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The bone Gla protein osteocalcin is expressed in cranial neural crest cells

Rotem Kalev-Altman[1,2,3], Veatriki Fraggi-Rankis[1,4], Efrat Monsonego-Ornan[2] and Dalit Sela-Donenfeld[1*]

Abstract

Background Osteocalcin is a small protein abundant in the bone extracellular-matrix, that serves as a marker for mature osteoblasts. To become activated, osteocalcin undergoes a specific post-translational carboxylation. Osteocalcin is expressed at advanced stages of embryogenesis and after birth, when bone formation takes place. Neural crest cells (NCCs) are a unique cell population that evolves during early stages of development. While initially NCCs populate the dorsal neural-tube, later they undergo epithelial-to-mesenchymal-transition and migrate throughout the embryo in highly-regulated manner. NCCs give rise to multiple cell types including neurons and glia of the peripheral nervous system, chromaffin cells and skin melanocytes. Remarkably, in the head region, NCCs give rise to cartilage and bone.

Finding: Here we report that osteocalcin is detected in cranial NCCs. Analysis of chick embryos at stages of cranial NCC migration revealed that osteocalcin mRNA and protein is expressed in pre-migratory and migratory NCCs in-vivo and ex-vivo. Addition of warfarin, an inhibitor of osteocalcin carboxylation, onto neural-tube explants, reduced the amount of NCC migration. These results provide the first evidence of osteocalcin presence in cranial NCCs, much before they give rise to craniofacial skeleton, and propose its possible involvement in the regulation of NCC migration.

Keywords BGP, OST, Neural tube, Delamination, Cell motility, Chondrogenesis, Hindbrain, Warfarin

Introduction

Osteocalcin, also known as bone Gla protein (BGP), is synthesized mainly by osteoblasts as the most abundant non-collagenous bone protein. It undergoes vitamin K-dependent post-translational modification, where three glutamic acid (Glu) residues undergo γ-carboxylation to form γ-glutamic acid residues (Gla). Vitamin K is essential for gamma-carboxylation by acting as a cofactor for the enzyme gamma-glutamyl carboxylase (GCCX), which catalyzes this reaction [1–5]. Newly carboxylated osteocalcin binds with high affinity to calcium ions in the bone hydroxyapatite, the major mineral crystal in the bone extracellular matrix (ECM) [6, 7]. Although osteocalcin is widely used as a marker for bone turnover [8–10], its role in bone mineralization remains unclear. Osteocalcin is used as marker to assess bone

*Correspondence:
Dalit Sela-Donenfeld
dalit.seladon@mail.huji.ac.il
[1]Koret School of Veterinary Medicine, The Robert H. Smith Faculty of Agriculture, Food and Environment, The Hebrew University, Rehovot 76100, Israel
[2]The Institute of Biochemistry and Nutrition, The Robert H. Smith Faculty of Agriculture, Food and Environment, The Hebrew University, Rehovot 76100, Israel
[3]The Section of Genetic Medicine, The Department of Medicine, The University of Chicago, Chicago, IL 60637, USA
[4]Smart Assays Biotechnologies Ltd, Weizmann Science Park, Ness Ziona 7414003, Israel

formation before and after birth [11, 12]. However, its expression and role during early stages of development, before bones are formed, is unexplored.

Neural crest cells (NCCs) are a unique population of vertebrate embryos. While initially located at the dorsal neural tube as an integral part of the neural ectoderm, later they detach as mesenchymal cells, migrate throughout the embryo and differentiate into a plethora of derivatives, including peripheral neurons and Schwan cells, skin melanocytes, adrenal medulla chromaffin cells, etc [13–16]. Remarkably, NCCs from the head region also give rise to also contribute to the formation of craniofacial bones and cartilage [17–24].

NCC development is governed by a complex gene regulatory network, including signaling pathways, transcription factors and downstream effector genes, which mediate their timely specification, migration and differentiation [25–32]. Interestingly, several molecules that participate in early stages of cranial NCC development, also control skeletal formation at later prenatal and postnatal stages. For instance, Sox9 and collagen type 2a1, two main regulators of chondrogenesis, and various matrix-metalloproteases (MMPs), which control cartilage/bone ECM remodeling, regulate NCC migration in young embryos [33–44].

Here we report that osteocalcin is expressed in cranial NCCs when detaching from the neural tube and raise the possibility that impairing with its carboxylation may interfere with this process.

Materials and methods

Embryos

Lohman chick eggs were incubated at 38°C. Embryos of the desired stages were washed in PBS and either collected for RNA preparation (snap frozen in liquid nitrogen), fixed in 4% paraformaldehyde (PFA, Sigma-Aldrich, Israel) for immunofluorescence, in 50:50 acetone: methanol (Sigma-Aldrich, Israel) for 10 min at -80°C for immunohistochemistry, dehydrated in 100% methanol and stored at -20°C for in-situ hybridization, or proceeded for explant preparation.

Ex-vivo explants

Neural tubes from the hindbrain level of 5–8 somite-old embryos, which consist promigratory NCCs, were isolated from the adjacent tissues with 2% pancreatin in PBS (Sigma-Aldrich, Israel), transferred to PBS supplemented with 5% newborn calf serum (Biological industries, Israel) to stop enzymatic activity, and washed in serum-free CHO-S-SFM II media (Gibco BRL, MD USA) [41, 43, 45, 46]. Neural primordia were placed onto 50 µg/ml fibronectin-coated 3 mm plates (Sigma-Aldrich, Israel) and incubated for overnight in CHO-S-SFM II media (Gibco BRL, MD USA) supplemented with 50µM or 100µM Warfarin. As a control, DMSO was added to the medium at equivalent concentrations to those used to dissolve Warfarin (0.062% or 0.125%). Explants were fixed in 4% PFA and preceded for immunofluorescence. Alternatively, neural tubes and migrating NCCs were separately collected for RNA extraction. Each experiment was performed at least three times in quadruplicate cultures.

In-situ hybridization

In-situ hybridization (ISH) was performed on whole embryos or paraffin sections as described elsewhere [43], using DIG-labeled mRNA antisense probes against chick *collagen type 2a* (*Col2a*) [44], *foxd3* [40], or *osteocalcin (OST) (*NM_205387; primers: Left: CTGCTCACATTCA GCCTCTG; Right: CCTCCTGGAAGCCGATCT; probe size *232*). Staining was detected using alkaline phosphatase (AP)-coupled anti-DIG antibody and NBT/BCIP substrate (Roche, Switzerland).

Immunofluorescence and immunohistochemistry

For immunofluorescent staining fixated embryos or neural tube explants were washed with PBST (PBS+0.1% Triton X-100) and put in blocking buffer (3% BSA/PBS) for 1 h or 10 min, respectively, at room temperature (RT). Samples were then incubated with mouse-anti osteocalcin (OST) (1:100, Abcam, CA, USA), rabbit-anti Sox10 (1:200, Abcam, USA) or mouse-anti-HNK-1 (1:300, BD Pharmingen, USA), at 4°C for overnight. Secondary anti-mouse/rabbit Alexa 488/594 (1:500, Molecular Probes, CA, USA) were added at RT for 2 h. Following PBS washes, embryos/explants were incubated for 5 min at room temperature in PBS with DAPI (1:400; Sigma-Aldrich), washed in PBS and mounted with fluoro-gel mounting medium (Bar-Naor, Israel) for microscope evaluation. For immunohistochemistry staining, fixated embryos were washed in PBS-T (PBS-0.5% Triton) for 5 min, incubated in 6% H_2O_2 for 2 h at RT, blocked with PBS-T+10% goat serum for 2 h at RT and incubated with rabbit-anti OST (1:100, (1:100, Santa Cruz Biotechnology, CA, USA) overnight at 4°C. Following PBS-T washes, goat anti rabbit HRP secondary antibody (1:300, Sigma MO, USA) was added for 40 min at RT and after further washes, embryos were stained with AEC substrate to reveal HRP activity.

RT-PCR

Total RNA was purified from isolated neural-tubes at the level of midbrain and hindbrain, either before their culturing, from neural-tube explants after overnight incubation, from NCCs that migrated after overnight incubation, or from whole embryos. RNA was extracted using RNeasy Plus Micro kit (QIAGEN, Hilden, Germany). Samples were prepared from 6–8 neural-tubes/

NCC explants or from 5–8 embryos. 300ng or 1µg RNA was reversed-transcribed using High-Capacity cDNA reverse transcription kit (Applied Biosystems, CA, USA). cDNA was amplified with goTaq polymerase Ready Mix (Promega, WI, USA) using chick OST primers (Forward 5'ATGGTGCGCAGGCAGAAG-3'; Reverse 5'GGGG CTCAGCTCACACACCTC-3'). cDNA from chicken growth-plate was used as a positive control. PCR program was: 5 min at 95°C, followed by 35 cycles of 30 s at 95°C, 30 s at 60°C, 20 s at 72°C and finally 10 min at 72°C.

Data analysis

Quantification of NCC migration was conducted using ImageJ 1.410 software [41]. Values were normalized relatively to control averaged values. Areas of NCC migration were calculated as a mean of 5 explants/treatment, using the following formula: total area occupied by migrating NCCs, divided by the area of the neural-tube explant. The average ratio of treated versus control samples was tested for significance using unpaired student's *t*-test using JMP software (SAS Institute, USA).

Microscopy

Samples were imaged using SZX17 stereomicroscope (Olympus), CTR 4000 confocal microscope (Leica), or Eclipse E400 upright microscope (Nikon) with DP70 CCD camera (Olympus) or DFC300FXR2 camera (Leica).

Results

Osteocalcin is expressed in cranial NCCs

As NCCs give rise to the craniofacial skeleton, we examined whether the skeletal marker osteocalcin is expressed in chick embryonic NCCs. First, expression of *osteocalcin* mRNA was analyzed by RT-PCR at various stages (Fig. 1A, Supp. Figure 1). RNA was extracted from whole embryos at the following stages: 2–6 somite staged (ss), when cranial NCCs are specified and before they start migrating; 8-12ss, when cranial NCCs are extensively migrating; 13-16ss, when cervical and upper-trunk NCCs also start migrating; and 18ss and 25ss, when trunk NCCs are mainly migrating. Chicken growth plate cDNA served as positive control [12]. *Osteocalcin* mRNA was detected in all examined embryos, indicating that this bone-marker is expressed at early stages, much before skeletal development initiates. Although this type of analysis could not determine the specific cell types expressing *osteocalcin*, cranial NCCs were candidates as the first cells to be fated to skeletal lineages during development [14–21]. Hence, we next examined *osteocalcin* mRNA in isolated NCCs, by using hindbrain explants from 5-8ss embryos. This procedure is based on the isolation of neural tube primordia at stages when NCCs have already been specified but are still in their pre-migratory phase. Following the seeding of the neural primordia, NCCs begin to detach from the neural tube and migrate as mesenchymal cells around the explant [41, 45, 46]. *Osteocalcin* was found to be expressed in isolated hindbrains, before or after their culturing ex-vivo, as well as in purified NCCs that migrated in the dish (Fig. 1A), suggesting its expression pre-migratory and migratory hindbrain NCCs.

To determine the spatiotemporal expression pattern of *osteocalcin* in cranial and cervical NCCs before and during their migration, ISH was utilized on embryos of 6ss to18ss (Fig. 1B). In embryos of 6ss, *osteocalcin* expression was detected in the rostral neural tube folds, suggesting its presence in pre-migratory NCC (Fig. 1Ba, arrows). In embryos of 10-18ss, when cranial NCCs undergo migration, *osteocalcin* was found in typical NCC migratory domains (Fig. 1Bb-e arrows). Transverse sections confirmed *osteocalcin* expression in pre-migratory and migratory cranial NCCs (Fig. 1Bf, g, arrows). Notably, *osteocalcin* was also expressed in the neural-tube basal plate, indicating for possible other roles of osteocalcin in neural development. Evaluating the expression patterns of two well-known NCC markers, *foxd3* and *collagen-2a*, revealed their comparable patterns to that of *osteocalcin* in cranial NCCs (Fig. 1Bh-k, arrows).

To further validate these results, protein expression of osteocalcin was examined together with that of Sox10, an additional typical marker for migratory NCCs [35, 41, 47]. Co-immunofluorescence staining was performed on embryos of 10ss and 18ss, when cranial NCC migration initiates and proceeds, respectively (Fig. 1C). Osteocalcin was detected in typical NCC migratory domains in the vicinity of the cranial neural tube with large overlapping to Sox10 expressing cells (Fig. 1Ca-f, arrows). Osteocalcin, but not Sox10, was also detected in rostral neural tube, concomitantly with the ISH data. Punctuated osteocalcin expression was also shown in the dorsal neural tube at vagal and upper-trunk axial levels (Fig. 1Ca, c,e asterisks), possibly in pre-migratory NCCs that are about to initiate migration. To further confirm the staining specificity, immunohistochemistry staining was conducted in 14ss embryos, using a different osteocalcin antibody (Fig. 1D). Expression of osteocalcin was shown in migratory streams of midbrain and hindbrain-derived NCCs (Fig. 1Da, c, arrows). Control embryos labeled only with secondary antibody presented a background staining without expression in NCCs (Fig. 1Db). Additional embryos were also stained for HNK1, a widely used marker for migratory NCCs [41], revealing typical patterns of migratory NCCs which resemble those of osteocalcin-expression NCCs (Fig. 1Dd, arrows), further validating that migrating NCCs express osteocalcin. Altogether, these findings indicate that osteocalcin mRNA and protein is expressed in early stages of cranial NCC migration.

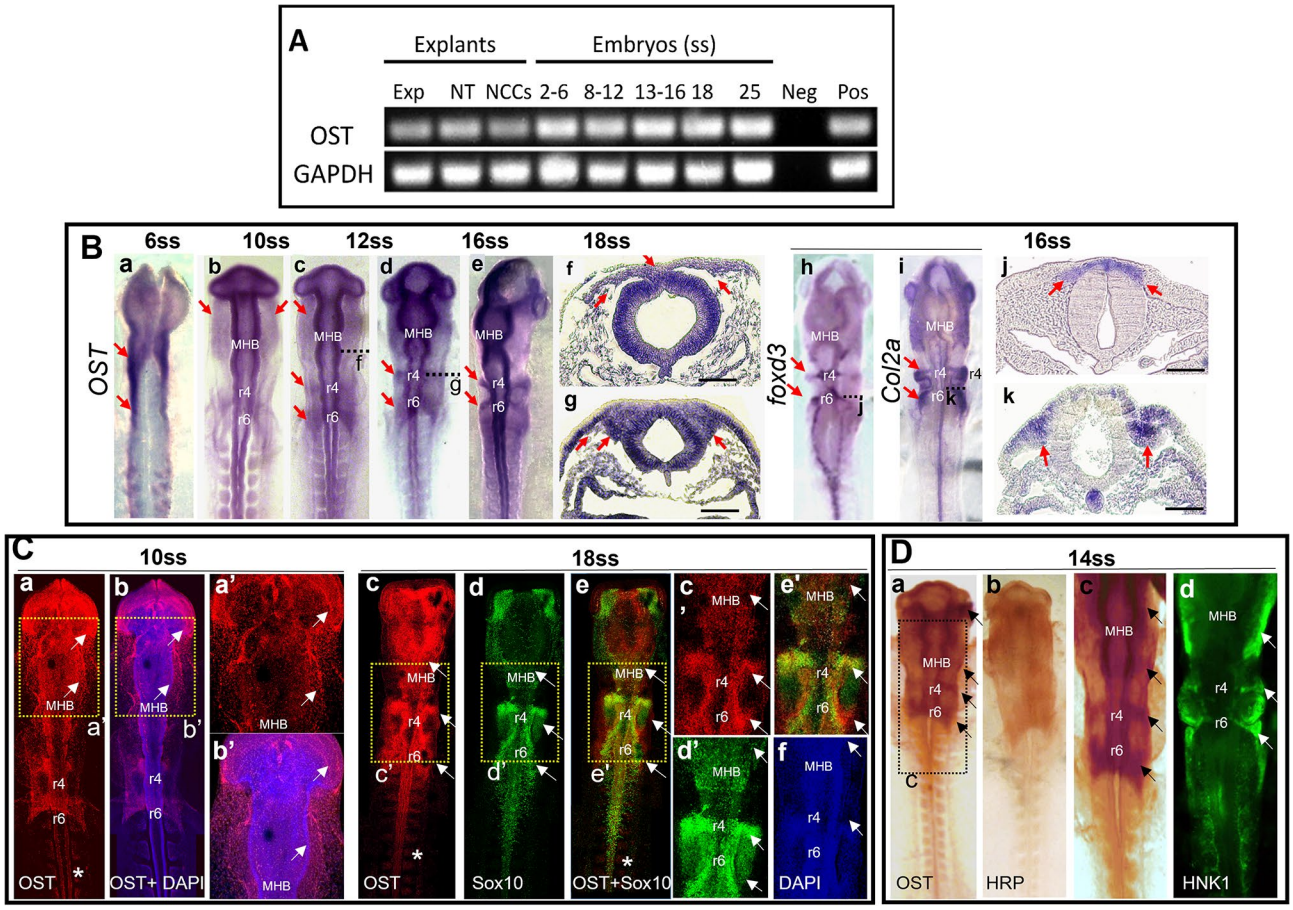

Fig. 1 Osteocalcin is expressed in cranial NCCs. (**A**) RT-PCR analysis on RNA purified from whole embryos at different somite stages from neural tube explants obtained from hindbrains of 5-8ss, either before (Exp) or after culturing (NT), and from NCCs that migrated around the NT explant (NCCs). Primer pairs were directed against 100 bp sequences of chick *osteocalcin*. Negative control did not contain cDNA (Neg). Positive control was obtained from chicken growth plate. Original gels are shown in Sup. Figure 1. (**B**) In-situ hybridization analysis on embryos or sections from different stages labelled with RNA probes against *osteocalcin* (a-g), *foxd3* (h, j) or *Col2a* (i, k). Broken lines in (c. d, h, i) represent location of transverse sections shown in (f, g, j, k). Red arrows indicate pre-migratory or migratory NCCs. Bar = 100 µm. (**C**) Immunofluorescence analysis on whole embryos at different stages stained for osteocalcin (a-c', e, e'), Sox10 (d-e') or DAPI (b, b', f'). Merged images of OST + DAPI are shown in (b, b'). Merged images of OST + Sox2 are shown in (e, e'). Higher magnifications of boxed areas in (a-e) are presented in (a'-e', respectively). White arrows indicate migratory NCCs, Asterisks represent pre-migratory NCCs. (**D**) Immunohistochemical analysis of whole-mounted embryo at 14ss stained for osteocalcin (a, c), HNK1 (d), or secondary antibody only (b). Detection was made using HRP (a-c) or Alexa-488 (d)-conjugated antibodies. Higher magnifications of cranial area in (a) is presented in (c). Black and white arrows indicate OST or HNK1 expressing NCCs, respectively. Abbreviations: ss, somite stage; NT, neural tube; NCC, neural crest cells; Exp, explant; Neg, negative; Pos, positive; OST, osteocalcin; Col2a, collagen type 2a; MHB, midbrain-hindbrain boundary; r, rhombomere.

Based on the presence of *osteocalcin* transcripts in NCC explants (Fig. 1), we next determined whether osteocalcin is also found in isolated NCCs. Explants obtained from the hindbrain level of 5–8 somite-old embryos, stages where pre-migratory NCCs reside in the neural primordia, were cultured overnight before undergoing co-immunofluorescence staining for osteocalcin and Sox10 proteins and for the nuclei dye DAPI (Fig. 2). A bright-field view of a typical explant demonstrates the neural tube surrounded by migratory NCCs (Fig. 2k). Co-labeling for Sox10 and osteocalcin was evident in all migrating NCCs (Fig. 2a-e', see also Fig. 2f-J for confirmation of osteocalcin-staining specificity). As expected from a transcription factor, Sox10 was expressed in the DAPI-stained nuclei (Fig. 2b", e', asterisks) whereas osteocalcin, a secreted protein, was detected in the cytoplasm, (Fig. 2a",e', asterisks). Notably, as explants contain solely neural primordia without any additional tissue, this ex-vivo staining further validates that osteocalcin is specifically expressed in migrating NCCs.

Warfarin inhibits NCC migration in explants

Warfarin (4-hydroxy-3-(3-oxo-1-phenylbutyl)-2 H-chromen-2-one, CAS RN 81-81-2), is a vitamin K-antagonist which has a well-documented activity to prevent gamma-carboxylation in numerous biological contexts, resulting in the accumulation of vitamin K-dependent proteins in their undercarboxylated inactive form [48, 49]. As

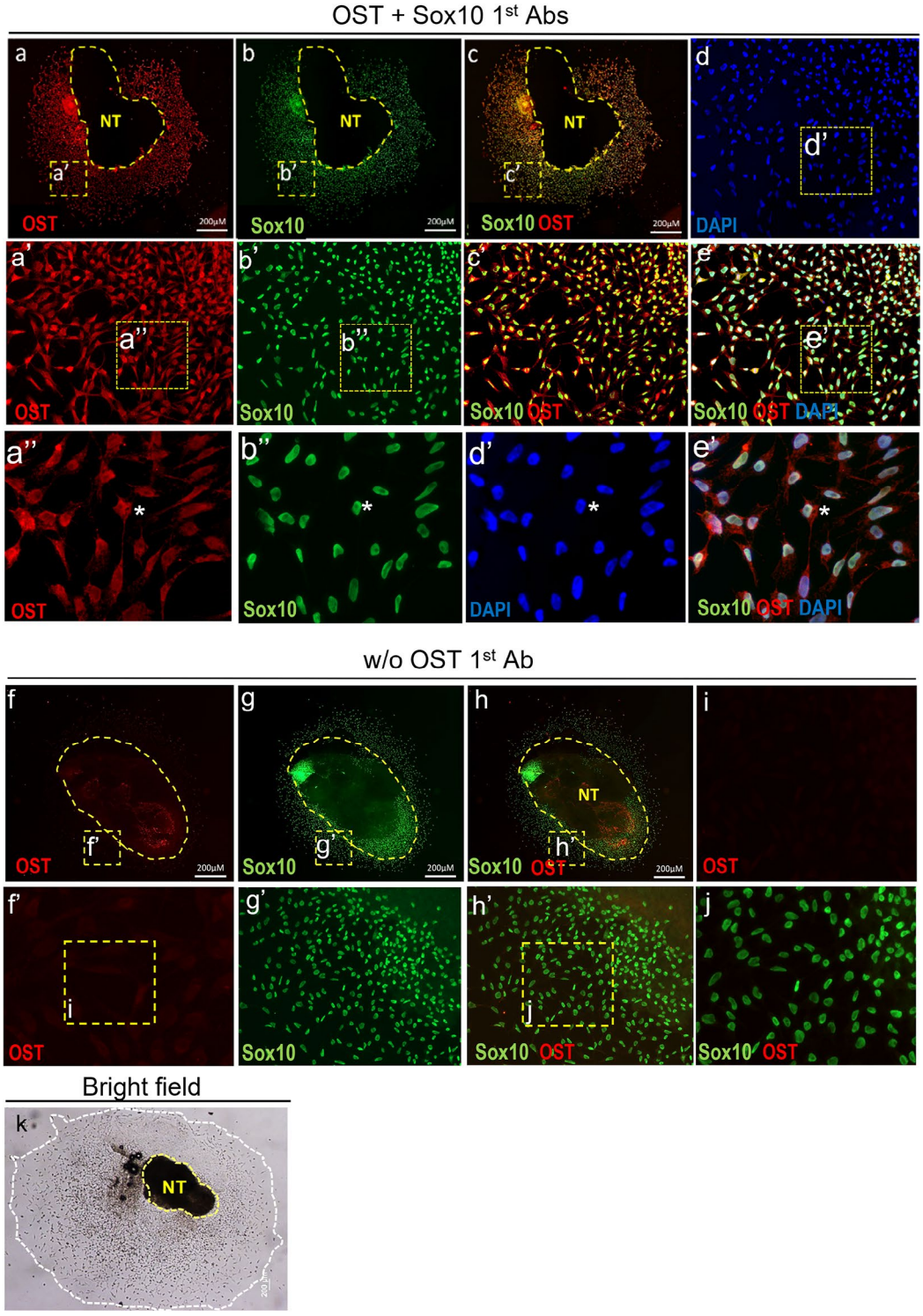

Fig. 2 Osteocalcin is expressed in explanted NCCs. (**a-j**) Immunostaining views of neural tube explants obtained from hindbrains of 5-8ss and stained for osteocalcin (a-a''), Sox10 (b-b'',g, g'), DAPI (d, d'), or secondary antibody without anti-osteocalcin antibody (f, f'). Merged image of (a, b) is shown in (c), of (a',b') is shown in (c'), of (a',b',d) is shown in (e), of (a'',b'',d') is shown in (e'), of (f, g) is shown in (h) and of (f',g') is shown in (h'). Magnifications of the boxed areas in (a-d, f-h) are represented in (a'-d',f'-h', respectively). Magnification of boxed areas in (a',b',e',g',h') and shown in (a'',b'',e'', i, j, respectively). (**k**) Bright-field view of a typical explant. White asterisks indicate a single NCC with nuclear staining (DAPI+), nuclear expression of Sox2 and cytoplasmic expression of osteocalcin. In all images, broken yellow line marks the neural tube. Broken while line in (k) marks the area of NCC migration. Abbreviations: OST, osteocalcin; NT, neural tube; w/o, without; Ab, antibody. Bar = 200 μm

osteocalcin undergoes this post-translational modification to become active [1–5], treatment with Warfarin is a commonly strategy used to prevent osteocalcin activation, leading to its remaining in its uncarboxylated form [11, 50–53]. To test the effect of Warfarin on NCC migration, hindbrain-derived explants were cultured in control media or in media containing 50μM or 100μM warfarin. These concentrations were selected based on published studies which have used similar doses in embryos, embryonic stem cells, neuronal cells, and in vivo [11, 54–57]. Migration of NCCs was evaluated by staining for the classical markers HNK1 and Sox10. Control explants revealed typical distribution of NCCs around the neural tube (Fig. 3a, c). Warfarin treatment caused a marked decrease in NCCs that dispersed around the explant (Fig. 3b, d). Quantification of these results revealed a dose-dependent effect of Warfarin on NCC migration, which led to 30–50% reduction upon addition of 50μM or 100μM Warfarin, respectively (Fig. 3e, f). Notably, no cell toxicity was evident in the Warfarin-treated explants, concomitantly with previous studies using similar or higher Warfarin concentrations [11, 54–56, 58]. These data support the possibility that carboxylated osteocalcin plays a role in NCC migration ex-vivo, as its presumed inhibition by Warfarin results in decreased migration. Yet, the effect of Warfarin may also be attributed to inhibiting gamma- carboxylation in other proteins that regulate NC migration, rather than-, or in addition to-, osteocalcin, which are yet to be identified.

Discussion

Our demonstration of osteocalcin expression in cranial NCCs is one of the first documentations of this bone-related factor in early development. As the emergence of craniofacial skeleton relies on the cranial NCC migration, which if perturbed, leads to major craniofacial defects [59–62], identifying new NCC molecules may help to better understand NCC's ontogeny in health and disease.

Several regulators of cartilage/bone formation have been reported to hold central functions in early NCC development; Sox9, the key chondrogenesis-inducing factor, is an important factor driving specification and delamination of NCCs [38, 63–65]. Col2a1, one of the earliest chondrogenic markers, is expressed in migrating cranial NCCs and mediates their differentiation to skeletogenic lineage [33, 34, 37, 39]. Similarly, we have

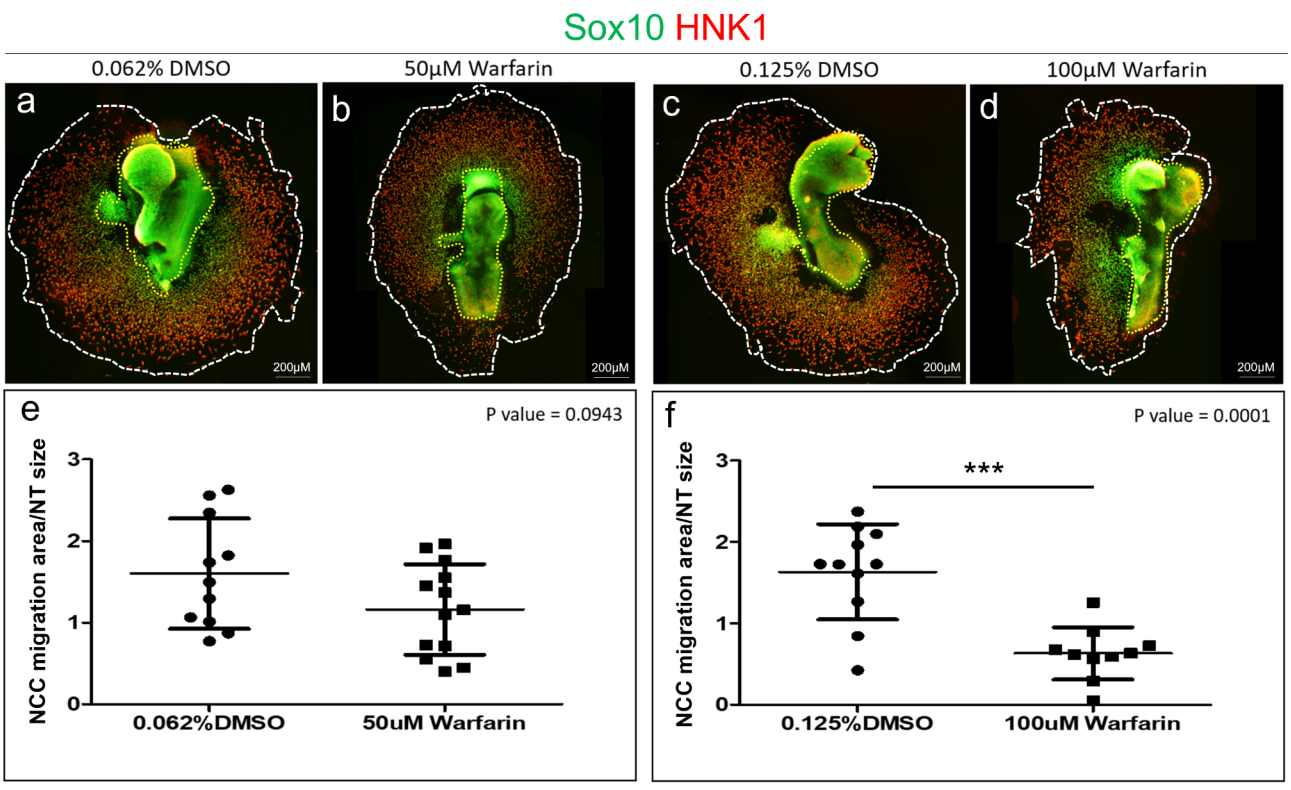

Fig. 3 Warfarin reduces NCC migration ex-vivo. (**a-d**) Views of HNK1 (red) and Sox10 (green)-immunolabelled explants that were isolated from the hindbrain level and 5–8 ss and grown for 16 h in control media added with two concentrations of DMSO (a, c; $n = 11$ for each), or with Warfarin-added media in different concentrations (b, d; $n = 13$ for b, $n = 10$ for d). Dashed yellow/white lines represent borders of the area occupied by migrating NCCs/NT-explant area. (**e, f**) Quantification of NCC migration in explants added with Warfarin versus control. Error bars represent mean and standard deviation. *P* values are indicated. Abbreviations: NCC, neural crest cells; NT, neural tube

previously uncovered that MMP2/9/16, central ECM modulators which govern chondrogenesis and bone formation, are expressed in cranial NCCs and promote their migration through degradation of the neural-tube basal lamina [36, 40–42, 44, 66–69]. As our data supports the involvement of yet another bone-regulatory factor in NCCs, it is likely that the embryo utilizes similar set of genes to regulate early NCC development of as well as their later differentiation [70]. Interestingly, osteocalcin has also been reported in several slightly more advanced NCC-derived structures, such as dorsal root ganglia, mandibular and maxillary processes of mice embryos, and branchial arche cells of fish [71–73]. These studies reinforce our finding that osteocalcin, which is seminal for bone development/homeostasis, is present during early development of craniofacial skeletal progenitors.

Addition of Warfarin led to a reduction in NCC migration. Since Warfarin prevents posttranslational carboxylation of osteocalcin, a perquisite process for osteocalcin activation in bone [11, 50, 51], our data raises the hypothesis that osteocalcin has to undergo carboxylation in order to promote NCC migration. As osteocalcin is a migration-promoting factor in cultured osteoclasts, tumorigenic cell lines and malignant skeletal tumors [74, 75], comparable activities of osteocalcin in different cell types are suggested. Although the mechanism through which osteocalcin may act on NCCs is unknown, one possibility is that it controls their metabolic state, akin to the metabolic alteration reported in osteocalcin knockout mice [76]. Alternatively, osteocalcin was shown to increase cytosolic calcium levels [74]. As calcium is an important cofactor for cadherins [77], the fundamental regulators of NCC EMT [32, 36, 40, 78, 79], it is possible that osteocalcin regulates calcium levels in NCCs, which in turn modulate cadherin function. Nevertheless, as Warfarin prevents vitamin K-dependent carboxylation in several types of proteins [49], future studies are required to directly determine the role of osteocalcin, and/or or other gamma-carboxylated proteins, in NCCs and the mechanism by which they regulate cranial NCC migration. Interestingly, extended exposure of zebrafish embryos to Warfarin was found to result in craniofacial malformations and alterations in expression of NCC-related genes [54, 57]. In humans, Warfarin administration during gestation has also been related to fetal skeletal malformations, at both cranial and trunk levels [80–82]. Since osteocalcin is a main bone protein, it is possible that the observed Warfarin-related malformations in fish and mammals are linked, at least partially, to the accumulation of inactive osteocalcin in NCC-related processes. Yet, it remains to be investigated whether osteocalcin expression and/or activity is evident in fish and mammalian NCCs, or whether other Warfarin-affected proteins mediate these phenotypes.

Limitations

The mechanism through which Warfarin inhibits NCC migration remained unknown. Analyzing the expression levels/patterns of key genes that regulate NCC migration in control and Warfarin-treated explants will help in clarifying the downstream pathways affected by Warfarin. Moreover, the use of loss-of function techniques to specifically eliminate osteocalcin in NCCs will be needed for demonstrating its direct role in this process. Finally, unraveling whether osteocalcin is also involved in the regulation of trunk NCC migration will provide a more comprehensive understanding on its general or region-specific role.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s13104-024-06990-7.

Supplementary Material 1

Author contributions

RKA, VFR, EMO and DSD conceptualized the study and designed the methodology, RKA and VFR conducted the experiments and prepared the figures, RKA, VFR, EMO and DSD analyzed the data, RKA and DSD led the writing of the manuscript, VFR and EMO reviewed and edited the drafts. All authors read and approved the final manuscript.

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Data availability

The datasets used and/or analysed during the current study are provided within the manuscript or will be available from the corresponding author on reasonable.

Declarations

Ethics and consent to participate:

Not applicable.

Competing interests

The authors declare no competing interests.

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