Summary

Cyclooxygenase-2 (COX-2) – a widely known pro-inflammatory enzyme, was studied for its presence, nature, activity, and plausible functions in the embryonic stages of chick of *Gallus domesticus*, specifically at the ones proven to be devoid of inflammatory responses (Kain et al., 2014). This research also focussed on exploring the downstream effectors of COX-2, responsible for the outcomes of its inhibition in the embryonic life. Further, one of the numerous processes in which COX-2 was found to be participating – limb development, was studied in-depth for evaluation of the underlying mechanisms of its effect.

Lastly, the work was extended to investigate the implicational aspects of the fundamental biology, to rationalise the current idea of using COX-2 specific pharmacological inhibitors for managing the condition arising in the case of an X-linked disorder – muscular dystrophy. The thesis is overall divided into five chapters, out of which, the first one discusses the general introduction of pathways and processes worked upon, in this research. The second one outlines the methods used for deriving conclusions out of the work, planned based on three main objectives of this research. These objectives are addressed sequentially in chapters 3, 4, and 5 in this thesis.

The study was envisaged considering involvement of COX-2 in the regenerating phenomena as observed in *Hamidactylus flavivirdis* and *Poecilia latipinna*, during previous studies in the lab (Buch et al., 2017; Anusree et al., 2011). The pathways functioning in case of regenerating system may differ from those working during the embryonic development of the organisms (Purushothaman et al., 2019). With the motive to understand the extent at which COX-2 could be involved in driving the mechanisms of embryogenesis, first objective of the thesis was designed to follow only its presence by studying the gene, protein, activity, and localization, in the tissues of embryo until organogenesis. In order to generate a fundamental knowledge which can be further extrapolated on humans, domestic hen, *Gallus domesticus*, was used as a model, owing to its striking transcript level similarities with the former, for the molecules involved in basic processes of life (Hilliers et al., 2014).

Fertile eggs of Rhode Island Red breed were duly incubated to harvest the embryos at several stages between day-1 to day-12 of development, for identifying the basal level of COX-2. Gene expression study revealed that COX-2 was present at extremely low level until day-9, when it surged, and settled back to the basal level on day-10. In contrast to that, its protein level

and activity were higher and synchronised with each other (similar protein and activity trends). The downfall of COX-2 protein and activity on day-2, was followed by a gradual decrease till day-10, which are the pivotal stages of organogenesis. Although the gene expression and activity did not run parallel, the latter showed consensus with protein level. Localization studies revealed that COX-2 shows widespread distribution in the whole embryo of chick.

As widely accepted, a relatively smaller structure as an embryo, aspiring to become a comparatively huge organism in short time-span, does not carry unnecessary load along with it as an approach to conserve the energy of packaging. Provided the negligible gene expression of COX-2, it is proposed in this research that, it is carried along from egg (maternal inheritance) in the form of m-RNA or protein. The work has a scope of a further investigation about the origin and mobility of COX-2 protein in the embryonic stages. All in all, the otherwise widely known as inducible isoform of COX enzyme, COX-2 acted more or less constitutive during embryonic life of chick, which was not exactly like a housekeeping protein due to its undulating pattern in the course of development rather than its constant stable presence overall. The modulation in COX-2 activity and protein expression has a scope of a more elaborate study, to understand spatially specific patterns of its function. The upstream regulators of COX-2 protein also remain unknown till date for an embryonic phase. In the post-embryonic life, it is known to be stimulated by various inflammatory cytokines (Herschman, 2003).

Immunolocalization of COX-2 using pNPP-alkaline phosphatase reaction, enables us to derive the conclusions about its spatial distribution at various stages of embryogenesis. COX-2 protein was present since the very first day of development, which got more concentrated to certain tissues with the growing embryonic age. Day-1 embryos showed sparse distribution of COX-2 all over the length of the embryo and around its periphery. The somites and growing optic vesicles especially showed higher coloration as compared to all other places in day-1 embryo. It got more localized to heart tube and in tissues around somites in the day-2 embryo, further getting concentrated to head, eyes, and allantoic vesicle on day-3. The completely looped heart of day-4 showed the presence of COX-2 in walls and even in the interiors of heart. Day-5 embryos expressed COX-2 in the whole length of developing limbs. At this stage, limb length as well as skeleton is found in growing stage. The most important cross-talk between apical ectodermal ridge (AER) and mesenchyme, maintained by fibroblast growth factor (FGF) signalling leads to the heightened cellular proliferation in the limbs (Gilbert, 2007). The presence of COX-2 in epithelium throughout the limb development raised

the speculation of its impact on FGF signalling, which was studied in detail and described in chapter-5.

COX-2 was further localized in the tissues of developing kidneys during day-6 of embryonic life, the timepoint which also showed its presence in the cells at the posterior-most tip of embryos, near tail. The embryos grow in length overall via increased proliferation of cells marking the edges of their bodies, as compared to the lesser proliferation rate towards the central regions of embryos, as the rate of cell-cycle is affected by several factors during axis development (Cairns, 1977). Presence of COX-2 in the proliferating regions defines its role in regulating proliferation rate directly or indirectly (via other factors such as FGFs) in the whole embryos. Its presence in kidney can be related to the available literature, suggesting its effects on kidney functions post-embryonically (Harris, 2000; Goetz Moro et al., 2017). Moving further, day-7 onwards COX-2 got localized continuously in the developing nerve cord and testis till day-10 as per the observations of current work. Deciphering its presence and role in the developing chick after day-10, needs further investigation, while the current research suggested the critical functions of COX-2 in the embryogenesis for the first time.

An attempt was made to inhibit COX-2 activity so as to understand its functional aspects in early embryogenesis (during organogenesis phase) using a pharamacological inhibitor - etoricoxib, which was more than hundred folds specific to COX-2 than to COX-1 (Lu et al., 1995). Inhibition first of all, led to the alleviation of COX-2 activity, which was quantified using an ELISA kit (chapter-3). The total COX activity however, did not diminish completely, which led to the curiosity about the activity status of another isoform of COX-1 and its functional contribution during the same stages. The experiments revealed that there was no conspicuous expression of COX-1 at transcript and protein level during day-1 to day-10 of (control) chick embryos. There was a sudden surge in activity of COX-1 during inhibition of COX-2 especially at day-2, day-6, and day-10 (when COX-2 activity further decreased), resulting in partial maintenance of total COX activity. Just to cross-check that such variations in activity were the result of etoricoxib administration, the control and treated embryonic homogenates were checked for the presence of etoricoxib peak in the liquid chromatographic profile based on the mass spectrometric characteristics (chapter-3). The results showed that there was no etoricoxib-like compound in the control embryos, and that the suspected peak of etoricoxib in treated embryos matched the peak generated in the standard solution.

From the experiments done so far, it was pretty evident that there must be a cross-talk between COX-2 and the genes driving organogenesis in chick embryo. However, before getting into the details of which of the pathways are affected when COX-2 is inhibited, it was necessary to pinpoint the downstream embryonic effector of COX-2. The major effectors of COX-2 in biological systems are four prostanoids, namely Prostaglandin E_2 , Prostaglandin D_2, Prostaglandin $F_{2\alpha}$, and Thromboxane A_2.

In order to reveal the embryonic effector of COX-2, literature was reviewed for the methods available so far. It was found that, ELISA methods were increasingly inaccurate for measuring the concentrations at nanogram level. The available LC-MS/MS methods were derived for the liquid samples, such as urine, serum, and cerebrospinal fluid (Brose et al., 2013). Some other methods were worked out on brain and spinal cord samples, which are differently composed organs comprising a lot of interstitial and connective tissues (Golovko and Murphy, 2008), unlike early embryonic tissues of chick. The available methods resolved only few of the major prostanoids at one time by liquid chromatographic conditions (Cao et al., 2008; Brose et al., 2013), while there was a need to resolve all the four prostanoids efficiently for their accurate quantification, from the sample which may contain a mixture of prostanoids as well as isoprostanes. Thus, this work was extended to standardization of prostanoids' LC-MS/MS from chick samples as described in chapter-4. A rapid methodology to extract prostanoids from soft embryonic tissue was derived using day-2 chick embryos. Standardization of appropriate liquid chromatography parameters and identification of unique mass spectrometric characteristics of these prostanoids was done at The Metabolomic Innovation Centre (TMIC), University of Alberta. This study for the first time, dictates the complete methodology to isolate and quantify four major prostanoids from the embryonic samples of chick of domestic hen.

The standardized method for LC-MS/MS of prostanoids finally derived the inference that PGE_2 is the only prostanoid out of the four major ones, which is decreased significantly with the declining COX-2 activity via etoricoxib administration (chapter-4). Having known the embryonic effector of COX-2, the focus was then shifted to find out the plausible connection of COX-2 with developmental pathways for one of the organs, the limbs. Limbs were selectively studied after screening various anomalies observed in the etoricoxib treated embryos (chapter-5), ranging from defects related to vascularization, craniofacial sculpture, ventral body wall closure to the aberrations in limb development. Two types of limb structural defects were most frequently present in the etoricoxib-treated embryos, namely limb-length reduction/increase, as well as lack of web digestion in hindlimb. To address the mechanisms

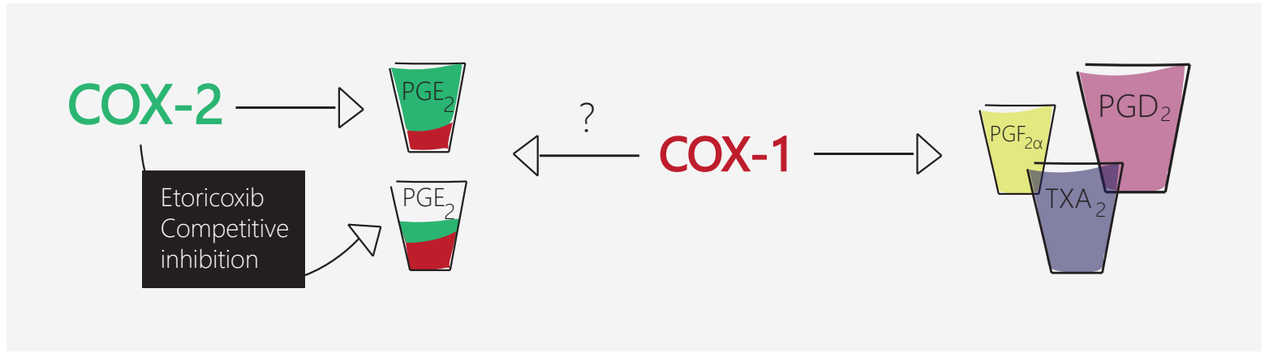
of these defects, the genes related to this kind of development were listed down, out of which, FGF9 and sonic hedgehog were found to be modulated in case of treated embryos as compared to their respective controls in forelimb as well as hindlimb of chick embryos. Interesting outcomes such as generation of excessive skeletal components was observed in day-7, which could be correlated to the significant upsurge of FGF9 during the same time point, in case of COX-2 inhibited embryos. Lower than normal level of FGF9 has already been correlated with decreased number of skeletal components in one of the earlier studies (Mariani et al., 2008).The lack of web digestion was correlated with modulation in expression level of several genes following COX-2 inhibition, which included FGF9, GLI3, gremlin1, noggin, sonic hedgehog, FGF8, bone morphogenetic proteins (2 and 7), as well as tissue inhibitors of matrix metalloproteinase3, ultimately leading to deranged and displaced expression of cleaved Caspase3 protein in the hindlimb autopods of treated embryos (chapter-5). This study empirically proved the crosstalk between COX-2 and limb developing genes for the first time.

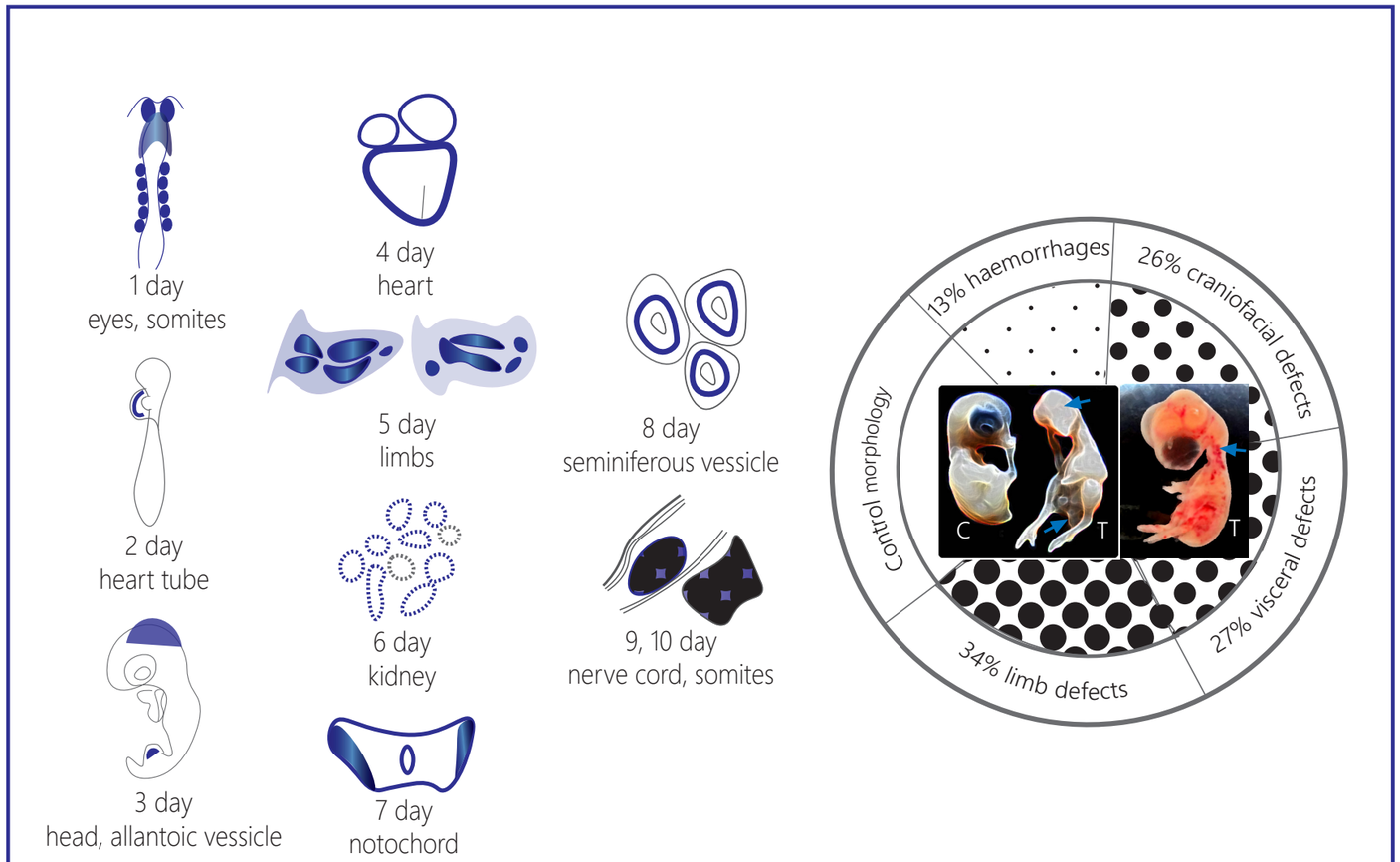
Finally, the principle function of COX-2 in embryogenesis, was taken further to the disease biology, by checking how COX-2 inhibition could have affected the dystrophic cells. For this part of objective 3, the standardization was carried out to generate chick embryonic dystrophic skeletal muscle cells in-vitro (chapter-5). Herein, the cells were isolated from 11 days old chick embryonic limbs, and were plated using the explant method of cell culture (Urja et al., 2018). They were added with anti-α-dystroglycan antibody, to disrupt the sarcoplasmic assembly of dystrophic proteins. The dystrophic cells developed in this set up, were then used to check the effect of etoricoxib, which showed that inhibition of COX-2 could downregulate TGFβ1 factor, which is usually associated with inflammation and low contractibility of myotubes, leading to hindrance to muscle functions. However, these cells did not show increased expression of any of the myogenic proteins, namely MYOD, MYOG, and LAMA2. Therefore, it was inferred that more research is necessary before concluding the managerial effects of COX-2 specific inhibitors on the dystrophic symptoms. This study however, successfully developed a novel in-vitro model for study of disease biology of muscular dystrophy (Urja et al., 2018).

In summary, this work generates a fundamental understanding of nature and functions of second isoform of COX enzyme along with the revelation of its functional effector (PGE_2), by relating it to organogenesis phase of chick embryonic development. The attempt of quantifying the main prostanoids from their mixture present in the embryonic samples, was first of its kind. The specific inhibitor of COX-2 caused developmental anomalies in various

organs, out of which, the in-depth experimentation on limb development revealed the role of COX-2 induced PGE_2 in limb lengthening and web digestion. Ultimately, the existence of crosstalk between COX-2 and limb developing genes was established. In the last segment of the work it was found that etoricoxib cannot act as potent medicine against dystrophy due to its inefficiency in upregulating myogenic genes, which were rather decreased by it in absence of COX-2 activity.

NATURE AND FUNCTIONS OF CYCLOOXYGENASE-2 IN EMBRYOGENESIS

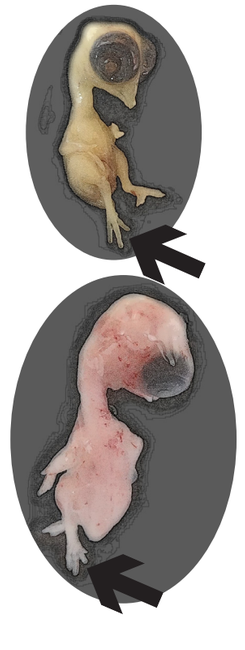




COX-2 immunolocalization in control embryos

Web digestion

	Control			Treated		
	Day-7	Day-8	Day-10	Day-7	Day-8	Day-10
TIMP3						
CASP3						
FGF8						
SHH						
SMO						
BMP2						
BMP7						
FGF9						
GLI3						
GREM1						
NOG						



Limb length

	Control				Treated			
	Day-6	Day-7	Day-8	Day-10	Day-6	Day-7	Day-8	Day-10
FGF9 FL								
FGF9 HL								
SHH FL								
SHH HL