Diverse roles of the tumor necrosis factor family member TRANCE in skeletal physiology revealed by TRANCE deficiency and partial rescue by a lymphocyte-expressed TRANCE transgene

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Tumor necrosis factor-related, activation-induced cytokine (TRANCE), a tumor necrosis factor family member, mediates survival of dendritic cells in the immune system and is required for osteoclast differentiation and activation in the skeleton. We report the skeletal phenotype of TRANCE-deficient mice and its rescue by the TRANCE transgene specifically expressed in lymphocytes. TRANCE-deficient mice showed severe osteopetrosis, with no osteoclasts, marrow spaces, or tooth eruption, and exhibited profound growth retardation at several skeletal sites, including the limbs, skull, and vertebrae. These mice had marked chondrodyplasia, with thick, irregular growth plates and a relative increase in hypertrophic chondrocytes. Transgenic overexpression of TRANCE in lymphocytes of TRANCE-deficient mice rescued osteoclast development in two locations in growing long bones: excavation of marrow cavities permitting hematopoiesis in the marrow spaces, and remodeling of osteopetrotic woven bone in the shafts of long bones into histologically normal lamellar bone. However, osteoclasts in these mice failed to appear at the chondroosseous junction and the metaphyseal periosteum of long bones, nor were they present in tooth eruption pathways. These defects resulted in sclerotic metaphyses with persistence of club-shaped long bones and unerupted teeth, and the growth plate defects were largely unimproved by the TRANCE transgene. Thus, TRANCE-mediated regulation of the skeleton is complex, and impacts chondrocyte differentiation and osteoclast formation in a manner that likely requires local delivery of TRANCE.

The binding of TRANCE to its receptor (TRANCE-R), itself a member of the tumor necrosis factor receptor family (4–6), activates similar intracellular signaling cascades in primary cultures of both dendritic cells and osteoclasts (9). The TRANCE–TRANCE-R interaction leads to the activation of NF-xB, ERK, c-Src, PI3-K, and Akt/PKB-dependent signaling cascades, presumably thereby inducing the survival and activation of osteoclasts necessary to resorb bone.

To investigate TRANCE’s diverse effects in vivo, we generated TRANCE-deficient mice by homologous recombination, and then crossed these mice to mice expressing the TRANCE transgene specifically in a majority of lymphocytes (T and B cells). Here, we report the skeletal manifestations in the TRANCE-deficient mice and in this partial genetic “rescue.” The phenotypic effects of the transgene in the skeleton varied considerably, from beneficial to no improvement, depending on the tissue location, implying that normal skeletal growth and maintenance depend in large part on a controlled, local supply of TRANCE.

Materials and Methods

Generation of TRANCE-Deficient or Transgenic Mice. We generated TRANCE knockout (KO) mice by homologous recombination, in which nucleotides 699–1,089 of exon 5 (amino acids 185–316) plus the additional 1,123 bp of 3’ untranslated region has been deleted. For this, we used the replacement vector, pPNT-TRANCE-RV1.2, which was constructed as follows: a 1.8-kb BamHI–KpnI genomic short arm for antisense insertion 5’ to PGKNeoA of the pPNT vector, and an 8.2-kb Sp3–Sp3I for 3’ antisense ligation next to PGKneoA. E14.1 embryonic stem (ES) cells were electroporated with pPNT-TRANCE-RV1.2. Two targeted ES cell clones carrying a single integration event were isolated and were microinjected into C57BL/6 blastocysts to produce germline chimeras. Both TRANCE KO mouse lines showed identical phenotypes, and only one line was used for further analysis and breeding with TRANCE transgenic mice. For TRANCE transgenic mice, we have generated several transgenic mouse lines overexpressing TRANCE under the control of the murine CD4 enhancer/promoter lacking the CD4 untranslated region has been deleted. For this, we used the replacement vector, pPNT-TRANCE-RV1.2, which was constructed as follows: a 1.8-kb BamHI–KpnI genomic short arm for antisense insertion 5’ to PGKNeoA of the pPNT vector, and an 8.2-kb Sp3–Sp3I for 3’ antisense ligation next to PGKneoA. E14.1 embryonic stem (ES) cells were electroporated with pPNT-TRANCE-RV1.2. Two targeted ES cell clones carrying a single integration event were isolated and were microinjected into C57BL/6 blastocysts to produce germline chimeras. Both TRANCE KO mouse lines showed identical phenotypes, and only one line was used for further analysis and breeding with TRANCE transgenic mice. For TRANCE transgenic mice, we have generated several transgenic mouse lines overexpressing TRANCE under the control of the murine CD4 enhancer/promoter lacking the CD4

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Abbreviations: TRANCE, tumor necrosis factor-related, activation-induced cytokine; TRANCE-R, TRANCE receptor; TRAP, tartrate resistant acid phosphatase; KO, knockout.

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silencer (10). Among three independent transgenic lines obtained, only one line, TRANCE.TG-1, which expressed transgenic TRANCE in both B and T cells, was used in this study. Transgenic TRANCE expression was detected in ~60% of T and B cells from these mice. The genotype of wild-type or null allele of TRANCE was determined by PCR analysis. Primer composition and concentration, together with the product size and PCR conditions used are as follows: 5′-TRANCE (0.5 μM), 5′-CCAACTGATCTGCTATCTCTG-3′; 3′ WT (0.2 μM), 5′-GTGGCAGCATGCTATC-3′ (345bp); 3′ NeoRec (0.2 μM), 5′-ATTGCACCGCAATCGCATTT-3′ (575bp); at 94°C for 5 min; 30 cycles (94°C for 30 s; 58°C for 45 s; 72°C for 1 min); and at 72°C for 7 min.

Mice carrying the TRANCE transgene were typed by staining peripheral blood lymphocytes with TRANCE-R-hlgG1 as described (11).

**Tissue Preparation and Histology.** Tibiae and femora were dissected and processed for histological and histochemical analyses as described previously (12). Briefly, some bones were processed for thin sections by fixation in 2.5% glutaraldehyde in 0.1 M sodium cacodylate followed by decalcification in 10% disodium EDTA, after which they were dehydrated with ethanol and embedded in epoxy (Epon; Polysciences). Some tissues were processed for tartrate resistant acid phosphatase (TRAP) enzyme histochemistry with controls, as described (12), by fixation in 2.5% glutaraldehyde in 0.1 M cacodylate supplemented with 7% glucose, dehydrated, and embedded in glycolmethacrylate. Brief treatment with 0.1% toluidine blue was used for histological staining. Some bones were processed without demineralization steps to assess the degree of mineralization in bones and cartilage.

**Radiological Analysis.** X-rays were taken at various ages of whole skeletons, of dissected limbs, and of skulls dissected at the midline and examined for skeletal phenotype, including the presence/absence of marrow spaces and erupted teeth (13, 14). The thickness of epiphyseal growth plates and of residual subepiphysial sclerosis in transgenic animals was assessed visually. Measurements of bone dimensions were made on radiographs projected at an enlargement of approximately 10× to permit greater precision. Tibial length was measured between the proximal and distal growth plates as described (15). Tibial width was measured at the thickest part of the proximal epiphysis. Skull length was measured both from the posterior-most surface of the occipital bone to the posterior surface of the incisor (total length) and from the anterior edge of the tympanic bulla to the posterior surface of the incisor (anterior length). Standard statistical parameters and tests for significance of differences were obtained with EXCEL 97 (Microsoft).

**Results**

**Skeletal Growth: Radiological Evaluation.** Radiographic examination showed the overall skeletal impact of deleting the endogenous TRANCE gene as seen in TRANCE KO mice, and then of “rescuing” the TRANCE KO mice with a lymphocyte-expressed TRANCE transgene as seen in TRANCE-null mice expressing the TRANCE transgene (hereafter called TRANCE TG/KO mice) (Fig. 1). The long bones of the hind limb, the vertebral column, the pelvis, and the skull were all shorter in the TRANCE KO mice than in the wild-type animals, the teeth did not erupt, and the epiphyseal growth plates (radiolucent band in ko Inset, arrow) were abnormally thick. The TRANCE transgene improved some, but not all, of the defects in TRANCE KO mice. Most notably, marrow spaces were restored in the appendicular and axial skeleton, and growth plate thickness appeared closer to normal. Strikingly, however, there remained dense, sclerotic regions of unresorbed mineralized cartilage at the ends of the marrow cavities (Inset in tg/ko, Fig. 1), and the teeth did not erupt.

To assess these effects more quantitatively, radiographs were enlarged by projection, and a series of measurements was made on animals of the different genotypes at ages ranging up to 3 mo. Fig. 2 shows tibial lengths of 2-mo-old animals measured in this way. In TRANCE KO animals, the tibia was only 68% of the normal length at 1 mo and 65% of the normal length at 2 mo, and TRANCE TG/KO mice showed only a marginal improvement, measuring 70% and 77% of normal at 2 and 3 mo, respectively. On the other hand, the width of the proximal tibia was not significantly different from normal in either the TRANCE KO or TG/KO animals at 1 mo, but was greater than normal at 2 and 3 mo (TRANCE KO mice, 131% of normal at 2 mo, P < 0.001; TRANCE TG/KO mice, 137% at 3 mo, P < 0.002). Skull lengths were also measured, both total length and the length from the anterior margin of the tympanic bulla, to assess relative contributions of different centers of skull growth. Both dimensions were affected similarly in the TRANCE KO and TG/KO animals, measuring about 87% of the normal length at 1 mo. We also measured the length of the same segment of the vertebral column (10 vertebrae moving caudally from the first sacral vertebra) in mice of each genotype. The TRANCE KO animals had significantly shorter spinal columns than wild-type animals (77% at 1 mo, P < 0.001; 74% at 2 mo, P < 0.01). Interestingly, the TRANCE TG/KO animals showed improvement in this parameter, measuring 93% of normal by 3 mo of age, not significantly different from wild-type (P > 0.1). We also carried out these tests comparing wild-type mice and mice expressing the transgene in a wild-type background, and there were no significant differences (data not shown). In summary, the impact of the lymphocyte-expressed TRANCE transgene on growth varied with skeletal site, having no effect on either length or width of long bones or skull, while causing a substantial improvement in vertebral growth by 3 mo of age.
Skeletal Growth: Microscopic Evaluations. To understand better the reasons behind the failure of long bone growth in the TRANCE KO and TG/KO animals, we performed histological analyses. Fig. 3 shows demineralized, 1-μm sections of proximal tibiae from animals at 1 mo of age. The growth plates of both TRANCE KO and TG/KO animals lack the normal, columnar organization of chondrocytes, and have markedly reduced proliferating zones and increased hypertrophic zones, although the hypertrophic zones of TRANCE TG/KO animals were less expanded. An orderly transition from bone to cartilage at the chondroosseous junction failed to occur in both TRANCE KO and TG/KO animals. The mineralized cartilage and bone that filled the diaphysis in TRANCE KO mice was histologically indistinguishable from the sclerotic tissue remaining at the metaphyseal ends of the TRANCE TG/KO long bones (asterisks, Fig. 3), and suggests that material has never been resorbed or remodeled. These growth plate abnormalities provide a likely, histologically identifiable, cellular explanation for the growth defects observed in both the TRANCE KO and TG/KO animals.

To examine in more detail the cellular basis for the skeletal phenotype of the TRANCE KO and TG/KO mice, we used enzyme histochemistry to visualize TRAP, an osteoclast-specific marker (16). Fig. 4 shows this comparison. In a and b, the proximal tibia of a wild-type mouse shows normal endochondral bone features, with areas 1–4 marked in a as key regions of osteoclast activity: 1, above the growth plate at the bottom of the epiphysis; 2, immediately below the growth plate at the chondroosseous junction; 3, along the trabeculae of the primary spongiosa; and 4, on the periosteal surface of the metaphysis, where the flared ends of the bone must be narrowed during growth. b shows TRAP-positive cells in all these areas in the wild-type mouse. c shows that no osteoclasts are seen in the TRANCE KO mouse. We have examined more than 75 similar sections from 8 individual TRANCE KO mice and found no evidence of TRAP-positive cells in any of them, a finding consistent with earlier reports (7). In e, the histology of the TRANCE TG/KO mice confirms the radiologic evidence shown in Fig. 1. A layer of dense, mineralized cartilage and bone underlies the growth plate, with an abrupt transition to a marrow space devoid of trabeculae. In d, osteoclasts are seen only in areas 1 and 3, i.e., above the growth plate and at the lower boundary of the sclerotic metaphysis. Examination of nondemineralized sections confirmed the presence of mineral in this region (data not shown). Both the chondroosseous junction and the metaphyseal periosteum are devoid of osteoclasts in the TRANCE TG/KO animals. In f, a higher magnification histological section confirms that the TRANCE TG/KO mice are producing bona fide osteoclasts. The image is from area 3, the boundary between the marrow space and the sclerotic metaphysis, and shows an active, multinucleated osteoclast resorbing the mineralized matrix. The venous sinus in the adjacent area appears normal, as does the abundant, active marrow that develops once a cavity is excavated.

Because osteoclasts only appear on the endosteal surface of long bones from TRANCE TG/KO mice without involvement of periosteal osteoclasts (see above, and Fig. 4), the question arises as to whether the bone of the diaphysis undergoes normal remodeling. As shown in Fig. 5, this appears to be the case. In the TRANCE TG/KO mice, normal, lamellar bone replaced the nonremodeled woven bone seen in the TRANCE KO animals. We noted abundant osteocysts occupying lacunae within the layered extracellular matrix, a layer of healthy-looking, active osteoblasts lining much of the endosteal surface, and the lamellar organization of the bone evident from the endosteal surface through to the periosteal side.
Discussion

The results reported here (i) provide quantitative data on retarded skeletal growth at multiple sites in TRANCE KO mice; (ii) confirm that such mice have osteopetrosis and lack osteoclasts and erupted teeth (7); and (iii) demonstrate that a lymphocyte-delivered transgenic TRANCE has a positive effect on osteoclast differentiation and function in only a subset of their normal skeletal sites of action.

The growth retardation of endochondral bones in TRANCE KO and TG/KO mice correlates with a unique chondrodystrophy at the growth plates, i.e., a marked reduction of the proliferating chondrocyte zone and an increase in the zone of hypertrophy. During normal skeletal growth, a remarkable constancy in the relative length of metaphyseal trabeculae is maintained at both ends of long bones as the central marrow cavity expands longitudinally (17). This is accomplished by a highly coordinated differentiation of chondrocytes in growth plates and the formation and resorption of bone in the metaphysis. Chondrocyte proliferation, hypertrophy, mineralization of vicinal matrix, and apoptosis in the orderly columns of growth plates is precisely balanced by vascular invasion of lacunae at the chondroosseous junction, removal of more than half of these columns at this site, deposition of bone onto the remaining mineralized cartilaginous scaffolds beginning at the chondro-osseous junction, and removal of both mineralized cartilage and bone at the advancing marrow cavity ends of metaphyseal trabeculae (3, 18, 19). The mechanism by which vertebrates achieve this precisely regulated control of longitudinal bone growth has been the subject of intensive investigation over the past decade, and substantial progress in delineating the control of long bone growth has been made by a number of workers. Central to our current understanding of growth plate regulation is a feedback loop in which Indian hedgehog and parathyroid hormone-related protein act in concert to prolong the proliferative stage of growth plate chondrocytes, thereby delaying chondrocyte hypertrophy, and permitting normal long bone growth to proceed (20–23). Our data show that, in TRANCE KO mice, growth is retarded, chondrocyte differentiation is deranged, and bone resorption is absent. The ability of lymphocyte-delivered transgenic TRANCE to rescue marrow cavity appearance and expansion, but without an effect on resorption of the mineralized cartilaginous scaffold at the chondroosseous junction or on the chondroytidyse, indicates that correcting a specific subset of the effects of the TRANCE-deficient state requires local delivery of the molecule. Exactly how this TRANCE-mediated growth plate regulation is integrated with the current models is an important question raised by the present results.

Fig. 4. Osteoclast distribution in proximal tibiae of wild-type (wt), TRANCE KO (ko), and TRANCE TG/KO (tg/ko) mice. (a and e) Toulidine blue stained, Epon-embedded sections from 1-mo-old wild-type and TRANCE TG/KO animals. (b–d) Osteoclasts (red) visualized by TRAP histochemistry on similarly oriented sections from wild-type (wt), TRANCE KO (ko), and TRANCE TG/KO (tg/ko) animals, respectively. Key areas of osteoclast activity in growing long bones are indicated by numbers 1–4 in a, and represent: 1, lower margin of epiphysis; 2, chondroosseous junction; 3, ends of metaphyseal trabeculae; and 4, metaphyseal periosteum. Osteoclasts are found in all four areas in wild type (b). No osteoclasts are present in TRANCE KO mice (c). In TRANCE TG/KO mice (d), osteoclasts are present in areas 1 and 3 only (arrows), with none in either the chondroosseous junction or the metaphyseal periosteum (asterisks). Marrow cavities (m) are seen in wild-type and TRANCE TG/KO, but not TRANCE KO mice. (f) A typical, multinucleated osteoclast resorbing mineralized cartilage (c) in area 3 of the TRANCE TG/KO mouse (s, venous sinus; m, marrow). (Original magnification: a–e, ×205; f, ×1,300.)
Regulation of the width of long bones at their ends during development is controlled by periosteal osteoclasts in the metaphyseal region that resorb bone formed by osteoblasts in the periosteal collar, which surrounds the lower growth plate and the subjacent metaphysis (18). This resorption narrows centripetally the external contours in the metaphyseal region to meet the narrower cylindrical shaft in the diaphysis. Without resorption at this site, where there are no osteoclasts in either TRANCE KO or TG/KO mice (site 4 in Fig. 4 c and d), the bulbous ends of long bones remain significantly wider than normal, a condition we observed in these mice. On the other hand, growth in width of the diaphyseal shafts of long bones is accomplished by coordinating periosteal bone formation with endosteal resorption. This remodels the woven bone originating from the growth plate into secondary, lamellar bone. Because endosteal osteoclasts and bone resorption are restored in TRANCE TG/KO mice, the weak, woven, primary diaphyseal bone found in TRANCE KO mice (Fig. 5b) is remodeled to lamellar bone in TRANCE TG/KO mice (Fig. 5c).

Recovery of normal length of sacral and caudal vertebrae by the third month in TRANCE TG/KO mice, whose hind limb bone lengths did not recover, may be related to their developmental timing. The general development of the mammalian embryo proceeds in a cranio-caudal direction (24) with the hind limb bones forming earlier than these vertebrae. Thus, it is likely that these long bones were under the influence of a TRANCE-free environment longer than the vertebrae and less responsive to recovery. The hypothesis that skeletal structures appearing later in development are more amenable to rescue should be tested in TRANCE TG/KO mice and could have important developmental implications for this molecule.

The absence of erupted teeth in TRANCE TG/KO mice was an unexpected finding of these studies. Tooth eruption depends on bone resorption (25); namely, the creation of eruption pathways in specific sites by osteoclasts. Timing is crucial because delayed the activation of resorption beyond the first postnatal day in osteopetrotic (toothless, tl) rats prevents eruption of incisors (26). Eruption of the molars occurs later, but resorption must begin before the third or the eleventh day to permit eruption of the first or second mandibular molars, respectively. Consistent with the absence of tooth eruption in TRANCE KO or TG/KO mice, no osteoclasts were found in the eruption pathways of the first molars when sections from mandibles of the 3-day-old TRANCE KO or TG/KO mice were examined, whereas numerous TRAP-positive osteoclasts were identified from those of wild-type mice (data not shown). Because the lymphocyte-directed expression of TRANCE in TRANCE TG/KO mice began in utero, the lack of tooth eruption implies that a local supply of TRANCE is required for osteoclastogenesis in these sites.

In summary, the results reported here demonstrate that TRANCE plays a key role in the developmental role in the two principal skeletal tissues, cartilage and bone. TRANCE is essential for normal differentiation of two of the major cellular constituents of the skeleton, osteoclasts and chondrocytes. These findings expand our knowledge of skeletal effects of TRANCE beyond its role in regulating bone resorption to an unidentified mechanism coordinating chondrocyte differentiation to ensure their orderly progression during longitudinal bone growth. Thus, the incomplete rescue of TRANCE’s functions in the skeleton when supplied by lymphocytes implies that local microenvironmental delivery is essential for normal skeletal growth and development.

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Fig. 5. Cortical bone remodeling is rescued in TRANCE TG/KO mice. Comparison of remodeling of diaphyseal cortical bone in wild-type (wt), TRANCE KO (ko) and TRANCE TG/KO (tg/ko) mice. Periosteal surface is toward the top and endosteum toward the bottom in all panels. The TRANCE KO mice show typical osteopetrotic woven bone that has never been remodeled, with mineralized cartilage cores (c, purple) covered by bone (blue). Normal remodeled, lamellar bone (L) lacking cartilage cores is seen in the wild-type and TRANCE TG/KO diaphyses, with healthy, cuboidal osteoblasts (●) lining the endosteal bone surface, osteocytes in lacunae (arrows), and active marrow (m) in both specimens. Toluidine-blue-stained, 1-μm Epon sections, tibial diaphyses of 30-day-old animals. (Original magnification, ×1,300.)

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