6

SUMMARY

Abnormal head shape, cleft palate, and cleft lip are among the most frequently observed congenital disabilities, accounting for approximately one-third of congenital malformation. According to the Centers for Disease Control and Prevention (CDC), craniofacial defects occur in approximately one in every 1600 to 2500 live births (Mossey & Catilla, 2003). Developmental biologists have tried to answer the reason behind congenital craniofacial defects in the past decade.

The cranial neural crest cells are major contributors to vertebrate craniofacial structures. These cells arise during neural tube closure and migrate to various regions of the developing embryo. A population of cranial neural crest cells which migrate to pharyngeal arches are important for facial prominence and followed by face development (Cordero et al., 2011; Sperber, 2006). Mutagenesis study identified zebrafish mutants of COX-2 exhibiting various craniofacial anomalies, and these promise to reveal many new essential functions in the formation of COX-2 in craniofacial structures (Grosser et al., 2002; Pini et al., 2005). Multiple studies using various types of animal models have increased the knowledge of craniofacial development. However, multiple signalling molecules such as Wnts, BMPs, FGFs and transcriptional factors SOX10, MSX1 and MSX2 play important roles in normal craniofacial morphogenesis (Paiva et al., 2010; Nie et al., 2006; Foerst-Potts & Sadler, 1997). Alterations in the expressions of these molecular regulations lead to abnormal craniofacial development. Targeted mutagenesis in animal models such as mice has provided important information regarding single gene defects in craniofacial development.

Apart from these established molecules, there are other facilitators which influence the developmental processes in a developing chick. Here we tried to understand one such molecule, COX-2, an enzyme which was not explored in the developing chick embryo. In the past decade, developmental and evolutionary biologists have realized that human embryogenesis resembles more closely that of birds and rodents than was previously

believed (Johnston & Bronsky, 1995). Despite the apparent differences in adult facial appearance between humans, mice, and chickens, the molecular specification and assembly of embryonic facial features are pretty similar.

Chick embryo serves as an excellent model for the craniofacial development of vertebrates due to its external development. The migration pattern of various cells, including neural crest cells, are similar in chicks and mammals. It gives us freedom of genetic manipulation, and as the development is external, the cellular events can be easily recorded under the microscope. Along with that, molecular signalling is similar to mammalian systems, and single-cell tracking is also possible with different developmental stages conferring a developmental biologist a certain advantage over the mice embryos. COX-2 is an important contributor to placenta and endometrium formation in mammals, the deletion and inhibition of COX-2 lead to no implantation or defective implantation of mice embryos; therefore, COX-2 was not studied much in mice during early developmental processes (Shah & Catt, 2005). Our focus was early craniofacial patterning, where the neural crest cells delaminate, migrate and differentiate, and various tissue folding and thickening occur for facial prominence development. For all the mentioned events, HH10 to HH20 developmental stages of chick embryos were selected for the study.

For decades, COX-2 was known as an inducible isoform of the COX enzyme family responsible for PGE_2 production and induced under certain conditions such as inflammation, hormones, etc. However, a recent study showed that COX-2 is present in a developing embryo devoid of inducing factors (Kain et al., 2014). This research also focused on exploring the downstream effector of COX-2, responsible for the outcome of its inhibition in embryonic development. Previous studies from the lab have shown the presence of COX-2 in various organs such as neural tube, limbs, heart and somites in a developing chick embryo (Verma et al., 2021).

Initially, COX-2 is expressed in the developing neural tube of the embryo. The neural tube gives rise to a particular cell population known as neural crest cells, which are known to express COX-2. The roles performed by the COX-2 in neural crest cells were checked by inhibiting the COX-2 using etoricoxib. The absence of COX-2 activity leads to various array of craniofacial defects, which were investigated in detail at the HH10 to HH20 stages.

The majority of craniofacial defects are related to the symmetry of the forehead region and facial primordia, neural tube closure, pharyngeal arch formation, optic cup development

and defective heart tube. Each defect was studied in detail to understand the participation of COX-2 in the patterning of those tissues.

Initially, when COX-2 was inhibited from the HH1 stage of embryonic development, the neural tube failed to close, and due to that, neural crest cells were unable to delaminate. Few embryos have defective closing of the neural tube, and when those embryos were analysed through sections, it indicated that a very less number of neural crest cells were formed, and they were unable to migrate. These observations made us curious to discover the molecular interactions of COX-2 in the delamination and migration of the neural crest cells. On the molecular side, altered levels of N-cadherin and E-cadherin, along with Vimentin under inhibition of COX-2, affect the neural crest cell delamination from the neural tube. Additionally, COX-2 interacts with TWIST & regulates the expression of neural crest survival factors like MSX1, SOX9 and FOXD3. As the delamination is hampered under COX-2 inhibition, and less number of neural crest cells were generated, and those were unable to migrate to destined places due to a dearth of vital molecules like such as MSX1, SOX9 and FOXD3 during HH10 to HH20 developmental stage. The lower number of neural crest cells leads to defective craniofacial structure formation in a developing embryo (Liu et al., 2020).

Different types of neural crest populations also get affected under COX-2 inhibition, and one such type is the cardiac neural crest cells. Around the HH10 stage, cardiac neural crest cells arise and migrate to the developing heart tube where it participates in the looping of the heart tube (Yelbuz et al., 2002). Inhibition of COX-2 after neural tube closure and neural crest cell formation (HH8) leads to defective heart looping and trabeculation via affecting the cardiac neural crest cells. The expression pattern of GATA family genes got altered in COX-2-inhibited embryos at the HH12 stage, which shows defective looping in the heart tube. The alterations in GATA4, GATA5 and GATA6 affect the cardiac neural crest cell proliferation and differentiation into developing heart tube, which was clearly observed in etoricoxib-treated embryos of the HH12 stage. Further, the heart tube starts differentiation into heart chambers and presumptive ventricle walls start trabeculation by HH20 developmental stage (van den Hoff et al., 2004). At stage HH20, the COX-2 inhibition showed defective trabeculation in ventricles walls in the developing embryo. This observation indicates the active participation of COX-2 in the trabeculation of ventricle walls which is majorly done by cardiac neural crest cells. Inhibition of COX-2 alters the molecular regulators of cardiomyocyte formation such as TBX20, HAND2 and MYOCD

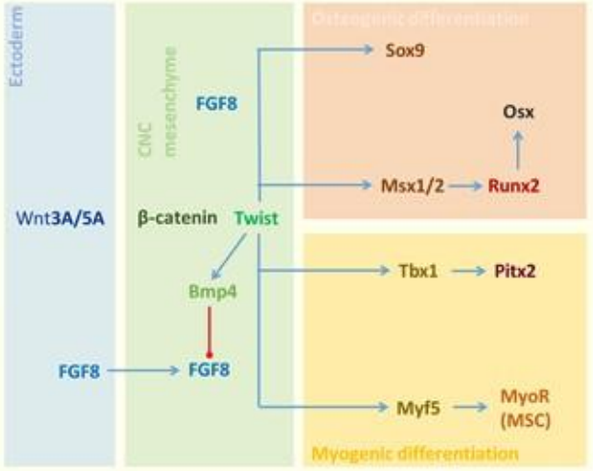
in both (HH12 & HH20) the developmental stages. As a result of COX-2 inhibition, neural crest cell's proliferation as well as the differentiation to cardiomyocytes to form the trabecula in ventricles was hampered to a larger extent. This was further substantiated when the histological section of heart tissue at HH20, showed deviant morphological characteristics as compared to the control embryo's heart tissue.

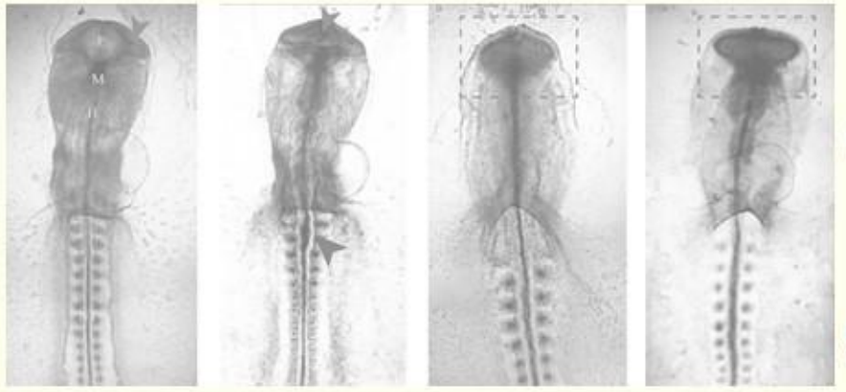
Further, inhibition of COX-2 in a developing embryo has shown a common defect in patterning of the eye, such as a single optic cup, absence of optic vesicles (single or both) and defective lens placode. As the eye is a complex tissue originating from different germ layers, and interaction of those germ layers in a temporal manner leads to a functional eye formation. Three major developmental stages (HH12, HH14 and HH16) were taken into consideration to understand the participation of COX-2 in eye patterning. At the HH12 stage, optic vesicles were formed, followed by the formation of optic cups from optic vesicles at the HH14 stage, and at HH16 optic cup differentiates into retina and lens was also formed. Also, at the HH12 stage, the embryo had a defective optic vesicle compromising the morphological features of the etoricoxib-treated embryos. These defective morphologies of optic vesicles were an outcome of the altered molecular regulation of WNT, SHH and BMP in the absence of COX-2.

As the molecular regulation has altered, the progression of the optic vesicle to the optic cup was delayed in many embryos under inhibition of COX-2. Due to the delay in the optic vesicle formation, defective reciprocal induction between the optic vesicle and surface ectoderm led to defective optic cup formation at the HH14 stage. The major regulators of optic cup patterning, such as FGF8, BMP7, Delta and PAX6, altered their expression pattern under COX-2 inhibition. Further defective optic cups were unable to interact with surface ectoderm and led to the defective retina and lens formation, which was clearly observed at the HH16 stage embryos treated with etoricoxib. The embryos have shown the absence of an optic cup, and many embryos have size reduction in the optic cup along with undifferentiated retina layers under COX-2 inhibition at the HH16 stage. This indicates the participation of COX-2 in optic cup differentiation to retina and lens patterning, which was found out through gene expression analysis and protein levels expression of regulatory molecules such as PAX6, SIX3, VSX2, MITF and OTX2 in both (control and treatment) groups of embryos. The alterations in the molecular regulations in the absence of COX-2 were reflected in the morphology of the developing eye in all three stages of embryonic development.

In conclusion, this entire study gives a fundamental understanding of COX-2 in the organogenesis of craniofacial structures in chick embryo, which resembles many of vertebrates. The attempt of understanding COX-2 expression and interactions in neural crest cells is a novel finding in the field of neural crest biology. An in-depth experiment with inhibition of COX-2 at various developmental stages gave an idea of COX-2 interaction with different types of neural crest cells and their temporal regulation in the patterning of organs. Single inhibition of COX-2 comes up with an array of craniofacial defects that indicates the interaction pattern of COX-2 in a developing embryo. The interactions of COX-2 with multiple signalling molecules occurs based on spatiotemporal variations, which results in COX-2 integrating various cellular events during organogenesis. The promising set of results obtained here adds to the current knowledge of craniofacial development and opens up a new research avenue to explore more regarding craniofacial developmental defects and a cure for the same.

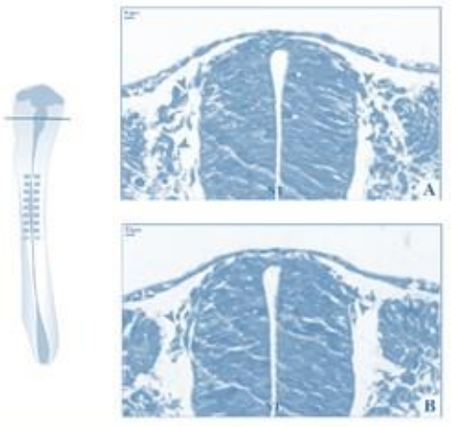
The current piece of work has been shown in a pictorial representation on the next page.

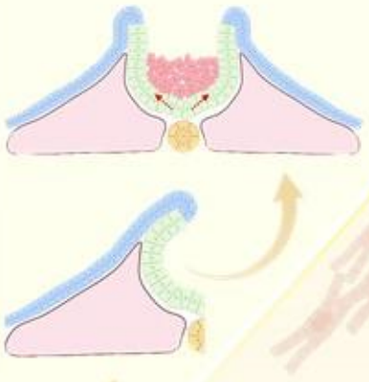




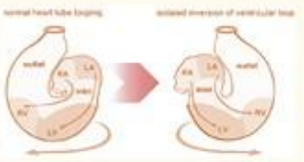
Wnt3A
Etoricoxib
MSX1
FoxD3

TGF-β
Etoricoxib
Twist
N-cadherin
E-cadherin
Vimentin





COX-2
Etoricoxib
WNT11
GATA6
GATA5
GATA4
SHH



Neural crest cells

COX-2
Etoricoxib
BMP4
Cl.Casp3
TBX20
PCNA
MyoCD