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Expression Analysis of *nha-oc/NHA2:* a Novel Gene Selectively Expressed in Osteoclasts

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Abstract

Bone resorption by osteoclasts is required for normal bone remodeling and reshaping of growing bones. Excessive resorption is an important pathologic feature of many diseases, including osteoporosis, arthritis, and periodontitis (Abu-Amer, 2005). On the other hand, deficient resorption leads to osteopetrosis which is characterized by increased bone mass and may lead to bone deformities or in severe cases to death (Blair and Athanasou, 2004; Del Fattore et al., 2006).

Recently, we identified a gene, *nha-oc/NHA2*, which is strongly up regulated during RANKL-induced osteoclast differentiation *in vitro* and *in vivo. nha-oc/NHA2* encodes a novel cation/proton exchanger that is strongly expressed in osteoclasts. The purpose of this work was to further validate the restricted expression of *nha-oc/NHA2* in osteoclasts by *in situ* hybridization. Our results showed that *nha-oc* is expressed predominantly in bone. In the head, expression was found in the supraoccipitale bone, calvarium, mandible, and maxilla. Furthermore, *nha-oc* positive cells co-express the osteoclast markers TRAP and *cathepsin k*, confirming *nha-oc/NHA2* osteoclast localization. However, only a subset of *cathepsin k*-expressing cells is positive for *nha-oc/NHA2*, suggesting that *nha-oc* is expressed by terminally differentiated osteoclasts.

Keywords

Osteoclast Specific; Cation Proton Exchanger; Bone Resorption

1. RESULTS AND DISCUSSION

The electro-neutral exchange of Na^+/H^+ or K^+/H^+ down their concentration gradients (antiport activity) is present in all phyla and kingdoms (Burckhardt *et al*., 2002; Demaurex and Grinstein, 1994; Ritter *et al*., 2001). Sodium-proton antiporters (NHAs) belong to a superfamily of proteins called cation-proton antiporters (CPA), responsible for this activity. CPA activity is essential to control processes such as intracellular pH, cell volume, and reuptake of sodium across several epithelia. During the course of a microarray screening aimed at identifying genes whose expression was upregulated during RANKL-induced osteoclastogenesis, we identified

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a novel gene, *nha-oc/NHA2* (Okamatsu *et al*., 2004). *nha-oc/NHA2* is the mouse orthologue of a previously identified but uncharacterized human gene, HsNHA2 (Brett *et al*., 2005). *nha-oc/NHA2* encodes a novel CPA2 member that is unique in that it is the first metazoan and tissue-restricted CPA2 characterized to date. Multi-tissue Northern-blot analysis showed no expression of *nha-oc/NHA2* in heart, brain, spleen, lung, liver, skeletal muscle, kidney, or testis (Figure 1A). Furthermore, RT-PCR amplification of RNA from a panel of mouse tissues showed *nha-oc/NHA2* expression restricted to bone (Figure 1B).

Using an *in vitro* system of osteoclast differentiation we characterized the expression of several osteoclast-specific genes during RANKL stimulation of RAW 264.7 monocytes for 5 days (Battaglino *et al*., 2002). In this system TRAP mRNA expression can be detected after 1 day of RANKL stimulation, while *cathepsin k* mRNA expression is detected after 3 days (Figure 1C). Under these conditions, only very weak expression of *nha-oc* could be detected (not shown). To increase the sensitivity of the detection we performed Northern Blot analysis using polyA$^+$ RNA. Under these conditions, expression could be clearly detected at day 3, peaking after 5 days (Figure 1D), indicating that *nha-oc* is expressed in later stages of osteoclastogenesis.

To evaluate the specific expression of *nha-oc/NHA2 in vivo*, we performed *in situ* hybridization analysis in one- (Figure 2) and three-day old (Figure 3) mouse head sections. We chose the neonatal head because it is a site of active osteoclastogenesis, bone growth and remodeling. *nha-oc/NHA2* expressing cells are clearly detected around tooth buds, in the mandible, maxilla, calvarium and the occipital bone. In addition, adjacent sections were stained for TRAP or hybridized to a *cathepsin k* probe, to correlate with osteoclast localization. Figures 2 and 3 illustrate co-expression of TRAP, *cathepsin k*, and *nha-oc* in the head. Co-expression was also observed in other bones, such as the clavicle and femur (Figure 4). In the femur, *nha-oc* expressing cells are found in a region adjacent to the growth plate. Taken together, these results confirm that *nha-oc* is highly and selectively expressed in osteoclasts.

Figure 4 also shows a magnification of an *in situ* hybridization performed on the third molar of a one-day old mouse. The white arrows indicate mononucleated cells. We noted that while *cathepsin k* expression could be seen in both mononucleated and multinucleated osteoclasts surrounding the 3rd molar, *nha-oc* expression was restricted to a subset of *multinucleated* cells, suggesting that *nha-oc* expression is restricted to mature osteoclasts.

Thus, the experiments described here show that the *nha-oc/NHA2* gene is primarily expressed in osteoclasts. Given the fact that *nha-oc*/NHA2 orthologues (including the human HsNHA2) make up a family of genes highly conserved in *vertebrates*, we anticipate that these other orthologues will exhibit a similar expression pattern. Supporting this notion, we have data showing equivalent restricted osteoclast expression of the rat *nha-oc* (R. Battaglino, unpublished data).

2. MATERIALS AND METHODS

2.1. Animals

All protocols using rodents were approved by the Institutional Animal Care and Use Committee and were in compliance with all federal and local guidelines. Mice were of the C57BL6 strain.

2.2. Cells

RAW 264.7 (TIB-71) mouse macrophage/monocytes were purchased from ATCC. Cells were cultured in DMEM/1.5g/l sodium bicarbonate (JRH Biosciences, Lenexa, KS) supplemented with 10% non heat-inactivated FBS (BioWhitaker). To induce osteoclast differentiation, cells

were cultured in the presence of 50 ng/ml soluble RANKL (PeproTech Inc., Rocky Hill, NJ) for up to 5 days with changes of medium and RANKL every other day.

2.3. Northern Blotting and RT-PCR

Total RNA was extracted from cells and tissues using the TRIAZOL reagent (Invitrogen, Carlsbad, CA). For RT-PCR, 2μg of total RNA were reversed-transcribed into cDNA using SuperScript II (Invitrogen, Carlsbad, CA) following the manufacturer's instructions. One-twenty fifth of the cDNA was used as a template for the PCR reaction, using the Platinum PCR Supermix (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. We used the following primers that amplified a 400bp NHA-oc/NHA2 fragment. This fragment was also used as a probe for Northern blot hybridization:

NHAoc/S (400bp) 5' GAA CTA CGG GAA GAG AGA GTC AT 3'

NHAoc/AS (400bp) 5' GGG ATA TTC CTC AAG AGA AAC C 3'

For Northern blot analysis, either 10 μg total RNA or 1.5 μg poly A^+ RNA, isolated using the PolyATtract mRNA Isolation System III (Promega, Madison, WI), was separated on a 1.2% agarose denaturing gel and transferred onto nylon membranes. A dsDNA NHA-oc/NHA2 probe was generated by RT-PCR from RANKL-stimulated RAW 264.7 total RNA and ^{32}P-labeled using the Rediprime™ II DNA Labeling System (GE Healthcare, Piscataway, NJ). The probes for β-actin, TRAP, cathepsin K and MMP-9 were generated by RT-PCR, as described by Battaglino (Battaglino et al., 2004). The membranes were hybridized to the radio labeled probes overnight at 42°C in hybridization solution (50% formamide, 5X SSC, 5X Denhardt's solution, 0.5% SDS and 100 μg/ml salmon sperm DNA), washed twice to remove unbound probe in 1X SSC, 0.2% SDS at 50°C for 20 minutes, and exposed to X-ray film. For tissue expression studies a Mouse Multiple Tissue Northern (MTN) blot was utilized (BD Biosciences, San Jose, CA). Hybridization and washes were performed according to the manufacturer's instructions.

2.4. In situ hybridization

^{35}S- or Digoxigenin (DIG)- labeled riboprobes, complementary RNAs (cRNAs), were transcribed from the following DNAs (cDNAs). The full length open reading frame for both 1640-bp mouse *nha-oc* cDNA and 997-bp mouse *cat-k* cDNA were subcloned into pCR II TOPO vector (Invitrogen, Carlsbad, CA) after PCR amplification using the following primer pairs:

nha-oc: (caccatggaggatgaagataagacagc, ggcttccgtaatggaatcctc)

cat-k: (ggatgtgggtgttcaagtttctg, gaatcacatcttggggaagc)

Sense and antisense cRNAs were synthesized from the linearized plasmids using the MAXIscript SP6/T7 kit (Ambion, Austin, TX), and DIG RNA labeling mixture (Roche, Indianapolis, IN). The sense RNA encoding the mouse *nha-oc* and the anti-sense RNA encoding the mouse *cat-k* was used as negative and positive controls respectively.

DIG-labeled hybridization was performed as follows: sections were deparaffinized with xylene and hydrated with decreased concentrations of ethanol. After washing with phosphate buffered saline (PBS), sections were digested with 1 μg/mL proteinase K (RT, 20 min) in TE (10 mM Tris-HCl and 2 mM ethylenediaminetetraacetic acid, EDTA, pH 7.4), washed again with PBS, and treated with 4% paraformaldehyde (PFA)/PBS (10 min). Sections then were rinsed in water, acetylated with 0.25% acetic anhydride in the presence of triethanolamine (0.1M, 10 min), incubated in PBT (3 min) three times, rinse with water, and air-dried. Hybridizations were performed in a humidified chamber in a solution containing 5x saline-sodium citrate (SSC) and 50% formamide (18 h, 70 °C).

After hybridization, sections were washed briefly with 5x SSC at 65 °C, 50% formamide-1x SSC (65 °C, 30 min), and with TNE (10 mM Tris-HCl, pH 7.6, 500 mM sodium chloride, and 1 mM EDTA) (37 °C, 10 min). Sections were then treated with 10 μg/mL RNase A in TNE (37 °C, 30 min). After being washed with TNE (10 min), sections were incubated once with 2x SSC (65 °C, 20 min), and twice with 0.2x SSC (65 °C, 20 min).

Sections were subsequently washed three times with MABT (100 mM maleic acid, 150 mM sodium chloride, 0.1% Tween-20, pH 7.5) (5 min), and blocked with 2% normal sheep serum and 2% Boheringer Blocking Reagent (Roche, Indianapolis, IN) in MABT (1 h). Finally, sections were incubated with anti-DIG antibody conjugated with alkaline phosphatase (Roche, Indianapolis, IN) at 4 °C overnight. After washing with MABT (5 min, 3 times), signals were detected with BM Purple (Roche, Indianapolis, IN) following the manufacturer's instructions.

Micrographs were taken with a Stemi SV11 microscope (Carl Zeiss MicroImaging, Inc. Thornwood, NY) and a Leica DMLS microscope (Leica Microsystems). The images were captured using Zeiss Axiovision 3.1 software (Stemi SV11) and DC Twain V4.0.2.0 software (Leica DMLS).

2.5. Histology

For tartrate resistant acid phosphatase (TRAP) staining, tissue sections were deparaffinized in xylene and rehydrated in decreasing concentrations of ethanol. After washing sections with PBS, Napthol AS-MX phosphate and Fast Red Violet LB Salt (Sigma, St. Louis, MO) were used as a substrate of TRAP and sections were stained according to manufacture's instructions. Adjacent sections were stained with Hematoxylin/Eosin

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References

1. Abu-Amer Y. Advances in osteoclast differentiation and function. Curr Drug Targets Immune Endocr Metabol Disord 2005;5:347–355. [PubMed: 16178795]
2. Battaglino R, Fu J, Spate U, Ersoy U, Joe M, Sedaghat L, Stashenko P. Serotonin regulates osteoclast differentiation through its transporter. J Bone Miner Res 2004;19:1420–1431. [PubMed: 15312242]
3. Battaglino R, Kim D, Fu J, Vaage B, Fu XY, Stashenko P. c-myc is required for osteoclast differentiation. J Bone Miner Res 2002;17:763–773. [PubMed: 12009006]
4. Blair HC, Athanasou NA. Recent advances in osteoclast biology and pathological bone resorption. Histol Histopathol 2004;19:189–199. [PubMed: 14702187]
5. Brett CL, Donowitz M, Rao R. Evolutionary origins of eukaryotic sodium/proton exchangers. Am J Physiol Cell Physiol 2005;288:C223–C239. [PubMed: 15643048]
6. Burckhardt G, Di Sole F, Helmle-Kolb C. The Na+/H+ exchanger gene family. J Nephrol 2002;15 (Suppl 5):S3–21. [PubMed: 12027219]
7. Del Fattore A, Peruzzi B, Rucci N, Recchia I, Cappariello A, Longo M, Fortunati D, Ballanti P, Iacobini M, Luciani M, Devito R, Pinto R, Caniglia M, Lanino E, Messina C, Cesaro S, Letizia C, Bianchini G, Fryssira H, Grabowski P, Shaw N, Bishop N, Hughes D, Kapur RP, Datta HK, Taranta A, Fornari R, Migliaccio S, Teti A. Clinical, genetic, and cellular analysis of 49 osteopetrotic patients: implications for diagnosis and treatment. J Med Genet 2006;43:315–325. [PubMed: 16118345]
8. Demaurex N, Grinstein S. Na+/H+ antiport: modulation by ATP and role in cell volume regulation. J Exp Biol 1994;196:389–404. [PubMed: 7823036]
9. Okamatsu Y, Kim D, Battaglino R, Sasaki H, Spate U, Stashenko P. MIP-1 gamma promotes receptor-activator-of-NF-kappa-B-ligand-induced osteoclast formation and survival. J Immunol 2004;173:2084–2090. [PubMed: 15265944]

10. Ritter M, Fuerst J, Woll E, Chwatal S, Gschwentner M, Lang F, Deetjen P, Paulmichl M. Na(+)/H(+)exchangers: linking osmotic dysequilibrium to modified cell function. Cell Physiol Biochem 2001;11:1–18. [PubMed: 11275678]

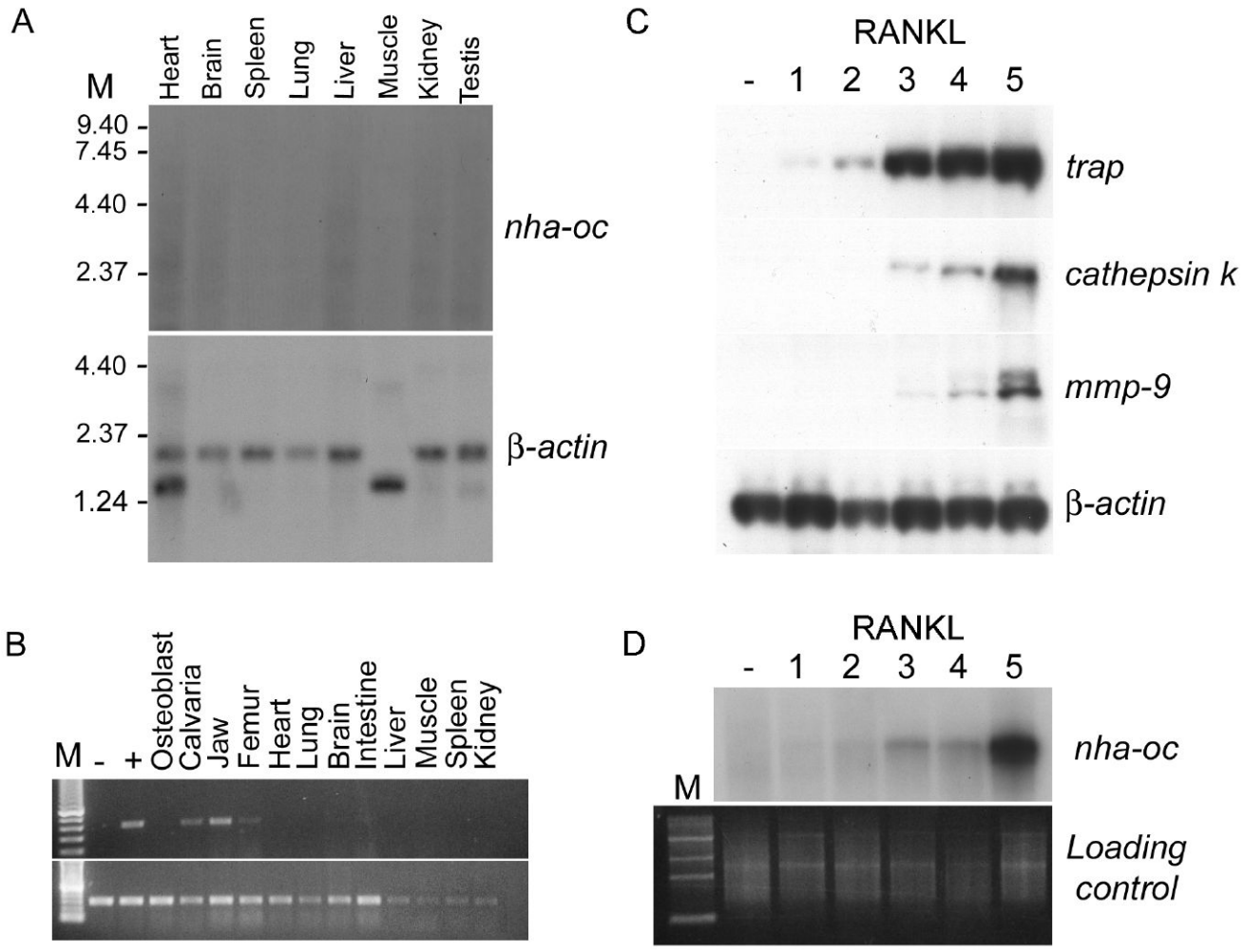

Figure 1.

nha-oc/NHA2 is highly and selectively expressed in osteoclasts.

(A) A multiple tissue polyA+ RNA blot hybridized to a specific probe detects no *nha-oc/NHA2* expression in heart, brain, spleen, lung, liver, skeletal muscle, kidney, or testis (top panel). The blot was stripped and reprobed with a β-actin probe (bottom panel). M: MW marker.

(B) Top panel: RT-PCR amplification detects *nha-oc/NHA2* expression in bone (calvaria, jaw, femur) and RAW 264.7- derived osteoclasts (lane +). No *nha-oc/NHA2* expression was seen in heart, lung, brain, intestine, liver, skeletal muscle, spleen, kidney, undifferentiated RAW 264.7 cells (lane -) or primary osteoblasts. Bottom panel: β-actin control. M: 100 bp ladder

(C) Northern blot analysis of unstimulated RAW 264.7 cells (lane -) or RAW 264.7 cells stimulated with RANKL for up to 5 days (lanes 1 to 5) using osteoclast gene -specific probes. A distinctive temporal expression pattern can be observed for each gene (TRAP, cathepsin k and MMP-9).

(D) A polyA+ Northern blot analysis of RNA (1.5 μg/lane) from unstimulated RAW 264.7 cells (lane -) or RAW 264.7 cells stimulated with RANKL for up to 5 days (lanes 1 to 5) using a *nha-oc/NHA2*-specific probe. *nha-oc/NHA2* expression can be strongly detected at 5 days. The Ethidium Bromide stained gel is shown as a loading control. M: MW marker

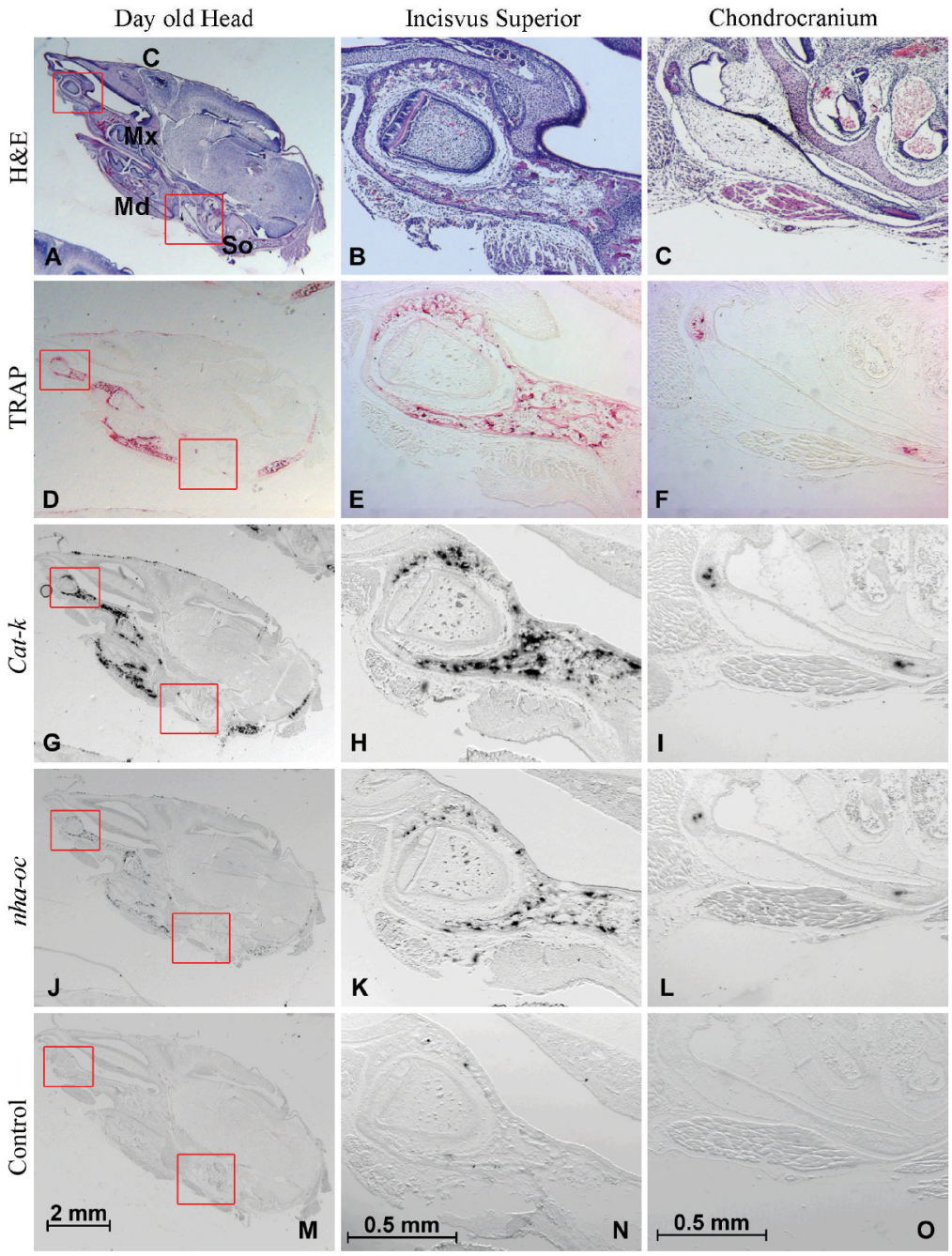

Figure 2.

nha-oc/NHA2 is expressed in osteoclasts in one-day old mouse head.

Mouse head sections were fixed and stained with Hematoxylin/Eosin (A, B, C); TRAP (D, E, F); hybridized to a *cathepsin k* specific probe (G, H, I); *nha-oc/NHA2* specific probe (J, K, L) or *nha-oc/NHA2* sense negative control probe (M, N, O).

In full heads (A, D, G, J and M) as well as in details from the Incisivus superior (B, E, H, K and N) or the chondrocranium (C, F, I, L and O) TRAP, *cathepsin k* and *nha-oc/NHA2*-expressing cells colocalize. C: calvaria, Mx: maxilla, Md: mandible, So: supraoccipital.

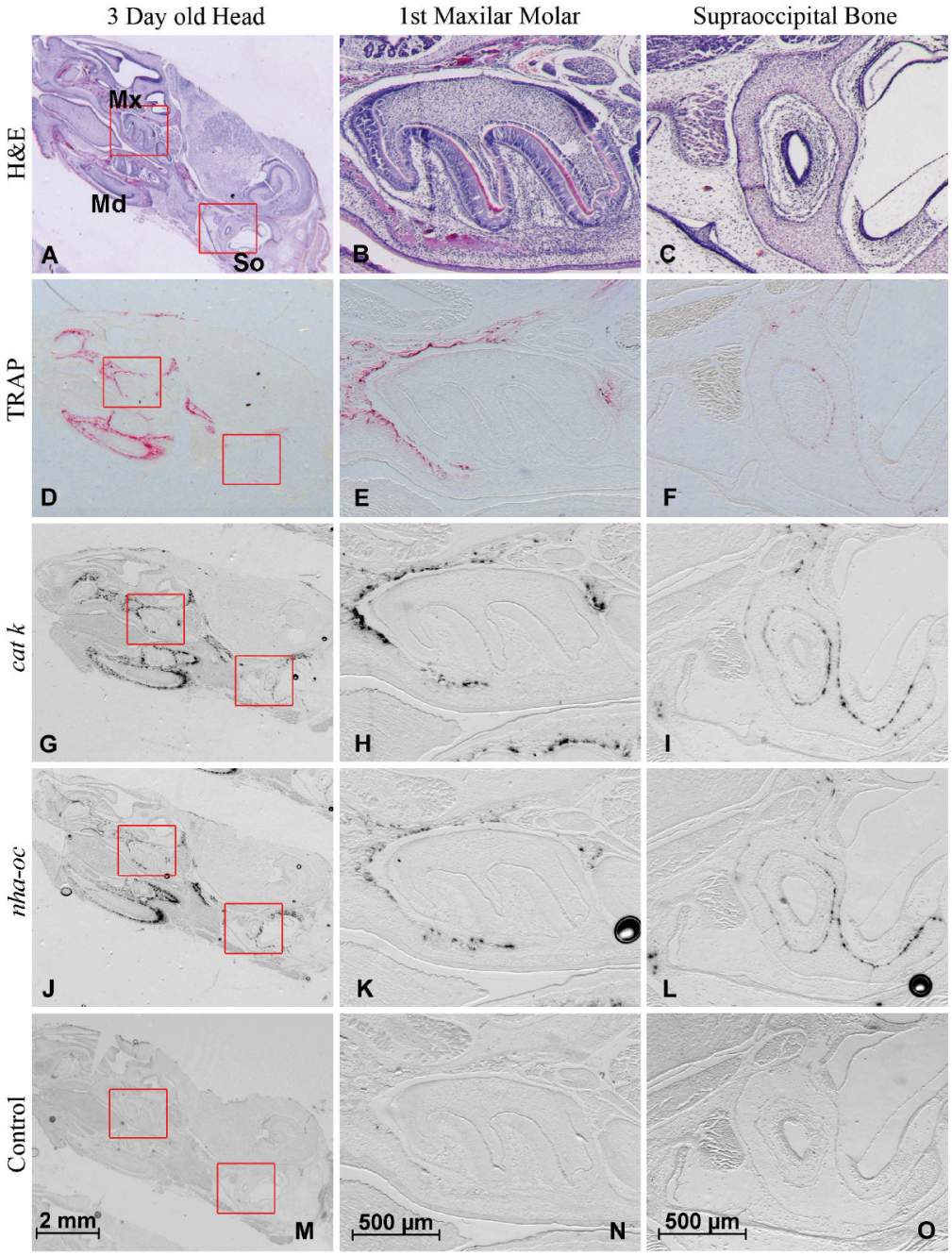

Figure 3.

nha-oc/NHA2 is expressed in osteoclasts in three-day old mouse head.

Mouse head sections were fixed and stained with Hematoxylin/Eosin (A, B, C); TRAP (D, E, F); hybridized to a *cathepsin k* specific probe (G, H, I); *nha-oc/NHA2* specific probe (J, K, L) or *nha-oc/NHA2* sense negative control probe (M, N, O).

In full heads (A, D, G, J and M) as well as in details from the first superior molar (B, E, H, K and N) or the occipital bone (C, F, I, L and O) TRAP, *cathepsin k* and *nha-oc/NHA2*- expressing cells colocalize. Mx: maxilla, Md: mandible, So: supraoccipital.

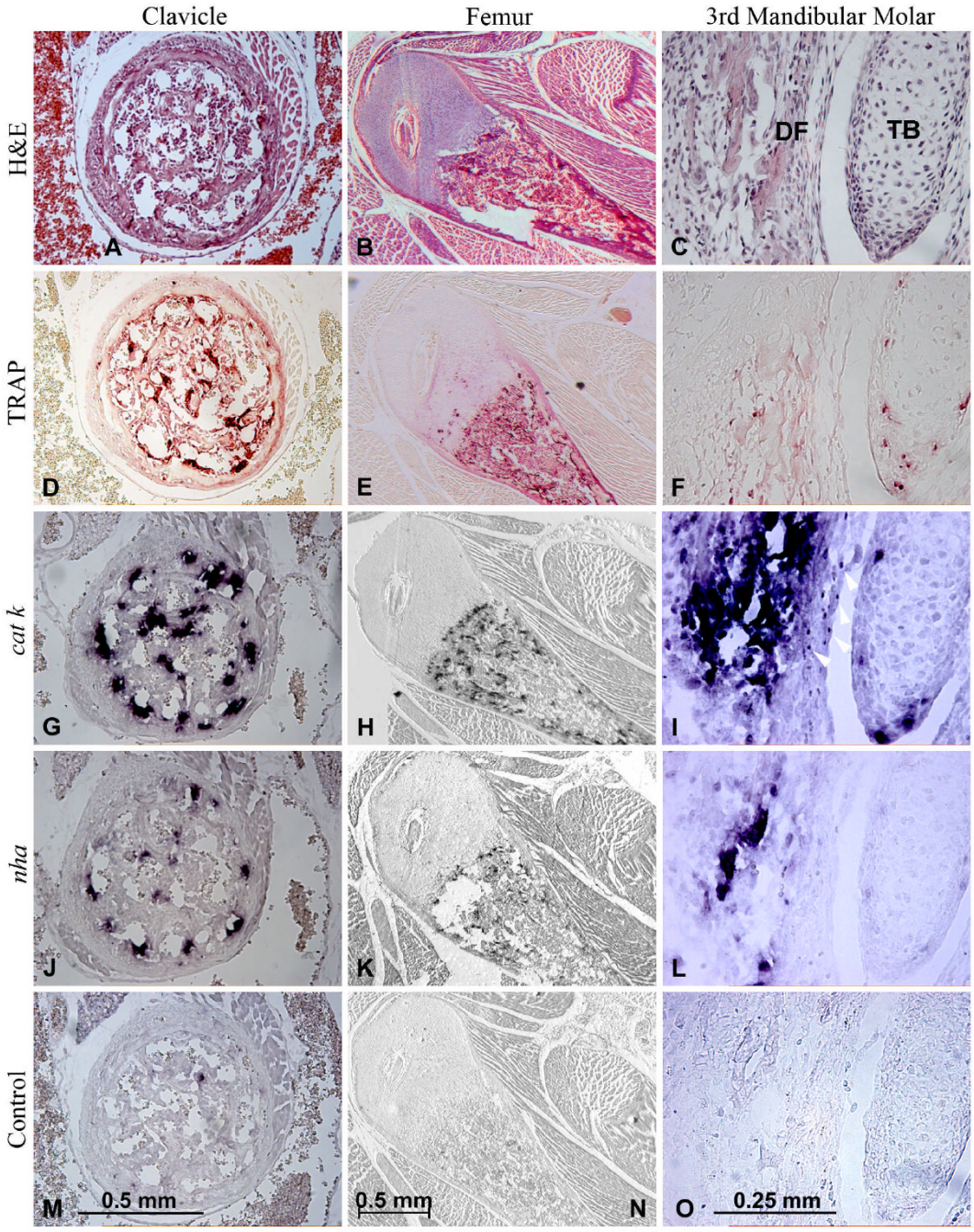

Figure 4.

nha-oc/NHA2 is expressed in osteoclasts in long bones of one-day old mice.

Mouse full body and head sections were fixed and stained with Hematoxylin/Eosin (A, B, C); TRAP (D, E, F); hybridized to a *cathepsin k* specific probe (G, H, I); *nha-oc/NHA2* specific probe (J, K, L) or *nha-oc/NHA2* sense negative control probe (M, N, O). In the clavicle (A, D, G, J and M; magnification 200X) as well as in the femur (B, E, H, K and N) TRAP, *cathepsin k* and *nha-oc/NHA2*- expressing cells colocalize. A detail of the third molar (C, F, I, L and O; magnification 400X) shows colocalization of TRAP, *cathepsin k* and *nha-oc/NHA2* expressing cells. White arrows (I) indicate mononucleated *cathepsin k*-expressing cells, absent in L. DF: dental follicle, TB: tooth bud.