1

Introduction

The real question concerning metazoan ontogeny is just how a single cell gives rise to the requisite number of differentiated cell lineages with all the right inductive developmental interactions required to reproduce the form of the mature organism……

(Moss, 2002)

DEVELOPMENT

Origin of the word **development** can be traced back to the mid seventeenth century, getting its roots from the Latin language. In Biology, the term development relates to the emergence of a multicellular organism from a single-celled **zygote** and all the processes performed by the body to maintain the morphological, physiological and anatomical feature of itself through the lifetime (Gilbert, 2007). These processes do not include only the growth, multiplication and differentiation, but also cell death in the form of apoptosis and necrosis. Development in the organisms can be divided into two main divisions, namely – embryonic development and post-embryonic development.

Post-embryonic development deals with all those processes that occur after the birth or emergence of an organism. It covers the areas of disease biology, regeneration, and ageing. Research in these areas has direct applicability for the synthesis of pharmacological agents, regenerative medicines, and beauty products, respectively (Giacomoni, 2005; Mao and Mooney, 2015; Mohs and Greig, 2017). That is the reason for more research being done in post-embryonic field rather than in the embryogenesis.

Embryology is a part of the developmental biology, which deals only with the studies that unfold the mystery of emergence of a multicellular organism from a relatively simpler one-celled zygote. An embryologist critically looks upon one or all the processes transpiring between the fertilization and birth. A chick egg when laid has some thousands of cells arranged in one layer. Within three weeks inside an incubator with ambient temperature and humidity factors, it develops into a beak-bearing self-emerging creature, which has always kept embryologists curious about the complex set of functions, those one-layered cells

acquired in the small course of time. Some of the distinct features of cells of the dynamic embryo are division, migration, differentiation, and death. All the events meticulously in a spatiotemporal manner organized by some set of organizers, which are nothing but a cluster of cells (Anderson et al., 2016). Based upon the common pattern of progression most embryos of invertebrates and vertebrates follow, embryonic development is divided into stages, namely fertilization, cleavage, blastulation, gastrulation, and organogenesis. Majority of the creatures we observe around us – the turtles, frogs, lizards, snakes, crocodiles, birds, and even us – we all belong to a single taxon sharing quite an extent of similarity in these described processes of embryogenesis. The taxon we belong to is called 'Tetrapoda' (Hickman, 1961). Some of the common characteristics and trends of tetrapod embryogenesis are as described in the following section.

EMBRYONIC DEVELOPMENT OF TETRAPODS

Tetrapoda, a superclass of phylum Chordata, includes organisms bearing four limbs. These four limbs could be modified in various structures according to the habitats and requirements of an organism. There are four classes in Tetrapoda, namely amphibia, reptilia, aves, and mammalia (Hickman, 1961). The embryonic development in these organisms starts with the cleavage of a single zygotic cell into numerous cells. Later the cells migrate to various locations deriving signals to pursue the fates for the formation of specific organs. The large-scale migrations occur in precise patterns, and the process is called **Gastrulation**. The cells determined to form a specific organ then further differentiate as per their fates, and this marks the initialization of the **Organogenesis** process (Hickman, 1961). It is this period of embryogenesis which gets affected the most by teratogens and external environment. This phase is the longest in case of the organisms exhibiting direct development (Bleyl and Schoenwolf, 2010). All these developmental stages are not well-compartmentalized in reality but are divided for ease of the understanding of the lengthy embryogenesis.

Embryonic development of tetrapods is known to be sharing many similarities in its structural and molecular details. The mention of phenotypic similarities can be traced back to 1824 (Baer, 1837). However, the finer details of conserved molecules started to be observed and identified quite later in the history (Kalinka et al., 2010). Only the genes that were associated with human diseases were studied in depth in other vertebrate models and were found conserved before the molecular systemics gained momentum (Fougerousse, 2002; Rugarli, 2002). Some of the most conserved genes and proteins acting as the morphogens,

signal initiators, secondary messengers, and regulators in embryogenesis are described in the following section.

CONSERVED PLAYERS OF EMBRYONIC ORCHESTRATION

An embryo grows following the signals for growth and differentiation. Growth, as well as differentiation, does not occur randomly, instead, it follows the directional patterns as per the polarity. An initial signal, drawing an anterior-posterior polarity in embryonic body-line arrives from fibroblast growth factors (FGFs). FGF proteins dictate a group of epiblast cells to get converted to neuronal derivatives in chick embryo (Streit and Stern, 1999). They are also found to be involved in neural induction in other organisms like mammals and amphibians (Harland, 2000). The proteins regulating left-right symmetry – Nodal and Pitx2 are also conserved among vertebrate classes (Semina et al., 1996; Ryan et al., 1998). Homeobox genes act as polarity developing regulators in a lower organism like *Drosophila* as well as in higher animals like amphibians, birds, and even in humans (Hunt and Krumlauf, 1992). Growth of the cells is always accompanied by some amount of cell death to provide characteristic shapes to particular organs. Cell death in embryonic life occurs majorly via an apoptotic mode. Caspase enzymes are widely known for their common apoptotic roles in all the vertebrates. Their homologous relatives *ced* proteins act on the same process in *Coenorhabditis elegans.* The homologues of gap genes - Orthodenticle and Empty spiracle participate in head morphogenesis in primitive and higher organisms like fly and mammals (Schinko et al., 2008). Other than these evolutionarily conserved molecules, some intermediate structures of embryonic lives are same in the organisms belonging to the separate classes. For instance, Hensen's node is even observed in mammalian development. This structure is called 'node' in mammals and was first identified in rabbits (Knoetgen et al., 2000). The discovery of these conserved structures and molecules were not always emerged from the core aim of a work conducted. In one of the experiments, rotating the eggs at initial stages caused duplication of the organizer in amphibian embryos (Rowning et al., 1997). The similar pattern of duplication was observed in fishes as well (Fluck et al., 1998). The observation of the same phenomenon in two different species belonging to separate classes led the path to the discovery of microtubules and its conserved functioning across various phyla. There are many such molecules which play the same roles across the phyla sculpting their differently growing embryos.

The players, now well-known for their actions in embryonic development, were once only the culprits for many pathological conditions. Research in the field of embryogenesis is less frequent because of its limited applicability. Such fundamental research is usually either curiosity-driven or accidental.

DISCOVERIES OF MOLECULES OF EMBRYOGENESIS

Discovery of the players forming the kernels of a developing embryo has often been accidental. Molecules either identified to be subversive in perilous diseases or lethal defective forms are traced back to their roles in careful integration of events of embryogenesis. For instance, various isoforms of WNTs function in polarity development, sculpting the cusps of teeth, muscle development, limb axis determination and urinogenital development (Duan and Bonewald, 2016). However, the identification of this gene can be traced back to research in cancer biology in 1982. The gene was found to be an oncogene and was named as '*int*' initially (Nusse et al., 1984). Yokoyama and colleagues discovered a gene which can reverse organ phenotypes if faulty while working on some other aspect. Here, nonspecific tyrosinase was added randomly to the fetus, which in turn inhibited the protein necessary for the normal sculpture (Yokoyama et al., 1993). The fact that estrogenic compounds can be generated from plastic containers, food items, and contraceptive creams – was discovered after a so-called failed experiment at Tufts University Medical School, wherein the control group cells produced excess estrogen due to the water and serum containers (Gilbert, 2007). A large family of FGFs including 23 members functioning in almost all aspects of organisms from angiogenesis to metabolism and from cellular growth to tissue repair was discovered slowly after the discovery of one of these family members by Armelin from a pituitary extract as a mere growth stimulator of fibroblast cells (Armelin, 1973; Hui et al., 2018). However, the roles of FGF family members in embryonic development were studied and identified fifteen years after FGF discovery in vertebrate embryos (Amaya et al., 1993). After a series of studies concluding a homogenate from the liver and kidney of Newt, swim bladder of fish and whole embryos of the chick – as a mesodermal instructor to isolated ectodermal cells since 1954 (Yamada, 1958; Kawakami et al., 1977; Brito, 1982), it was finally in 1987 when few scientists could isolate and determine FGF as the major inductive molecule for this process. Pioneer studies revealed their roles during mesodermal fate specification, but in the current time, it is known to affect most of the differentiation, pattern formation, migration, growth-related processes during vertebrate embryonic development (Kimelman and

Kirschner, 1987; Slack et al., 1987; Smith, 1987). The similar example is the discovery of Cyclooxygenase (COX). It was about 130 years later than the usage of anti-inflammatory drugs, Vane identified the mechanism of action of aspirin-like drugs was to suppress COX derived Prostaglandins (Vane, 1971). Nevertheless, the roles of COX in embryogenesis started to unfold much later (Stanfield, 2003). Even afterwards, the information is limited to the statistical correlations drawn between consumption of pain-killers (inhibitors of COX) and miscarriages (Li et al., 2018).

CYCLOOXYGENASES

Cyclooxygenase (COX) enzymes are also known as Prostaglandin-endoperoxide synthases (PTGS; E.C.1.14.99.1). As the name suggests, these enzymes are involved in the biosynthesis of endoperoxides which further produce prostaglandins (Simmons et al., 2004). Endoperoxides are the cyclic compounds bearing the peroxide residue in the rings. One such endoperoxide- Prostaglandin H_2 (PGH_2) is an intermediate of many ultimate products collectively known as Prostanoids. PGH_2 is synthesized by COX from the membrane fatty acid Arachidonic acid (AA) with the help of Phospholipases. Phospholipases are another group of enzymes functioning for cleaving the fatty acids from the membrane phospholipid molecules (Simmons et al., 2004). In this manner, a 20-carbon omega-6 polyunsaturated fatty acid AA is converted to many Prostanoids via PGH_2 with the help of phospholipases and cyclooxygenases (figure 1).

Cyclooxygenase enzymes are found to occur in three forms in most Vertebrates. These three are called COX-1, COX-2 and COX-3. Structurally and functionally these three isoforms share a high level of similarity. Two of the three – COX-1 and COX-2 are observed to have an overlapping function in some physiological conditions (Hla et al., 1999). However, each of them also performs some unique physiological roles. The common roles are on account of their structural similarities. COX-1 and COX-2 contain 576 and 581 amino acid residues, respectively (Gierse et al., 1996). Both of them contain three common oligosaccharides, which have a high amount of mannose in them (Gierse et al., 1996).

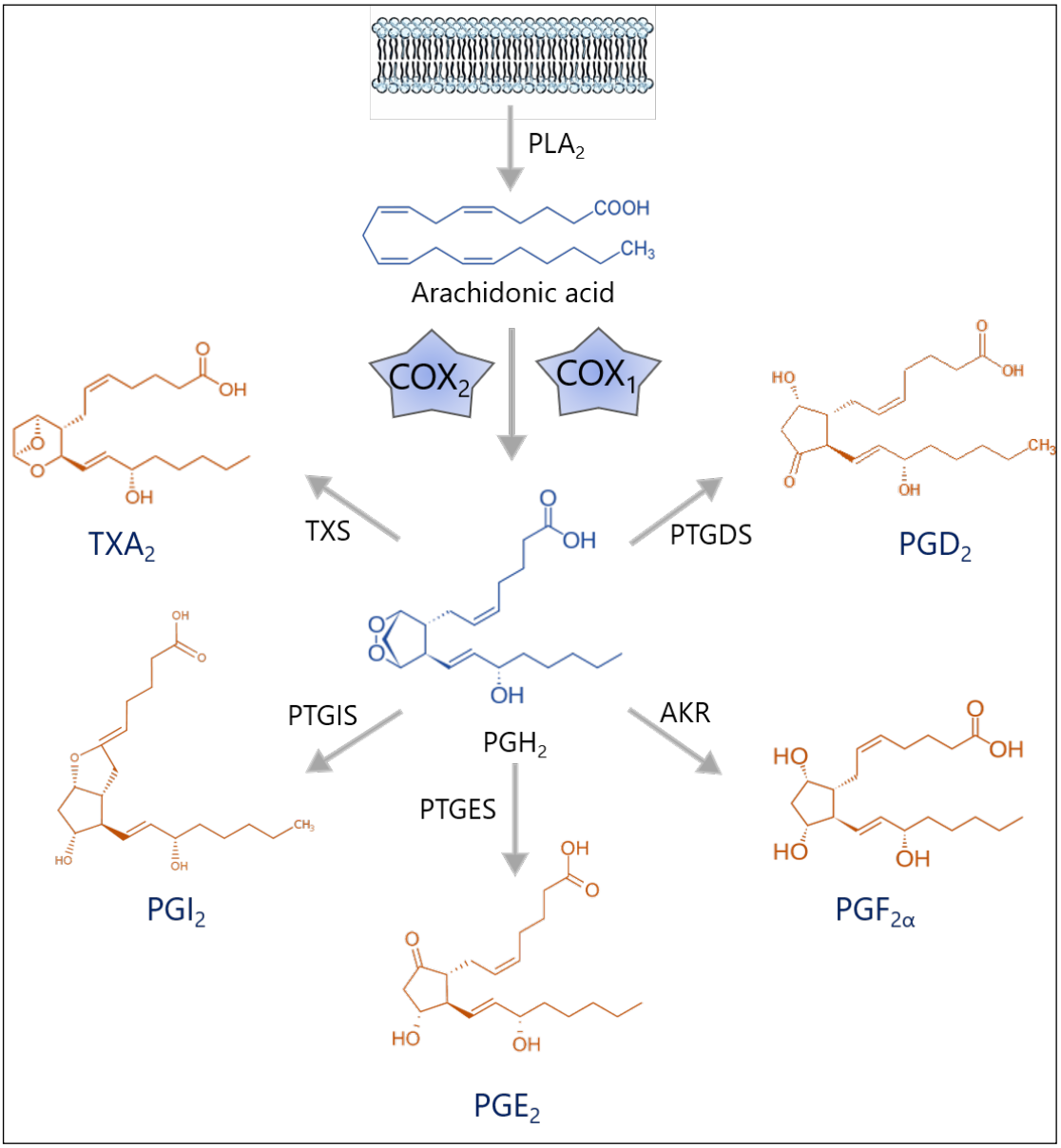

Figure 1: *Cyclooxygenase pathway. COX-1 and/or COX-2 converts membrane phospholipid Arachidonic acid into various prostanoids via the action of phospholipase. The conversion is mediated by tissue-specific enzymes unique for each prostanoid to be synthesized. PLA_2: phospholipase A_2, COX-1 and -2: cyclooxygenase-1 and -2, PGH_2: prostaglandin H_2, TXA_2: thromboxane A_2, TXS: thromboxane synthase, PGD_2: prostaglandin D_2, PTGDS: prostaglandin D synthase, $PGF_{2\alpha}$: prostaglandin $F_{2\alpha}$, AKR: aldoketo reductase, PGE_2: prostaglandin E_2, PTGES: prostaglandin E synthase, PGI_2: prostacyclin, PTGIS: prostaglandin I (prostacyclin) synthase.*

However, the fourth oligosaccharide in COX-2 is unique, which takes part in its degradation. Both the isoforms superimpose well owing to their similarity being a bit more than 50%. These isozymes are produced from different segments of DNA. The resulting mRNAs are different in sizes as well. COX-1 mRNA is about 2.8 kb, and that of COX-2 is about 4 kb. The mRNA of COX-1 is more stable than COX-2. Therefore, COX-1 is relatively long-lived and is involved in homeostatic functions unlike COX-2, which is usually degraded soon after it is produced for some inducible functions in adults (Bakhle and Botting, 1996).

COX-1 is known as a constitutive COX isoform which maintains the basal level of prostanoids. 'Prostanoids' is the term for the prostaglandins and thromboxanes, the molecules acting at small quantities in the near vicinity of their production. Such basal levels of prostaglandin E_2 (PGE_2) and prostacyclin (PGI_2) protect the intestinal lining in most animals and in humans as well (Ricciotti and FitzGerald, 2011). Prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) produced via COX-1 regulates reproductive activity in females, which include ovulation and parturition (Sugimoto et al., 1997).

COX-2 was discovered in a laboratory of Brigham Young University by Professor Daniel Simmons research group (Xie et al., 1991). Before the discovery of COX-2, it had already been targeted by a drug invented by Dupont company which was known as DuP-697. Functional details of COX-2 were revealed much later after it was being inhibited by the medicines for suppressing inflammatory state of the organisms. This isoform is mainly identified to be inducible via inflammatory mediators. It then mediates the production of several prostanoids, which rise above their basal levels. Increase in systemic temperature and regional pain at the injury site – are the inflammatory responses generated via PGs (Ricciotti and FitzGerald, 2011). These are targeted by the most used drugs in the world – the non-steroidal anti-inflammatory drugs (NSAIDs) of the new generation. These novel NSAIDs were the outcomes of the necessity for minimizing ulcers caused as side-effects of the early NSAIDs reducing pain and fever otherwise.

NSAID

It has been over a hundred years since the humankind started using NSAIDs due to their efficiency in reducing pain and fever without knowing the biochemical basis of their pharmacological effects. The first ever used NSAID was salicylic acid, derived from the extract of the plants like Myrtle and Willow which were then known for their antipyretic and

analgesic effects (McMurry et al., 2008). Felix Hoffman, son of a rheumatoid arthritis patient, was acetylating salicylic acid with the hope of improving its strength with least toxic effects. He acquired the strategy of acetylation from the synthetic pattern of fever-relieving drug phenacetin, discovered by the Bayer medicine company he used to work for. Salicylic acid was known for its side effects of stomach ache and intolerance in the patients using it before this discovery. Once acetylated, the new form of drug was directly tested in humans and showed decreased toxicity. It was later tested in animals and marketed as Aspirin ('A' for Acetylation and 'spirin' for *Spirea*- genus of the shrub used for isolation of Salicylic acid). After few mechanism revealing studies in Guinea pigs, finally Vane identified Aspirin as a Prostaglandin-suppressing agent in 1971 (Collier and Shorley, 1963; Collier, 1969; Vane, 1971; Cadavid, 2017). Identification of the mechanism of action of Aspirin led to the invention of many NSAIDs in that era. Slowly and gradually, the usage of NSAIDs increased and their side effects like ulceration and aplastic anemia started appearing (McCarthy and Chalmers, 1964; Hudson and Hawkey, 1993; Wallace, 2000).

In 1999, the newer generation of pain-killers started appearing in markets. These were the specific COX-2 inhibitors (Hawkey, 1999). The initial COX-2 inhibiting NSAIDs also used to suppress COX-1 activity to certain extent. However, more research has led to the development of more specific COX-2 inhibitors (figure 2). The NSAID used for the current study is a COX-2 specific inhibitor known as '*Etoricoxib*'. Etoricoxib is widely used (in 80 countries worldwide) as pharmacological agent to cure pain and fever. This chemical compound was invented by Fischer and Ganellin in 2006.

Chemically called as – 5-chloro-6-methyl-3-[4-(methylsulfonyl) phenyl]-2,3-bipyridine - Etoricoxib (ARCOXIA2) possesses a hundred-fold more affinity for COX-2 than COX-1. Its short half-life (approximately 22 hours in humans), as well as its metabolism pattern, are its essential characteristics contributing to the greater specificity (Birmingham and Buvanendran, 2014). Etoricoxib is metabolized via hydroxylation and oxidation in the biological system. None of its metabolites further interacts with any of the COX isozymes. They neither inhibit nor promote COX-1 and COX-2 activity (Brooks and Kubler, 2006).

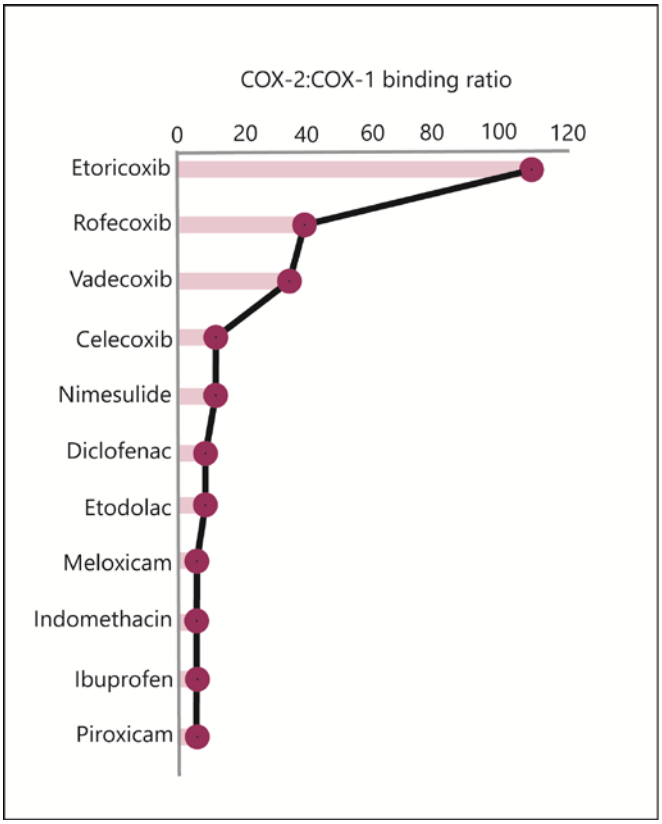

Figure 2: *Binding ratios of various non-steroidal anti-inflammatory drugs (NSAIDs) to COX-2 and COX-1*

Due to its least gastrointestinal repercussions, etoricoxib has been used for managing symptoms in many disorders. It is prescribed in musculoskeletal disorders such as lower backache, rheumatoid arthritis, and osteoarthritis (Brooks and Kubler, 2006). It is also consumed in case of the lower abdominal painful muscle cramps of menstruation (dysmenorrhea) (Ranong and Sukcharoen, 2007). Use of etoricoxib in the field of orofacial pain and headache is a more recent addition to its existing uses (Benoliel et al., 2010). Nevertheless, its repeated consumption to combat orofacial pain caused oral mucosa in several patients (Edel et al., 2018). The cases of skin infections and erythema due to etoricoxib intake have also been noticed (Straube, 2010). Frequent etoricoxib usage is found to cause blood pressure destabilization, heart attacks, and strokes (Walker, 2018). The underlying mechanisms of such side-effects are still under investigation, as their occurrence has recently been identified. However, it is clear that the effect of COX-2-inhibition must be hampering the levels of one or more **prostanoids**, which are elevated in particular circumstances such as inflammation.

PROSTANOIDS

The principal effectors of COX enzymes are prostanoids in all the vertebrates. The five primary prostanoids are PGI_2, PGD_2, PGE_2, $PGF_{2\alpha}$, and TXA_2. Structurally these prostanoids are very similar to each other. In biological systems, they are synthesized in picograms or nanograms as and when required (Pilbeam et al., 2008). A meagre quantity of prostanoids is always present for housekeeping functions in the maintenance of adult life. Embryonic presence and functionality of prostanoids are least studied and recorded so far. These heterocyclic compounds are not very stable in body fluids while their levels are maintained by COX enzymes working closely with dehydrogenases (Thill et al., 2009).

Prostanoid	**Site of production**	**Functions**
Prostacyclin (PGI_2)	Heart, Vascular endothelial cells	Inhibition of platelet and leukocyte aggregation
	Gastrointestinal tract	Mucosal protection
	Reproductive organs of the female	Inhibition of uterine contractions
Prostaglandin D_2 (PGD_2)	Central nervous system	Vasodilation, edema Initiation of allergic response
	Gastrointestinal tract, Uterus	Muscle relaxation
Prostaglandin E_2 (PGE_2)	Kidney	Uterine muscular contractions, Edema, erythema
	Reproductive organs of the female	Cervical ripening
	Endothelial cells lining the preoptic area	Thermoregulation
Prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$)	Reproductive organs of the female	Vasoconstriction, Uterine muscular contractions
	Hippocampus	Neurogenesis
	All tissues	Regulation of COX-2 production via the feedback loop
Thromboxane A_2, B_2 (TXA_2, TXB_2)	Platelets	Platelet aggregation, Vasoconstriction, Bronchoconstriction

Table 1: *Derived from Chung-Davidson et al., 2008; Takeuchi and Amagase, 2017; Tan and Knight, 2018*

The prostanoids belong to the class eicosanoids which bear numerous other heterocyclic compounds produced in the lower quantities. Among the major prostanoids – PGE, PGD, PGF, and PGI are the families classified in the group called the prostacyclopentanes. The prostanoids which are structurally slightly different from the prostacyclopentanes are divided into another group known as thromboxanes (TX). Prostacyclopentanes possess cyclopentane rings, whereas TX compounds possess bicyclic oxetane ring (Bhagwat et al., 1985). Each of the prostacyclopentane group members, PGE, PGD, PGF, and PGI contain further the isoforms such as – PGE_1, PGE_2; PGD_1, PGD_2; PGF_1, PGF_2, PGF_3; PGI_1, and PGI_2. The group TX contains two isoforms, namely TXA_2, and TXB_2. All of these isoforms are not produced using AA in biological systems. Some other precursor fatty acids for prostanoids are – eicosapentaenoic acid, and dihomo-γ-linoleinic acid. Once the precursor fatty acid is liberated using phospholipases from the lipid bilayer, COX enzymes along with the peroxidases (POX) converts them into an unstable intermediate PGH_2. This short-lived molecule is further converted to the specific PG isoform according to the needs of the tissue. Their production is performed by tissue-specific synthases which are unique for each of the prostanoid (Hara, 2017). Prostanoids are regulated at the level of those tissue-specific enzymes in most cases. These are broadly known as synthases. The basal concentrations for the prostanoids are in picograms which increases to nanograms when required for special functions other than tissue homeostasis as described in the table 1. This thesis is focussed on PGE_2, PGD_2, $PGF_{2\alpha}$, and TXA_2. PGE_2 has been referred to as inflammatory prostanoid and TXA_2 as platelet aggregator (Kuehl and Egan, 1980). $PGF_{2\alpha}$ has been related to the process of labour since the 1970's (Anderson, 1973). PGD_2 and PGE_2 together function to maintain the sleep-wake rhythms (Hayaishi, 2000). After the initial studies working on their mechanisms of action in homeostatic functions, the focus has been shifted to finding their roles in disease progression.

Studies since the beginning of the current century are indulged in connecting the prostanoids and cancer. It is now known that COX-2 derived prostanoids function for the recruitment of angiogenic factors during tumor progression (Salvado et al., 2012). It is recently found to be an accelerating agent in the course of skeletal muscle regeneration (Ho et al., 2017). In the adult kidney cells, PGE_2 is secreted as stimulated by vasopressin, whose effect is mediated for alleviating NaCl reabsorption by Henle's loop (Vance and Vance, 2008). The property of PGE_2 to cause severe pain is inhibited by NSAIDs. This is known as hyperpathia or hyperalgesia and is a commonly occurring phenomenon in case of

inflammation (Ma, 2010). Overall, the prostanoids function to decrease the harm in the alarming conditions, the side effects of which are pain and fever, cured using NSAIDs.

Apart from all their unique functions executed during the post-embryonic life, the least is known about their essentiality in embryogenesis phase of life. There are multiple reports which suggest that the pain-killers reducing the activity of COX-2 can cause problems related to pregnancy or child-health in humans. The studies involving animal models suggest plausible roles of the prostanoids in feather development and kidney development. However, the focus has always been towards roles of COX in disease biology and cure. Work presented here deals with functions of COX-2 in embryonic life, predicting its roles in the vertebrate organogenesis.

ANIMAL MODELS FOR STUDY OF EMBRYOGENESIS

There are numerous species of animals, which are used over the years for the study of embryogenesis. To name a few, sea urchin, flies, zebra fishes, frogs, and mice are the most used organisms. Selection of animal model, however, depends upon factors such as availability of the particular organisms in the vicinity of the study site, suitability of the organism to the local climatic conditions, facilities that the laboratory possess for the designed study, and cost of the organisms (Davidson et al., 1987). Nonetheless, there are some specific requirements based on the study type, such as low genetic variability and randomness, as well as the possibility of extrapolation of the results to the ultimate targeted group of animals. In case of embryogenetic studies, the selection is usually based on the length of embryogenic life, requirements of the embryo for embryonic progression under experimental conditions, ease of observation of the progression closely, ease of genetic/ metabolic manipulation, and minimum randomness in characteristics of embryos at different stages of development. Amongst the vertebrates, the chick embryo bears numerous such characters as described in the following paragraph. The chicken embryos are being utilized as models since as early as the time of Aristotle (Hamburger and Hamilton, 1951).

The domestic hen belonging to the genus '*Gallus'* has been reared by humans since ancient times. A large number of eggs are produced by a hen in its lifetime. Stages of embryonic development are well-defined for this organism if they are incubated at a particular temperature and humidity conditions (Hamburger and Hamilton, 1951; Eyal-Giladi and Kochav, 1976). The investigations related to the effect of any chemical on

embryogenesis can be studied without the interference of maternal metabolism. The embryos can be isolated at any developmental stage without sacrificing mother as in case of the animals with internal development. It is rather easy to expose the embryos to the test chemicals via several available routes such as injection in the yolk, albumen, and air cells. Other than these benefits, chick embryo serves as an appropriate model to study the functions of COX-2 other than inflammation because the embryo does not show any inflammatory activity until three weeks of development (Janković et al., 1975; Kain et al., 2014). Thus, there is no interference of inflammatory activity while studying other roles of COX-2 by inhibiting it. The specific breed to work on was decided based on the availability in the near vicinity. Development of embryo starts from inside the hen, which gets completed till blastulation while being inside the mother's body. When the egg is laid, it is in gastrulation state. In the hot days of summer, the local temperatures make the continuous development possible for these eggs. Due to the delay in receiving them after they are laid, one may lose the ideal '0' stage (as defined by Hamburger and Hamilton, 1951) before incubation in the lab starts. Therefore, it becomes necessary to select a breed which is locally available for the work. Rhode Island Red eggs were used for this research derived from government poultry unit soon after they were laid.

DEVELOPMENT OF CHICK EMBRYO

A chick is hatched of a macrolecithal egg after an incubation period of around 21 days, provided with 37 °C temperature and 70 – 75 % relative humidity. Owing to the large quantity of yolk as being a macrolecithal egg, a cleavage furrow divides only a part of the egg, in a discoidal manner. A freshly laid egg of domestic hen contains around 20 thousand cells arranged in more than a single layer bearing a cavity in the center termed as the subgerminal cavity (Kotpal, 2010). The cells are, at this time, in gastrulation state as the process of fertilization gets completed inside an oviduct before the hard-calcareous shell covers the egg. This embryo, which are just laid has two distinct regions, namely '*area opaca*' which surrounds the light-shaded '*area pellucida*' which will later form almost all the tissues of the growing chick. The cells at the connecting edge of these two areas form a '*marginal belt*' and participate in fate determination of other cells (Azar and Eyal-Giladi, 1979). The first structure seen in this embryo is the primitive streak which appears as early as 15 hours after the incubation starts. The apical region of primitive streak starts thickening and forming an organizer of chick embryo – the **Hensen's node**. The cells migrating from all the regions

to the interior pass from Hensen's node through a groove of primitive streak known as **primitive groove** and get inside the blastocoel with the determination of structures they are about to form. The embryo then specifies the polarity by following the cues received in the form of paracrine or autocrine factors to be oriented dorso-ventrally, anterio-posteriorly, as well as proximo-distally. The first sophisticated organ to develop in the embryo is the heart. The fully functional heart of a smaller size can be observed on day-3 of incubation. However, it pumps since day-2 when it is a mere heart tube. A uniformly folded embryonic heart is entirely sculpted in its adult-kind symmetry on day-5 of embryogenesis (Gilbert, 2007). Day-2 (HH 12) also shows the presence of one or both the limb buds. Interestingly, the limbs are sculpted for the longest time during the embryogenesis of chick. Its development starts from late day-2 and keeps growing till day-12 of embryogenesis. Limbs at day-10 (HH 34) are completely developed feather-less miniatures of the adult limbs. Day-12 (HH 38) limbs show the presence of feathers as well. However, the embryonic eyes begin their development as early as day-3 and keep developing till day-10 (Lindner et al., 2017). The structural details, along with the molecular networks of the embryonic life of chick, have been the interest of scientists since 460 BC (Wolpert, 2004).

The earliest debate about embryogenesis in chick was about the origin of embryo itself. The hypothesis of Needham and Hughes (1934) was that the embryo develops from the yolk and derives the nutrition from the albumen. They dictated that hatching of the chick was dependent upon the amount of egg white (albumen) and the chick hatches as soon as the nutrition inside the egg gets over. Later, Aristotle studied the incubation process more thoroughly and derived a conclusion about the chick being developed because of the heat (Ross and Smith, 1908). He hypothesized that the chick develops from albumen and the yolk provides nutrition (Steno and May, 1950). Two theories surfaced then, namely 'theory of preformation' and 'theory of epigenesis' (Wolpert, 2004). The preformation theory suggested that the egg contained a small animal which only grew in size to make a large hatching chick which came out of shell deprived of nutrition. In 1673, a book was published with photographs of embryos observed using simple microscope (Malpighi, 1673). The blood vessels and somites were marked in embryos isolated from eggs, which were recently laid. Therefore, the scientists then believed the preformation would have occurred as the structures were believed to be present since the eggs were laid. It was observed that the yolk was continuous with the intestine of the developing chick, therefore, they believed that the yolk and intestines developed at the same time in the mother even before fertilization occurred via

preformation (Steno and May, 1950; Wolpert, 2004). Finally, it was proved by Wolff that intestines of chicks were not formed since before the eggs were laid, and the theory of preformation was false (Aulie, 1961). Whether being the theories of generation of foetus, or the details of organogenesis, chick has served as an efficient model in the field of embryogenesis. The unique features of the growing chick embryo were identified and divided into stages for convenience of experimental design by several scientists. In 1889, an atlas was prepared with scientifically correct drawings of chick embryos. This atlas was however incomplete showing the embryos only till ninth day of incubation (Duval, 1889). Keibel divided the stages as per the morphological details as early as in the year 1900 (Keibel, 1900). This division did not register the older embryos and showed very few illustrations. Additionally, it was published only in the German language, which is why it became less popular later.

Hamilton and Hamburger (1951) divided the stages of embryos from the eggs that were freshly laid till their incubation was complete. However, much of the development such as cleavage, hypoblast formation, and discrimination of area pellucida-opaca occurs inside the genital tract of hen, Eyal-Giladi and Kochav (1976) studied and divided the embryonic stages from much earlier before the eggs were laid. At day-0 of incubation, when eggs are just laid, they are at stage-0 as per Hamilton-Hamburger classification, and at stage-X as per Eyal-Giladi and Kochav. The stages described by the latter are always written with roman numerals. Both of these stages the HH (Hamburger-Hamilton) and EGK (Eyal-Giladi and Kochav) are used and cited in the studies so far. The work presented in this thesis focused on the development of embryos after the eggs were laid. Therefore, stages described by Viktor Hamburger and Howard Hamilton were followed for the experimentation.

HAMILTON-HAMBURGER STAGES OF CHICK EMBRYOS

The stages were divided based on the characteristics of embryos on a particular day of incubation (table 2). These scientists also considered the variations arising due to the delay in the initiation of incubation, genetic differences such as breed-dependent rapidness in hatching (white-leghorn chicks typically hatch a day earlier than other breeds), seasonal differences in the vigor of eggs, minor temperature fluctuations in incubators, and relative humidity fluctuations due to the size and type of incubators. Therefore, the stages possess typical characteristics but overlapping time points, especially in the case of early embryos (day-0 to

day-4). The stages used for the current study are HH 1 to HH 38. The description of these stages is summarized below for their known general characteristics.

HH Stage	**Major characteristic of embryo**	**Embryonic Age (in Days Post Laid)**	**Other stage-specific characteristics of the embryo**
1	Pre-Streak	Less than 12 hours	Embryonic shield formed due to aggregation of the cells at the posterior region of blastoderm, area pellucida and area opaca visibly discriminable
2	Initial-Streak	Less than 12 hours	Presence of short conical primitive streak at the posterior region of area pellucida
3	Intermediate-Streak	12 – 13 hours	Length of primitive streak till the center point of the area pellucida
4	Definitive Streak	18 – 19 hours	Maximum length of primitive streak
5	Head-Process	19 – 22 hours	A notochord extending from Hensen's node towards anterior edge of area pellucida
6	Head-Fold	23 – 25 hours	A blastoderm-fold at the anterior end of the embryo
7	One somite	23 – 26 hours	The first visible somite, actually being second one (first somite still not developed fully), neural folds at head
8	Four somites	26 – 29 hours	Blood islands visible
9	Seven somites	29 – 33 hours	Primary optic vesicles
10	Ten somites	33 – 38 hours	Three brain vesicles
11	Thirteen somites	40 – 45 hours	Cranial flexure, heart bent to right
12	Sixteen somites	45 – 59 hours	Well established optic vesicle and optic stalk
13	Nineteen somites	48 – 52 hours	Enlarged telencephalon
14	Twenty-two somites	50 – 53 hours	Cranial flexure complete, body rotated at somites 7 – 9
15	Limb primordia	50 – 55 hours	Lateral body fold extends to somites 15 – 17
16	Tail bud	51 – 56 hours	Flat primordium of leg, oval third cleft of visceral arches
17	Epiphysis	52 – 64 hours	Nasal pits
18	Allantois	65 – 69 hours	Closed amnion, rightward turned tail bud

HH Stage	Major characteristic of embryo	Embryonic Age (in Days Post Laid)	Other stage-specific characteristics of the embryo
19	Equal maxillary and mandibular processes of visceral arches	68 – 72 hours	Unpigmented eyes
20	Grey eyes	70 – 72 hours	Completely rotated body, maxillary arch exceeds in length from that of mandibular processes
21	Maxillary process extension to the middle of the eye	3½ days	Faint eye pigmentation
22	Somites extension to tail-tip	3½ days	Distinct eye pigmentation
23	Limbs	3½ – 4 days	Stout limbs as long as wide
24	Demarcated toe plate	4 days	Toes not demarcated in hind limbs; Wing digital plate not demarcated
25	Elbow and Knee joints	4½ days	Faint grooves between hindlimb toes
26	Demarcation of first three toes	4½ – 5 days	Third and fourth visceral clefts now not visible
27	Small beak	5 days	Grooves between first, second, and third toe visible; barely visible beak; eye size smaller than the forebrain knob
28	Second and third toe longer than others	5½ days	Visibly grown beak with lower beak still not protruded out; eye size equals to or larger than the forebrain knob
29	Second digit of wing longer than others	6 days	Eye visibly larger than forebrain knob; wide space between upper and lower beak
30	Visible first toe	6½ days	Forebrain protuberance smaller in size; forelimbs with distinct digits
31	Feather papillae on hindlimb stylopod	7 days	Web between first and second digit
32	Web digestion	7½ days	Early signs of web digestion both in forelimb and hindlimb by thinning of webs; First digit of forelimb visible with thinner interdigital membrane; All toes visible with thin interdigital membrane; Head knob almost flattened
33	Three rows of feather germs on tail	7½ – 8 days	Third digit of forelimb detaching from others with thin membrane in between;

HH Stage	Major characteristic of embryo	Embryonic Age (in Days Post Laid)	Other stage-specific characteristics of the embryo
			Upper and lower beak coming together
34	Limb elongation	8 days	Highest growth in length of stylo, zeugo and autopods of both limbs; Forebrain knob absent; early signs of feather germs visible tracts
35	Feather papillae at sternum	8 – 9 days	Nictitating membrane migrating towards scleral papillae
36	Feathers papillae arranged in multiple rows around the umbillicus	10 days	Dorsal body line, limbs, and tail feather-germs; egg tooth on beak; labial groove visible; nictitating membrane approaching cornea
37	Feather primordia around eyes	11 days	Onset of cornification in hindlimb dorsal side; pads on plantar surface; nictitating membrane reached anterior edge of cornea
38	Primordia of scales on hindlimbs	12 days	Eyelids leaving only a smallest area of cornea exposed

Table 2: *The major characteristics of the stages of chick embryos according to the classification of Hamburger-Hamilton (1951).*

Development of chick embryo conveniently occurs without complete dependence on water due to some unique features of the eggs of the species of this class. These features part these organisms from the water-dependent tetrapods like amphibians. This particular type of egg is called an **amniotic egg** which is also observed to be laid by reptiles (Hickman et al., 1984).

AN AMNIOTIC EGG

Eggs of all the amniotes viz. birds, reptiles and mammals, bear the four common fetal membranes, namely chorion, allantois, amnion and yolk sac. The amniote eggs protect the embryos from desiccation which is a necessity to the vertebrates which are independent of an aquatic system for laying eggs.

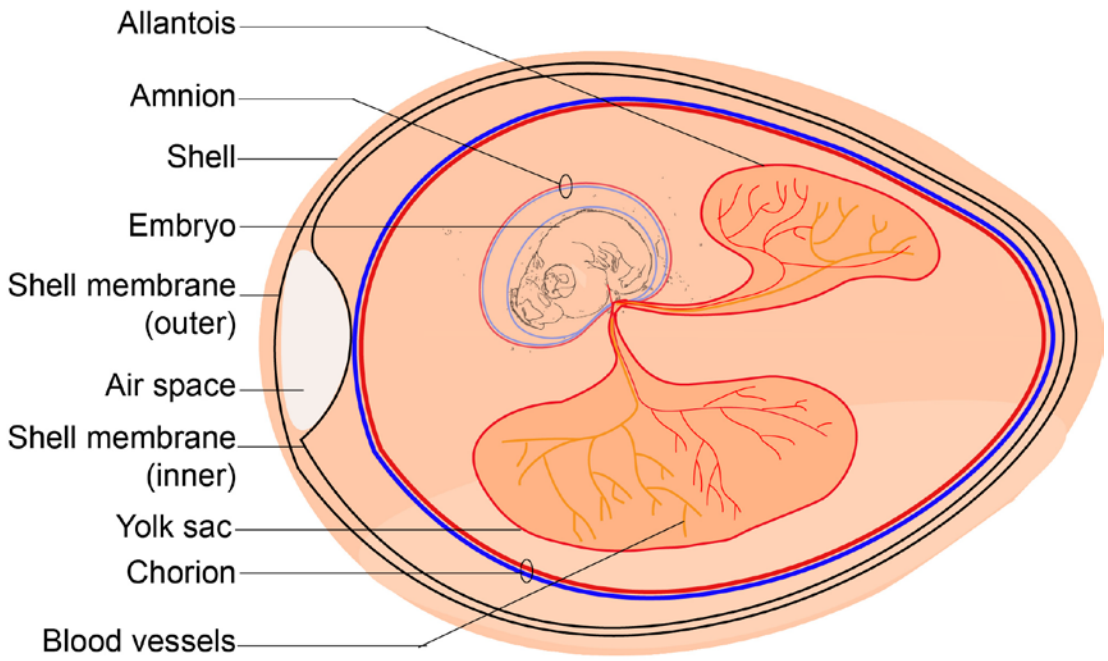

Figure 3: *A typical amniote egg. The outermost layer is a calcareous shell. Next to the shell toward the interior are the shell membranes – outer, and inner. Both the shell membranes are wide apart forming the air space at the blunt edge of the egg. The further interior is the extraembryonic membrane – the chorion. The embryo is covered with another embryonic membrane – the amnion. The third membrane – the yolk sac is rich in blood vessels. The allantois is connected to the embryo with the help of a stalk.*

Reptilia, aves and mammalia are the classes of vertebrates collectively known as amniotes. Egg of domestic hen and most birds have a typical structure consisting of a shell, double-layered shell membrane with an air-cell at a blunt end, thin and thick albumen arranged in concentric layers surrounding the yolk and chalazae positioning the embryo proper (figure 3). There are various methods of inoculation in eggs of birds – the injection routes like yolk sac, chorioallantoic membrane, amniotic cavity, allantoic sac and air cell. The air cell method is the safest as far as the side-effects are concerned wise. The normal development of chick embryo inside this kind of egg can be traced time to time from outside using the traditional candling method. The new methods of open culture are developed for better observation of what goes on when the chick embryo develops. In those methods, the embryo is emptied in flask over a solution added with antibiotic and antimycotic to prevent infection. The whole procedure is performed in sterile chambers or rooms. The calcium and other minimal requirements are to be supplied, and one can observe the normal development of embryos outside the shells (Tahara and Obara, 2014). Such a system is known as a shell-less culture system, which can even be used to more easily add the test chemicals or coated beads on the embryo and documenting the real-time changes with the help of digital cameras.

The drawback of this visually appealing experiment type is that getting rid of an infection or contaminants in the exposed egg is a real challenge. Also, the higher mortality rate can mislead the experimenter to judge the real effects of test chemicals. The more straightforward system than this is the windowing and observing live embryos throughout the incubation (Korn and Cramer, 2007). The drawback is that mortality is high in this case too. Most commonly, the disturbances occurred during the windowing process, and repeated observations are the culprits. Therefore, the traditional time lapse study utilizing the whole egg is largely preferred. Owing to the excellent features of this organism as a model, numerous researches have been conducted utilizing the chick embryos. Some of which are listed below.

CONTRIBUTIONS OF CHICK EMBRYO TO THE DEVELOPMENTAL CONCEPTS

An embryo transforms into a hatching chick undergoing numerous processes, some of which are identified and well-defined by embryologists. As an organism chick has been one of the major contributors in the field of vertebrate embryology.

The mechanism of neural tube formation from the ectoderm of chick embryo was the first-ever study revealing cell's ability of **competence** to the **induction** (Waddington, 1934). Competence is the primary developmental process occurring in all the organisms for development of several structures such as a primitive streak, lens, and nasal structures (Waddington, 1936; Fujiwara et al., 1994).

The concept of **regulative development** got established with the experiments conducted in chick embryos (Spratt and Hass, 1960). It was observed that a chick embryo cut into several (about 50 thousand) pieces could generate the whole normal embryos from each of the pieces. Such a developmental pattern was later observed in most of the vertebrate organisms.

The major question of differential development on the left and right sides of the embryo was solved using the chick embryo as a model. The early-identified players behind a left/right asymmetrical development are Nodal, Shh, and Activin-receptor – which were identified first in the chick embryos (Wolpert, 2004).

The character of the chick embryo, most useful for the division of the developmental stages, is the somite. Formation of somites occurs via expression of specific genes in a cyclic

manner. These genes are called the **oscillating genes**. The first oscillating gene was discovered in the chick embryo, which was responsible for somite formation, namely hairy (Gibb et al., 2010). Such rhythmically expressing genes were then identified not only in the vertebrates, but in the invertebrate organisms as well.

The signalling cascade of expression and repression signals for several *pax* genes in case of spinal cord development was first identified using the tissue grafting experiments in chick embryos (Tanabe and Jessell, 1996). Not just the molecular details, the most of what is known about embryogenesis of humans is derived from the work in chick embryos. The early study in the field of neural development can be traced back to 1959 when the formation of nissl substance was visualized using the microscope (Bellairs, 1959). Role of bone morphogenetic protein (BMP) in the neural development was established in the same experimental model for the first time (Faure et al., 2002). Our molecular-level understanding of the embryogenetic players is only due to the studies in model organisms, and the chick has played a central role in it. Owing to the considerable similarity in the genes and proteins of chicken and human, the work in chick embryos can be extrapolated to add in the knowledge of human development (Hillier et al., 2014).

ORIGIN OF THE PROBLEM

About three decades down the line since its discovery by Vane and colleagues, COX-2 is still debated upon for its nature, whether being constitutive or inducible. Most articles in the earlier era referred to this isoform as an inducible isoform of COX. Stanfield and coworkers pointed out the presence of COX-2 protein in mice embryonic and fetal tissues such as heart, kidney skin, and cartilage (Stanfield et al., 2002). However, the exact mechanism of its action in embryogenesis remains to be studied. On the other hand, COX-2 specific NSAIDs are recorded to cause the birth abnormalities as recorded in epidemiological studies (Li et al., 2018). Studies also dictate its ability to cause congenital disabilities, even if consumed at the time of conception (Li et al., 2003). Therefore, it is crucial to unearth the mechanisms by which such developmental artefacts are triggered via COX-2 inhibition at any stage around the zygote formation or even at the egg cell (ovum) production. Vertebrate embryonic development always surprises the researchers in the way the pathways work entangled to each other. The simultaneous line of work in the lab proved that COX-2 could also affect other signaling pathways during appendage regeneration in the northern house gecko (Buch

et al., 2018). The genes, whose expression is modulated by COX-2 in that case, are also active during embryonic life. This initial knowledge about COX-2 and the associated processes and pathways in post-embryonic life led to the curiosity of COX-2 being able to modulate similar pathways during embryogenesis.

For understanding plausible roles of COX-2 in the vertebrate system, the chick was selected as a model in the current study. Inhibition of COX-2 in chick led to axial deformities in mice (Shim et al., 2010). These results suggested the plausible roles of COX-2 in embryogenesis of mammals. However, the least information is revealed so far in this direction. An ability of both the isoforms to compensate for each other's actions is also debated upon vigorously in the scientific world. The only abundant product of COX-2 is PGE_2, which has been identified to participate in most COX-2 driven biological processes. The ever-debated question about nature of COX-2 led to the path of this research work, which focused on its embryogenic activities performed via PGE_2. Nonetheless, the possibility of simultaneous action of prostanoids such as PGD_2, $PGF_{2\alpha}$, and TXA_2 was also studied.

OBJECTIVES OF THIS STUDY

This work aimed at identifying unknown roles of COX-2 derived PGE_2 during the embryonic life of the domestic hen. The objective was divided into the following specific aims.

1. To ascertain the nature of COX-2, whether constitutive or inducible, during the embryonic life of the chick of the domestic hen
2. To identify the key prostanoid effectors of COX-2 in the embryonic development of the hen
3. To study the roles of COX-2 induced PGE_2 (identified based on the results of objective 2) in the development of limbs

REVIEW OF LITERATURE

COX-2 induced PGE_2 is involved in inflammatory pain sensitization in post-embryonic life of vertebrates. A careful scan through the available literature revealed that only sparse information is available about the roles of this molecule during embryonic life. It is suspected to be involved in some cellular processes during the early and later phases of embryonic development. These processes involve proliferation, migration, and differentiation. One of

the studies proved the regulatory role of PGE_2 over differentiation of keratinocytes (Evans et al., 1993). Jacoby and coworkers found that inhibition of COX-2 by SC236 reduced cell growth by arresting them at the G_2-M phase of the cell cycle (Lanza-Jacoby et al., 2004). COX-2 inhibition via NS-398 reduced growth of cancerous cells – human esophageal adenocarcinoma cells – via alleviating levels of FGF2 expression (Baguma-Nibasheka et al., 2007).

Ample literature can be cited for the roles of COX-2 and PGE_2 in the developmental processes before pregnancy and after birth. For instance, it is known now to be participating in the decidualization, ovulation, implantation, as well as fertilization processes in mammals (Dinchuk et al., 1995; Lim et al., 1997). Some reports are available proving roles of COX-2 in organogenesis. Its inhibition impairs closure of ductus arteriosus in mice (Loftin et al., 2000). Moreover, in the studies conducted in our lab proved COX-2 derived PGE_2's roles in the process of regeneration. These studies were carried out in the northern house gecko (*Hemidactylus flaviviridis*). These roles were directly correlated with the process of cellular growth and inflammation. COX-2 was found to affect the Wnt-β-catenin pathway during the process of wound healing as well as blastema stages of epimorphosis (Buch et al., 2017). Another study in the same line proved the interaction of COX-2 with MMP (matrix metalloproteinase) and FGF (fibroblast growth factor) in the tail of lizard (Buch et al., 2018).

RATIONALE OF THE STUDY

The mysteriously acting COX-2 and the prostanoids as its effectors are known to participate in several developmental processes post-embryonically. However, the curiosity to know its roles in embryogenesis led to this research.

Etoricoxib as a test chemical

The investigation aiming the functions of the second isoform of COX could not have been accomplished without the test chemical that is explicitly acting on COX-2. Etoricoxib came as an option having the highest specificity to COX-2 binding relative to COX-1 binding in comparison with other available NSAIDs (figure 2). Etoricoxib competitively inhibits COX-2 enzyme letting the transcripts of the same unharmed. This allows one to decide the modulations in the expression pattern of other affected pathways. At the same time, the COX-2 activity is reduced while still having less lethality of the subject organisms, i.e. chicks. The

genetic modulations often lead to irreversible changes in multiple organ systems and massive mortality. Besides, this kind of genetic chimera is tedious to develop and challenging to maintain until the required timepoints in incubation.

The choice of the COX-2 pathway

The inhibitors of COX-2 are the most prescribed medicines of the current times across the globe (Wongrakpanich et al., 2018). The marketing of any medicine is preceded by an array of experimentation in several models of drug testing. However, investigation of the dose and frequency-dependent accumulative effects on the embryonic development is often omitted due to the delay it might cause in doing so. The real-life situations also vary from the lab system in a way that every organism's physiology varies according to the varying environmental conditions. The combination of the medicine with smoke, alcohol, or another known teratogen may be elevating the side effects in the real-time cases. Many such reasons lead to confusion about the plausible side effects of the safe-proven medicines in humans and other animals. The COX pathway is poorly studied for several reasons so far. One that its effectors are synthesized at very low concentrations by the human body. Most prostanoids at their active state are present in nanograms per ml concentration range. Besides, their half-lives are few seconds. This makes it difficult to evaluate with simple biochemical methods. Overall, the less studied molecule COX-2 when evaluated for its roles in the regeneration process, showed up being upstream of other pathways such as Wnt/β-catenin, MMP, and FGF. Owing to the gaps in the knowledge of embryogenesis players in vertebrates, COX-2 was studied as the candidate in this process.