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INFECTION

The role of *Staphylococcus aureus* small colony variants in intraosseous invasion and colonization in periprosthetic joint infection

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Aims

This study aimed to explore the role of small colony variants (SCVs) of *Staphylococcus aureus* in intraosseous invasion and colonization in patients with periprosthetic joint infection (PJI).

Methods

A PJI diagnosis was made according to the MusculoSkeletal Infection Society (MSIS) for PJI. Bone and tissue samples were collected intraoperatively and the intracellular invasion and intraosseous colonization were detected. Transcriptomics of PJI samples were analyzed and verified by polymerase chain reaction (PCR).

Results

SCVs can be isolated from samples collected from chronic PJIs intraoperatively. Transmission electron microscopy (TEM) and immunofluorescence (IF) showed that there was more *S. aureus* in bone samples collected from chronic PJIs, but much less in bone samples from acute PJIs, providing a potential mechanism of PJI. Immunofluorescence results showed that SCVs of *S. aureus* were more likely to invade osteoblasts in vitro. Furthermore, TEM and IF also demonstrated that SCVs of *S. aureus* were more likely to invade and colonize in vivo. Cluster analysis and principal component analysis (PCA) showed that there were substantial differences in gene expression profiles between chronic and acute PJI. Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis showed that these differentially expressed genes were enriched to chemokine-related signal pathways. PCR also verified these results.

Conclusion

Our study has shown that the *S. aureus* SCVs have a greater ability to invade and colonize in bone, resulting in *S. aureus* remaining in bone tissues long-term, thus explaining the pathogenesis of chronic PJI.

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Keywords: *Staphylococcus aureus*, Small colony variant, Periprosthetic joint infection

Article focus

- The present work focuses on the role and mechanism of small colony variants (SCVs) of *Staphylococcus aureus* intraosseous invasion and colonization in periprosthetic joint infection (PJI).

Key messages

- Our results showed that *S. aureus* SCVs have a greater ability to invade and colonize in bone.
- The underlying mechanism might be that SCVs could reduce the expression level of chemokines in bone tissue compared with normal phenotype, so that immune cells such as neutrophils cannot be

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recruited quickly and effectively to clear *S. aureus*, resulting in *S. aureus* remaining in bone tissues long-term, thus explaining the pathogenesis of chronic PJI.

Strengths and limitations

- This was the first study of the role and mechanism of SCVs of *S. aureus* intraosseous invasion and colonization in PJI.
- However, we did not explore all the molecular mechanisms involved in PJI, which need further study.

Introduction

Periprosthetic joint infection (PJI) is a serious complication after arthroplasty with a reported incidence of between 1% and 3%.[1,2] Although this is not very high, with the advent of an ageing society, and the increasing number of patients undergoing arthroplasty, the rate of PJI will probably rise. The incidence of PJI after revision surgery varies from 3% to 10%,[1,2] and those patients who suffer recurrent infection may need multiple operations involving debridement, long-term antibiotic treatment, amputation, and occasionally their infection can result in death. *Staphylococcus aureus* is a gram-positive cocci,[3] and is one of the most common pathogenic microorganisms of PJI. The specific pathogenesis of *S. aureus* PJI is not yet fully understood, and can be difficult to treat.[4] Thus, further investigation of the pathogenesis of *S. aureus* PJI would improve the understanding of PJI and provide a basis for developing new therapeutic strategies.

Previous studies have shown that *S. aureus* can escape the host's immune system and not respond to treatment, while remaining present in the tissues for a long period,[5-7] resulting in chronic infection. *S. aureus* can adhere to implant surfaces and form biofilms, while abscesses can be formed in the skin mucosa and muscle soft-tissues, which help *S. aureus* to effectively escape the host's immune system and antibiotic treatment.[8,9] The main treatment strategies for *S. aureus* PJI include thorough debridement to remove abscesses along with removal of prostheses, to remove the biofilms, followed by the administration of sensitive antibiotics for several weeks. However, even after thorough debridement, removal of prosthesis, and sufficient antibiotic treatment, a number of *S. aureus* PJIs do not respond to treatment.[10] This suggests that there are other mechanisms involved in *S. aureus* PJI.

Recently, several studies showed that the colonization of *S. aureus* in bone tissue was a novel mechanism of PJI. de Mesy Bentley et al[11] reported that *S. aureus* can invade the osteocyte lacuna-canalicular network (OLCN), and then colonize, migrate, and proliferate. In addition, in vitro studies have found that *S. aureus* can invade non-professional phagocytes such as osteoblasts, osteoclasts, and osteocytes, colonizing and proliferating over a long period, resulting in an intracellular infection.[12-14] On one hand, this mechanism enables *S. aureus* to survive an attack by the host's immune system as well as by antibiotics. On the other hand, *S. aureus* could obtain nutrition in OLCN and proliferate, resulting in recurrent infection after antibiotic therapy.[15] It is apparent, therefore, that the intraosseous colonization by *S. aureus* plays an important role in orthopaedic infections, such as chronic osteomyelitis and chronic PJI, although the mechanism by which *S. aureus* promotes its intraosseous colonization is not clear.

Small colony variants (SCVs) were first found in *S. aureus* and other coagulase-negative staphylococci, which were characterized by small morphology, slow growth, and low metabolism.[16] Recently, it has been reported that SCVs are closely related to recurrent and persistent infection by *Staphylococcus.* These include infections related to medical instruments, musculoskeletal infections, and respiratory infection in patients with cystic fibrosis, among others.[17,18] Kalinka et al[19] found that when comparing patients with acute or chronic osteomyelitis, the proportion of SCVs in *S. aureus* isolated from chronic osteomyelitis was higher. Sendi et al[18] and Kahl et al[20] have also isolated SCVs from chronic PJIs. The characteristics of PJI caused by *S. aureus* SCVs include a long duration of infection and a high recurrence rate, and its clinical efficacy is inferior to that caused by normal *S. aureus* phenotype.

As the strains of *S. aureus* which were isolated from chronic infection often appear in the form of SCVs in vitro, with the characteristic of slow growth and low metabolism,[16,17,21] we hypothesized that *S. aureus* might enhance its ability to colonize in bone tissue by means of SCV phenotype transformation, resulting in chronic PJI.

Methods

Clinical samples collection. All patients who were diagnosed with PJI, caused by *S. aureus*, and who underwent revision surgery in our centre were included in the study. PJI was diagnosed according to the MusculoSkeletal Infection Society (MSIS) criteria[1]: 1) a sinus tract communicating with the prosthesis; 2) a pathogen isolated by culture from two separate tissue or fluid samples obtained from the affected prosthetic joint; and 3) four of the following six criteria exist: i) elevated ESR and CRP ; ii) elevated synovial fluid white blood cell (WBC) count; iii) elevated synovial fluid neutrophil percentage; iv) presence of purulence in the affected joint; v) isolation of a microorganism in one periprosthetic tissue or fluid culture; and vi) > five neutrophils per high-powered field. Patients were classified as acute PJI (≤ three months) and chronic PJI (> three months).

In order to further explore the host molecular mechanisms involved in the process of *S. aureus* SCV bone colonization, bone tissues from acute and chronic *S. aureus* PJI cases were collected for transcriptome profile analysis. Clinical characteristics of PJI patients are shown in Table I.

With the approval of the Ethics Committee of First Affiliated Hospital, Fujian Medical University, synovial fluid and at least five periprosthetic tissues were collected intraoperatively from each patient, some of which were used for pathological examination, while the rest were

Table I. Demographic characteristics.

ID	Sex	Age, yrs	BMI, kg/m²	Joint	Diagnosis	Pathogen
1	Female	58	23.2	Hip	Acute PJI	*S. aureus*
2	Male	57	22.8	Hip	Acute PJI	*S. aureus*
3	Female	60	23.5	Hip	Acute PJI	*S. aureus*
4	Male	59	23.1	Hip	Chronic PJI	*S. aureus*
5	Female	56	23.3	Hip	Chronic PJI	*S. aureus*
6	Female	61	23.6	Hip	Chronic PJI	*S. aureus*

PJI, periprosthetic joint infection; *S. aureus*, *Staphylococcus aureus*.

transferred for microbial culture. The colony morphology was observed, the colony size was measured, and the growth time was recorded. Bone tissues were also collected intraoperatively, and stored at -80°C for subsequent experiments.

RNA extraction. In total, 1 ml of TRIzol (15596018; Invitrogen, Thermo Fisher Scientific, USA) was added into tissue samples, stood for five minutes at room temperature, centrifuged at 4°C for ten minutes (12,000 r/min), and then the supernatants were collected in a new eppendorf (EP) tube (Axygen, Corning, USA). Next, 200 µl chloroform (MilliporeSigma, USA) was added, vortexed for 15 seconds, stood at room temperature for 15 minutes, and centrifuged at 12,000 r/min at 4°C for ten minutes. The upper water phase was transferred to RNase-free tubes (Axygen), equal volumes of isopropyl alcohol (CAS 67-63-0; Aladdin, China) were added, and then turned upside down for three times, stood at room temperature for ten minutes, centrifuged at 12,000 r/min at 4°C for ten minutes, and the supernatants were removed; finally, RNAs were deposited at the bottom of the EP tubes.

Real-time quantitative polymerase chain reaction. After RNAs were extracted, these were reverse-transcribed into complementary DNA (cDNA) according to the manufacturer's instructions (K1691; Thermo Fisher Scientific). The products were stored at -80°C for later experiments. Real-time quantitative polymerase chain reaction (PCR) was performed according to the instructions of the quantitative PCR kit (AQ132-11; TransGen Biotech, China). The primers were designed and synthesized by Sangon Biotech (China), and the sequences are listed in Supplementary Table i. The whole sampling processes were completed in the ice box, and finally amplified on a PCR instrument (Quantitative PCR instrument 7500; Applied Biosystems, USA).

Co-culture of *S. aureus* and osteoblast. Next, 1 ml of *S. aureus* suspension (1.5×10^8 CFU/ml) was prepared, centrifuged for one to three minutes at 3,000 r/min, and washed three times with 0.85% sodium chloride (NaCl). After that, the *S. aureus* was labelled by LIVE/DEAD Bacterial Viability and Counting Kit (MA0390; Dalian Meilun Biotechnology, China). Briefly, 3 µl of meilungreen (3.34 mM) nucleic acid dye was added into 987 µl of 0.85% NaCl, then 10 µl of the above *S. aureus* suspension was added and incubated for 15 minutes at room temperature in the dark, and then centrifuged at 10,000 r/min for one to three minutes, removed supernatants, and resuspended with 0.85% NaCl. Human fetal osteoblast (hFOB) cells were cultured in six-well plates in Dulbecco's Modified Eagle Medium (DMEM) (Shanghai Basalmedia Technologies, China) supplemented with 1% fetal bovine serum (FBS) (Thermo Fisher Scientific) as per the manufacturer's instructions, and infected by labelled *S. aureus* at multiplicity of infection (MOI) 100 for six hours in the dark. After that, lysostaphin (L7386; MilliporeSigma) was added to kill the extracellular *S. aureus*.

Establishment of PJI models. PJI models were established in C57BL/6 mice, which weighed approximately 22 to 24 g. In brief, titanium wire was selected as the material to simulate the prosthesis. The end of the prosthesis was bent into a right angle as the stem of the prosthesis, the stem was taken as the shaft, and the main part was tightly wound vertically into a disc shape. Different phenotypes of *S. aureus* suspension were prepared with 0.85% NaCl. After abdominal anaesthesia, an incision was made in the middle of the mouse knee, and the articular cartilage from the distal femur and tibial plateau was removed by a chainsaw, the prosthesis was installed, and the wound was washed and sutured. After the primary healing of the wound, the prepared *S. aureus* suspension was injected into the joint cavity. Then we observed the local incision of mice and judged whether the PJI models were successful or not. Mice without *S. aureus* were selected as controls. We have included an ARRIVE checklist to show that we have conformed to the ARRIVE guidelines.

Immunofluorescence (IF). Bone tissues were fixed overnight with paraformaldehyde (CAS 30525-89-4; Aladdin) and decalcified with ethylenediaminetetraacetic acid (EDTA) (CAS 60-00-4; MilliporeSigma). A cryotome (Thermo Cryotome FSE Cryotome; Thermo Fisher Scientific) was used to slice the bone tissue, about 10 mm thick. After that, slices were washed with phosphate-buffered saline (PBS) three times. Anti-*S. aureus* antibody (ab37644; Abcam, USA) was added and incubated overnight at 4°C. After washing with PBS three times, five minutes each time, then with secondary antibodies, FITC Goat Anti-Mouse IgG (H + L) (AS001; ABclonal, China) FITC was added and incubated in a shaker at 37°C for two hours. After again washing with PBS three times, five minutes each time, 4′,6-diamidino-2-phenylindole (DAPI) (MA0127; Dalian Meilun Biotechnology, China) was added and incubated for five minutes. After once again washing with PBS three times, five minutes each time, the tissues were photographed with Zeiss LSM800

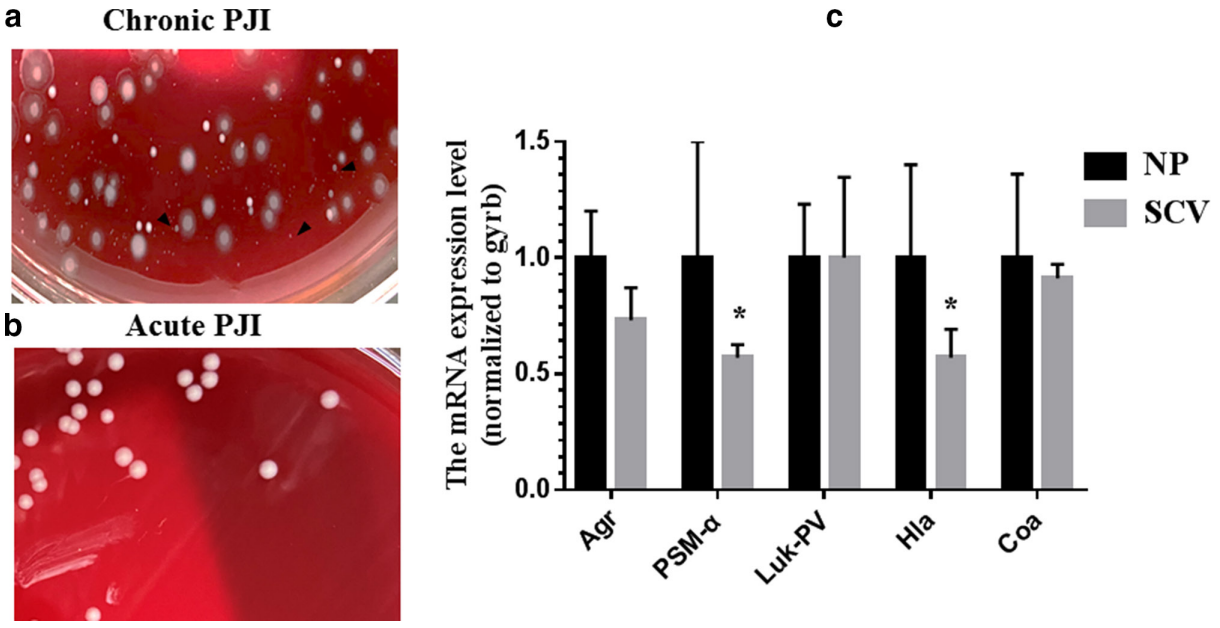

Fig. 1

a) and b) Colony morphologies of acute and chronic periprosthetic joint infection (PJI) microbial cultures: small colony variants (SCVs) could be isolated from chronic PJI cultures (indicated by black arrows). c) Expression levels of virulence factors in *Staphylococcus aureus* normal phenotype (NP) and SCVs. *p < 0.05. Agr, accessory gene regulator; Coa, coagulase; gyrb: gyrase subunit B; Hla, α-haemolysin; Luk-PV, Panton-Valentine leukocidin; mRNA, messenger RNA; PSM-α, phenol-soluble modulin-α.

confocal laser scanning microscope (Carl Zeiss, Germany) and quantified by Image-Pro Plus software (Media Cybernetics, USA).

Transmission electron microscopy (TEM). Fresh bone tissues about 1 × 1 × 1 mm^3 were collected and decalcified with EDTA (CAS 60-00-4; MilliporeSigma), prefixed with 3% glutaraldehyde (CAS 111-30-8; Aladdin), 1.5% paraformaldehyde (CAS 30525-89-4; Aladdin), and 0.1 M PBS (pH 7.2) at 5°C for several days, then rinsed with 0.1 M PBS (pH 7.2) more than three times, 1% osmic acid and 1.5% potassium ferrocyanide at 4°C for two hours, and 0.1 M PBS (pH 7.2) more than three times, successively. Afterwards, samples were dehydrated with 50% alcohol for ten minutes, 70% alcohol-saturated uranium acetate dye solution at 4°C overnight, 90% alcohol for ten minutes, 90% alcohol-acetone for ten minutes, 90% acetone for ten minutes, and anhydrous acetone for ten minutes, three times, successively. Then these samples were saturated with anhydrous acetone and epoxy resin with a ratio 1:1 for 1.5 hours, epoxy resin for three hours at 35°C, then epoxy resin for 24 hours at 35°C, 12 hours at 45°C, and two to three days at 60°C, successively. The ultrathin sections (90 to 100 nm) were obtained with ultramicrotome Leica EM UC7 (Leica, Germany), and were dyed with uranium acetate for 20 minutes, washed with distilled water, dyed with lead citrate for five to ten minutes, and washed with distilled water. Finally, the images were obtained using a transmission electron microscopy (TEM) device (Tecnai Spirit, FEI Company, USA).

Transcriptomic analysis. In order to further explore the host's molecular mechanisms involved in the process of *S. aureus* colonization, acute and chronic *S. aureus* PJI bone tissues were collected for transcriptomic analysis. The steps included RNA extraction and labelling, microarray hybridization, washing, and scanning.

The expression of genes was analyzed by DESeq (Anders & Huber, Germany),[22] and the thresholds for screening differentially expressed genes were fold change (FC) > 1.5 and p < 0.05. R package pheatmap (R Foundation for Statistical Computing, Austria) was used to draw the heat map of differential gene clustering. According to the expression level, principal component analysis (PCA) was carried out on each sample by using the prcomp function of R language. PCA of transcript profiles showed the variability between the samples (closer samples were more similar).

Bioinformatics analysis and verification. Gene ontology (GO) enrichment analysis included three modules: molecular function; cellular component; and biological process. Database for Annotation, Visualization and Integrated Discovery (DAVID) is a tool for collecting and analyzing a variety of biological information, and in this study DAVID was used to perform the GO analysis. Kyoto Encyclopedia of Genes and Genomes (KEGG) database could analyze the metabolism, environmental information processing, cell processes, biological systems, human diseases, and other processes related to specific genes from the perspective of molecular level, which could substantially improve our understanding about some types of diseases. Thus, KEGG was also used in this study to understand the process of *S. aureus* colonization; all these results were also verified by PCR.

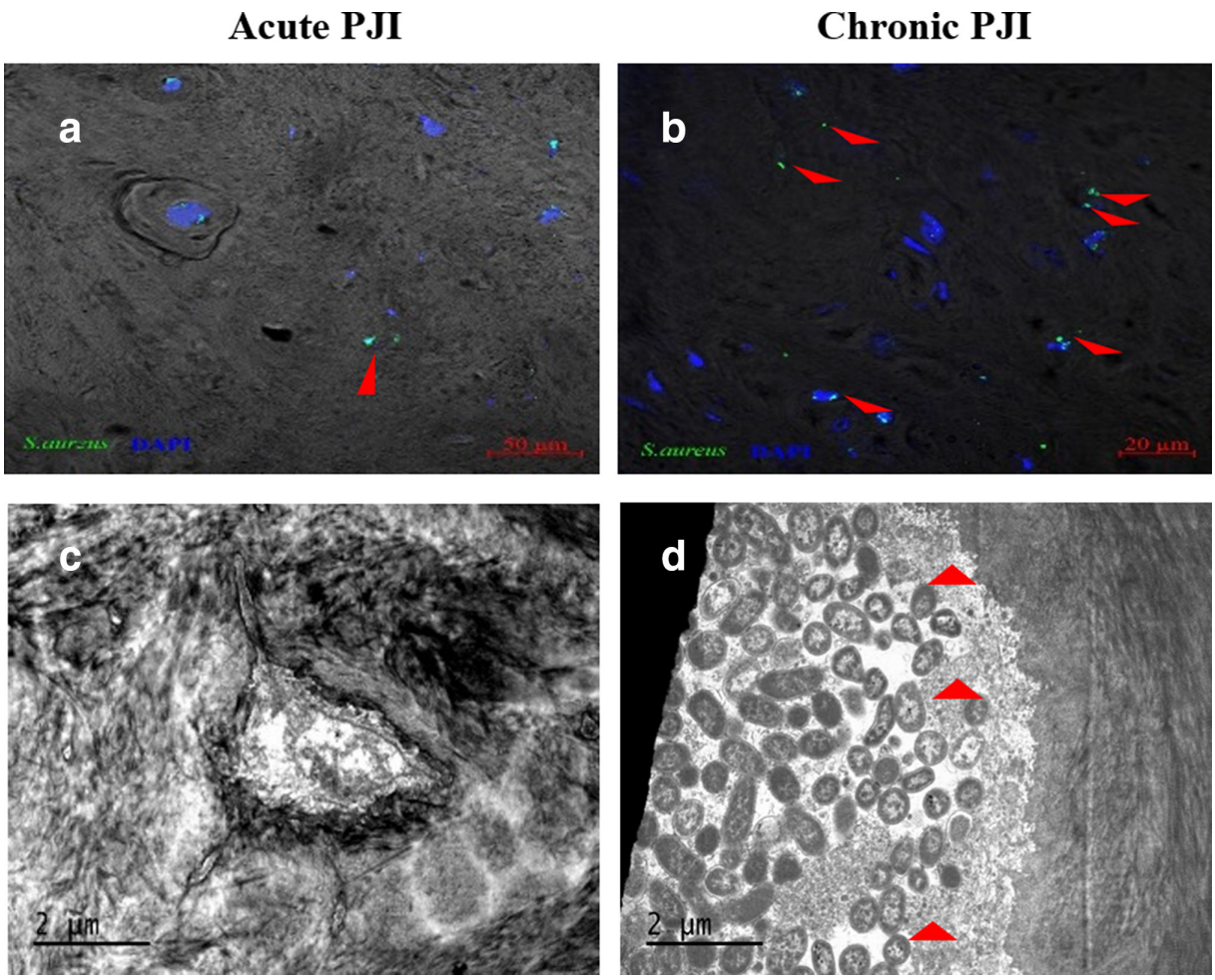

Fig. 2

a) to b) Immunofluorescence and c) to d) transmission electron microscopy results of acute and chronic *Staphylococcus aureus* periprosthetic joint infection (PJI) bone tissues: there was more *S. aureus* colonization in chronic PJI bone tissues than in acute PJI (indicated by red arrows). DAPI, 4′,6-diamidino-2-phenylindole.

Statistical analysis. Data are expressed as means and standard deviations. Independent-samples *t*-test was used to analyze the differences between the two groups, and $p \leq 0.05$ indicates statistical significance. All data were analyzed in SPSS 11.0 (SPSS, USA) and GraphPad Prism 7.0 (GraphPad Software, USA).

Results

Isolation and identification of *S. aureus* SCVs. In the present study, patients were divided into acute PJI and chronic PJI, then synovial fluid and periprosthetic tissues were collected intraoperatively for microbial culture, whose colony morphologies were observed and growth times recorded. The presence of more small colonies was observed from chronic *S. aureus* PJI microbial culture results, the diameters of small colonies were largely smaller than that of the normal phenotype, and their growth was slow, which conformed to SCV growth characteristics (Figures 1a and 1b). To further identify whether these small colonies were SCVs, the expression levels of some virulence factors such as accessory gene regulator (Agr), phenol-soluble modulin-α (PSM-α), Panton-Valentine leukocidin (Luk-PV), α-haemolysin (Hla), and coagulase (Coa) were compared between *S. aureus* normal phenotype and small colonies. The results showed that the expression levels of PSM-α and Hla in small colonies were lower than those of normal phenotype (Figure 1c), thus these small colonies were identified as SCVs. However, fewer SCVs were obtained from acute *S. aureus* PJI. These results suggest that *S. aureus* may be involved in the pathogenesis of chronic PJI through phenotype transformation into SCV.

Colonization of *S. aureus* in bone tissues. Bone tissues were collected intraoperatively from acute and chronic *S. aureus* PJI, which were incubated with anti-*S. aureus* antibody after decalcification, and the colonization of *S. aureus* was detected by IF. The results showed that the *S. aureus* colonization could be easily observed in chronic *S. aureus* PJI bone tissues, while only a small amount of *S. aureus* colonization could be observed in acute *S. aureus* PJI bone tissues. In order to further confirm the bone colonization of *S. aureus*, bone tissues were decalcified and

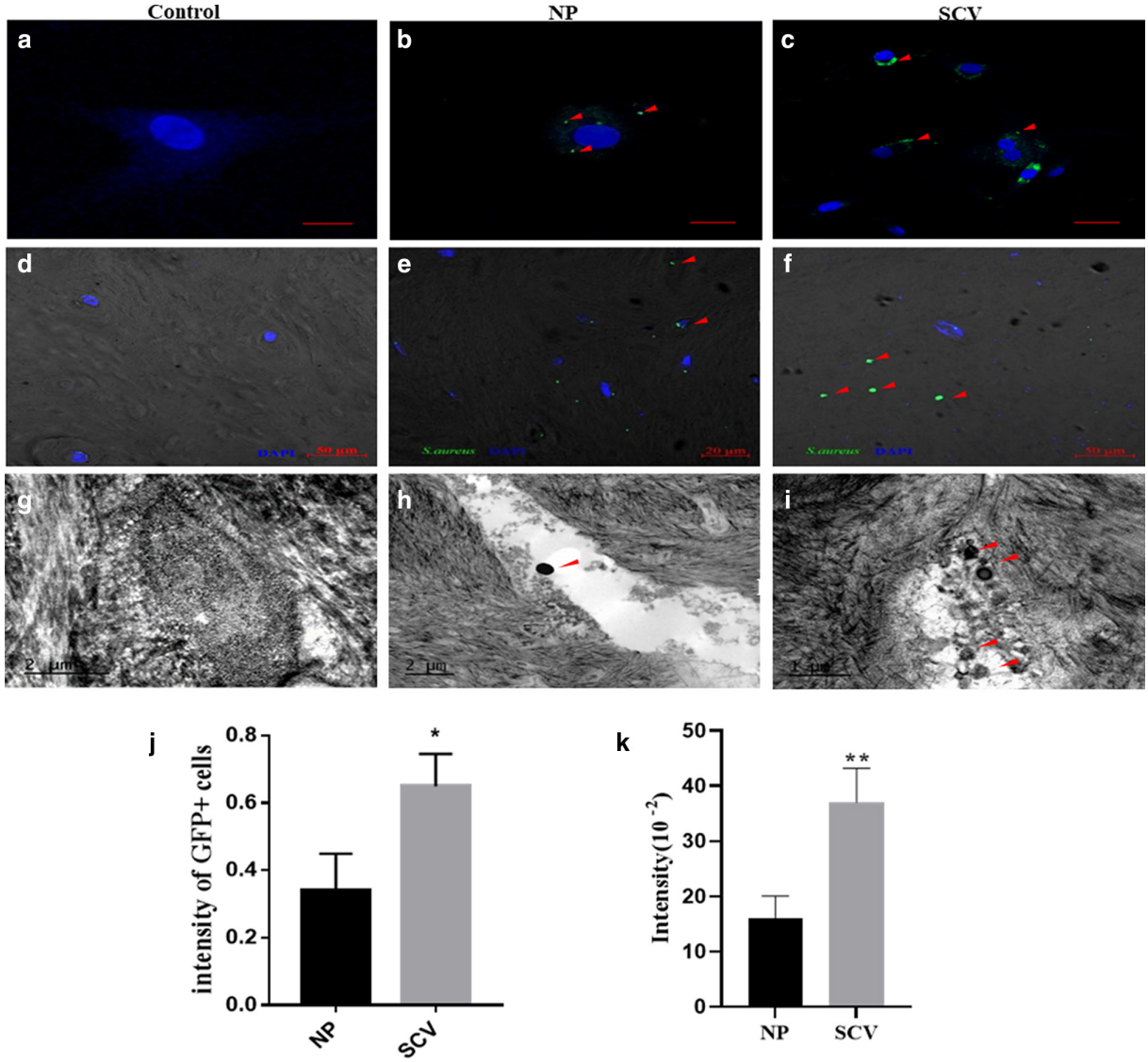

Fig. 3

Comparison of *Staphylococcus aureus* small colony variant (SCV) and normal phenotype (NP) intracellular invasion and intraosseous colonization ability. a) to c) Intracellular invasion ability detected by immunofluorescence (IF) and quantification (j). d) to f) Intraosseous colonization ability detected by IF and quantification (k), and by transmission electron microscopy (g to i). a) and b) Scale bar 20 μm; c) scale bar 50 μm; d) to f) scale bar 50 μm; g) and h) scale bar 2 μm; i) scale bar 1 μm. *p < 0.05; **p < 0.01. DAPI, 4′,6-diamidino-2-phenylindole; GFP, green fluorescence protein.

then observed by TEM. The results also showed that significant *S. aureus* colonization was observed in chronic *S. aureus* PJI bone tissues, while less *S. aureus* colonization could be observed in acute PJI bone tissues (Figures 2a to 2d).

The internalization and colonization ability of different phenotypes of *S. aureus* in vitro and in vivo. In order to explore the internalization ability of *S. aureus* with different phenotypes in vitro, *S. aureus* normal phenotype and *S. aureus* SCV suspension were prepared respectively, and then infected hFOB cells in vitro after being labelled by green fluorescence. The internalization and colonization of *S. aureus* in osteoblasts were detected by IF. The results showed that when compared with normal phenotype, *S. aureus* SCVs had a greater invasion ability and were more easily internalized by osteoblasts (Figures 3a to 3c and 3j).

In order to further verify the differences in colonization ability between different phenotypes of *S. aureus* in vivo, *S. aureus* normal phenotype and *S. aureus* SCV suspension were prepared, and PJI models were established, respectively. Then, the periprosthetic tissues were collected for microbial culture, and the tibia and femur were collected for *S. aureus* colonization detection. IF and TEM results showed that compared with *S. aureus* normal phenotype, more *S. aureus* colonization could be significantly observed in bone tissues of *S. aureus* SCV PJI models (Figures 3d to 3i and 3k). All these results show that *S. aureus* SCV phenotypic transformation could

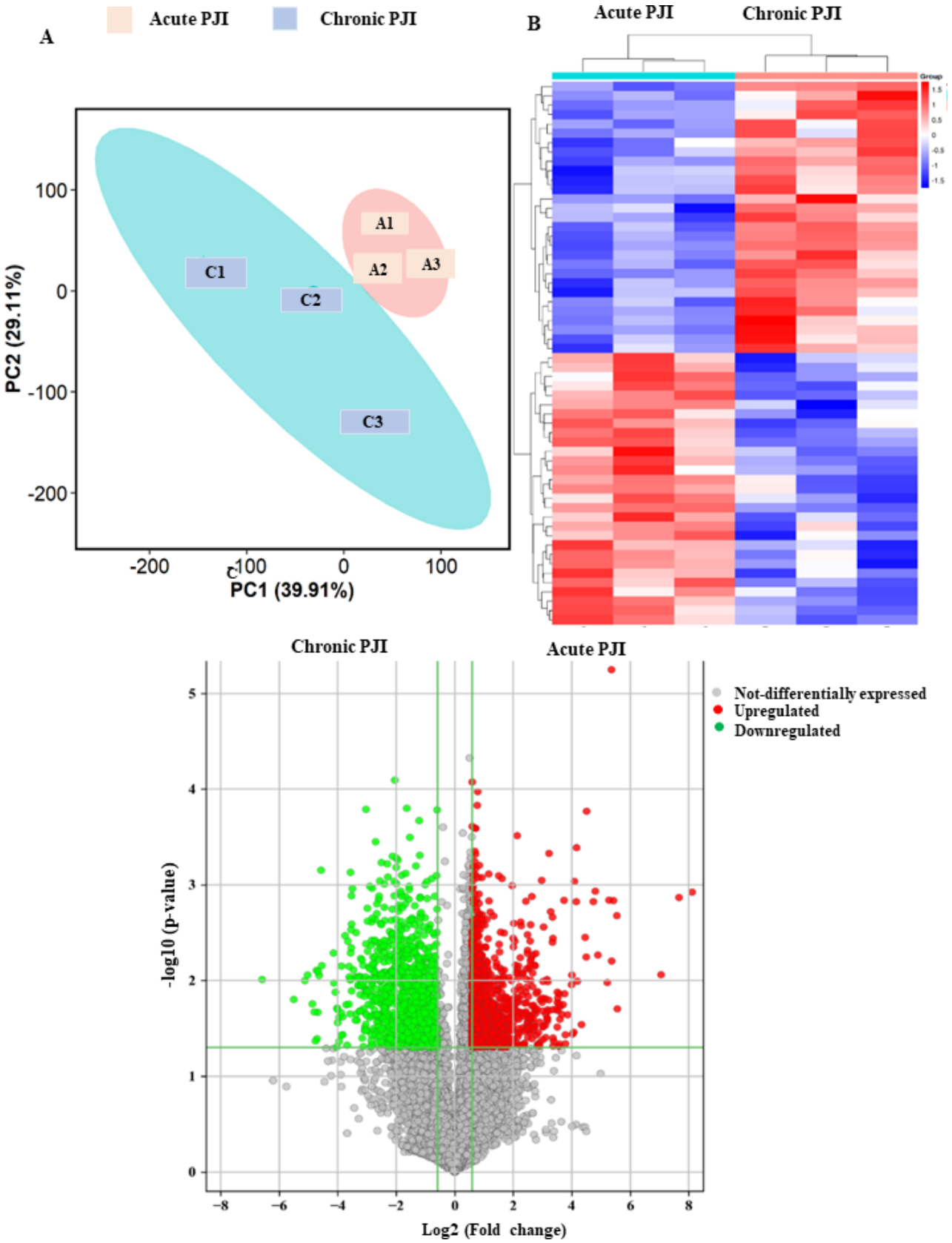

Fig. 4

a) Principal component analysis (PCA), b) heat map, and c) volcano map of differentially expressed genes between acute and chronic periprosthetic joint infection (PJI).

Table II. The top ten differentially expressed genes between acute and chronic periprosthetic joint infections.

Gene symbol	Fold change (log2-scaled)	Regulation	Chrom	p-value
HLA-DRB5	8.118054739	up	chr6	0.001
CSF2	7.67026642	up	chr5	0.001
SAA2	7.054877446	up	chr11	0.008
MMP3	5.561197839	up	chr11	0.020
IL1A	5.544776784	up	chr2	0.002
OSM	5.426615179	up	chr22	0.001
CCL18	5.364543389	up	chr17	0.006
CXCL6	5.359087801	up	chr4	< 0.001
MARCO	5.276307417	up	chr2	0.001
C15orf48	5.218072819	up	chr15	0.010
HBG2	-6.583738974	down	chr11	0.001
IBSP	-6.20561178	down	chr4	0.111
DEFA4	-5.752653666	down	chr8	0.128
FABP4	-5.499750702	down	chr8	0.016
CTD	-5.122057024	down	chr11	0.010
CATG	-5.026436892	down	chr9	0.009
EPHA3	-4.863809654	down	chr3	0.018
OMD	-4.801034264	down	chr9	0.021
ENTPD3	-4.759807978	down	chr3	0.043
CRISPLD1	-4.726284078	down	chr8	0.008

CATG – at least one of the ten top ranked transcripts is Coding Potential Assessment Tool (CPAT) coding and max Open Reading Frame (ORF) size ≥ 300, not annotated as noncoding in GENCODE.
CCL18, C-C motif chemokine ligand 18; C15orf48, chromosome 15 open reading frame 48; CRISPLD1, cysteine rich secretory protein LCCL domain containing 1; CSF2, colony stimulating factor 2; CTD, C-terminal domain; CXCL6, C-X-C motif chemokine ligand 6; DEFA4, defensin alpha 4; ENTPD3, ectonucleoside triphosphate diphosphohydrolase 3; EPHA3, EPH receptor A3; FABP4, fatty acid binding protein 4; HBG2, haemoglobin subunit gamma 2; HLA-DRB5, major histocompatibility complex, class II, DR beta 5; IBSP, integrin binding sialoprotein; IL1A, interleukin 1 alpha; MARCO, Homo sapiens macrophage receptor with collagenous structure; MMP3, matrix metallopeptidase 3; OMD, osteomodulin; OSM, oncostatin M; SAA2, serum amyloid A2.

promote its intracellular internalization and intraosseous colonization.

Different gene expression profiles in chronic and acute *S. aureus* PJI cases. The cluster analysis and PCA results show that there were significant differences in gene expression profiles between acute and chronic PJIs (Figures 4a and 4b). The differentially expressed genes were screened based on the thresholds of FC > 1.5 and p < 0.05, and finally 2,592 significant differential expression genes were screened out (including 1,456 upregulated genes and 1,136 downregulated genes) (Figure 4c). The top ten upregulated and downregulated genes are shown in Table II. Notably, in acute PJI bone tissues, the expression of chemokines, including CCL18 and CXCL6, was significantly higher than that of chronic PJI bone tissues.

Bioinformatics analysis and verification. In order to clarify the molecular biological processes involved in the process of *S. aureus* SCV colonization, the differentially expressed genes underwent further GO and KEGG pathway analysis. GO analysis showed that these differentially expressed genes were mainly enriched in alpha-chemokine receptor (CXCR) binding, chemokine activity, and chemokine receptor binding in molecular function modules (Figure 5a). Also, KEGG pathway enrichment analysis showed that the differentially expressed genes were mainly enriched in cytokine-cytokine receptor interaction, chemokine signalling pathway, Wnt signalling pathway, etc. (Figure 5b). To further verify the results of the bioinformatics analysis, RNAs were extracted from acute and chronic *S. aureus* PJI bone tissues, and the expression levels of some typical inflammatory chemokines were detected by quantitative PCR. The results showed that the expression levels of CCL5, CXCL8, CXCL9, and CXCL11 in chronic *S. aureus* PJI bone tissues were significantly lower than those in acute *S. aureus* PJI (Figure 5c).

Discussion

PJI is a catastrophic complication after joint arthroplasty, with the number of PJI patients increasing year by year. At present, treatment strategies for PJI mainly include: debridement, antibiotics, irrigation and retention (DAIR); one-stage revision; two-stage revision; and long-term oral antibiotics.[23,24] For acute PJI, DAIR is the treatment of choice, while for chronic PJI, one-stage or two-stage revision is advocated. In both one- and two-stage revision, it is necessary to remove all of the prosthesis, the abscess, and necrotic tissues. After thorough debridement, a new prosthesis or an antibiotic-loaded spacer can be implanted, and appropriate antibiotics are administrated to eradicate the infection.[23,24] However, in chronic PJI even following this treatment plan there remains a high failure rate after DAIR of between 30% and 50%,[25] and after revision surgery, along with prosthesis removal, debridement and antibiotic therapy, the failure rate varies

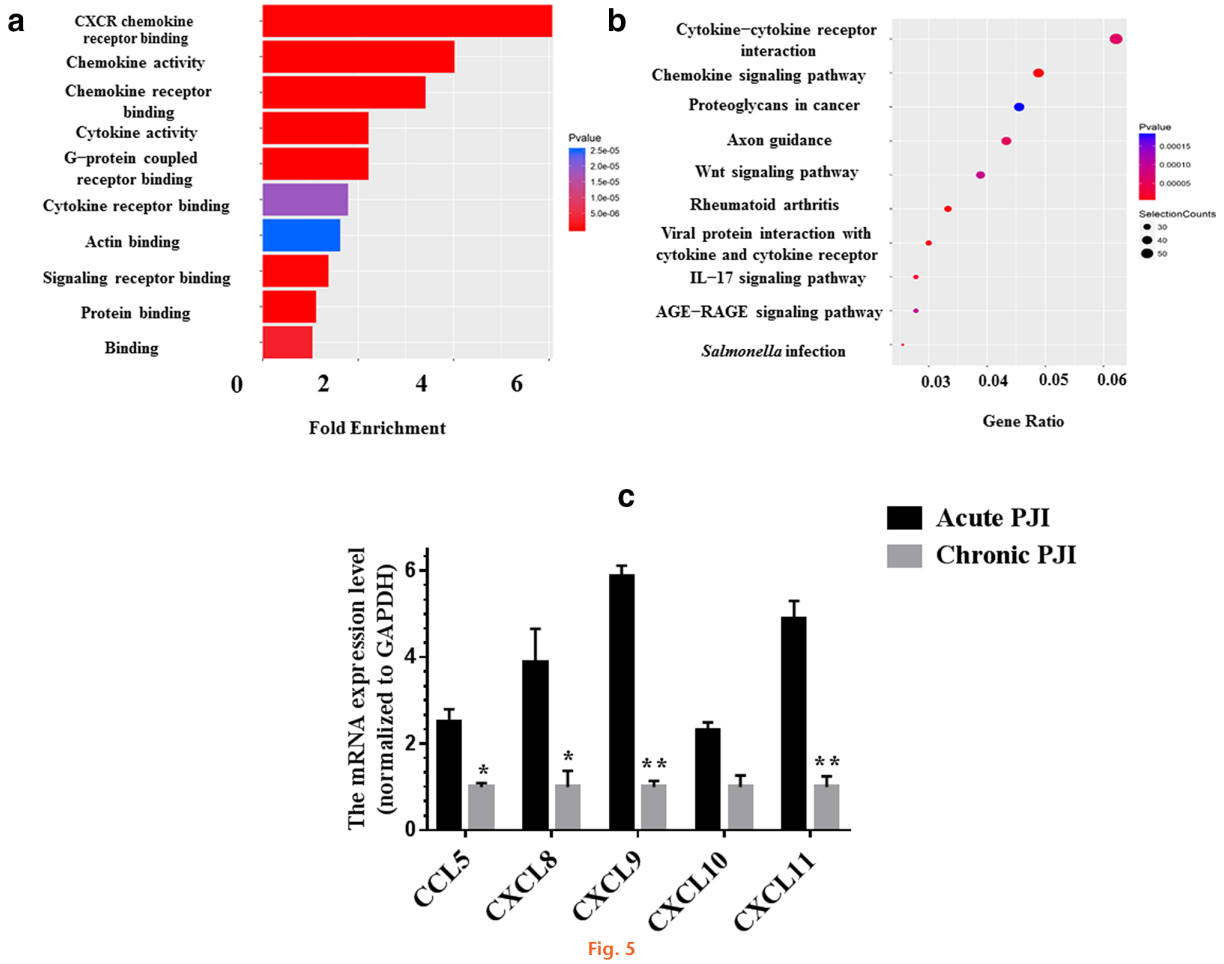

Fig. 5

a) Gene Ontology analysis and b) Kyoto Encyclopedia of Genes and Genomes pathway analysis of differentially expressed genes. c) Expression levels of chemokines in acute and chronic *Staphylococcus aureus* periprosthetic joint infection (PJI) bone tissues. *p < 0.05; **p < 0.01. CCL, C-C motif chemokine ligand; CXCL, C-X-C motif chemokine ligand; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; IL, interleukin; mRNA, messenger RNA.

between 1% and 20%.[26,27] For these recurrently infected patients, regular debridement and long-term antibiotic treatment are the main line of treatment. Indeed, some patients eventually need amputation to prevent septic shock, and others may even die as a result of PJI.

S. aureus SCV is a subgroup of *S. aureus* under special conditions, which has the characteristics of small colony and slow growth. Previous studies have shown that more SCVs would be present in biofilms,[18,19] and most biofilms formed in chronic infections, thus suggesting that SCVs might be related to chronic infections. In addition, it has been reported that SCVs are common in staphylococcal PJI and osteomyelitis.[18,19] In this study, we have shown that colonies with small diameter and slow growth were isolated from chronic *S. aureus* PJI, and the expression level of virulence factors was decreased, but we found that fewer SCVs were isolated from patients with acute *S. aureus* PJI. Loss et al[17] also isolated SCVs from chronic PJI patients, and their findings are consistent with ours.

Previous studies have reported that *S. aureus* can be colonized in OLCN, deform, and migrate.[11] OLCNs have become another residence for *S. aureus*, within which *S. aureus* could get nutrition and proliferate, and avoid the attack of the host's immune system, participating in the pathogenesis of chronic osteomyelitis.[11,14] In this study, we collected bone tissues from acute and chronic *S. aureus* PJI, and detected the *S. aureus* colonization by IF and TEM. Yang et al[13] also reported that IF and TEM both showed obvious *S. aureus* colonization in chronic PJI bone tissues, while only a small amount of *S. aureus* colonization could be found in acute PJI bone tissues, which suggests that *S. aureus* intraosseous colonization is involved in the pathogenesis of chronic PJI. However, the mechanism by which *S. aureus* promotes intraosseous colonization remains unclear.

It has been reported that compared with normal phenotype, *S. aureus* SCV phenotype had greater intracellular invasion ability, which could invade non-professional phagocytes and colonize,[6] but it is not clear whether *S. aureus* SCV also has greater intraosseous colonization ability. Therefore, the isolated *S. aureus* SCV and *S. aureus* normal phenotype were used to infect osteoblasts in vitro, and the results confirmed that *S. aureus* SCV had a greater ability to

invade osteoblasts. In addition, the PJI models were so constructed and infected by SCV and normal phenotype, respectively, so that the intraosseous colonization of *S. aureus* was detected. The results show that SCV had stronger intraosseous colonization ability than normal phenotype. These findings indicate that *S. aureus* SCVs had a greater ability for intracellular invasion and intraosseous colonization.

It is possible that in patients with chronic PJI, *S. aureus* could promote intraosseous colonization through SCV phenotype transformation, thus participating in the process of chronic PJI pathogenesis.

In order to further study the host's molecular mechanism involved in the process of *S. aureus* intraosseous colonization, we applied transcriptomic profile analysis and bioinformatics analysis. The results showed that there were marked differences in gene expression profiles between acute and chronic *S. aureus* PJIs. GO analysis showed that these differentially expressed genes were mainly enriched in CXCR chemokine receptor binding in the molecular function modules, and KEGG pathway analysis showed that compared with acute PJI, the differentially expressed genes were mainly enriched in cytokine-receptor interaction and chemokine signalling pathway. Also, the quantitative PCR results verified the decreased expression of chemokine in chronic PJI bone tissues.

Chemokine is a cytokine or protein secreted by leucocytes or connective tissue cells, which can induce the directional chemotaxis of nearby reaction cells. Chemokines are important for immune system balance, cell recruitment, and the movement of leucocytes under physiological or inflammatory conditions, which play an important physiological role in the biology of leucocytes. For example, CXCL8, also known as interleukin 8, could be secreted by monocytes, T cells, and endothelial cells. CXCL18 is the main neutrophil chemotactic factor in the human body and the main mediator of inflammation. A previous study has shown that CXCL8 could combine with G protein-coupled receptors CXCR1 and CXCR2 expressed on the surface of neutrophils, mast cells, and $CD8^+$ T cells. After the interaction initiated, they could further recruit neutrophils and activate neutrophil activity.[28] Granulocyte chemotactic protein 2 (GCP-2)/CXCR6 is a CXC chemotactic factor expressed in macrophages, epithelial cells, and mesenchymal cells under inflammation. It could also play a role in recruiting and activating neutrophils by binding to CXCR1 and CXCR2 receptors. In addition, studies have shown that CXCR6 also has antibacterial activity, which is beneficial for skin mucosa to clear microorganisms.[29]

Neutrophils and macrophages are the main reactive cells of *S. aureus* infections. Within a few minutes of *S. aureus* invasion, neutrophils are recruited to the infected site under the action of chemokines and other cytokines, and kill the invading *S. aureus* by phagocytizing the *S. aureus*, secreting antibacterial peptides and reactive oxygen species. Finally, neutrophils undergo programmatical death and are cleared by macrophages. At the same time, neutrophils can secrete cytokines and chemokines to recruit and activate more neutrophils and other immune cells to kill invading pathogens.[30,31] Therefore, chemokines play an important role in the recruitment and activation of neutrophils after *S. aureus* infection.

In this study, transcriptomic profile analysis showed that decreased expression of chemokines such as CCL8 and CXCL6 occurred in chronic PJI bone tissues. Bioinformatics analysis also showed the differentially expressed genes mainly involved in chemokine-related pathways. Also, PCR confirmed the lower expression of typical chemokines such as CXCL8 and CCL5 in chronic PJI bone tissues. Based on our findings, we conclude that the mechanism of *S. aureus* SCV intraosseous colonization might be described as the following: compared to infecton by *S. aureus* normal phenotype, the expression levels of chemokines in the infected site were lower during infection by *S. aureus* SCV, thus lowering the recruitment and activation of neutrophils and other immune cells, making *S. aureus* unable to be effectively cleared by the host and producing colonization of bone tissues for a long period of time, resulting in chronic PJI.

In summary, *S. aureus* SCV has strong potential for intracellular invasion and intraosseous colonization, and its mechanism might be that the expression level of host chemokines is low after infection by *S. aureus* SCV, resulting in immune cells such as neutrophils not being effectively recruited to clear *S. aureus*, with the consequence that *S. aureus* remains and colonizes the host for a long time.

Supplementary material

Table showing primer sequences used in this study. An ARRIVE checklist is also included to show that the ARRIVE guidelines were adhered to in this study.

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