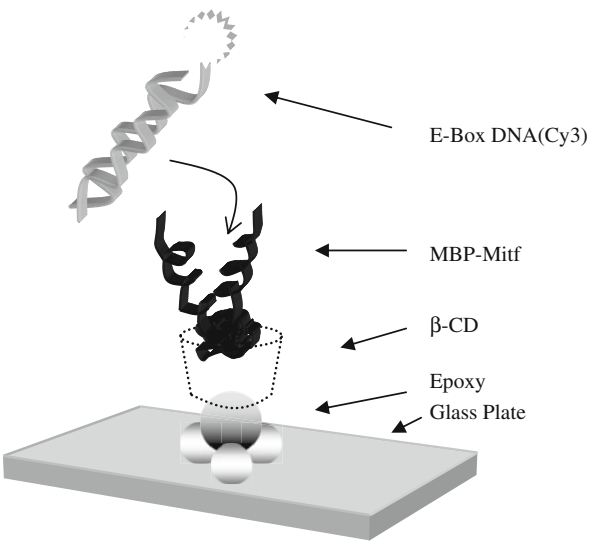

Fig. 1 Schematic illustration of fluorescence-based microarray. The fusion protein (MBP–Mitf) was immobilized on β-CD-coated glass plate. Cy3-labeled oligomers were spotted on Mitf-immobilized plates

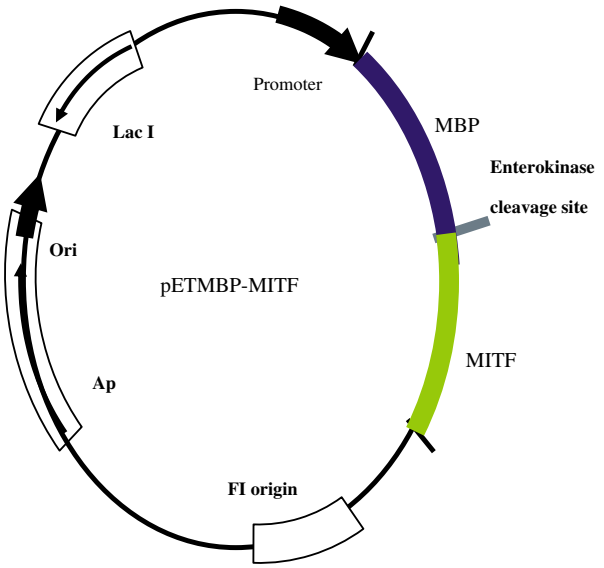

Fig. 2 Construction of plasmid pET–MBP–Mitf. The gene for Mitf was fused to the MBP and subcloned into a pET vector, which contains enterokinase cleavage site

the Mitf fusion protein was not detected, although binding of Mitf to surface was detected (data not shown). This is due to the fact that the mass of 22 base E-box DNA is too light to detect the mass difference of binding. Additional work is under progress to increase the weight of DNA oligomer, for example, by binding of gold particle to DNA.

As SPR and SPRi are little effective for detecting binding interaction between Mitf and DNA, fluorescence-based protein array was performed. In this system, an epoxy-treated glass plate and Cy3-labeled E-box DNA, instead of gold thin film and unlabeled DNA, were employed for monitoring binding interaction between Mitf and E-box DNA. As a control, BSA with a concentration of 10 mg/ml was spotted on the epoxy slide chip. As shown in Fig. 4, the control spots are almost invisible on the fluorescence image, thus indicating that the protein binding is specific. Next, we examined whether Mitf–MBP fusion system allowed for a quantitative assay to be performed. Human Mitf fusion protein was spotted on a chip at five different concentrations. The fluorescence intensity increased linearly as the amount of Mitf increased to 500 μg/ml and gradually saturated as it increased further (Fig. 5). The intensity difference, which as the average value of the spot intensity minus the background intensity calculated from the image in Fig. 5 using an image analysis software (GenePix 4.1, molecular devices, USA), was plotted against the concentration of DNA (Fig. 5). The lowest visible protein concentration was found to be about 31.2 μg/ml in the fluorescence imaging. This result suggests that the fluorescence intensity has a linear correlation with the fusion protein concentration up to 500 μg/ml, showing the possibility of measuring concentration-dependent binding intensities.

For a reliable and reproducible experiment, we have confirmed optimum concentration of the MBP–Mitf, immobilized on glass slide whose surface was coated with β-CD, and E-box DNA bound to Mitf. On the surface of immobilized Mitf, Cy3-labeled oligonucleotides were spotted with varying concentrations and detected by a fluorescence scanner. At optimal concentration of Mitf, oligonucleotide DNA was shown to be bound to Mitf in a dose-dependent manner (Fig. 6). Kinetics of DNA binding with Mitf showed

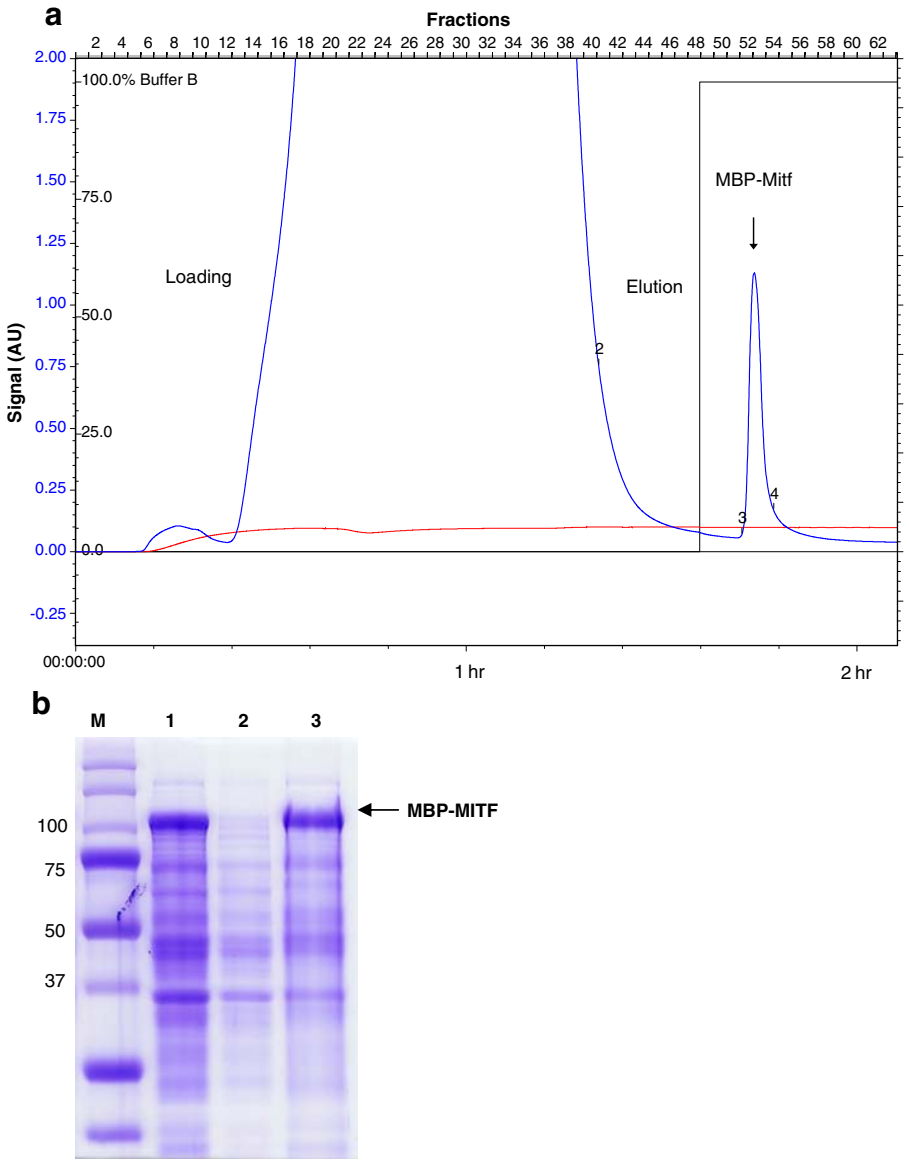

Fig. 3 Analysis of partially purified MBP–Mitf protein. **a** Chromatogram of affinity column chromatography packed with MBP exocellulose (Bioprogene, Korea). **b** SDS-PAGE analysis of partially purified MBP–Mitf fusion protein (*M* MW standard, *1* total fraction, *2* insoluble fraction, *3* soluble fraction)

Langmuir isotherm and its kinetic constants were determined by manipulating the equation into liner form and its constant were determined graphically by the same method used in Lineweaver–Burk plot (Ki constant, 17.2 μM; Max. intensity, 1.04×10^5). To determine if the interaction between Mitf and E-box is sequence-specific, we modified E-box DNA (CATGTG → CTTGAG). When this modified E-box DNA (CTTGAG) was added to authentic E-box DNA (CATGTG), fluorescence intensity was decreased (Fig. 7), indicating

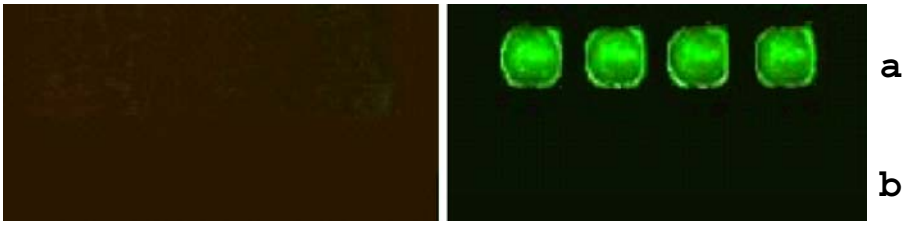

Fig. 4 Fluorescence imaging analysis of partially purified MBP–Mitf fusion protein. Spotting of unlabeled (*left*) and Cy3-labeled (*right*) MBP–Mitf binding to the β-CD glass slide. **a** 120 μg/ml; **b** BSA (10 mg/ml)

that Mitf–DNA binding is sequence-specific, and protein–DNA binding was inhibited by a competitive inhibitor.

These results show that this method was sensitive and could analyze multiple reagents simultaneously. Therefore, we suggest this fluorescence-based protein–DNA chip is applicable to the high-throughput screening (HTS) inhibitor screening system. In conclusion, we constructed a protein chip containing Mitf and suggested its potential as a screening tool for depigmenting agents.

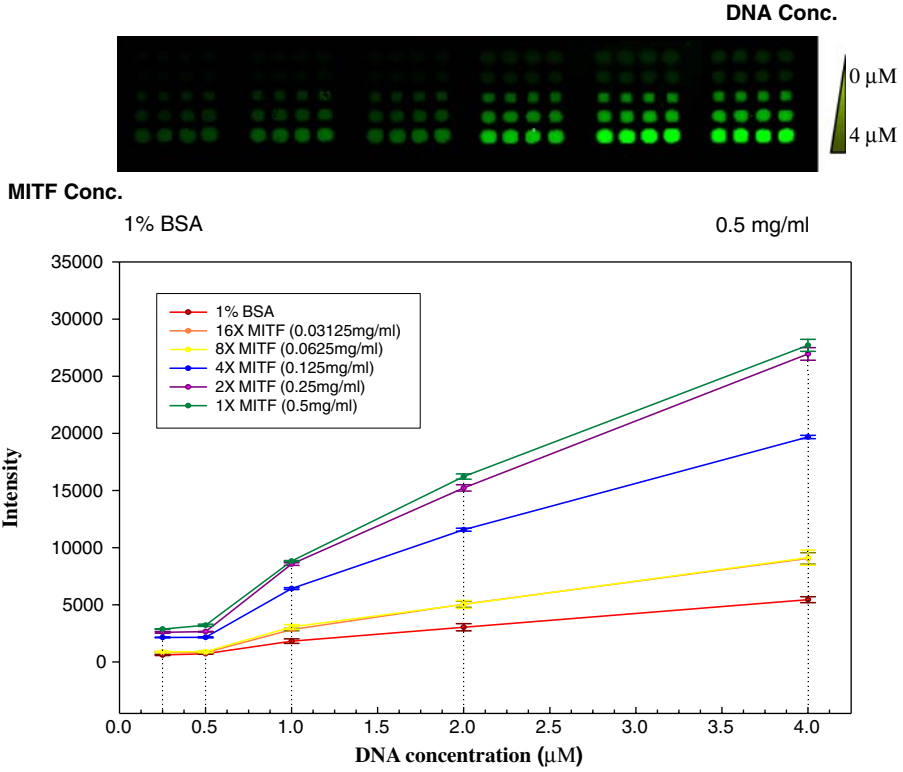

Fig. 5 Effects of concentrations on the binding of Mitf and E-box DNA. Each data point is an average of four spots. For the fluorescence intensity measurements, spots were visualized by emission wavelength at 532 nm

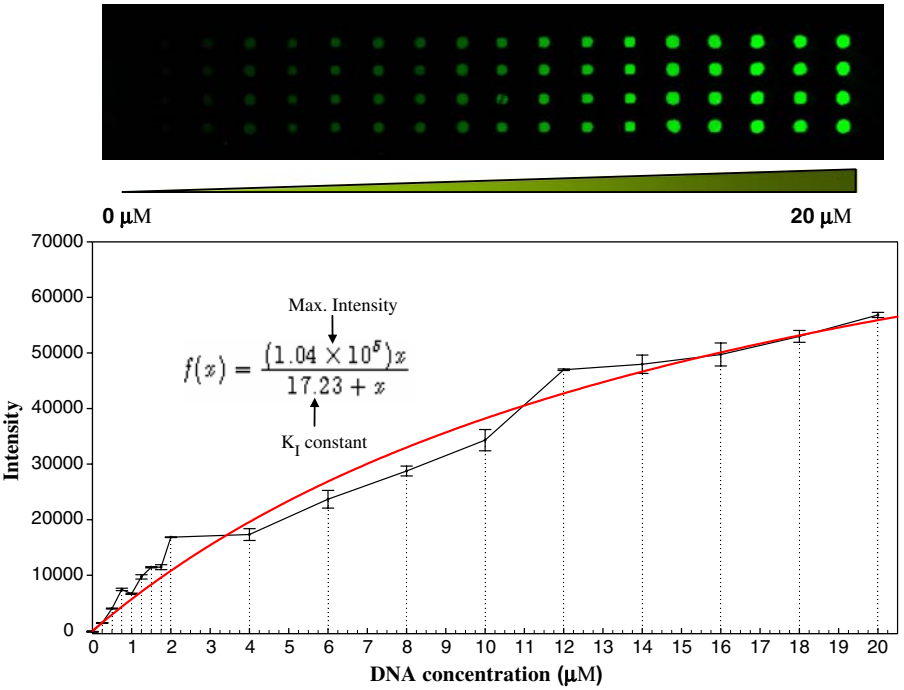

Fig. 6 Kinetic analysis of Mitf–DNA binding on a glass chip. Photo shows four rows of replicate

Discussion

Protein chip technology provides a new and useful tool for high-throughput screening of drugs and cosmetic ingredients because of its high performance and low sample consumption [17]. The binding interaction between the transcription factor (Mitf) and target DNA was investigated. Mitf is a key regulatory factor of melanogenesis and binds to E-box (CATGTG) within promoter of pigmentation-related genes such as tyrosinase, TRP-1, and TRP-2. Therefore, we aimed to determine if an inhibitor of protein–DNA binding could be developed as skin whitening agents and evaluated its potential as a tool of HTS.

In this study, we confirmed that fluorescence is the most effective method for monitoring in vitro binding between Mitf and E-box. MBP–Mitf fusion protein was constructed via pGEX–Mitf to immobilize on β-CD-treated surface, as maltose binding protein (MBP) can specifically bind to β-CD [18]. We assessed the MBP–protein chip on the glass and gold chip activation chemistry, which formed exclusive specific bonds with MBP–Mitf and binding β-CD. Then, nonspecific binding was tested by BSA. First, we tried SPR analysis to detect Mitf on the chip. We conducted SPR analysis of real time detection β-CD chip and MBP–Mitf. SPRi analysis produced the same results as SPR. We concluded from the specific binding of MBP–Mitf and β-CD that MBP-fusion protein binds specifically to the β-CD surface (data not shown). Then, E-box DNA (CATGTG) specifically binds to the MBP–Mitf. The SPR was sufficient to quantify only Mitf but not to detect Mitf–DNA binding because no difference was indicated between the two cases

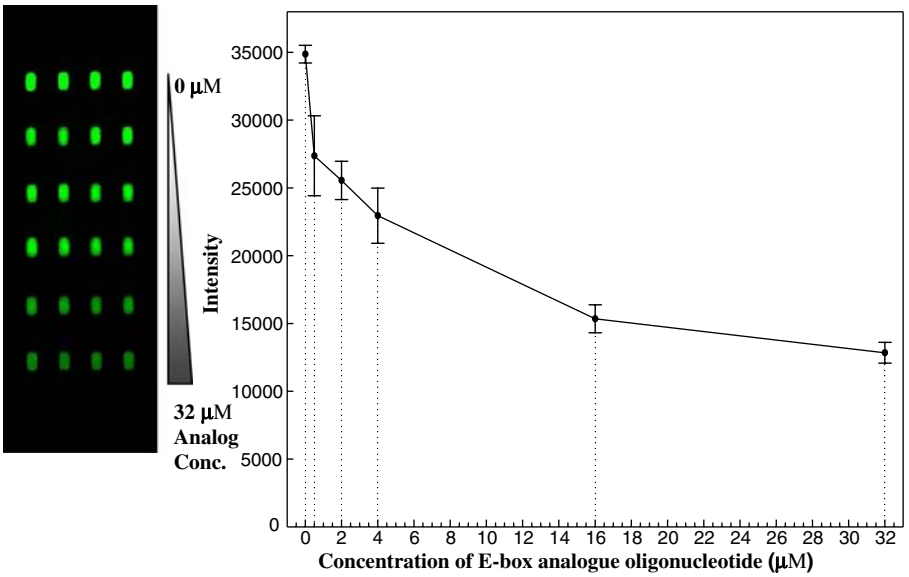

Fig. 7 Effects of E-box analogue (CTTGAG) on Mitf-DNA binding. Analogue was added to 4 μM authentic E-box DNA (CATGTG)

(data not shown). If we developed a different strategy of elevating Mitf on the layer of DNA, the binding Mitf on a DNA immobilized chip could be detected by the SPR. This work is under progress.

Next, we performed fluorescence-based microarray. Cy-3-labeled DNA was spotted on the immobilized MBP–Mitf on β-CD-treated glass plate (Fig. 4). Optimum concentration of Mitf and DNA were determined by changing the concentration of the protein and DNA (Fig. 5). Langmuir isotherm and inhibition constant were determined (Fig. 6). After optimizing the construction, a reported inhibitor should be used to confirm the effectiveness as an inhibitor screening tool. There was work demonstrating that binding inhibition by dominant negative of Mitf (Mitf-DN) decreased the expression of tyrosinase. However, no small inhibitors of Mitf–DNA binding were reported [11].

Instead, E-box mutant oligomer was employed to elucidate whether this interaction was sequence specific or not. E-box oligomer mutant revealed an inhibitory effect (Fig. 7). Mutant dsDNA is a binding competitor that acts by specifically blocking the binding of dsDNA with the protein. We confirmed that a competitor was interfering with binding of Mitf and the target DNA. This approach offers the potential for high-throughput determination of transcription factor binding profiles and inhibitor screening systems.

In conclusion, we have developed a fluorescence-based HTS methodology for the screening of depigmenting agents. This screening method allows for an easy, fast, and compact strategy. We have also demonstrated this method is dependent on the concentration of both the immobilized protein and DNA oligomers. This approach could be possibly used to screen the inhibitors of Mitf–DNA binding. Finally, these results demonstrate that the Mitf protein chip has potential to be used as a HTS tool for in vitro screening using fluorescence.

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