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Calvarial bone development and suture closure in *Dicer* deficient mice

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Abstract

Objective—To evaluate whether lack of *Dicer* during calvaria development would lead to dysmorphology of calvaria and suture closure in mice.

Materials and Methods—A conditional *Dicer* deficient under *Osx* promoter mouse was employed in this study. The 4- and 10-week old conditional *Dicer* deficient mice, control littermates and *Osx*-cre transgenic mice were studied for calvarial bone morphology and suture closure. Dry skull, microcomputed tomography (μ CT), histological and gene expression studies were investigated to evaluate the effect of *Dicer* deficiency on calvarial bone morphology and their related genes during calvaria development.

Results—The results elucidated that complete suture closure was observed in 10-week old conditional *Dicer* deficient mice while incomplete closure suture was observed in age-matched *Osx*-cre control mice. The μ CT and histological sections demonstrated complete fusion of posterior frontal suture and dysmorphic calvarial bones in *Dicer* deficient mice compared to the ones in their littermates and age-matched *Osx*-cre control mice. Gene expression study demonstrated significantly increased expression of suture and calvarial bone related genes i.e. *Tgf-beta family*, *Bmp3*, *Msx2*, *Alx4*, *Runx2* and *Osx* in *Dicer* deficient mice during suture closure time.

Conclusions—The results suggest mature miRNAs are important for suture closure and calvarial morphology during calvaria development.

Keywords

Calvaria; *Osx*-cre; *Dicer*; MicroRNA; Suture closure

Introduction

Disruption of normal skull ossification and development leads to numerous pathological conditions for children's health. Premature ossification of one or more sutures of the skulls leads to an abnormal skull shape called craniosynostosis (CS) (1). On the other hand, delayed ossification of calvarial bones or suture closure leads to a condition called enlarged parietal foramina (EPF) (2). The most common type of CS involves suture closure of one

suture (simple or non syndromic CS), and etiology of these disturbances is not fully understood. Multiple suture synostoses (syndromic CS) are often associated with syndromes of genetic origin (1). Calvarial osteogenesis and the differentiated state of the bone/suture interface are strictly regulated at posttranscriptional, translational and posttranslational levels, which transmit signals across nuclear membrane into cytoplasm, activating a number of signaling pathways, which subsequently affect gene expression (3, 4). MicroRNA (miRNAs, miRs) are small non-coding RNAs that orchestrate complex posttranscriptional regulatory networks essential to the regulation of gene expression (5). Each miRNA appears to regulate the expression of tens to hundreds of genes to efficiently coordinate multiple cellular pathways (6-8), and homeostasis of ECM of tissues including bone, cartilage and ligaments (9-15). To be functional and mature miRNAs, *Dicer*, an endoribonuclease: is needed because it cleaves the miRNA precursor during miRNA biogenesis (16). *Dicer* inactivation in osteoprogenitor cells, osteoblasts and osteoclasts interfere physiological bone formation, resorption and its homeostasis (11-13). *Dicer* ablation in mice under neural crest specific promoter was lethal perinatally and resulted in malformation of the midbrain and cerebellum with severe abnormalities during craniofacial morphogenesis and organogenesis (17, 18). The aim of this study is to evaluate whether lack of *Dicer* in calvarial cells during calvarial bone development and suture closure would lead to abnormal calvarial bone morphology and suture closure.

Materials and Methods

Animals

A conditional *Dicer* deficient carrying *Osx* promoter mouse (*Osx-cre/Dicer*^(fl/fl), *Dicer* deficient) model was employed in this study. *Osx-cre/Dicer*^(fl/fl) is a *Dicer* floxed mouse (8) carrying *Osx/TatetOCre* promoter (19) for time/tissue-dependent *Dicer* deletion purposes. The study was carried out in 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 animal care committee, the University of - (ACC 13-084).

The conditional *Dicer* deficient mice have been bred as follows:

$$Osx\text{-}cre \times Dicer^{(fl/fl)} > Osx\text{-}cre/Dicer^{(fl/WT)}$$

$$Osx\text{-}cre/Dicer^{(fl/WT)} \times Dicer^{(fl/fl)} > Osx\text{-}cre/Dicer^{(fl/fl)} \text{ (25\% of number pups)}$$

Osx-cre/Dicer^(fl/fl) mice lack *Dicer* protein, which the *Dicer* gene is floxed in the mice. Because the *Osx* promoter is expressed in calvarial bones (20, 21), in *Osx/TatetOCre* system, cre recombinase is generated in the absence of tetracycline analog and removes the *Dicer* gene in calvarial bones. Mice carrying floxed alleles of *Dicer* were described previously and purchased from Jackson Laboratory (Bar Harbor, ME) (8). Doxycycline water was fed to the mice until the pups were born to inhibit cre recombinase activity then normal water was fed afterwards to turn on the cre recombinase activity postnatally. Tail genotyping was performed using specific pairs of primers (Table I). Western blot analysis of calvarial bones' extract from *Dicer* deficient and control littermate was performed. *Osx-cre* transgenic mice were used as another set of controls in this study. To validate the expression of *Osx* promoter in calvarial bones, we performed β -galactosidase staining in calvaria of

Osx-cre/*Rosa26* mouse (22) to demonstrate the expression and distribution of *Osx* promoter in the calvarial bones.

Dry skull and microcomputed tomography

Dry skulls from the *Osx*-cre/*Dicer*^(f/f) mice, their control littermates and the *Osx* cre mice at postnatal day 70 (P70) (n=4) were prepared using *Dermestes* beetles. Briefly, soft tissues were removed from the animal heads. The dry skulls were gently placed with *Dermestes* beetles at 30°C under close observation. Once the soft tissues of skulls were completely removed, the dry skull, and especially the posterior frontal suture (PFS) of each animal was examined and photographed under a stereomicroscope at 2× magnification. The skulls were subjected to a desktop micro-computed tomography (μCT) 50 system (Scanco Medical AG, Bruttisellen, Switzerland). The x-ray source voltage was 70 kVp with the source current at 114 μA, and the integration time at 300 ms. The skulls were placed in a holder and scanned with the resolution of 10 μm/voxel.

Beta-galactosidase staining and histological analysis

For β-galactosidase staining, defleshed animal heads of *Osx*-cre/*Rosa26* mouse at P28 were fixed with 4% paraformaldehyde at 4°C. The samples were washed then incubated in a staining buffer containing 1 mg/ml X-gal at 37°C overnight. The calvarial bones were subjected to a demineralizing solution at 4°C until the demineralization was completed. The mouse skulls at P70 (n=3) from the *Osx*-cre/*Dicer*^(f/f) mice, their control littermates and the *Osx*-cre mice were also subjected to demineralizing solution at 4°C. The demineralized samples were stained with H&E or β-galactosidase staining.

Gene Expression and western blot study

Total RNAs were isolated from calvarial bones (n=3) of the *Osx*-cre/*Dicer*^(f/f), control littermate mice and the age-matched *Osx*-cre mice and cDNA was generated with Sprint RT Complete kit (Clontech, Mountain View, CA). Real-time PCR was performed using sequence specific sybergreen primers (Table I) and the ABI Prism 7000 Sequence detection system (Applied Biosystems, Foster City, CA). Samples were normalized using β-actin. The analyses were performed in triplicate for three independent experiments. Relative expression levels were calculated using the 2^{-Ct} method (23). Equal amounts of protein extracts from calvaria of the *Osx*-cre/*Dicer*^(f/f) and the control littermate mice at age P28 in a lysis buffer were subjected to SDS-PAGE (Biorad, Hercules, CA), and transferred to a PVDF membrane. The membrane was incubated with rabbit anti-mouse *Dicer* antibodies (Abcam, Cambridge, MA).

Statistical Analysis

The data were analyzed by SPSS version 21 (SPSS Inc, Chicago, IL). The distribution of the raw data was evaluated using Shapiro-Wilk test of normality. Kruskal-Wallis analysis was performed to investigate the mean difference among groups. Posthoc analysis by Mann-Whitney test was performed with statistical significance at 0.05.

Results

Genotyping and verification of expression

The *Osx*-cre/*Dicer*^(f/f) mouse was viable (Fig. 1A). The localization of the *Osx* promoter expression in osteoblasts was revealed as β -Gal positive area (blue color, arrow) (Fig. 1B). Genotyping of *Dicer* deletion under *Osx* promoter mice was shown in Fig. 1C and western blot of mouse calvaria exhibited significantly lower amount of *Dicer* protein in *Osx*-cre/*Dicer*^(f/f) mouse's calvaria compared to that of the age- and gender-matched control littermate.

Calvarial bone morphology and suture closure

The skulls from 10-week old *Osx*-cre/*Dicer*^(f/f), littermate control mice and *Osx*-cre mice were compared using dry skull analysis, microcomputed tomography (μ CT) and H&E staining. The μ CT demonstrated the complete closure of posterior frontal suture (PFS) in the *Osx*-cre/*Dicer*^(f/f) and littermate control mice while the PFS in the age-matched *Osx*-cre mice did not close completely (Fig. 2A,D,G). The dry skull showed complete suture closure in the *Osx*-cre/*Dicer*^(f/f) and littermate control mice (Fig. 2B, E, H). Histomorphological analysis demonstrated complete closure of the posterior frontal suture in the *Osx*-cre/*Dicer*^(f/f) and littermate control mice (Fig. 2C and F) in contrast to incomplete closure in the *Osx*-cre mice (Fig. 2I). The H&E staining sections showed a fused bony bridge formation on the telencephalic aspect of the calvaria in the *Osx*-cre/*Dicer*^(f/f) and littermate control mice (Fig. 2C and F), while the frontal bone arch was interrupted by a soft tissue interface in the *Osx*-cre mice. The interrupting soft tissue extended from the periosteum to the dura mater (Fig. 2I). There was no significant difference of bone volume and bone mineral density of the calvaria from the *Osx*-cre/*Dicer*^(f/f) and littermate control mice but a significant decrease was found in the *Osx*-cre calvaria ($P < 0.05$) (Fig. 2J and K). Calvarial bone thickness of the *Osx*-cre/*Dicer*^(f/f) mice was significantly increased when compared to those of littermate control and the *Osx*-cre mice ($P < 0.05$) (Fig. 2M).

Gene expression study

Dicer deficiency resulted in significant increases in *Msx2* and *Alx4* transcriptional factors as well as *CcnD1* gene (Fig. 3A). In addition, *Dicer* deficiency resulted in significant increases bone transcription factors (*Runx2* and *Osx*) but not bone mineralization marker gene expression (*Ibsp* and *Ocn*) (Fig. 3B). *Tgf-beta* family and *FgfR3* were up-regulated in the *Osx*-cre/*Dicer*^(f/f) mice's calvarial bones (Fig. 3C and D).

Discussion

The skeletal elements of the skull are derived from embryonic mesoderm and cranial neural crest (24, 25). *Dicer* has been reported as indispensable for mouse development and survival (6, 8). *Dicer* ablation in mice under *Wnt1*-cre was lethal perinatally and resulted in a loss of neural crest-derived craniofacial bones (17, 18, 26). Conditional deletion of *Dicer* in osteoprogenitors by *Col1a1*-Cre compromised fetal survival (13) and *in vivo* excision of *Dicer* by *Osteocalcin*-Cre in mature osteoblasts generated a viable mouse with increased bone mass in long bones and vertebrae (13). In the present study, we showed that conditional

deletion of *Dicer* in calvarial osteoblasts and suture cells under *Osx*-cre postnatally caused increased calvarial bone volume, thickness and mineral density and restored suture defects in *Osx*-cre mouse. Literatures reported that *Osx*-cre transgenic mice developed craniofacial bone development defects as delayed calvarial mineralization in postnatal day 6 (P6) mice (21) and hypomineralization phenotype and persisted suture patency defect in P21 mice (20). In mouse, all calvarial sutures, except for the posterior section of the interfrontal suture, remain patent. The posterior frontal suture (PFS) fuses between P20-P45 (27). Our study showed that PFS defect and hypomineralization in the *Osx*-cre mice persisted in adult mice at P70. However in the *Osx*-cre/*Dicer*^(flfl) mice, the calvarial bone hypomineralization and PFS suture defects were not observed implicating that increased bone mass and suture closure was rescued by *Dicer* deficiency. The increased calvarial bone mass, thickness and bone mineral density in the *Osx*-cre/*Dicer*^(flfl) mice and suture defect rescue was observed by μ CT, histological and dry skull analyses. Water containing doxycycline was withdrawn from all *Osx*-cre/*Dicer*^(flfl), littermate control and *Osx*-cre mice at the day of birth (P0). The effect of doxycycline should be washed out when the skulls were studied at 10 weeks (P70) and the phenotype of suture was observed only in the *Osx*-cre mice not in *Osx*-cre/*Dicer*^(flfl) or littermate control. We speculated that *Dicer* deficiency affected calvarial bone phenotypes and suture closure by increased expression of crucial bone and suture transcriptional factors i.e. *Runx2*, *Osx*, *Msx2*, *Alx4* and *Tgf- β* family. Overexpression of *Runx2* and *Msx2* was reported to promote suture obliteration (28, 29). *Dicer* was under-expressed in *Runx2* deficient mice implicating *Dicer* was regulated by *Runx2* (30). Surprisingly, in this study we discovered that *Runx2* expression was up-regulated in the absence of *Dicer* during calvarial development as well.

Conclusions

We demonstrated that conditional deletion of *Dicer* with *Osx**TATetOCre* promoter postnatally led to increased calvarial bone mass, thickness and bone mineral density in *Osx*-cre/*Dicer*^(flfl) mice and rescued suture defect phenotype in *Osx*-cre transgenic mice.

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Fig. 1. Beta-galactosidase and Dicer expression in cranial bone and sutures: Comparison of a conditional *Dicer* deficient carrying *Osx* promoter mouse (*Osx-cre/Dicer*^(f/f)) mouse and its littermate (A); Histological calvarial sections of β -gal stained and Eosin counterstained of the calvaria in the posterior-frontal region of *Osx-cre/R26R* mouse sections. Note the β -gal positive calvarial osteoblasts were shown with arrows (B); Genotyping of *Osx-cre* transgene (upper panel, left) and floxed *Dicer* (upper panel, right) (C). Western blot of control littermate and the *Osx-cre/Dicer*^(f/f) calvarial bone/suture at age postnatal day 28. Dicer protein was recognized as a band above 220 kDa. β -Actin was used as loading reference (C).

Fig. 2. Microcomputed tomographs, posterior frontal suture of dry skull samples and H&E staining of suture of mouse's calvaria: Control littermate (A,B,C); *Osx-cre/Dicer^(f/f)* (D,E,F); *Osx-cre* mouse (G, H, I). Mean and standard deviation of bone parameters and measurements of mouse's calvaria: Bone volume fraction (bone volume/tissue volume) (J); Bone mineral density (K); Calvaria width at frontal bones (L); Calvaria thickness at frontal bones (M). *, #, \$ ($P < 0.05$); Df/df C-: littermate control; Df/df C+: *Osx-cre/Dicer^(f/f)*; C+: *Osx cre*.

Fig. 3.

Comparison of mean and standard deviation of gene expression levels from mouse's calvaria and sutures: Suture associated genes (A); Bone transcriptional factors and markers (B); Tgf-β family (C); Fgf-receptor family (D). *, #, \$ (P<0.05); Df/df C-: littermate control; Df/df C+: *Osx-cre/Dicer^(flf)*; C+: *Osx-cre*.

Table I
Primer sequences for PCR analysis

	Forward	Reverse
Dicer flox	5' cctgacagtgcggtcctcaag	5' catgactcttcaactcaaat
R26R	5' aaagtcgctctgagttgttat	5' gcgaagagtttgtctcaacc
		5' ggagcggagaaatggatatg
<i>Osx-cre</i>	5' gtcgatgcaacgagtgtgagc	5' cgaacatcttcaggttctcgg
	5' catgctagcagctcggagaaac	5' atgctagacctgggcagccata
β -actin	5' gatcattgctcctcctgagc	5' acatctgctggaaggtggac
CcnD1	5' ggctgcgatgcaaggcctga	5' cagcggagcagtcgggtca
FgfR1	5' gccctcacgctctcctcca	5' gcagccctcgccaagtgtt
FgfR2	5' aacagcggacaccccgatgc	5' gcccgagcaaccttcccc
FgfR3	5' cggcacgacctactgactg	5' ggtgtactccccgcgtcct
Ibsp	5' ggaaccacaggggagggga	5' tccataggttcggcggt
Msx2	5' cacagtccgggctctcgt	5' cggggagtatctcggggct
Ocn	5' agatgccaahcccagcggc	5' ctgaggtccaaggttagcgcc
Runx2	5' gattgagggccgcgcaga	5' gcgggcagcagcagcacag
Osx	5' aaggcagcatccacgcagg	5' tcacctggccctgaccacc
Tgf β 1	5' ggactctccactgcaagac	5' gactggcgagccttagtttg
Tgf β 2	5' ctgcaggagaaggcaagccgg	5' tggcggggatggcattttcgg
Tgf β 3	5' cccaacccagctccaagcg	5' acagccattcagcgggtgcc
Twist1	5' ccaggtctgcagagcgacg	5' ccaggccccctccatctcc