Visualisation of developmental ossification using trace element mapping†

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Endochondral ossification is the process by which bone is deposited during development, growth and repair of the skeleton. The regulation of endochondral ossification is extremely important as developmental flaws can result in severe skeletal abnormalities. However, until recently the limitations of available methodologies have restricted our understanding of this fundamental physiological process. The analysis of chemical elements that are intimately associated with discrete biochemical stages of ossification within bone could provide new insight to such processes at the atomic level. In this study we present detailed characterisation of the elemental inventory within actively ossifying bone during development in mice using synchrotron microfocus X-ray techniques. X-ray fluorescence imaging showed differential distributions of Zn, Sr and Ca, which may be correlated with the processes of cartilage replacement (Zn), active ossification (Sr) and fully ossified tissues (Ca). Quantification of these trace elements confirmed their relative distributions. These results represent the first detailed visualisation of local endochondral ossification processes using trace elemental mapping. Such studies have far reaching applications not only in the medical field, but to our understanding of the evolution of the bony skeleton given that trace element inventories have been shown to be preserved through deep time (millions of years).

Introduction

The endoskeleton is one of the most significant evolutionary developments in the history of multicellular life on Earth and is a defining character of the phylum Chordata. Its development imparts significant evolutionary advantages allowing for a wide range of body forms and expansion into almost all ecological niches, providing aid in both structural support and biochemical processes (mineral homeostasis). Despite the significant scientific interest and implications for many fields such as evolutionary biology, materials science and medicine, the intricacies of bone development are still poorly understood.1,2 This is partially due to the sheer complexity of the processes needed to maintain and regulate bone growth and remodelling.1–4 Of particular interest is the role of trace elements (biologically important, but less than 0.1% by weight composition) in these processes as several metals such as Zn are key regulators associated with biomolecules during bone development. In this study we utilise the association of trace element inventories with growth stages in bone to visualise local endochondral ossification during development.

Endochondral ossification

Endochondral ossification is a key process during skeletal growth and development (with exception to the skull).3,4 In the epiphyseal (growth) plates, chondrocytes (cartilage-forming cells) proliferate as stacks of newly formed flattened cells that synthesise cartilage matrix. This allows the epiphyseal cartilages towards the ends of the bones to be pushed apart and bones to grow in length. As the cartilage progresses, it is replaced by ossified (mineralised) bone along what is known as the ossification front.3 The cycle of cartilage replacement with bone (ossification front) becomes organized into the growth plate, occupying the space between the epiphyseal cartilages and growing bone.5,7 A unique feature of growing bone is the zoning that spatially occurs within a growth plate, with early stage chondrocytes furthest from the ossification front. These zones of cells are named after their predominant activity (active, resorption, etc.). In addition to the growth plate, there are also areas of secondary ossification in most bones developing in the middle of the epiphyseal cartilage that remains after the growth plate becomes established.6,7
The complicated series of cell proliferation, specialization, migration and death that occurs at the growth plate are controlled and moderated by an equally impressive assortment of growth hormones and proteins, including Insulin-like Growth Factor 1 (IGF-1), Transforming Growth Factor beta (TGF-β), Bone Morphogenetic Proteins (BMPs), Matrix Metalloproteins (MMPs), osteopontin, osteonectin and osteocalcin. These in turn are regulated by a suite of metal-based biomolecules that utilize elements such as Zn, Fe, and Sr. Zinc is required for the growth, development, and maintenance of healthy bone by stimulating osteoblasts, inhibiting osteoclasts function and enhancing bone protein synthesis (which increases bone mass and growth). It is an important constituent of MMPs, which remove cartilage during bone development, and is a cofactor for alkaline phosphates (ALPs), which are responsible for the removal of phosphate groups. Iron is important for procollagen enzymes, which are essential for hydroxylation of proline and lysine, precursors of collagen, and is also known to enhance the stability of the apatite lattice in bones and teeth. Strontium has been found to promote bone nodule formation by increasing osteoblastic marker expression in ALPs, bone sialoprotein, and osteocalcin. In bone resorption, Sr stimulates the production of osteoprotegerin, resulting in an inhibition of osteoclast proliferation and differentiation. Strontium also interrupts the sealing zone of osteoclasts, the site at which they secrete hydrogen peroxide to dissolve bone, inhibiting their ability to demineralize bone.

As trace metals can be associated with different phases of ossification, we hypothesize that the localized processes of bone development can be observed by spatially resolving these trace elements across the ossification front and growth plate areas of developing bones. Until recently, investigations into such chemical/histological correlations have not been attempted. This is largely because commonly available techniques are unable to resolve the dilute variations (ppm levels) in chemistry at the necessary resolution (micron). However, recent work applying synchrotron-based X-Ray Fluorescence (SR-XRF) elemental mapping has shown the ability to resolve the chemical variations associated with fine scale histological features in extant and fossil bone. These studies focus on mature (adult) bone, which despite having reached a “steady state” of growth, still exhibit differential distributions of trace elements crucial for bone deposition and remodelling. Here we present elemental mapping on one-day old postnatal mice (Mus musculus) to visualise the trace element moderation of the complicated bone development/ endochondral ossification pathways, and precise quantification to correlate elemental map distributions. X-ray absorption spectroscopy is performed to determine how these trace elements that are key during ossification are bound within the developing skeleton, which can also be used as a reference for identifying original bone chemistry in the fossil record. We also mapped a 50 million year old fossil fish to show how this method can be applied to archaeological and palaeontological specimens.

Methods
Samples
One-day old mice (M. musculus) were purchased from the Monkfield Nutrition distributor (Fig. S1†). Age was based on the information given by this distributor. The upper limb was removed and skinned using a dissecting microscope and sterile scalpel, and then freeze-dried for XRF analyses. A fossil fish (Knightia eoceana; UM 868) was collected from Fossil Lake basin of the 50 million year old Green River Formation of Wyoming, USA (Fig. S2†). No additional sample preparation was performed to either the extant mouse or fossil fish (embedding, polishing, etc.) as such procedures could adversely affect and contaminate their natural elemental characters of the samples.

Synchrotron
The skinned M. musculus forelimb was mapped at the undulator microfocus beamline, I-18, at the Diamond Light Source (DLS; Oxfordshire, UK) using the experimental setup of ref. 18–20. Maps were made using 5.5 μm diameter beam size produced via a system of Kirkpatrick-Baez focusing mirrors. The sample was mounted on an x-y-z stage and rastered at a 45° angle to the incident X-ray beam with a four element Si drift detector Vortex set at 90° scattering angle. Mapping, point analyses, and spectroscopy at beamline I18 is completed for high atomic weight elements (high-Z; from Ca and higher) in air with an incident beam energy of 17 keV and for light atomic weight elements in a He purged atmosphere with an incident beam of 3.15 keV (low-Z; Si to Cl). Maps of individual elements were created using the ROI imaging tool in PyMCA freeware by deconvoluting the X-ray emission energy of an element in the recorded Energy Dispersive Spectroscopy (EDS) spectra.

Quantification was performed by taking a full EDS spectrum for 30 seconds at specific locations of interest. Three measurements were taken for each location to account for microscale heterogeneity within the bone tissue. PyMCA was used to fit EDS spectra, calibrated using a Durango apatite mineral standard, and well-constrained using previous quantification data (Table S1†). Sr X-ray Absorption Near Edge Structure spectroscopy (XANES) were also collected at beamline I18. XANES were collected in fluorescence mode, with the specimen set at 45° to the incident X-ray beam. The energy of the X-rays was calibrated using a SrCO3 standard, defined at 16105 eV. Background subtraction and data normalization of the XANES were performed using Athena from the software package Demeter 0.9.20.

The Knightia eoceana fossil fish was mapped at the bending magnet Core-XAS beamline, B18, at the Diamond Light Source (DLS; Oxfordshire, UK). The radiation was monochromated with a Si(111) double crystal, the low energy cut off in emission from the bending magnet precluded higher harmonics. Maps were collected with an incident beam energy of 20 keV, using a 100 μm beam size produced via a focussing toroidal mirror. The sample was mounted in air on an x-y stage and rastered at a 45° angle to the incident X-ray beam with a four element Si drift detector (Vortex) at 90°. Full MCA spectra were collected with a resolution of 100 microns (both vertically and horizontally) across the sample, with a collection time of 17 ms per point.
Fig. 1  Optical image of *M. musculus* forelimb compared to microfocus elemental maps of Zn, Sr and Ca. The box on the optical image represents the area mapped. In elemental maps, brighter areas represent relatively higher concentrations, with brightness scales set for each element (e.g. ppm Zn versus weight percent Ca). The overlay of Zn (green), Sr (red) and Ca (blue) shows how these three different elements can be correlated with the different stages of ossification. Zn is present in both the cartilage of the carpal area, as well as the ossified tissues of the distal phalanges and proximal forelimb. Sr is concentrated in the ossifying tissues and the ossifying front, but not in the carpal area. Ca is restricted to ossified tissues. Scale bar is 1 mm.
Environmental scanning electron microscopy (ESEM)

ESEM data was obtained on the same *M. musculus* specimen from the synchrotron analyses to compare elemental distributions and concentrations and to calculate the attenuation factor caused by soft tissue (periosteum) to add to synchrotron calculations. Specimens were scanned using a FEI Quanta FEG650 ESEM fitted with a Bruker XTrace XRF attachment (Rh Kz incident beam at 20.2 keV). Incident electron beam images were collected at 1.2 kV accelerating voltage. XRF maps were collected for over 24 hours to optimise counting statistics for the low levels of Zn within the sample. Calibration of the XRF was provided by analysis of a Durango apatite standard. Data were processed using the Esprit 2.1 software provided by the manufacturer.

**Results**

**XRF mapping**

Synchrotron-based XRF mapping revealed details of the bones in the *M. musculus* forelimb in Ca, P, Zn, and Sr, which were able to resolve the microscale histological structures such as trabeculae (Fig. 1). A false colour overlay of these element maps highlights differential distributions associated with the tissue types found within the distal ulna/radius, carpals, and proximal phalanges (Fig. 1). Zinc is present in both the cartilage of the carpal area and within the ossified forearm and phalanges. Strontium extends from slightly beyond the ossified areas into the cartilage. Calcium is concentrated only in the ossified areas. Phosphorus shows the same distribution within the ossified tissues as Ca, though such a distinction is difficult to resolve without overlaying P with another low-Z element such as S (Fig. S3†).

Due to detection limitations, ESEM-based XRF mapping was only able to successfully map Ca and P within the ossified tissues and no clear zonation pattern could be discerned for Zn (Fig. 2).

**Quantification**

Synchrotron XRF quantification shows that Zn and Sr concentrations were highest around the edge of developing bones, while Ca concentrations increased from the wrist (cartilage) to the centre of developing bones (ossified tissue; Table 1). Thus quantification confirmed the association of Zn, Sr and Ca with the different ossification zones identified in XRF maps. Also of

![Fig. 2](image-url)

*Fig. 2* ESEM back-scatter (A) and overlay XRF map (B) of P (red) and Ca (blue) taken from the same *M. musculus* analysed at the synchrotron. Note that the field of consists of the entire forelimb and thus represents a larger scanned area than seen in Fig. 1. Phosphorus and Ca are both correlated within the ossified bones. No clear zonation pattern could be discerned from the Zn maps (24 hour map). The green of the false colour image is a result of background fluctuation. Scale bar is 1 mm.

<table>
<thead>
<tr>
<th>Element</th>
<th>Synchrotron</th>
<th>ESEM</th>
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<tr>
<td></td>
<td>Durango standard</td>
<td><em>M. musculus</em> wrist</td>
</tr>
<tr>
<td>P</td>
<td>18.2%</td>
<td>22%</td>
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<tr>
<td>S</td>
<td>5750</td>
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Fig. 3  Sr K-edge XANES of SrCO$_3$ (red) and ossified bone (black). Sr in the ossified bone shows an appearance of a “shoulder” in the spectra (see F2) between 16 125–16 138 eV, which is assigned to hydration of Sr, compared to a decreased intensity at similar energy (see F1) when Sr is solely surrounded by carbonate molecules.
note, Fe is shown to be concentrated around the edge of developing bone (Table 1).

Quantification of Zn and Sr in bone and soft tissue using the ESEM were unsuccessful as the concentrations of both were below the detection limits of quantification for this instrument. However, the ESEM shows that the bone and cartilage is still covered in a thin layer (25 μm) of soft tissue that is most likely the periosteum (layer of connective tissue around bones; Table S2†). These variables were added as a filter layer when fitting the synchrotron XRF data in PyMCA.

Spectroscopy

Strontium XANES of the ossified part of the bone was different compared to the SrCO3 standard (Fig. 3) and showed strong similarities to the spectra of fully hydrated Sr species.24 The appearance of a “shoulder” at feature F2 in the spectra suggests this is a fairly crystalline hydrated Sr species, which indicates that Sr is surrounded by hydroxyl molecules. This also suggests that hydration plays a role in the substitution of Sr for Ca in the hydroxyapatite (mineral component of bone) of the ossified bone matrix.

Discussion

Synchrotron-based microfocus elemental mapping and quantification along the forelimb in a one-day old M. musculus revealed a discrete distribution of elements associated with bone growth, specifically Zn, Sr, and Ca. By comparing these distributions to stained histological studies of skeletal development25 we can now correlate these elements to bone versus cartilage and determine their significance in the development process.

Zinc is distributed throughout all the skeletal elements, both cartilage and bone, with slightly elevated levels around the edges of developing bones (ossification front; Fig. 1 and Table 1). This is consistent with the current literature as Zn has been associated with the mineralization of bone at the growth plate region via other quantification methods22 and in the areas between mineralized and unmineralized zones of osteons.10,11,13,14,18 In addition, it is known that Zn is also expressed between the first and second week of fracture healing in rats, where it increases the expression of osteocalcin needed for hard callus formation; the replacement of cartilage to ossified tissue.15 As fracture healing recapitulates the stages of bone development, it is unsurprising that Zn would be associated with the similar processes of cartilage to bone replacement in developing bones.26

Strontium is present in the ossified tissue and also appears to extend past the fully ossified areas of the bone into the cartilage and is locally concentrated between ossified bone and cartilage, suggesting that this element is important at the ossification front (Fig. 1 and Table 1). This is similar to the findings of Maciejewska et al., who found higher levels of Sr within younger individuals’ decreases with age, suggesting a decrease in Sr as the bones become more ossified. This Sr anomaly is also seen in fracture healing, where the bias for Sr uptake in trabecular bone coupled with the relatively high speed of incorporation suggests that the stage of fracture healing most affected by Sr is the repair stage, during the formation of the bony callus.27–29 This inferred use of Sr as a “quick fix” for bone ossification is further strengthened by the XANES data, as a hydrated Sr species would enhance the substitution potential of Sr within the mineral structure, making it easy to quickly take in and dispel Sr as needed for the ossification process.

Calcium is most concentrated in the centre of the developing bones suggesting it is present in the ossified tissue (Fig. 1 and Table 1). This association is based on histological studies, which have shown that the long bones of the forelimb are completely ossified except for the ends in one-day postnatal mice, but the carpals remain cartilaginous until around one week postnatal.30 It should be noted that caution is needed when comparing the distributions of light elements, such as Ca, with higher energy elements such as Sr, because the higher energy Sr fluorescence has a much deeper escape depth. This is especially important if zonation is not perpendicular to the surface plane of the object or if there is significant compositional heterogeneity perpendicular to the surface. Our analytical protocol and geometry minimized this issue by removing the integument and imaging the chemistry perpendicular to the growth direction.

Fig. 4 Example of preserved chemistry in the bones and scales of a 50 million year-old fish from the Green River Formation. As in the extant M. musculus, Sr and Zn correlate within the skeletal elements. Due to significant amounts of Ca in the geological matrix, this element is not clearly resolved in the fossil tissues. Scale bar is 1 cm.
The correlation between bone/cartilage and trace elements was only possible due to the sensitivity of new techniques such as synchrotron X-ray fluorescence allowing the assessment of the low element concentrations (ppm) and fine scale histological features (microns) associated with such tissues. As highlighted earlier, the endoskeleton was one of the most significant evolutionary developments in the history of life on earth. Considering the results presented here for extant mouse, it may be possible to use these same techniques to gain insight into the evolutionary development of this key biological process. Indeed, it has recently been found that the same correlations observed in the extant mouse can be observed in archaeological samples of humans (e.g. Swanston et al.1), the ancient remains of fossil mammals (e.g. 19 million year old manatee fossils)18 and even dinosaurs (Allosaurus, 145 million years old).19 To widen the taxonomic range in which these observations can be demonstrated, we present here new synchrotron XRF maps of a 50 million year old fish (Knightia oceana, a relative of the herring), which also show differential distribution of the elements Zn and Sr within the ossified tissues of the skeleton (Fig. 4). Just as in the extant M. musculus, Zn and Sr are clearly elevated within ossified tissues (vertebrae, ribs and skull) and even assist in resolving the scale pattern. This adds clear and strong support to the notion that these biologically important trace elements can be preserved in situ over deep time and we can use these to identify discrete biochemical pathways in extinct organisms. It also demonstrates that such elemental inventories in bone tissue are constrained across the vertebrate group. This opens up new opportunities for the study of physiological process in extinct animals, resulting in exciting new studies on ancient biochemistry, organismal biology, fossilisation and the evolution and development of the bony skeleton.

Conclusion
Elemental mapping of one-day postnatal mice revealed the precise distribution of trace elements crucial during bone development. These elements can be associated with three distinct phases of endochondral ossification: the beginning ossification front of bone deposition onto the cartilage scaffold (Zn), the mineralization of bone tissue (Sr) and the final ossified tissue (Ca). The distributions of the trace elements permit the spatial mapping of these specific processes, allowing for a better understanding of the localised controls on ossification that have not been seen before. These correlations could only be made through the use of synchrotron-based analysis, which provides both the resolution and sensitivity required to map biologically important trace elements. In addition, these techniques can also be applied to the fossil record, shedding light on the evolutionary development of this fundamental biological process.

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