Circular RNA circIKBKB promotes breast cancer bone metastasis through sustaining NF-κB/bone remodeling factors signaling

Yingru Xu1,2†, Shuxia Zhang1,2†, Xinyi Liao1,2, Man Li1,2, Suwen Chen1,2, Xincheng Li1,2, Xingui Wu1,2, Meisongzhu Yang1,2, Miaoling Tang1,2, Yameng Hu1,2, Ziwen Li1,2, Ruyuan Yu1,2, Mudan Huang1,2, Libing Song1,3 and Jun Li1,2*

Abstract

Background: Breast cancer (BC) has a marked tendency to spread to the bone, resulting in significant skeletal complications and mortality. Recently, circular RNAs (circRNAs) have been reported to contribute to cancer initiation and progression. However, the function and mechanism of circRNAs in BC bone metastasis (BC-BM) remain largely unknown.

Methods: Bone-metastatic circRNAs were screened using circRNAs deep sequencing and validated using in situ hybridization in BC tissues with or without bone metastasis. The role of circIKBKB in inducing bone pre-metastatic niche formation and bone metastasis was determined using osteoclastogenesis, immunofluorescence and bone resorption pit assays. The mechanism underlying circIKBKB-mediated activation of NF-κB/bone remodeling factors signaling and EIF4A3-induced circIKBKB were investigated using RNA pull-down, luciferase reporter, chromatin isolation by RNA purification and enzyme-linked immunosorbent assays.

Results: We identified that a novel circRNA, circIKBKB, was upregulated significantly in bone-metastatic BC tissues. Overexpressing circIKBKB enhanced the capability of BC cells to induce formation of bone pre-metastatic niche dramatically by promoting osteoclastogenesis in vivo and vitro. Mechanically, circIKBKB activated NF-κB pathway via promoting IKKβ-mediated IκBα phosphorylation, inhibiting IκBα feedback loop and facilitating NF-κB to the promoters of multiple bone remodeling factors. Moreover, EIF4A3, acted acting as a pre-mRNA splicing factor, promoted cyclization of circIKBKB by directly binding to the circIKBKB flanking region. Importantly, treatment with inhibitor eIF4A3-IN-2 reduced circIKBKB expression and inhibited breast cancer bone metastasis effectively.

Conclusion: We revealed a plausible mechanism for circIKBKB-mediated NF-κB hyperactivation in bone-metastatic BC, which might represent a potential strategy to treat breast cancer bone metastasis.

Keywords: Bone metastasis, Breast cancer, circIKBKB, Bone remodeling factor, NF-κB

Background

Female breast cancer (BC) has become the leading cause of global cancer incidence worldwide [1]. The majority of BC fatalities are due to complications from recurrent or tumor metastases in distant organs [2, 3]. Thereinto, the most common site of BC metastasis is the bone, occurring in 70% of patients with metastatic BC [4, 5].
Currently, the clinical interventions for patients with BC-bone metastasis (BC-BM) are bisphosphonates (BPs) or denosumab (an anti-receptor activator of nuclear factor kappa B ligand (RANKL) monoclonal antibody), which disrupt the vicious cycle by targeting osteoclasts [6]. However, these drugs have no effect on the patient survival rate and also cause severe side effects, even leading to BC metastasis to visceral organs [7–9]. Therefore, understanding the mechanisms of BC-BM is essential to develop innovative therapeutic strategies and improve patient outcomes.

BC-BMs mostly are osteolytic metastases that are characterized by aberrant bone destruction caused by increased osteoclasts-mediated bone resorption in the tumor-bone interface [10]. Accumulating evidence has proven that cancer-secreted factors play direct or indirect roles in the activation of osteoclasts, i.e., to absorb the bone matrix in bone compartments, which establishes a “bone pre-metastatic niche” to support cancer bone metastasis [5, 9, 11–13]. In turn, the degraded bone matrix released various bone matrix-bound growth factors that benefit the seeding and expansion of metastatic tumor cells in bone, which forming a “vicious cycle” [9].

Hyperactivation of nuclear factor kappa B (NF-κB) signaling has been reported to be involved in cancer bone-metastasis via upregulation of multiple bone-remodeling factors, such as RANKL, parathyroid hormone-related protein preproprotein (PTHrP), macrophage colony stimulating factor (M-CSF) and granulocyte–macrophage colony stimulating factor (GM-CSF) [14–18]. Systemically blocking NF-κB activity via 1κB kinase (IKK) inhibitors or nondegradable NF-κB inhibitors alpha (IκBα) dramatically inhibited osteolytic bone metastasis of BC in vivo [18]. However, systemic inhibition of NF-κB activity might also promote tissue injury via inducing cell apoptosis and could even promote hepatocarcinogenesis by inducing reactive oxygen species (ROS) accumulation and liver damage [19, 20]. Therefore, investigating the mechanism of NF-κB hyperactivation in BC would provide effective target for BC-BM treatment.

Circular RNAs (circRNAs) are a class of widespread circular RNAs that are covalently closed single stranded circular transcripts formed by precursor mRNA backsplicing or skipping events, which have no 5’ caps or 3’ poly(A) tail [21]. Clinical evidence and animal studies have demonstrated that aberrant expression of circRNAs contribute to carcinogenesis and development of BC [22–24]. In the present study, we identified a novel circRNA, circIKBKB (hsa_circ_0084100), derived from the IKKKB gene (encoding inhibitor of NF-κB kinase subunit beta), which plays an important role in promotion of osteoclastogenesis and BC-BM through inducing bone pre-metastatic niche formation via specific upregulation of multiple bone remodeling factors. Importantly, treatment with an inhibitor of eukaryotic translation initiation factor 4A3 (EIF4A3), the circIKBKB cyclization factor, reduced circIKBKB expression and effectively inhibited BC-BM. Taken together, our results uncover a plausible mechanism underlying the intrinsic metastatic property of BC to bone and might represent an attractive therapeutic target in the treatment of BC-BM.

Methods

Patient information

This study, which complied with all relevant ethical regulations for work with human participants, was conducted on a total of 20 tumor-adjacent normal breast tissues and 331 paraffin-embedded BC samples, including 295 primary BC tissues (237 non-BM/BC and 58 BM/BC) and 36 bone-metastatic BC tissues (at bone), that were histopathologically and clinically diagnosed at the Third Affiliated Hospital and Sun Yat-sen University Cancer Center from 2005 to 2018. The study protocols were approved by the Institutional Research Ethics Committee of Sun Yat-sen University for the use of these clinical materials for research purposes. All Patients’ samples were obtained according to the Declaration of Helsinki and each patient signed a written informed consent for all the procedures.

RNA sequencing of circRNA extracted from human BC tissues

The total RNA was extracted from six BC (without BM) and six BC (with BM) tissues using TRIzol reagent (Takara, Dalian, China) and further purified by rRNA depletion, followed by cDNA synthesis and RNA amplification, according to the manufacturer’s instructions. The RNA-seq libraries were constructed and sequenced utilizing the Illuma HiSeq2500 platform (Illumina, San Diego, USA).

ASO-mediated knockdown and ASO in vivo treatment

The antisense oligonucleotides (ASOs) targeting circIKBKB were obtained from Ribobio (Guangzhou, China). Transfections with ASOs (50 nM) were performed with Lipofectamine RNAiMAX according to the manufacturer’s instructions. RNA and protein were harvested for analysis 72 h after transfection. For in vivo treatment experiment, 10 nmol ASOs were delivered into mouse through tail vein injection twice a week.

Xenografted tumor models

All of the animal procedures were approved by the Sun Yat-sen University Animal Care Committee. Intracardiac injections of luciferase-expressing BC cells (1 × 105) were conducted in nu/nu nude mice (5 weeks old) for bone metastasis assays. IKK-I VI (1 mg/kg, iv) or eIF4A3-IN-2
We then further examined the circular characteristics of circIKBKB. As shown in Fig. S1a-e, sanger sequencing indicated that the back-splice junction of circIKBKB was from exon 5 to exon 3 of IKBKB, reverse transcription PCR (RT-PCR) analyses using convergent and divergent primers showed that circIKBKB was only amplified by divergent primers from cDNA but not from gDNA. Using random or oligo DT primers indicated circIKBKB had no poly-A tail. In addition, circIKBKB was more stable than linear IKBKB mRNA upon RNase-R/actinomycin D treatment.

The expression of circIKBKB was then examined using in situ hybridization (ISH), with a specific probe targeting the junction sequence of circIKBKB, in 20 normal breast tissues and 331 clinical BC tissues, including 295 primary BC tissues (237 non-BM/BC and 58 BM/BC) and 36 bone-metastatic BC tissues (at bone). As shown in Fig. 1b, circIKBKB signals were undetectable in normal breast tissues, and only marginally detectable in primary non-BM/BC tissues but increased in primary BM/BC tissues (with BM) and were elevated markedly in bone-metastatic BC tissues (at bone). Importantly, ISH statistical analysis revealed that patients with high circIKBKB-expressing BC had significantly shorter bone-metastasis-free survival than those with low circIKBKB-expressing BC ($P < 0.001$; Fig. 1c). Taken together, these results suggest that circIKBKB overexpression correlates with BC-BM.

To determine the role of circIKBKB in BC-BM, we investigated the effect of silencing circIKBKB by using antisense oligonucleotide (ASO) targeting the circIKBKB junction sequence, on bone-metastasis of SCP2 cells, which is a bone-tropic breast cancer cell line with high circIKBKB expression compared to low bone-metastatic breast cancer cell lines, including MCF7, SKBR3, MDAMB-231 and 4175 (Fig. 1d). The quantitative real-time PCR (qRT-PCR) showed that circIKBKB-ASO treatment significantly reduced expression of circIKBKB, but not IKBKB mRNA, in bone-metastatic tumors (Fig. 1f).

Prominently, compared to ASO-control treated mice, circIKBKB-ASO treatment resulted in less and delayed osteolytic bone metastasis of breast cancer in vivo. a H&E analysis (left) and Volcano plot analysis (right) of dysregulated circRNAs from circRNAs deep sequencing comparing BC tissues with or without bone metastasis ($n = 6$). b ISH analysis (left) and quantification (right) of circIKBKB expression in 20 normal breast tissues and 331 clinical BC tissues, including 295 primary BC tissues (237 non-BM/BC and 58 BM/BC) and 36 bone-metastatic BC tissues (at bone). c Kaplan–Meier analysis of bone metastasis-free survival curves in patients with BM/BC with low vs high expression of circIKBKB ($n = 58$; $P < 0.001$; log-rank test). d Real-time PCR analysis of circIKBKB expression in the indicated cells. GAPDH served as a loading control. e Normalized BLI signals of bone metastases and Kaplan–Meier bone metastasis-free survival curve of mice from the indicated experimental groups ($n = 8$/group). f Left: BLI, μCT (longitudinal and trabecular section) and histological (H&E and TRAP staining) images of bone lesions from representative mice. Scale bar, 50 μm. Right: Quantification of circIKBKB and IKBKB expression and μCT osteolytic lesion area and TRAP $+$ osteoclasts along the bone-tumor interface of metastases from experiment in the left panel. g Quantification of bone parameters from representative mice in (f). BV/TV, bone/tissue volume ratio; BS/TV, bone surface/ tissue volume ratio, Tb. n, trabecular number; Tb. sp, trabecular separation; Tb. th, trabecular thickness; TBPf, trabecular bone pattern factor. Each error bar represents the mean ± SD of three independent experiments. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.
Fig. 1 (See legend on previous page.)
incidence of bone metastases and significant reduction of metastasis burden in SCP2 cell-injected mice (Fig. 1e, f). Statistical analysis of micro-computed tomography (μCT) revealed that circIKBKB-ASO-treated mice displayed significantly decreased BM lesions/osteolytic areas, accompanied with relatively increased the volume/number/thickness of trabecular and reduced trabecular separation and bone pattern factor (Fig. 1g). Importantly, we found that circIKBKB-ASO treatment significantly reduced the tartrate-resistant acid phosphatase (TRAP)$^+$-osteoclasts along the bone-tumor interface compared to those in control mice (Fig. 1f). Taken together, these results indicate that silencing circIKBKB inhibits BC-BM.

**CircIKBKB overexpression promotes osteolytic bone metastasis of breast cancer in vivo.**

To further investigate the effect of circIKBKB overexpression on BC-BM, BC cell line MCF-7, with low circIKBKB expression, and MDA-MB-231, with moderate circIKBKB expression, were established to stably express circIKBKB and firefly luciferase reporter (Fig. 1d). Ectopically expressing circIKBKB did not alter the level of IKBKB mRNA in these BC cell lines (Fig. S2a).

In vivo bone metastasis model monitored by bioluminescence imaging (BLI) showed that the mice intracardially injected with circIKBKB-overexpressing BC cells exhibited earlier bone metastatic onsets and larger bone metastatic tumor-burden (Fig. 2a, b). μCT analysis indicated that, compared to the vector-control mice, circIKBKB/mice displayed larger osteolytic bone lesions together with significant modulation of bone parameters, such as decreased trabecular volume/number/thickness and increased trabecular separation/bone pattern factor (Fig. 2b and Fig. S2b). Meanwhile, we observed dramatically increased numbers of TRAP$^+$-osteoclasts represented along the bone-tumor interface in circIKBKB/mice (Fig. 2b), suggesting that circIKBKB-overexpressing BC cells might possess a strong capability to induce the formation of bone pre-metastatic niche.

**Overexpression of circIKBKB in BC cells induces osteoclastogenesis.**

Consistent with the abovementioned hypothesis, we observed that the numbers of TRAP$^+$-multinucleated mature osteoclasts and TRAP enzymatic activity were drastically increased in pre-osteoclasts (pre-OC) treated with conditioned media (CM) from BC/circIKBKB cells (CM-BC/circIKBKB) (Fig. 2c). However, stimulated with CM-BC/circIKBKB had no effect on the differentiation of pre-osteoblasts (pre-OB), as indicated by no obvious change in the number of ALP$^+$-osteoblasts and RANKL/OPG ratio (Fig. 2c and Fig. S2c). These results indicate that overexpressing circIKBKB in BC cells induces osteoclastogenesis. Indeed, we found that CM-BC/circIKBKB treatment induced the expression of multiple osteoclastogenesis-related markers, including FBJ osteosarcoma oncogene (C-fos), acid phosphatase 5, tartrate resistant (Acps), calpepsin K (Ctsk), nuclear factor of activated T cells 1 (Nfat-c1) and dendrocyte expressed seven transmembrane protein (Dc-stamp), and facilitated the fusion of pre-osteoclasts, accompanying with increased podosome (actin ring) formation (Fig. S2d and Fig. 2d).

Importantly, bone resorption assay showed that CM-BC/circIKBKB-treated osteoclasts possessed higher bone-resorbing activity (Fig. 2e), resulting in an elevated the level of bone matrix-released transforming growth factor beta (TGF-β) and consequently promoted BC cells proliferation (Fig. S2e). Taken together, these results indicate that upregulation of circIKBKB in BC cells induces osteoclastogenesis.

**Silencing circIKBKB in BC cells reduces osteoclastogenesis.**

Consistent with in vivo results that circIKBKB-ASO treatment decreased the number of TRAP$^+$-osteoclasts along the bone-tumor interface in SCP2/mice (Fig. 1g), silencing circIKBKB significantly reduced the inductive effect of CM/SCP2 cells on osteoclastogenesis, as indicated by reduced TRAP$^+$-multinucleated mature osteoclasts and TRAP enzymatic activity, as well as decreased the expression of osteoclastogenesis-related markers.
Fig. 2 (See legend on previous page.)
Correspondingly, the stimulatory effects of CM/SCP2 on the fusion events and bone-resorbing activity of osteoclasts were abolished by circIKBKB downregulation (Fig. S3d, e). These results provide further evidence that circIKBKB plays a crucial role in induction of osteoclastogenesis.

Overexpression of circIKBKB activates NF-κB signaling pathway in BC cells
To further clarify the mechanism underlying circIKBKB-mediated osteoclastogenesis, signal finder signal transduction 45-pathway reporter array was performed in vector- and circIKBKB-transduced BC cells. Strikingly, the NF-κB transcriptional activity was most significantly induced in both circIKBKB-overexpressing cells compared to control cells (Fig. 3a), suggesting that circIKBKB might be involved in modulation of NF-κB pathway. In line with this hypothesis, overexpressing circIKBKB also significantly increased, but silencing circIKBKB decreased, the NF-κB-driven luciferase activity, the NF-κB/DNA binding activity, the NF-κB nuclear level and the level of K48-linked polyubiquitinated IκBα (Fig. 3a-f). However, the promotive effect of circIKBKB on NF-κB pathway was drastically abrogated by ectopically expressing a specific NF-κB inhibitor (IκBα-mu) (Fig. S4a). These results suggest that circIKBKB overexpression activates NF-κB pathway in BC cells.

Blocking NF-κB inhibits circIKBKB-induced osteoclastogenesis and bone metastasis
Next, we examined the contribution of NF-κB signaling to circIKBKB-induced osteoclastogenesis and bone metastasis. We first tested the effect of IKK-β inhibitor VI (IKK2-I VI), which dramatically inhibited the NF-κB pathway in SCP2 and MDA-MB-231/circIKBKB cells (Fig. S4b). In vitro experiments showed that compared to vehicle treatment, IKK2-I VI treatment considerably abolished the inductive effect of CM from SCP2 and MDA-MB-231/circIKBKB cells on osteoclastogenesis and bone-resorbing activity (Fig. 3g-h). Furthermore, we found that IKK2-I VI treatment significantly decreased the incidence of bone metastasis, reduced the size and number of osteolytic bone lesions and lessened the number of TRAP⁺-osteoclasts along the bone-tumor interface (Fig. 3i and Fig. S4c, d). Similar to effect of IKK2-I VI, blocking NF-κB via overexpression of IκBα-mu also showed inhibitory effects on circIKBKB-induced osteoclastogenesis and bone metastasis (Fig. S4e-h). Therefore, these data demonstrate that NF-κB signaling activation is essential for circIKBKB-induced osteoclastogenesis and bone metastasis.

CircIKBKB activates NF-κB pathway both in cytoplasm and nuclear
To investigate the mechanism underlying circIKBKB-mediated NF-κB activation, we first examined the circIKBKB localization using fluorescence in situ hybridization (FISH) and subcellular fractionation assays. As shown in Fig. 4a, circIKBKB was distributed in both cytoplasm and nucleus. Interestingly, RNA pull-down assay following mass spectrometry (MS) showed that circIKBKB interacted with multiple key components in NF-κB signaling, including IκKα, IκKβ, IKKγ, p65, p50 and IκBα (Fig. 4b). However, RNA pull-down assays revealed that cytoplasmic circIKBKB could interact with both IκKα/IκKβ/IκKγ and p65/p50/IκBα complexes, whereas nuclear circIKBKB only interacted with p65/p50 complex (Fig. 4c), suggesting that circIKBKB might regulate NF-κB signaling in both cytoplasm and nuclear. Furthermore, in vitro binding assays showed that circIKBKB directly interacted with recombinant p65 and active IKKβ, but not with other recombinant proteins, suggesting that circIKBKB activated the NF-κB pathway through direct interaction with p-IKKβ and p65 (Fig. 5a). Consistent with this hypothesis, silencing IκKβ abrogated the interaction of circIKBKB with IκKα/IκKβ/IκKγ and p65/p50/IκBα complexes, whereas IκKβ abolished the association of circIKBKB with IκKα/IκKγ and IκKβ and silencing p65 abolished the association of circIKBKB with p50 and IκBα (Fig. 5b). RNA pull-down assays further demonstrated that circIKBKB interacted with constitutively active IκKβ (177E/181E) even without TNFα treatment but not with
Fig. 3 (See legend on previous page.)
the kinase-deficient IKKβ (177A/181A) mutant in TNFα-treated cells (Fig. 4d).

Moreover, we found that p-IKKβ could form complex with circRNA formed by IKKBK exon 3/4 or exon 3/5, but not with exon 4/5, indicating that exon 3 of circIKKBK was the binding region with IKKβ (Fig. 4e). However, deletion of either exon 3 or 5 abolished the circIKKBK/p65 interaction. These results suggest that the exon 3–5 back-splice junction is necessary for the circIKKBK/p65 complex formation (Fig. 4e).

CircIKKBK sustains NF-κB signaling via inhibition of IκBα negative feedback

Since RNA pull-down assays showed that the N-terminal DNA-binding domain of p65, which is the IκBα-binding region, was the region that bound to circIKKBK (Fig. 4f). We speculated that circIKKBK and IκBα might competitively interact with NF-κB. Consistent with previous reports that IκBα forms a complex with NF-κB that removes NF-κB from DNA and translocates NF-κB back to the cytoplasm [25–27], overexpressing circIKKBK decreased the p65/IκBα complex formation, increased the level of DNA-bound NF-κB and persisted the TNF-α-induced nuclear accumulation of NF-κB. The opposite effects were observed in circIKKBK-silenced cells (Fig. 4g-i, Fig. S5c, d). Moreover, electrophoretic mobility shift assay (EMSA) indicated that the endogenous NF-κB DNA-binding activity after TNF-α treatment was significantly prolonged in circIKKBK-transduced cells compared to vector-control cells (Fig. 4j). Collectively, our results demonstrate that circIKKBK overexpression sustains NF-κB activity in BC cells.

CircIKKBK recruits NF-κB to the promoters of multiple bone remodeling factors

Next, we sought to identify the potential factors involved in circIKKBK-induced osteoclastogenesis and bone metastasis. qPCR analysis revealed that among total 25 bone remodeling factors, the mRNA levels of M-CSF and GM-CSF, which are also downstream targets of bone remodeling factors, the mRNA levels of M-CSF and GM-CSF were elevated in the CM from BC/circIKKBK cells and reduced in the CM from circIKKBK-silenced cells (Fig. S6b, c). Importantly, blocking NF-κB via overexpressing IκBα-mu drastically inhibited the induced effects of circIKKBK on M-CSF and GM-CSF expression (Fig. S6c, d). These results further support the crucial role of NF-κB in circIKKBK-induced osteoclastogenesis and bone metastasis.

Although overexpressing circIKKBK increased and silencing circIKKBK reduced the enrichment of NF-κB/p65 on M-CSF and GM-CSF promoters, ablating p65 did not change the level of circIKKBK on M-CSF and GM-CSF promoter (Fig. 5b, c). As we already demonstrated that circIKKBK was associated with NF-κB in the nuclear (Fig. 4c). Therefore, we hypothesized that circIKKBK might bind and recruit NF-κB to M-CSF and GM-CSF promoters. Analysis using the JASPAR (http://jaspar.genereg.net) and RNAInter (http://www.rna-society.org/rnainter/IntaRNA.html) platforms showed that the binding sites of circIKKBK and NF-κB on either M-CSF promoter or GM-CSF promoter are very close to each other and near to transcriptional start site (TSS), which was further confirmed by ChIP and ChIRP assays (Fig. 5d, e). Furthermore, we found that either using RNA interference (RNAi)-mediated silencing of M-CSF or GM-CSF or treatment with neutralizing anti-M-CSF or anti-GM-CSF antibody significantly abolished the inductive effect of circIKKBK on osteoclastogenesis and the bone-resorbing activity of osteoclasts (Fig. 5f, g and Fig. S6e, f). Therefore, these results indicate that circIKKBK recruits NF-κB to upregulates multiple bone remodeling factors, consequently inducing the formation of bone-metastatic environment.

EIF4A3 increases circIKKBK expression via direct promotion of circIKKBK cyclization

To identify the potential factor(s) involved in circIKKBK cyclization, an RNA pull-down assay was performed using circIKKBK pre-mRNA, which was prepared via in vitro transcription, and followed the mass
Fig. 4 (See legend on previous page.)
spectrum-based proteomics analysis (Fig. 6a). All together 35 proteins were identified to be potent circIKBKB pre-mRNA-interacting proteins, including 3 pre-mRNA splicing factors, which were PTBP1, EIF4A3 and FUS (Fig. 6a). Further qRT-PCR analyses revealed that silencing EIF4A3 significantly reduced, but overexpressing EIF4A3 increased, the circIKBKB expression in BC cells (Fig. S7a, b and Fig. 6b, c). Moreover, overexpressing EIF4A3 did not affect the expression level of IκBKB (Fig. 6c), suggesting that EIF4A3 might be involved in circIKBKB cyclization.

We then examined whether EIF4A3 directly interacted with circIKBKB pre-mRNA. Circinteractome (https://circinteractome.nia.nih.gov/index.html) analysis showed 8 putative binding sites of EIF4A3 in the upstream and downstream regions of circIKBKB pre-mRNA (Fig. 6d). RNA immunoprecipitation (RIP) assays indicated that EIF4A3 was only associated with the putative binding sites near to exon 3 and exon 5 in circIKBKB pre-mRNA (Fig. 6d). These results were confirmed by RNA pulldown assays using in vitro transcript RNA fragments of the circIKBKB pre-mRNA (Fig. 6e). Therefore, these results demonstrate that EIF4A3 directly binds to the circIKBKB pre-mRNA and induces circIKBKB cyclization.

**EIF4A3 promotes osteoclastogenesis through circIKBKB/NF-κB signaling**

Consistent with the inductive effect of EIF4A3 on circIKBKB expression as showed in above, overexpressing EIF4A3 significantly activated NF-κB signaling, as showed by increased NF-κB-luciferase activity and nuclear NF-κB level, and enhanced capability of BC cell to induce osteoclastogenesis, as indicated by elevated M-CSF and GM-CSF expression and the number of TRAP⁺-cells (Fig. 6f-i and Fig. S7c, d). However, the promotive effect of EIF4A3 on NF-κB signaling and osteoclastogenesis was dramatically abolished by either overexpressing IκBα-mu or silencing circIKBKB (Fig. 6f-i and Fig. S7c, d). Taken together, these results indicate that overexpression EIF4A3 promotes osteoclastogenesis through circIKBKB/NF-κB signaling.

**EIF4A3 overexpression correlates with breast cancer bone-metastasis**

To further confirm whether EIF4A3 contributed to BC bone-metastasis clinically, IHC staining was performed in 20 normal breast tissues and 331 clinical BC tissues, including 295 primary BC tissues (237 without BM and 58 with BM) and 36 bone-metastatic BC tissues (at bone). As shown in Fig. 7a, compared to normal breast tissues, EIF4A3 expression was moderately increased in BC tissues (without BM) and primary BC tissues (with BM) but strongly elevated in bone-metastatic BC tissues (at bone). Importantly, patients with high EIF4A3-expressed BC had significantly shorter bone-metastasis-free survival than those with low EIF4A3-expressed BC (P < 0.001; Fig. 7b). These results suggest that EIF4A3 overexpression is clinically associated with BC bone-metastasis.

Moreover, we examined whether the abovementioned EIF4A3/circIKBKB/NF-κB axis identified in the in vitro and in vivo studies was clinically relevant in bone-metastatic BC tissues. The expression of circIKBKB was examined by ISH assay and the expression of NF-κB p65, M-CSF and GM-CSF were examined by IHC assay in 36 bone-metastatic BC tissues. Statistical analysis revealed that the EIF4A3 level was strongly correlated with the expression of circIKBKB (P < 0.001), nuclear p65 (P < 0.001), M-CSF (P < 0.001) and GM-CSF (P = 0.003). These data provided clinical evidence that the EIF4A3/circIKBKB/NF-κB axis-mediated upregulation of M-CSF and GM-CSF induced bone pre-metastatic niche formation, and consequently resulted in bone metastasis and poorer clinical outcomes in human breast cancer (Fig. 7c).

**Blocking EIF4A3 inhibits circIKBKB/NF-κB signaling and suppresses osteoclastogenesis**

Consistent with the biological role of EIF4A3 in activation of circIKBKB/NF-κB signaling and the induction of osteoclastogenesis as shown in above, downregulation of EIF4A3 expression via RNAi technology or inhibition of EIF4A3 activity via treatment with eIF4A3-IN-2, a highly selective and noncompetitive EIF4A3 inhibitor,
Fig. 5 (See legend on previous page.)

- **a** circIKBKB vs Vector
- **b** ChIP: p65
  - M-CSF
  - circIKBKB
  - MDA-MB-231
- **c** SCP2
  - M-CSF promoter
  - GM-CSF promoter
- **d** circIKBKB
  - p65 binding site
  - circIKBKB binding site
- **e** circIKBKB
  - p65 binding site
  - circIKBKB binding site
- **f** Scramble, M-CSF si, GM-CSF si, M-CSF si + GM-CSF si
  - CM from MDA-MB-231/circIKBKB
- **g** CM from MDA-MB-231/circIKBKB
  - Vehicle, M-CSF si
  - No. of resorption pit/ bone slice
reduced circIKBKB expression and NF-κB-driven luciferase activity, and decreased M-CSF and GM-CSF in both transcriptional and translational levels (Fig. S8a-d). Meanwhile, we also observed that EIF4A3 inhibition also significantly decreased the capability of BC cells to induce osteoclastogenesis, as indicated by reduction the number of TRAP$^+$-multinuclear osteoclasts and TRAP activity and lessened bone-resorbing activity (Fig. S8e).

Pharmaceutical inhibition of EIF4A3 blocks bone-metastasis of BC cells in vivo

Finally, the in vivo effect of inhibition of EIF4A3 via eIF4A3-IN-2 treatment on BC-BM was examined. We first tested whether eIF4A3-IN-2 treatment could inhibit BC-BM, which eIF4A3-IN-2 treatment was started at 2 days after intracardial injection of SCP2 cells. As shown in Fig. S8f and g, eIF4A3-IN-2 treatment significantly delayed bone metastasis and reduced the onsets of bone-metastases and bone-metastasis burden compared to vehicle treatment. The eIF4A3-IN-2-treated mice displayed less osteolytic areas and number of TRAP$^+$-osteoclasts in bone surface area (Fig. 7d). These results suggested that eIF4A3-IN-2 treatment might prevent BC-BM. Furthermore, we examined the therapeutic effect of eIF4A3-IN-2 on BC-BM, which eIF4A3-IN-2 treatment was started when bioluminescence signal of bone-metastatic tumors reached $2 \times 10^5$ p/sec/cm$^2$/sr (Fig. 7e). After 5 weeks treatment, the vehicle-treated mice exhibited rapid progress of bone metastasis, which showed more bone metastases and larger bone-metastatic tumor burden, accompanying with severe osteolytic bone lesions and higher numbers of TRAP$^+$-osteoclasts along the bone-tumor interface. Strikingly, eIF4A3-IN-2 treatment dramatically reduced the onsets of bone metastases and decreased bone-metastatic tumor burden compared to vehicle treatment (Fig. 7e). Therefore, our results demonstrate that pharmaceutical inhibition of EIF4A3 not only prevents the initiation of BC-BM but also suppresses the progression of BC to bone-metastasis.

Discussion

The overall survival of patients with BC has been improving significantly for the past two decades due to the marked progress in diagnostic techniques, multidisciplinary treatments, and implementation of surveillance programs. However, this concurrently provided time for tumor progression within the extramammary organs and the formation of metastatic foci at distant sites. The most common site of metastasis is the bone, which occurs in 65–80% of patients with metastatic breast cancer. Bone metastases not only dramatically reduces life expectancy to just 2–3 years following diagnosis but also severely affects the quality of life of patients by inducing skeletal-related events (SREs). However, bone metastasis of BC appears incurable clinically and the 5-year survival rates for these patients are below 25%. Currently, the main therapeutic interventions are focusing on disruption of the osteolytic cycle by targeting osteoclasts to extenuate and prevent SREs. Bisphosphonates and denosumab have been used to treat patients with BC-BM and could delay of the onset of SRES in patients [28] but also cause severe side effects, such as osteonecrosis of the jaw and hypercalcaemia [29, 30]. Therefore, the development of novel therapeutic strategies for treating patients with BC-BM is urgently required. Herein, we reported that blocking EIF4A3 by eIF4A3-IN-2 significantly decreased circIKBKB expression and effectively prevented the initiation of BC-BM and also suppressed the progression of BC-BM. Therefore, our results might represent a new strategy for the treatment of BC-BM.

Breast cancer cells preferentially metastasize to specific organs, known as “organotropic metastasis”. The formation of primary tumors-induced microenvironment in distant organs/tissue sites, named pre-metastatic niche, has been demonstrated to be critical for subsequent engraftment and survival of metastatic cells, even determining metastatic organotropism [31, 32]. Consistently, tumor-produced cytokines have been proven to promote osteoclast-mediated bone resorption directly or indirectly, resulting in the release of bone matrix-stored...
Fig. 6 (See legend on previous page.)
growth factors, which further expanding tumors and generating a “vicious cycle” that supports bone metastasis [33, 34]. These studies suggest that identification of the specific factors that induce the formation of the bone pre-metastatic niche would be helpful for determining metastatic development before bone metastasis formation. In this study, we found that expression of GM-CSF and M-CSF, among 25 previous reported bone remodeling factors, was most significantly increased in conditioned media from circIKBKB-overexpressing breast cancer cells. Importantly, treatment with neutralizing anti-M-CSF or anti-GM-CSF antibodies significantly abolished the inductive effect of circIKBKB on osteoclastogenesis and the bone-resorbing activity of osteoclasts. These results indicated that GM-CSF or M-CSF were the key factors in conditioned media to exert on osteoclastogenesis. Consistent with our findings, Park BK et al. reported that GM-CSF was a key factor to promote osteolytic bone metastasis of breast cancer by stimulating osteoclast development [18]. Meanwhile, it has been also reported that M-CSF protein secreted from breast cancer cells also contributed to osteoclast formation and repression of M-CSF suppressed osteoclastogenesis and tumor-induced osteolysis in an orthotopic breast cancer bone metastasis mouse model [17, 35]. We further demonstrated that circIKBKB could directly associate with the promoters of M-CSF and GM-CSF and upregulate M-CSF and GM-CSF levels. Thus, our results unveiled a decisive role of circIKBKB in promotion of bone metastasis by inducing pre-metastatic niche formation and suggested that circIKBKB might be a useful biomarker for evaluating bone metastasis in BC.

Interestingly, as downstream target of transcriptional factor NF-κB [36, 37], either M-CSF or GM-CSF could also positively feedback to activate NF-κB signaling pathway [38, 39], which suggested that M-CSF and GM-CSF might regulate each other. Indeed, two previous studies have reported that GM-CSF treatment could upregulate endogenous M-CSF in human monocytes [40, 41]. Herein, we found that overexpressing circIKBKB significantly activated NF-κB pathway, resulting in significant upregulation of M-CSF and GM-CSF, and silencing either M-CSF or GM-CSF or treatment with neutralizing anti-M-CSF or anti-GM-CSF antibody significantly abolished the inductive effect of circIKBKB on osteoclastogenesis and the bone-resorbing activity of osteoclasts. Therefore, we speculated that overexpressing induced M-CSF and GM-CSF expression via activation of NF-κB signaling, and circIKBKB-induced M-CSF and GM-CSF might feeded back to further strengthen the circIKBKB-mediated NF-κB activation, which led to osteoclastogenesis and breast cancer bone metastasis.

Constitutive overactivation of NF-κB signaling has been found in multiple cancer types, including BC. Functioning as natural NF-κB inhibitor, IκBa is also one of the transcriptional targets of NF-κB. The activated NF-κB-mediated resynthesis of IκBa removes NF-κB from chromatin and exports NF-κB back to the cytoplasm, which forms feedback loop to prevent constitutive NF-κB overactivation [25–27]. However, how BC cells override the NF-κB/IκBa negative feedback loop, which maintains the hyperactivated NF-κB, remains unclear. In the current study, we found that circIKBKB interacted with the NF-κB N-terminal, which is the IκBa-binding region. Overexpression of circIKBKB decreased the p65/IκBa complex formation, persisted the TNF-α-induced nuclear accumulation of NF-κB and increased the level of DNA-bound NF-κB, indicated that circIKBKB competitively interacted with N-terminal of NF-κB with IκBa. Thus, our finding represents a novel mechanism in which circRNA mediated NF-κB hyperactivation through disrupting the NF-κB/IκBa negative feedback loop in BC. Considering that hyperactivation of NF-κB frequently found in many types of cancer, it is worthy to further investigate the expression and role of circIKBKB in other cancers.
Fig. 7 (See legend on previous page.)
Conclusions
Disrupting the interaction between the circulating breast cancer cells (seeds) and bone microenvironment (soil), which form a vicious cycle to support metastatic cancer cells proliferation and survival in the bone tissue, will be beneficial for a large group of patients with bone metastasis. Herein, we demonstrated that a novel circRNA circIKBKB played a vital role in inducing bone pre-metastatic niche formation via sustaining NF-κB/bone remodeling factors signaling, consequently leading to breast cancer bone metastasis. Considering the high stability and abundance of circRNA, further investigation of the expression and role of circIKBKB in other cancers will not only provide valuable insights to better understand the mechanism driving the pre-metastatic niche formation but also may develop novel therapeutic strategies for treatment of human cancers.

Abbreviations
BC: Breast cancer; circRNAs: Circular RNAs; BPs: Bisphosphonates; RANKL: Anti-receptor activator of nuclear factor kappa B ligand; BC-BM: BC-bone metastasis; NF-κB: Nuclear factor kappa B; PTHrP: Parathyroid hormone-related protein preproprotein; IL-6: Interleukin 6; M-CSF: Macrophage colony stimulating factor; GM-CSF: Granulocyte–macrophage colony stimulating factor; IKK-β kinase; IkBα: Nuclear factor kappa B inhibitor alpha; ROS: Reactive oxygen species; IKKBK: Inhibitor of nuclear factor kappa B kinase subunit beta; EIF4A3: Eukaryotic translation initiation factor 4A3; non-BM/BC: BC tissues without BM, BM/BC: Bone-metastatic BC tissues; RT-PCR: Reverse transcription PCR; qRT-PCR: Quantitative real-time PCR; RT-PCR: Reverse transcription PCR; ISH: In situ hybridization; ASO: Antisense oligonucleotide; BLC: Bioluminescence imaging; mCT: Micro-computed tomography; TRAP: Tartrate-resistant acid phosphatase; C-fos: FBJ osteosarcoma oncogene; ApcS: Acid phosphatase S, tartrate resistant; Ctsk: Cathepsin K; Nfat-c1: Nuclear factor of activated T cells 1; DC-stamp: Dendrocyte expressed seven transmembrane protein; pre-oc: Pre-osteoclasts; CM: Conditioned media; ALP: Alkaline phosphatase; OPG: Osteoprotegerin; IF: Immunofluorescence; TGF-β: Transforming growth factor beta; IκBα-mu: Nuclear factor kappa B inhibitor; IKK2-IVI: IKK-β inhibitor VI; FISH: Fluorescence in situ hybridization; MS: Mass spectrometry; EMSA: Electrophoretic mobility shift assay; TSS: Transactional start site; ChIP: Chromatin immunoprecipitation; RNAi: RNA interference; RIP-RNA Immunoprecipitation; SRES: Skeletal-related events.

Supplementary Information
The online version contains supplementary material available at https://doi.org/10.1186/s12943-021-01394-8.

Additional file 1: Table S1. The original sequencing results of all differentially expressed circRNAs in six breast cancer (without BM) and six breast cancer (with BM) tissues.

Additional file 2: Table S2. Primers, Probes, siRNA, shRNA and ASOs used in this study.

Additional file 3: Table S3. Pathways of the Cignal Finder 4S-Pathway Arrays.

Additional file 4. Supplementary Methods and Figures.

Acknowledgements
Thanks to Prof. Guohong Hu of the Chinese Academy of Sciences and Shanghai Jiao Tong University School of Medicine for kindly providing the SCP2 and 4175 cell lines. This work was supported by Natural Science Foundation of China; Guangzhou Science and Technology Plan Projects; Natural Science Foundation of Guangdong Province.

Authors’ contributions
YX and SZ contributed equally to this work. YX and SZ designed the experiments and analyzed data. XL and ML performed the xenograft tumor experiments. SC and XL performed in vitro cell line studies. XL, ML and MH performed staining, immunohistochemical and pathological analysis. MY, M.T., Y.H. and Z.L. performed the ChIP ChIRP immunoprecipitation, western blot and real time PCR. RY and X.W. analyzed mass spectrometry data. LS and J.L. supervised the whole study and wrote the paper. The author(s) read and approved the final manuscript.

Funding
This work was supported by Natural Science Foundation of China (No. 820300378, 81830082, 82072609, 81620104 and 82030128), Guangzhou Science and Technology Plan Projects (201803010098), Natural Science Foundation of Guangdong Province (2018B030311009, 2016A030308002, 2018B030311060 and 2019A1515110118).

Availability of data and materials
The datasets used and/or analyzed during the current study are available within the manuscript and its supplementary information files.

Declarations

Ethics approval and consent to participate
All Patients’ samples were obtained according to the Declaration of Helsinki and each patient signed a written informed consent for all the procedures. All of the animal procedures were approved by the Sun Yat-sen University Animal Care Committee.

Consent for publication
Not applicable.

Competing interests
All authors state that there are no competing interests in this study.

Author details
1 Program of Cancer Research, Key Laboratory of Protein Modification and Degradation and Guangzhou Institute of Oncology, Affiliated Guangzhou Women and Children’s Hospital, School of Basic Medical Sciences, Guangzhou Medical University, Guangzhou 510623, China. 2Department of Biochemistry, Zhongshan School of Medicine, Sun Yat-sen University, Guangzhou 510080, China. 3State Key Laboratory of Oncology in South China, Collaborative Innovation Center for Cancer Medicine, Sun Yat-sen University Cancer Center, Guangzhou 510080, China.

Received: 8 April 2021   Accepted: 17 July 2021

Published online: 29 July 2021

References
1. Sung H, Ferlay J, Siegel RL, Laversanne M, Soerjomataram I, Jemal A, Bray F. Global cancer statistics 2020: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA Cancer J Clin. 2021. https://doi.org/10.3322/caac.21660.
2. Weigelt B, Peterse JL, Van’t Veer LJ. Breast cancer metastasis: markers and models. Nat Rev Cancer. 2005;5:591–602. https://doi.org/10.1038/nrc1670.
3. Lu J, Steeg PS, Price JE, Krishnamurthy S, Mani SA, Reuben J, Cristofanilli M, Dontu G, Buda L, Valero V, et al. Breast cancer metastasis: challenges and opportunities. Cancer Res. 2009;69:4951–3. https://doi.org/10.1158/0008-5472.CAN-09-0099.
4. Weibauer KJ, Guise TA, McCauley LK. Cancer to bone: a fatal attraction. Nat Rev Cancer. 2011;11:411–25. https://doi.org/10.1038/nrc3305.
5. Akhtar M, Mansuri J, Newman KA, Guise TM, Seth P. Biology of breast cancer bone metastasis. Cancer Biol Ther. 2008;7:3–9. https://doi.org/10.4161/cbt.7.1.5163.
6. Mundy GR. Metastasis to bone: causes, consequences and therapeutic opportunities. Nat Rev Cancer. 2002;2:584–93. https://doi.org/10.1038/nrc867.
