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Research

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Enhancement of osteogenic induction by LL37 modified with collagen binding domain in vitro and in vivo

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Abstract:

Bone defect diseases, particularly induced by inflammation, render a challenge for designing ideal drug-loading scaffold that could facilitate bone repairing and eliminate inflammatory pathogens. LL37 is considered as promising alternative loading drug due to its broad-spectrum antimicrobial effect and various bio-functions including osteogenic induction. In this study, we synthesized modified LL37 by adding collagen binding domain (CBD), which aim to provide a specific binding onto collagen and slow-releasing pattern. The modified peptide was proved to exhibit similar biological activities to nature LL37 on rat BMSCs including promoting migration activity, anti-inflammatory activity and osteogenic induction *in vitro*. Ectopic bone formation experiment further confirmed the angiogenesis and osteoinduction activities *in vivo*. Collectively, LL37-CBD may be a potential loaded drug for preventative and curative applications in Inflammation-induced bone diseases, exerting dual strategies including anti-inflammatory and osteogenic effects.

Keywords: LL37, collagen binding domain, osteoinduction

Background

Inflammation-induced bone diseases such as periodontitis and rheumatoid arthritis are characterized by severe bone loss [1, 2]. Restoration of these inflammatory bone defects is a major clinical and economic concern. Besides the exciting progress in the development of bone substitutes, the loading drugs which aim to enhance the bioactivity of substitutes or accelerate the regeneration are attracting increasing interest. Antimicrobial peptides (AMPs) are a class of functional molecules of the innate immune system with various bioactivities. Among these, several members were found and one of which has attracted increasing attention named LL37. It is the only human member of the cathelicidin family, formed from the last 37 amino acid residues of the C-terminus of human cationic antimicrobial peptide 18 (hCAP-18) [3]. LL37 is widely expressed in several bodily fluids and tissues such as epithelium and skin [4]. Along with its potent and broad-spectrum antimicrobial activity against both Gram-positive and Gram-negative bacteria, LL37 also exhibits multiple bio-functions including LPS-neutralizing effect and cell migration promotion, cytokine production, wound healing [3]. In recent years, LL37 has been found to accelerate bone formation via regulating angiogenesis, recruiting stem cells [5] and even promote osteogenic differentiation of bone mesenchymal stem cells (BMSCs) [6]. Moreover, it performed a significant inhibition of osteoclastic bone resorption in the LPS-stimulated calvarial osteolysis model [6]. These multiple biofunctions owned by LL37 indicate promising potential applications

including bone tissue regeneration especially combined with infection.

While the clinical translation of LL37 is opposed by its susceptibility to protease degradation, potential toxicity, poor bioavailability and other limitations [7-9]. Thus, lots of works have been focused on overcoming the above limitations including the peptide modification or combination with suitable delivery systems. Typically, Poly (lactic-co-glycolic acid) (PLGA) nanoparticles loading LL37 would significantly promote wound healing compared to the individual administration [10]. In addition, LL37-nanopore titania exhibited the enhancement on antibacterial ability and bone-formation [11]. However, these vehicles displayed a comparatively poor binding pattern. It often happens that the loading systems exhibit initial drug-releasing burst which may lead to the premature loss or unwanted side-effects topically since the binding is mainly depend on interstitial capture or electrostatic attraction [12-14].

Given the facts that collagen is a component of the extracellular matrix (ECM) and one of the most extensively used as bone scaffolds due to its excellent properties. Several previous studies have described the collagen-tethering of biomolecules or antimicrobial drugs via various short sequences named Collagen Binding Domain (CBD) in order to achieve specific binding and slow releasing manner [15-17]. Visser et al [16] produced a modified rhBMP-2 with an additional CBD and showed not only that the rhBMP2–CBD had an increased affinity to collagen, but also it maintained its osteoinductive activity. Furthermore, the idea has been proposed that CBD-mediated delivery of LL37

onto collagen scaffolds [17]. While the potential applications of this system for bone tissue engineering was not investigated. Accordingly, we aim to introduce LL37 modified with CBD (LL37-CBD) as loading drug onto collagen scaffold and explore its bone regenerating effect. It is expected that this composite substitute could exert a more durable and prominent promotion on bone regenerating.

Results:

1. Synthesis of LL37-CBD and preparation of the collagen scaffold

The modified peptides were functionalized on its C-terminus with the collagen binding domain selected from human vWF and synthesized at 95.22% purity confirmed by HPLC result. Mass Spectrometry revealed that the relative molecular mass of LL37-CBD was 6665.64 which mainly accorded with the estimated calculation. Pure collagen scaffold was prepared as a round disk with 8 mm diameter and 2 mm thickness (Fig. 1).

Fig. 1 Prepared collagen scaffold. Pure collagen scaffold was prepared as a round disk(A). SEM images of morphology of collagen scaffolds(B). Scale bar=100 μ m

2. CBD sequences altered secondary structures of LL37 peptides

The amphiphilic secondary structures were investigated via CD spectroscopy, as figure shown (Fig. 2). In pure water, the spectra of LL37-CBD and LL37 nearly overlapped, indicating similar secondary structures in aqueous solution for these two peptides. While in 50% TFE solution, both two spectra performed relatively steeper peaks compared to the spectra in water at 208 and 222 nm (red arrow), which means more significant alpha helices would be formed in this membrane-like environment. The content of specific secondary structures (helix, beta strand, turn, random) were identified based on CD spectral data. In aqueous solution, there are slight differences between LL37 and LL37-CBD on the content of secondary structures. The contents of helices were significantly increased in 50% TFE. Similar contents could be calculated on helix and turn structure, while differences can be seen on the contents of beta and random structure in 50% TFE between LL37 and LL37-CBD.

Fig. 2 Circular Dichroism (CD) analysis of LL37 and LL37-CBD in pure water and 50% TFE. Representative CD spectra for 40 μ M LL37 and LL37-CBD (①: LL37 in 50% TFE, ②: LL37-CBD in 50% TFE, ③: LL37-CBD in water, ④: LL37 in water) (A). Based on the CD spectra results, the contents of secondary structures including helices, beta, turn and random were calculated(B).

3.Modification with CBD increases the adsorbing amount of LL37 and improves retention on collagen.

The collagen-binding abilities of LL37-CBD and unmodified LL37 were evaluated by ELISA as described above. With the modification of CBD on its terminus, we hypothesized that modified LL37 would be more specifically adsorbed on collagen and cause a slower release process over time. As shown in Fig. 3A, after the 2h incubation, a significantly greater amount of LL37-CBD had been adsorbed on the collagen compared to unmodified LL37.

Subsequently, we measured the cumulative releasing curves for both peptides (Fig. 3B). In general, the total cumulative release of LL37-CBD and LL37 was 10.8% and 38.4%, respectively, after 2 weeks. Interestingly, the releasing amounts in the initial 12h were the most significant for both groups. Except for this initial rapid release profile, the overall releasing curve was almost horizontal. Also, greater amounts of LL37 were released between each time interval compared to LL37-CBD ($p<0.05$).

Fig. 3. In vitro collagen-binding abilities of LL37-CBD on collagen. The loading amount of LL37-CBD on per milligram collagen was 0.91 ± 0.09 nmol and that is 0.38 ± 0.06 nmol for LL37 (A); Total cumulative release of LL37-CBD and LL37 after 2 weeks was 10.8% and 38.4%, respectively (B) (* $p<0.05$).

4. LL37-CBD exhibits less cytotoxicity and similar migrated activity

We presumed that the modification with CBD may not cause excessive change of its biological activity. Thus, we firstly used CCK-8 assay to evaluated the

influence of LL37-CBD on the cell viability (Fig. 4). As expected, the LL37-CBD exhibits similar proliferation on BMSCs at the concentrations below 10 μ M. Interestingly, at the concentration of 14 μ M, LL37-CBD displayed less cytotoxicity ($p<0.05$). Also, compared to the control group, a significant greater amount of cell on 6 μ M treating LL37-CBD group can migrate into the lower well of transwell plate (Fig. 5A, B, C). And quantitatively, there was statistic difference between the control group and the other two groups($p<0.01$) (Fig. 5D).

Fig. 4 Cell viability of BMSCs treated with three different concentrations and times of peptides. CCK-8 assay demonstrated that LL37-CBD and LL37 both

similarly stimulated BMSCs proliferation at the low concentration. LL37-CBD displayed less cytotoxicity at 14 μ M concentration(* p <0.05).

Fig. 5 LL37-CBD promotes BMSCs migration. The cells in response to different treatments went through a Transwell membrane and were stained subsequently. Representative fields from each group were shown(50 $\times$, bar=250 μ m)(A:LL37-CBD; B:LL37; C:Control).The calculated fold of migrated cells in five random microscopy fields (ration to the control) is shown (D) (* p <0.05).

5. Inhibition of LPS inducing cytokines expression

BMSCs were stimulated with LPS (1 μ g/ml) with or without the peptides (1 or 5 μ M) and the change of cytokines were determined by ELISA kit. Comparing

with the control group, quantitative results showed that 1 μ M LL37-CBD or LL37 can partially inhibit cytokines expression induced by LPS, while 5 μ M of that caused a marked decrease in the TNF- α and IL-1 expression, which almost reach the control level (Fig. 6). The results correlate with the potency of the peptides even modified with CBD to inhibit the binding of LPS to BMSCs.

Fig. 6 LPS-binding activity of LL37-CBD and LL37. The cytokines production were represented as ratio to the control without any additive. Results showed that 5 μ M LL37-CBD and LL37 can almost entirely inhibit the TNF- α (left) and IL-1 (right) expression induced by 1 μ g/ml LPS (* p <0.05).

6. LL37-CBD shows similar ability to promote BMSC osteogenic differentiation comparing with LL37 in vitro

LL37 showed enhancement in the osteogenic differentiation of BMSCs in our previous research. As shown in the Fig. 7, LL37-CBD and LL37 treatment group

showed stronger and more extensive alizarin red staining of calcium nodules than the control group. Osteogenic differentiation was further confirmed by the determination of alkaline phosphatase and osteogenesis related mRNA expression assayed by RT-PCR. The ALP expression was detected both in 7 and 14 days of induction, which both exhibited enhanced expression levels compared with the controls (Fig. 8). The relative expressions of bone formation-related genes were detected by RT-PCR. ALP, OCN and Runx2 were increasingly expressed in LL37-CBD and LL37 group in both 3 days and 7 days induction (Fig. 9). The western blot assay showed that the expression of bone-related proteins including Runx2 and COL1A were significantly higher both in LL37 and LL37-CBD treatment group (Fig. 10).

Fig. 7 Representative results of the Alizarin Red S staining in 14 days (A/a=control, B/b=3 μM LL37-CBD, C/c=3 μM LL37). Scale bar=200 μm.

Fig. 8. The quantitative determination of ALP expression with the co-culture of LL37-CBD. The LL37-CBD group significantly increased ALP activity compared with the controls. Data were presented as the mean $\pm$ S.D(* $p < 0.05$).

Fig. 9 mRNA expression by quantitative real time RT-PCR in 3 days and 7 days, respectively. The treatment of LL37-CBD or LL37 can cause an increasing expression of ALP, OCN and Runx2 comparing with control group

(* $p < 0.05$)

Fig. 10. Expression of osteogenic proteins in the control group, LL37 treatment group and LL37-CBD treatment group. Western Blotting images of three different treatment groups (A). Western Blotting analysis for the relative gray value (B). Three replicates were carried out for each group and the results were expressed as means $\pm$ standard deviation (Comparison between the treatment group and the control group, * $p < 0.05$).

7. Histological analysis

In the 4-weeks groups, the implants exhibited a fraction of preservation of raw materials while in the 8-weeks groups, the samples were almost completely degraded and absorbed. The infiltration of inflammation cells was evident. The effect on angiogenesis of LL37 can be verified since the experimental groups formed more new blood vessels than control group. And among the experimental groups, the groups added with LL37-CBD appeared to promote more evident angiogenesis (Fig. 11).

Furthermore, osteoinduction evidence was identified by immunohistochemical

staining. As a significant marker of osteogenic differentiation, the expression of OCN and Runx2 was detected. Despite that there was no obvious sign of new bone matrix formation, the results showed that positive staining for OCN and Runx2 were visible in the LL37 groups and most obvious in the LL37-CBD groups. And there was virtually no obvious positive staining in the control groups at 4 weeks. Also, the differences were further validated via quantitative analyses, which showed increasing expression in LL37-CBD groups while no significant difference existed between 0.4 and 0.9 nmol/mg LL37-CBD (Fig. 12).

Fig. 11 H&E staining of the implants from different groups on 4 weeks and 8 weeks. Significant bone matrix was unobservable in all groups. The implants added with LL37-CBD displayed more significant vascular scattered distribution (green arrows) than the one with LL37 or control groups. Scale bar=50 μ m.

Fig. 12 Immunohistochemical staining for OCN and Runx2. Representative staining of the implants from different groups (A). Scale bar=50 μ m. The quantitative analysis of the positive staining area indicates that more significant staining scores were present in the LL37-CBD groups (B) (* p <0.05).

Discussion

As expected, the CBDs provided specific binding onto I-type collagen, in accordance with other similar researches [16, 17]. Despite the desirable slow-release pattern as it shown, the releasing amount in the initial 12h was 64.3% and 32.8% of total 2-weeks cumulative release for LL37-CBD and LL37 group, respectively. It may be explained that the most of the initial releasing was extra detained peptides on the outer surface via interstitial capture. The differences between two groups were significant which may indicate the application potential for loading drugs for implanted scaffold. Instead of premature and excessive releasing, the loading peptides modified with CBDs can be released gradually into the surrounding extracellular matrix along with the gradual degradation of collagen.

It is crucial that the adding of CBD domain does not interfere with the original biofunction. A great number of studies on CBD-mediated collagen attaching including growth factors [18-20], bone morphogenetic protein (BMP) [16, 21] and streptavidin [22], most of which confirmed that the modified molecule can still remain its major activities even with CBD linking. While, for some macromolecules, the modification with CBD may cause unexpected changes or even the loss of their original biologic activity. The reasons were speculated that the auxiliary modification may interfere with the characteristic spatial configuration of macromolecules like epidermal growth factor (EGF) [23, 24]. From this perspective, the choose of small molecule compounds becomes

another option. As we know, the bioactivities of LL37 are highly influenced by structural characteristics. In our study, we found that LL37-CBD exhibited similar proliferated and chemotactic activity toward BMSCs as unmodified LL37 at the low concentration. While, LL37-CBD displayed less cytotoxicity at peptide concentration of 14 μ M. The most-widely accepted mechanism of AMPs for their cytotoxicity or antimicrobial action is permeabilization followed by membrane disruption [25, 26]. And this insertion into the bilayers depends on the random C-terminus [27]. From the CD results, in the 50% TEF solution, the modification of CBD sequence mainly decreased the random structure, which may account for the lower cytotoxicity at high concentration of LL37-CBD. On the other hand, lots of studies have tried to identify the essential region of LL37 that is responsible for the bioactivity or antimicrobial effect. Interestingly, it was found out that the N-terminal fragment is more likely to be involved in immunoregulation, chemotaxis, peptide oligomerisation and proteolytic resistance [28, 29]. Therefore, in this study, the CBD was added into the C-terminal tail, thus the chemotactic effect and proliferative effect were not altered significantly.

Several studies have found that LL37 shows a strong binding affinity and neutralizing capacity for lipopolysaccharide (LPS) through electrostatic and hydrophobic interaction [30-32], and that is physiologically relevant since LPS composes the external structure of membrane for most Gram-negative bacteria. Also, the common inflammatory mediators such as TNF- α , IL-1 β and RANKL

can be over-secreted induced by LPS [33], leading to most inflammatory conditions which may advance to inflammatory bone diseases or septic shock. It is the concave hydrophobic surface of LL-37, consisted of phenylalanine located at position 5, 6, 17 and 27, that directly recognize and bind to LPS [28, 34]. Moreover, as shown by the other research [35], the neutralizing between the LL37 and the LPS is not enough, it is also necessary to dissociate the LPS aggregates as well as competing with it for the CD14 receptor in the cell. Besides, it was speculated that LL37 may exert its neutralization via regulating the NF- κ B pathway induced by activation of LPS-binding Toll-like receptors [36]. Therefore, it is remarkable that the modification of CBD does not affect the bioactivity of the LL37, even the ligand-receptor binding capacity. LL37-CBD maintained the similar neutralizing capacity against LPS and 5 μ M of these peptides can almost inhibit the secretion of TNF- α and IL-1 β induced by 1 μ g/ml LPS, giving a potential application of LL37-CBD for the inflammation-related bone repairing conditions.

The P₂X₇ receptor (P₂X₇R) is a ligand-gated plasma membrane ion channel [37]. It was generally agreed that ATP was the only physiological agonist. While, over the last several years, increasing evidences suggest that ATP might not be the only agonist, especially at inflammatory sites [38]. P₂X₇R can be activated by various agonists including LL37[39-41] via poorly understood mechanisms of action. It has been confirmed that the osteoblastic differentiation of BMSCs was mainly mediated by the P₂X₇ receptor [42-45]. This was demonstrated by

several classic experiments, which used different kinds of receptor agonists of P_2X_7R to achieve osteoblastic differentiation [42, 44]. Based on these studies, our previous research [6] also found out that LL37 can promote the osteoblastic differentiation via the activation of P_2X_7R . The exact binding manner between LL37 and P_2X_7R remains unknown. It was assumed that the functional interaction between them involves transmembrane segment-mediated binding, interestingly, was irrespective of their chirality or helix sense [40]. As shown in our results, the LL37-CBD displayed similar osteogenic activity *in vitro*, indicating the CBD does not interfere the ligand-receptor binding capacity and osteogenic activity of LL37.

Previously, the *in vivo* osteoinductivity of LL37 has not been proven. With the positive results achieved from the *in vitro* experiments, the ectopic bone formation experiment was employed to further verify the osteoinductivity of LL37. It's logical that the initial vascularization formed within the implant can provide indispensable nutrients and maintain tissue viability. LL37 was found to assist the vascularization via recruit vascular endothelial precursor cells to the damaged area and promote cell proliferation. Furthermore, LL37 can directly induce angiogenesis mediated by formyl peptide receptor-like 1 expressed on endothelial cells [46]. The HE staining results showed that only the implants with LL37-CBD displayed significant sign of angiogenesis. While the angiogenic effects were impaired on unmodified LL37 groups due to the premature loss of peptides. Similarly, immunohistochemical staining showed that both the high

and low dosage of LL37-CBD caused a significant increase in OCN and Runx2 staining positive expression areas, which was consistent with the preceding results. Hence, the enhancement of osteoinduction of LL37-CBD loading collagen comparing to that with LL37 demonstrated the significance of specific retention of loading drugs, which can be controlled released accompanied by the gradual degradation of bone restorative materials. On the other hand, significant newly formed bone matrix was unobservable, which might result from the relatively fast degradation of the collagen scaffolds.

Conclusion

In this work, we have demonstrated that the addition of the CBD achieved a more durable retention on collagen and did not modify the native LL37's biological activities including osteogenic induction *in vitro*. Furthermore, ectopic bone formation experiment confirmed the osteoinduction performance of LL37-CBD loading collagen scaffold. The versatile biological properties of LL37-CBD produced the idea that apply the modified LL37 for bone tissue engineering especially inflammatory bone defects.

Materials and Methods

LL37-CBD synthesis

A conserved sequence derived from human von Willebrand factor (vWF) was selected (WREPSFCALS) [18] and the modified antimicrobial peptide LL37-

CBD was synthesized from Bioyeargene Biotechnology Inc (Wuhan, China) using Solid Phase Peptide Synthesis (SPPS) technique. A FLAGTM domain (DYKDDDDK) linker sequence was included to preserve flexibility [17, 47, 48]. High-performance liquid chromatography (HPLC) and Mass Spectrometry (MS) were used to confirm the peptide purity and relative molecular mass, respectively.

Circular Dichroism (CD) Spectroscopy

CD spectra were employed to analyze the secondary structures of peptides or proteins. Spectra were taken from 190-280 nm for solutions of 40 μ M LL37 or LL37-CBD in pure water and 50% vol/vol trifluoroethanol (50% TFE) solution, respectively, through the CD spectrometer (JASCO, Japan). The 50% TFE solution was used to simulate the hydrophobic environment of cell membrane. Then, the contents of secondary structure components were analyzed using DICHROWEB software.

Peptide loading and release

Type I collagen was extracted from rat tail tendons according to the method described in our previous study [49]. In brief, the tendons were dissolved in acetic acid and centrifuged. The supernatant was dialyzed for 5~7 days in dipotassium phosphate solution. Then the collected hydrogel was rinsed deionized water and lyophilized to form homogeneous paste, transferred to a

columniform mold with same dimensions to set (8 mm diameter, 2 mm thickness). The morphology of materials was examined using scanning electron microscopy (SEM, JSM6460, JEOL, Japan).

The solidified collagen scaffolds were immersed with peptides (LL37 or LL37-CBD) dissolved in sterile phosphate buffered saline (PBS, pH 7.4) and rotated on a Rotator (Heidolph Duomax 1030, German) for 2h. Subsequently, the collagen scaffolds were rinsed with PBS twice which was added into its corresponding immersed solution. LL37 ELISA Kit (CUSABIO Biotech, Wuhan, China) was used to measure peptide concentrations before the loading and after the rinsing, through that the peptide binding amount can be calculated. Then, the scaffolds with either LL37-CBD or LL37 were immersed in release medium (sterile PBS supplemented with 1% wt/v bovine serum albumin) and rotated on the rotator. At specific time points, release medium was collected and replaced with fresh medium. Peptide concentrations in the collected medium were measured using ELISA Kit, which can be compared with the initial absorbing amount of peptide to determine cumulative release.

Isolation and Culture of BMSCs

BMSCs were prepared as previously described [50]. Briefly, under sterile conditions, both the tibia and femur bones of 3-week-old Sprague–Dawley rats were isolated, with careful removal of the attached muscles thereafter. And then the marrow was flushed out by using Dulbecco's Modified Eagle's

Medium/Nutrient Mixture F-12 (DMEM/F-12, Gibco, CA, USA) containing 10% fetal bovine serum (FBS, Gibco, CA, USA), 2 mmol/L L-glutamine and 100 U/ml penicillin/streptomycin. The cell suspension was seeded and then cultured at 37°C with 5% CO₂. Nonadherent cells were removed by changing fresh medium after 24 h. Adherent cells were passaged with TrypLE. In this study, cells at passages 3~5 were employed.

Cell proliferation assay

To investigate the effects of LL37-CBD on the cellular proliferation of BMSCs. CCK-8 assay was performed using the Cell Counting Kit (CCK)-8 (Dojindo, Kumamoto, Japan) as per the manufacturer's instructions. First, different concentrations of LL37 or LL37-CBD was administrated to the culture medium of the BMSCs (5×10^4 cells/well) for 12, 24 and 48 h prior to adding the CCK-8 solution. Then, the optical absorbance (OD) of the CCK-8 reagent was measured at 450 nm with a microplate reader (Infinite 200, Tecan, Switzerland). BMSCs group alone was used as a control group.

Migration assay

Transwell plates (8 µm pore size; Costar, Corning, NY, USA) were used to perform the cell migration assay in accordance with the manufacturer's recommendations. Briefly, 100 µl serum-free suspension (5×10^5 cells/mL) of

BMSCs was seeded to each upper well. 6 μ M LL37-CBD, LL37 or equal amount of solvent was placed in the upper wells as well. Growth medium with 10% serum was added in the lower well. After incubation for 6 h at 37°C, the lower cells were fixed with 4% paraformaldehyde for 15 min and incubated with 0.1% crystal violet to stain the migrated cells. Then the migrated cells in microscopy fields were recorded using an inverted microscope (Axio Observer Z1, Zeiss, Oberkochen, Germany) after rinse with PBS 2~3 times. Whereafter, the cells in five random microscopy fields were counted using the inverted microscope.

Lipopolysaccharide (LPS)-neutralizing activity

BMSCs (5×10^4 cells) were cultured in 12-well plates. LPS (Invitrogen, Carlsbad, CA, USA) at 1 μ g/ml from *Porphyromonas gingivalis* with or without LL37-CBD or LL37 (1 μ M and 5 μ M) were added into each well and then cultured at 37°C. Untreated culture media were used as control group. At specific time point (12h, 24h, 48h), supernatants were collected and employed to measure the release of IL-1 and TNF- α by ELISA kit according to a protocol provided by the manufacture (R&D Systems). The experiment was performed in triplicate.

Alizarin Red staining

BMSCs at passages 3 were seeded to 6-well plate and 6 μ M LL37-CBD or LL37 was administrated in the osteogenic medium, followed by medium changes every three days. After 14 days' osteogenic induction, the cells were fixed with

4% paraformaldehyde for 15 min and incubated with Alizarin Red (Cyagen, USA) for 10 min. Excess stain was eliminated by three washes using PBS.

Alkaline phosphatase (ALP) activity determination

With osteogenic induction for 7 and 14 days, the BMSCs were treated with Triton for 30 min and the supernatants were collected. The alkaline phosphatase activity was tested according to the manufacturer's protocol (Jiancheng, Nanjing, China). The product was determined by evaluating the absorbance at 520 nm using a microplate reader.

Real-time PCR

Total RNA was extracted from the cells using Trizol reagent (Invitrogen, Carlsbad, CA, USA). The first-strand cDNA was reversely transcribed using 2 µg of total RNA and PrimeScript™ RT Master Mix (TaKaRa, Kusatsu, Japan) and then amplified with SYBR Premix Ex Taq II (TaKaRa, Kusatsu, Japan), which was carried out on a Light Cycler 480 (Roche, Basel, Switzerland). The specific oligonucleotide primers used are provided in Table 1. For The cycling profile for the real-time PCR (45 cycles) was as follows: 95°C for 10 min, 95°C for 15 sec, and 60°C for 60 sec. the relative expression of genes was normalized of that of GAPDH. Three independent samples in each group were analyzed and three parallel repeats were analyzed for each sample.

Table 1 Primers used for real-time polymerase chain reaction.

Gene	Forward primer sequence (5'–3')	Reverse primer sequence (5'–3')
ALP	TATGGCTCACCTGCTTCACGG	GCTGTCCATTGTGGGCTCTTG
Runx2	TTAGGGCGCATTCTCATCC	GTCAGAGGTGGCAGTGTCAT
OCN	GCCCTGACTGCATTCTGCCTCT	TCACCACCTTACTGCCCTCCTG
GAPDH	GCAAGTTCAACGGCACAG	GCCAGTAGACTCCACGACAT

Western blot analysis

After 14 days' osteogenic induction, the cells were washed with ice-cold PBS and lysed using RIPA buffer (Pierce, MA, USA) supplemented with protease and phosphatase inhibitors (Calbiochem, Darmstadt, Germany). Cell homogenates were obtained by gently scraping the cells from each well and centrifugated at 15000 g for 10min. The protein concentrations from the supernatant were determined by using the BCA Protein Assay Kit (ComWin Biotech, Beijing, China). Total protein samples were denatured by heating at 95°C for 10 min in 5x SDS-PAGE sample loading buffer (Beyotime, Shanghai, China) before loading. Thirty micrograms of the protein sample were separated by SDS-polyacrylamide gel electrophoresis at 110 V for 90 min and transferred to 0.45-μm polyvinylidene fluoride (PVDF) membranes at a constant current of 300 mA for 60 min. After blocked in 5% Bovine Serum Albumin (BSA) at room temperature, the membranes were incubated with rabbit anti-GAPDH antibody (1:1000) (Cell Signaling Technology, Boston, USA), mouse anti-COL1A (1:100),

mouse anti- Runx2 (1:200) (Santa Cruz Biotechnology, Dallas, USA) overnight at 4°C. The washed bands were incubated for 1 h with the corresponding secondary antibody (Santa Cruz Biotechnology, Dallas, USA). The ECL luminophore (ThermoFisher, Massachusetts, USA) was prepared under dark conditions and the bands were visualized using an enhanced chemiluminescence detection system (Amersham Biosciences, Piscataway, NJ, USA). The relative intensity of protein bands was quantified using ImageJ software (National Institutes of Health, MD, USA).

Establishment of ectopic subcutaneous models

Twenty-four male Sprague–Dawley rats (250~300 g in weight) were used. The rats were anesthetized by intraperitoneal injection of sodium pentobarbital (120 mg/kg). Hair around incisions was shaved and the operative regions were disinfected with povidone iodine. Subcutaneous implantation was performed through four longitudinal incisions near the midline of the dorsal skin (Fig. 13). Then subcutaneous pockets were prepared with a blunt instrument and received implants from four different groups (Group1-unloading collagen scaffold, Group2-0.4 nmol/mg LL37 loading scaffold, Group3-0.4 nmol/mg LL37-CBD loading scaffold, Group4-0.9 nmol/mg LL37-CBD loading scaffold). The wounds were closed with absorbable suture after implantation. The postoperative intraperitoneal injection of penicillin was applied to prevent infection (8×10^4 U/each). The animals were sacrificed after eight weeks. The

subcutaneous implant samples were separated and fixed in 4% paraformaldehyde for subsequent histological assays.

Fig. 13 The surgical procedure of ectopic subcutaneous models. A subcutaneous pocket was prepared with a blunt instrument (A). The implantation of collagen scaffold (B). The wound was stitched closed (C). Each rat received four incisions for different implantation (D).

Histological assays

After embedding the samples in paraffin, 5 μm -thick serial sections were prepared. Hematoxylin and eosin (H&E) staining were performed according to the instructions. Briefly, cell nuclei were stained by Mayer's hematoxylin and cell plasma and extracellular matrix were stained by eosin.

For immunohistochemical staining, osteogenic markers (Runx2 and OCN) were detected. Selected slides were dewaxed, rehydrated and endogenous peroxidase activity eliminated. Non-specific proteins were blocked with 3% BSA for 30 minutes. Then the slides were incubated with Runx2 antibodies (Affinity) and OCN antibodies (Servicebio) overnight at 4 °C. The secondary antibodies (HRP labeled) incubation was employed after removing the primary antibodies. The antibody complexes were visualized by Diaminobenzidine (DAB) chromogenic agent and the images were captured using a slice scanner (Aperio AT2, Leica). The quantification procedure was conducted by Aperio ImageScope software.

Statistical analysis

All data were analyzed using the SPSS software package and expressed as the mean $\pm$ standard deviation (SD) with three replications. The level of significance was determined by one-way analysis of variance (one-way ANOVA) followed by a Bonferroni post hoc test for multiple comparisons. A p value < 0.05 was considered statistically significant.

Declarations

Ethics approval and consent to participate

The in vivo experimental protocols were reviewed and approved by the Ethics Committee of Sun Yat-Sen University, China (Approval No. SYSU-IACUC-2019-000327).

Consent for publication

Not applicable

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests" in this section.

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Authors' contributions

XL and SC performed most experiment work and the writing of manuscript. JQ and QZ participated in animal experiments. ML and XM were major contributor in the collection and analysis of experimental data. SM and YZ committed in study design and final revision of this manuscript. All authors read and approved the final manuscript.

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Figures

Figure 1

Prepared collagen scaffold. Pure collagen scaffold was prepared as a round disk(A). SEM images of morphology of collagen scaffolds(B). Scale bar=100μm

Figure 2

Circular Dichroism (CD) analysis of LL37 and LL37-CBD in pure water and 50% TFE. Representative CD spectra for 40 μ M LL37 and LL37-CBD (▨: LL37 in 50% TFE, ▩: LL37-CBD in 50% TFE, ▤: LL37-CBD in water, ▥: LL37 in water) (A). Based on the CD spectra results, the contents of secondary structures including helices, beta, turn and random were calculated(B).

Figure 3

In vitro collagen-binding abilities of LL37-CBD on collagen. The loading amount of LL37-CBD on per milligram collagen was 0.91 ± 0.09 nmol and that is 0.38 ± 0.06 nmol for LL37 (A); Total cumulative release of LL37-CBD and LL37 after 2 weeks was 10.8% and 38.4%, respectively (B) (*p < 0.05).

Figure 4

Cell viability of BMSCs treated with three different concentrations and times of peptides. CCK-8 assay demonstrated that LL37-CBD and LL37 both similarly stimulated BMSCs proliferation at the low concentration. LL37-CBD displayed less cytotoxicity at 14 μM concentration(*p<0.05).

Figure 5

LL37-CBD promotes BMSCs migration. The cells in response to different treatments went through a Transwell membrane and were stained subsequently. Representative fields from each group were shown(50 $\times$, bar=250 μ m)(A:LL37-CBD; B:LL37; C:Control).The calculated fold of migrated cells in five random microscopy fields (ration to the control) is shown (D) (* p <0.05).

Figure 6

LPS-binding activity of LL37-CBD and LL37. The cytokines production were represented as ratio to the control without any additive. Results showed that 5 μM LL37-CBD and LL37 can almost entirely inhibit the TNF-α (left) and IL-1 (right) expression induced by 1 μg/ml LPS (*p<0.05).

Figure 7

Representative results of the Alizarin Red S staining in 14 days (A/a=control, B/b=3 μ M LL37-CBD, C/c=3 μ M LL37). Scale bar=200 μ m.

Figure 8

The quantitative determination of ALP expression with the co-culture of LL37-CBD. The LL37-CBD group significantly increased ALP activity compared with the controls. Data were presented as the mean $\pm$ S.D(* $p < 0.05$).

Figure 9

mRNA expression by quantitative real time RT-PCR in 3 days and 7 days, respectively. The treatment of LL37-CBD or LL37 can cause an increasing expression of ALP, OCN and Runx2 comparing with control group (* $p < 0.05$)

Figure 10

Expression of osteogenic proteins in the control group, LL37 treatment group and LL37-CBD treatment group. Western Blotting images of three different treatment groups (A). Western Blotting analysis for the relative gray value (B). Three replicates were carried out for each group and the results were expressed as means $\pm$ standard deviation (Comparison between the treatment group and the control group, $*p < 0.05$).

Figure 11

H&E staining of the implants from different groups on 4 weeks and 8 weeks. Significant bone matrix was unobservable in all groups. The implants added with LL37-CBD displayed more significant vascular scattered distribution (green arrows) than the one with LL37 or control groups. Scale bar=50 μ m.

Figure 12

Immunohistochemical staining for OCN and Runx2. Representative staining of the implants from different groups (A). Scale bar=50 μ m. The quantitative analysis of the positive staining area indicates that more significant staining scores were present in the LL37-CBD groups (B) (* p <0.05).

Figure 13

The surgical procedure of ectopic subcutaneous models. A subcutaneous pocket was prepared with a blunt instrument (A). The implantation of collagen scaffold (B). The wound was stitched closed (C). Each rat received four incisions for different implantation (D).