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Original Article

Adenovirus mediated gene delivery of α-calcitonin gene-related peptide facilitates osseointegration of implant in ovariectomized rat

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ABSTRACT

Background: Osteoporosis, which adversely affected osseointegration of dental implants, became prevalent with the entry of ageing era. Recent studies indicated that α-calcitonin gene-related peptide (α-CGRP) played a role in modulating osteoporosis. However, due to the plasma half-life of α-CGRP being estimated at merely 7–10 min, it was difficult to utilize a conventional method that administered sufficient α-CGRP to the implant site. This present study aimed to investigate the efficacy of an α-CGRP transgene in promoting implant osseointegration in ovariectomized (OVX) rats.

Method: The osteoporosis rat model was established through bilateral ovariectomy, following which the subjects were categorized into three distinct groups: the α-CGRP transgene group, the empty virus vector group, and the blank control group. Ad-CGRP-EGFP was locally administered into the bone defect site prior to implant placement in the OVX rats. 7 and 28 days after implantation, the femurs were isolated for molecular and histological analyses, micro-CT analysis and biomechanical test.

Result: Bone marrow stromal cells (BMSCs) transduced with Ad-CGRP-EGFP could continuously express α-CGRP more than 28 days in vitro. Successful transgene expression was confirmed through cryosectioning and Western blot analysis 7 days after implantation in vivo. The results indicated a substantial decrease in the quantity of TRAP + cells in the α-CGRP transgene group. Additionally, quantitative real-time RT-PCR and Western blot analysis revealed a significant elevation in the expression levels of Runx2 and ALP, coupled with a notable reduction in the expression of cathepsin K and RANKL *($P<0.05$)*. Moreover, the α-CGRP transgene group exhibited a significant enhancement in osteointegration and restoration of implant stability in OVX rats ($P<0.01$) 28 days after implantation.

Conclusion: This study implied a great therapeutic potential of α-CGRP in osseointegration of titanium implants in OVX-relative osteoporosis rats, offering valuable insights for guiding bone regeneration strategies under osteoporotic conditions.

1. Introduction

Osseointegration, or direct bone-to-implant contact (BIC) has been claimed to be the clinical condition allowing the functional loading of dental implants [1]. Despite the insertion procedure of implant has became standard and various strategies have been used to expedite bone-implant integration, osseointegration at the titanium implant surface still remain a challenge [2,3].

Osteoporosis impacts millions, particularly the elderly and postmenopausal women [4]. The local metabolic imbalance around implants in osteoporotic conditions can compromise the stability of the implant [5,6]. Compared to individuals without osteoporosis, those with the condition have been reported to experience higher rates of implant failure and significant marginal bone loss around the implants [7]. Numerous studies have been conducted to enhance the process of osseointegration, including implant surface modification and the topical or systemic administration of

Abbreviations: α-CGRP, α-calcitonin gene-related peptide; OVX, ovariectomized; BMSCs, Bone marrow stromal cells; BIC, bone-to-implant contact.

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substances [8,9], with the primary objective of promoting the recruitment and differentiation of osteoprogenitor cells, such as bone marrow stromal cells (BMSCs), or inhibiting the differentiation of osteoclasts.

Recent studies have indicated that α-calcitonin gene-related peptide (α-CGRP) is a crucial molecule with diverse biological functions in the regulation of bone homeostasis, vascular remodeling, and aging [10,11]. α-CGRP is expressed in both bone tissues and peripheral nerves innervating bone tissue [12]. In OVX-induced osteoporosis, the level of α-CGRP in the femur is decreased [13]. Correspondingly, antagonizing the α-CGRP receptor impedes bone formation and bone mass in diet-induced obesity. Mice deficient in α-CGRP exhibit decreased bone formation and osteopenia [14]. Those studies suggest that application of exogenous α-CGRP could have a positive effect in bone metabolism. However, due to the short half-life of proteins [15] and the fact that high doses often result in adverse side effects, conventional therapeutic methods find it challenging to deliver sufficient α-CGRP to implant sites. Previous studies of ours have indicated that genetically modified engineered bone tissue using an adenovirus transgene is a promising approach for repairing large bone defects or promoting osseointegration [16].

Considering the aforementioned evidence, in this study, we devise a gene therapy strategy to achieve the localized, continuous delivery of α-CGRP around the implant. Adenovirus-mediated α-CGRP was administered to the bone defect prior to the placement of titanium implant in the femur of OVX rats. We aim to investigate the efficacy of α-CGRP in enhancing osteointegration of titanium implant in OVX rat model. Meanwhile, the potential molecular mechanisms are assessed.

2. Materials and methods

2.1. Construction of replication defective adenovirus vector

The AdEasy Vector System was utilized for the construction of the Ad-EGFP adenoviral vector, which incorporated the EGFP reporter gene originating from pEGFP-C. The recombinant adenovirus transfer vector, encoding for α-CGRP, was produced through a series of sub-cloning procedures as previously delineated [17]. The Ad-EGFP served as a control for the empty virus vector. The adenovirus plaque forming units of the construct were 1×10^{10}/ml.

2.2. In vitro gene transduction detection

BMSCs were isolated and cultured in accordance with previously established protocols [18], and subsequently utilized to examine the bioactivity of the transgene. The cells were segregated into three distinct groups based on their treatment: the α-CGRP transgene group, the empty virus vector group, and the virus-free group. These cells were cultured at 37 °C in an atmosphere enriched with 5 % CO_2 for a duration of four weeks.

Given the co-expression of EGFP within the reconstructed vectors, the bioactivity of the vectors was assessed three days post-transduction using inverted fluorescence microscopy. Concurrently, real-time RT-PCR was executed to verify the expression of α-CGRP. The sequences of the primers employed for amplification are detailed in Table 1, with GAPDH serving as an internal control. To quantify the expression levels of α-CGRP over time, measurements were taken on days 1, 3, 5, 7, 14, 21, and 28 within the Ad-CGRP-EGFP group.

2.3. Implant surgery in the OVX rat model

A total of eighty female Sprague-Dawley rats, aged 12 weeks and weighing 260 ± 50 g, were utilized for the investigation. Following a week-long acclimatization period, the rats underwent either bilateral ovariectomy (OVX) (n = 70) or sham OVX (n = 10) surgical procedures. The methodology for these surgeries has been previously detailed [16,19]. Adherence to the animal use and care protocol was in accordance with the guidelines set forth by the Animal Care Committee of Shandong Provincial Hospital, affiliated with Shandong First Medical University. Cylindrical, threadless titanium implants, measuring 10 mm in length and 1 mm in diameter, were procured from the School of Materials Science and Engineering at Jinan University. These implants were not subjected to any surface treatments, such as porous coating or etching.

Upon the completion of six months, ten rats were randomly selected from the OVX group. Serum estradiol levels, HE staining, and Micro-CT examination were conducted to verify the presence of osteoporosis, in comparison with the sham group animals. The remaining OVX rats were subsequently divided into three distinct groups (n = 20) based on varying experimental treatments. Each rat underwent bilateral femur implantation. The implantation surgery was executed as previously outlined [2,20]. In brief, after induction of anesthesia via intraperitoneal injection of chloral hydrate, all rats were subjected to the creation of a 1 mm-diameter channel leading to the medullary canal through the intercondylar region of the distal femur metaphysis. These procedures were conducted with the aid of cooled sterile saline irrigation. Ad-CGRP-EGFP vectors at a concentration of 1×10^8 pfu were injected into the defects, while two other groups received either Ad-EGFP or sterilized PBS, respectively. The muscular and dermal layers were meticulously sutured, and antibiotics were administered for three consecutive days post-surgery.

The rats were euthanized at 7 and 28 days post-implantation. Concurrently, the femurs were extracted for cryo-sectioning, Western blot analysis, TRAP staining, real-time RT-PCR, histological evaluation, micro-CT analysis, and biomechanical testing.

Table 1
The primers used for RT-PCR analysis in this study.

Gene	Forward primer	Reverse primer
α-CGRP	AGATGAAAGCCAGGGAGCTG	AGGTCTTGTGTGTACGTGCC
Cathepsin K	CAGCTTCCCCAAGATGTGAT	TGGAGGACTCCAGCGTCTAT
RANKL	AACATGACGTTAAGCAACGGA	AACAGGGAAGGGTTGGACACAC
Runx2	CGCCTCACAAACAACCACAG	AATGACTCGGTTGGTCTCGG
ALP	CCTGGACCTCATCAGCATTT	AGGGAAGGGTCAGTCAGGTT
GAPDH	TCCATGACAACTTTGGTATCG	TGTAGCCAAATTCGTTGTCA

2.4. In vivo gene transduction assessment

Seven days subsequent to implantation, cryo-sectioning and Western blot analysis were executed to assess the expression of the α-CGRP transgene in vivo. In the cryo-sectioning process, the isolated femurs were subjected to fixation at 4 °C with continuous agitation for a duration of three days in a 4 % Paraformaldehyde solution. Subsequently, the specimens were embedded in an optimal cutting temperature compound, and horizontal sections measuring 10 μm were meticulously prepared using a model SP1600 microtome (Leica Microsystems, Wetzlar, Germany). All operations were conducted in darkness. Confocal laser scanning microscopy was employed to detect the activity of the reconstructed vector.

Western blot analysis was conducted in accordance with a previously established study protocol. The sampled femurs were pulverized and homogenized using liquid nitrogen. Proteins extracted from the crushed bones were lysed with the aid of a Nuclear and Cytoplasmic Protein Extraction Kit (Signalway Antibody, Shanghai, China). The proteins were then separated by means

of 10 % SDS-PAGE, transferred onto a nitrocellulose membrane. The membranes were subjected to incubation with a primary antibody, followed by incubation with horseradish peroxidase-conjugated secondary antibodies (both sourced from Cell Signalling Technology), and subsequently underwent chemiluminescence signal development. Following this, the membranes were stripped and re-probed with a mouse anti β-actin primary antibody.

2.5. Real-time RT-PCR and TRAP staining

Seven days after implantation, the femurs were isolated for TRAP staining and Real-time RT-PCR. In the process of TRAP staining, the femurs were separated and subsequently fixed in a 10 % neutral-buffered formalin solution for a duration of one week. The implants were meticulously excised following a decalcification period of 30 days in a 10 % EDTA solution at a temperature of 4 °C. Post-decalcification, the femurs underwent dehydration in an ascending ethanol series and were subsequently embedded in paraffin. Sections of tissue measuring 6 μm in thickness were prepared and affixed to glass slides. A commercial TRAP staining kit (sigma 389) was employed for the staining procedure. TRAP + cells in the peri-implant region were identified by the presence of dark purple granules within the cytoplasm indicative of TRAP activity. For the analysis of TRAP staining, 10 images were randomly captured from a single section. The overall percentage of TRAP activity is quantified as the ratio of the TRAP-positive stained area to the total area, expressed as a percentage, which correlates with osteoclast activity.

For the execution of real-time RT-PCR, total RNA was isolated from the frozen metaphyseal bone adjacent to the implant (extending 1 mm both mesially and distally from the implantation site), which had been rapidly frozen using liquid nitrogen and homogenized in Trizol (Invitrogen). The detailed methodology for real-time RT-PCR has been previously delineated. The sequences of the primers utilized for the amplification of rat α-CGRP, Runx2, ALP, Cathepsin K, and RANKL are enumerated in Table 1, with GAPDH serving as the reference gene.

2.6. Micro-CT scanning

Twenty eight days after implantation, the femurs were excised and subsequently fixed in a 10 % neutral-buffered formalin solution for the purpose of micro-CT scanning. The femurs, inclusive of the implant, were scanned in a direction perpendicular to the long axis employing a micro-computed tomography device (Scanco Medical, Bassersdorf, Switzerland), configured at 70 kV, 114 mA, and an integration time of 700 ms. In the three-dimensionally reconstructed images, the segmentation of bone tissue and implants was executed using a threshold procedure ($\rho = 1.2$, support = 1, with the threshold for bone set at 205 and that for the implant at 700). A region of interest (VOI) measuring 0.25 mm in area was selected around the implant. The following parameters were evaluated within the VOI: the ratio of bone volume to total volume, the mean trabecular thickness, the mean trabecular number, connectivity density, and the mean trabecular separation.

2.7. Histomorphometric analysis

Twenty-eight days post-implantation, tissue sections measuring 10 μm in thickness were prepared as previously described and subjected to aniline blue staining. In each group, ten images were captured using a Nikon Eclipse E600 microscope, and the area of newly formed bone within a 0.1 mm radius surrounding the implant was quantified using Image-Pro Plus 6.0 software. Bone contact to the whole implant was represented as the percentage of osteointegration (%OI).

2.8. Biomechanical test

Twenty-eight days post-implantation, the femurs with the implant were surgically extracted. A total of six femurs from each group were included in this analysis. Approximately 15 mm of the distal femur, embedded in a custom-designed mold with self-curing plastic, underwent compressive strength testing along the axis of the implant (Instron 5566; Instron, Norwood, MA, USA). The pull-out test was conducted at a compression rate of 1 mm/min, and the ultimate compressive stress and interfacial shear strength were recorded.

2.9. Statistical analysis

Data are expressed as means ± SD and statistical analyses were performed using GraphPad Prism 5. One-way analysis of variance and an unpaired student's t-test were employed to evaluate differences between the Ad-CGRP-EGFP group, the Ad-EGFP group, and the virus-free group. All tests were two-tailed, and values of $P<0.05$ were considered statistically significant.

3. Results

3.1. Related tests of adenovirus transduction in vitro

Three days after transduction, the green fluorescent signals emanating from the co-expressed EGFP were observed under fluorescence microscopy within the Ad-CGRP-EGFP group and the Ad-EGFP group, signifying successful in vitro transduction (Fig. 1-a). The outcomes of the RT-qPCR analysis revealed that the expression of α-CGRP in the Ad-CGRP-EGFP group was markedly elevated ($P<0.01$) (Fig. 1-b), and progressively ascended to peak levels, approximately attained by day 7. The over-expression persisted for over 28 days subsequent to a singular transduction event (Fig. 1-c).

3.2. Identification of successful Ad-CGRP-EGFP transduction in vivo

The generation of green fluorescent signals by EGFP serves as evidence of successful transduction. Fig. 2(a–c) illustrate the pronounced fluorescence surrounding the implant in both Ad-CGRP-EGFP and Ad-EGFP groups, seven days post-implantation. In contrast, the virus-free group exhibited only background autofluorescence. Additionally, Western blot analysis substantiated that α-CGRP expression was significantly elevated in the Ad-CGRP-EGFP transgene group at the local implantation site, as compared to the control groups ($P < 0.01$) (Fig. 2-g).

3.3. Expression of potent osteogenic transcription factors and osteoclastogenesis relative gene profile

Seven days after implantation, TRAP staining was executed to ascertain the overexpression of α-CGRP in relation to osteoclast differentiation at the bone-implant interface (Fig. 2d–f). Fig. 2h indicated a substantial diminishment in TRAP-active cells within the Ad-CGRP-EGFP group relative to the control groups ($P<0.01$). These results substantiate that α-CGRP transgene modification at the local implantation site impedes osteoclast differentiation.

Seven days post-implantation, Real-time RT-PCR and Western blot analysis were conducted to assess the specific gene expression profiles within the samples subsequent to α-CGRP transgene modification, with the results delineated in Fig. 3. It was observed that α-CGRP modification led to a significantly elevated expression

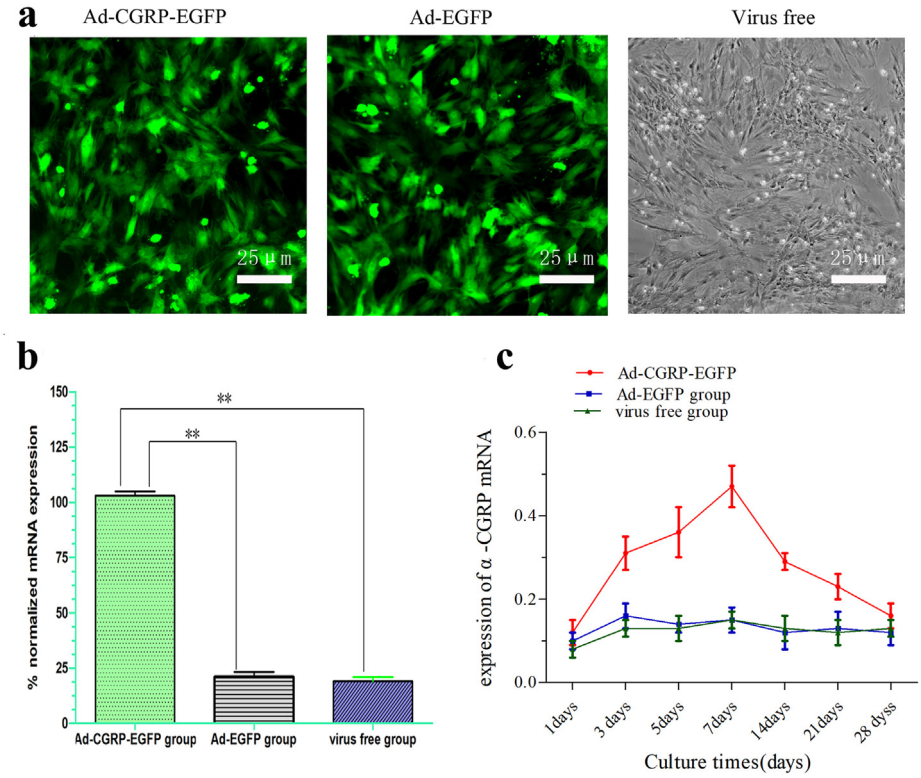

Fig. 1. (a)Representative images of green fluorescence under the inverted fluorescence microscopy. Original magnification × 400. (b) The results of RT-qPCR showed that the expression of α-CGRP in Ad-CGRP-EGFP group was significantly increased*(P<0.01)* (c) Depicts the trend of α-CGRP mRNA expression on days 1, 3, 5, 7, 14, 21, and 28. Data were expressed as mean ± SD, $^{**}P < 0.01$.

of Runx2, ALP in comparison to the control groups *(P<0.01)*. Notably, the expression of Runx2, which is pivotal for osteoblast differentiation and bone mineralization, exhibited a 150 % increase relative to the control groups. Intriguingly, we also discerned significantly reduced expressions of CPK and RANKL within the α-CGRP modification group *(P<0.05)*. These findings suggest that the overexpression of α-CGRP has the potential to enhance osteoblast differentiation and attenuate osteoclastogenesis.

3.4. Histological analysis of bone regeneration

Twenty eight days after implantation, the implants were effectively integrated with the host bone in the OVX group (Fig. 4a–c). Lamellar bone was observed encircling the implant. Histomorphometric analysis revealed that bone-to-implant contact (% OI) in the Ad-CGRPG-EGFP group experienced a statistically significant increase of 86.3 % in comparison to the other control groups *(P<0.01)*. Conversely, there was no statistically significant difference between the Ad-EGFP group and the virus-free group (Fig. 4d). The persistent overexpression of α-CGRP exerted a substantial influence on osseointegration at the implant surface.

Qualitative micro-CT transverse and longitudinal 3-D images provided additional insights into the osseointegration and trabecular microarchitecture in the vicinity of the implant (Fig. 5a and b). The findings indicated that the α-CGRP transgene group exhibited a greater bone volume and trabecular bone surrounding the implant in comparison to the control groups. It is plausible to deduce that the sustained overexpression of α-CGRP via gene therapy could represent a viable method to enhance the osteointegration of implants within an osteoporotic status. The results of the quantitative analysis have been summarized (Fig. 5c–g).

3.5. Biomechanical testing after transgene therapy

The push-out test was conducted to evaluate the biomechanical stability of implant after 28 days transgene modification treatment, with the results presented in Table 2. In OVX rats, Ad-CGRP-EGFP group demonstrated well-fixed mechanical stability that required more force to dissociate the implants compared with the control groups ($P < 0.01$). The maximal push-out force experienced an increase of 161.9 %, and the ultimate shear strength saw an enhancement of 234.3 %.

4. Discussion

Titanium implants have been extensively utilized as an efficacious remedy for edentulous conditions. The purpose of many dental and orthopedic implants is the complete and direct integration of the implant to bone, a process known as osseointegration [21]. Although the insertion procedure of dental implants has become standard and various strategies have been used to accelerate bone-implant integration, there are still many local and systemic conditions, including osteoporosis, that jeopardize the osseointegration. Consequently, we devise a gene therapy strategy to facilitate the continuous infusion of α-CGRP locally surrounding the implant and also evaluate its potential molecular mechanisms to accelerate osseointegration under osteoporotic conditions.

The present study introduces a novel method for the delivery of therapeutic proteins through adenovirus-mediated α-CGRP transduction, achieving persistent and localized expression. In vitro the expression of α-CGRP sustain for over 4 weeks after a single injection and elevate progressively with the peak level achieved approximately on day 7. In vivo, recombinant vectors were

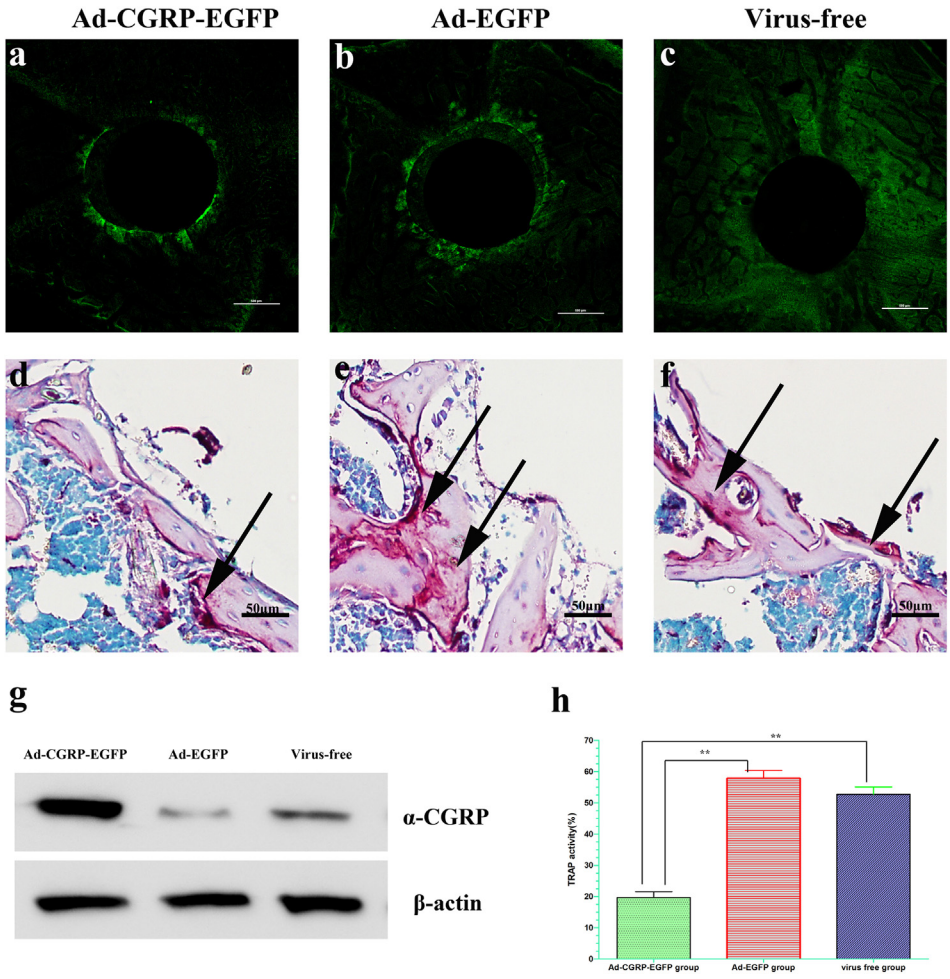

Fig. 2. (a–c) Depicts the pronounced fluorescence encircling the implant against the backdrop of bone tissue autofluorescence in both the Ad-CGRP-EGFP and Ad-EGFP groups. The vectors exhibited successful in vivo transduction. (d–f) Depicts representative images of TRAP staining around the implant 7 days post-implantation. The black arrow indicates the TRAP-positive stained area. Original magnification × 100. (g) Western blot analysis confirms that α-CGRP expression was significantly elevated in the Ad-CGRP-EGFP transgene group compared to control groups at the local implantation site ($P < 0.01$). (h) The Ad-CGRP-EGFP group displayed notably diminished staining for TRAP activity. Data are expressed as mean ± SD. **$P < 0.01$.

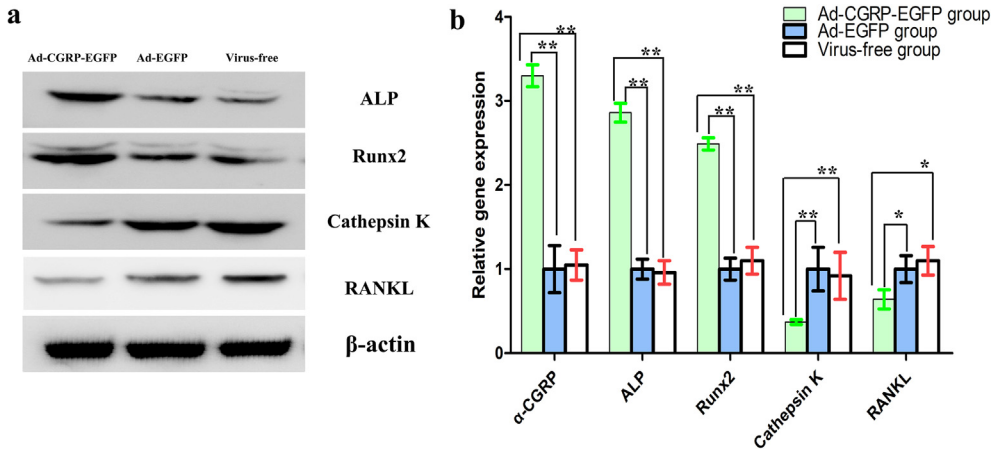

Fig. 3. (a) Western blot analysis of expression levels of ALP, Runx2, Cathepsin K, RANKL, with the bone isolated at a distance of 0.25 mm from the implant. (b) Specific gene expression profiles in the samples subsequent to α-CGRP transgene modification, 7 days post-implantation. Data are presented as mean ± standard deviation. **$P < 0.01$, *$P < 0.05$.

administered into slightly undersized cavities prior to the press-fitting of implants. The vectors diffuse in the marrow cavity surrounding the defect and transduce the exogenous α-CGRP gene into the osteogenesis-related cells. Cryo-sectioning results 7 days post-implantation indicated co-expression of EGFP in both the Ad-CGRP-EGFP and Ad-EGFP groups, corroborating that the recombinant

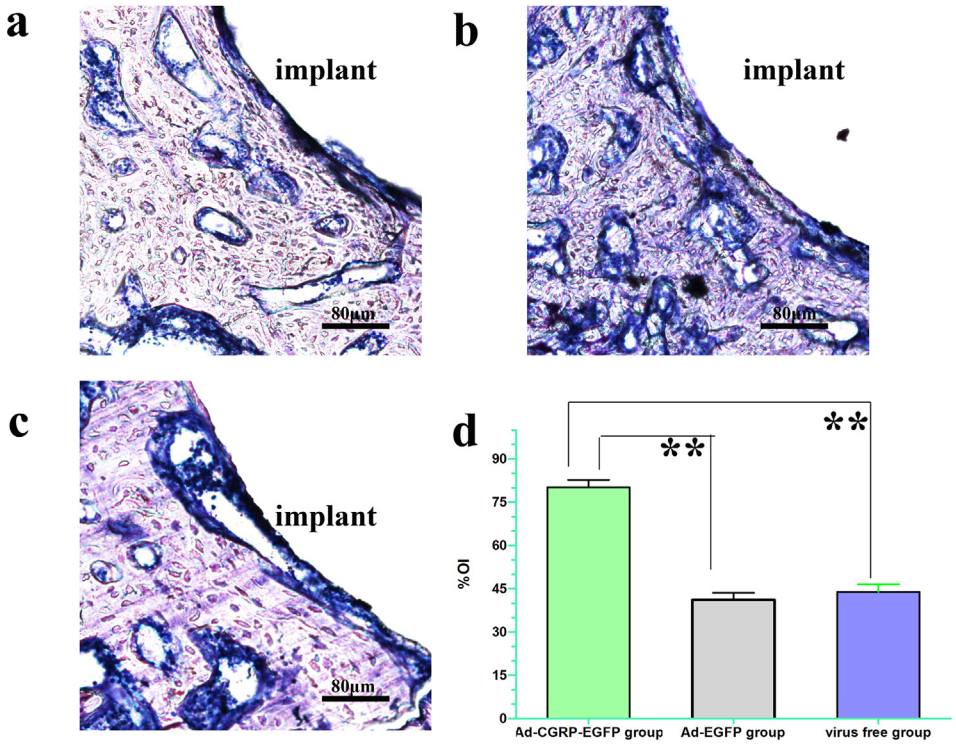

Fig. 4. (a–c) (a–c) Depicts the histological characteristics of the proximal femur with implants 28 days post-implantation, captured at an original magnification of × 100. (d) %OI signifies the percentage of bone-to-implant contact. The data are presented as mean values alongside standard deviations. **P < 0.01.

adenovirus vector functioned effectively in the vicinity of the implant post-injection. Additionally, α-CGRP immunoblot analysis of bone isolated around the implant 7 days post-implantation further confirmed successful transduction. The notably elevated expression of osteogenic factors Runx2 and ALP at the same time point in the Ad-CGRP-EGFP group, which are pivotal in the proliferation and differentiation of BMSCs into osteoblasts and in the formation of bone matrix during bone remodeling, suggested that α-CGRP overexpression could regulate osteogenesis via BMSCs differentiation. The data from this study corroborate previous findings by Jia et al., which indicated that CGRP stimulates the proliferation and migration of BMSCs, enhances the expression of ALP and Runx2, and thereby increases the BMD of rats in vivo [22]. As a result, significant new bone formation and bone-to-implant contact were observed in the α-CGRP modified group 28 days post-implantation. Further analyses using Micro-CT and mechanical testing confirmed the regeneration of bone surrounding the implant. Compared to the Ad-EGFP and virus-free groups, the α-CGRP modified group exhibited an 80.2 % increase in bone volume to total volume ratio, a 55.1 % increase in trabecular number, a 68.7 % increase in trabecular thickness, and a 38.4 % decrease in trabecular separation. These findings substantiate that α-CGRP can expedite bone formation by promoting BMSCs osteogenic differentiation and mineralization.

Furthermore, recent investigations have indicated that the RANKL signaling pathway played a significant role in bone homeostasis modulated by CGRP and may represent a crucial target for the treatment of osteoporosis [23]. He et al. demonstrated that CGRP reduces the expression of RANKL in osteoblasts while simultaneously increasing the expression of osteocalcin and osteoprotegerin [24]. Intriguingly, in our present study, with a reduction in TRAP positive stains and a diminishment of the gene expression levels of RANKL and CPK, the exogenous α-CGRP transgene effectively suppressed osteoclastogenesis. These outcomes suggested that α-CGRP could function as an inhibitor of bone resorption.

The OVX rats employed in our research served as a model for surgical menopause, characterized by a marked decline in estrogen levels [25]. Numerous studies had indicated that the fracture healing process in OVX models was compromised during both the early and late stages of repair, evidenced by diminished bone mineral density and reduced bone strength [26]. Following implantation, a local imbalance in bone metabolism ensues, resulting in structural alterations to the trabecular architecture (porosity) and a decrease in both organic and inorganic bone composition in the vicinity of the implants [6,27]. To enhance dental implant osseointegration in the context of osteoporosis, the present study proposed a biochemical strategy aimed at the sustained expression of α-CGRP. Additionally, the potential advantages of utilizing this bioactive molecule to expedite osseointegration under osteoporotic conditions were evaluated.

Many researches had demonstrated that α-CGRP played a pivotal role in numerous physiological and pathological processes, including bone homeostasis and angiogenesis [28]. Bone was recognized as a common target for α-CGRP, with evidence suggesting that α-CGRP significantly influenced osteoclast-mediated bone resorption and osteoblast-mediated bone formation [29]. Appelt et al. had indicated that the expression of CGRP and its receptor was elevated in the callus during bone regeneration [30]. Studies conducted by Wakabayashi et al. and Zhang et al. had revealed that the level of α-CGRP decreases in the femur but increased in the spinal cord of animals subjected to ovariectomy [13,31]. Consequently, augmenting local α-CGRP application might represent a promising strategy to enhance bone regeneration in vivo and to improve the desirable implant foundation, potentially accelerating implant osseointegration. However, given the significant role of inflammation in CGRP-related bone disorders and the fact that anti-IL-6R antibody and alendronate (ALN) treatment

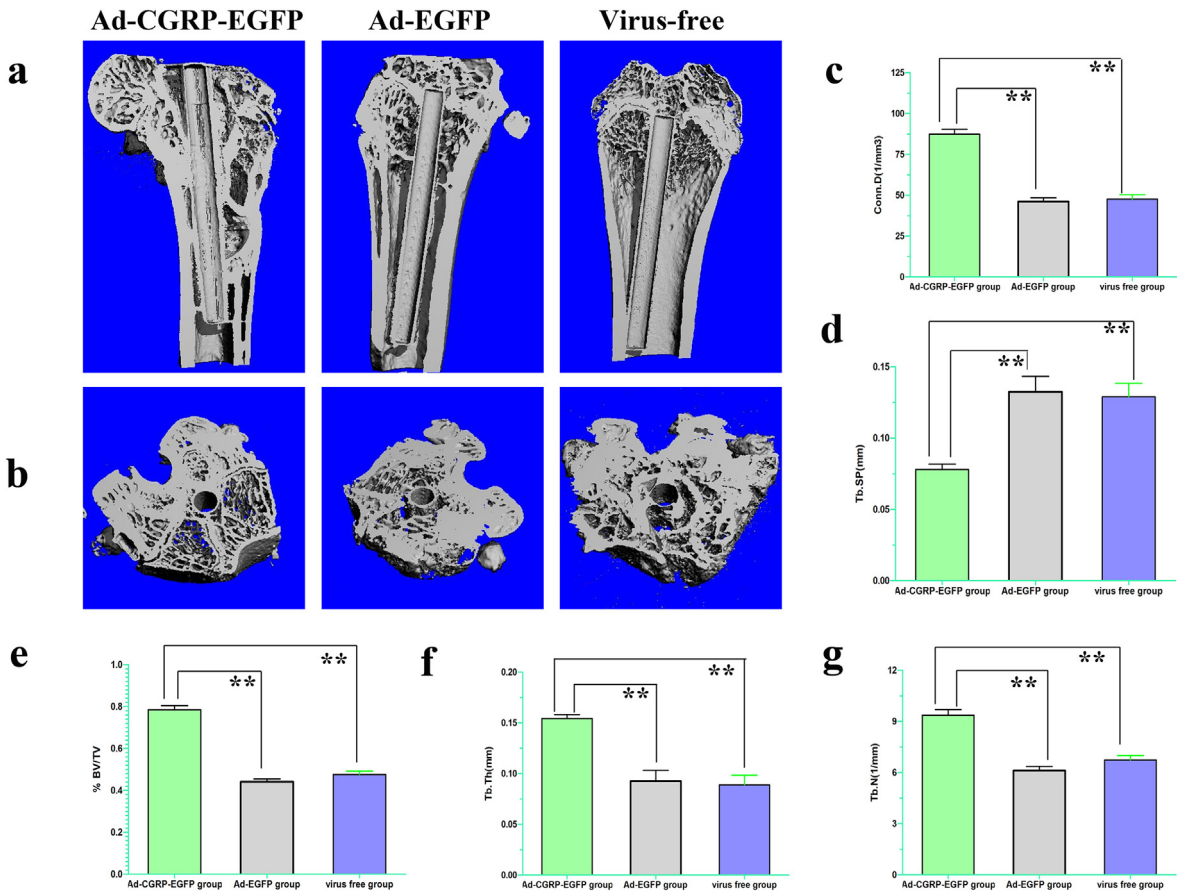

Fig. 5. (a, b) Longitudinal and transverse three-dimensional images of the femur with the implant in the OVX rats, and the three-dimensional microarchitectural indices of the volume of interest (VOI) analyzed using micro-computed tomography (micro-CT). The calcitonin gene-related peptide (CGRP) modification exhibited a significant reversal in the diminishment of peri-implant bone volume within the OVX groups. (c) Conn. D (connectivity density). (d) Tb. Sp (trabecular separation). (e) BV/TV (bone volume/tissue volume). (f) Tb·Th (trabecular thickness). (g) Tb·N (trabecular number). Data are presented as mean ± standard deviation (s.d.). **P < 0.01.

Table 2
The maximum force and the corresponding interfacial shear strength in the push-out test.

OVX rats	Parameters	
	Maximal push-out force(N)	Ultimate shear strength (N/mm^2)
Ad-CGRP-EGFP	75.7 ± 8.82**	10.61 ± 2.32**
Ad-EGFP	28.6 ± 5.68	3.17 ± 0.69
Virus-free	29.3 ± 5.12	3.28 ± 0.97

Data are expressed as mean ± SD, n = 6 femurs/group.
**P<0.01 vs control groups (Ad-EGFP and PBS) in the OVX rats.

had been shown to effectively mitigate mechanical hyperalgesia and CGRP upregulation in the spinal cord induced by OVX [32], further exploration was warranted. Many cell signal pathways of α-CGRP regulation in bone homeostasis had been put forward, such as the Wnt signaling pathways, MAPK signaling pathways et al. [29]. There remained considerable scope for further investigation in this area.

Recombinant adenoviral vectors employed in this study had been extensively utilized as gene delivery vehicles in applications related to wound healing and bone repair. Compared to the array of carriers utilized in research, recombinant adenoviral vectors could be easily purified to high titers and were capable of infecting a broad spectrum of both dividing and non-dividing cells [33]. Cryosectioning results revealed strong green fluorescence on the Ti-implant with a distinct margin, indicating minimal viral diffusion and demonstrating high transduction efficiency in vivo. As a non-integrating virus, the vectors did not integrate with the host chromosome, thereby minimizing the risk of long-term health complications. In our experiment, the recombinant adenovirus vectors exhibited a slower attainment of peak α-CGRP expression but sustained production for over 4 weeks. Efficient gene expression within bone was readily achieved through a simple injection, and the investigation into vector-mediated transduction allowed for repeated administrations. Our results indicated that the α-CGRP adenovirus vector system expressed target-gene in an effective, appropriate and sustained manner, resulting in enhancement of titanium implant osseointegration over long periods of time.

In summary, a localized adenovirus-mediated gene transduction system had been developed and implemented in this study. Local administration of α-CGRP not only significantly enhanced osteogenic differentiation but also inhibited osteoclastogenesis, thereby accelerating new bone formation and enhancing the osseointegration of

titanium implants. Our complementary findings offered new insights into the osteoporotic bone repair process and suggested that α-CGRP might serve as an agent for treating bone defects in osteoporosis condition. The study demonstrated a potent use of the adenovirus-mediated transduction above in osseointegration and bone regeneration.

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Declaration of competing interest

Yin Guozhu, Li Jia and Zhang Shizhou conceived and designed this study. Yin Guozhu wrote the manuscript. Yin Guozhu, Wu yihua, Dai li, Zhang Tianqi and You Zhu did the experiments, analyzed the data and prepared the figures.

The authors have no conflict of interests to declare.

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