ELUCIDATING THE ROLE OF CYCLOOXYGENASE-2 IN THE MORPHOGENESIS OF CRANIOFACIAL STRUCTURES IN DOMESTIC CHICK

[SYNOPSIS OF THE Ph.D. THESIS]

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INTRODUCTION

Facial development is one of the most complicated yet remarkable embryonic events. It is primarily derived from seven outgrowths or prominences, namely single frontonasal processes (FNP) and lateral nasal, maxillary and mandibular prominences in pairs, which are the distinguishing facial features (Cordero *et al.*, 2011). FNP leads to the formation of the midface, which includes the forehead, a ridge of the nose and the primary palate, while parts of the nose develop from lateral nasal prominence, along with a significant contribution from maxillary and mandibular processes to construct the operative mouthparts (Szabo-Rogers *et al.*, 2010). The construction of different prominences involves various cell clusters that contribute to craniofacial development. Cranial neural crest (CNC) cells and cranial mesoderm play a pivotal role in craniofacial patterning (Trainor and Krumlauf, 2001; Tapadia *et al.*, 2005; Rinon *et al.*, 2007).

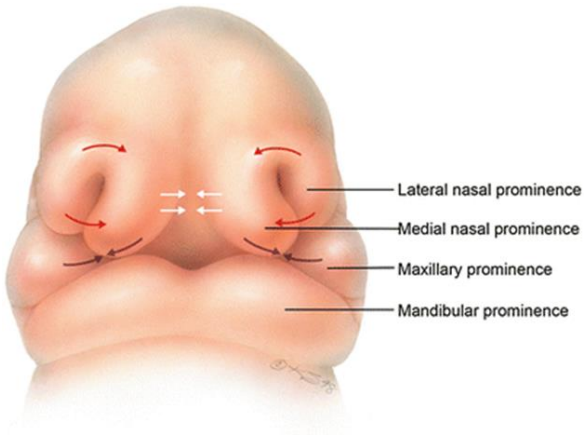

Figure 1. Facial prominences of six-week old mouse embryo

During the neurulation in the embryo, cells from the dorsal ectodermal ridge of the neural tube start delaminating. These neural crest cells are highly proliferative and undergo epithelial-mesenchymal transition (EMT) (Cousin, 2017). Neural crest (NC) cells have four basic types - Cranial neural crest cells(CNCs), cardiac neural crest cells, visceral neural crest cells and

trunk neural crest cells (Basch *et al.*, 2006). Cranial neural crest cells derived from cranial paraxial mesoderm (CPM) and neural ectoderm, eventually differentiate into various cell types to form neurons, facial skeleton, glial cells and pigment cells (Le Douarin and Kalcheim, 1999; Martik and Bronner, 2017).

Moreover, for the successful sculpting of facial structures, the event of EMT in CNCs plays a directorial role. This in turn is governed by multiple paracrine factors like BMPs, Wnts, FGFs, Slug and RhoB (Prasad *et al.*, 2019). Wherein, neuroectodermal cells (ectodermal plate) constitutively express Wnt1/3a, BMP4 and FGF8 (Patthey *et al.*, 2009), leading to the expression of Slug gene in the neural tube formation. Slug performs two functions here; on one hand it inhibits the E-cadherin and on the other hand activates RhoB and Rac1. These cytoskeleton modifying proteins modulate the motility of delaminated cells and allow them to migrate (Mayor and Carmona-Fontaine, 2010).

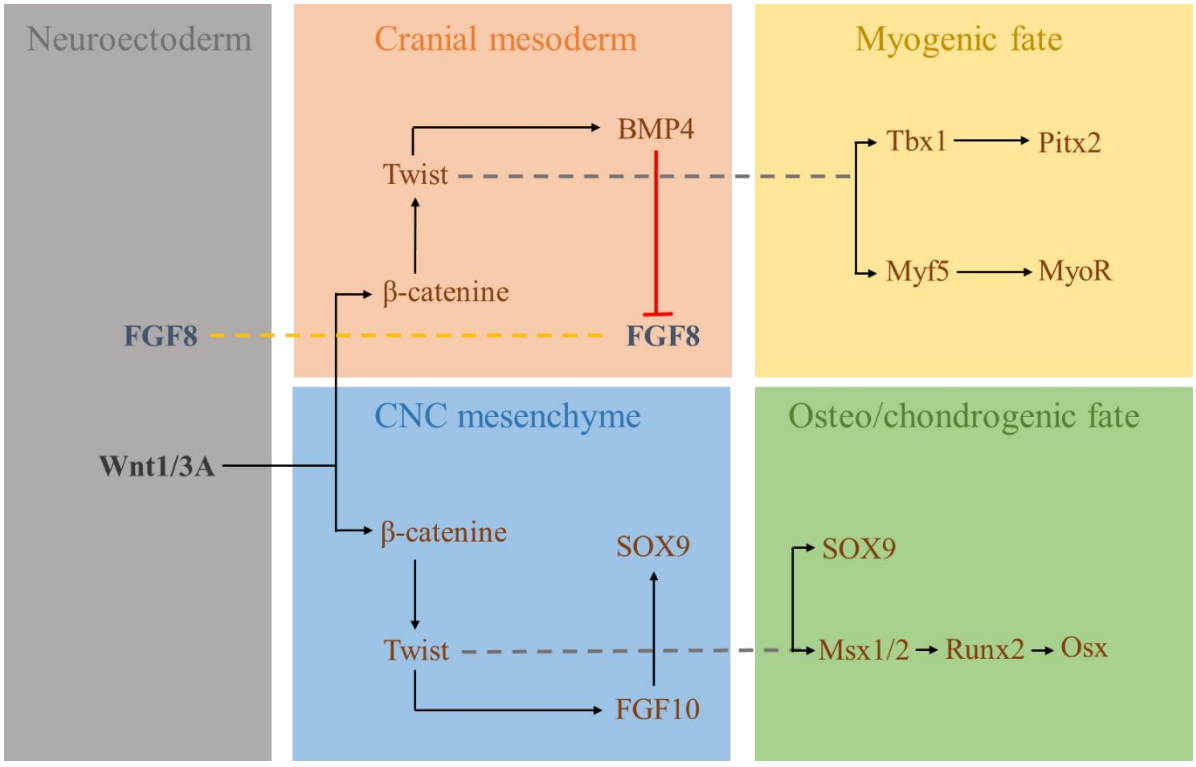

Figure 2. Regulatory pathway of neural crest cell induction and differentiation (Xiaochen Fan et al., 2016).

Additionally, the pluripotent nature of the CNCs sustained by Sox, Slug, FoxD3 and Msx1 impede further differentiation of cells (Kelsh 2006; Betancur *et al.*, 2010; Tran *et al.*, 2010). Cranial neural crest cells reside in pharyngeal arches where interaction between CNCs,

mesodermal cells and ectodermal cells leads to the formation of skeletal elements, muscle tissues as well as connective tissues.

Simultaneously, expression of Twist and Tbx1 in the pharyngeal mesenchyme is required for proper segregation of CNCs in the pharyngeal arch 1 (PA1) and pharyngeal arch 2 (PA2) (Soo *et al.,* 2002; Vitelli *et al.,* 2002; Evans and Francis-West, 2005). Pharyngeal arches express Shh and various Hox genes, that help the CNCs in craniofacial patterning (Dworkin *et al.,* 2016). Craniofacial muscle formation is also under the regulation of CNCs' interaction with cranial mesoderm (Grammatopoulos *et al.,* 2000; Olsson *et al.,* 2001; Ericsson *et al.,* 2004; Buchmann-Moller *et al.,* 2007).

Parallelly, craniofacial development involves the formation of multiple sensory organs, and one of them is the eye. Vertebrate eye development is complex interaction between neuronal ectoderm and mesoderm. The early patterning of vertebrate eye is regulated by Shh, Pax6, Otx and Vsx genes (Gilbert and Barresi, 2017). Various other regulatory molecules like Twist1, Wnt1, Wnt3A, and FGF8 participate in successful formation of eye.

Any imperfection in the above discussed molecular interaction can lead to a range of craniofacial deformities like microphthalmia, anophthalmia, cleft palate, jaw deformities, malformed or missing teeth and defects in the ossification of facial or cranial bones and facial asymmetries. Craniofacial development is widely studied to reveal the reasons behind such deformities and focusing on the operative signaling cascades in the CNCs.

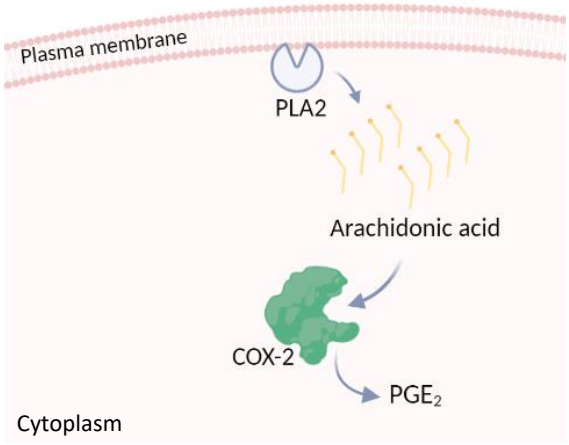

Figure 3. Cyclooxygenase-2 pathway. Membrane phospholipid arachidonic acid is metabolized by cyclooxygenases-2 to prostaglandin E_2 via a short-lived intermediate prostaglandin H_2 (Ricciotti *et al.*, 2011).

Previous studies from the lab and an extensive literature survey elucidated the role of Cyclooxygenases (COX) in governing the function of these pivotal signaling cascades (Dinchuk *et al.*,1995; Seta *et al*., 2004; Harris *et al.,* 2005; Buch *et al.,* 2017). COXs convert the cell membrane-derived Arachidonic acid into the precursor of all the operative prostaglandins, PGH_2. This conversion is catalyzed by two major isoforms namely, COX-1 and COX-2. Leading the conversion of PGH_2 subsequently into various prostanoids by the selective action of tissue-specific enzymes as per the requirement on the cellular front (Rouzer and Marnett, 2003; Harris *et al.,* 2005).

Several studies revealed that inhibition of COX-2 during post-embryonic development leads to improper tissue formation and irregular cell differentiation (Evans *et al.,* 1993; Sharma and Suresh, 2008; Buch *et al.*, 2017).

Along with its induced activity during physical insults, COX-2 also plays a vital role in cellular processes like cell migration, proliferation and differentiation (Evans *et al.,* 1993; Wu *et al.,* 2006; Jana *et al.,* 2016). These cellular processes are operative during embryonic and post-embryonic development; hence, one can assume active participation of COX-2 during both the stages. Also, COX2 levels have been found be elevated in metastatic cancers where it induces EMT to allow migration of tumor cells (Chen *et al.*, 2001). A similar kind of the event occurs during NC delamination (Singh *et al.,* 2007). For these reasons, neural crest cells are widely studied, that might help uncover the mechanisms behind the metastasis of cancerous cells. Malignant tumors also show up-regulation of FGFs, FGFR and BMPs along with a hike in levels of COX-2 (Williams *et al.,* 1999). Thus, literature study indicates COX-2 playing an essential role in the EMT process.

ORIGIN OF PROBLEM

Previous studies in the lab have revealed that Cyclooxygenase-2 (COX-2) plays a specific role in orchestrating the early events that regulate the morphogenesis of the regenerating tail in lizards (Buch *et al.,* 2017). Subsequently, it was observed that COX-2, through one of its metabolic intermediates, facilitates the proper sculpting of appendages in chick during embryonic development (Unpublished). However, recently we have noticed that, ablation of

COX-2 activity using a specific pharmacological inhibitor can lead to abnormal patterning of craniofacial features in chick embryos. Therefore, it was thought worth evaluating the role of COX-2 in the successful realization of cranial architecture of domestic chick.

Various animal models are used for studying Craniofacial development. Scientists are widely using *Danio rerio*, *Xenopus laevis* and *Gallus domesticus*. Chick embryo is one of the most preferred animal models since it is phylogenetically and genetically closer to the mammalian system. Moreover, its tissue and organ development is similar to that of mammals and regulatory genes are also identical for a particular type of construction (Brugmann *et al.*, 2015). A significant advantage of using the chick embryo as a model organism is that it shows external development; hence the maternal metabolites do not display any effects. Therefore, the effects of various compounds can be accurately checked. Secondly, the developmental stages are well studied and classified (Hamilton and Hamilton, 1993). Additionally, Chick embryo genome has been sequenced and many of the genes are extensively examined for developmental abnormalities.

HYPOTHESIS

Based on our preliminary observations it is hypothesized that COX-2, through its metabolic product $PGE_{2,}$ regulates the signaling cascades that facilitates the morphogenesis of cranial features like beak and eye.

OVERALL OBJECTIVE

This study was attempted to understand the mechanisms by which COX-2 orchestrates the proper formation of cranial features like beak and eye in domestic chick *Gallus domesticus*.

In order to achieve this, the following parallel studies will be conducted, which can be treated as specific aims.

1. To unearth the role of COX-2 in the migration of cranial neural crest cells during chick embryogenesis.

2. To study the role of COX-2 in the differentiation and patterning of an eye in the domestic chick.

3. To elucidate the mechanisms by which COX-2 expedites the development of beak in chick.

MATERIALS & METHODS

Egg procurement

Fertilised Rode Island Red chicken eggs were obtained from a Government Poultry farm in Vadodara, Gujarat. Eggs were disinfected with betadine and divided into two experimental groups control and treatment. The eggs were incubated in humidified incubator at 37 ±2°C with relative humidity of 60-70% till the stages considered for our study. All the embryos were staged, according to Hamburger and Hamilton (1951).

Dosing of embryos

Eggs from the control group were kept in an incubator after disinfecting, while eggs from the treatment group were candled to localize air sac and marked using a pencil. Thereafter, under a laminar airflow chamber (LAF), 3.5µg/50µl dose of etoricoxib (a COX-2 specific inhibitor) was inserted in the air sac. After dosing, the eggs were kept in the incubator till the desired developmental stage.

Isolation of embryos

The embryos of day1, day2 and day3 were isolated using the filter ring method (Chapman *et al.*, 2001). Isolated embryos were observed under the microscope for identification of HH stages. The morphological defects were noted and captured by using Catcam camera. Mortality was also measured in isolated embryos of control and treatment groups.

COX activity assay

Embryos were collected from both groups on day1, day2 and day3 in 0.1 M Tris-EDTA buffer. Further, 10% tissue homogenate was used for a spectrophotometric kit-based COX assay (Cayman Chemical Co. USA; ID: 760151). The specific activity was calculated in nmol/min/100g tissue weight. The method deployed in this assay utilized the peroxidase activity of COX, wherein the appearance of the oxidized form of N,N,N',N'-tetramethyl-p-phenylenediamine (TMPD) was measured at 590 nm. TMPD was provided as a ready-to-use substrate in the kit, while the sample prepared from the isolated tissues acted as the enzyme cocktail.

PGE_2 estimation

Embryos from both the groups on day1, day2 and day3 were collected and homogenized in autoclaved PBS (1g tissue/4ml PBS). To this homogenate, 15% v/v methanol was added and prostanoids were allowed to dissolve in the alcohol for 1 hour. These homogenates were centrifuged at 4°C and 2000g for 5 min. Consequently, the supernatant was collected for the kit-based estimation assay of PGE_2 (R & D Lab Systems, USA; ID: KGE004B). Herein, the PGE_2 present in the sample competes with the horseradish peroxide (HRP) labeled PGE_2 in a sequential competitive binding immunoassay. The color developed due to the competitive enzyme activity is 450nm. The estimated levels of PGE_2 were calculated in pg/ml.

Gene expression

Total RNA was extracted by the TRIzol method followed by the quantification of total RNA which was done by Qubit assay (Invitrogen, USA). The RNA samples from both groups were used for cDNA synthesis. cDNA of both groups were synthesized using 1µg of RNA by reverse transcriptase PCR. Real-time PCR was performed with Roche single-colored multichannel detection system LC96. The primers of Wnt3a, TGF-β, MSX1, Twist, CDH1, CDH2, Vimentin, PCNA and Caspase3 were used for the same. The amplification reaction mixture consists of 5µl SYBR green, 1µl of specific primer set, 1µl of cDNA and 3µl of nuclease-free

water. The fold change of various molecules among the groups was calculated using Livak and Schmittgen method (Livak and Schmittgen, 2001).

Western blot

Embryos from control and treatment groups on day1, day2 and day3 were collected in lysis buffer along with protease inhibitor and processed at 8000rpm for 20 min. The total protein was estimated using Bradford's method by assaying 10% homogenate (Bradford, 1976). Further, an equal amount of the protein extract was allowed to electrophorese on 12% gel of SDS-PAGE at 100 volts. The gel components were then allowed to be transferred on PVDF membrane via semi-dry western-blot transfer at 100mA for 20min. This membrane was used to develop immunoblots using specific antibodies against the E-cadherin, N-cadherin, Vimentin, FoxD3, PCNA, Sox2 and Cl.caspase3. Herein GAPDH was considered as a loading control for both the groups. The blots were developed using Streptavidin conjugated Alkaline phosphate (ALP) as an enzyme and BCIP-NBT as substrate (Sigma-Aldrich, USA). The bands on the membrane were quantified by ImageJ software.

Whole mount immune-localization

Embryos of day2 and day3 of the control group were collected in NBF (neutral buffer saline) and processed after 2 hours of fixation. The entire embryos were placed on cavity slides and immune localization was performed. Embryos were permeabilized for 45min, followed by antigen retrieval for 20min. After that, embryos were washed with PBST and non-specific sites were blocked by blocking buffer for 1hour at room temperature. The primary antibody of COX-2 was added to the embryo and kept at 4°C overnight, followed by the secondary antibody for 1 hour. The color was developed using Streptavidin conjugated ALP as an enzyme and BCIP-NBT as substrate. The embryos were washed with PBST once the color was produced, images were taken using CatCam software.

RESULTS

COX activity assay

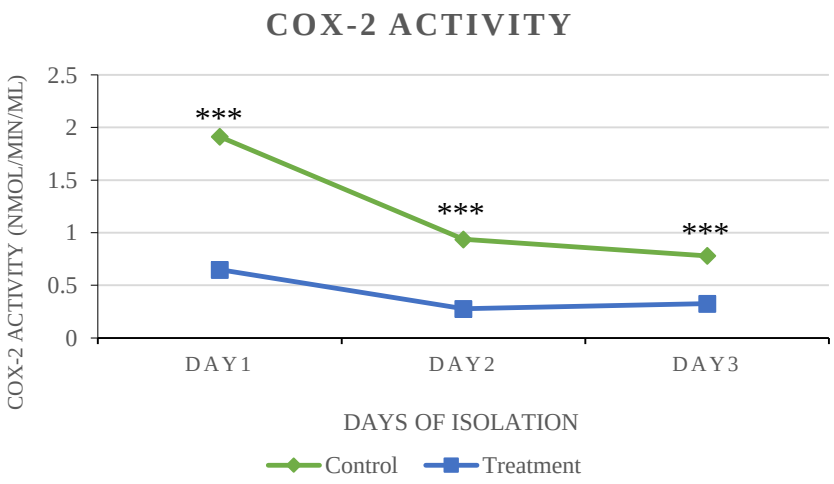

Figure 4. COX-2 activity during early development of chick embryo in control and etoricoxib-treated embryo of day1, day2 and day3; ***p≤0.001.

This study compares COX-2 activity on various days in control and treated embryos (Fig. 4). In etoricoxib treated embryos, COX-2 activity showed a significant decrease during initial patterning of the face compared to a normal embryo of day1. On day2 and day3, etoricoxib treated embryos showed a substantial drop in COX-2 activity compared to control embryos on respective days. This observation points toward the plausible defective patterning of a craniofacial region during early days of development. The comparison drawn here further directs us to check the end product of COX-2 reaction, which is the reason for resultant modulations, namely PGE_2.

PGE_2 estimation

The levels of PGE_2 were compared on the selected days of the study in control and treated groups (Fig. 5). A decrease in PGE_2 levels was observed in day1 etoricoxib treated embryos compared to control embryos.

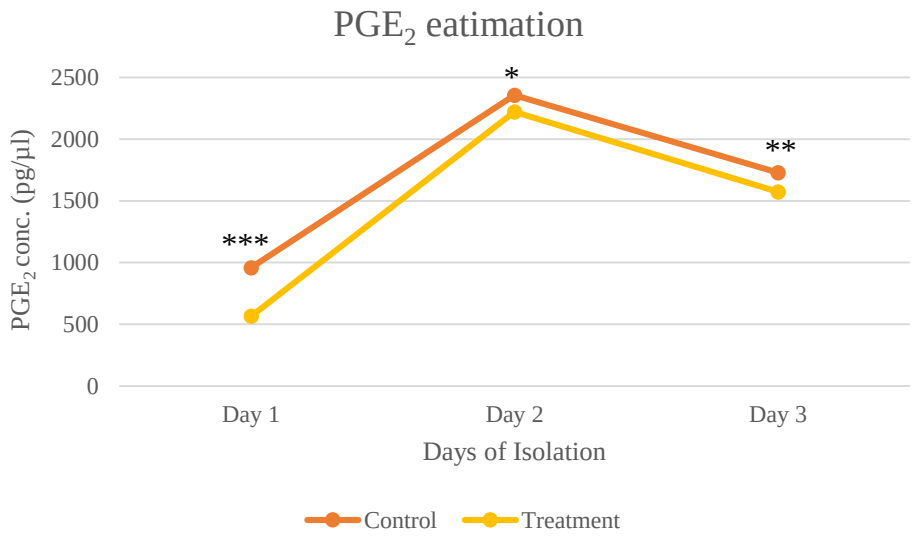

Figure 5. PGE_2 estimation in control and treatment embryos of day1, day2 and day3; *p≤0.05, **p≤0.01, ***p≤0.001.

Interestingly, the level of PGE_2 hiked in day2 control embryos compared to the control embryo of day1. Whereas in case of day2, etoricoxib treated embryos have low levels of PGE_2 as compared to control. Similarly, a reduction was observed in PGE_2 level on the day3 etoricoxib treated embryos compared to the control of the same day.

Morphology

Developing embryo of chick having distinct craniofacial features on day wise manner which is clearly visible in the day1, day2 and day3 control embryos. The initial development of normal embryos on day1 shows the closing of neural folds and patterning of the brain initiated at this stage (Fig. 6A). However, etoricoxib treated embryos of day1 showed defective patterning of the brain and neural folds were unable to close (Fig. 6B-C).

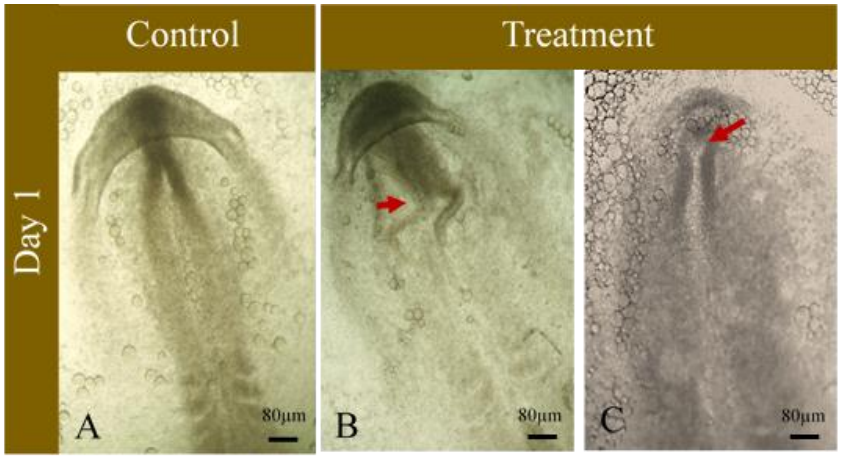

Figure 6. Morphology of control and etoricoxib treated embryos on day1. (A) control embryo of day1, (B) defective neural tube curved at the base showed by red arrowhead, (C) neural tube closure failure, two neural folds are apart from each other showed by red arrowhead.

Further, normal embryo development on day2 has distinct rhombomers, optic vesicles, and heart tube (Fig. 7A). In case of COX-2 inhibited day2 embryos, abnormal brain vesicles and rhombomers formation, poorly developed or complete absence of optic vesicles, and overall length of embryos reduced drastically (Fig. 7B-C).

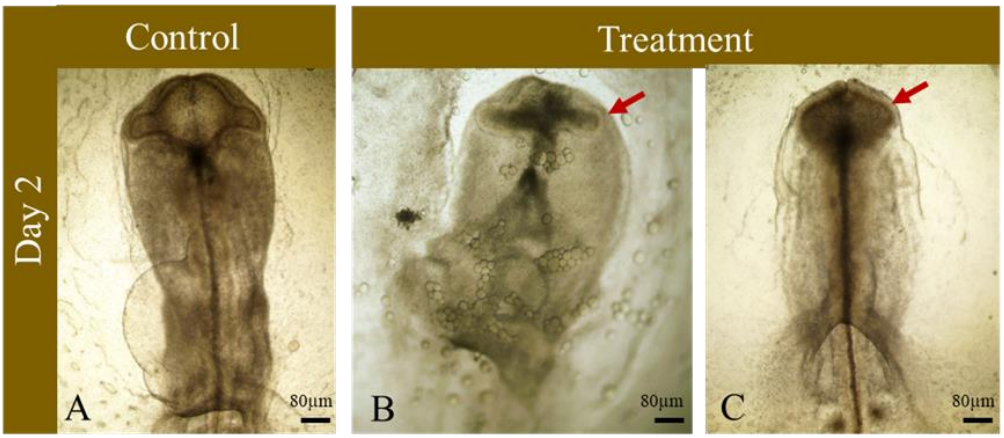

Figure 7. Morphology of day2 control and treated embryos. (A) Day2 control embryo with distinct head regions and optic vesicles, (B) Etoricoxib treated embryo with defective optic vesicles (red arrowhead) and deformed head regions, (C) Poorly developed embryo, absence of optic vesicles (red arrowhead) and head regions.

As the normal embryo develops further, on day3, it shows cervical flexure, optic cup, enlargement of the forebrain region and development of pharyngeal arches (Fig. 8A). On the contrary, the treatment group of embryos has abnormal head formation, absence of optic cup and cervical flexure (Fig. 8B-C). Embryos of day2 and day3 show the loss of symmetry in the craniofacial region under COX-2 inhibition.

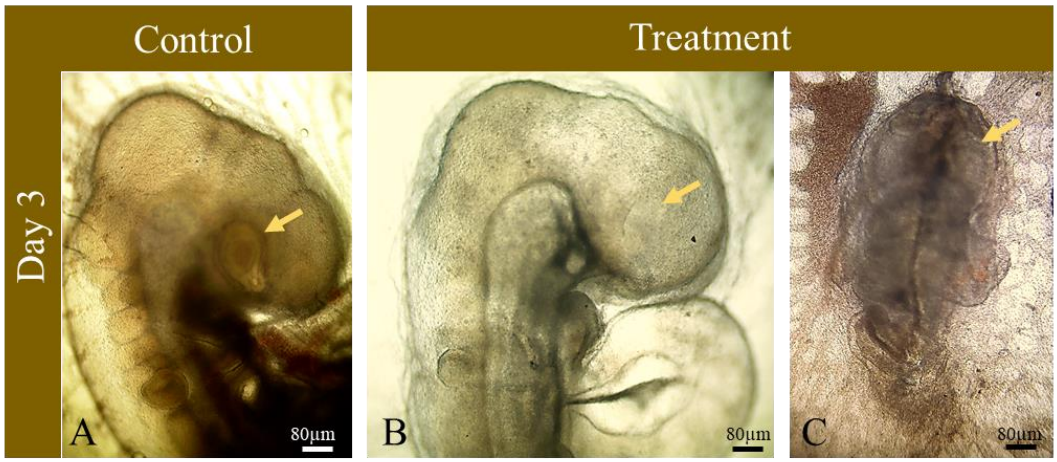

Figure 8. Morphology of day3 control and treated embryo. (A) day3 control embryo with well-developed optic cup and cervical flexure (yellow arrowhead), (B) complete absence of optic cup in treated embryo (yellow arrowhead), (C) symmetry of head region lost and only optic vesicles are formed (yellow arrowhead).

Immunolocalization

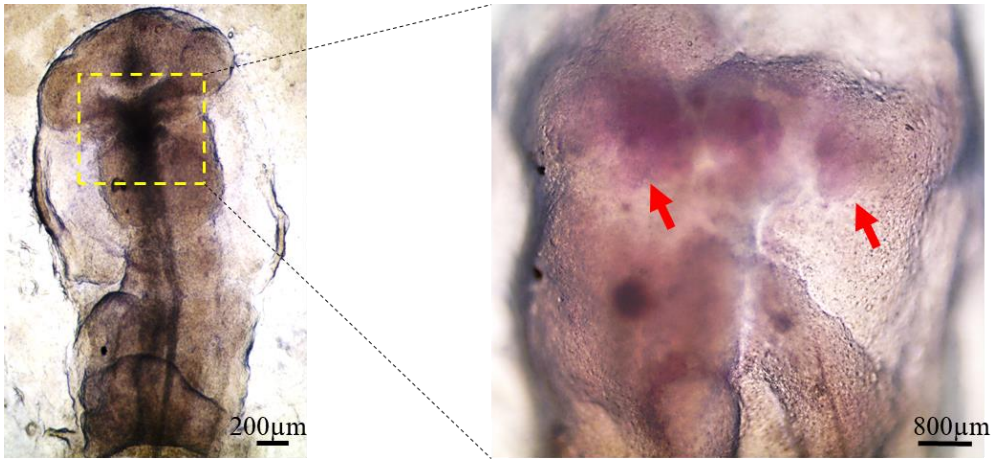

Figure 9. Immunolocalization of COX-2 in day2 control embryo. COX-2 localized at midbrain region showed by red arrowhead.

COX-2 was localized in the control embryos of day2 and day3 and the subsequent microscopic analysis distinctly represented its site of expression. In the day2 embryo, a strong patchy expression at the forebrain region and optic vesicles was observed (Fig. 9). Apart from the head, COX-2 was localized at somites and the outer layer of heart tube.

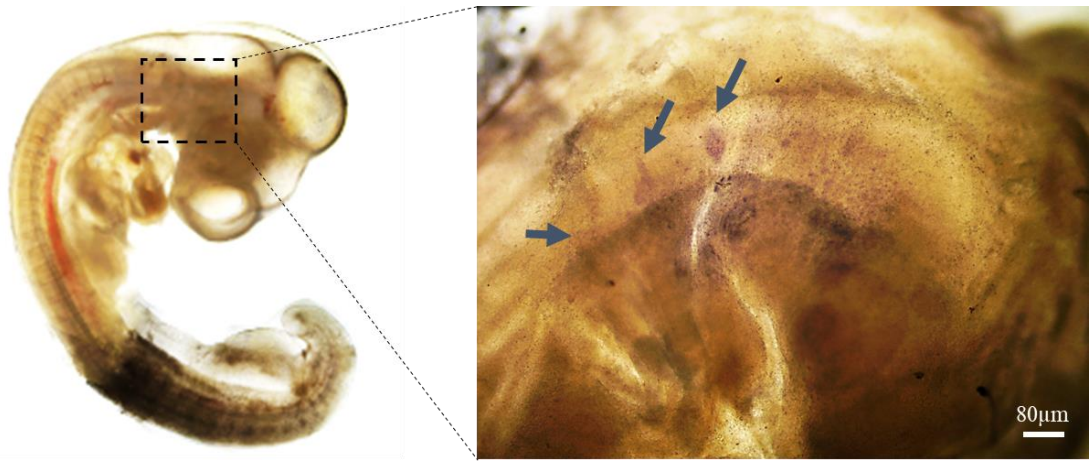

Figure 10. Whole mount immunolocalization of COX-2 in day3 control embryo. Phalangeal arches have expression of COX-2 showed by blue arrowhead.

The intensity of COX-2 expression faints marginally and becomes more specific to the organ as the embryo grows. The hindbrain region and pharyngeal arches had high expression of COX-2 in day3 control embryos (Fig. 10).

Western blot

Protein expression status was checked for the major regulators of craniofacial patterning in both control and treated groups of embryo to understand the molecular regulators under COX-2. Western blot results showed imminent alteration in the levels of various mediators in etoricoxib treated embryos (Fig. 11). E-cadherin level in etoricoxib treated embryos increased as compared to control on day1. A similar trend was observed for E-cadherin in day2 and day3 etoricoxib treated embryos. Alongside, protein expression of N-cadherin and PCNA was checked, which showed gradual decline in day2 and day3 etoricoxib treated embryos compared to control.

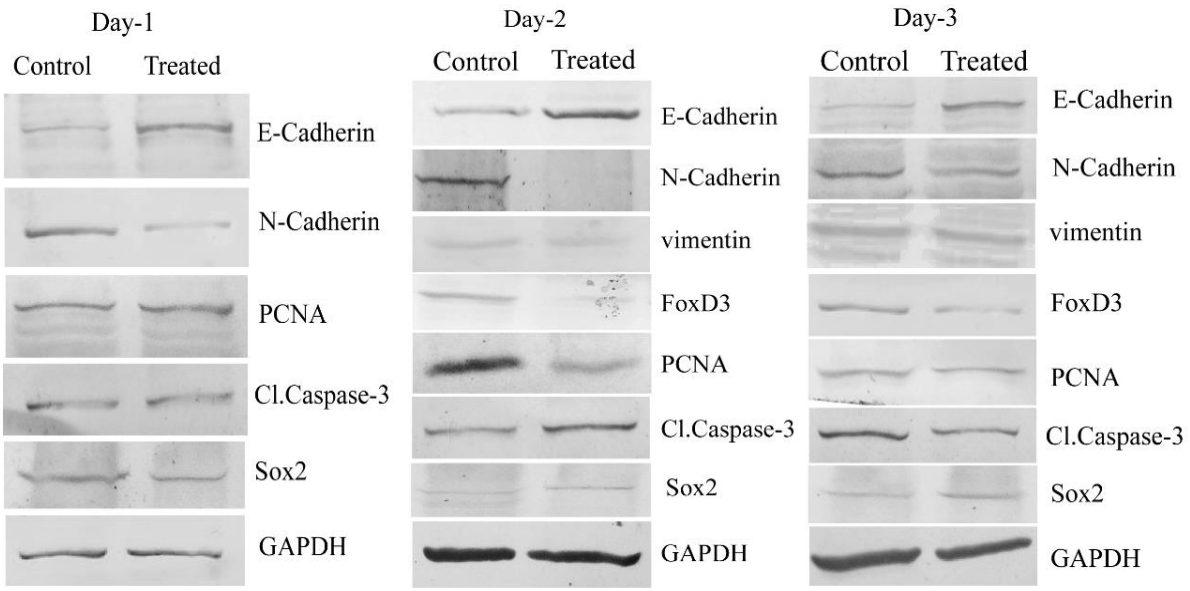

Figure 11. Western blot of E-cadherin, N-cadherin, vimentin, PCNA, FoxD3, Cl.caspase3 and Sox2 for day1, day2 and day3 in control and treated group of embryo.

Further, Vimentin, FoxD3 and SOX2 indicated low expression levels in day1, day2 and day3 COX-2 inhibited embryos (Fig. 11). The levels of cleaved caspase3 increased in day1, day2 and day3 embryos under COX-2 inhibition.

Quantitative Real-time PCR

The expression pattern of the various regulatory molecules of craniofacial patterning was validated through quantitative real-time PCR. These molecules were majorly considered for their distinct roles in forming different structures in the craniofacial region. All three days of developing embryos showed significant alterations in the expression of these molecules under inhibition of COX-2. Wnt3a showed upregulation during neural crest cell formation in normal developing embryo of day1, which was downregulated upon COX-2 inhibition at the same stage. TGF-β is also active in neural crest cell formation; however, its transcript showed reduction upon etoricoxib treatment in day1 embryos.

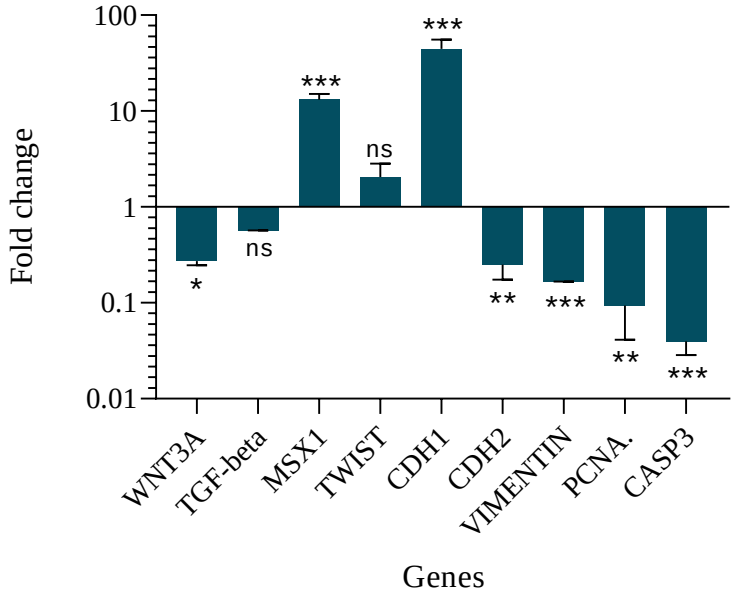

Figure 12. mRNA expression pattern of the genes involved in the regulation of neural crest cell migration in etoricoxib-treated embryo. Values are expressed as fold change (mean ±SEM). Fold change values are compared with the control embryo for all genes (ns: non-significant, *: $p \leq 0.05$, **: $p \leq 0.01$, ***: $p \leq 0.001$).

Further, cadherin levels, especially CDH1(E-cadherin) expression, increased ten folds, whereas CDH2(N-cadherin) expression declined two folds under the etoricoxib treatment compared to the control embryos of day1. Along with cadherins, transcript level of Vimentin significantly decreased when the activity of COX-2 was inhibited in day1 embryos. MSX1 and Twist function as survival factors for neural crest cells, which showed elevated expression in day1 treated embryos compared to control embryos. The cell number is majorly regulated by proliferation and apoptosis during embryonic development. Here we measured the marker of proliferation PCNA and apoptosis Cl.caspase3 in day1 control and treated embryos, which showed a decline in transcripts of both the genes under COX-2 inhibition (Fig. 12).

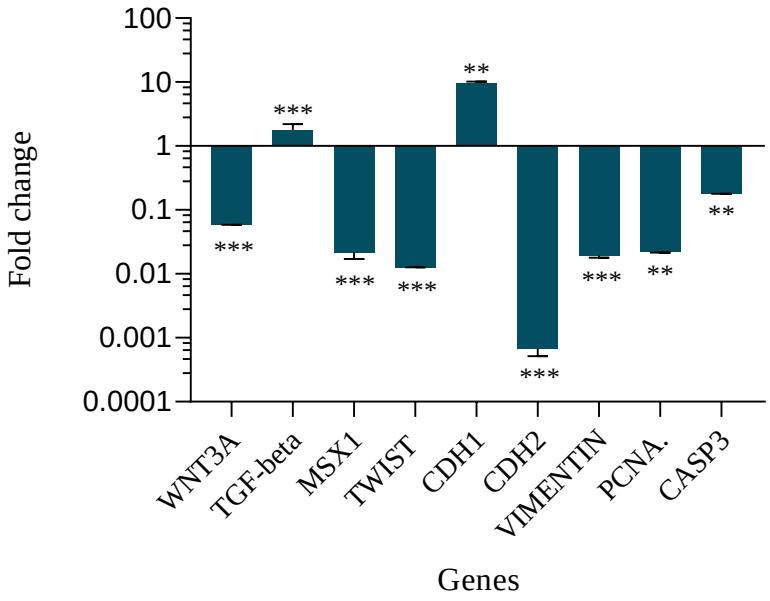

Figure 13. Transcript-level expression of genes involved in the migration of neural crest cells in etoricoxib-treated Day-2 embryo. Values are expressed as fold change (mean ± SEM). Fold change values of treated embryo are compared with respective controls for all the genes (**: $p \leq 0.01$, ***: $p \leq 0.001$).

Similarly, the day2 embryos of the control and treatment groups were analysed for gene expression studies which showed the level of Wnt3a decreased under etoricoxib treatment. The treated embryos of day2 showed a low level of TGF-β and Msx1 transcripts compared to control embryos. On the other hand, CDH1 transcripts showed 12 folds rise under the exposure of COX-2 inhibitor, whereas CDH2 again declined 15 folds under the same condition in day2 embryo. As observed earlier, Vimentin and PCNA mRNA levels decreased significantly (Fig. 13) in the treatment group embryos compared to control embryos. However, the apoptotic marker Cl.caspase3 showed three-folds hike in expression when treated with etoricoxib (Fig. 13).

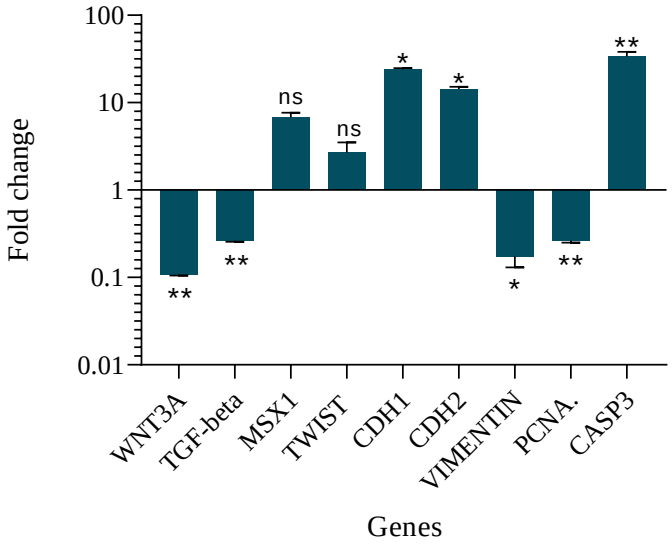

Figure 14. Transcript level of genes regulating head formation and neural crest cell migration in the etoricoxib-treated Day-3 embryo. Values are expressed as fold change (mean ± SEM). Fold change values of treated embryo are compared with respective controls for all the genes (ns: non-significant, *: $p \leq 0.05$, **: $p \leq 0.01$).

Interestingly on day3, transcript levels of MSX1, Twist and CDH2 marginally increased in the treatment group of the embryo as compared to control embryos. The primary regulator Wnt3a and TGF-β showed a constant decrease when treated with etoricoxib on day3. However, CDH1 showed increased expression throughout all three days of development under etoricoxib treatment (Fig. 14). This hints towards the altered morphology observed in etoricoxib treated embryos. Etoricoxib treatment decreased the transcript levels of Vimentin and PCNA in day3 embryo. Further, the Cl.caspase3 transcripts showed a steady rise when COX-2 activity was inhibited in day3 embryos.

Thus, the altered transcript levels of craniofacial patterning indicate COX-2 interacts with Wnt3a, CDH1, CDH2, twist, PCNA and Cl.caspase3 during early embryonic development.

DISCUSSION

COX-2 in neural crest cell formation and survival

Neural crest cells are major contributors to the craniofacial patterning of developing vertebrate embryos (Tapadia *et al.,* 2005). Neural crest cells known to arise during neural tube closure. The process of neural crest cell formation and their migration to different regions of the developing embryo is regulated by various signalling molecules such as Wnts, BMPs, TGF-β and FGFs (Patthey *et al.,* 2009). Here we are trying to understand the role of one such molecule COX-2, which interacts with multiple pathways regulating cellular events such as proliferation, migration, differentiation, and apoptosis. Previous studies in the lab have shown the interaction of COX-2 with Wnt/β-catenin during post embryonic development of the lizard tail (Buch *et al.,* 2017). Further, COX-2 was widely studied in gastric cancer where it effects cellular events such as migration and proliferation by interacting with Wnt3a, TGF-β. (Nuñez *et al.,* 2011; Greenhough *et al.,* 2009). The maximum amount of cellular processes such as cell proliferation, migration, differentiation and apoptosis occurs at embryonic development. A recent study showed the presence of COX-2 during the early development of chick embryo (Verma *et al.,* 2021). To understand the exact role of COX-2 in craniofacial patterning, COX-2 was inhibited by etoricoxib a COX-2 specific competitive inhibitor. Our study in domestic chick embryo during day1, day2 and day3 shows that COX-2 contributes to the morphogenesis of neural crest cells.

During day1 the neural folds fuse and from the apical region of the neural tube from which neural crest cell starts emerging out under Wnt and TGF-β signalling (García-Castro *et al.,* 2002; Kalkan *et al.,* 2009). Wnt3a and BMP7 are expressed on the ridge of the neural folds. Wnt3a by β-catenine signalling activates cytoskeletal changes and maintain the population of neural crest cells (Steventon *et al.,* 2005). However, in our study, when COX-2 was inhibited, the neural folds failed to close or undergo improper closure of the neural tube, affecting the neural crest cell formation. The decreased activity of COX-2 affects the Wnt3a transcript levels, which further alters the downstream molecules such as MSX1 that function as a survival

factor for newly formed neural crest cells (Ishii *et al.,* 2005). With the inhibition of COX-2, levels of MSX1 decrease along with that SOX2, a pre-migratory neural crest cell marker also declines, which indicates that NCC do not survive when they are formed. Reports show that Wnt3a regulates the expression of Twist, a transcription which further leads to downstream gene express of CDH1 and CDH2 in pre-migratory neural crest cells (Taneyhill, 2008). Delamination of the neural crest cells is majorly due to decreased cell adhesion molecules like E-cadherin (CDH1) and an increase in N-cadherin (CDH2). These changes are vital for epithelial-mesenchymal transition. As per our observations, levels of CDH1 remains high and CDH2 decreases, indicating neural plate border cells cannot form neural crest cells by following the Wnt3a signalling.

Overall, the COX-2 interacts with Wnt3a and TGF-β signalling, which plays an essential role in formation of NCC by regulating the downstream molecules such as MSX1, Twist, CDH1 and CDH2.

COX-2 in migration of neural crest cells

Once neural crest cells delaminate from the neural tube, they migrate to various parts of the developing embryo. The migration of neural crest cells is regulated by multiple signalling molecules such as Wnt, TGF-β, FGF, etc. Wnt3a governs one such pathway along with TGF-β, which have roles in the delamination of the neural crest (Le Douarin *et al.,* 2003; De Calisto *et al.,* 2005). MSX1 and Twist are required for the survival of neural crest cells while migrating to different regions of the developing embryo (Ishii *et al.,* 2005; Steventon *et al.,* 2005). Further, Sox2, FoxD3 and PCNA are essential for the proliferation of the migrating neural crest cells and also maintain their pluripotency (Shimotake *et al.,* 1995; Wakamatsu *et al.,* 2004; Stewart *et al.,* 2006). To know the contribution of COX-2 in neural crest cell migration, transcript levels of above mentioned molecules were measured under the inhibition of the COX-2 in day2 embryo. COX-2 inhibited day2 and day3 embryos showed morphological defects such as defective optic vesicle development and malformed head region, indicating molecular glitch when COX-2 is not active. Transcript levels of Wnt3a and TGF-β were decreased significantly, altering the downstream molecules. Further, decrease in levels of CDH2 and Vimentin indicates neural crest cells are still adhered to the neural tube. The levels

of CDH1 remains high in treated group of embryos when compared with the control, indicating neural crest cells retain epithelial characteristics.

With declined levels of Sox2 and FoxD3 in the treated group compared to control embryos, neural crest cells could not survive and their proliferation decreased. Further, in etoricoxib-treated embryos, the population of migrating neural crest cells declined due to the increased level of Cl.caspase3 and lowered level of PCNA. These results vividly express that COX-2 is required for maintaining the neural crest cell population during migration in developing embryo.

CONCLUSION

Present study used COX-2 inhibitor, etoricoxib, to understand the role of COX-2 in craniofacial development. The etoricoxib treated group of embryos has morphological abnormalities in the craniofacial region. Chick embryo, a model for developmental studies, has helped in examining the molecular mechanism responsible for the anomalies observed during early development (day 1, day 2 and day 3) of COX-2 inhibited embryos. The central role of COX-2 appears to be involved in the delamination, maintaining cell population and migration of neural crest cells. By inhibiting COX-2, the correct migration and levels of signalling molecules required for migration and delamination, such as Wnt3a, TGF-β, MSX1, CDH1 and CDH2, are altered significantly. Abnormal signalling can profoundly affect the craniofacial patterning as observed in the present study.

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