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Osteoblast derived-neurotrophin-3 induces cartilage removal proteases and osteoclast-mediated function at injured growth plate in rats

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Abstract

Faulty bony repair causes dysrepair of injured growth plate cartilage and bone growth defects in children; however, the underlying mechanisms are unclear. Recently, we observed the prominent induction of neurotrophin-3 (NT-3) and its important roles as an osteogenic and angiogenic factor promoting the bony repair. The current study investigated its roles in regulating injury site remodelling. In a rat tibial growth plate drill-hole injury repair model, NT-3 was expressed

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Study design: YS, XZ and CX. Study conduct: YS. Data collection: YS, SC, LZ, RC, CF, YP, QT, and MH. Data analysis: YS and CX. Data interpretation: YS, SC, BF, LB, SG, DC, YX, LC, CP, XZ, JX, and CX. Drafting manuscript: YS and CX. Revising manuscript content: YS, SC, XZ, JX, and CX. Approving final version of manuscript: YS, SC, LZ, RC, CF, MH, YP, QT, LB, SG, DC, YX, LC, BF, CP, XZ, JX, and CX. YS and CX take responsibility for the integrity of the data analysis.

Disclosure

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prominently in osteoblasts at the injury site. Recombinant NT-3 (rhNT-3) systemic treatment enhanced, but NT-3 immunoneutralization attenuated, expression of cartilage-removal proteases (MMP-9 and MMP-13), presence of bone-resorbing osteoclasts and expression of osteoclast protease cathepsin K, and remodelling at the injury site. NT-3 was also highly induced in cultured mineralizing rat bone marrow stromal cells, and the conditioned medium augmented osteoclast formation and resorptive activity, an ability that was blocked by presence of anti-NT-3 antibody. Moreover, NT-3 and receptor TrkC were induced during osteoclastogenesis, and rhNT-3 treatment activated TrkC downstream kinase Erk½ in differentiating osteoclasts although rhNT-3 alone did not affect activation of osteoclastogenic transcription factors NF-κB or NFAT in RAW264.7 osteoclast precursor cells. Furthermore, rhNT-3 treatment increased, but NT-3 neutralization reduced, expression of osteoclastogenic cytokines (RANKL, TNF-α, and IL-1) in mineralizing osteoblasts and in growth plate injury site, and rhNT-3 augmented the induction of these cytokines caused by RANKL treatment in $RAW_{264.7}$ cells. Thus, injury site osteoblast-derived NT-3 is important in promoting growth plate injury site remodelling, as it induces cartilage proteases for cartilage removal and augments osteoclastogenesis and resorption both directly (involving activing Erk½ and substantiating RANKL-induced increased expression of osteoclastogenic signals in differentiating osteoclasts) and indirectly (inducing osteoclastogenic signals in osteoblasts).

Keywords

Growth plate injury; Injury site remodeling; Signal crosstalk; Neurotrophic factors

1. Introduction

Childhood bone fractures are common with about 50% of children and adolescents unfortunately experiencing a bone fracture [1]. Located at the weakest part of a long bone, the growth plate cartilage (which is responsible for bone growth) is highly prone to injuries (involved in 20% of all fractures) [2]. Yet, the injured growth plate is often “undesirably” repaired by bony tissue, causing bone growth defects (limb length discrepancy and angulations). Currently, correction of these bone defects relies on extremely invasive and sometimes ineffective surgical procedures, and there is a strong need to gain mechanistic understanding underlying the “faulty” repair and develop a preventive treatment [3]. Although previous work in animal models of growth plate repair has identified repair responses (inflammatory, fibrogenic, osteogenic and remodelling) and of some molecular pathways involved [3–18], mechanisms for the faulty repair still require further elucidation.

In a recent study, neurotrophin-3 (NT-3) and its high affinity receptor TrkC were found to be highly induced at the injury site and endogenous NT-3 was found to promote bony repair [18]. Increasing evidence now suggests that, apart from their known functions regulating the development/maintenance of the nervous system, neurotrophins or NTs [including nerve growth factor (NGF), brain-derived growth factor (BDNF), neurotrophin-3 (NT-3) and NT-4] and their receptors [including their common low-affinity p75 receptor and specific high-affinity Trk receptors (TrkA for NGF, TrkB for BDNF & NT-4, and TrkC for NT-3)], also have roles in the bone and cartilage as well as skeletal repair events [18–20]. NTs and receptors are expressed in bones [21] and bone repair cells [18,22,23]. Local application of

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NGF promoted bone healing [24,25]. Recently, in a rat tibial growth plate and bone injury model, we observed increased mRNA expression for neurotrophins NGF, BDNF, NT-3 and NT-4 and their Trk receptors (with NT-3 and its receptor TrkC showing the highest induction) at injury site [18]. Furthermore, NT-3 has been shown to induce the critical osteogenic factor bone morphogenetic protein (BMP-2) and key angiogenic factor vascular endothelial growth factor (VEGF), and NT-3 was found to promote bone formation and vascularization and thus bony repair at the injured growth plate. However, it remains unknown whether and how the injury site-induced NT-3 is involved in regulating the growth plate injury site remodelling.

Previous studies in rat models demonstrated that bony repair at the injured growth plate involves both cartilage and bone formation and osteoclastic remodelling of the newly formed bone trabeculae at the injury site [8,9,12]. During skeletal repair injury site remodelling, cartilage tissue initially formed needs to be removed by osteoclast/chondroclast-mediated resorption and/or degradation by cartilage proteases collagenase-3 (MMP-13) and gelatinase-9 (MMP-9) [26,27]. Then, the initial bone formed and bone converted from cartilage tissue require further remodelling (by resorption and bone formation) [26.28]. Skeletal repair events are known to be tightly regulated by locally produced molecules, such as inflammatory cytokines, osteogenic factors (particularly BMPs [29]), angiogenic factors (particularly VEGF [30]), and osteoclastogenesis regulators [including the major osteoclastogenesis stimulator RANKL (receptor activator of nuclear factor kappa-B ligand) and inhibitor osteoprotegerin (OPG)] [31]. Evidence is emerging that remodelling of the skeletal injury sites is orchestrated by crosstalk between osteoclast precursors/osteoclasts and repair cells (osteoblasts and chondrocytes, which produce cross-talking signals [26,28]). However, further work is required to identify and characterise cross-talk signals that regulate skeletal repair and injury site remodelling.

The current study investigated potential functions and action mechanisms of injury site-derived NT-3 in injury site remodelling in a rat proximal tibial growth plate repair model and in in vitro models of osteoclastogenesis and resorption.

2. Materials and methods

2.1. Rat drill-hole growth plate injury and NT-3 intervention treatments

To investigate roles of NT-3 in injury site remodelling during growth plate injury repair, a proximal tibial drill-hole growth plate injury model was used in both hind legs of 6-week-old male normal Sprague Dawley rats as described [6], with approval from the Animal Ethics Committee of SA Pathology/CHN (South Australia). On days 3 and 7 after injury, some injured rats received intraperitoneal injection of a normal sheep serum IgG or a sheep neutralising anti-NT-3 serum IgG [32] (both at 1 mg/kg per injection) (n=12) as described [18]. On day 3 post-injury, some injured rats received subcutaneous implantation (dorsally between the scapulae) of an Alzet micro-osmotic pump (DURECT, Cupertino, CA) delivering 1.2mg/ml rhNT-3 (R&D Systems, Minneapolis, MN) (n=12) or saline control (n=12) as described [18]. On day 10 (within bony tissue formation phase, receiving 1 week infusion) and day 28 (within bone remodelling phase, receiving 2-week infusion) [3,6] post-

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injury, animals were sacrificed by CO_2 overdose (n=6 per treatment group per time point). A group of uninjured control animals of the same age were sacrificed on day 14 (n=6).

2.2. Analysis of osteoclast density within the growth plate injury site

For histological studies, the right tibial specimens of the above rat experiment were fixed in Zamboni's fixative for 24 h, decalcified in 14.5% EDTA in phosphate buffer (pH 7.4) at 4 °C for 14 days, and processed for paraffin embedding for obtaining paraffin sections (4 pm thick) mounted on positively charged SuperFrost Plus™ glass slides. To examine treatment effects on osteoclast presence and bone remodelling within the growth plate injury site, tartrate-resistant acid phosphatase (TRAP) histochemical staining was conducted on paraffin sections as described [33,34]. The stained sections were then photographed under a light microscope at X100 original magnification. The number of osteoclasts (TRAP-stained cells with at least 3 nuclei) within the growth plate injury site was counted by image analysis on serial sections (100 μm apart) throughout the injury site from each rat of all groups (n=6), and results were averaged and are presented as number of osteoclasts/mm^2 trabecular bone area within the injury site.

2.3. NT-3 and TrkC immunohistochemical analyses in rat tibia

Cellular localization of NT-3 and TrkC in rat bones was determined immunohistochemically. Briefly, de-waxed and rehydrated sections of rat proximal tibia of normal rats or rats at days 10 or 28 after growth plate injury (n=6) were treated in 3% hydrogen peroxide/PBS for 30 min and then heated for 1.5 h at 70 °C in 0.1 M citric buffer (pH 6). After blocking in 10% pig serum and 1% bovine serum albumin (BSA) in PBS, sections were incubated with the rabbit polyclonal anti-human NT-3 at 2.5 μg IgG/ml (Chemicon, Temecula, CA) or the rabbit polyclonal anti-human TrkC at 1:300 (Abcam, Cambridge, MA, USA) overnight at 4 °C, followed by incubation with a biotin-labelled swine anti-rabbit secondary antibody (1:400, Dako, Botany, NSW, Australia). After washes, sections were incubated with ABC-complex reagents (1:500, Dako) and reaction colour was developed by HRP enzymatic reaction with DAB kit (Dako) which was followed by haematoxylin counter-staining [6,18]. Replacement of the primary antibody with a normal rabbit IgG of the same concentration was used as a negative control.

2.4. Rat bone marrow stromal cell or calvarial osteoblast cultures and in vitro osteogenesis

For examining expression of NT-3 and other NTs during osteogenic cultures, rat bone marrow stromal cells (rBMSCs) were isolated from bone marrow mononuclear cells of long bones of normal 7-week-old male Sprague Dawley rats as described [35]. In addition, osteoblasts were isolated from new born Sprague-Dawley rat (< 3 day old) calvarias by sequential enzymatic digestion as described [36]. Mineralizing cultures assays were then set up with the isolated rBMSCs [35] and calvarial osteoblasts (passage 2) [36] for collecting culture-conditioned medium and examining mRNA expression profiles of NTs and receptors at different stages of in vitro osteogenesis. Conditioned medium was collected/stored at −20 °C and cultured cells were harvested at days 0, 3, 7, 10 and 14, and stored at −80 °C until RNA extraction and RT-PCR assessment of mRNA expression of NTs and receptors as well as osteogenesis-related genes (Runx2, osterix and osteocalcin) (as described below).

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Some cultures were also processed for examining calcium deposition and formation of mineralized matrix nodules, which were identified morphologically by Alizarin red S staining as described [35].

Furthermore, for investigating roles of NT-3 in modulating expression of osteoclastogenesis-regulatory genes during in vitro osteogenesis of rBMSCs, some cells were treated with a rabbit anti-NT-3 or normal rabbit IgG (7.5 μg/ml), rhNT-3 (100ng/ml) or saline control (n=3 wells/group) from day 3 until the end of osteogenic culture (day 14) with medium refreshed twice weekly [18], and the treated and untreated cells were used for RT-PCR analyses (as described below) for RANKL and OPG as well as osteoclastogenic cytokines including tumour necrosis factor (TNF-α) and interleukins (IL-1β and IL-6) [33].

2.5. Osteoclast formation from ratbone marrow cells and murine $RAW_{264.7}$ osteoclast precursor cells

To investigate the potential function of NT-3 in regulating osteoclast formation, in vitro osteoclastogenesis assays were performed using primary rat bone marrow cells or mouse monocyte macrophage (osteoclast precursor) $RAW_{264.7}$ cell line cells. For primary cultures, bone marrow was flushed out from both femurs and tibiae of normal rats and non-adherent hematopoietic cells were collected after overnight culture. Then the collected cells were cultured in 48-well trays at 1×10^6 cells/well in triplicate in basal medium supplemented with or without 10 ng/ml macrophage colony-stimulating factor (M-CSF) (Peprotech, Rehovot, Israel). After 24 h, medium was replaced with a basal medium supplemented with either rhNT-3 (100 ng/ml)+M-CSF (10ng/ml), rhNT-3 (100 ng/ml)+M-CSF (10 ng/ml) +RANKL (30 ng/ml), or just positive control M-CSF (10 ng/ml)+RANKL (30 ng/ml) [37], and cells were cultured for another 8 days with medium replenished every 3 days. Cells were then collected at day 9 and analysed by RT-PCR for mRNA expression of osteoclast receptors RANK (receptor activator of nuclear factor-KB) and OSCAR (osteoclast-associated receptor) as described [34], or fixed in 4% formaldehyde and processed for TRAP staining to identify/quantify multinuclear osteoclasts formed ($TRAP^+$ cells containing three or more nuclei/mm^2 culture area) [33,34].

To compare effects of treatments with anti-NT-3 or rhNT-3 at the early or late stage of osteoclast formation, rat bone marrow cell osteoclastogenic cultures in the presence of M-CSF and RANKL as above were treated with or without rhNT-3 (100 ng/ml), anti-NT-3 (7.5 μg/ml), or normal rabbit IgG (7.5 μg/ml) during the early stage (from day 1 to day 4) or late stage (from day 6 to day 9) of differentiation (n=6 per group). Cells were then collected at day 4 or day 9 and analysed as above.

To examine potential effect of osteoblast-derived NT-3 in comparison to rhNT-3 on osteoclastic differentiation, RAW264.7 macrophage cells (RIKEN, Tsukuba, Ibaraki, Japan) at 1.5×10^3 cells/well in 96-well plates were exposed to basal medium with or without RANKL (100 ng/ml), rhNT-3 (100 ng/ml), or conditioned medium (CM) collected from day 0 or day 14 primary osteogenesis culture (see above), CM+normal rabbit IgG (7.5 μg/ml), or CM+anti-NT-3 (7.5 μg/ml). The cells were then cultured for 5 days with medium replaced every other day. Cells were collected for RT-PCR analysis for the gene expression of

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osteoclastogenesis-related markers OSCAR and cathepsin K (see below) or stained for TRAP to identify TRAP-positive multi-nucleated osteoclastic cells as described above.

2.6. Osteoclastic mineral resorption assay

To examine potential effect of osteoblast-derived NT-3 in comparison to rhNT-3 on osteoclastic mineral resorption activity, $RAW_{264.7}$ cells were cultured in basal medium as above for 11 days on a Corning® osteo assay surface 96-well microplate (Corning, Tewksbury, MA), which was coated with crystalline synthetic calcium phosphate thin films that mimic native bone mineral. Cells were cultured in the presence or absence of RANKL (100 ng/ml) with day 0 or 14 CM from primary osteogenesis culture, or CM+normal rabbit IgG (7.5 μg/ml), or CM+anti-NT-3 (7.5 μg/ml), or rhNT-3 (20 or 100 ng/ml). After culture, the cells were removed with 12.5% sodium hypochlorite prior to von Kossa staining to visualize the resorption area. Resorption pits were visualized under a light microscope and analysed by Cell^F multifluorescence and imaging software (Olympus Soft Imaging Solutions, Notting Hill, VIC, Australia).

2.7. Quantitative RT-PCR gene expression studies

Quantitative RT-PCRT assays were used to analyse treatment effects on expression of regulatory genes at the growth plate injury sites or from above cell culture experiments. For examining effects on expression of molecules involved in callus remodelling at injured growth plate, after the metaphyseal bone of the left proximal tibia was carefully snapped apart from the epiphysis (n=6/group/time point), growth plate injury site tissue (red in appearance and well separated from the adjacent uninjured pale-colored cartilage) or growth plate tissue from the control animals were collected and stored at −80 °C prior to RNA extraction as described [18]. Similarly, RNA was isolated from the above cell culture experiment samples. After being treated with DNase 1, RNA was used to synthesize cDNA using random decamers (Gene-Works, Adelaide, Australia) and Super-Script™ III RT kit (Invitrogen). Then SYBR green-based PCR assays (in triplicate) were run with these cDNA samples using specific rat (Table 1) or mouse (Table 2) primer pairs for each target gene and endogenous control gene Cyclophilin A as described [18]. Molecules being assessed for mRNA expression include cartilage removal-related collagenolytic matrix metalloproteases (MMP-9 and MMP13), osteoclastogenesis-related markers (osteoclast receptors RANK and OSCAR) and regulatory molecules (RANKL, OPG, TNF-α, IL-1, and IL-6), NTs and receptors, as well as osteogenesis-related genes (Runx2, osterix and osteocalcin). All primers were designed using PRIMER BLAST and supplied by GeneWorks. Relative gene expression (calibrated in relation to CT values of Cyclophilin A) was calculated using the $2^{-\Delta\Delta CT}$ method.

2.8. NF-κB and NFAT luciferase reporter gene assays

Reporter assays were also carried out to examine whether rhNT-3 can directly influence activation of osteoclastogenic transcription factors. The nuclear factor-kappaB (NF-κB) and the nuclear factor of activated T-cells (NFAT) signaling pathways have long been recognized critical for osteoclastic formation, function, and survival [38–40]. To examine whether NF-κB or NFAT activity is influenced by anti-NT-3 or rhNT-3 treatment during osteoclastic formation, NF-κB and NFAT activation luciferase reporter assays were employed using

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RAW264.7 osteoclast precursor cells which were stably transfected with NF-κB luciferase reporter construct [33] or a NFAT luciferase reporter construct [41]. For the NF-κB assay, transfected cells were seeded at 1.5×10^5/well in 48-well plates and left overnight to allow attachment. Cells were then pre-treated with normal rabbit IgG (7.5 μg/ml), anti-NT-3 (7.5 μg/ml), rhNT-3 (5, 25 or 100 ng/ml) or PBS control for 1h before being stimulated with or without RANKL (25 ng/ml) for another 6 h. The treated cells were harvested and lysed, and the luciferase activities in lysates were measured using the Promega Luciferase Assay System (Promega, Sydney, Australia) and a BMG Polar Star Optima luminescence reader (BMG, Germany). For the NFAT assay, transfected cells were seeded at 5×10^4/well and grown until 80% confluence. Cells were first pre-treated with rhNT-3 at different doses (0, 5, 25, 50, or 100 ng/ml) for 1 h and then incubated with or without RANKL (100 ng/ml) for a further 24 h prior to luciferase activity measurement.

2.9. Western blot phosphorylation analyses of TrkC downstream kinases in osteoclast lineage cells

As a step to demonstrate direct action of NT-3 and examine potential activation of TrkC downstream kinases in osteoclast lineage cells, $RAW_{264.7}$ osteoclast precursor cells (at 80% confluence) were firstly serum-starved for 2h and then cultured in α-MEM with or without rhNT-3 (100 ng/ml) for 5, 15, or 30 min, or with 100 ng/ml rhBDNF for 15 min as a comparison, and in the absence or presence of RANKL (100 ng/ml). After 3 washes with cold PBS, cells were lysed for 30 min at 4 °C in RIPA buffer containing protease inhibitor and phosphatase inhibitor cocktails (Roche, Basel, Switzerland). Supernatants were harvested after centrifuging for 15 min at 13000 rpm and protein concentrations quantified. After being denatured, proteins were separated through a 4–15% Criterion™ SDS-PAGE gel (Bio-Rad, NSW, Australia), transferred onto nitrocellulose membrane, incubated with rabbit antibodies against phosphorylated (p-) or total (t-) Erk½ (extracellular signal-regulated kinases-½, Cell signaling, Beverly, MA), p-Akt or t-Akt (also known as protein kinase B, Santa Cruz, Dallas, Texas), or sheep anti-mouse GAPDH (as a loading control, Osenses, Adelaide, Australia), and then incubated with HRP-conjugated secondary antibodies (Dako) and finally processed for signal development using an ECL kit (Thermo Fisher Scientific, Melbourne, Australia) as described [18].

2.10. Statistical analysis

Statistical analysis was conducted using SPSS (IBM Corp, New York, NY) and Graphpad Prism (6.01 for Windows, La Jolla, CA). Data are presented as mean ± SD. Cell culture data were from representative results from one of three independent reproducible experiments. Statistical significance within matched groups was assessed using oneway ANOVA (for time course studies without intervention treatments) or two-way ANOVA (for studies with different time points and intervention treatment groups) with Bonferroni's or Tukey's correction for multiple comparisons. A p-value < 0.05 was accepted as statistically significant. For graphic representation, for time course tissue culture study PCR data, groups with *, **, or *** are different from the untreated control (UT), respectively at $p < 0.05$, 0.01, or 0.001. For some group comparisons (saline vs rhNT-3 infusion; normal serum vs anti-NT-3 serum), groups linked by a horizontal bars/lines are different (at $p < 0.05$, ^ or *; $p < 0.01$, ^^ or **; or $p < 0.001$, ^^^ or ***).

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3. Results

To examine potential effects on the non-injured areas/bones of the systemic treatment with rhNT-3 or the anti-NT-3, further analyses of micro-computer tomography (μ-CT) data [18] at the non-injured areas of tibia metaphysis were performed. Results showed that systemic treatment with rhNT-3 or the anti-NT-3 had no significant effects in altering the trabecular bone volume fraction and trabecular structural parameters (trabecular number, thickness and separation) at both day 10 and day 28 post-injury (Supplement Fig. 1). This suggests that these systemic treatments do not affect the structure and bone volume of the non-injured bone within the 4-week experimental time window. Thus, this study has focused analyses on NT-3 functions at the growth plate injury site.

3.1. NT-3 in callus remodelling

Recently, we have shown the critical osteogenic and angiogenic roles of endogenous NT-3 in the bony repair of injured growth plate (with anti-NT-3 treatment being found to significantly reduce bone volume fraction and formation of new blood vessels) [18]. Following treatment with the neutralising anti-NT-3 IgG, less bone tissue was found present, bony trabeculae were not well formed, and the injury sites also had larger amounts of mesenchymal and cartilaginous callus tissues when compared to those from the normal serum IgG-treated group at days 10 and 28 [18]. Moreover, compared to saline-treated controls, injury sites from the rhNT-3 protein-treated rats showed less cartilaginous/mesenchymal tissue remnants but a larger amount of trabecular bone, which appeared to be more remodelled with presence of a larger area of bone marrow tissue in between the trabeculae [18]. These findings suggest that NT-3 is important for the remodelling of the growth plate injury site, and that NT-3 blockade delayed the bony repair and injury site remodelling.

To further investigate functions of NT-3 at the growth plate injury site remodelling during bony repair, effects on osteoclastic density were compared among the different treatment groups in the current study. Anti-NT-3 antibody treatment inhibited, but rhNT-3 enhanced, densities of $TRAP^+$ multinuclear osteoclasts at the injury sites on both day 10 ($p < 0.05$ anti-NT-3 vs normal IgG, and rhNT-3 vs saline) and day 28 ($p=0.08$ anti-NT-3 vs normal IgG, $p < 0.05$ rhNT-3 vs saline) (Fig. 1A, B).

Moreover, treatment effects on expression of genes related to osteoclastic density/remodelling as well as cartilaginous remodeling were examined by injury site qRT-PCR analyses. Consistently, similar trends of effects were obtained for the expression of osteoclast receptors RANK and OSCAR (Fig. 1B). In addition, anti-NT-3 treatment was found to inhibit, but rhNT-3 was found to enhance, injury site gene expression of cathepsin-K (the major protease of osteoclasts responsible for calcified tissue resorption) and collagenase-3 (MMP-13) and gelatinase-9 (MMP-9) (Fig. 1C), the two collagenolytic MMPs that are critical for the early stage of callus remodelling (cartilage removal) during bone fracture healing [27,42,43]. Our data suggest that NT-3 has a role in remodelling of both soft and hard calluses during growth plate bony repair.

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3.2. Prominent expression of NT-3 at injury repair site and in osteoblasts

Previously, we observed that, at the injury repair site, NT-3 and its receptor TrkC showed the highest induction (NT-3 > 100-fold, other NTs < 35 fold) [18]. As a step to investigate mechanisms how NT-3 participates in injury site remodelling, NT-3 immunohistochemistry was carried out. Strong NT-3 protein immunostaining was observed at the growth plate injury sites on both days 10 and 28, particularly in osteoblasts on the newly formed bone (Fig. 2A), which has a similar staining pattern as in the normal metaphysis trabecular bone. In addition, NT-3 staining and TrkC immunostain were also observed in osteoclasts on the trabecular bone surface at the injury site (more description later and in Fig. 6C).

Consistent with the most prominent induction of NT-3 and its receptor TrkC at the growth plate injury site, NT-3 and TrkC were also found to be most prominently induced during in vitro osteogenesis of rat bone marrow stromal cells (rBMSCs), among NTs (NT-3 60 fold vs others 9–20 fold) and NT receptors (TrkC >4.5 fold vs other receptors < 2.5 fold) (Fig. 2B). Likewise, in osteogenic culture of osteoblasts isolated from neonatal rat calvarial bone, NT-3 and receptor TrkC were found to be induced dramatically particularly during the maturation and mineralizing stages (up to 45 folds for NT-3 vs lower than 2.5 folds for other NTs; and up to 140 folds for TrkC vs lower than 2.5 folds for other Trks) (Fig. 2C).

Thus the above findings indicate that NT-3 is prominently induced during osteogenesis and at the injury site osteoblasts. Our data presented below demonstrates that osteoblast-derived NT-3 promotes osteoclastogenesis and resorption in vitro and participates in callus remodelling during growth plate bony repair.

3.3. Osteoblast-derived NT-3 augments RANKL-induced osteoclastogenesis

To examine whether osteoblast-derived NT-3 can enhance osteoclastogenesis, in $RAW_{264.7}$ cell osteoclastogenic culture assays, similar to rhNT-3 control, osteoblast-derived NT-3 (from conditioned medium from day-14 osteogenic culture vs day-0 culture) by itself did not promote formation of multi-nuclear osteoclasts in the absence of RANKL (Fig. 3A). Treatment with the anti-NT-3 neutralising antibody also did not affect osteoclast formation without the presence of RNAKL. However, in the presence of RANKL, day-14 conditioned medium augmented RANKL-induced osteoclastogenesis (formation of $TRAP^+$ multinuclear cells) ($p < 0.05$ vs RANKL control alone) (Fig. 3B–C) and expression of marker genes (osteoclast receptor OSCAR and protease cathepsin K) (Fig. 3D). This effect of the conditioned medium was abolished by the presence of anti-NT-3 antibody in the culture ($p < 0.05$ vs normal IgG) (Fig. 3B–D). Furthermore, presence of anti-NT3 neutralising antibody substantially suppressed RANKL-induced formation of multi-nuclear osteoclasts ($p=0.08$, RANKL vs RNAKL+anti-NT3) and significantly attenuated RANKL-induced expression of osteoclast protease cathepsin K ($p < 0.05$, RANKL vs RNAKL+anti-NT3). These findings suggest that osteoblast-derived NT-3 can act as a potential crosstalk signal and have a direct role for augmenting osteoclastogenesis.

3.4. Osteoblast-derived NT-3 augments RANKL-induced mineral resorption

Furthermore, when the above experimental treatments involving the conditioned medium were applied to the RAW264.7 cell osteoclastic mineral resorption assays, similar patterns of

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treatment resorption outcomes were observed as with osteoclast formation outcomes above, where day-14 conditioned medium augmented RANKL-induced mineral resorption (Fig. 4A–D), an effect that was abolished in the presence of anti-NT-3 neutralising antibody (Fig. 4B–D). These findings suggest that osteoblast-derived NT-3 can act as a potential regulatory signal for osteoclastic mineral resorption.

3.5. rhNT-3 augments RANKL activity in osteoclastogenesis

To investigate roles of NT-3 on osteoclast formation further, effects of rhNT-3 treatment on osteoclastogenesis in rat bone marrow cells were examined in vitro. Although NT-3 by itself (in the presence of MCSF but absence of exogenous RANKL) was able to enhance expression of osteoclastic receptor molecules RANK and OSCAR ($p < 0.05$ vs NT-3-free control), it was not able to promote osteoclastogenesis (Fig. 5A–B). However, in the presence of RANKL, addition of NT-3 (during the 9-day culture) augmented ability of RANKL in promoting formation of TRAP-stained multinuclear osteoclastic cells ($p < 0.05$ vs RANKL alone) and expression of osteoclast receptors particularly OSCAR ($p < 0.05$ vs RANKL alone; but $p > 0.05$ for RANK) (Fig. 5A–B).

To address whether NT-3 exerted this stimulation effect during the early or late stage of osteoclastogenesis, comparisons were made for the effects on osteoclastogenesis between the early (days 1–4) or late (days 6–9) stage treatments with anti-NT-3 or rhNT-3 in the presence of M-CSF and RANKL. The results showed that early stage treatment with rhNT-3 increased the RANK expression (by 24%, $p < 0.05$, compared to untreated cells), and that the anti-NT-3 treatment decreased expression of RANK ($p < 0.05$, compared to normal IgG) in day 4 collected cells (Fig. 5C). Effects on OSCAR were less obvious. In day 9 cultured cells, early stage anti-NT-3 treatment more obviously and significantly suppressed the expression of RANK and OSCAR (by 76% decrease in RANK, $p < 0.05$ and 72% in OSCAR, $p < 0.01$), while rhNT-3 elevated levels of both RANK and OSCAR mRNA (by 83% increase in RANK and 92% in OSCAR, both $p < 0.01$) when compared to the untreated group. After the late stage treatment, anti-NT-3 group still maintained the ability to decrease RANK and OSCAR mRNA levels (by 75% in RANK and 63% in OSCAR, both $p < 0.01$) (Fig. 5C). Interestingly, treatment with rhNT-3 during this late period did not have obvious effects on expression of RANK and OSCAR. There were no obvious changes in these markers between the normal IgG-treated group and untreated group.

3.6. Induction of TrkC during osteoclast differentiation and rhNT-3-induced activation of TrkC downstream kinases

As a step to study the underlying mechanisms for the NT-3 promoting effect on osteoclastic differentiation, firstly, expression of neurotrophins and their receptors was profiled by qRT-PCR in the late stage of osteoclastogenesis (day 5) in $RAW_{264.7}$ osteoclastic precursor cells (Fig. 6A), and during the early stage (day 4) and late stage (day 8) of osteoclastogenesis from rat bone marrow non-adherent cells (Fig. 6B). As shown in Fig. 6A, when $RAW_{264.7}$ cells were differentiated to osteoclasts (with significant induction of osteoclast markers cathepsin K and OSCAR at day 5 vs day 0), although NT-3 and TrkC were not found to be the most prominently induced neurotrophin or receptor, they were induced about 2–2.5 folds (vs the highest induced NT-4 at 14 folds and the highest induced TrkB at 8 folds). During

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the osteoclastogenesis from rat bone marrow non-adherent cells, there were obvious and significant inductions of osteoclast markers RANK (about 10 and 5 folds respectively on days 4 and 8) and OSCAR (12 folds on day 8) when compared to day 0 control. NGF, BDNF, NT-3, and NT-4 showed no inductions on day 4 but significant inductions on day 8 (3.4, 5.8, 4.4, and 4 folds, respectively). While there were no inductions for TrkA and p75, significant inductions were detected in TrkB (174 and 269 folds on day 4 and day 8) and TrkC (2.1 and 7.2 folds on day 4 and day 8, respectively). The inductions of NT-3 and its high-affinity receptor TrkC (as well as BDNF and TrkB) during osteoclastogenesis suggest potential direct roles of NT-3 and BDNF in enhancing osteoclastogenesis. Furthermore, while these results show some substantial differences in expression patterns of NTs and receptors in osteoclast differentiation between $RAW_{264.7}$ osteoclast precursor cells and bone marrow non-adherent cells, expression of NT-3 and TrkC in osteoclasts was confirmed in vivo by their immunostaining in bone-resorbing osteoclasts at both normal metaphyseal trabecular bone and at growth plate injury sites in rats (Fig. 6C).

Secondly, since NT-3 has been shown to stimulate Erk½ and Akt activation in mineralising osteoblasts [18], two major kinases downstream of Trk receptors also activated by NTs in nerve cells [44], here we examined whether rhNT-3 or BDNF treatment could directly activate these two kinases during osteoclastogenesis in RAW264.7 osteoclast precursor cells in the presence or absence of RANKL. As shown by Western blot analyses (Fig. 7A), rhNT-3 treatment even in the absence of RANKL was found to activate the major kinase Erk½ downstream of Trk receptors, showing increased phosphorylation of Erk½ (but not Akt) 30 min after exposure when compared to the non-treated controls (0 min). While RANKL alone obviously activated these two kinases (at NT-3 time point “0”), there were no obvious differences in the levels of phosphorylation of Erk½ and Akt between rhNT-3 alone treatment and the rhNT-3+RANKL combination treatment (at NT-3 treatment “5, 15, and 30 min” time points). Furthermore, like NT-3 at 15 min following exposure, BDNF treatment did not obviously change the activation levels of these two major kinases. These results suggest that rhNT-3 has a direct effect on differentiating osteoclastic cells and that activation of TrkC downstream kinase Erk½ is involved in NT-3 effect in osteoclastogenesis.

3.7. Lack of NT-3 direct effect in NF-κB or NFAT activation in osteoclastic precursor cells

Furthermore, we also examined any direct functions of rhNT-3 in activating two critical osteoclastogenic transcription factors NF-κB and NFAT (both downstream of RANKL-RANK interaction) using osteoclast precursor $RAW_{264.7}$ cells which stably express the NF-κB or NFAT luciferase reporter gene construct. As shown in Fig. 7B, without the stimulation of RANKL, treatment with varying doses of rhNT-3 or with normal IgG or anti-NT-3 did not significantly alter the activation levels of NF-κB or NFAT when compared to PBS control. In the presence of RANKL, the NF-κB activation in all treatment groups was remarkably increased (around 3000–4000%) when compared to PBS alone (without RANKL addition) group. Similar results were obtained with NFAT activation in the presence of RANKL. However, in the presence of RANKL, the levels of NF-κB or NFAT activation did not exhibit any significant differences in cells treated with anti-NT-3 or different concentrations of rhNT-3 when compared with normal IgG-treated or untreated cells. This finding suggests

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that the positive effect of NT-3 on osteoclastogenesis may not directly involve the promotion of NF-κB or NFAT activation.

3.8. NT-3 has direct action on osteoblasts and induces osteoclastogenic cytokines

Since NT-3 was found significantly induced at the bony repair site and prominently expressed by the osteoblasts lining the newly formed trabeculae at the injury site (Fig. 2A), to investigate a potential indirect mechanism for the NT-3 osteoclastogenic effect via osteoblasts, potential roles of NT-3 in regulating expression of osteoclastogenic signals in osteoblasts were examined. In osteoblastic mineralising cells cultured from bone marrow stromal cells, rhNT-3 treatment was found to promote induction of TNF-α, IL-1β and RANKL/OPG ratio ($p < 0.05$ vs untreated control) (Fig. 8A). Conversely, anti-NT-3 treatment during the osteogenic culture suppressed expression of IL-1β and the RANKL/OPG ratio ($p < 0.05$ vs normal IgG) and had a slight tendency to inhibit TNF-α ($p=0.24$ vs normal IgG). These findings suggest that NT-3 has a direct action on osteoblasts to promote expression of osteoclastogenic signals.

To examine whether these cytokines can also be induced by NT-3 in the osteoclast lineage cells, similar experiments were performed with $RAW_{264.7}$ osteoclast precursor cells (Fig. 8B). Analyses showed that RANKL treatment in RAW cells significantly induced IL-1β and RANKL/OPG ratio ($p < 0.05$). While rhNT-3 alone had no obvious effects in inducing the pro-osteoclastogenic cytokines, rhNT-3+RANKL combination treatment augmented induction of TNF-α ($p < 0.01$, rhNT-3+RANKL vs untreated; $p=0.19$, rhNT-3+RANKL vs RANKL), IL-1β ($p < 0.001$, rhNT-3+RANKL vs untreated; $p < 0.05$, rhNT-3+RANKL vs RANKL), IL-6 ($p < 0.01$, rhNT-3+RANKL vs untreated; $p=0.11$, rhNT-3+RANKL vs RANKL), and RANKL/OPG ($p < 0.001$, rhNT-3+RANKL vs untreated; $p=0.10$, rhNT-3+RANKL vs RANKL). Furthermore, interestingly, the presence of the anti-NT-3 neutralising antibody significantly suppressed the induction of TNF-α and IL-1β caused by RANKL treatment (Fig. 8B), suggesting a role of NT-3 in the RANKL-induced increased expression of these cytokines.

3.9. NT-3 promotes expression of osteoclastogenic cytokines at the injury repair site

To further investigate mechanisms for NT-3 osteoclastogenic effect during callus bone remodelling, gene expression analyses showed that treatment with the anti-NT-3 serum IgG decreased, but rhNT-3 treatment increased, expression of osteoclastogenic cytokines (TNF-α and IL-1β) (Fig. 9A) and RANKL/OPG ratio (Fig. 9B) at the growth plate injury site particularly on day 28 ($p < 0.05$ to $p < 0.001$ anti-NT-3 IgG vs normal serum IgG, rhNT-3 vs saline). Treatment with the anti-NT-3 serum IgG also decreased expression of osteoclastogenic cytokine IL-6 particularly on day 28 ($p < 0.001$ antiserum IgG vs normal serum IgG) (Fig. 9A). These effects correlated with the treatment effects on osteoclastic density and repair site remodelling observed (Fig. 1).

4. Discussion

Mechanisms for the faulty bony repair and bone bridge formation at injured growth plate cartilage remain largely unclear. In a recent study, NT-3 and its receptor TrkC were found to

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highly induced, and NT-3 was found to enhance osteogenesis and angiogenesis and promote the bony repair at the growth plate injury site [18]. In the current study, we demonstrated that injury site-derived NT-3 participates in the injury site remodelling. We demonstrated that NT-3 is important for the growth plate injury site remodelling, as NT-3 blockade delayed the bony repair and injury site remodelling. We showed that blocking NT-3 reduced, but rhNT-3 treatment enhanced, growth plate injury site expression of MMP-13 and MMP-9 (critical for endochondral bone growth [45] and for cartilage callus remodelling [27,42,43]), of cathepsin-K (major osteoclast protease for calcified tissue resorption), and of osteoclastogenic cytokines and RANKL/OPG ratio. Moreover, NT-3 blockade inhibited but rhNT-3 treatment enhanced osteoclast presence during the bony repair. These data suggest that injury site-derived NT-3 has a role in soft and hard callus remodelling during repair at the injury site.

In the current study, we have shown that rhNT-3 promotes, but anti-NT3 reduces, RANKL-induced osteoclast formation. We also showed that both NT-3 and its high affinity receptor TrkC are induced during osteoclast differentiation particularly during the maturation stage, and that both NT-3 and TrkC are expressed in osteoclasts in normal bone and at growth plate injury site. Consistent with this, our Western blotting analyses revealed that NT-3 can activate TrkC downstream kinase Erk½ in $RAW_{264.7}$ osteoclast precursor cells. Furthermore, we showed that both NT-3 and TrkC are expressed in osteoclasts in both normal bone and injury repair sites. These findings suggest that NT-3 has a direct role in osteoclast differentiation. A potential function of neurotrophins in osteoclastic formation/recruitment has been reported recently. Using human peripheral blood mononuclear cell culture, an in vitro study indicated that NGF had the ability to induce osteoclastic formation and lacunar resorption, and such effects were not affected when osteoprotegerin (OPG, an osteoclastogenesis inhibitory factor) being added into the cultures to neutralize RANKL function, suggesting a RANKL-independent mechanism involved [46]. Similarly, addition of BDNF into the peripheral blood mononuclear cell culture appeared to stimulate the formation of osteoclasts, and such effects can be inhibited by neutralising anti-BDNF antibody [47]. In the current study, we demonstrated that NT-3 augments RANKL-induced osteoclast formation (an effect that was abolished by anti-NT-3 and was associated with induction of TNF-α and IL-1β in osteoclasts formed from $RAW_{264.7}$ osteoclast precursor cells). Thus previous studies and data from our current study together suggest that neurotrophins can have a direct role in promoting osteoclastic formation and participating in osteoclastic bone remodelling.

Furthermore, we demonstrated that NT-3 was mainly expressed in osteoblasts in the newly-formed bony trabeculae at the injury site and NT-3 and TrkC were also found to be most highly induced (among all NTs and Trk receptors) during osteoblast differentiation and mineralization. Moreover, osteoblast-derived NT-3 (osteoblast-conditioned medium) was found to augment RANKL-induced osteoclastogenesis and resorption in osteoclastogenic culture. These results have confirmed the possibility of osteoblast-derived NT-3 as a signal for osteoclastogenesis, implying the important role of injury site osteoblast-derived NT-3 as a regulatory signal for osteoclastic recruitment and injury site remodelling during the bony repair. Nonetheless, while roles of endogenous NT-3 in regulating injury site repair [18] and remodelling (the current study) were observed using the immuno-neutralization approach,

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future studies with pharmacological and/or genetic interventions (including being delivered during the repair phase and/or during the remodelling phase) are required to further elucidate and prove the roles of NT-3 in growth plate injury repair and remodelling.

In the current study, NT-3 itself did not activate two critical osteoclastogenic transcription factors NF-κB or NFAT (both downstream of RANKL-RANK interaction) and to cause osteoclast formation, although NT-3 was found to augment the RANKL effect in osteoclastogenesis and mineral resorption. Previously, it was found that BDNF had no effect on NF-κB activation [48], although, through the action of p75 receptor (but not Trk receptors), NGF (but not BDNF nor NT-3) was able to induce activation of NF-κB in Schwann cells or in transformed fibroblasts overexpressing p75 [49]. Thus, the osteoclastogenic effect of NT-3 does not involve a direct effect in activating osteoclastogenic transcription factors NF-κB or NFAT. On the other hand, we further showed that NT-3 can activate TrkC downstream kinase Erk½ in osteoclast precursor cells. Thus, the positive effect of NT-3 on osteoclastogenesis may not directly involve the promotion of NF-κB or NFAT activation but it involves activation of TrkC downstream kinase Erk½ in differentiating osteoclastic cells. However, further studies are required to address whether NT-3 treatment alters other osteoclast-specific transcription factors or signaling pathways than Erk½, Akt, NF-κB and NFAT that we analysed in the current study.

Furthermore, in the current study, NT-3 was found to induce osteoclastogenic cytokines (TNF-α, IL-1β and RANKL/OPG ratio) at the injury remodelling site and in cultured mineralizing osteoblasts, suggesting that NT-3 may also promote osteoclastogenesis *indirectly* via stimulating expression of pro-osteoclastogenic factors in osteoblasts. Recently, suggesting NT-3 has direct effects on osteoblasts, TrkC was shown to be expressed by osteoblasts at the injury site as well as in normal bone and cultured osteoblasts, and NT-3 was shown to stimulate activation of TrkC downstream kinases Erk½ and Akt in mineralising osteoblasts [18]. These findings suggest that NT-3 derived from osteoblasts is an osteoclastogenic factor that not only has a direct effect in osteoclast precursors or osteoclasts, activating Erk½ and augmenting RANKL activity in osteoclastogenesis and mineral resorption, but also has an indirect role in osteoclastogenesis via inducing osteoclastogenic cytokines and RANKL in osteoblasts. Consistently, BDNF was also found to induce RANKL but suppress OPG in bone marrow stromal cells through stimulating Erk½ and Akt activation, contributing to osteoclastogenesis [48]. While Erk½ activation is known to be important in osteoblast differentiation, future studies are required to examine whether the NT-3-induced Erk½ and Akt signaling pathways in osteoblasts are directly involved in NT-3 action in promoting expression of osteoclastogenic signals in osteoblasts and thus indirectly enhancing osteoclastic formation and resorption. Similarly, further studies are required to elucidate potential roles of the osteoclastogenic cytokines induced by NT-3 in mediating the NT-3 function in osteoclastogenesis.

In conclusion, although the 4-week rhNT-3 or anti-NT-3 systemic treatment did not significantly alter the trabecular bone structure/volume parameters at the non-injured metaphysis areas of tibias, the current study indicates that locally produced NT-3 (most prominently expressed in osteoblasts) is an important factor having a major involvement in regulating remodelling of calluses during growth plate bony repair. It promotes growth plate

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injury site expression of MMP-13 and MMP-9 (important for cartilage callus remodelling) and presence of bone-resorbing osteoclasts. Furthermore, osteoblast-derived NT-3 has both direct and indirect roles in promoting osteoclastogenesis, as it can activate Erk½ in differentiating osteoclasts and augment RANKL activity in osteoclastogenesis and mineral resorption, and it can induce osteoclastogenic cytokines and RANKL in osteoblasts and can augment RANKL-induced induction of osteoclastogenic cytokines in osteoclast precursor cells. Thus, while our recent study suggests that injury site-induced NT-3 is an osteogenic and angiogenic factor upstream of BMP-2 and VEGF promoting bony repair at injured growth plate [18], the current study suggests that NT-3 derived from the injury site (particularly osteoblasts) is also important in regulating the callus remodelling during the bony repair, inducing cartilage proteases for cartilage callus removal, directly augmenting RANKL osteoclastogenic effect in differentiating osteoclasts, and inducing osteoclastogenic signals in osteoblasts and thus indirectly promoting osteoclast recruitment/function. This new knowledge will add significantly to our understanding of factors and mechanisms controlling growth plate injury repair/re-modelling responses and may potentially lead to future studies exploring therapeutic targets for preventing the faulty bony repair.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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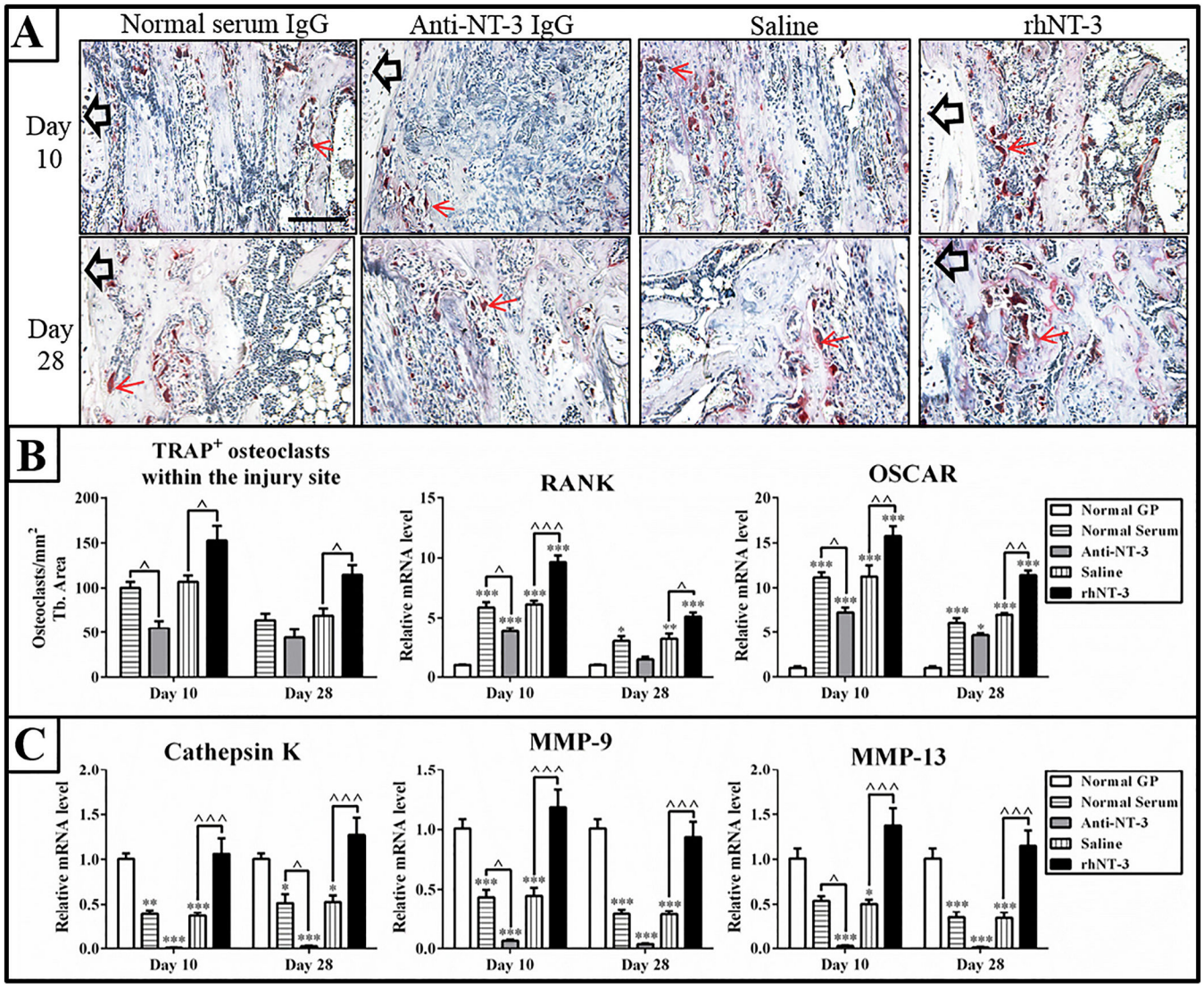

Fig. 1.

Effects of anti-NT-3 or rhNT-3 treatment on osteoclast density and mRNA expression of osteoclast markers and proteases involved in the remodelling of growth plate injury sites. (A) Representative TRAP-stained histological sections of day 10 and day 28 growth plate injury sites of rats of different treatment groups. Arrows indicate $TRAP^+$ multinucleated osteoclasts on bony trabeculae. Scale bar=200 μm. (B) Quantification of injury site osteoclasts ($TRAP^+$ osteoclasts/mm^2 trabecular bone area) on sections and mRNA expression of osteoclast receptor genes RANK and OSCAR by quantitative RT-PCR analysis. (C) mRNA expression levels for osteoclastic protease cathepsin K and cartilage degradation enzymes MMP-9 and MMP-13. Values were means ± SD of n=6 rats per group.

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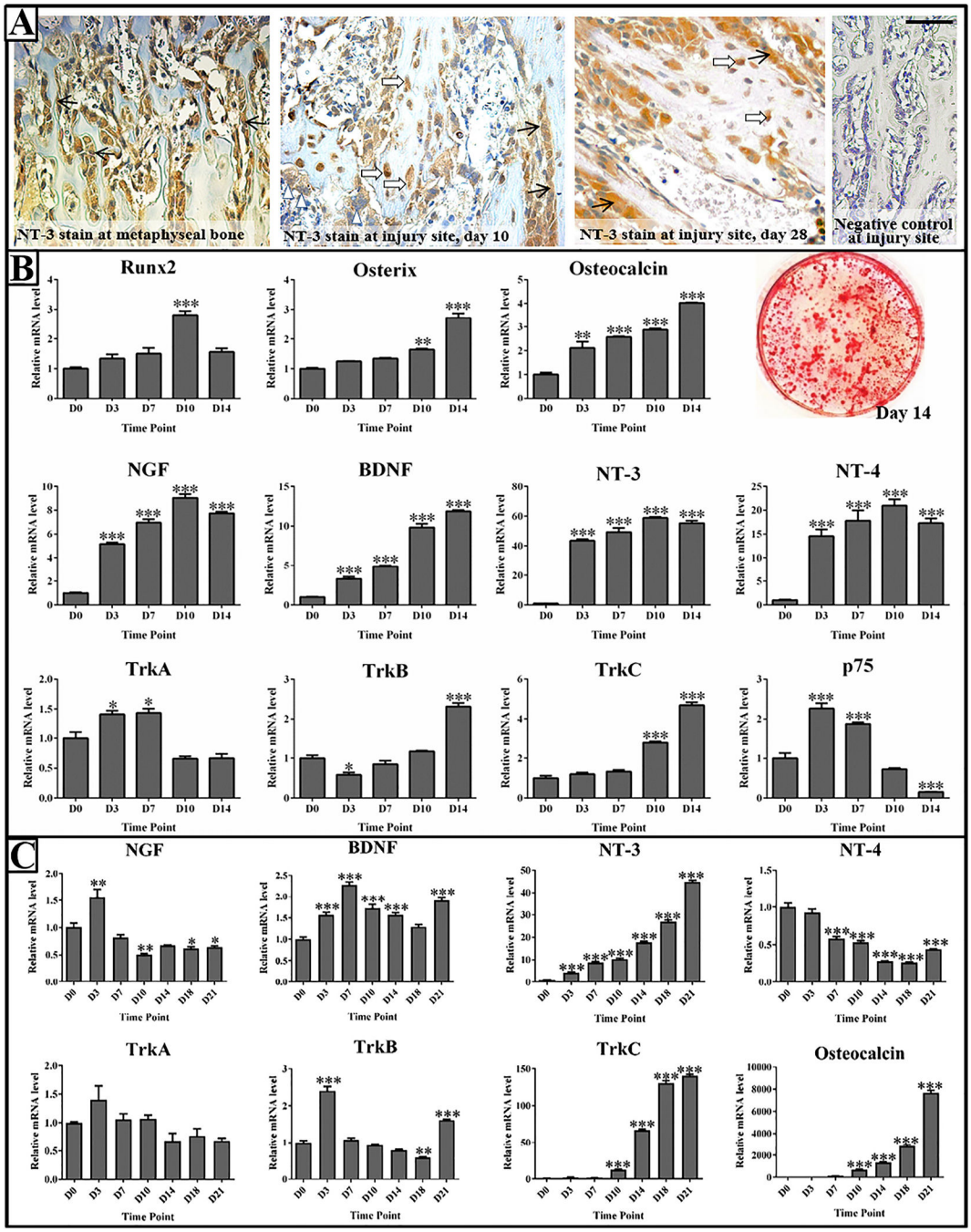

Fig. 2.

Expression of NT-3 at the growth plate injury site and during in vitro mineralization of rat bone marrow stromal cells and rat calvarial osteoblasts. (A) Immunolocalization of NT-3 or negative control (using a normal IgG) in the injured growth plate at days 10 and 28 after injury or in normal metaphysis bone. Scale bar=50 μm. Arrows point to positive staining. (B) A representative image of in vitro osteogenesis assay (day 14) as stained by Alizarin red S (ARS) and quantitative RT-PCR time course analysis [on days (D) 0, 3, 7, 10 and 14] of bone specific genes Runx2, osterix and osteocalcin, neurotrophins (NGF, BDNF, NT-3 and

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NT-4) and their receptors (TrkA, TrkB, TrkC and p75) during in vitro mineralization culture of rat bone marrow stromal cells. (C) Quantitative RT-PCR time course expression analyses of neurotrophins and Trk receptors and osteocalcin during in vitro osteogenic culture of neonatal rat calvarial osteoblasts. Data are expressed as means ± SD fold increase (n=3 wells per time point) over day 0 control.

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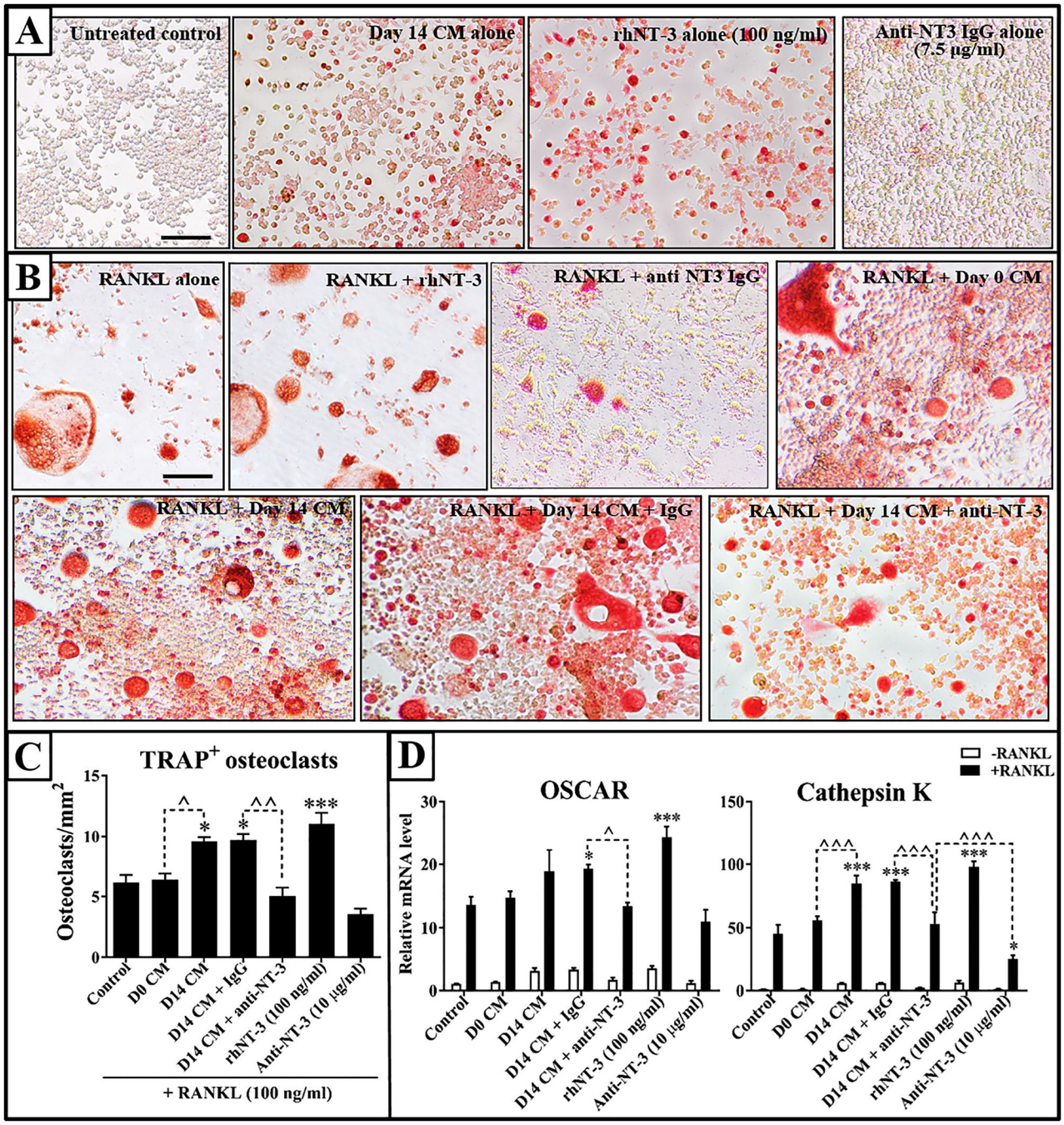

Fig. 3.

Effects of treatment with 100ng/ml rhNT-3 or osteoblast-derived NT-3 (from rat bone marrow stromal cell day-14 osteogenic conditioned medium (CM) compared to day-0 medium) on osteoclastic differentiation in 5-day $RAW_{264.7}$ osteoclastic precursor cell cultures in the presence or absence of 100 ng/ml RANKL and 7.5 μg/ml anti-NT-3 IgG or normal rabbit IgG. (A-B) Representative images of various cultures stained by TRAP. Scale bars=100 μm. (C) Quantification of $TRAP^+$ multinucleated cells per mm^2 of culture area of

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various treatment groups after RANKL stimulation. (D) Quantitative real-time PCR analyses of OSCAR and cathepsin K mRNA expression.

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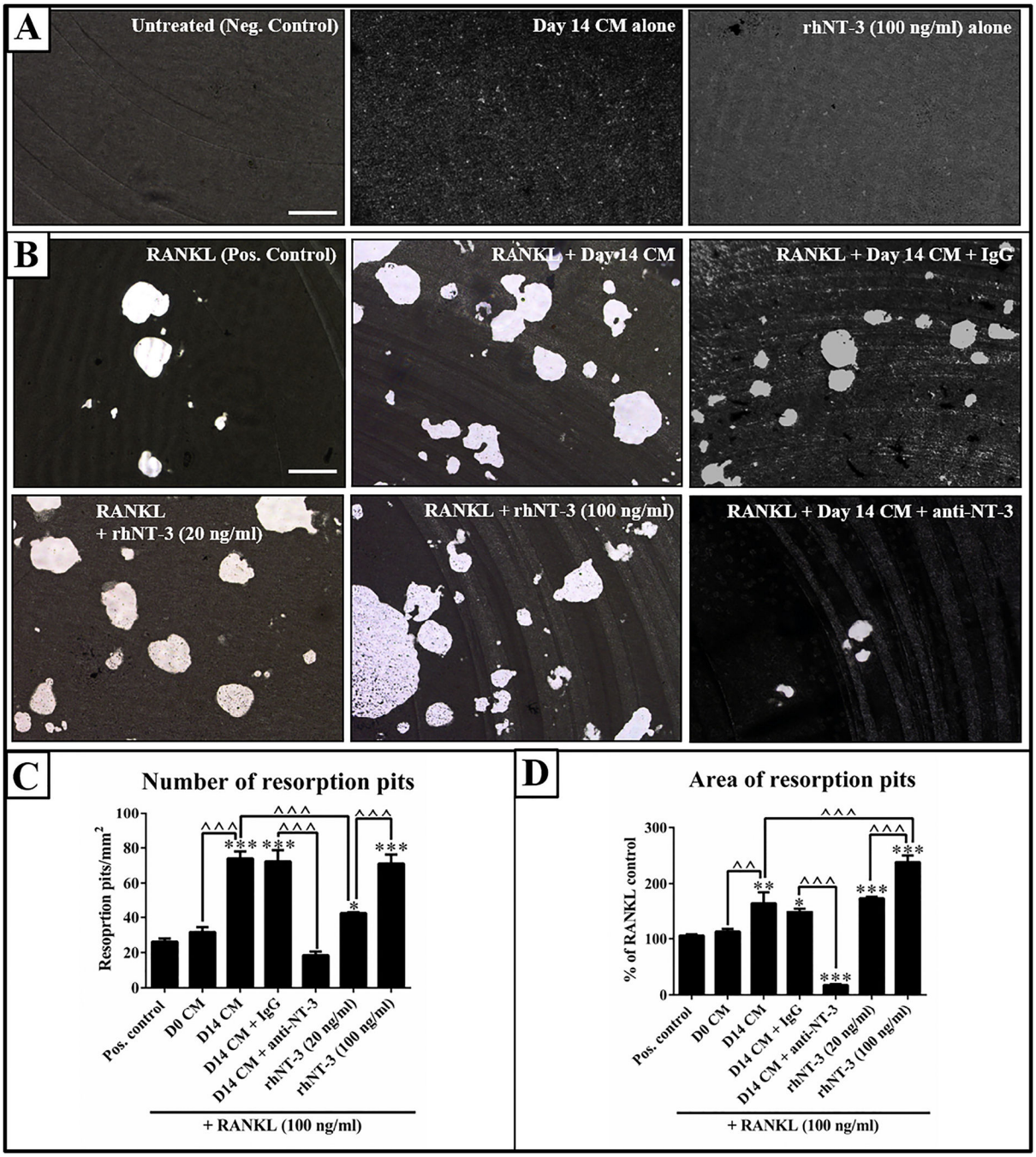

Fig. 4.

Effects of treatment with 100ng/ml rhNT-3 or osteoblast-derived NT-3 (from rat bone marrow stromal cell day-14 osteogenic conditioned medium (CM) compared to day-0 medium) on resorption pit formation ability of osteoclasts formed from 11-day $RAW_{264.7}$ osteoclastic precursor cell cultures in the presence or absence of 100 ng/ml RANKL, 7.5 μg/ml anti-NT-3 IgG or normal rabbit IgG. (A-B) Representative images of resorption pits formed and as visualized by von Kossa staining in various cultures after cultured cells were removed. Scale bars=100 μm. (C-D) Resorption activity was assessed under light

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microscopy and quantified by counting the number of resorption pits or measuring the pit area using Cell^F software. Data shown are means ± SD of quadruplicate wells.

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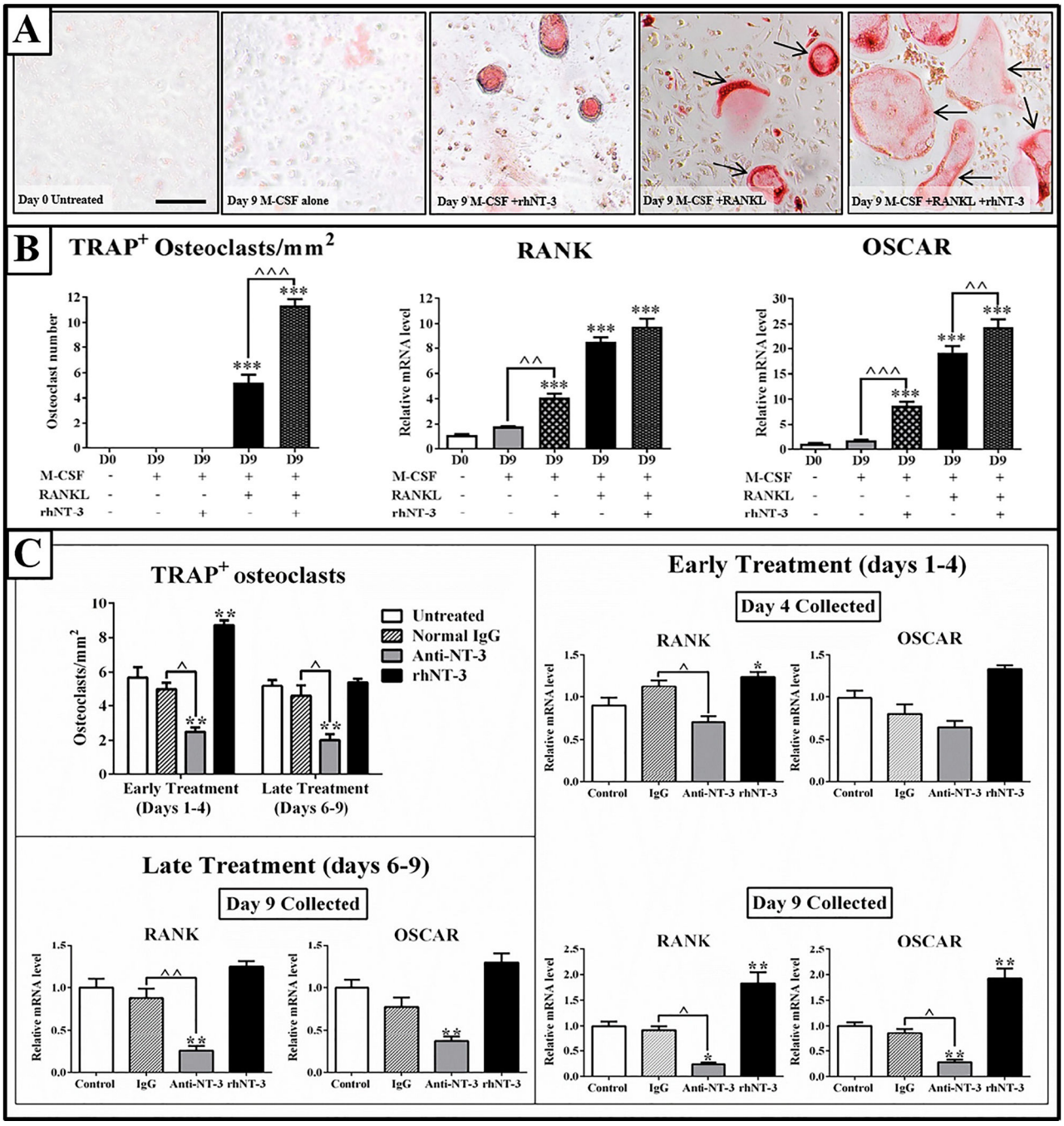

Fig. 5.

Effects of rhNT-3 or anti-NT-3 treatment on osteoclastogenesis in rat bone marrow cells. (A) Representative images of TRAP-stained (black arrows) osteoclastic cultures (scale bar=80 μm) and (B) average numbers of $TRAP^+$ multinucleated osteoclasts formed/mm^2 of culture area and RT-PCR mRNA expression analyses of osteoclast receptors RANK and OSCAR, on day 0 untreated normal rat bone marrow cells or on day 9 following treatment with M-CSF (10 ng/ml) alone, rhNT-3 (100 ng/ml) alone, RANKL (30 ng/ml) alone, or rhNT-3+RANKL. (C) Comparison of the effects of early (days 1–4) or late (days 6–9) stage

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treatments with anti-NT-3 or normal rabbit IgG (7.5 μg/ml) or rhNT-3 (100 ng/ml) on osteoclastogenesis after M-CSF (10 ng/ml)+RANKL (30 ng/ml) stimulation. $TRAP^+$ multi-nucleated osteoclasts were counted at day 9 of culture, and mRNA expression of RANK and OSCAR was analysed at day 4 or 9 of culture.

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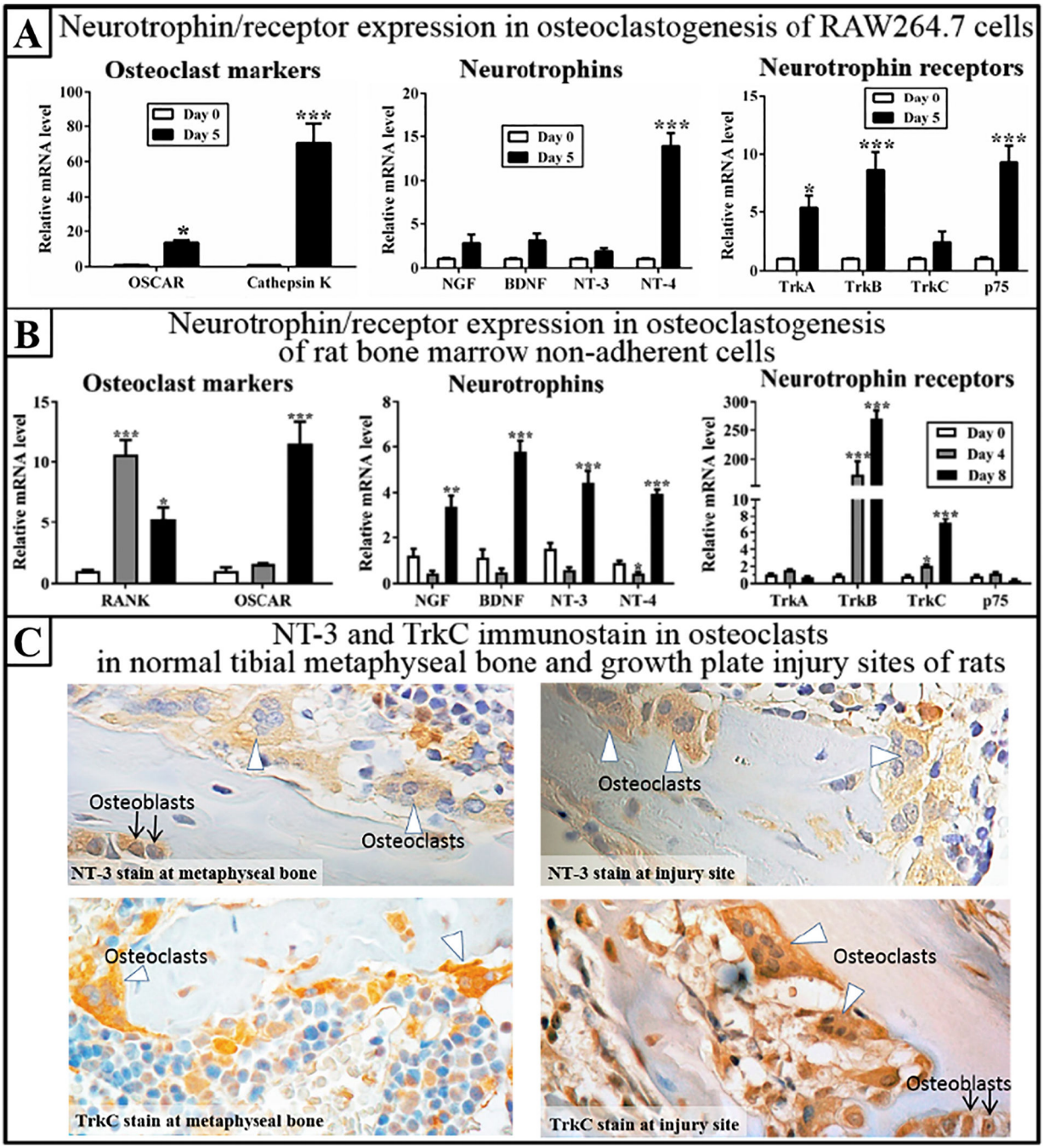

Fig. 6.

Induction of NT-3 and TrkC during osteoclastogenesis in vitro and their immunolocalization in osteoclasts in vivo. (A) Changes in mRNA expression of osteoclast markers (OSCAR and cathepsin K), neurotrophins and receptors as examined by quantitative RT-PCR in days 5 vs 0 of osteoclast differentiation culture from $RAW_{264.7}$ pre-osteoclastic cells. (B) Changes in mRNA expression of osteoclast markers (RANK and OSCAR), neurotrophins and receptors as examined in days 4 or 8 of osteoclast differentiation culture from rat bone marrow non-adherent cells. (C) Immunolocalization of NT-3 and TrkC in osteoclasts in normal tibial

metaphyseal bone and growth plate injury sites of rats (arrows pointing to positive stain in osteoblasts, and triangles pointing to positively-stained osteoclasts).

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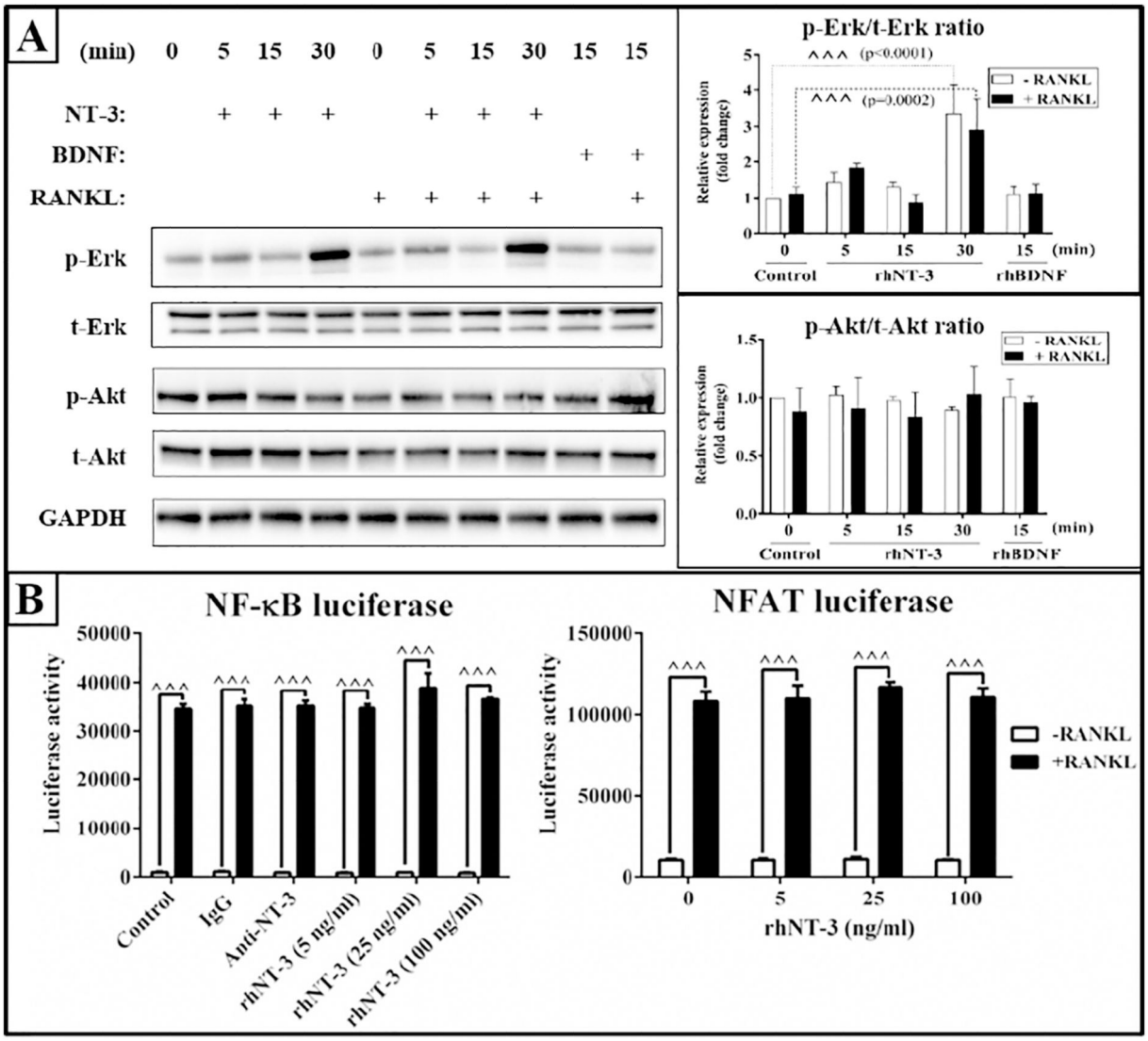

Fig. 7.

Effects of rhNT-3 treatment in activating TrkC downstream kinases Erk½ and Akt and osteoclastogenic transcription factors NF-κB or NFAT. (A) Western blot analyses examining protein expression of phosphorylated (p) and total (t) Erk½ or Akt (with GAPDH as an internal control) in $RAW_{264.7}$ osteoclast precursor cells after being treated for 0, 5, 15 or 30 min with rhNT3 (at 100 ng/ml) or for 15 min with 100 ng/ml rhBDNF as a comparison, and in the absence or presence of RANKL (25ng/ml) (n=3 wells/group). (B) Quantification of NF-κB or NFAT luciferase activity in $RAW_{264.7}$ pre-osteoclastic cells (stably expressing a NF-κB or NFAT luciferase reporter construct) after being treated with normal IgG, anti-NT-3 or varying doses of rhNT-3 (5, 25 or 100 ng/ml) for 1 h, followed by exposure to RANKL or control. Data represent mean values of triplicates ± SD.

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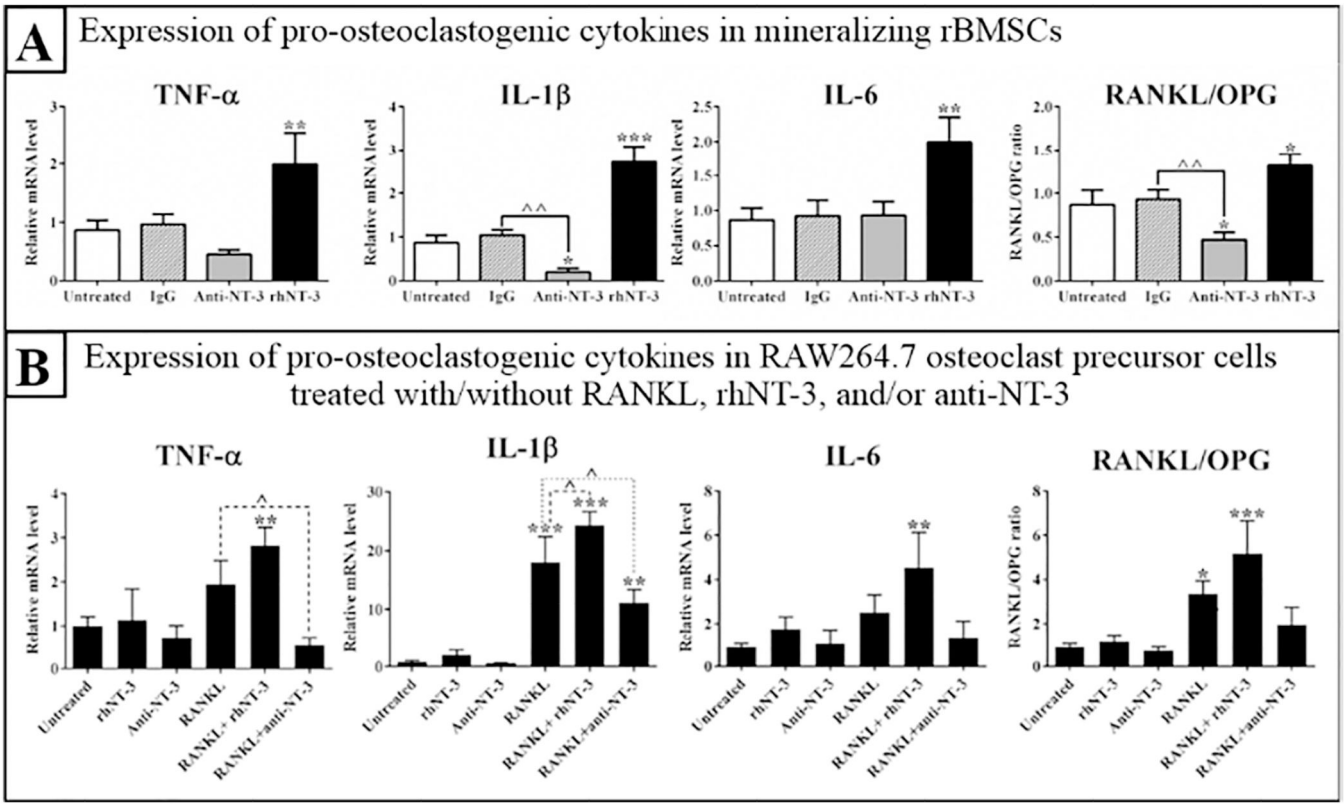

Fig. 8.

Changes in mRNA expression of osteoclastogenesis-related genes (TNF-α, IL-1β, IL-6 and RANKL/OPG ratio as quantitated by real time RT-PCR) in rat bone marrow stromal cell osteogenic cultures (rBMSCs) at day 14 of osteogenic induction (A) and in $RAW_{264.7}$ osteoclast precursor cells at day 5 of osteoclastogenic induction (B) with/without treatment with 7.5 μg/ml anti-NT-3 IgG or normal rabbit IgG, or 100ng/ml rhNT-3. Data represent mean values of triplicates ± SD.

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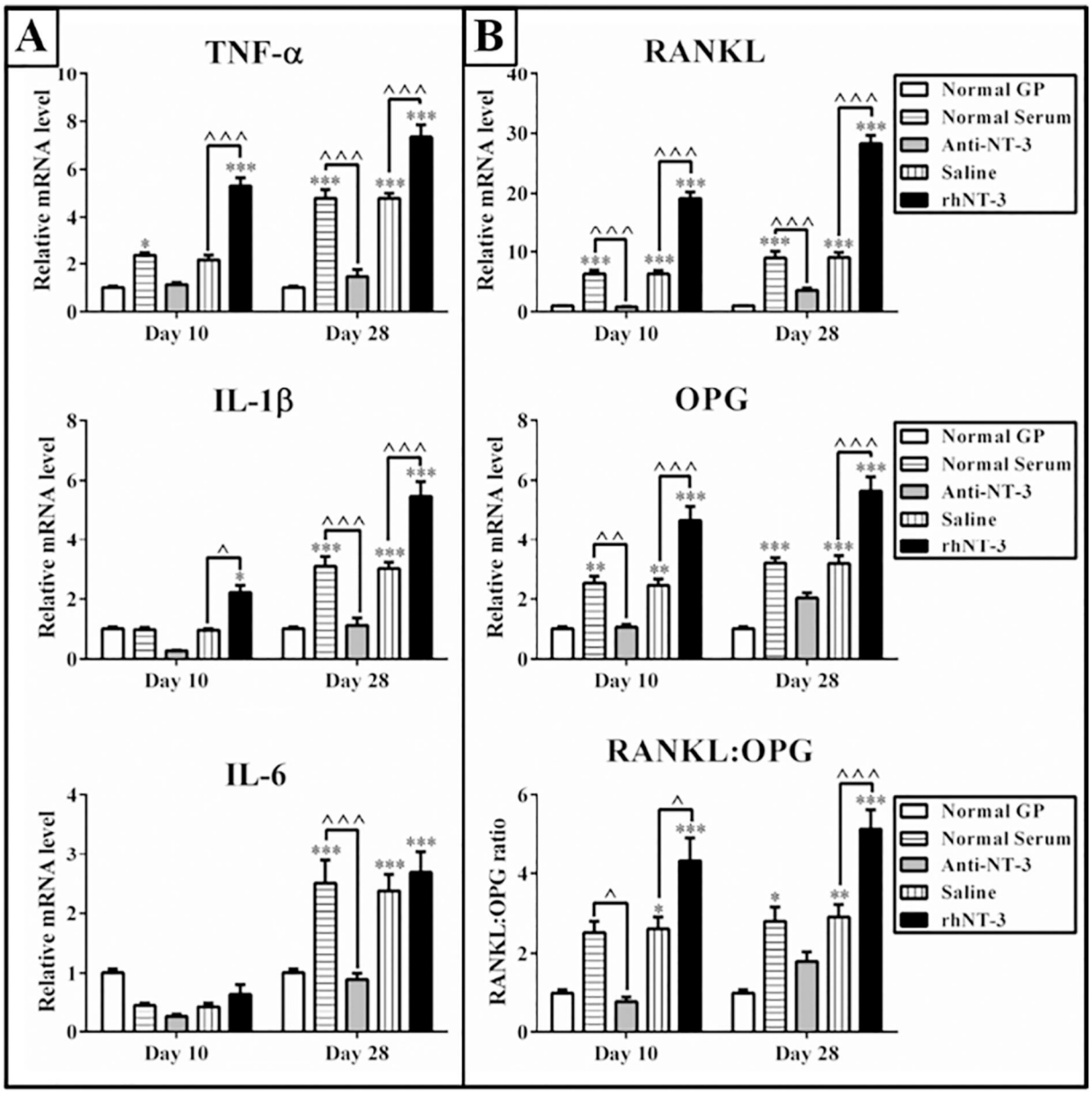

Fig. 9.

Effects of treatment with normal serum or anti-NT-3 serum IgG, rhNT-3 or saline control on mRNA expression of osteoclastogenesis regulatory molecules at the growth plate injury sites at days 10 or 28 after injury. (A) Pro-inflammatory cytokines TNF-α, IL-1β, and IL-6; (B) RANKL, OPG and RANKL/OPG ratio. Data (means ± SEM, n=6) were obtained by real-time RT-PCR are expressed as fold change in relation to uninjured normal growth plate (GP) controls and after being normalized to the internal standard Cyclophilin-A.

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Table 1

Rat primer sequences used for quantitative RT-PCR analyses.

Gene	Forward primer (5′–3′)	Reverse primer (5′–3′)
RANK	GGGAAAACGCTGACAGCTAATC	GGTCCCCTGAGGACTCCTTATT
OSCAR	CTGGTCATCAGTTCCGAAGG	CTATGATGCCCAAGCAGATG
Cathepsin K	GACTCAGAGTGCCACCTTCG	ACTGGCCCTGGTTTTTGACT
MMP-9	TCGAAGGCGACCTCAAGTG	GCGGCAAGTCTTCGGTGTAG
MMP-13	TTCACTTTGAGGACACGGGG	ACACGTGGTTCCCTGAGAAG
TNF-α	ATGGCCCAGACCCTCACACTCAGA	CTCCGCTTGGTGGTTTGCTACGAC
IL-1β	GTTTCCCTCCCTGCTCTGACA	GACAATGCTGCCTCGTGACC
IL-6	GATACCACCCACAACAGACC	GCCATTGCACAACTCTTTTCT
RANKL	CCGTGCAAAGGGAATTACAAC	GAGCCACGAACCTTCCATCA
OPG	GGAGACACAGCTCGCAAGA	GCACACTGCTTTCACAGAGG
Runx2	TCACAAATCCTCCCCAAGTGG	GAATGCGCCCTAAATCACTGA
Osterix	GCTTTTCTGTGGCAAGAGGTTC	CTGATGTTTGCTCAAGTGGTCG
Osteocalcin	GCTGGCCCTGACTGCATTCTG	ATTCACCACCTTACTGCCCTCCTG
NGF	GACTCCAAGCACTGGAACTC	CACGCAGGCTGTATCTATCC
BDNF	GCAGGGGCATAGACAAAAGG	TGAACCGCCAGCCAATTCTC
NT-3	TAAAGAAGCCAGGCCAGTCA	AGTCAGTGCTCGGACGTAGG
NT-4	CACTGGCTCTCAGAATGCAA	CAAGCGGTGTCGATCCGAAT
TrkA	CTGGTACCAGCTCTCCAACACT	CGCATGATGGCGTAGACATCAG
TrkB	GATCAAGACTCTGTGAACCTCA	TTGTAGAACCACTGAAGTGCTG
TrkC	GGTTCCAGCTCTCTAACACA	CAGCACCCCAGCATGACATC
p75	TGGGCCTTGTGGCCTATATTGC	ATGCCACTGTCGCTGTGCAGTT
Cyclophilin A	GAGCTGTTTGCAGACAAAGTTC	CCCTGGCACATGAATCCTGG

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Table 2

Mouse primer sequences used for real-time RT-PCR analyses.

Gene	Forward primer (5′–3′)	Reverse primer (5′–3′)
OSCAR	TGGTGTTGCTTCATTTCCTCAG	GGAAAAGGTTGACCCTGCCT
Cathepsin K	GGACGCAGCGATGCTAACTA	TGGGCAGAGAGAAGGGAAGT
Cyclophilin A	ATCTGCACTGCCAAGACTGA	TGGTGATCTTCTTGCTGGTC