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**Usage of dexamethasone increases the risk of cranial neural crest dysplasia in the chick
embryo**

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Abstract

Dexamethasone (Dex) is commonly used in the treatment of a variety of benign and malignant conditions. Unfortunately, although it has a variety of teratogenic effects, it remains used in clinical practice for pregnant women mainly due to limited alternatives. However, there is limited knowledge of the mechanisms that lead to the observed teratogenic effects. In this study, the effects of Dex during embryogenesis on neural crest development were evaluated in the early chick embryos. First, we demonstrated that 100 μ L 10⁻⁶ M Dex treatment leads to craniofacial developmental defects, and also retards embryo growth and plausibly can cause embryo demise. Second, we demonstrated that Dex represses the production of HNK-1, PAX7 and AP-2 α labeled cranial neural crest cells (CNCCs), the progenitor cells of the craniofacial skeleton. Third, double immunofluorescent staining of pHis3/PAX7 and AP-2 α /c-Caspase3 revealed that Dex promotes cell apoptosis but does not change cell proliferation rates. Last, FGF signaling molecules were inhibited by Dex treatment. Dex also inhibited NCCs production by repressing *Msx1* expression in the developing neural tube and by altering expression of epithelial-mesenchymal transition-related adhesion molecules and cell migration genes. Overall, we obtained experimental evidence that Dex treatment during embryogenesis disrupts cranial neural crest (CNC) development which in turn causes defective cranial bone development.

Key words: dexamethasone (Dex); cranial neural crest (CNC); skeletogenesis; *Fgf*

INTRODUCTION

As a long-acting glucocorticoid, dexamethasone (Dex) is often clinically used to treat certain kinds of arthritis, severe allergies, asthma and occasionally as part of chemotherapy treatments (Hanaizi et al., 2015). However, it is noteworthy that glucocorticoids are potentially teratogenic (Rajadurai and Tan, 2003; Uno et al., 1990). It has been previously demonstrated that Dex treatment has a role in the cellular and molecular mechanisms associated with the development of cleft palate (Azziz and Ladda, 1990). Other defects such as encephalocele, meningocele and minor cranial skeletal abnormalities can develop in rhesus macaques when exposed to triamcinolone acetonide or Dex during gestation (Jerome and Hendrickx, 1988). There have also been some reports that Dex impairs palatogenesis in murine embryonic palatal shelves (Lu et al., 2008; Natsume et al., 1986). Meyer-Bahlburg et al. demonstrated alteration in cognitive and motor development of children from women who took Dex prenatally (Meyer-Bahlburg et al., 2004). These birth defects are closely associated with the development of the neural crest, a special cell population during embryonic development. Given that, we focused on the effects of prenatal use of Dex on neural crest generation, which remains controversial.

As a group of ecto-mesenchymal cells, neural crest cells (NCCs) derive bilaterally from the margins of the dorsal-side of the neural tube and extensively migrate shortly before and after neural tube closure into specific locations. Subsequently, they differentiate into many tissue derivatives within developing embryos (Smith et al., 2013). Due to these extraordinary abilities, NCCs are sometimes referred to as the fourth embryonic germ layer. NCCs undergo the processes of neural induction, delamination, epithelial-mesenchymal transition (EMT)

and migration to give rise to the different tissues (Hall, 2008). The NCCs arising from the lateral tips of embryonic diencephalon, mesencephalon and rhombencephalon are defined as cranial neural crest (CNC), which is distinct from the cardiac and trunk neural crest (Chai and Maxson, 2006). Unlike trunk neural crest cells which show minimal skeletogenic capacity, cranial neural crest cells (CNCCs) possess the ability to form cartilage and bone. During craniofacial development, the CNC contributes to all of the craniofacial skeleton (Cordero et al., 2011; Schneider, 1999).

We previously reported that hyperglycemia impairs the ability of NCCs to differentiate into cranial bone (Wang et al., 2015), and imidacloprid exposure suppresses NCCs generation (Wang et al., 2016). These reports show that NCCs are sensitive and vulnerable to external insults when they initially delaminate and migrate within the embryos during spatiotemporal development. Several families of signaling molecules, including FGF, BMP, and WNT, are implicated in the accurately controlled development of NCC and its derivatives (Hall, 2008; Simoes-Costa and Bronner, 2015; Smith et al., 2013). Various signaling families play different roles on NCC differentiation (Fantauzzo and Soriano, 2015). Of these, the fibroblast growth factor (FGF) family remains very important (Basch and Bronner-Fraser, 2006). *Fgf8*, a member of FGF family, is involved in the induction, patterning, migration, and differentiation of NCCs, and promoting the development of chondrogenic, odontogenic, adipogenic, and neurogenic progenitors (Shao et al., 2015). Additionally, research has shown that *Fgf8* promotes neural crest derived mesenchyme survival and induces the developmental program required for the first branchial arch morphogenesis, which later develops into a number of craniofacial skeletal elements including jaws and teeth (Trumpp et al., 1999).

In this study, we used the classical chick embryo model (Li et al., 2013) to explore the possible negative effects of Dex treatment on the CNC, and to elucidate the cellular and molecular mechanism involved.

MATERIALS AND METHODS

Chick embryos. Fertilized leghorn eggs were acquired from the Avian Farm of South China Agriculture University (Guangzhou, China). Dex (Sigma, MO, USA) was administered using two different protocols. The first was early embryo treatment, in which the fertilized eggs, at Hamburger-Hamilton (HH) stage 0 (Chapman et al., 2001), were incubated in EC culture (Streit, 2008) in the presence of DMSO (control) or Dex (10^{-6} M, dissolved in 0.1% DMSO) inside a humidified incubator (Yiheng Instrument, Shanghai, China) set at 38 °C and 70% humidity. The embryos from these incubated eggs were harvested for analysis when they reached the desired developmental HH stages (Solloway et al., 1998). In the second model for later stage embryos, 100 μ L of DMSO or Dex (10^{-6} M) were carefully injected into a small hole made in the air chamber of the pre-incubated HH4 embryo eggs. After treatment, the embryos were further incubated 13 days before being harvested for analysis.

Alizarin red staining of whole embryos. The craniofacial skeleton was visualized in 13-day (E13) chick embryos by staining with alizarin red dyes (Solarbio, Beijing, China). Briefly, the embryos were fixed in 95% ethanol for 3 days, then the skin and viscera were carefully removed before post-fixation for an additional 1 day. The embryos were then pretreated in 0.5% KOH (Jielong, Guangzhou, China) for 48 hours before they were stained

with the alizarin red dye suspended in 0.5% KOH for 3 days. Finally, the embryos were cleared in a graded series of glycerol. The craniofacial skeleton was photographed using a stereomicroscope (Olympus MVX10, Japan).

Immunofluorescent staining. Chick embryos were harvested after incubation and fixed in 4% PFA (Newprobe Bioscience Technology Co., Ltd, Beijing, China) overnight at 4 °C. Immunofluorescent staining for whole-mount embryos or primary explants were performed using: HNK-1 (1:200, Sigma, USA), PAX7 (1:200, DSHB, USA), AP-2 α (1:100, DSHB, USA), phospho-Histone3 (pHIS3; 1:200, Santa Cruz, USA), and cleaved-Caspase3 (c-Caspase3, 1:200, Cell Signaling Technology, USA) antibodies. Briefly, the fixed chick embryos were incubated with the primary antibodies at 4 °C overnight on a shaker. After extensive rinsing in PBST (0.1% Tween-20), the embryos were treated with the corresponding Alexa Fluor® 555 or 488 labeled secondary antibody (1:1000, Invitrogen, USA) at 4 °C overnight on a shaker. For double immunofluorescent staining, the antibodies were incubated one after the other. All the embryos were later counterstained with DAPI (1:1000, Invitrogen) at room temperature for 1 hour. Subsequently, the stained embryos were sectioned at 10 μ m using a cryostat (Leica CM1900). At least 6 embryos were employed in each group. For each embryo, three planes were selected evenly in the region beyond the rhombencephalon and before the heart. Subsequently, three sections were selected from each plane.

***In situ* hybridization.** Whole-mount *in situ* hybridization of chick embryos was

performed according to a standard *in situ* hybridization protocol (Henrique et al., 1995). Previously published Digoxigenin-labeled probes were synthesized to detect *Fgf8* and *Msx1* mRNAs (GEISHA ISH Analysis) (Lu et al., 2014; Wang et al., 2016). Whole-mount stained embryos were photographed and then frozen sections at a thickness of 20 μ m were prepared from these embryos for histological analysis.

RNA isolation and quantitative-PCR (q-PCR) analysis. Total RNA was isolated from HH10 chick embryos heads (N > 25) using a Trizol kit (Invitrogen, USA) according to the manufacturer's instructions. First-strand cDNA synthesis and SYBR[®] Green qPCR assay was performed using a PrimeScript[™] RT reagent kit (Takara, Japan). All specific primer sequences used are described in Supplementary Figure S1 (Gilmour et al., 1995; Smith and Fauquet, 1984). Reverse transcription and amplification reactions were performed in Bio-Rad S1000[™] (Bio-Rad, USA) and ABI 7000 thermal cyclers, respectively. The housekeeping gene *GAPDH* was run in parallel to confirm that equal amounts of RNA were used in each reaction. The expression of the genes was normalized to *GAPDH*, and the expression level was compared by $\Delta\Delta$ Ct. The q-PCR result was representative of three independent experiments.

Primary NCC cultures. NCCs were prepared from the cranial neural tubes isolated from chick embryos, according to methods previously described (Etchevers, 2011). Briefly, fertilized chick eggs were incubated until the 7-9 somite stage (HH 9-9+). The neural tube was then dissected from the head region of the embryo and explanted into 3.5 mm dishes

containing DMEM and 10% FBS for 6 hours at 37 °C and 5% CO₂, to allow the explants to adhere. The explants were then incubated until a few NCCs were observed migrating out of the neural tubes. At this point, 1 mL of culture medium containing DMSO (Control) or Dex (10⁻⁶ M) was introduced into the cultures. The treated explants were allowed to develop for 48 hours and then the areas containing the migrant NCCs were measured and analyzed using Image-Pro Plus 7.0 (IPP, Media Cybernetics, MD, USA). After incubation, the NCCs cultures were washed with pre-warmed PBS and fixed in PFA.

Scratch wound migration assay. A “scratch wound” was created by scraping a monolayer culture of HEK293 cells using a sterile 100 µL pipette as described previously (Cheng et al., 2012). The “wounded” cultures were then incubated in DMEM plus 10% FBS for 48 hours. At selected time intervals, HEK293 cells that have migrated into the wound areas were photographed using an inverted microscope, and the migrating distance from the edge of scratch wound was measured using IPP software. The assays were performed thrice using triplicate culture wells.

Photography. Following immunofluorescent/*in situ* hybridization staining, whole mount embryos and regions of interest were photographed using a stereo-fluorescence microscope and processed using Olympus software package Image-Pro Plus 7.0. The embryos were then sectioned at 10 µm on a cryostat microtome (Leica CM1900), photographed using an Olympus IX51epi-fluorescent microscope (at 200× and 400×) and analyzed using a CW4000 FISH Olympus software.

Data analysis. All data analyses and graphics were performed using the Graphpad Prism 5 software (Graphpad Software, CA, USA). The results were presented as the mean value ($\bar{x} \pm$ SE). All data were analyzed using the two-tailed *t* test to establish difference between the experimental and control groups. $P < 0.05$ was considered to be significantly different.

RESULTS

Dex treatment increases the risk of developmental defects of the craniofacial skeleton in chick embryos

Exposing early chick embryos with 10^{-6} M Dex increased the incidence of shortened coracoids (Figs. 1B, E) or curved coracoids (Figs. 1C, F) in comparison to controls (Figs. 1A, D). Additionally, 13 day-old chick embryo skulls were stained with alizarin red dye following 10^{-6} M Dex treatment to investigate which craniofacial skeletal elements were affected by Dex treatment (Figs. 1G-I). This demonstrated that there were distinct developmental defects on the maxillary bone (mx) in embryos treated with 10^{-6} M Dex in comparison to control embryos (Figs. 1G'-I', L). The alizarin red staining results demonstrated that maxillary bone formation was impaired which might imply part or full absence of the bone (shown by arrows in Figs. 1H', I'). The rest of the skull showed no obviously detectable malformation except for overall developmental delay. Dex treatment also increased the mortality (Figure 1J) and reduced the body weight (Control: 6.703 ± 0.172 g, $n = 9$; Dex: 5.329 ± 0.129 g, $n = 17$; $P = 0.000$; Figure 1K) of the embryos.

As Figure 2 shows, the chick embryos in the control group would develop to HH10 (Figs.

2A-D) after 42 hours of incubation, but some of the chick embryos exposed to Dex were still at HH8 for the same incubation time (Figs. 2E-H). The extent of the chick embryo' development was quantified by measuring the length of the embryos' anterior-posterior axis when both groups developed to the desired stage of HH10. The anterior-posterior length of embryos treated with Dex was shorter than the control embryos (Control: 4.190 ± 0.031 mm, $n = 28$; Dex: 3.833 ± 0.063 mm, $n = 28$; $P = 0.000$; Figure 2K). Generally, the normal chick embryos' incubation time-points of 0h, 18h, 28h and 42h in EC culture corresponded to the developmental stages HH0, HH4, HH7-8 and HH10, respectively (Figure 2). Therefore, maturation was evaluated by the ratio of the number of embryos which reached the desired developmental stage at different developmental time-points. The maturation rate in Dex-treated embryos was lower than the controls (18h-Control: 0.893 ± 0.022 , $n = 45$; Dex: 0.705 ± 0.011 , $n = 45$; $P = 0.001$; 24h-Control: 0.900 ± 0.028 , $n = 45$; Dex: 0.559 ± 0.006 , $n = 45$; $P = 0.000$; 42h-Control: 0.890 ± 0.031 , $n = 45$; Dex: 0.632 ± 0.011 , $n = 45$; $P = 0.001$; Figure 2J), suggesting that Dex treatment of chick embryos retards the growth velocity of the chick embryos. Lastly, exposing to Dex at HH0 increased the mortality of chick embryos compared to controls (Figure 2I).

Dex treatment suppresses CNC production during chick embryo development

Since the craniofacial bones are mainly derived from CNC during the early stages of embryogenesis (Santagati and Rijli, 2003), it is reasonable to assume that the developmental defects of the craniofacial skeleton mentioned above are due to faulty NCCs generation and differentiation. We evaluated the expression of three NCCs-associated markers including

HNK-1 (marker for migratory NCCs), PAX7 (marker for pre-migratory and migratory NCCs) and Ap-2 α (marker for CNCCs), in the presence or absence of Dex during gastrula development (Figure 3). First, the HNK-1 immunofluorescent staining showed that the areas of HNK-1⁺ migratory NCCs were significantly reduced in presence of 10⁻⁶ M Dex – as demonstrated in whole-mount preparations (Figs. 3A-D) and transverse histological sections (Control: $(2.629 \pm 0.317) \times 10^4 \mu\text{m}^2$, n = 6; Dex: $(0.873 \pm 0.162) \times 10^4 \mu\text{m}^2$, n = 6, P = 0.001; Figs. 3B1-B2, D1-D2, M). Similar findings were seen with the number of PAX7⁺ NCCs in whole-mount preparations (Figs. 3E-H) and transverse sections (Control: 114.778 ± 11.638 , n = 6; Dex: 69.907 ± 8.879 , n = 6, P = 0.011; Figs. 3F1-F2, H1-H2, N). Likewise, the number of Ap-2 α ⁺ CNCCs were also reduced by Dex treatment in both whole-mount preparations (Figs. 3I-L) and transverse sections (Control: 128.667 ± 6.265 , n = 6; Dex: 86.333 ± 5.162 , n = 6; P = 0.000; Figs. 3J1-J2, L1-L2, O). These immunofluorescent staining results imply that Dex treatment represses the production and migration of CNC.

Dex treatment does not affect cell proliferation but promotes apoptosis of developing CNC

We studied whether or not cell proliferation and apoptosis are involved in the reduction of NCCs production in presence of Dex. Using double immunofluorescent staining for PAX7 and pHIS3 or AP-2 α and c-Caspase3 co-expression, we demonstrated that there was not a significant difference between the number of CNCCs co-expressing PAX7 and pHIS3 between Dex-treated embryos and control embryos in both whole-mount preparations (Figs. 4A-D) and transverse sections (Control: 3.474 ± 0.547 , n = 6; Dex: 3.538 ± 0.527 , n = 6; P

=0.935; Figs. 4B1-B3, D1-D3, I). However, the number of CNCCs co-expressing AP-2 α and c-Caspase3 was significantly increased in the Dex-treated embryos than in controls in both whole-mount preparations (Figs. 4E-H) and transverse sections (Control: 1.333 ± 0.211 , n = 6; Dex: 3.833 ± 0.401 , n = 6; P = 0.000; Figs. 4F1-F3, H1-H3, J). We further calculated the percentage of PAX7 and pHis3 double positive cells, AP-2 α and c-Caspase3 double positive cells to the whole PAX7 positive cells in control and Dex-treated embryos respectively, the results showed the same trend as the quantitative analysis of double positive cell numbers (Control: $3.265 \pm 0.482\%$, n = 6; Dex: $3.502 \pm 0.482\%$, n = 6; P = 0.736; Fig. 4K; and Control: $1.065 \pm 0.197\%$, n = 6; Dex: $4.593 \pm 0.641\%$, n = 6; P = 0.000; Fig. 4L). These results suggest that Dex treatment increases CNC apoptosis but does not exert obvious effect on cell proliferation.

Dex treatment inhibits expression of FGF signaling in neural tubes of chick embryos

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We performed *in situ* hybridization to determine if *Fgf8* mRNA expression is inhibited in early chick embryos when exposed to 10^{-6} M Dex. The results demonstrated that *Fgf8* expression was dramatically inhibited when treated with Dex in comparison to controls in both whole-mount preparations (Figs. 5A-D) and transverse sections (Figs. 5B1-B2, D1-D2). Using q-PCR, we determined that *Fgf8* (Control: 1.000 ± 0.139 , n = 3; Dex: 0.520 ± 0.086 , n = 3; P = 0.043), *Fgf10* (Control: 1.000 ± 0.084 , n=3; Dex: 0.584 ± 0.116 , n = 3; P = 0.044), *Fgfr1* (Control: 1.000 ± 0.048 , n = 3; Dex: 0.542 ± 0.051 , n = 3; P = 0.003), *Fgfr3* (Control: 1.000 ± 0.046 , n = 3; Dex: 0.469 ± 0.071 , n = 3; P = 0.003), *Sox2* (Control: 1.000 ± 0.043 , n = 3; Dex: 0.6301 ± 0.077 , n = 3; P = 0.014) and *Sox3* (Control: 1.000 ± 0.029 , n = 3; Dex:

0.685 ± 0.108, n = 3; P = 0.048) expressions were correspondingly repressed in Dex-treated embryos as compared with control embryos (Figure 5E). Only the expression of *Fgfr2* was not affected by Dex treatment (Control: 1.000 ± 0.331, n = 3; Dex: 0.722 ± 0.032, n = 3; P = 0.450; Figure 5E).

To further confirm the influence of FGF signaling on NCCs production, we exposed half of the embryo slide to 100 μM SU5402 (fibroblast growth factor receptor-specific tyrosine kinase inhibitor), while other side of embryo slide was used as a control. This method has been described in detail in our previous publication (Li et al., 2013). This showed that HNK-1⁺ staining area of SU5402-exposed side of embryos were significantly reduced in both whole-mount (Control: (1.380 ± 0.101) × 10⁴ μm², n = 7; SU5402: (1.052 ± 0.867) × 10⁴ μm², n = 7; P = 0.029, Figs. 5G, H) and transverse sections (Control: (2.839 ± 0.434) × 10⁵ μm², n = 6; SU5402: (1.807 ± 0.085) × 10⁵ μm², n = 6; P = 0.041, Figs. 5G1-G2, I) compared to controls. These findings further validate that FGF signaling is involved in Dex-induced inhibition of the migration of NCCs.

Dex treatment restricts the generation of NCCs from neural tube explant cultures

We isolated explants from the chick neural tube and cultured them in the presence or absence of 10⁻⁶ M Dex for 18 hours (Figs. 6A-B). There were fewer NCCs emanating from the neural tube explants in the Dex-treated explants than in controls (Control: 0.869 ± 0.021, n = 8; Dex: 0.587 ± 0.032, n = 8; P = 0.000; Figs. 6A1-A3, B1-B3, C). We demonstrated that the majority of the cells emanating from the neural tube were NCCs due to expression of HNK-1 (Figs. 6A2-A3, B2-B3). In addition, the scratch wound assay showed that Dex exposure inhibited

HEK293 cell migration, which was reflected in the migratory distance of “wound” closure between the control and Dex-treated groups after 48 hours incubation (12h-Control: $341.932 \pm 4.536 \mu\text{m}$, n = 6; Dex: $242.690 \pm 2.239 \mu\text{m}$, n = 6; P = 0.000; 24h-Control: $393.612 \pm 7.395 \mu\text{m}$, n = 6; Dex: $270.695 \pm 6.437 \mu\text{m}$, n = 6; P = 0.000; 36h-Control: $441.493 \pm 6.237 \mu\text{m}$, n = 6; Dex: $335.413 \pm 4.485 \mu\text{m}$, n = 6; P = 0.000; 48h-Control: $468.000 \pm 6.020 \mu\text{m}$, n = 6; Dex: $361.998 \pm 4.338 \mu\text{m}$, n = 6; P = 0.000; Figs. 6D-D3, E-E3, F).

In order to investigate how Dex inhibits NCCs production, we performed *in situ* hybridization to detect the *Msx1* expression. Dex treatment reduced *Msx1* expression in the dorsal side of neural tubes compared to controls in both whole-mount preparations (Figs. 7A-D) and transverse sections (Figs. 7B1-B2, D1-D2). In addition, using q-PCR, we also found that Dex treatment caused the reduction of EMT and cell migration-related genes including *Msx1* (Control: 1.000 ± 0.077 , n = 3; Dex: 0.577 ± 0.057 , n = 3; P = 0.012), *Bmp4* (Control: 1.000 ± 0.085 , n = 3; Dex: 0.455 ± 0.111 , n = 3; P = 0.018), *Slug* (Control: 1.000 ± 0.107 , n = 3; Dex: 0.598 ± 0.056 , n = 3; P = 0.029), *RhoB* (Control: 1.000 ± 0.059 , n = 3; Dex: 0.564 ± 0.116 , n = 3; P = 0.029), *Cadherin-6B* (Control: 1.000 ± 0.104 , n = 3; Dex: 0.568 ± 0.079 , n = 3; P = 0.029) and *Laminin β 2* (Control: 1.000 ± 0.113 , n = 3; Dex: 0.491 ± 0.085 , n = 3; P = 0.023), indicating that these gene expression suppressions may contribute to the abnormal cell development induced by Dex treatment. However, the expression of *N-Cadherin* (Control: 1.000 ± 0.044 , n = 3; Dex: 1.919 ± 0.109 , n = 3; P = 0.002) was up-regulated with Dex exposure (Figure 7E).

DISCUSSION

Whether or not Dex treatment causes bone deformities remains a controversial issue. Glucocorticoid exposure was found to cause the osteogenic phenotype in fibrous dysplasia (Stanton et al., 1999). However, Bolt et al. reported that Dex treatment did not change the body composition in preterm infants when it was used to treat chronic lung disease (Bolt et al., 2002). Furthermore, Gilmour et al. demonstrated that Dex did not affect body composition of term gestational age infants despite the negative influences on glucose metabolism and growth patterns after drug exposure (Gilmour et al., 1995). Nevertheless, Dex exposure was shown to cause a high teratogenic risk in our previous experiments (Cheng et al., 2014; Cheng et al., 2016). Despite the awareness of potential teratogenicity, Dex remains in clinical practice during the perinatal and neonatal periods, in scenarios where better options are not available. The more we understand about the mechanisms of the numerous birth defects induced by Dex usage, the more we may be able to avoid the teratogenic effects of Dex in the future.

Intramembranous ossification is the main form of osteogenesis of the craniofacial skeleton, where bone formation directly stems from mesenchymal cells. In addition, the mesenchymal cells in the cranial region are principally derived from CNC (Santagati and Rijli, 2003). In this study, we first found that exposing chick embryos to Dex caused deformities or absence of the maxillary bone, and increased the occurrence of shortened or curved coracoids (Figure 1). Maxillary derivatives continued to form, including jugal, quadratojugal, maxillary and palatal bones (MacDonald et al., 2004). The impairment or absence of the maxillary bone led to an asymmetric development of the palate, which subsequently resulted in curved coracoids. This demonstrated that there is suppression of

normal cranial bone formation when exposed to Dex. Meanwhile, the negative effects of Dex treatment may not be limited to osseous findings since delayed embryo development and high mortality rates were also found in the presence of Dex exposure (Figure 2). Therefore, it can't be excluded that the shortened coracoids (shown in Figs. 1B, E) were associated with an overall developmental delay in the embryos. The complexity of embryogenesis means that minor anomalies or delays during development can result in severe malformations.

Due to the abnormal cranial bone formation described in this study, we decided to study the process of CNC development of chick embryos in presence/absence of Dex using *in vivo* and *in vitro* models. CNCCs are multipotent progenitor cells that are capable of differentiating into numerous types of tissue derivatives. We assessed the CNC production in developing chick embryos (Figure 3). Additionally, we demonstrated that Dex exposure suppressed the amount of HNK-1⁺, PAX7⁺ and AP-2 α ⁺ NCCs at an early chick embryonic stage (Figure 3), which indicates that Dex exposure interferes with the process of generation (PAX7⁺) and migration (HNK-1⁺) of CNCCs (AP-2 α ⁺). This also implies that Dex-induced defects in the maxillary bones of the skull might at least partially be due to the poor production of CNC. Prior *in vitro* experimental evidence revealed that glucocorticoid hormones could induce catecholamine anabolism in various adrenergic derivatives of the neural crests (Smith and Fauquet, 1984). The differences between that report and our observations in this study are likely because of the different microenvironments of *in vitro* compared to *in vivo* studies at the very early embryonic stages. This could be partially supported by observations from the development of the rat sympathetic nervous system in response to environmental stimuli (Teitelman et al., 1979). Another possible reason for these

differing results are the different embryonic developmental stage evaluated in this study. In their experiments, trunk neural crests explants taken from HH11-12 quail embryos were treated with glucocorticoids before migration had begun, while the earlier HH4 chick embryos were exposed to Dex in our experiments. However, whether it is the differentiating process from NCCs into mesenchymal cells or the subsequent process of osteogenesis that is impaired by Dex treatment still needs to be studied.

Besides pre-migration/migration and differentiation of the CNC, cell proliferation and apoptosis are also involved in the neural crest development. Therefore, we further evaluated the effect of Dex treatment on cell proliferation and apoptosis using double-labeled NCCs with either pHis3/PAX7 or c-Caspase3/AP-2 α (Figure 4). We found that Dex treatment did not greatly affect cell proliferation (pHis3⁺/PAX7⁺), but it promoted cell apoptosis (c-Caspase3⁺/AP-2 α ⁺), suggesting that CNC are more susceptible to apoptosis rather than to decreased cell proliferation when exposed to Dex during the early developmental stage. Meanwhile fewer AP-2 α ⁺ cells were seen in the Dex-treated embryos, which also implied that CNC production was affected. Our observations in the CNC are generally consistent with reports from sheep placenta, where Dex treatment acts as an imbalance factor for placental survival and apoptosis during development (Braun et al., 2015).

Given our observed Dex-induced alteration of CNC generation, we would like to further explore whether or not Dex treatment could affect the known signaling pathways that play a crucial role during organization of cranial skeleton (Mishina and Snider, 2014). The molecular mechanisms inducing chondrogenesis and osteogenesis in CNC, are cranial-specific pathways which are distinct from those operating in mesodermal cells, which

produce the remainder of the skeleton. Nonetheless, *Fgf* molecules are expressed at nearly every stage of bone formation within the craniofacial bones, the axial skeleton, and the limb bones (Kronenberg, 2003). Therefore, the expression of *Fgf* genes as well as neural plate markers were examined by *in situ* hybridization and q-PCR. Here, our results indicate that Dex treatment represses *Fgf8* expression as well as *Fgf10*, *Fgfr1*, *Fgfr3* and *Sox2-3*, and in turn blocking the FGF signaling with SU5402 inhibits the production of CNC (Figure 5), which suggests that Dex-induced disturbance of FGF signaling might be responsible for the abnormal CNC development.

Another logical question is if the important signal-regulated events (delamination, EMT and migration) of NCCs generation are affected by Dex treatment. We demonstrated that fewer NCCs emigrated and with a lower migratory ability in the presence of Dex (Figure 6), indicating that the EMT from pre-migratory to migratory NCCs or migratory ability of NCCs are deleteriously affected by Dex treatment. The negative effects of Dex exposure, shown by neural tube *in vitro* explants and scratch assays, include both cell proliferation/apoptosis and migration. To further confirm this we subsequently assessed the expressions of EMT-related or cell migration-related genes. These results demonstrated reduced expressions of *Msx1*, *Bmp4*, *Slug*, *RhoB*, *Cadherin-6B* and *Laminin β 2* genes. However, there was an increase of *N-Cadherin* expression in the presence of Dex (Figure 7). *Msx1* has been reported to modulate the EMT process in vertebrate organogenesis. *Msx1*^{-/-} mice exhibit a number of craniofacial abnormalities including an arrest of tooth development, an absence of periodontal intramembranous bone and the alveolar process (Davidson, 1995; Satokata and Maas, 1994). *Bmp4*, a downstream target of *Msx1*, plays an important role in the initiation

and differentiation of NCCs as well as during alveolar bone formation (Zhang et al., 2003). *Cadherin-6B* (epithelial *Cadherin-6B*) is an adhesion molecule that is response for the EMT of chick CNC (Kerosuo and Bronner-Fraser, 2012). Together these data imply that Dex might directly or indirectly interfere with these EMT regulatory transcription factors, adhesion molecules and cell migration gene expressions. Thereby, inhibiting the EMT of CNC and causing normal migration to not properly occur.

Taken together (Figure 8), we showed that there are developmental defects of the premaxilla when the early chick embryos were treated with Dex. Next, we revealed that Dex treatment inhibits the generation of CNC in early chick embryo development. The increase of NCC apoptosis in presence of Dex could partially explain the phenotype seen. In addition, abnormal expressions of EMT regulatory transcription factors, adhesion molecules and cell migration-related genes might also contribute to the phenotypes induced by Dex treatment. This study explored the causes of the abnormal generation of chick CNC, the progenitor cells for cranial osteogenesis, in the presence of Dex treatment. Although, further work to describe the precise molecular biological mechanisms involved is required.

SUPPLEMENTARY DATA

Supplementary Data: The sets of primers used for q-PCR in this study.

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CONFLICT OF INTEREST

The authors declare no conflict of interests.

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FIGURE LEGENDS

FIG. 1. Dex exposure suppresses craniofacial skeleton development of chick embryos.

18-hour pre-incubated chick embryos were exposed to 100 μ L either Dex (10^{-6} M) or DMSO

(control) *in vivo* for 13 days. (A-C) Representative head lateral views of the control (A) and 10^{-6} M Dex-treated (B-moderate deformity; C-severe deformity) chick embryos. (D-F) Representative head front views of the control and 10^{-6} M Dex-treated (E-moderate deformity; F-severe deformity) chick embryos. (G-I) Inferior (palatal) view after lower beak removed of the control (G) and 10^{-6} M Dex-treated (H-moderate deformity; I-severe deformity) embryos, stained with alizarin red. (G'-I') High magnification images were taken from the sites indicated by dotted squares in G-I, respectively. (J) Bar chart showing the comparison of embryo survival rates between control and Dex-treated groups. (K) Bar chart showing the comparison of embryo weights between control and Dex-treated groups. (L) Bar chart showing the frequency of deformity between control and Dex-treated groups. Abbreviation: j, jugal; mx, maxillary bone; p, palatine bone; pp, palatine process of maxillary bone; pnc, prenasal cartilage; pmx, premaxilla; pt, pterygoid and qj, quadratojugal. White arrows in A and D indicated the coracoids. Black arrows in G'-I' showed the normal, impaired and absent maxillary bone respectively. Scale bars = 0.5 mm in A-F and 1 mm in G-I, G'-I'.

FIG. 2. Decreased growth of chick embryos occurs in presence of Dex. Chick embryos were exposed to DMSO or 10^{-6} M Dex for 18, 28 and 42 hours in EC culture. Ten embryos were assigned to each treatment group. (A-D) Representative bright-field images of chick embryos in the control group at 0h (A), 18h (B), 28h (C) and 42h (D). (E-H) Representative bright-field images of chick embryos in the Dex-treated group at 0h (E), 18h (F), 28h (G) and 42h (H). (I) Bar chart showing the comparison of the embryo survival rates between control and Dex-treated groups. (J) Bar chart showing the comparison of embryo maturation rates at

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different developmental time-points between control and Dex-treated groups. (K) Bar chart showing the comparison of embryo lengths (longitudinal axis) between control and Dex-treated groups developed to desired stage of HH10. Scale bars = 0.5 mm in A-H.

FIG. 3. CNC production of chick embryos is suppressed by Dex exposure. Chick embryos were exposed to DMSO or 10^{-6} M Dex until HH10 and then immunofluorescently stained for HNK-1, PAX7 and AP-2 α expression. (A-B) Embryo head images of bright-field (A) and HNK-1 fluorescent staining (B) in the control group. (B1-B2) Transverse sections produced from B as indicated by the dotted line. Sections were counterstained with DAPI (B2). (C-D) Embryo head images of bright-field (C) and HNK-1 fluorescent staining (D) in the Dex-treated group. (D1-D2) Transverse sections produced from D as indicated by the dotted line. Sections were counterstained with DAPI (D2). (E-F) Embryo head images of bright-field (E) and PAX7 fluorescent staining (F) in the control group. (F1-F2) Transverse sections produced from F as indicated by the dotted line. Sections were counterstained with DAPI (F2). (G-H) Embryo head images of bright-field (G) and PAX7 fluorescent staining (H) in the Dex-treated group. (H1-H2) Transverse sections produced from H as indicated by the dotted line. Sections were counterstained with DAPI (H2). (I-J) Embryo head images of bright-field (I) and AP-2 α fluorescent staining (J) in control group. (J1-J2) Transverse sections produced from J as indicated by the dotted line. Sections were counterstained with DAPI (J2). (K-L) Embryo head images of bright-field (K) and AP-2 α fluorescent staining (L) in Dex-treated group. (L1-L2) Transverse sections produced from L as indicated by the dotted line. Sections were counterstained with DAPI (L2). (M) Bar chart showing the comparisons

of HNK-1⁺ areas in the traverse sections between the control and Dex-treated embryos. (N-O) PAX7⁺ (N) and AP-2α⁺ (O) cell numbers in the traverse sections between the control and Dex-treated embryos. Scale bars = 100 μm in A-L and 50 μm in B1-B2, D1-D2, F1-F2, H1-H2, J1-J2, L1-L2.

FIG. 4. Dex treatment promotes apoptosis of chick CNC. (A-D) Control (A-B) and Dex-treated (C-D) embryos were double immunofluorescently stained for pHIS3 (red) and PAX7 (green). A and C are bright-field while B and D are fluorescent images. (E-H) Control (E-F) and Dex-treated (G-H) embryos were double immunofluorescently stained for AP-2α (red) and c-Caspase3 (green). E and G are bright-field while F and H are fluorescent images. (B1, D1, F1, H1) The transverse sections were taken from the levels indicated by dotted lines in B, D, F and H respectively. (B2, D2, F2, H2) The B1, D1, F1 and H1 images merged with DAPI staining respectively. (B3, D3, F3, H3) High magnification images were taken from the sites indicated by dotted squares in B2, D2, F2 and H2 respectively. (I) Bar chart showing the comparison of the average cell numbers of PAX7 and pHIS3 double positive cells per section in control and Dex-treated embryos. (J) Bar chart showing the comparison of the average cell numbers of AP-2α and c-Caspase3 double positive cells per section in control and Dex-treated embryos. (K) Bar chart showing the comparison of the percentage of PAX7 and pHIS3 double positive cells to the PAX7 positive cells per section in control and Dex-treated embryos. (L) Bar chart showing the comparison of the percentage of AP-2α and c-Caspase3 double positive cells to the PAX7 positive cells per section in control and Dex-treated embryos. White arrows in B3, D3 and F3 and H3 indicated the PAX7 and pHIS3 double

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positive cells, AP-2 α and c-Caspase3 double positive cells respectively. Scale bars = 100 μ m in A-H and 50 μ m in B1-B3, D1-D3, F1-F3, H1-H3.

FIG. 5. Dex treatment restricts FGF signaling in the cranial neural tubes of chick embryos, and HNK-1⁺ CNCCs migration is suppressed by SU5402. (A-C) *In situ* hybridization was performed to detect *Fgf8* expression in chick embryos, which were exposed to DMSO (control) (A) or Dex (C). (B, D) High magnification images were from the sites indicated by dotted squares in A and C, respectively. (B1, D1) Transverse sections of the stained embryos at levels indicated by dotted lines in B and D, respectively. (B2, D2) High magnification images from the sites indicated by dotted squares in B1 and D1, respectively. (E) q-PCR showing extent of *Fgf8*, *Fgf10*, *Fgfr1*, *Fgfr2*, *Fgfr3*, *Sox2* and *Sox3* expression in control and Dex-treated embryos. (F-G) Representative images of HNK-1 immunofluorescent staining in the cranial regions of SU5402-treated half-side chick embryos. F demonstrates bright-field image and G shows fluorescent image. (G1-G2) Transverse sections of the stained embryos were taken at the level indicated by dotted lines in G. G1 is the control side while G2 is the SU5402-treated side of the embryo. (H) Bar chart showing the comparison of the area of HNK-1⁺ staining in the control and SU5402-treated whole-mount embryos. (I) Bar chart showing the comparison of the area of HNK-1⁺ staining in the transverse sections of control and SU5402-treated embryos. Scale bars = 500 μ m in A, C; 100 μ m in B-B1, D-D1, F-G and 50 μ m in B2, D2, G1-G2.

FIG. 6. Dex treatment represses CNC production and migration in cranial neural tubes

explants. (A-B) Representative bright-field images of control (A) and Dex-treated (B) cranial neural tube explants cultured for 48 hours. (A1-B1) Higher magnification of bright-field image showing cells migrating out of the explants as indicated by dotted squares in control (A1) and Dex-treated group (B1). (A2-A3, B2-B3) Higher magnification of HNK-1 fluorescent staining (A2, B2) and merged with DAPI (A3, B3) as indicated by dotted squares in in control and Dex-treated group. (C) Bar chart demonstrates the comparison of the ratio between area of HNK-1⁺ staining and total area in control and Dex-treated cranial neural tube explants. (D-E) Representative images of the scratch-wound assay in HEK293 cells at 0h incubation from the control (D) and Dex-treated (E). (D1-D3, E1-E3) Representative images of the scratch-wound assay in HEK293 cells at 24h (D1, E1), 36h (D2, E2) and 48h (D3, E3) incubation from the control and Dex-treated groups. (F) Bar charts showing the migratory distance of HEK293 cells along with incubation time in the presence or absence of Dex. Scale bars = 500 μm in A-B, D-D3, E-E3 and 100 μm in A1-A3, B1-B3.

FIG. 7. Dex treatment inhibits EMT-related gene expressions of CNC. (A, C) *In situ* hybridization showing the extent of *Msx1* expression in the cranial regions of control (A) and Dex-treated (C) embryos, respectively. (B, D) High magnification images were taken from the sites indicated by the dotted squares in A and C respectively. (B1-B2, D1-D2) Transverse sections (B1, D1) and higher magnification (B2, D2) images were taken from the levels indicated by dotted lines in B, D respectively. (E) q-PCR data showing the extent of *Msx1*, *Bmp4*, *Slug*, *RhoB*, *Cadherin-6B*, *Laminin β 2* and *N-Cadherin* mRNA expression in control and Dex-treated embryos. Scale bars =500 μm in A, C; 100 μm in B, D; 50 μm in B1-B1 and

D1-D2.

FIG. 8. Model depicting the hypothesis for Dex treatment-induced dysplasia of CNC.