Ameloblastin Inhibits Cranial Suture Closure by Modulating Msx2 Expression and Proliferation

Phimon Atsawasuwan1,2, Xuanyu Lu1, Yoshihiro Ito1, Youbin Zhang1, Carla A. Evans2, Xianghong Luan1,2*

1 Brodie Laboratory for Craniofacial Genetics, Department of Oral Biology University of Illinois College of Dentistry, Chicago, Illinois, United States of America, 2 Department of Orthodontics University of Illinois College of Dentistry, Chicago, Illinois, United States of America

Abstract

Deformities of cranial sutures such as craniosynostosis and enlarged parietal foramina greatly impact human development and quality of life. Here we have examined the role of the extracellular matrix protein ameloblastin (Ambn), a recent addition to the family of non-collagenous extracellular bone matrix proteins, in craniofacial bone development and suture formation. Using RT-PCR, western blot and immunohistochemistry, Ambn was localized in mouse calvarial bone and adjacent condensed mesenchyme. Five-fold Ambn overexpression in a K14-driven transgenic mouse model resulted in delayed posterior frontal suture fusion and incomplete suture closure. Moreover, Ambn overexpressor skulls weighed 13.2% less, their interfrontal bones were 35.3% thinner, and the width between frontal bones plus interfrontal suture was 14.3% wider. Ambn overexpressing mice also featured reduced cell proliferation in suture blastemas and in mesenchymal cells from posterior frontal sutures. There was a more than 2-fold reduction of Msx2 in Ambn overexpressing calvariae and suture mesenchymal cells, and this effect was inversely proportionate to the level of Ambn overexpression in different cell lines. The reduction of Msx2 expression as a result of Ambn overexpression was further enhanced in the presence of the MEK/ERK pathway inhibitor O126. Finally, Ambn overexpression significantly reduced Msx2 down-stream target gene expression levels, including osteogenic transcription factors Runx2 and Osx, the bone matrix proteins Ibisp, Coll, Ocn and Opn, and the cell cycle-related gene CcnD1. Together, these data suggest that Ambn plays a crucial role in the regulation of cranial bone growth and suture closure via Msx2 suppression and proliferation inhibition.

Citation: Atsawasuwan P, Lu X, Ito Y, Zhang Y, Evans CA, et al. (2013) Ameloblastin Inhibits Cranial Suture Closure by Modulating Msx2 Expression and Proliferation. PLoS ONE 8(4): e52800. doi:10.1371/journal.pone.0052800

Editor: Chi Zhang, University of Texas Southwestern Medical Center, United States of America

Received September 4, 2012; Accepted November 21, 2012; Published April 4, 2013

Copyright: © 2013 Atsawasuwan et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Funding: This study has been generously supported by grant DE19155 from the National Institute of Dental and Craniofacial Research (http://www.nidcr.nih.gov/). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

Introduction

During skull development, calvarial bones expand from initial ossification centers toward the suture interface between adjacent bones [1]. Developmentally, this gradual intramembranous ossification is accomplished by the differentiation of ectomesenchymal cells into calvarial osteoblasts [2]. On a molecular level, membranous bone ossification is regulated by a number of growth and transcription factors as well as extracellular matrix proteins, including fibroblast growth factor receptors FGFR1 and FGFR2 [3], transcription factors CBFA1/RUNX2 [4–6], MSX2 [7], TWIST [8], [9], and matrix proteins osteopontin and tenasin [10], [11]. Abnormal changes in these factors not only affect bone thickness and mineralization, but also the expansion of bone along the ossification center/suture axis, resulting in either synostosis or open foramina.

From a clinical perspective, disturbances in normal skull ossification result in a number of pathologies with often dramatic consequences for children’s health. Premature ossification of one or several sutures of the skull leads to an abnormal skull shape or retarded skull growth (Craniosynostosis, CS). The most common type of CS involves disturbances in the formation of a single suture (simple or non syndromic CS), and its etiology remains unexplained. Multiple suture synostoses (syndromic CS) are often associated with syndromes of genetic origin i.e. Apert syndrome, Crouzon syndrome, Pfeiffer syndrome and Saethre-Chotzen syndrome [12]. The incidence of non-syndromic CS in United States is about 34.3/100,000 live births while syndromic CS is rare; their incidence is about 1.5/100,000 [13]. The treatment of CS is composed of multiple surgical interventions to correct the abnormal shape of the head, give space for the brain to grow in a normal fashion and prevent the development of intracranial pressure leading to neurological and brain damage complications or death [14]. Complications from surgical treatment can lead to infection, re-ossification, and death from hemorrhage [15].

Cranial osteogenesis and the differentiated state of the bone/suture interface are regulated by extracellular matrix molecules that transmit signals across the cell membrane into the cytoplasm, activating a number of signaling pathways, which subsequently affect gene expression [16], [17]. One of the recently discovered members of the bone extracellular matrix is ameloblastin (AMBN), a glycoprotein previously only associated with the extracellular enamel matrix [18], [19]. Recent studies have noted Ambn expression also in dentin, cementum, pulp, cranial bones, and primary osteoblasts [20–25]. Moreover, Ambn has been shown to regulate Mxs2 expression in ameloblasts [18], [26], and...
Msx2 mRNA expression was dysregulated in Ambn deficient mice [26]. In the present study, we hypothesized that Ambn might affect the growth of craniofacial bones and the patency of cranial sutures through its effect on Msx2 and possibly other mechanisms. Here we have tested this hypothesis using Ambn transgenic mice and in vitro models. Our studies reveal a distinct effect of Ambn on craniofacial bone growth and suture closure, and suggests possible mechanisms of action.

Materials and Methods

Transgene constructs and transgenic mice

Two transgenic constructs were generated using a modified pSKII- transvector in which the K14 promoter, the polyA signal (a generous gift from Dr. Elaine Fuchs, Rockefeller University), the β-globin intron, the mouse Ambn coding region, or the LacZ gene was inserted. The β-globin intron was used to ensure that the transgenes were transcribed properly. The transgenic fragments were freed from pSKII-K14-AMBN or pSKII-K14-LacZ by digesting the constructs with Sac I and Hind III, gel purified and microinjected into mouse zygotes [19]. Mice used in the present study included human keratin 14 (K14) promoter-driven Ambn transgenic mice (AmbnTg, E18.5, P1, 20, 35 and 60 days of age, n = 14) and K14 promoter-driven LacZ transgenic mice (P1, n = 3).

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Committee on the Ethics of Animal Experiments of the University of Illinois at Chicago (Permit Number: 11–077). For further analysis, mice were euthanized in a CO2 chamber followed by cervical dislocation, and calvarial bone and suture tissues were collected for fixation or RNA/protein extraction.

Genotyping. Genotyping was carried out using tails collected from AmbnTg or LacZ transgenic heterozygous litters [19]. The tails were lysed in DirectPCR (Tail) buffer (Viagen, Los Angeles, CA) at 50°C overnight and then 85°C for 40 min. PCR was performed using a set of specific primers for K14 promoters: a forward primer 5’-GCTTAGCCAGGTTACAGAG 3’ and a reverse primer 5’-CAGAGGGGTTAAATGCAGAG 3’. After 35 reaction cycles, aliquots of the PCR products were separated on a 2.0% TAE (Tris acetate EDTA) agarose gel, stained with ethidium bromide, and photographed under UV light [19].

Table 1. Primer sequences for RT-PCR analysis.

| Gene    | Forward Primer             | Reverse Primer            |
|---------|---------------------------|---------------------------|
| AMBN    | 5’-aaacccaccaacacactgag   | 3’-cattggtcctccgagatattg |
| β-ACTIN | 5’-gacctgtccctctgagc      | 3’-acatctgtgtaggaagggac  |
| IBSP    | 5’-gaacctaggtgacggacagac  | 3’-gaacccctgtagaccagaca  |
| CCND1   | 5’-cagaggccactagcgagcggt | 3’-cgcgaggtctgtagctctct |
| Coll A  | 5’-ccactcattagagctctgattg| 3’-tccgcttccagctgagatg |
| MSX2    | 5’-agcatatatgcctctccacac| 3’-caaggctagagctgggattg |
| OCN     | 5’-tcgctgtctccatccagcc  | 3’-ttggcactaccaagcttgag |
| OPN     | 5’-tccaagagagaccaagagaga| 3’-ttggtcctgtctgttccag |
| OSX     | 5’-cctgtctctccatcagcttc | 3’-gggaacacggcgaagatgat |
| RunX2   | 5’-ccacacactcactcaccagc | 3’-ttggtcctgtctgttccag |

Figure 1. Ambn expression in cranial bone and dura mater. (A) RT-PCR analysis of Ambn mRNA expression in the posterior frontal suture region from 1, 3, 10, and 20 days postnatal WT mice. The β-Actin gene was used as internal control. (B) Western blot analysis of Ambn protein expression in postnatal day 3 posterior-frontal (PF) suture tissues and teeth. Two bands at 55 and 50 kDa were recognized. β-Actin was used as loading reference. (C) Immunostained sections of cranial bone and dura mater from 3 day postnatal wildtype (WT) mice demonstrated Ambn protein localization in calvarial bone (cb), dura mater (dm), and condensed mesenchymal cells (me). Note the high levels of Ambn protein in the dura mater and in calvarial osteoblasts. Bar = 20 μm.

doi:10.1371/journal.pone.0052800.g001

Figure 2. LacZ and Ambn transgene expression in cranial bone and sutures. (A and B) Whole mount β-gal staining of wildtype (WT) and K14-LacZ overexpressing mouse skulls. The β-gal blue signal was localized in cranial bones and sutures of the Ambn overexpressor. (C and D) Histological sections of β-gal stained and Eosin counterstained skulls were prepared from the coronal plane of the calvaria in the posterior-frontal region. Note the β-gal positive calvarial osteoblasts in the Ambn transgenic mouse sections. No blue staining of β-gal was detected in the WT skull section. Bar for A and B=1 mm, C and D = 20 μm. (E) Western blot analysis of protein extracts from WT and Ambn transgenic calvarial bone and sutures at age postnatal day 3. The Ambn protein level in the transgenic mouse (right lane, upper panel) was 5-fold higher than that in the control mouse (left lane, upper panel) after normalization with β-actin (lower panel).

doi:10.1371/journal.pone.0052800.g002
Dry skull preparation, whole mount X-gal staining and alcian blue/alizarin red staining

Dry skulls of three adult mice from wild-type and Ambn transgenic mice at postnatal day 60 (n = 3) were prepared using Dermestes beetles. Briefly, the crude muscle and soft tissues were removed from the animal heads and left cool-dry for several days. The dry skulls were gently placed in a container with Dermestes beetles for several days at 30°C under close observation. Once the soft tissues of skulls were completely removed, the dry skulls were collected and cleaned meticulously with a soft brush. The dry skull, and especially the cranial suture of each animal was examined and photographed under a stereo microscope. Width of the posterior frontal suture of each animal was measured with a digital caliper and recorded in mm units. For whole mount X-gal staining, de-skinned animal heads were fixed with 4% paraformaldehyde in PBS at 4°C overnight. The samples were rinsed with PBS at room temperature 3 times for 15 minutes each and then incubated in the dark with a staining buffer containing 0.05 mM K₃Fe(CN)₆, 0.05 mM K₄Fe(CN)₆, 1 mM MgCl₂ and 1 mg/ml X-gal at 37°C for 7 hours. For whole mount alcian blue/alizarin red staining, three embryos from the wild type and Ambn transgenic groups at embryonic day 18.5 (n = 3) were fixed and dehydrated with 70%, 100% ethanol and acetone. The samples were stained with saturated alcian blue (Sigma, St Louis, MO) in 95% ethanol for 2 days, destained with 95% ethanol, and rehydrated. Subsequently, samples were then stained with saturated alizarin red S (Sigma) in 0.5% potassium hydroxide (KOH) solution for 2 days, destained in 0.5% KOH solution until all soft connective tissue turned clear and then stored in 80% glycerol.

Tissue processing

Calvaria and cranial sutures from wildtype, Ambn or LacZ transgenic mice at age of embryonic 18 and postnatal 35 days (n = 3) were dissected and fixed with 10% formalin at 4°C and demineralized in a solution containing 45% EDTA, 4.5% NaOH and 1% formalin at 4°C until the demineralization was completed. The demineralized samples were embedded in paraffin, cut in 5 μm sagittal sections and mounted on glass slides. Thereafter, the samples were deparaffinized, rehydrated, and stained with Hematoxylin and Eosin or subjected to immunohistochemistry.

Immunohistochemistry

Sections were deparaffinized, rehydrated, and treated with 6% peroxide and methanol followed by a brief incubation in 10 mM sodium citrate buffer with 0.05% Tween 20 at pH 6.0 for antigen retrieval. Sections were then incubated in 2% bovine serum albumin (BSA) for 30 minutes at room temperature to block nonspecific binding of the antibody. After blocking, sections were first incubated with affinity purified anti-Ambn antibody [19],[27] at dilution of 1:200, and then with anti-rabbit secondary antibody (Abcam, Cambridge, MA) at dilution of 1:2000. The expression of Ambn protein was visualized with a Histomouse Broad Spectrum AEC kit (Invitrogen, Carlsbad, CA) under a light microscope. As a negative control, non-immune rabbit serum was used instead of the primary antibody.

Figure 3. Delayed posterior frontal suture closure in Ambn overexpressing mice. (A and B) Whole mount alcian blue/alizarin red staining of wildtype (WT) (A) and Ambn transgenic (TG) (B) animals at embryonic stage day 18. The interfrontal gap was wider in the transgenic skulls compared to controls. (C and D) Higher magnification of the skulls shown in A and B. Note that the margins of the frontal bone osteogenic front were not well defined, and the interfrontal and parietal spaces were wider in transgenic mice compared to controls. (E and F) Dried skulls of WT and TG animals at 35 days postnatal. The PF suture was closed in WT mice (E), but remained patent in transgenic mice (F). (G and H) Higher magnification of the skulls shown in A and B. In TG mice, sutures remained open and adjacent bones overlapped (H). Cross-sections of the PF suture revealed the ossified areas of WT mice (I) and TG mice (J) at age 35 day postnatal. The PF suture was sealed by ossification in WT mice, while the left and right parietal bones were linked by soft tissue in TG mice.

doi:10.1371/journal.pone.0052800.g003
BrdU and TUNEL staining

For BrdU staining, pregnant mice at E18 were injected with BrdU (100 mg/kg) intraperitoneally prior to euthanization. Calvaria and cranial sutures were dissected and processed for paraffin sections. The sections were deparaffinated, rehydrated, and stained with biotinylated anti-BrdU antibody. The BrdU positive cells were detected with the streptavidine-biotin system (Invitrogen). For TUNEL staining, sections were first exposed to proteinase K (20 μg/ml) at ambient temperature for 10 min, and then incubated with TdT staining solution at 37°C for 1 hour according to the manufacturer’s instruction (Promega, Madison, WI). The fluorescence-dUTP-labeled DNA was visualized using a fluorescence microscope.

Cell Culture

The mouse suture mesenchymal cells were isolated from the underlying dura mater and overlying pericranium of posterior frontal and sagittal sutures of wildtype and Ambn transgenic mice at postnatal 3 days, and maintained in α-minimum essential medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin and 25 ng/ml Amphotericin B in a 5% CO₂ atmosphere at 37°C. The medium was changed twice a week. To study the effects of Ambn on cell proliferation and differentiation, suture mesenchymal cells from wildtype and Ambn transgenic mice were subjected to regular culture medium or a mineralization induction medium containing 50 μg/ml ascorbic acid and 2 mM β-glycerophosphate, and cultured for 2–21 days. Upon terminating the culture, cells were used for MTT assay, alkaline phosphatase activity test, alizarin red staining, or RNA and protein preparation.

Alkaline phosphatase and in vitro mineralization assay

After 7 days of culture in osteogenic media, cells were washed and stained with alkaline phosphatase substrate (Roche Diagnostics, Indianapolis, IN) to verify early osteogenic activity. After 21 days of culture in osteogenic media, the cells were fixed with methanol, stained with 10% alizarin red solution, and mineralized nodules were identified as red spots on the culture dish.

Cell proliferation assay

Prior to termination of culture, cells were incubated in MTT solution (2 mg/ml of MTT in DMEM with 2% FBS) for 4 hours. To quantify proliferative activity, the MTT stained cells were lysed in HCL/Isopropanol, and the absorbance was detected at 570 nm with background subtraction at 630 nm [28].
RNA extraction and RT-real time PCR

Total RNAs were isolated from mouse suture tissues or cultured cells using the TRIZOL LS Reagent (Invitrogen) according to the manufacturer’s instructions. Two micrograms of total extracted RNA was applied toward cDNA generation with the Sprint RT Complete kit (Clontech, Mountain View, CA). To quantify the mRNA expression levels of transcription factors and bone marker genes, real-time PCR primers were designed based on EMBL/GenBank searches (shown in Table I). Real-time PCR was performed using sequence specific sybergreen primers and the ABI Prism 7000 Sequence detection system (Applied Biosystems, Foster City, CA). Reaction conditions were as follows: 2 min at 50°C (one cycle), 10 min at 95°C (one cycle), and 15 sec at 95°C, and 1 min at 60°C (40 cycles). Samples were normalized using GAPDH. The analyses were performed in triplicate for three independent experiments to confirm reproducibility of the results. Relative expression levels were calculated using the 2–ΔΔCt method [29], and values were graphed as the mean expression level ± standard deviation.

Protein extraction and western blot analysis

Calvaria from 3 transgenic and 3 control animals at age P20 were collected and homogenized under liquid N2. Equal amounts of protein extracts in a lysis buffer containing 100 mM Tris HCl pH 9.0, 200 mM KCl, 25 mM EGTA, 36 mM MgCl2, 2% deoxycholic acid and 10% DTT v/v were subjected to SDS-polyacrylamide gel electrophoresis (Biorad, Hercules, CA), and the separated proteins were transferred to a PVDF (Polyvinylidene Difluoride) membrane (Immobilon P®, Millipore, Billerica, MA). The membrane was incubated with rabbit anti-mouse Ambn [19],[27], Msx2, or CcnD1 antibodies (Abcam). Immune complexes were detected with goat anti-rabbit IgG horseradish peroxidase-conjugated secondary antibody (Molecular Probes, Carlsbad, CA) and enhanced chemiluminescence reagents (Pierce Biotechnology, Rockford, IL). The amount of protein expression was compared after normalization with the amount of β-actin as an internal calibrator in each lane.

Statistical analysis

Quantitative data were presented as means ± SD from three independent experiments and compared using Kruskall-Wallis one-way analysis of variance. The difference between groups was considered statistically significant at P<0.05.

Results

AMBN is expressed in calvarial bone

Previous studies have demonstrated that the extracellular matrix protein Ambn is expressed in cranial tissues including enamel [30], cementoblasts [22], and cranial bones [24]. To examine the function of Ambn in cranial bone and suture formation, Ambn expression in mouse posterior frontal suture structures was
characterized. RT-PCR analysis of samples ranging between postnatal days 1–20 revealed Ambn mRNA expression in postnatal cranial sutures (Fig. 1A). Western blotting recognized 50 and 55 kDa bands positive for Ambn in calvarial bone extracts (Fig. 1B). Using immunohistochemistry, Ambn protein was localized in calvarial bone, dura mater, and adjacent condensed mesenchyme (Fig. 1C).

Five-fold AMBN overexpression in a K14-driven transgenic mouse model

Gain of function mice are useful models to mimic syndromes of genetic origin such as suture synostoses (syndromic CS) [31]. We therefore generated Ambn transgenic mice using the human K14 promoter to drive Ambn gene expression [19]. In addition, K14-LacZ transgenic mice were created to verify the location of the enforced Ambn gene expression [19]. Whole mount β-gal staining of the K14-driven LacZ transgenic skull revealed dark blue staining in parietal, frontal, maxillary, ethmoid and occipital bones and in calvarial sutures (Fig. 2B), when compared to wildtype mice (Fig. 2A). Histological analysis demonstrated β-gal blue staining in calvarial osteoblasts in transgenic mouse sections (Fig. 2D), but not in control mouse sections (Fig. 2C). When compared on western blots and after normalization with β-actin, the amount of Ambn protein from calvarial bones and posterior frontal (PF) sutures was 5 times higher in the Ambn transgenic mice than in wildtype controls (Figs. 2E and F).

Posterior frontal suture fusion was delayed in Ambn transgenic mice

To determine the effect of Ambn on cranial suture closure, skulls from Ambn transgenic and wildtype mice were compared using alcian blue/alizarin red staining for cartilage and bone mineralized tissue. Ambn transgenic mice at embryonic day 18.5 exhibited wider gaps of interfrontal and interparietal spaces (Figs. 3B and D) compared to those of their controls (Figs. 3A and C) (n = 3). There was less bone density and ossification along the suture (Fig. 3D), and the osteogenic margins of frontal bones were disorganized and not well defined (Fig. 3D), when compared to the wildtype control (Fig. 3C). This phenotype was indicative of a delayed development of the frontal bones. Skull samples of adult Ambn transgenic animals (age 60 days postnatal) revealed the patency of the posterior frontal sutures, revealing spanned gaps between the frontal bones (Figs. 3F and H), while the controls exhibited complete suture closure (Figs. 3E and G). The posterior frontal suture gap width in the Ambn transgenic mice was 0.12–0.42 μm (Fig. 3J), compared to 0.02–0.04 μm in controls (Fig. 3I) (n = 3).

Ambn overexpressor skulls weighed less, and their interfrontal bones were thinner and wider

To further characterize the phenotypes of Ambn transgenic mice, dried skull samples from wildtype and Ambn transgenic mice at age of postnatal day 60 were compared, resulting in at least three significant differences. First, there was a significant 13.2% reduction in weight of Ambn transgenic skulls compared to WT controls (Fig. 4A). Second, skull width of Ambn transgenic animals was 14.3% increased when measured at the interfrontal bone/
coronal suture interface (Figs. 4B). Third, there was a significant 33.3% reduction in interfrontal bone thickness in transgenic mice (Fig. 4D). Interestingly, there was no significant difference in the length of skulls (data not shown), and in the length of the posterior frontal suture, when comparing wildtype and transgenic groups (Fig. 4C).

**Incomplete posterior frontal suture closure and reduced proliferation rates in Ambn transgenic mice**

Histomorphological analysis demonstrated complete closure of the posterior frontal suture in control mice (Fig. 5A) in contrast to incomplete closure in transgenic mice (Fig. 5B). The micrograph was a fused bony bridge formation on the telencephalic aspect of the calvaria in control mice (Fig. 5A), while the frontal bone arch was interrupted by a soft tissue interface in the *Ambn* transgenic mice (Fig. 5B). The interrupting soft tissue extended from the periosteum to the dura mater in vertical direction (Fig. 5B). BrdU staining of the developing posterior frontal suture (E18) revealed 29% less proliferating cells in *Ambn* transgenic mice. The number of BrdU positive cells per 0.01 mm² in the control group was 124+/− 5.6 while the number in *Ambn* TG was 88+/− 2.8. In contrast, the number of TUNEL positive cells was 1.3-fold higher in *Ambn* overexpressors (Figs. 5D and F) when compared to wildtype mice (Figs. 5C and E).

**Ambn inhibited mouse suture mesenchymal cell proliferation and differentiation in vitro**

To determine how Ambn affects suture development, suture mesenchymal cells were isolated from underlying dura mater and overlying pericranium of posterior-frontal sutures of wildtype and transgenic mice. Suture mesenchymal cells from wildtype mice expressed basal Ambn protein levels (Fig. 6A), while the cells from two different transgenic mouse lines over-expressed Ambn protein at about 4.05 (TG1) and 2.5 (TG2) times higher levels (Figs. 6A and 7B). To further examine whether Ambn expression levels affect cell function, proliferation of suture mesenchymal cells was analyzed using the MTT assay. There was an inverse relationship between Ambn expression levels and cell proliferation capacity, with highest MTT OD value in wildtype cells, followed by TG2 and then TG1 cells (Fig. 6B). In addition, there was a substantial reduction in alkaline phosphatase activity and Alizarin red staining in the two cell lines from *Ambn* overexpressor mice, TG1 and TG2 (Figs. 6C and D). Remarkably, the high Ambn overexpressing cell line TG1 had a stronger effect on Alizarin red-detected mineralization inhibition than the less Ambn overexpressing cell line TG2, suggesting that Ambn over-expression inhibited suture mesenchyme osteogenic cell differentiation in a dose-dependent manner (Figs. 6D).

**Ambn regulated Msx2 and downstream gene expression**

Ambn overexpressor mice displayed posterior frontal suture patency, a phenotype previously reported in Msx2 deficient mice [32]. In addition, Ambn over-expressing suture mesenchymal cells displayed lower proliferative capacity and delayed differentiation similar to the effects of Msx2 deficiency on mesenchymal cell function [32]. Therefore, Msx2 expression in suture tissues as well as suture mesenchymal cells from *Ambn* transgenic and wildtype mice was examined. Western blot analysis revealed that Msx2 and CcnD1 expression in calvarial bones and PF suture tissues of *Ambn* transgenic mice were downregulated (Fig. 7A). Msx2 mRNA expression in suture mesenchymal cells from wildtype and transgenic mice was reverse proportional to Ambn mRNA expression, when comparing cells from two different transgenic lines (Fig. 7B and C). To determine whether Ambn affects expression of Msx2 down-stream target genes, expression levels of osteogenic transcription factors Runx2 and Osx, bone matrix proteins Ibsp, Coll, Ocn and Ocn, as well as the cell cycle-related gene CcnD1 were detected using quantitative RT real time PCR. Our data demonstrated that Ambn overexpression resulted in significant decreases in bone transcription factor and bone mineralization marker gene expression in a dose-dependent manner (Figs. 7D and E). Similarly, CcnD1 expression was downregulated in TG1 and TG2 cells. Together, these studies indicate that Ambn inhibits cell proliferation and reduces Msx2 and downstream gene expression in vivo and in vitro.

**Discussion**

Transgenic animal models are ideal biological systems to test the effect of forced gene expression on the development of a phenotype. In the present study we have used the human *Keratin 14* promoter to overexpress the extracellular matrix protein Ambn in calvarial osteoblasts and suture mesenchymal cells. Keratin 14 (*K14*, Cytokeratin 14 = CK14) is commonly known as an epithelial protein encoded by the *KRT14* gene. However, K14 expression in calvarial osteoblasts has been previously reported and confirmed by Western blot [33]. In addition, reports of keratins in non-epithelial cell lineages have become more frequent in the literature, including mouse studies related to keratins involved in endodermal and mesoderm adhesion in early embryogenesis [34] (Vijayaraj et al. 2010, reports on K19 in dental papilla and dental pulp [35] [Webb et al. 1995], microarray and western blot detection of K18 in cementoblasts [36] (Dangaria et al. 2011), findings of K19 and K8 in mature striated muscle [37] (Shah et al. 2012), and K8 and K18 expression in mesenchymal progenitors of regenerating limbs [38] (Cocoran and Ferretti 1997). Here, *K14* expression in calvarial osteoblasts was further confirmed by β-gal staining for the *K14* LacZ transgene in *K14* LacZ transgenic mice using both histology and whole mount staining. The *K14* promoter system has become a promoter of choice in our and other laboratories because of its efficient expression of many transgenes [39],[40]. *K14*-driven overexpression of Ambn in mouse calvaria resulted in a robust 5-fold enhancement of Ambn levels in calvarial tissue extracts.

Our study indicates that Ambn was expressed in developing calvarial bone and sutures. This finding matches other reports of Ambn expression during bone formation [25],[41] and expands its original concept as an ameloblast-specific gene product [42]. However, when compared to the developing enamel matrix, expression levels were less, suggesting that Ambn may not act directly on bone development by controlling crystal growth as it does in the enamel matrix [29],[43], but rather indirectly as a signaling molecule affecting the expression of transcription factors and extracellular matrix signaling pathways [23],[44],[45]. This concept is supported by findings reported in the present study that already small differences in *Ambn* concentration have significant effects on cell behavior, including cell proliferation and mineralization.

Our human *K14*-driven Ambn overexpressor mice suffered from delayed and/or incomplete cranial suture closure and displayed patency of the posterior frontal suture. Moreover, frontal bones of *Ambn* overexpressors weighed less and were thinner, suggestive that Ambn was involved in tissue growth or mineralization or both. We interpret these findings to indicate that Ambn plays an important role in the growth and development of cranial bones and subsequent suture closure. Our concept that *Ambn* affects mineralized tissues outside of teeth is supported by earlier studies.
related to the effect of *Ambn* on bone [41]. In our study, the phenotype of incomplete suture closure in *Ambn* overexpressing mice is explained by two related in vitro findings, namely (i) a significant reduction in suture mesenchymal cell proliferation and (ii) a downregulation of the cell proliferation marker cyclin D1 (*CcnD1*). In addition, studies in our and other laboratories have also provided evidence for the inhibitory effect of *Ambn* on cell proliferation, e.g. an increase of the cell proliferation inhibitors p21 and p27 in *Ambn* overexpressing ameloblasts [26],[46]. In contrast, there have also been reports suggesting that *Ambn* promotes bone healing by enhancing progenitor cell proliferation [41] (Tambursten et al. 2011) and regulates osteogenic differentiation [47] (Iizuku et al.). We propose that such findings may be due to different concentrations of *Ambn* acting either as a signaling molecule or a structural matrix protein [48] (Diekwisch 2011).

Another factor responsible for the inhibition of suture closure by *Ambn* overexpression is the transcription factor Msx2, a causative gene involved in human cranial suture pathologies such as CS and EPF [49],[50]. Our finding that Msx2 is downregulated by *Ambn* gene involved in human cranial suture pathologies such as CS and Ambn overexpression is the transcription factor Msx2, a causative factor of Ambn affecting craniofacial suture morphogenesis and growth. Cells Tissues Organs 181: 127–135. These data suggested that the extracellular matrix protein Ameloblastin and Cranial Bone Development

Evidence from previous studies suggests that the effects of *Ambn* on Msx2 suppression and calvarial osteoblast proliferation inhibition may be related, as it has been shown that Msx2 increases the expression of *CcnD1*, inhibits cell differentiation [52], and influences the number of proliferative osteogenic cells in growth centers of the developing mouse skull [53]. Others have reported that calvarial osteoblasts derived from Msx2 deficient mice had a lower rate of proliferation and demonstrated accelerated osteoblastic differentiation at a later stage when compared to osteoblasts derived from wildtype mice [54]. Our finding that suture mesenchymal cells from transgenic mice reduced *CcnD1* and osteogenic gene expression under subconfluent condition supported a potential connection between the role of *Ambn* in osteogenic differentiation and its role in cell proliferation inhibition. Together, our data indicate that *Ambn* affects calvarial osteoblast proliferation and differentiation through suppression of the transcription factor Msx2.

**Conclusions**

These data suggested that the extracellular matrix protein *Ambn* plays a crucial role in cranial suture closure. The effect of *Ambn* on suture closure may be explained by the inhibitory effect of *Ambn* on the transcription factor Msx2 and its effect on cell proliferation, resulting in reduced growth and differentiation of calvarial osteoblasts as well as delayed suture closure in mice.

**Author Contributions**

Conceived and designed the experiments: X.Luan. Performed the experiments: PA Yi X.Lu YZ. Analyzed the data: PA X.Lu. Wrote the paper: PA CE X.Luan.

**References**

1. Bruce JA (1941) Time and order of appearance of ossification centers and their development in the skull of the rabbit. Am J Anat 68: 41–67.

2. Hall BK (2005) Bones and Cartilage: development skeleton biology. San Diego: Academic press. 769p.

3. Ornitz DM, Marie PJ (2002) FGF signaling pathways in endochondral and intramembranous bone development and human genetic disease. Genes Dev 16: 1446–1465.

4. Komori T, Yagi H, Nomura S, Yamaguchi A, Sasaki K, et al. (1997) Targeted disruption of Chd1 results in a complete lack of bone formation owing to an early arrest in osteoblast formation. Cell 99: 753–764.

5. Lee B, Thirunavukkarasu K, Zhou L, Pastore L, Baldini A, et al. (1997) Missense mutations abolishing DNA binding of the osteoblast-specific transcription factor OSM2/CBF1 in cleidocranial dysplasia. Nat Genet 16: 307–310.

6. Mundlos S, Otto F, Mundlos C, Mulliken JB, Alysworth AS, et al. (1997) Mutations involving the transcription factor CBF1A cause cleidocranial dysplasia. Cell 89: 773–779.

7. Dodig M, Tadic T, Kroneberg MS, Dacie S, Liu YH, et al. (1999) Ectopic Msx2 overexpression inhibits and Msx2 antiternine stimulates calvarial osteoblast differentiation. Dev Biol 209: 298–307.

8. el Ghouzzi V, Le Merrer M, Perrin-Schmitt F, Lajeunie E, Benit P, et al. (1997) Combined report of problems and complications in 793 craniofacial operations. Plast Reconstr Surg 64: 198–203.

9. Howard TD, Paznekas WA, Green ED, Chiang LC, Ma N, et al. (1997) Mutations of the TWIST gene in the Saethre-Chotzen syndrome. Nat Genet 15: 36–41.

10. Kopher RA, Mao JJ (2003) Suture growth modulated by the oscillatory Notch signaling molecule or a structural matrix protein [48] (Diekwisch 2011).

11. Lu X, Ito Y, Kulkarni A, Gibson C, Luan X, et al. (2011) Ameloblastin is a cell adhesion molecule required for maintaining the differentiation state of ameloblasts. J Cell Biol 167: 973–983.

12. Lu X, Ito Y, Kulkarni A, Gibson C, Luan X, et al. (2011) Ameloblastin-rich extracellular matrix favors short and randomly oriented apatite crystals. Eur J Oral Sci 119 Suppl: 254–260.

13. Fukuimoto S, Kiha T, Hall B, Iehara N, Nakamura T, et al. (2004) Ameloblastin is a cell adhesion molecule required for maintaining the differentiation state of ameloblasts. J Cell Biol 167: 973–983.

14. Fukuimoto S, Kiha T, Hall B, Iehara N, Nakamura T, et al. (2004) Ameloblastin is a cell adhesion molecule required for maintaining the differentiation state of ameloblasts. J Cell Biol 167: 973–983.

15. Tambursten MV, Reseland JE, Spahr A, Brookes SJ, Kvalheim G, et al. (2010) Ameloblastin expression and putative autoregulation in mesenchymal cells suggest a role in early bone formation and repair. Bone 48: 406–413.

16. Adams JC, Watt FM (1993) Regulation of development and differentiation by the extracellular matrix. Development 117: 1183–1198.

17. Carinci P, Becchetti E, Bodo M (2000) Role of the extracellular matrix and growth factors in skull morphogenesis and in the pathogenesis of craniosynostosis. Int J Dev Biol 44: 715–723.

18. Lu X, Ito Y, Kulkarni A, Gibson C, Luan X, et al. (2011) Ameloblastin-rich extracellular matrix favors short and randomly oriented apatite crystals. Eur J Oral Sci 119 Suppl: 254–260.

19. Fukuimoto S, Kiha T, Hall B, Iehara N, Nakamura T, et al. (2004) Ameloblastin is a cell adhesion molecule required for maintaining the differentiation state of ameloblasts. J Cell Biol 167: 973–983.

20. Lu X, Ito Y, Kulkarni A, Gibson C, Luan X, et al. (2011) Ameloblastin-rich extracellular matrix favors short and randomly oriented apatite crystals. Eur J Oral Sci 119 Suppl: 254–260.

21. Fukuimoto S, Kiha T, Hall B, Iehara N, Nakamura T, et al. (2004) Ameloblastin is a cell adhesion molecule required for maintaining the differentiation state of ameloblasts. J Cell Biol 167: 973–983.

22. Lu X, Ito Y, Kulkarni A, Gibson C, Luan X, et al. (2011) Ameloblastin-rich extracellular matrix favors short and randomly oriented apatite crystals. Eur J Oral Sci 119 Suppl: 254–260.

23. Lu X, Ito Y, Kulkarni A, Gibson C, Luan X, et al. (2011) Ameloblastin-rich extracellular matrix favors short and randomly oriented apatite crystals. Eur J Oral Sci 119 Suppl: 254–260.

24. Lu X, Ito Y, Kulkarni A, Gibson C, Luan X, et al. (2011) Ameloblastin-rich extracellular matrix favors short and randomly oriented apatite crystals. Eur J Oral Sci 119 Suppl: 254–260.

25. Lu X, Ito Y, Kulkarni A, Gibson C, Luan X, et al. (2011) Ameloblastin-rich extracellular matrix favors short and randomly oriented apatite crystals. Eur J Oral Sci 119 Suppl: 254–260.

26. Lu X, Ito Y, Kulkarni A, Gibson C, Luan X, et al. (2011) Ameloblastin-rich extracellular matrix favors short and randomly oriented apatite crystals. Eur J Oral Sci 119 Suppl: 254–260.

27. Lu X, Ito Y, Kulkarni A, Gibson C, Luan X, et al. (2011) Ameloblastin-rich extracellular matrix favors short and randomly oriented apatite crystals. Eur J Oral Sci 119 Suppl: 254–260.

28. Lu X, Ito Y, Kulkarni A, Gibson C, Luan X, et al. (2011) Ameloblastin-rich extracellular matrix favors short and randomly oriented apatite crystals. Eur J Oral Sci 119 Suppl: 254–260.

29. Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using the 2–Delta Delta Ct method. Methods 25: 402–408.

30. Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using the 2–Delta Delta Ct method. Methods 25: 402–408.
30. Lee SK, Krebsbach PH, Matsuiki Y, Nanci A, Yamada KM, et al. (1996) Ameloblastin expression in rat incisors and human tooth germs. Int J Dev Biol 40: 1141–1150.

31. Shen K, Krakora SM, Cunningham M, Singh M, Wang X, et al. (2009) Medical treatment of craniosynostosis: recombination Noggin inhibits coronal suture closure in the rat craniosynostosis model. Orthod Craniofac Res 12: 254–262.

32. Satokata I, Ma L, Ohshima H, Bei M, Woo I, et al. (2000) Mx2 deficiency in mice causes pleiotropic defects in bone growth and ectodermal organ formation. Nat Genet 24: 189–195.

33. Wazen RM, Moffat P, Zaltz SF, Yamada Y, Nanci A (2009) A mouse model expressing a truncated form of ameloblastin exhibits dental and junctional epithelium defects. Matrix Biol 28: 292–303.

34. Zhang Y, Zhang X, Liu X, Anawasenjan P, Luan X (2011) Ameloblastin regulate cell attachment and proliferation through RhoA and p27. Eur J Oral Sci 119 Suppl: 280–285.

43. Lee SK, Kim SM, Lee YJ, Yamada KM, Yamada Y, et al. (2003) The structure of the rat ameloblastin gene and its expression in amelogenesis. Mol Cells 15: 443–450.

44. Fukumoto S, Yamada A, Nonaka K, Yamada Y (2005) Essential roles of ameloblastin in maintaining ameloblast differentiation and enamel formation. Cells Tissues Organs 181: 189–195.

45. Zhang Y, Zhang X, Liu X, Anawasenjan P, Luan X (2011) Ameloblastin regulate cell attachment and proliferation through RhoA and p27. Eur J Oral Sci 119 Suppl: 280–285.