1

INTRODUCTION

Craniofacial development has traditionally been a field of research in which many divergent views are expressed, and novel theories are put forward to explain observed normal and abnormal phenomena. Decades of investigation have shown that craniofacial development is an intricate and complex event that requires inductive and directive cell and molecular interactions. The series of events control the initiation, movement, and differentiation of various embryonic cell populations across a spatial and temporal time continuum that results in the correct outgrowth, patterning, and tissue integration required to make a face (Robert, 1993; Schilling, 1997).

Of all the new-born with birth defects, approximately one-third display anomalies of the head and face, including cleft lip or cleft palate, small or absent facial or skull bones, and improperly formed nose, eyes, ears, as well as teeth (Centers for Disease Control & Prevention-CDC, Mai et al., 2010-2014). Occasionally, craniofacial anomalies can cause infant mortality. They have serious lifetime functional aesthetic and social consequences that are devastating to both children and parents alike. The development of the human skull and face is a highly orchestrated and complex three-dimensional morphogenetic process. This involves hundreds of genes controlling the coordinated patterning, proliferation, and differentiation of tissue with multiple embryological origins. Any alteration in the interaction of signaling molecules leads to craniofacial defects in developing embryos (Choe & Crump, 2015; Francis-West & Crespo-Enriquez, 2016).

The journey of any organism from zygote to death, where a single cell will become a fully functional multicellular organism, exhibits aging, which are relatively slow progressive changes, known as development (Gilbert & Barresi, 2018).

The entire development of an organism is divided into two parts: embryonic and post-embryonic development. The phase of an organism between fertilization and birth is known as embryology. Development never ceases after birth, and a multicellular organism is

maintained through growth, regeneration, and aging, known as post-embryonic development (Gilbert & Barresi, 2018).

Post-embryonic development has direct applicability in fields like pharmacology, regenerative medicines, stem cell therapies, and cosmetics. However, embryonic development is more fundamental, where scientists are trying to solve the mystery behind a single cell's journey to a multicellular organism. The embryonic development is more important as it forms the various organs and systems that make an organism sustainable in the post-embryonic period.

Embryonic development

The sole way of developing from egg to adult is through embryonic development for all types of animals. The genotype is translated into phenotype and ensures the embryo develops like its parents.

One of the events of embryonic development is the generation of cellular diversity in a single organism through chemical signals, morphogen gradients, and the microenvironment of cells. A fertile ovum undergoes a series of extremely rapid mitotic divisions known as cleavage that generates blastomeres. These blastomeres change their relative positions and rearrange to form three germ layers: ectoderm, mesoderm, and endoderm (Eakin & Behringer, 2004). Cells from these three germ layers interact to form various tissues, followed by organogenesis. During organ formation, specific cells migrate from their origin to the final location, which is responsible for developing blood, lymph, pigment, and gamete formation (Johnston et al., 1977).

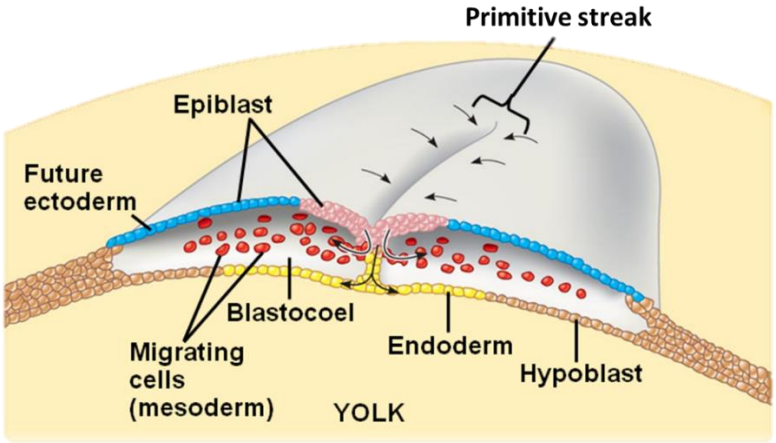

Figure 1.1: Differentiation of three germ layers in a developing embryo of chick (https://quizlet.com/559978056/gastrulation-to-neurulation-flash-cards/)

Organogenesis begins when the most dorsal mesoderm region cells start to condense to form a rod of cells known as a notochord (Fig 1.1). The cells of the notochord produce chemical signals that induce the neural tube formation by folding the ectodermal cells sheet above it (Karfunkel, 1974). On the other hand, Mesodermal cells adjacent to neural tube and notochord become condensed and segmented into somites. Further, during embryonic development, ectoderm initiates folding into a three-dimensional structure due to coordinated cell division, along with neural crest cells helping in the formation of craniofacial features of an organism (Finnell et al., 1998).

Craniofacial development

Craniofacial development is a complex interaction between cranial ectoderm and mesoderm. During this process, mesodermal tissue initially segregates into the prechordal plate, lateral palate mesoderm, and paraxial mesoderm (Trainor & Tam, 1995). Interaction between cranial mesoderm and ectoderm leads to the formation of facial prominences, which further develop into specific structures of the craniofacial region. Five facial prominences are formed, including a central frontonasal prominences (FNP) and paired maxillary and mandibular prominences (Shen et al, 1997; Sperber, 2006).

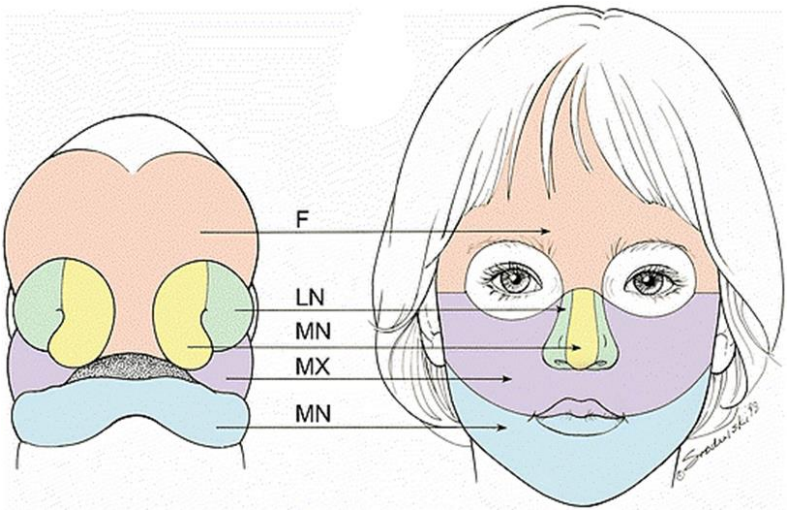

Figure 1.2: Facial prominences and their derived structures in vertebrates (F: frontal, LN: Lateral nasal, MN: Mid nasal, MX: Maxillary, MN: Mandibular) (https://entokey.com/embryology-and-anatomy-of-the-developing-face/#CR10)

FNP establishes the facial midline composed of the forehead, the bridge and tip of the nose (Fig 1.2). Early development of FNP is separated into medial nasal prominence and two lateral nasal prominences by the emergence and invagination of nasal pits (Sperber et al,

2006). Their development is regulated mainly by an interaction between forebrain neuroectoderm, neural crest cells, and facial ectoderm (Diewert et al., 1993). Maxillary prominence gives rise to the upper jaw (maxilla) and lateral parts of the upper lip. It starts growing medially and fuses with other facial prominences to establish seamless upper jaw and lip continuity. Medially, maxillary prominence will fuse to lateral nasal and medial nasal (a subdivision of frontonasal prominence) to form a structure like the nostril and upper lip. Mandibular prominence is a derivative of the first pharyngeal arch that eventually forms the lower lip and lower jaw. It begins as a bilaterally paired structure that grows medially and later fuses to create a seamless lower jaw (Kosaka et al., 1985). Maxillary prominence arises as a branch of branchial arch 1 and connects with mandibular prominences throughout the development. Integration of several complex embryonic processes and fusion of facial prominences is necessary for normal facial and oral cavity development. The palate is a vital part of the oral cavity, serving as the roof of the mouth and playing a role in important functions such as speaking, breathing, and eating. The development of the palate involves the formation of both primary (anterior) and secondary (posterior) palates. The primary palate is formed when facial prominences, such as the frontonasal prominence, fuse with medial nasal and maxillary prominences (Moxham., 2003). Secondary palate develops from the shelves originates from the maxillary processes, which are extensions of the first pharyngeal arch. The palatal shelves are in a vertical position on either side of the tongue, and they elevate and fuse horizontally to form the intact secondary palate (Li et al., 2017). Disruption in timing or positioning at any stage of the fusion process can result in a cleft lip or palate (Chang et al., 2015).

For initiating organogenesis, epithelial-mesenchymal interaction in the craniofacial area is essential. Early orofacial epithelium expresses inductive signals to the underlying mesenchyme or ectomesenchyme (Liu et al., 2010). In the meantime, mesenchyme and epithelium undergo morphogenesis in response to the inducing signal. This gives feedback to the epithelium for further development of facial prominences (Brugmann et al., 2010).

Molecular regulators of craniofacial development

Among vertebrates, gene expression patterns regulating craniofacial patterning are primarily conserved. The neural crest cells are primary contributors of craniofacial patterning. It forms cranial ectomesenchyme which interacts with pharyngeal ectoderm and makes facial prominences (Buchtova et al., 2010). BMPs and FGFs govern the

condensation of neural crest cells that form the cranial mesoderm (Schumacher et al., 2011; Endo et al., 2012). Moreover, *WNT3* directly increases cell proliferation within developing FNP and induces *BMP* expression in FNP neural crest cells (Brugmann et al., 2010). In the frontonasal process (FNP), juxtaposed domains of Sonic hedgehog (*Shh*) and *FGF8* form the frontal ectodermal zone (FEZ). This regulates the growth and polarity of the upper jaw (Hu et al., 2003; Eberhart et al., 2006; Hu & Marcucio, 2009). For example, the Dlx code confers jaw identity so that *Dlx5/6* expression specifies lower jaw identity across vertebrate taxa (Clouthier et al., 1998; Depew et al., 2002). Patterning and polarity of the facial prominences are highly regulated, and several regionally localized signaling centers appear to manage the correct structure formation.

The genetic mechanisms of craniofacial development have begun to be elucidated, with *WNT*, *FGF*, *BMP* and *SHH*, along with many other developmental signal pathways playing critical roles (Hu & Helms, 1999; Nie et al., 2004; Nie et al., 2006; Geetha-Loganathan et al., 2009).

WNT proteins have a range of functions during various developmental processes, such as proliferation, asymmetric division, patterning, and cell fate determination (Veeman et al., 2003; Logan and Nusse, 2004; Gordon and Nusse, 2006; Karner et al., 2006). During craniofacial development *WNT1, WNT2b, WNT3a, WNT4, WNT5a, WNT5b, Wnt9b* and *WNT11* ligands get expressed at different time points and functions through canonical (β-catenin dependent) or non-canonical (β-catenin independent) pathways. The zebrafish larva showed expression of Fzd2 and Fzd6 in the entire cranial region, and it participates in shaping of the head skeleton (Sisson & Topczewski, 2009). WNT2b, WNT3a, and WNT4 ligands are getting expressed ectodermally, whereas WNT5a and WNT5b are limited to mesenchyme (Geetha-Loganathan et al., 2009). WNT antagonist Dkk expression was also observed at the HH17-18 stage in chick embryos. It prevents the WNT ligand interaction with LRPs and expresses in ectodermal and mesenchymal tissues to regulate WNT signaling during patterning (Geetha-Loganathan et al., 2009; Shimomura et al., 2019). The deletion of Dkk1 is predicted to cause an increase in canonical WNT signaling and dramatic loss of the entire facial complex due to its role in the early patterning of the head (Mukhopadhyay et al., 2001). Further, targeted deletion of WNT3a causes death upon birth in mice due to mandibular defects (Louvi et al., 2007). Complete deletion of WNT5a causes truncation of the upper and lower jaw. WNT2b was initially restricted to the ectoderm, dorsal to the eye, that eventually extended ventrally to cover the maxillary prominence

(Person et al., 2010; Medio et al., 2012). In developing chicks at stage 21, *WNT11* transcripts are detectable in maxillary prominences, and two faint bands of expression are also seen in each of the cranial mandibular prominences (Chiquet et al., 2008). Thus, regulating the WNT signaling is essential for several aspects of facial morphology.

During craniofacial patterning, WNT signaling also stays in coordination with other regulatory molecules, such as FGF and BMP. Gene expression of several cell-survival and patterning factors, including *FGF8, FGF3* and *FGF17*, is dramatically diminished in the anterior neural ridge and/or the adjacent frontonasal ectoderm of the β-catenin conditional mutant mice. Wang and co-workers showed that the *FGF8* gene is transcriptionally targeted by WNT/β-catenin signaling during early facial and forebrain development (Wang et al., 2011). BMP and WNT together block the cranial myogenesis, whereas antagonist of WNT and BMP secreted by CNC promotes cranial muscle development in chick embryo (Tzahor et al., 2003).

Fibroblast growth factors (FGFs) are known to regulate cell proliferation and survival through canonical Receptor Tyrosine Kinase (RTK) signaling and regulation of gene expression and are also known to regulate cell–matrix during embryonic and post-embryonic development (Meyer et al., 2012) or cell–cell adhesion (Rasouli et al., 2018; Sun & Stathopoulos, 2018; Kurowski et al., 2019) through other less well-established mechanisms. There are 23 known FGF ligands that function through four types of receptors. All known FGF ligands *FGF2, FGF3, FGF8, FGF9* and *FGF10* express during craniofacial patterning of vertebrate embryo. The FGF expression is distinct in developing craniofacial regions, such as *FGF2* and *FGF4* get expressed in facial ectoderm and regulate differentiation of frontonasal and mandibular mesenchyme (Richman et al., 1997). *FGF8, FGF9* and *FGF10* are intensely expressed at nasal pits, whereas *FGF3, FGF15* and *FGF17* expression is restricted to the nasal pits' medial side (Bachler & Neubüser, 2001). Ectodermally expressed *FGF8* induces homeobox gene expression in ectomesenchyme, which is critical for the structure formation within the facial primordia (Cobourne & Sharpe, 2003). *FGFr1* and *FGFr2* are broadly expressed in the facial primordia (Wilke et al., 1997; Bachler & Neubüser, 2001), and the signal is integrated with Twist for differentiation of primordia (Rice et al., 2000). FGF8 signaling selectively induces the expression of *Pax9* in the posterior region of palatal mesenchyme during primary palate formation in mice. Loss of Pax9 due to altered FGF8 level results in a palatal shelf development defect and cleft palate (Hilliard et al., 2005).

Additionally, later during development, *FGF8* has strong synergistic effects with Shh on chondrogenesis in vitro, and that is sufficient to promote chondrogenesis in chick embryos (Tucker et al., 1999; Abzhanov & Tabin, 2004). FGF10/FGFr2b pathway regulates Shh expression in the palate and epithelial-mesenchymal interaction, which is mitogenic to the palatal mesenchyme, disruption in the pathway resulting in cleft palate in mice embryos (Rice et al., 2004). Furthermore, *FGF10* also appeared to be required for the survival of mesodermal cells and normal expression of *Jagged2* and *Tgfb3* in the palatal epithelia (Alappat et al., 2005). Jagged2 functions as ligand and involved in mediating cell-cell communication between the epithelial and mesenchymal cells of the developing palate (Casey et al., 2006). Along with that TGF-β3 contributes to the proper growth and elevation of the palatal shelves by influencing cellular processes such as proliferation and differentiation (Wattanaroonwong et al., 2000). However, facial muscle development requires signaling through FGFr4 (Michailovici et al., 2015). At this point, inhibition of FGFr4 leads to a dramatic loss of muscles; and arrests the muscle progenitor differentiation (Marics et al., 2002).

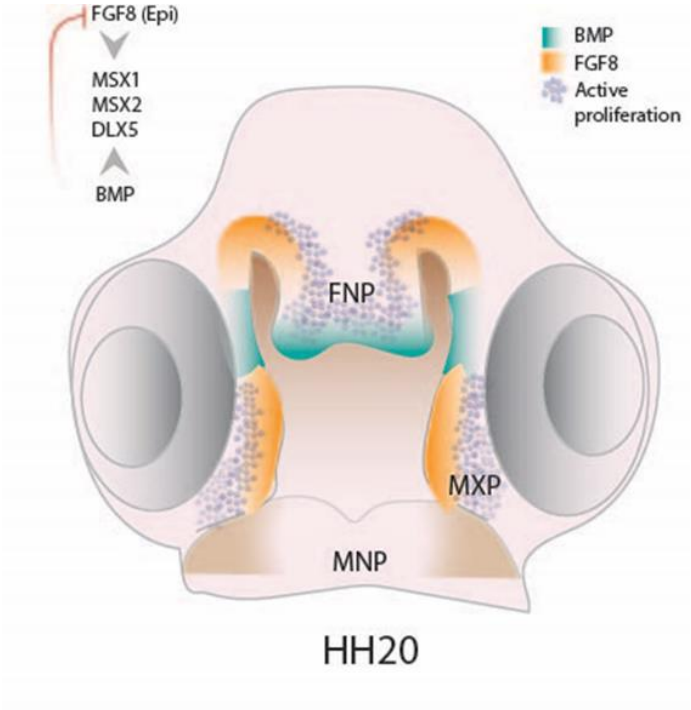

Figure 1.3: Molecular interactions amongst facial prominences at HH20 stage of development in chick embryo. FNP-Frontonasal prominences, MXP-Maxillary prominences, MNP-Mandibular prominences (adapted from Szabo-Rogers et al., 2008).

In chick embryos, the developmental stages are divided into Hamberger Hamilton (HH) stages, also known as HH stages. Craniofacial structures begin developing at HH6 and continue until HH40. Early in facial development, at HH20 stage, when the facial primordia

consist of undifferentiated mesenchymal buds, *BMP4* expression is restricted to the epithelium of the medial region of the mandibular primordia and the anterior part of the maxillary primordia i.e., the most distal tips (Fig 1.3). The epithelial expression of *BMP4*, at HH20 stage is associated with underlying mesenchymal expression of *BMP2*, *Msx1* and *Msx2* in chick (Francis-West et al., 1994, Wall & Hogan, 1995). BMP4 is critical for the fusion of lip and mesenchymal cell proliferation, whereas deficiency of BMP4 results in craniofacial anomalies, such as cleft lip and palate, in mice and humans (Liu et al., 2005; Suzuki et al., 2009).

At HH24 stage, the extended expression of *Msx1* in the mesenchyme of the maxillary primordium is associated with extended expression of Shh in the epithelium. In the early embryonic stage, the mandible develops around Meckel's cartilage, the first pharyngeal arch derivative. Meckel's cartilage is a transient structure providing early structural stability to the mandible prior to the development of the mandibular bone. Bmp2 and Bmp7 are expressed at the early stages of the developing Meckel's cartilage, while Noggin expression is continuous. During the development of the hindbrain, BMP4 induces apoptosis of the neural crest cells migrating from rhombomeres 3 and 5 (Graham et al., 1994). Later during development, Bmp4 regulates proximal-distal patterning as well as the timing of bone differentiation in mandibular mesenchyme (Liu et al., 2005; Merrill et al., 2008).

The symmetry of developing craniofacial structures is majorly governed by *Shh* signaling during facial primordia formation (Jeong et al., 2004). *BMP4* functions downstream of *Msx1* and controls the expression of *Shh* in the palatal epithelium. Shh in turn regulates the expression of *BMP2* in the mesenchyme to promote cell proliferation (Zhang et al., 2002). *Shh* is a critical factor for the development and survival of cells within pharyngeal arch 1, specifically neural crest cells that have colonized facial prominences. A loss of neural crest cell fidelity and survival within the arches PA1 largely causes Shh-mediated craniofacial defects. Interestingly, although the Shh receptor Patched is expressed throughout the mesenchyme of pharyngeal arch 1, the *Shh* expression remains largely restricted to epithelium, indicative of paracrine signaling operating within the arch. *Shh*$^{-/-}$ mice demonstrate normal early patterning of PA1 until E9.5, with no concomitant differences in the expression of markers which definitively demarcate the arch endoderm, mesoderm, ectoderm, and neural crest cells (*HoxA2, HoxA3, Dlx3*, and *AP2*), as well as markers of pouch identity *Pax1* and *FGF8*. However, within 24 hours, PA1 is greatly reduced in size,

indicative of global first arch atrophy (Kraus & Lufkin, 2006). Shh is an important regulator of both maxillary and mandibular development, primarily through its role in maintaining an adequate critical mass of CNCC-derived ecto-mesenchymal cells within PA1. *Shh* is expressed in the facial ectoderm, the neuro-ectoderm, besides the pharyngeal endoderm at various stages of development (Tapadia et al., 2005). Other than the facial skeleton, mutations in *Shh* also lead to facial defects of the eye, such as coloboma (missing eye tissue) and microphthalmia (abnormally small eyes) in mice embryos.

Moreover, many genes from a large class of homeobox containing transcription factors such as Dlx, Otx, Msx, and Sox appear to affect the condensation of neural crest cells (Duboule, 1994; Krumlauf, 1994). Dlx genes are expressed spatiotemporally throughout the neural tube, ectoderm, and neural crest derived mesenchyme of the face (Bendall & Abate-Shen, 2000). Enhancers of *Sox10* and *FoxD3* have cranial and vagal neural crest sites responsible for neural crest specifier transcription factors such as *Sox9* (Betancur et al., 2010). *Twist1* appears to be a part of the EMT program in mice, although it is expressed in mesoderm cells shortly after initiation of migration (Füchtbauer, 1995; Gitelman, 1997). *Twist1* mutant mice survive gastrulation and die from cranial defects (Chen & Behringer, 1995).

Novel contributors to craniofacial development

Along with the well-known signaling of craniofacial patterning, new regulatory molecules are added. The molecular regulators of developing embryos have been identified accidentally and often to know the reasons behind the congenital disabilities in new-borns. These molecules may be enhancers or promoter binding proteins, siRNA, miRNA, transcription factors, enzymes, etc. These various types of molecules were traced back to their role during the developmental period to understand congenital defects and their prevention.

The interest in studying the Snail gene family arises from its crucial role in arthropods' mesoderm and nervous system development. A similar variant is found in vertebrates, where it is essential for maintaining the stem cell population in developing embryos. However, it has shown invasiveness in human epithelial tumors (Côme et al., 2006).

Snail1, in collaboration with *Snail2*, has crucial functions in murine craniofacial development (Carver et al., 2001; Murray et al., 2007). *GATA3* is strongly expressed in the

mouse and chick embryo's maxillary and frontonasal regions (Ruest et al., 2004). A point mutation in *GATA3* leads to variable craniofacial defects in zebrafish, where the most common defect is macrostomia. The expression of *GATA3* is under the regulation of the BMP pathway (Zhang et al., 2016). *Hand1* and *Hand2* overlap in medial mandible development, promote progenitor cell proliferation and inhibit differentiation (Barbosa et al., 2007; Funato et al., 2009).

For instance, various isoforms of WNTs play their role in polarity specification, muscle sculpting, limb formation, and nervous system development (Loganathan et al., 2005; von Maltzahn et al., 2012; Sokol, 2015). However, the identification of WNTs was traced back to cancer biology in 1982, indicating the genes responsible for developmental processes that can also lead to disease conditions. A large family of FGFs includes 23 members and four different types of receptors functioning in all the diverse tissue remodeling and growth events. A series of experiments revealed that FGFs express themselves since the organism's embryonic development (Aigner et al., 2002). Later on, it was identified as a mesodermal instructor in vertebrate embryogenesis. Pioneer studies showed that FGFs are essential for various organ development, however, currently, it is widely known for cellular interaction, cell proliferation, cell migration, and patterning of organs (Slack et al., 1987, Smith, 1987).

A similar instance is about the discovery of Cyclooxygenase enzymes (COX), that was discovered as a target of the nonsteroidal anti-inflammatory drugs (NSAIDs) and found to play a primary therapeutic role in treating pain, fever, and inflammation (Smith & Voss, 2012; Haley & von Recum, 2019). However, the role of COX in embryogenesis was discovered much later in 2003 by Stanfield. COX-2 expression is observed in rat fetal organs, including skin, heart, cartilage, and kidney, during gestation days 15–20 (Stanfield & Khan, 2003). Despite the well-known expression of COX-2, its role during embryonic development has not been studied in detail.

Cyclooxygenases

Cyclooxygenase enzymes are also known as Prostaglandin endoperoxidase synthases (PTGS; E.C.1.14.99.1) and are known to convert free arachidonic acid into prostaglandins (Simmons et al., 2004). Arachidonic acid is released from the cell membrane when Phospholipase A-2 (PLA2) acts on the second position of phospholipids. All vertebrates investigated, including cartilaginous fishes, bony fishes, birds, and mammals, have two COX genes: one encoding the constitutive COX-1 and another the inducible COX-2. COX-

1 and COX-2 share approximately 60-65% amino-acid similarity; COX-1 orthologs (without the signal peptide) share approximately 70-95% amino-acid similarity across vertebrate species, and COX-2 orthologs share 70-90% similarity (Fig 1.4).

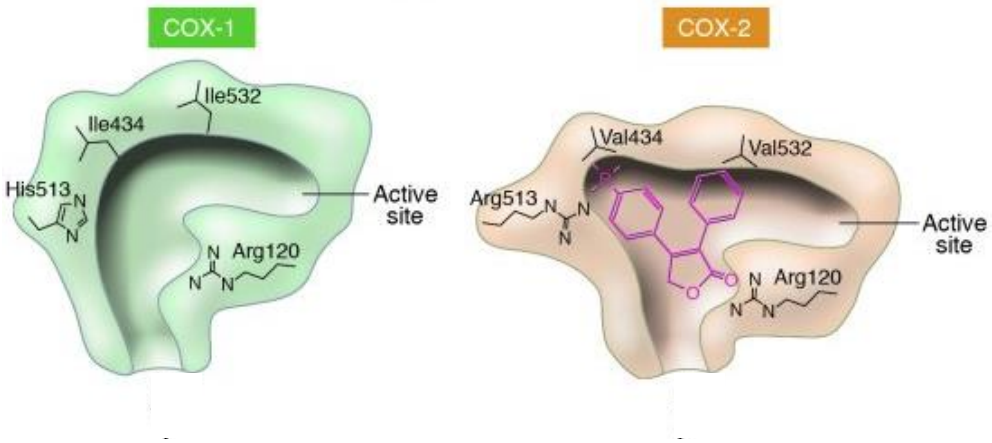

Figure 1.4: Structure of COX-1 and COX-2 along with enzymatic active sites (Grosser et al., 2006).

Both classes of COX are bifunctional enzymes with two distinct catalytic activities: cyclooxygenase (or bis-dioxygenase) activity and peroxidase activity. Prostaglandin isomers act upon G-protein-coupled receptors (Narumiya et al., 1999), and there are multiple receptors for some isoforms (such as Prostaglandin E_2). Prostaglandins are short-lived in vivo (with half-lives of seconds to minutes) and act in an autocrine or a paracrine rather than an endocrine fashion (Jabbour et al., 2002; Bygdeman, 2003).

Cyclooxygenases have short catalytic life spans (frequently 1-2 minutes at Vmax in vitro) because the enzyme is auto-inactivated (Newton et al., 1997; Fitzpatrick, 2004). The mechanism of auto-inactivation is unknown, but reactive tyrosyl radicals may cause internal protein modification (Chandrasekharan & Simmons, 2004).

Inhibition of COX-2

The COX-1 enzyme functions for regular homeostasis and maintains blood pressure, but COX-2 is mainly identified as an inflammatory mediator. Owing to its high affinity for modified arachidonic acid, COX-2 is capable of producing different types of prostanoids. These increase systemic temperature and regional pain at the injury site (Fig 1.5) (Ricciotti & FitzGerald, 2011). Different types of NSAIDs are used to overcome the inflammatory response generated by COX-2. NSAIDs inhibit the COX enzymes irrespective of isoform and reduce the production of prostanoids, reducing pain and inflammation. However,

inhibition of COX enzymes affects regular tissue functions and causes ulceration and aplastic anemia (McGettigan & Henry, 2000; Gor & Saksena, 2011).

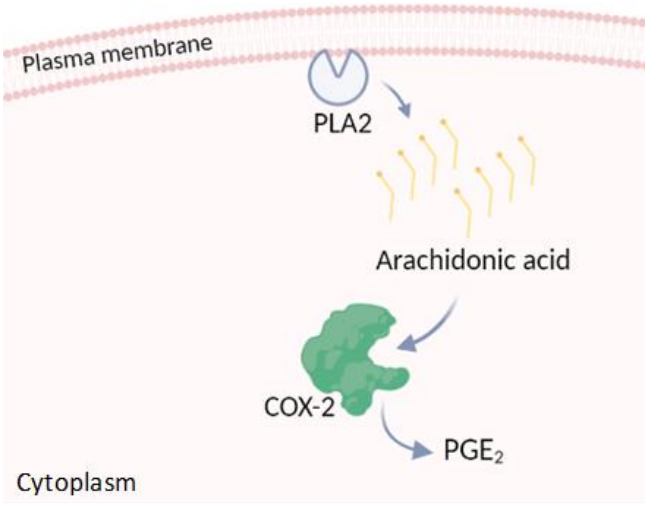

Figure 1.5: Mechanism of COX-2 enzyme to convert arachidonic acid into Prostaglandin E_2 (Ricciotti & FitzGerald, 2011); PLA2: Phospholipase A-2, PGE_2: Prostaglandin E_2.

Although NSAIDs block synthesis of all PG, they are efficacious and relatively safe drugs. However, a range of hazardous side effects, of which gastrointestinal toxicity is of primary clinical importance, preclude NSAID use in highly sensitive patients (Mattia & Coluzzi, 2005; Zarghi & Arfaei, 2011). Based on the COX-2 hypothesis, the apparent solution to the gastrointestinal toxicity of NSAIDs was the development of selective COX-2 inhibitors (Fig 1.6). The resultant massive effort in the pharmaceutical industry came up with new drugs, the coxibs, in the market within eight years after the discovery of COX-2 (Marnett., 2009). Among all the known coxibs, the Etoricoxib, invented by Fischer and Ganellin in 2010, is 100 times more specific to COX-2 than COX-1. The half-life of etoricoxib in humans is around 22 hours, and metabolites cannot bind to any COX isoforms (Brooks & Kubler, 2006).

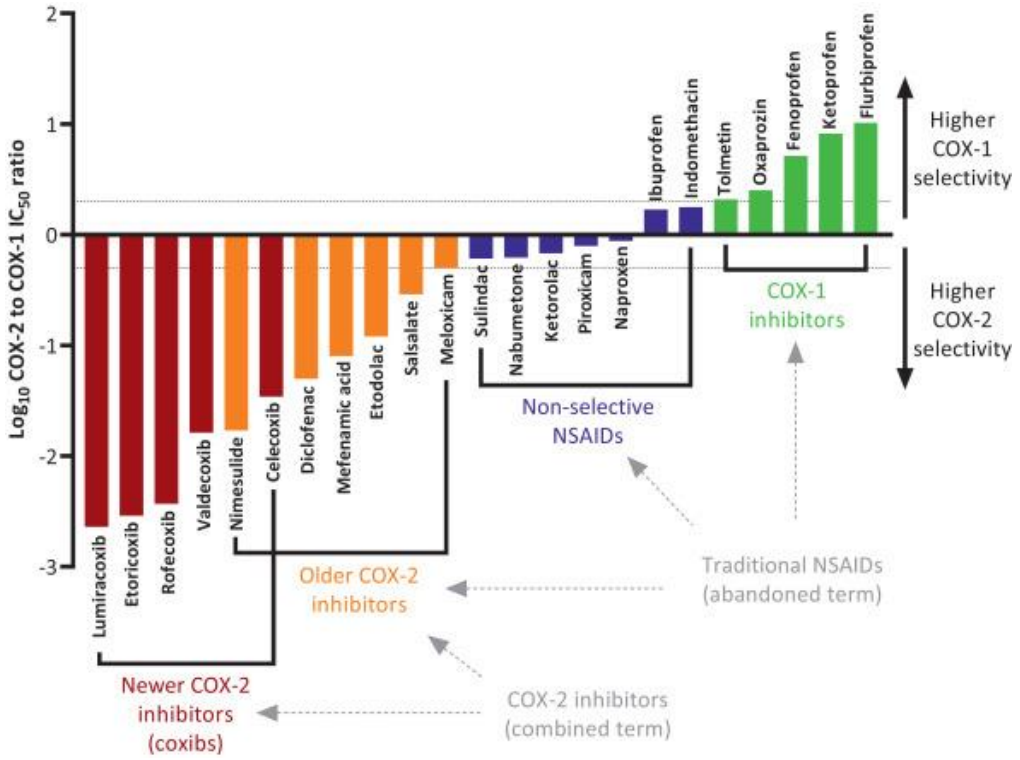

Figure 1.6: The selectivity of different types of COX-1 and COX-2 inhibitors (Bonnesen & Schmidt, 2021).

Etoricoxib is widely used as a pain killer in rheumatoid arthritis, spondylitis, gout, and osteoarthritis due to its high specificity. However, the frequent inhibition of COX-2 leads to blood pressure destabilization, heart attacks, and strokes in adults (Mukherjee et al., 2001; Roumie et al., 2008; Walker, 2018). As per the reports, very little information is available on COX-2 essentiality and functioning during embryogenesis (Yasojima et al., 1999; Stanfield et al., 2003). To find out the functioning of COX-2 during embryogenesis and organogenesis, various studies were initiated in different animal models such as zebrafish, chick, and mice (Mendias et al., 2004; Yang et al., 2005; Ishikawa et al., 2007). Xu and co-workers showed that COX-2 expresses during heart tube development in zebrafish (Xu et al., 2011). It has also been known that COX-2 is expressed in heart tube, limb buds, renal tissue, and craniofacial region (Verma et al., 2021), but its function in the development of various tissues is still unexplored.

To explore the roles of COX-2 in craniofacial development, we have used COX-2 specific inhibitor etoricoxib. Chick was used as an animal model to analyze the phenotypical defects, cellular events, and molecular alterations.

Animal Model: *Gallus gallus*

"A box without hinges, key, or lid, yet golden treasure inside is hid." – *The Hobbit.*

Various animal species, such as drosophila, *Caenorhabditis elegans*, zebrafish, frog, chick, and mice, are used for embryological studies. For embryological studies, animal model selection is based on the length of embryonic development, experimental conditions, ease of genetic manipulation, and minimum randomness in developmental stages. The chick embryo has been utilized as an embryological model since Aristotle's time and has fulfilled all the requirements of embryological study (Ruijtenbeek et al., 2002).

The chick embryo is accessible from the pre-gastrulation stage to organogenesis. Entire organogenesis can be observed by culturing live embryos on albumen agar plates and allowing possible manipulations in developing embryos. Patterns of organ development and cellular events are similar to mammalian organogenesis (Stern, 2005). The craniofacial structure development is well conserved in chick embryos. They resemble the same facial prominence growth as is observed in mammals. The genome linkage mapping suggested that the chicken genome is relatively closer to the human genome than the mouse genome (Hintermann et al., 2022). Owing to all the limitations of the mice embryo, for this study, we used chick embryo, where a few developmental stages were targeted, specific to craniofacial development.

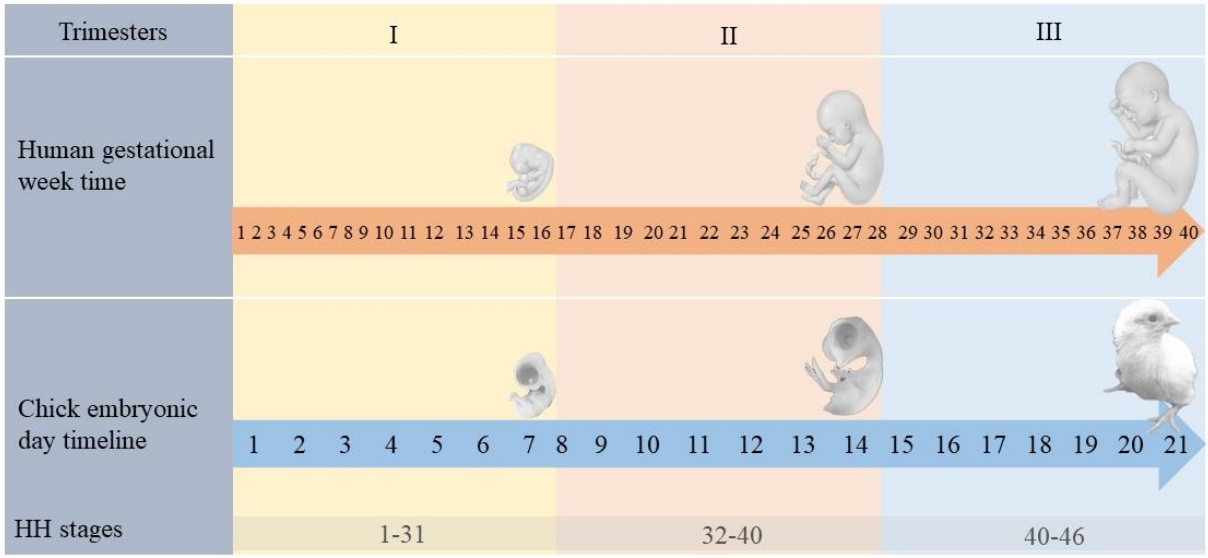

Figure 1.7: Comparison of Carnegie and Hamberger-Hamilton staging system for human and chick embryos (Bjørnstad et al., 2015).

The entire development of chick embryo is divided into 46 stages based on morphological characteristics of the embryo on a particular day of development (Hamburger & Hamilton, 1951). This classification of avian embryos allows us to correlate the developmental stages with the human embryo of 40 weeks (Fig 1.7) (Bjørnstad et al., 2015). Variations often arise in the developmental stages due to incubation delay, genetic differences, different breeds, and seasonal variation. Therefore, the stages have specific characters but overlapping time points, especially during the early developing embryo (day 1 to day 4).

HH12 to HH20 stages were considered for the craniofacial development study, along with a few late developmental stages (HH32-HH36). At HH12 (48hrs) developmental stage neural tube is closed, three brain vesicles are distinctly formed along with the optic vesicle, and a 'C' shaped heart tube is also formed (Hilfer, 1983). In chick embryo HH12 to HH20 stages are crucial for the migration of the neural crest cells along with initiation of eye development. Followed by HH14 stage, wherein cranial flexure is complete, which leads to the formation of pharyngeal arches and facial prominences. At the same stage, the heart tube turns and becomes 's' shaped (Hamburger & Hamilton, 1951).

Origin of the problem

Previous studies in the lab and around the globe revealed that COX-2 plays a specific role in orchestrating the cellular events that regulate the morphogenesis of the regenerating tail of the lizard (Buch et al., 2018). Subsequently, COX-2, through one of its metabolites, was observed to facilitate the sculpting of appendages in chick embryo (unpublished). However, one of the studies showed that inhibition of COX-2 by pharmacological inhibitor affects the cellular events and causes deformed neural tube patterning in developing embryo (Geliflimine, 2010). This role of COX-2 in cancer physiology is well studied and shows the interaction with major regulatory pathways such as WNT, FGF, and BMP. All these signaling pathways play a crucial role in different cellular events and occur at their highest during embryonic development, leading to a thought about the presence of COX-2 and its functions. However, in our preliminary study, chick embryos exposed to COX-2 specific pharmacological inhibitor, showed abnormal patterning in NCC derived heart and eye.

Therefore, the current study was envisaged, wherein the mechanistic insight into COX-2 inhibition on NCC derived heart and eye development was assessed in the chick embryo.

The results of this study are presented in the form of three chapters as follows:

Chapter 3 focuses on the neural crest cells, a significant player in craniofacial patterning in vertebrates. The inhibition of COX-2 hampered the neural crest formation and migration (HH6, HH12, HH20) in the developing embryos. The chapter revealed the interaction of COX-2 with various pathways related to migration and regulations.

Chapter 4 gives insights into COX-2 and cardiac neural crest cell interaction, vital for heart tube patterning at the HH12 stage. Further, at stage HH20, differentiation of cardiac neural crest cells to myocytes also required COX-2.

Chapter 5 aims toward the COX-2 interactions with eye development, a sense organ of the craniofacial region. The eye's early morphogenesis was focused on optic vesicle to optic cup development (HH12, HH14, and HH16) where the participation of COX-2 was found.