

# Promotion of minTBP-1-PRGDN on the attachment, proliferation and collagen I synthesis of human keratocyte on titanium

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## Abstract

• **AIM:** To investigate the influence of minTBP-1-PRGDN on the attachment, proliferation and collagen I synthesis of human keratocyte on titanium (Ti) surface.

• **METHODS:** The chimeric peptide RKLDPAPRGDN (minTBP-1-PRGDN) was synthesized by connecting RKLPPDA (minTBP-1) to the N-terminal of PRGDN, the influence of minTBP-1-PRGDN on the attachment, proliferation and collagen I synthesis of human keratocyte on Ti surface were tested using PRGDN and minTBP-1 as controls. The keratocytes attached to the surface of Ti were either stained with FITC-labeled phalloidin and viewed with fluorescence microscope or quantified with alamar Blue method. The proliferation of keratocytes on Ti were quantified with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide up-taking methods. The secretion of type I collagen was determined using an ELISA kit.

• **RESULTS:** The results showed that minTBP-1-PRGDN at a concentration of 100ng/mL was the most potent peptide to enhance the attachment of human keratocytes to the surface of Ti (1.40±0.03 folds,  $P=0.003$ ), to promote the proliferation (1.26±0.05 folds,  $P=0.014$ ) and the synthesis of type I collagen (1.530±0.128,  $P=0.008$ ). MinTBP-1 at the same concentration could only promote the attachment (1.13±0.04 folds,  $P=0.020$ ) and proliferation (1.15±0.06 folds,  $P=0.021$ ), while PRGDN had no significant influence ( $P>0.05$ ).

• **CONCLUSION:** Our data show that the novel chimeric

peptide minTBP-1-PRGDN could promote the attachment, proliferation and type I collagen synthesis of human keratocytes on the surface of titanium.

• **KEYWORDS:** minTBP-1-PRGDN; titanium; cell attachment; proliferation; type I collagen

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## INTRODUCTION

Corneal blindness is a major and widespread eye problem, with a population of approximately 10 million worldwide. The current treatment of corneal blindness is corneal transplantation. Although the success rate of corneal transplantation is higher than that of other tissue transplants, certain issues such as tissue availability, financial cost, risk of infection, potential complications, recurrent corneal graft rejection and cases of chemical burn can limit the use of keratoplasty<sup>[1,2]</sup>. Therefore, artificial cornea or keratoprosthesis is a desirable option for alternation<sup>[1,2]</sup>. An artificial cornea generally consists of a transparent optical central part and a biocompatible peripheral part that should allow the cellular integration from the human cornea. Titanium (Ti) has been used as peripheral part of keratoprosthesis owing to its good biocompatibility, resistance to corrosion and low allergenicity. However, due to the lack of thoroughly biointegration between Ti and human cornea, the major complication of this kind of keratoprosthesis is extrusion because of the melting of the human cornea around the prosthesis<sup>[3,4]</sup>.

Strategies have been developed to enhance the biocompatibility of Ti implant. It has been reported that synthetic surfaces coated with certain peptides such as laminin and fibronectin prompted cellular attachment<sup>[5-7]</sup>. The Arg- Gly- Asp (RGD) sequence is by far the most effective and most common employed peptide sequence for stimulating cell adhesion on biomaterials surface<sup>[8-10]</sup>. Stable linking of peptide to biomaterial is essential to promote strong cell adhesion because the formation of focal adhesions

only occurs when the ligands successfully withstand the cell contractile forces<sup>[11]</sup>. RGD peptides are covalently attached to the surface of biomaterials *via* functional groups like hydroxyl, amino, or carboxyl radicals.

Recently, peptide aptamers (*i.e.* binders) that interact with inorganic materials have been artificially created and used as a "glue" to link the surface of inorganic metals to various bimolecular stuffs<sup>[12]</sup>. A new peptide aptamer, named TBP-1 (RKLDPAPGMHTW) that explored by a linear 12-per peptide displaying phage library, has been proved to have the characteristic of special interacting with the surface of Ti. Its N-terminal, RKLPGA (minTBP-1), is sufficient for Ti binding<sup>[12-14]</sup>. We have recently synthesized a novel chimeric peptide, namely minTBP-1-PRGDN not only show the affinity to Ti surface but also facilitate the adhesion of MC3T3-E1 cells<sup>[15]</sup>.

In this study, we further explored the influence of minTBP-1-PRGDN on the attachment of human keratocytes to the surface of Ti and the proliferation as well as type I collagen synthesis activity of the keratocytes were also tested.

## MATERIALS AND METHODS

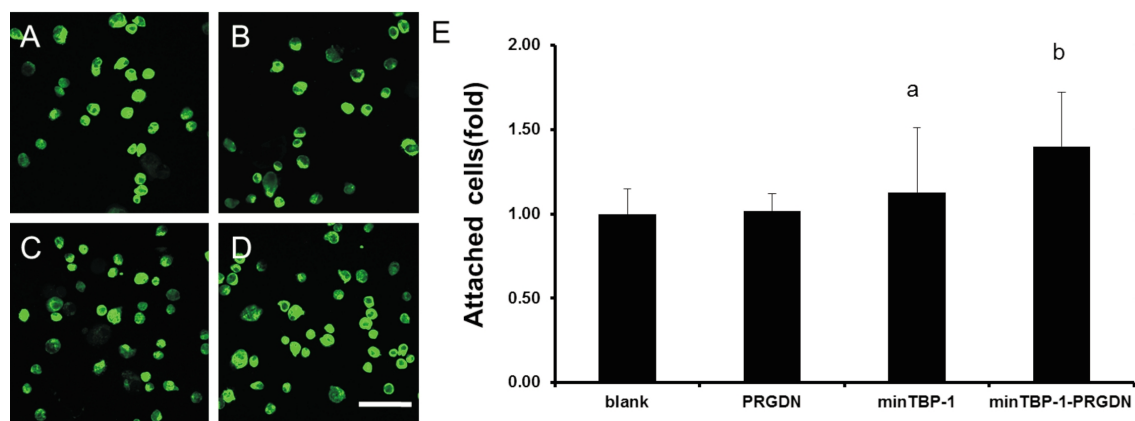
**Preparation of Peptides Coated Ti Disks** As described previously, Ti disks with 15-mm diameter and 1-mm thickness (Baoji Nonferrous Metal Industry Co., China) were wet-polished using 400-, 800- and 1200-grits silicon carbide paper sequentially and then cleaned with ultrasound successively in pure water, acetone, and 70% ethanol for 10min each<sup>[15]</sup>. The chimeric peptide RKLDPAPRGDN (minTBP-1-PRGDN, 1238.38g/mol) was synthesized by connecting RKLPGA (minTBP-1) to the N-terminal of PRGDN deriving from the consecutive sequence of human BSP (AAA60549.1, 285-289 residues). The other two peptides, minTBP-1 RKLPGA (698.83g/mol) and PRGDN (557.57g/mol) were also synthesized by China Peptides Co., Ltd. All peptides were synthesized using the solid phase peptide synthesis (SPPS) method, purified by high pressure liquid chromatography (HPLC) and identified by amino acid analysis. The lyophilized peptides were reconstituted in deionized water to achieve final peptide concentrations. Ti disks were just placed in the bottom of 24-well culture plate, with the same diameter as Ti disk to prevent the non-specific binding of cells to the bottom of culture plate. The disks were then coated overnight at 4°C with minTBP-1-PRGDN, minTBP-1 and PRGDN at a dosage of 100µg/mL respectively. The group of disks immersed in deionized water was used as blank control.

**Human Keratocytes Cell Culture** Human corneas from donors aged 20-65 years were obtained from the Red Cross Eye Bank of Wuhan (Wuhan, China) and managed in accordance with the Declaration of Helsinki and approved by the ethics committee of Tongji Medical College, Huazhong

University of Science and Technology. The time between death and enucleation was less than 6h, the corneas were cut down immediately after delivered to the Eyebank and preserved in Optisol medium before used for transplantation. Human keratocytes were isolated and cultured from the residual corneal tissue after transplantation as reported previously<sup>[16]</sup>. In brief, after the corneal epithelium was scrapped off using a cell scraper and the Descemet's membrane was peeled off, the remaining corneal stroma was cut into pieces of 0.5mm×0.5mm and digested with 1mg/mL collagenase in Dulbecco's Modified Eagle Medium (DMEM) at 37°C for 12h in a CO<sub>2</sub> incubator. Cells were then collected by centrifugation at 1 000g for 5min and cultured for 7d in 6-well plastic plates in DMEM containing 20mmol/L N-2-Hydroxyethylpiperazine-N-2-Ethane Sulfonic Acid (HEPES) buffer, 50µg/mL gentamicin, 1.25µg/mL amphotericin B and 10% fetal bovine serum (FBS).

**Cell Attachment to Ti Surface** Ti disks were pre-coated with minTBP-1-PRGDN, minTBP-1 or PRGDN peptides, and disks immersed in deionized water were used as blank control. Human cornea keratocytes were detached from tissue culture flasks as described above and reseeded onto Ti disks at a density of 2×10<sup>4</sup>/cm<sup>2</sup> substrate surface area in non-FBS culture medium. After further incubation for 1h, cells were rinsed three times with Phosphate buffered saline (PBS) in order to remove non-adhesive cells. The adhesive cells were stained with phalloidin- (fluorescein isothiocyanate) FITC (Sigma, P5282) as following procedures. Cells were fixed for 5min in 3.7% formaldehyde/PBS solution and then washed extensively in PBS. After being permeabilized by incubation in PBS containing 0.2% Triton X-100 for 5min, cells were washed again in PBS and stained with 1µg/mL phalloidin-FITC/PBS solution for 60min at room temperature. Then, disks were washed several times with PBS to remove unbound phalloidin conjugate and cell photos were taken with fluorescence microscope (OLYMPUS DP70, OLYMPUS BX 51TRF). Colorimetry using alamar Blue (Biosource International, Camarillo, USA) was adopted for the quantification of adhesive cells following manufacturer's instructions<sup>[14]</sup>. Adhesive cells were incubated in serum-free culture medium containing 10% fresh alamar Blue for another 5h. Cell numbers were measured as the differences in absorbance between 570nm and 600nm by an enzyme-linked immunosorbent assay (ELISA) plate reader (UQUA38).

**Keratocytes Proliferation on Ti Surface** For the cell proliferation assay, 5×10<sup>3</sup> keratocytes were seeded on each Ti disk coated with different kinds of peptide, *i.e.* minTBP-1-PRGDN, minTBP-1 or PRGDN peptides, and disks immersed in deionized water were used as control. The cell culture medium was changed every 3d. Samples were harvested 7d later, washed with PBS and incubated with 5g/L



**Figure 1 Promotion of minTBP-1-PRGDN on attachment of keratocytes to titanium surface.** The attached keratocytes were stained with FITC-labeled phalloidin and viewed under fluorescence microscope. The colorimetric methods using alamar Blue (Biosource International, Camarillo, USA) was adopted in the quantification of adhesive cells. The density of keratocytes on Ti disks were relatively low in blank control group (A), that in the PRGDN (B) was higher than the control without significant differences (E). The density of keratocytes in the minTBP-1 (C) and minTBP-1-PRGDN (D) groups were significant higher than that in the control (<sup>a</sup> $P < 0.05$ , <sup>b</sup> $P < 0.01$  vs blank control, all experiments  $n = 5$ ). Scale bar = 50  $\mu\text{m}$ .

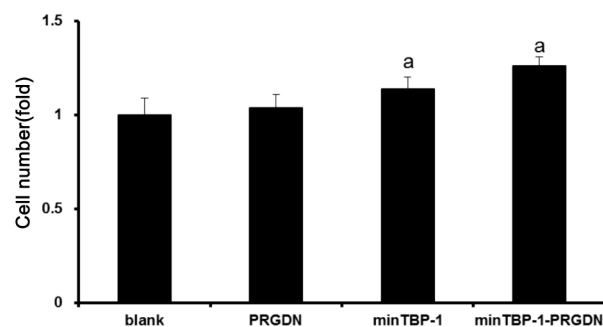
3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT; Sigma-Aldrich) in full culture medium. After 4h of incubation, the medium was removed and washed with PBS for three times. After the addition of 1 000  $\mu\text{L}$  Dimethyl Sulfoxide to each well, it was transferred into a new 96-well plate, and then the optical density was read in a microplate reader (Anthos 2010; Biochrom, Cambridge, UK) at 570nm.

**Type I Collagen Synthesis Assay** After 7d of culture on Ti disk coated with different kinds of peptides, levels of type I collagen secreted from keratocytes were determined using a sandwich ELISA kit (Sigma-Aldrich, St Louis, MO, USA) according to the manufacturer's instructions. Supernatants were collected and quickly read at 450nm. The concentration of type I collagen in the supernatant was quantified through a standard curve with rat recombinant type I collagen as the standard.

**Statistical Analysis** All experiments were performed at least in triplicate. Data sets were expressed as mean  $\pm$  SD and tested with Student's  $t$  test between two sets of experiments and one-way ANOVA in more than two sets of experiments. A  $P$ -value of less than 0.05 was considered statistically significant.

## RESULTS

**MinTBP-1-PRGDN Promoted the Attachment of Keratocytes to Ti Surface** Previously, our data showed that 100ng/mL was the most effective concentration for minTBP-1-PRGDN, minTBP-1 and PRGDN peptides binding to Ti disks, and was most effective in promoting the attachment of MC3T3-E1 cells to Ti surface [15]. In this experiment, the human keratocytes were seeded onto the disks in three groups and allowed to adhere for 1h, the cells binding on Ti disks with different pre-coatings were indicated by the absorbance of alamar Blue corresponding to cell



**Figure 2 Proliferation of keratocytes on Ti surface** To determine if minTBP-1-PRGDN could induce the proliferation of human keratocytes, cell numbers were assessed utilizing the MTT assay and compared to the blank control, which was set as 1. Compared to the control group, both 100ng/mL minTBP-1-PRGDN recombinant protein and minTBP-1 groups induced significant increase in the proliferation rate of keratocytes on the Ti surface over a 7-day period (<sup>a</sup> $P < 0.05$ ).

numbers. As shown in Figure 1, the density of keratocytes attached to the surface of Ti increased in all Ti disks coated with the peptide when compared to blank control (Figure 1 A-D), although quantified data analysis indicated statistical differences only existed among the minTBP-1 ( $1.13 \pm 0.04$  folds,  $P = 0.020$ ), minTBP-1-PRGDN ( $1.40 \pm 0.03$  folds,  $P = 0.003$ ) groups and the control (Figure 1E).

**MinTBP-1-PRGDN Stimulated the Proliferation of Keratocytes on Ti Surface** To determine if minTBP-1-PRGDN could induce the proliferation of human keratocytes, cell numbers were assessed utilizing the MTT assay and compared to the blank control. Figure 2 clearly showed both 100ng/mL minTBP-1-PRGDN ( $1.26 \pm 0.05$  folds) recombinant protein and minTBP-1 ( $1.15 \pm 0.06$  folds) groups induced dramatic increase in the proliferation rate of keratocytes on the Ti surface over a 7-d period when compared to the control group ( $P = 0.014, 0.021$ , respectively).

**MinTBP-1-PRGDN Promoted the Synthesis of Type I Collagen of Keratocytes** To determine if minTBP-1-PRGDN could induce type I collagen synthesis, the expression of type I collagen was analyzed with ELISA after incubation of keratocytes on each Ti disk coated with different kinds of peptides, *i.e.* minTBP-1-PRGDN, minTBP-1 or PRGDN peptides for 7d. The synthesis of type I collagen was significant higher in the MinTBP-1-PRGDN group ( $1.530\pm 0.128$ ) than that in the control group ( $1.003\pm 0.045$ ) (Figure 3) ( $P=0.008$ ), while the relative concentration of type I collagen in either minTBP-1 ( $1.070\pm 0.082$ ) or PRGDN ( $1.020\pm 0.040$ ) group was not higher than the control group. These results indicated that minTBP-1-PRGDN promoted the synthesis of type I collagen from keratocytes cultured on the surface of Ti.

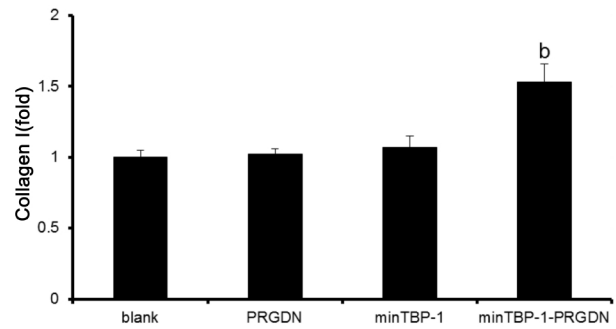
## DISCUSSION

The most critical complication for keratoprosthesis surgery is poor bioincompatibility, followed by extrusion, glaucoma and endophthalmitis<sup>[1-4]</sup>. Improvements on the design and materials of keratoprosthesis are therefore necessary to improve tissue biocompatibility and prevent extrusion. A biocompatible artificial cornea showing suitable tissue biointegration and adequate optical as well as mechanical properties could be a substitute for damaged corneas.

In the present study, experiments on cell attachment and proliferation confirmed the considerable adhesion and expansion of human keratocytes onto the Ti surface. The attachment of these cells, as well as the rate of cell proliferation and type I collagen synthesis, could be used as a criteria to evaluate the biointegration ability of the corneal implant.

MinTBP-1, isolated from a peptide phage display system, is a hexapeptide (RKLPDA) of the N-terminal of the 12-amino peptide TBP-1 (Arg- Lys- Leu- Pro- Asp- Ala- Pro- Gly- Met- His- Thr- Trp)<sup>[12]</sup>. In previous studies, minTBP-1 was affixed to the N-terminal end of ferritin or BMP-2, which endowed it specific binding ability to Ti<sup>[17,18]</sup>. To promote the binding of RGD motif onto Ti surface and thereby improve the cell attachment to biomaterials, we designed and synthesized an artificial peptide, minTBP-1-PRGDN through connecting RKLPDA (minTBP-1) to the N-terminal end of PRGDN. Our previous study showed that the novel peptide had double function of binding Ti surface and recognizing cells like MC3T3-E1 cells<sup>[15]</sup>.

The attachment of keratocytes to Ti implants is the first step for biointegration between human cornea and Ti keratoprosthesis. RGD which can be found in most extracellular matrix (ECM) including certain mineralized-related protein is the most widely used peptide mediating cell attachment to biomaterials<sup>[19]</sup>. It has been widely used to modify the surface of various biomaterials, such as Ti and hydroxyapatite. Adding residues to the N-terminal end of the



**Figure 3 Synthesis of type I collagen of keratocytes on Ti surface** The expression of type I collagen was analyzed with ELISA and compared among different groups after incubation of seven days. The synthesis of type I collagen was significantly higher in the minTBP-1-PRGDN group than that of the control group ( $P < 0.01$ ), while the relative concentration of type I collagen in either minTBP-1 or PRGDN groups was not higher than that of the control group.

RGD will not change the RGD function of mediating the cell attachment to materials<sup>[20]</sup>. Therefore, some chimeric peptides in the form of a combination were synthesized from two motifs for some specific aims<sup>[21]</sup>. In this study, minTBP-1-PRGDN was designed through adding minTBP-1 to N-terminal end of the PRGDN and the effect of peptide coating on cell behavior was investigated. As expected, minTBP-1-PRGDN improved cell attachment to Ti surface. Meanwhile, since the absorbance of minTBP-1 on the surface of Ti did not rely on nonspecific hydrophobic interactions, which generally led to the destruction of most protein structure, it suggested that the binding of peptide to minTBP-1 could reserve its original function without destroying spatial structure<sup>[14]</sup>. Although the RGD did not improve the attachment of keratocytes onto the Ti surface, several previous studies did find that the RGD loading Ti surface improved the osseointegration<sup>[11,20]</sup>. Type I collagen is the most abundant type of collagen in the human cornea, making it crucial indicator for the function of keratocytes during the healing of the cornea after damage<sup>[22]</sup>. Among the three groups, minTBP-1-PRGDN was the most effective to promote keratocytes attachment, proliferation and type I collagen synthesis, indicating that minTBP-1-PRGDN had successfully endowed Ti with the capacity of promoting the biointegration between keratocytes and Ti. While the issue of biocompatibility is clearly important, increasing cellular migration over the Ti back plate of the prosthesis as well as collagen stimulation may have adverse effects on the formation of retroprosthetic membrane, thus it should be important to treat the Ti frame but not the optical part of the keratoprosthesis in application.

In conclusion, our data shows that the novel chimeric peptide minTBP-1-PRGDN could not only enhance the attachment of human keratocytes to the surface of Ti, but also promote the proliferation and type I collagen synthesis during such

process. Further investigation on the effectiveness of minTBP-1-PRGDN on improving the biointegration between Ti and human cornea is promising to reduce the complication of keratoprosthesis surgery in the future.

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