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Original Article

Skull and cerebellar phenotypes in Dlx2 over-expressed mice

Ningjuan Ouyang, Jiewen Dai, Jianfei Zhang, Xiaofang Zhu, Hongliang Li, Xudong Wang, Guofang Shen

Department of Oral & Cranio-maxillofacial Surgery, Shanghai Ninth People's Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai Key Laboratory of Stomatology, No. 639 Zhizaoju Road, Shanghai 200011, China

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Abstract: Cranial neural crest stem cells (CNCCs), a subpopulation of neural crest stem cells (NCCs), can give rise to jaw bones and cartilage, odontoblasts, connective tissues and smooth muscle cells in craniofacial region. What's more, the development of cerebellum is also related to the migration of NCCs. Dlx2, a member of the homeodomain family of transcription factors, is expressed in the developing branchial arches within the mesenchyme and epithelium. Previous studies revealed that targeted deletion of Dlx2 in mice had its special roles during the developmental process of craniofacial structures of the first and second branchial arches. In our former study, by generating a transgenic mouse overexpressing Dlx2 in NCCs (Wnt1Cre::iZEG-Dlx2), we found that up-regulated expression of Dlx2 could cause cleft lip, mid-facial cleavage defects, nasal bone deformities and body weight decrease of mice, which might be a result of the disturbed migration, increased apoptosis and decreased proliferation as well as abnormal chondrogenesis and osteogenesis of CNCCs within the craniofacial region. In this study, by further evaluating the pattern of skulls and craniofacial synchondroses in the cranial base, as well as morphology of cerebellar in NCCs-specific Dlx2 overexpression mice, we observed abnormal sutures and defective synchondroses in the skull in Wnt1Cre::iZEG-Dlx2 mice, accompanied by deranged proliferation of chondrocytes in cranial bases. Meanwhile, cerebellar dysplasia was also detected in the Dlx2 overexpression mice. Together with previous studies, we found that the Wnt1Cre::iZEG-Dlx2 mice might be an ideal model as to explore better therapeutic methods for genetic craniofacial and cerebellar defects and malformations.

Keywords: Dlx2, overexpression, skull, cerebellum

Introduction

Neural crest stem cells (NCCs) are a population of multipotent migratory cells. They originate from the neural crest and migrate to various regions of the embryo, followed by differentiating into multiple cell types, including endocrine and para-endocrine cells, melanocytes, sensory and autonomic neurons, and glia [1]. In the craniofacial region, cranial neural crest cells (CNCCs) can give rise to jaw bones and cartilage, odontoblasts, connective tissues and smooth muscle cells. Several events can be seen in developmental process of craniofacial structures, such as migration of cranial neural crest into the branchial arches, epithelial-mesenchymal interactions that induce mesenchymal condensations into the appropriate size and location, and differentiation into diverse cell types [2]. For skull bones, previous studies revealed that most skull bones, such as parietal bone, were derived from mesoderm mesenchymal cells. However, frontal bone and Spheno-occipital synchondrosis were originated from NCCs.

The vertebrate brain consists of three neuromeric compartments in the anterior-posterior axis, including the forebrain, the midbrain, and the hindbrain, which is divided into eight rhombomeres (rhombomere1-rhombomere8, r1-r8). Previous studies showed that CNCCs might originate from the hindbrain, and also probably from the midbrain, and thereafter migrate into the branchial arch [3]. Meanwhile, the cerebellum derives from r1, allocated in the dorsal part of the forefront hindbrain segment, which is also called the midbrain-hindbrain boundary

(MHB). After a series of morphological changes, the cerebellar primordium appears, and various cerebellar neuron types are generated thereafter [4-6].

Dlx family genes encode the homeodomain transcription factors with structurally similarity to the *Drosophila* distal-less gene [7]. There are six members in this family that have overlapping expression patterns but different roles in the craniofacial development. Dlx2 is expressed in the developing branchial arches within the mesenchyme and epithelium. Dlx2-expressed CNCCs are found in the developing craniofacial mesenchyme and ganglia [3]. Targeted deletion of Dlx2 in mice has revealed its special roles during the developmental process of craniofacial structures of the first and second branchial arches [8]. Also, Dlx2 over-expression reduces neural crest cell migration and enhances cell to cell adhesion in both the neural tube and the branchial arch mesenchyme, as well as increasing the level of mesenchymal condensation [9]. In our previous study, by generating a transgenic mouse overexpressing Dlx2 in NCCs, we found that up-regulated expression of Dlx2 could cause cleft lip, midfacial cleavage defects, nasal bone deformities and body weight decrease of mice, which might be due to the disturbed migration, increased apoptosis and decreased proliferation as well as abnormal chondrogenesis and osteogenesis of cranial neural crest stem cells (CNCCs) within the craniofacial region. In this study, we evaluated the pattern of skulls and craniofacial synchondrosis in the cranial base, as well as morphology of cerebellar in NCCs-specific Dlx2 overexpression mice.

Materials and methods

Mouse strains

Wnt1-Cre transgenic C57BL/6J mice were acquired from the Jackson Laboratory (Bar Harbor, Maine, USA). iZEG-Dlx2 mice were preserved in our laboratory. Littermates were used as controls. Protocols were reviewed and approved by the Institutional Animal Care and Use Committees of the Shanghai Ninth People's Hospital, Shanghai Jiao Tong University School of Medicine.

Generation of Wnt1Cre::iZEG-Dlx2 double transgenic mice

P90 iZEG-Dlx2 transgenic mice were mated with P90 Wnt1-Cre transgenic mice to generate Wnt1-Cre::iZEG-Dlx2 double transgenic offsprings [10], which were genotyped via PCR using primers to detect Cre and EGFP. Cre-mediated recombination was detected by PCR analysis using the primers CAG-Dlx2. Identification of the Wnt1-Cre::iZEG-Dlx2 mice was performed as our previous description [11].

Skeletal preparations and Micro CT scans

Control and Wnt1Cre::iZEG-Dlx2 mice were collected, skinned and eviscerated before being transferred to 95% ethanol for 2 days. The Micro CT datasets were collected using an eXplore Locus MicroCT scanner (GE health, Milwaukee, Wisconsin, USA). P90 mice were anesthetized with pento-barbital sodium via intraperitoneal anesthesia. The slice thickness for micro CT scans was 0.04 mm. Reconstruction of 3D skulls and bone mineral density calculations were completed using GE MicroView software 2.2 (GE Healthcare, Milwaukee, Wisconsin, USA).

Histological analysis

Heads of mice were dissected free, and fixed in 4% paraformaldehyde (PFA). P90 mice were then demineralized in 0.5 M EDTA. The tissues were embedded in paraffin, and the section at thickness of 5 mm were cut and stained with H&E. For Alcian blue staining, slides were stained in an Alcian blue solution (0.03% Alcian blue, 70% ethanol, 20% acetic acid) for 20 min, followed by washing in running tap water prior to staining in a 0.3% Alizarin red solution for 30 min at 37. Finally, the stained slides were washed in running tap water, followed by clearing in xylene, and then mount with resinous mounting medium.

Immunohistochemistry

Tissues were embedded in paraffin and sectioned at a thickness of 5 mm. Antigen retrieval was performed using a bone antigen restoration liquid kit (Sunteam Biotech, China). Slides were then washed in PBS and blocked for 1 h with 3% BSA in PBS containing 0.2% Triton in room temperature. Sections were incubated with anti-Col2 and anti-Dlx2 (Abcam, 1/200; Abcam, 1/100), overnight at 4 . The secondary antibodies were Alexa Fluor 488 donkey anti-rabbit (Jackson), which were diluted 1/300 and incubated for 1 h at room temperature. Slides

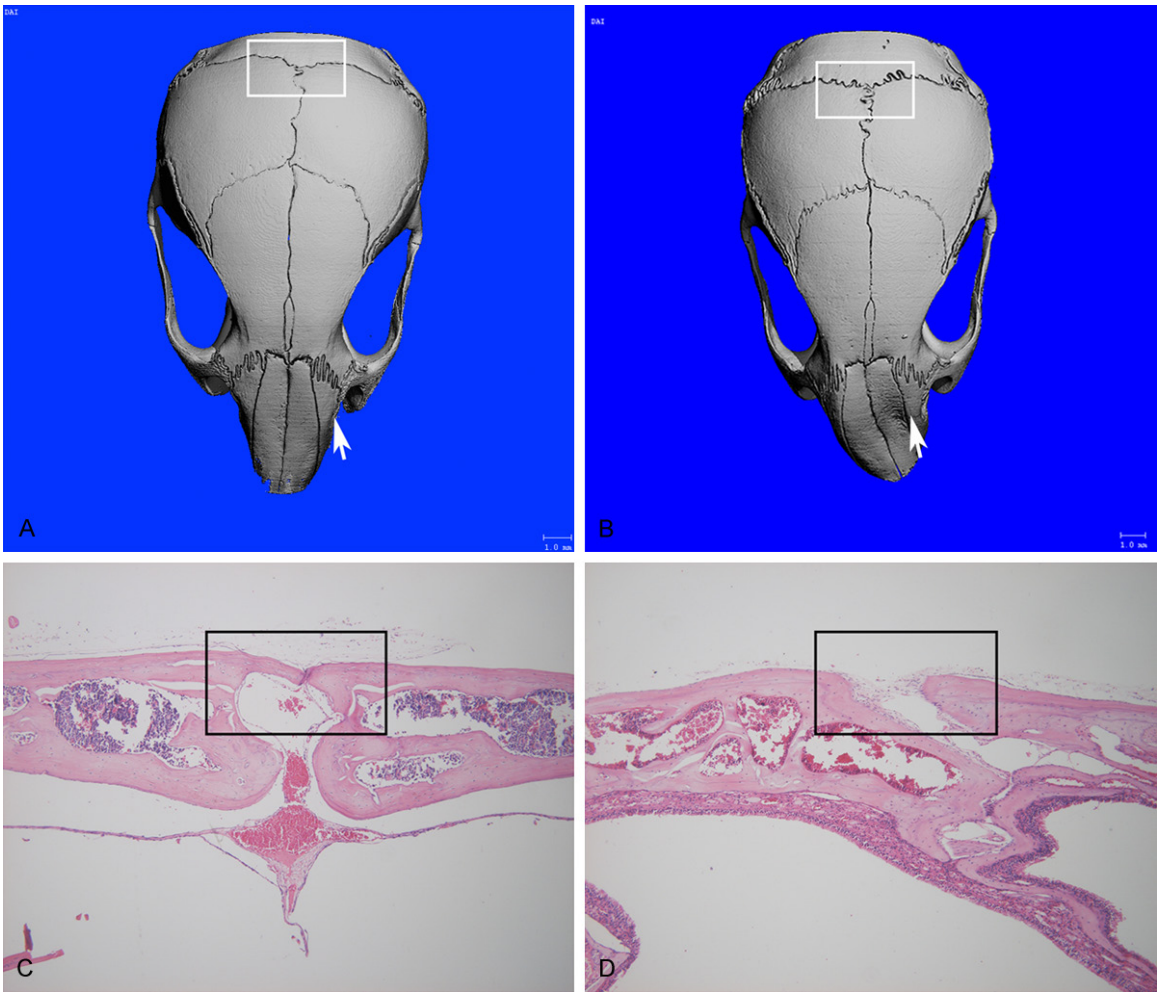

Figure 1. Top views of MicroCT three-dimension reconstructed images of iZEG-Dlx2 mice skull (A) and Wnt1Cre::iZEG-Dlx2 mice (B) showed that the nasal suture, the sagittal suture, the lambdoid suture, the coronal suture, and the posterior frontal suture all existed in both groups. Skulls in Wnt1Cre::iZEG-Dlx2 mice displayed serrated-edge sutures in the frontal and parietal bones, while the iZEG-Dlx2 mice skulls had straight sutures and smooth edges (indicated by the box). Also note that Wnt1Cre::iZEG-Dlx2 mice showed signs of suture inactivation, especially on the side where the maxilla was shorter (indicated by the arrowhead). Hematoxylin and eosin-stained coronal sections of the skull sutures showed that there was incomplete closure of the sutures in Wnt1Cre::iZEG-Dlx2 mice (D) compared to the iZEG-Dlx2 mice (C) (100×) (indicated by the box).

were mounted with Vectashield mounting medium containing DAPI (Invitrogen) and visualized under a fluorescence microscope.

Statistical analysis

Each experiment was performed a minimum of three times. Quantified evidence of the imaging data was obtained through Image-Pro Plus V6.0 (Media Cybernetics, Inc.). All data were expressed as mean ± SEM. The significance of differences was analyzed by independent sample t-test. A value of $P<0.05$ was considered statistically significant.

Results

General observation and abnormal sutures of skulls in Wnt1Cre::iZEG-Dlx2 mice

Skulls from P90 control (iZEG-Dlx2) and the NCCs-specific Dlx2 overexpression (Wnt1Cre::iZEG-Dlx2) mice were analyzed by Micro CT (**Figure 1A**, **1B**). The P90 Wnt1Cre::iZEG-Dlx2 mice were characterized by short snouts, as well as nasal and maxillary hypoplasia and deviation. In the frontal and parietal bones, skulls of the Wnt1Cre::iZEG-Dlx2 mice displayed serrated-edge sutures, while the iZEG-Dlx2 mice skulls had straight sutures and

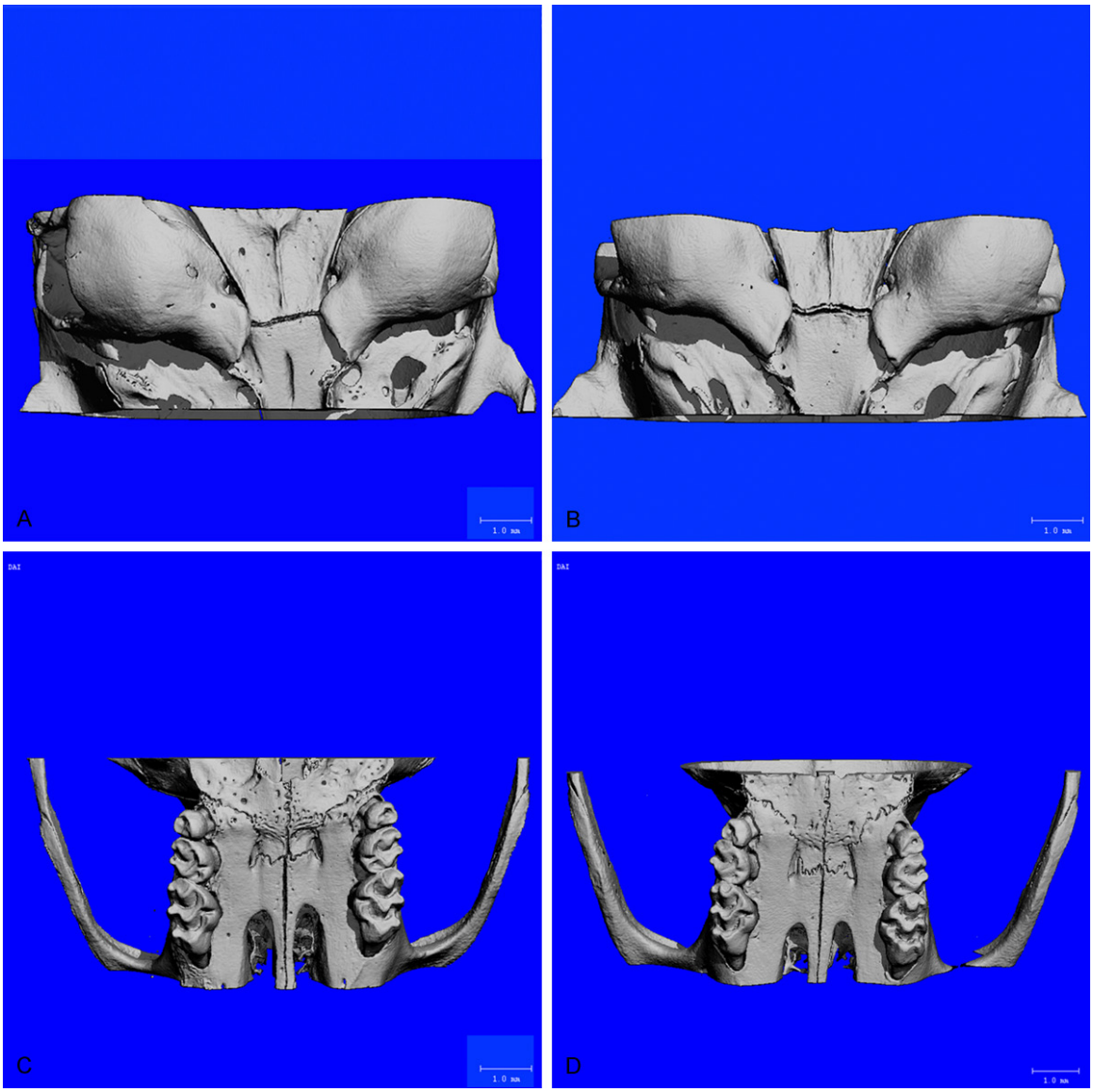

Figure 2. Bottom views of MicroCT three-dimension reconstructed images of iZEG-Dlx2 and Wnt1Cre::iZEG-Dlx2 mice skulls. The spheno-occipital synchondroses in P90 Wnt1Cre::iZEG-Dlx2 mice (B) were abnormal compared to its control littermates (A), as the distance between the synchondroses were partially increased and there was incomplete closure in certain parts of the synchondroses. Whereas no obvious difference was detected for presphenoid-maxilla synchondroses between the Wnt1Cre::iZEG-Dlx2 mice (D) and its littermates (C).

smooth edges. Meanwhile, the inter digitations was especially prominent in lambdoid suture of Wnt1Cre::iZEG-Dlx2 mice. This might be a sign of retarded mineralization during suture morphogenesis. What's more, premaxillary sutures in the Wnt1Cre::iZEG-Dlx2 mice showed signs of suture inactivation, especially on the maxillary hypoplastic side. The inactivation of sutures probably impaired anterio-posterior growth and lead to the twisting of the snout and maxilla to one side. Hematoxylin and eosin-stained sections of the skull sutures confirmed that there was incomplete closure of the sutures of Wnt1Cre::iZEG-Dlx2 mice compared to its littermates.

Wnt1Cre::iZEG-Dlx2 mice exhibited obvious craniofacial synchondrosis defects

Skulls from P90 Wnt1Cre::iZEG-Dlx2 mice were analyzed by Micro CT and were shown by bottom view. The spheno-occipital synchondroses

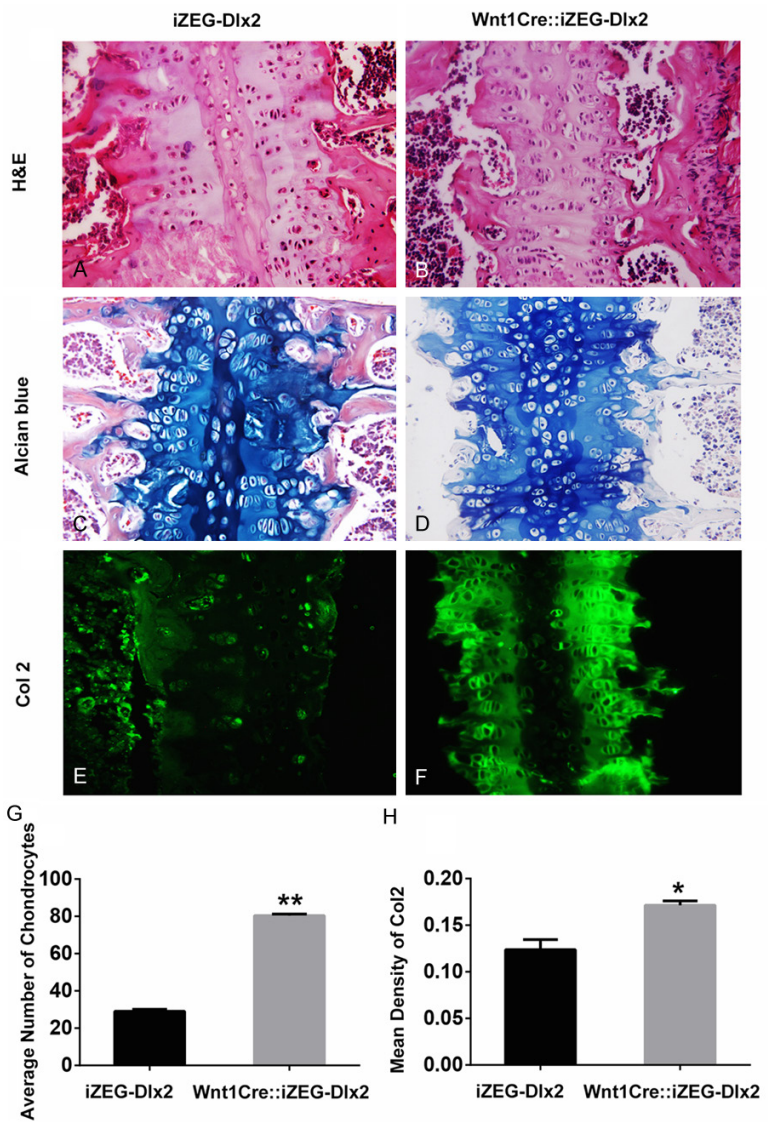

Figure 3. Parasagittal sections of H&E and Alcian blue staining showed more hypertrophic and prehypertrophic chondrocytes in the spheno-occipital synchondroses in P90 iZEG-Dlx2 (A, C), and more resting chondrocytes and proliferating chondrocytes in the spheno-occipital synchondroses in P90 Wnt1Cre::iZEG-Dlx2 mice (B, D, G). Spheno-occipital syn chondrosis in Wnt1Cre::iZEG-Dlx2 mice also exhibited a near absence of primary spongiosa (D), and more advanced bone fronts were apparent in iZEG-Dlx2 mice (B). Expression of Col2 in iZEG-Dlx2 mice was significantly reduced (E, H), as Col2 was mainly expressed in proliferating chondrocyte zones in Wnt1Cre::iZEG-Dlx2 (F). Values are mean± SEM (n=3). Significant differences between groups are noted by $*P<0.05$ and $**P<0.01$.

of the P90 Wnt1Cre::iZEG-Dlx2 mice were abnormal compared to its control littermates, as the width of the synchondroses were partially increased. In iZEG-Dlx2 mice, margins of the synchondroses were neat and would gradually fuse evenly across the entire length of the synchondroses. Whereas in Wnt1Cre::iZEG-Dlx2 mice, fusion of the synchondroses occurred unsynchronously, as incomplete closure in certain parts of the synchondroses could be detected (**Figure 2A**, **2B**). This indicated a delayed synchondrosis closure of the basicranial synchondrosis. However, there was no obvious difference between the presphenoid-maxilla synchondroses of the Wnt1Cre::iZEG-Dlx2 mice and its littermates (**Figure 2C**, **2D**).

Synchondrosis disorganization in cranial bases in Wnt1Cre::iZEG-Dlx2 mice

The synchondrosis consists of a resting zone in the middle, and two sets of maturation zones flanking aside, creating bone growth in two opposing directions simultaneously. They are the resting, proliferative, prehypertrophic, hypertrophic zones and primary bone spongiosa. All of them were arranged by increasing maturity. Parasagittal sections of H&E and Alcian blue staining showed that there were more hypertrophic and prehypertrophic chondrocytes in the spheno-occipital synchondroses in P90 iZEG-Dlx2 than Wnt1Cre::iZEG-Dlx2 mice. Meanwhile, more resting chondrocytes and proliferating chondrocytes in the spheno-occipital synchondroses of P90 Wnt1Cre::iZEG-Dlx2 mice were detected (**Figure 3A-D**, **3G**). This indicated a delayed onset of chondrocyte hypertrophy and endochondral ossification in Wnt1Cre::iZEG-Dlx2 mice. What's more, spheno-occipital synchondrosis of Wnt1-Cre::iZEG-Dlx2 mice showed that there was a near absence of primary spongiosa, and more advanced bone fronts were apparent in: iZEG-Dlx2 mice.

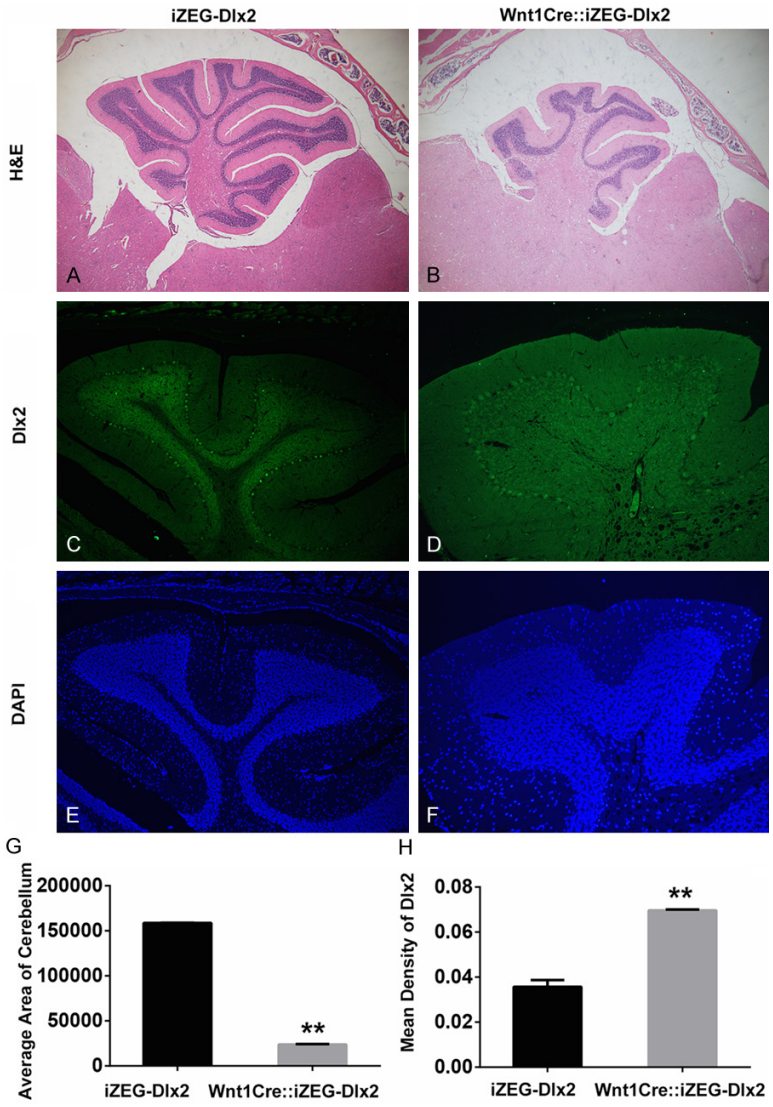

Figure 4. Hematoxylin and eosin-stained sagittal sections of cerebellum showed dysplasia and hypoplasiain P90 Wnt1Cre::iZEG-Dlx2 cerebellum (B) (40×). Cerebellum was smaller and the folia formation was less compared to the cerebellum of its littermates (A, G). The size and number of cerebellar folia decreased, as the size of the white matter increased. Expression of Dlx2 (C, D, H) and DAPI staining (E, F) in P90 iZEG-Dlx2 and Wnt1Cre::iZEG-Dlx2 cerebellum (200×). Values are mean ± SEM (n=3). Significant differences between groups are noted by **P*<0.05 and ***P*<0.01.

Immunofluorescence analyses of spheno-occipital synchondrosis showed that collagen type II (Col2), a marker of resting and proliferating chondrocytes was detected (**Figure 3E**, **3F**, **3H**). We found that in Wnt1Cre::iZEG-Dlx2 mice, Col2 was expressed in two opposing directions that were parallelled to each other, indicating the mirrored proliferative zones. While in iZEG-Dlx2 mice, there was a significant reduction of Col2 expressing proliferating chondrocytes, which was accordant with the results of H&E and Alcian blue staining.

Cerebellar dysplasia and hypoplasia in Wnt1Cre::iZEG-Dlx2 mice

Parasagittal sections of the cerebellum showed smaller gross morphology of Wnt1-Cre::iZEG-Dlx2 mice compared with iZEG-Dlx2 mice. At P90 the Wnt1Cre::iZEG-Dlx2 cerebellum was smaller and the folia formation was poor. The size and number of cerebellar folia decreased simultaneously, while the size of the white matter increased (**Figure 4A**, **4B**, **4G**). Immunofluorescence analyses showed that Dlx2 mainly expressed in the granule layer of cerebellum (**Figure 4C-F**, **4H**). The dysplasia and hypoplasia of cerebellum might imply the failure in allocation of neural crest cells in Wnt1Cre::iZEG-Dlx2 mice.

Discussion

The Dlx genes are of great importance in the development, patterning and morphogenesis of the branchial arches, as they play a dominant role in the development of the mandible and maxilla [12]. In Dlx1/2 knockout mice, maxillary process-derived parts were more severely affected than those of the mandible [7, 13], while our study showed the same results. We found that overexpression of Dlx2 lead to short snouts, and nasal and maxillary hypoplasia and deviation as well. In the meantime, overexpression of Dlx2 lead to serrated-edge sutures of the skull, indicating retarded osteogenesis. In addition, the twist of the snout might also be associated with the inactivation of sutures. Our previous study showed that F1 maxillary mesenchymal cells from Wnt1Cre::iZEG-Dlx2 embryos responded

weakly to osteogenic medium compared with control, exhibiting weaker ALP staining and fewer mineralized nodules, as well as lower expression of Runx2 and OCN [11]. Taking these findings together, it implied that the abnormal sutures and the retarded osteogenesis of skulls might be a result of the defective osteogenesis. Previous study also indicated that the Dlx genes might be important for the control of osteogenesis as Dlx5 and Dlx6 genes were expressed in all skeletal elements from the time of initial cartilage formation onward [14].

Furthermore, a delayed synchondrosis closure was detected in the basicranial synchondroses of Wnt1Cre::iZEG-Dlx2 mice indicating that overexpression of Dlx2 influenced not only the intramembranous bone but also the endochondral bones as well, which complemented our former study. Previously, we found that Dlx2 overexpression might promote the differentiation of CNCCs into early chondrocytes, but not into mature chondrocytes, and thus displayed abnormal chondrogenesis and defective osteogenesis [11]. On the other hand, the presphenoid-maxilla synchondroses remained unaffected. This might be explained by the distinct way of maxilla joining to the sphenoid bone and to the occipital bone or other basicranial elements, as the cranial basis derived from both ectoderm and mesoderm mesenchymal cells. Although previous studies also suggested that the cell fate of CNCCs was unchangeable as they were pre-programmed before their migration, evidence showed that CNCCs were capable of reprogramming in response to different environmental signals as well [15, 16]. In this study, We found that synchondrosis organization and chondrocyte proliferation were disorganized in cranial bases in Wnt1Cre::iZEG-Dlx2 mice. Additionally, the expression of Col2 increased compared to the control, demonstrating the increased number of proliferating chondrocytes. A possible mechanism is the alternation of CNCCs differentiation: CNCCs that would originally differentiate into osteoblasts might have differentiated into a chondrocyte lineage instead.

The cerebellum is classically defined by its role in sensory and motor control [17]. It is considered as one of the most elaborate parts of the central nervous system (CNS). Thus, the cerebellum is often used as a model for investigating the developmental processes of CNS [18]. The cerebellum is derived from the dorsal part of the anterior hindbrain and is divided into several folia. In sagittal section, each folia comprises distinct cellular layers with white matter beneath. Nuclei of the cerebellum lie within the white matter. The cerebellar layer patterning reflects the distribution of different cell types: the Purkinje cell layer is sandwiched between the internal granule cell layer and a molecular layer where the Purkinje cell dendrites and the granule cell axons interact. The basis of cerebellar morphology is the allocation of a territory in which its component cell types are specified. The area that will generate the cerebellum is allocated at about embryonic day (E) 8.5 in mice, which is during the early embryonic phase of hindbrain development. After the establishment of rhombomere boundaries, between E10.5 and E18.5, various cells are allocated along the dorsoventral axis [3]. Glutamatergic cells, for example, are allocated at the most dorsal interface between neural and non-neural boundary, known as the rhombic lip. During this stage, the basic categorization between GABAergic and glutamatergic cell types is also generated beyond the stereotyped Purkinje-granule cell circuit, creating the evolutionary change of cerebellum in the embryo [6]. Many genes have been detected and functionally identified during the processes of cerebellar embryonic development [19]. Previous studies have revealed that the expression of Otx2 and Hoxa2 was crucial for determining the caudal limits of cerebellar differentiation, while Gbx2 was required for the formation of cerebellum [20-23]. Meanwhile, Fgf8 was the major signaling molecule in the MHB, expressed in the Gbx2-positive domain and inhibits Otx2 expression [24, 25]. In this study, we detected cerebellar dysplasia in Wnt1Cre::iZEG-Dlx2 mice, indicating that overexpression of Dlx2 influence the morphology of cerebellum. Additionally, in previous study [11], we also found that E9.5 Wnt1Cre::iZEG-Dlx2 embryos showed a neural tube dysraphism deformity, which might share similar causes with the cerebellar dysplasia.

In summary, we observed abnormal sutures and defective synchondrosis in the skull in Wnt1Cre::iZEG-Dlx2 mice, accompanied by deranged proliferation of chondrocytes in cra-

nial bases. Meanwhile, cerebellar dysplasia could also be noted in the Dlx2 overexpression mice. Combined with previous studies, it implied that the Wnt1Cre::iZEG-Dlx2 mice might be an ideal model that contribute to explore better therapeutic methods for genetic craniofacial and cerebellar defects and malformations.

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Disclosure of conflict of interest

None.

Address correspondence to: Guofang Shen, Xudong Wang and Jiewen Dai, Department of Oral & Cranio-maxillofacial Surgery, Shanghai Ninth People's Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai Key Laboratory of Stomatology, No. 639 Zhizaoju Road, Shanghai 200011, China. Tel: +86-21-23271699; Fax: +86-21-82263136; E-mail: maxillofacsurg@163.com (GFS); xudongwang70@hotmail.com (XDW); daijiewen@163.com (JWD)

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