2

MATERIALS AND METHODS

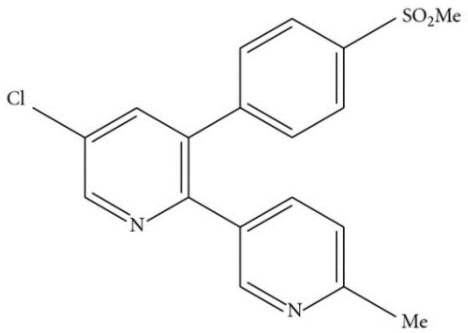
The objectives of the study were achieved using multiple methods in different sets of experiments. There are a few protocols commonly used for all the objectives, such as sterilisation of eggs, candling, grouping of eggs and dosing. These basic procedures were followed by specific protocols for the respective experiments, such as immunolocalisation, In situ hybridisation, enzyme activity assay, morphometry, qualitative and quantitative analysis of biomolecules (nucleic acid and proteins). The basic procedure included procurement of eggs followed by dosing. The dose of the COX-2 inhibitor was selected based on a previously done dose range study (Verma et al., 2021). The detailed protocol, as well as sample preparation for experiments, are described in the following sections.

Procurement of Animals

Fertilised Rhode Island Red eggs were obtained from government-run intensive poultry, Vadodara unit. The fertilised eggs were candled to locate the air sac and disinfected with Povidone Iodine solution (Win-Medicare, New Delhi, India), and were maintained at 25°C till the time of dosing. All the protocols were approved by Institutional Animal Ethical Committee (IAEC) under protocol number IAEC No. MSU-Z/IAEC/09-2020.

Test chemical

The inhibition of COX-2 was carried out using the pharmacological inhibitor Etoricoxib. It is more specific to COX-2 as compared to other available COX-2 inhibitors (Martina et al., 2005). Pharmacological grade etoricoxib was received as a gift from Sun Pharma Advanced Research Company, Vadodara. The physical and chemical properties of etoricoxib are as follows:

Chemical Name	5-chloro-2-(6-methylpyridin-3-yl)-3-(4-methylsulfonyl phenyl) pyridine
Molecular formula	$C_{18}H_{15}CIN_2O_2S$
Manufacturer	Sun Pharmaceutical Industries Ltd.
Physical appearance	Amorphous solid
Storage condition	Store in dark and cool place (4°C)
Chemical structure	
Solubility	Water 80µg/mL Polyethylene Glycol (PEG-400) 2mg/ml (Nayak & Panigrahi, 2012)

The previous study based on the dose range of Etoricoxib during the early developmental stages of the chick embryo indicated that the vehicle control did not show any deformities. The vehicle control embryos were identical to the control embryos (Verma et al., 2021).

Etoricoxib dose preparation: 3.5µg/50µl

- Deionised water (450 ml) was placed on a magnetic stirrer at medium speed at room temperature.
- The 0.035gm of Etoricoxib was weighed in 1ml MCT, and 1ml of deionised water was added. The mixture was vortexed for 5 minutes.
- The prepared mixture of Etoricoxib was added to the deionised water placed on the magnetic stirrer dropwise using a micropipette.
- The mixture was stirred for 2 hours, and the remaining volume was made up by adding deionised water.
- The prepared solution was stored at 4°C for further use. The shelf life of the solution is one month.

In ovo dosing

The eggs were divided into three groups: control, vehicle control and treatment. The treatment group of the eggs were subjected to 50µl (3.5µg) prepared dose of etoricoxib, whereas the vehicle control group of the eggs were subjected to 50µl of deionised water. Dosing in fertile eggs was carried out by the method described by Blankenship and co-workers (Blankenship et al., 2003).

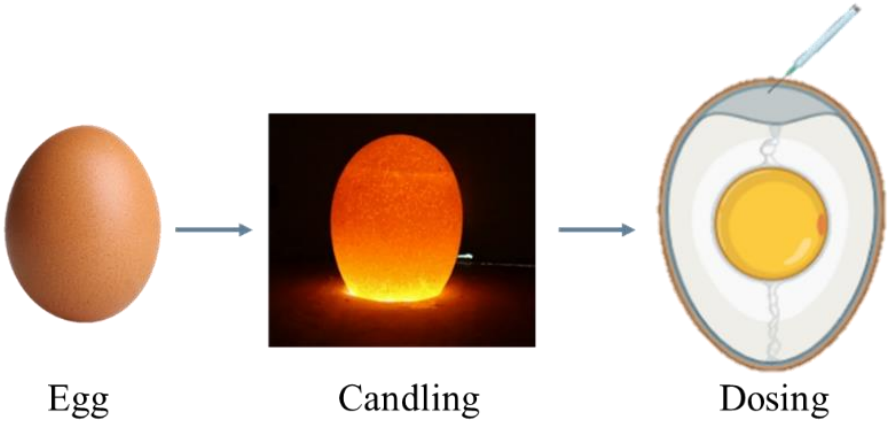


Figure 2.1: Candling and air cell dosing method for egg

The air cell was punctured using a sharp pointed sterile needle under the laminar air flow (Fig. 2.1). Immediately, 50µl of Etoricoxib or deionised water was injected using a 0.1ml sterile insulin syringe with a 36-gauge needle (BD, Haryana, India). The opening was quickly sealed after injection using molten paraffin wax. The control group of the eggs were not treated with anything. After dosing, the eggs were kept upright for 30min for the diffusion of Etoricoxib to the developing embryos from the air cell.

Incubation & embryo collection

Eggs from all three groups were kept in the incubator (Model no. 3940, Thermo-Fischer Scientific, USA) at 37±2°C and 60-70% relative humidity. Eggs were kept inclined at an angle of 45° and turned on an interval of 2 hours till the isolation time. The egg rotation is necessary to prevent the membrane from sticking to the eggshell. The incubation time was chosen based on the chick embryo developmental stage series given by Hamburger–Hamilton (1951).

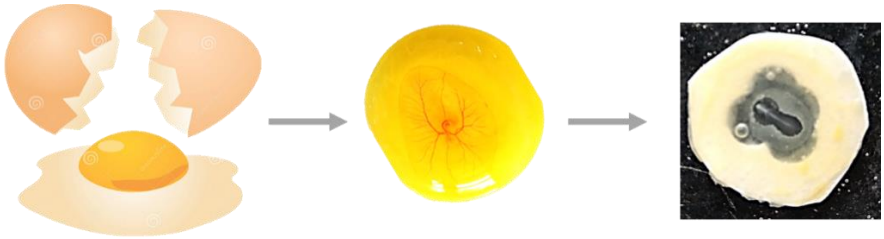


Figure 2.2: The isolation of embryos at the early stages of development was done by filter ring method (Chapman et al., 2001)

The entire study focused on early developmental stages, and the embryos were isolated at HH6 to HH20 stages based on the objectives. The embryos were isolated by the filter ring method due to their small size and fragile nature (Chapman et al., 2001). In the filter ring method, the eggs were horizontally placed for 5min before isolation. The eggs were cracked open horizontally in a petri plate, while taking care that embryo remained attached to the yolk (Fig 2.2). As a result of this, the embryos were found to be placed on the top of the yolk and surrounded by thick albumen. The latter was gently wiped out using Whatman filter paper (grade 3, GE HealthCare, USA). Removal of thick albumen exposed the vitelline membrane, beneath which the embryo grows. A filter ring of size 1cm to 3cm was placed on the vitelline membrane such that window came over the embryo and other sides adhered to the vitelline membrane. This filter ring was kept on the membrane for 10sec for the membrane to adhere firmly to the filter paper and then, the membrane was cut at the outer edges using a sterile scissor by holding the filter ring with blunt forceps on one corner. The filter ring was gently lifted with the embryo attached to the window. The embryo was washed in autoclaved PBS and the respective HH stage was identified by observing it under the light microscope (Magnus, New Delhi, India). These embryos were then collected in different storage solutions for further experiments.

Morphological analysis

The effects of COX-2 inhibition were studied through phenotype analysis of developing embryos. Craniofacial abnormalities were noted along with their frequencies. Deformities related to the neural tube, eye, beak and heart patterning were found to be the most recurrent.

The sample size was 30 eggs for the control, and 50 eggs for the treated group and experiments were repeated multiple times. The sample size was bigger for the treated group than the control group to compensate for mortality in the former one. Thus, it was

ensured that the sample size was even for all the experiments. The abnormal phenotypes of COX-2 inhibited embryos were further studied in detail and compared with control. The prime contributors of craniofacial patterning, neural crest cells' delamination and migration were analysed in HH6 to HH20 staged embryos. In addition to that, a type of neural crest cell known as cardiac neural crest cells, which participates in heart tube patterning were studied in HH12 to HH20 staged embryos. The early patterning of eye was analysed in HH12 to HH16 staged chick embryos. The evidence for phenotypic deformities was collected in the form of images using a camera (Nikon, USA) or a microscope (Leica DM2500, Germany).

COX activity assay

The total COX activity along with its isoform's activity was measured in all the experimental groups. The study primarily focused on the development of the skull and face, using early stages of embryonic development ranging from HH6 to HH24. At HH6, the neural crest cells, which play a major role in craniofacial development, begin to emerge. The migration and differentiation of these neural crest cells occur throughout various developmental stages. A colorimetric assay was performed to determine COX-1, COX-2 and total COX activity in stage HH6 to HH24 embryos. Negative control, standard controls and treatment samples were assayed as per the manufacturer's description (Cayman Chemicals, USA). The inhibition of COX-2 was measured by its activity in a developing embryo using the same assay. The peroxidase activity of COX-2 oxidises N,N,N′,N′-tetramethyl-p-phenylenediamine dihydrochloride (TMPD), which was measured at 590nm using a plate reader (Bio-Rad, USA). The specific isoform activities were calculated by dividing the total protein value by repeating the assay three times. The following formula was used for the COX-2 activity calculation.

$$\text{COX-2 activity} = \frac{\Delta A_{590}/5\text{min}}{0.000826\mu\text{M}^{-1}} \text{ X } \frac{0.21\text{ml}}{0.04\text{ml}} \div 2^{*} = \text{nmol/min/ml (U/ml)}$$

PGE_2 estimation

The activity of COX enzyme results in the production of various prostanoids, and amongst those the PGE_2 is the highest produced prostanoid. Levels of PGE_2 produced by the COX enzymes were estimated by PGE_2 sandwich ELISA kit as per the manufacturers' protocol (R & D systems, USA).

The embryos from different experimental groups were collected and homogenised in sterile double distilled water at a ratio of 1gm per 4ml. Extraction of prostanoid from the embryo homogenate was done by methanol. 15% v/v methanol was added to the homogenate and kept at 4°C for 1 hour. The mixture was centrifuged at 2000g for 5 min at 4°C, and the supernatant was collected for the PGE_2 assay.

This assay is based on the forward sequential competitive binding technique in which PGE_2 present in the sample competes with horseradish peroxidase (HRP) labelled PGE_2 for a limited number of binding sites on mouse monoclonal antibody. PGE_2 in the sample was allowed to bind to the remaining antibody sites. Following a wash to remove any unbound materials, a substrate solution was added to the wells to determine the bound enzyme activity. The colour development was stopped, and the absorbance was read at 450nm (Bio-Rad, USA). The intensity of the colour was inversely proportional to the concentration of PGE_2 in the sample.

Gene expression analysis

The expression pattern of various genes was analysed by following standard protocols such as RNA isolation, RNA quantification and quality check followed by cDNA synthesis and quantitative PCR.

I. RNA isolation

- Embryos were collected in a vial with 25mg tissue per ml TRIzol reagent (Ambion, Life Technologies, USA).
- Tissue was homogenised in a pre-cooled mortar pestle. Homogenised mixture was collected in a new tube and centrifuged at 8000g for 10min at 4°C to remove cell debris and tissue chunks.
- The supernatant was collected in a new tube which had 0.2ml of chloroform and incubated for 20min with intermittent mixing.
- The mixture was then centrifuged for 15min at 12,000g and 4°C to get three phases separated into a lower pink-red phenol-chloroform, buffy interphase and a colourless upper aqueous phase.
- The aqueous phase containing RNA was transferred to a new tube by angling the tube at 45°. 0.5ml Isopropanol was added to the aqueous phase. This mixture was allowed to stand for 1 hour with intermittent shaking at every 15min.

- The mixture was centrifuged at 12,000g for 20min at 4°C. Total RNA was precipitated into a white gel like pellet at the bottom of the tube.
- The supernatant was discarded, the pellet was resuspended in 1ml of 75% ethanol, and centrifuged for 5min at 7500g at 4°C.
- The supernatant was removed, the pellet was mixed in 100% ethanol, and centrifuged at 7500g for 5min at 4°C. The supernatant was discarded and the pellet was air dried for 10-20min.
- The pellet was resuspended in 20-50µl of RNase-free water by pipetting up and down.

II. RNA quality and quantification

The isolated RNA sample was checked for its quality through agarose gel electrophoresis. Dissolved RNA was diluted 1:10 times and quantified using Qubit Assay System as per the manufacturer's protocol (Promega, USA).

- RNA loading dye

Reagent	**Volume (µl)**
500 mM EDTA	8
40% Formaldehyde	72
Glycerol	200
Formamide	30
2.5% BPB	30
10X TBE	400

III.cDNA synthesis

- 1 µg of mRNA from every sample was used to prepare cDNA using High-capacity cDNA Reverse Transcription Kit (Applied Biosystems, USA). Following materials were mixed in the mentioned ratio for cDNA synthesis.

Materials	**Volume per vial (µl)**
10x RT Buffer	2.0
25xdNTP mix	0.8
10x RT Random Primer	2.0

RT enzyme	1.0
Nuclease-free water	4.2
RNA sample	10
Total volume	**20**

The following protocol was set in thermal cycler (Bio-Rad, USA) for cDNA synthesis.

Temperature	**Time (minutes)**
25°C	10
37°C	120
85°C	5
12°C	∞

Synthesised cDNA was stored at -20°C till further processing.

IV. Semi-quantitative PCR

- Synthesised cDNA was used for the gene expression analysis through semi-quantitative PCR. For the PCR reaction the following reagents were used:

Reagents	**Volume per vial (µl)**
cDNA sample	1.0
5 µm Forward Primer	1.0
5 µm Reverse Primer	1.0
Nuclease-free water	2.0
2x PCR master mix	5.0
Total volume	**10**

- The conditions for PCR were as follows:
  3 min at 95 °C followed by 35 cycles of 10 s at 95 °C, 30 s at 60 °C, and 30 s at 72 °C. Semi-quant PCR was performed in Thermal cycler T100 (Bio-Rad, USA).
- The PCR products were loaded on the agarose gel to allow separation of different sized components of the sample.

V. Quantitative PCR (qPCR)

- The selected group of genes were studied for their expression levels by performing the quantitative PCR in LightCycler 96 (Roche Diagnostics, Switzerland), using specific primers (Table 2.1).
- The reaction mixture of qPCR comprised the following components:

Reagent	**Volume (µl)**
Forward primer (5µM)	0.5
Reverse primer (5µM)	0.5
cDNA template	1.0
Nuclease-free water	3.0
2x SYBR green master mix	5.0
Total	**10**

18s rRNA was used as housekeeping control, and the following program was performed in LightCycler 96.

Temperature	**Time(seconds)**
95°C	100s
95°C	10s
60°C	30s X 35 cycles
72°C	30s
4°C	∞

- The data obtained from this experiment was used to calculate fold change using the Livak and Schmitten's fold change method (2001). The fold change values for each gene were plotted in a logarithmic scale. The data was analysed using student T-test by comparing the control and treatment group of embryos to establish statistical significance.

Protein expression analysis

The protein expression was checked by western blot technique. It included different steps such as protein isolation, estimation, quantification, sample preparation followed by SDS-PAGE.

I. Protein isolation

The isolated embryos were treated with lysis buffer to isolate the total protein. The embryos were homogenised in a pre-cooled mortar pestle. The homogenised solution was spun at 8000rpm at 4°C for 10min. The supernatant was collected in a new vial and the total protein concentration was measured through Bradford's assay (Bradford, 1976).

- Tris-Triton Lysis Buffer:

Reagent	**Concentration**
Tris base	50mM
NaCl	200mM
$CaCl_2$	10mM
Triton X-100	1%
Protease inhibitor (Sigma, USA)	1%
pH 7.5	

In the Bradford assay, the quantification standard curve was plotted for the observed OD of known concentrations of BSA. 1µl volume from the isolated protein was used for measurement of the concentration of protein in samples. The OD was measured at 595nm, and concentration was calculated using slope values.

	Sample(µl)	**Bradford Reagent(µl)**	**Water(µl)**
BSA	1-10	200	9-0
Isolated Protein	0.5/1	200	9.5/9

II. SDS-PAGE

The sample buffer (5x) was added to the final working concentration (2µg/µl) as per their quantity required for loading in SDS-PAGE apparatus. The mixture was heated at 80°C for 10min in a water bath and then stored at -20°C till further use.

SDS-PAGE assembly was setup using two glass plates and spacers. The plates were sealed at the bottom with 2% agarose gel. 4% stacking and 12% resolving gel were prepared and poured into SDS-PAGE setup, first resolving then followed by stacking. These gels were allowed to polymerise for 30min each.

The prepared gel was placed in a gel running unit filled with tank buffer. The samples were loaded along with a dual stain protein ladder (Bio-Rad, USA) and allowed to resolve proteins at 100V.

Resolved proteins were stained with Coomassie Brilliant Blue stain to check the quality of proteins.

III.Western blot

The resolved proteins on the SDS-PAGE gel were transferred to PVDF membrane (Bio-Rad, USA) via semidry transfer at 100mA for 20min. The membrane was stained with Ponceau to check the transfer efficiency and washed with distilled water for 3-4 times.

The washed membrane was used to develop immunoblot using antigen specific antibodies against the protein of interest. The membrane was blocked with blocking solution for 1 hour at room temperature. After blocking, primary antibody was added on the membrane and kept at 4°C overnight. On the next day unbound primary antibody was removed by multiple washes to the membrane and followed by the addition of secondary antibody for 1 hour at room temperature. Streptavidin-ALP conjugate was then added to the membrane which was followed by washing to remove the unbound conjugates. The substrate p-Nitrophenyl Phosphate (Pnpp) was added on the membrane for a colour development reaction. Distilled water was added to stop the colour reaction once the optimum colour developed. The images were taken using high resolution camera, and band intensity was measured in ImageJ software.

Immunohistochemistry (IHC)

The localisation of protein of interest in specific tissue regions was done by immunolocalisation in tissue sections. The embryos were collected in PBS and fixed for 1 hour at room temperature in 4% PFA prepared in PBS. Embryos were washed with PBS after fixation and passed through a sucrose gradient followed by embedding of the tissue in OCT (Sakura Tissue-Tek®, USA). Tissue sections of 8-10µm were

taken using cryostat microtome (Leica Biosystems, Germany) at -20°C. The sections were collected on frost-free slides and kept at 37°C for 1 hour to remove OCT from the tissue.

The tissue sections were blocked with 10% goat serum and 1% BSA prepared in PBST for 1 hour at room temperature. Primary antibodies diluted in blocking solution were added to tissue sections and incubated overnight at 4°C. The following primary antibodies were used: anti-COX2; anti-Pax6; anti-FGF8; anti-MyoCD (Table 2.2). The slides were washed with PBS multiple times to remove excess antibodies. Tissue sections were incubated with secondary antibody for 1 hour at room temperature, followed by washing of slides with PBS. The nuclear stain DAPI prepared in PBS (1:1000) was added to the sections and incubated for 10 mins. The slides were washed with PBS for 2-3 times and mounted with fluoroshield (Sigma-Aldrich, USA) at room temperature. The sections were imaged using Leica DM2500 fluorescent microscope (Leica, Germany) and FV3000 Confocal Laser Scanning Microscope (OLYMPUS, USA).

In situ hybridization

I) Cloning

The gene of interest was amplified using gene-specific primers through PCR. The amplified gene was extracted from the gel using QIAquick Gel Extraction Kit (QIAGEN, Germany), and the extracted PCR product was used for the ligation reaction.

Ligation of the vector and gene of interest was done using pGEM®-T Easy Vector Systems (Promega, USA). The kit contained pGEM-T vector, 2x ligation buffer and T4 DNA ligase enzyme. The reaction mixture for ligation reaction was as follow:

Reagents	**Volumes (µl)**
2x ligation buffer	5
pGEM-T vector (50ng)	1
PCR product	3
T4 DNAligase	1
Total	**10**

The ligation mixture was kept at 4°C for overnight incubation. After ligation of vector and gene of interest, the ligation mixture was used for transformation and cloning.

The transformation was done in chemically competent DHα cells, which were stored at -80°C. The cells were aliquoted for transformation reaction and kept on ice. In 50µl of DHα competent cells, 5µl ligation mixture was added and kept on ice for 30min. Followed by heat shock to cells at 42°C for 42 secs, after which the vials were quickly placed on the ice. 1ml of LB media without antibiotics was added to the transformation mixture to recover the cells and kept those vials at 37°C in an incubator shaker for 1 hour.

Once the recovery period was completed, the transformation mixture or cloning mixture was plated on the carb treated LB agar plates under the LAF. The plates were kept at 37°C for 16 hours to grow the transformed cell colonies. The plates were checked for colonies and after 16 hours few colonies from the plate were taken and processed for colony PCR to check the insertion of the gene. Once the colony with desired gene insert was found, that respective colony was used for the primary culture (5ml) of the cells to produce high number of plasmids.

The plasmid was isolated from the primary culture using QIAprep Spin Miniprep Kit (QIAGEN, Cat no. 27106X4, Germany). The quantity of plasmid was checked by Nano drop (ThermoFisher Scientific, USA), and 120ng/µl concentration was taken for the plasmid sequencing to find the alignment of the inserted gene. The results of plasmid sequencing were analysed, and the alignment of the gene was confirmed through the NCBI blast tool. Based on the alignment of the desired gene in plasmid, restriction enzymes were selected to linearise the plasmid. The following reagents were used for linearising plasmid:

Reagent	**Volume (µl)**
10x RE buffer	5
Plasmid (10µg)	X
Restriction enzyme	5
Nuclease free water	Y

Total	**50**

(X and Y are variable volumes according to the concentration of plasmid)

The plasmid linearisation mixture was incubated at 37°C for 3 hours, followed by precipitation of the linearised plasmid through the following steps:

- 5µl of 3M sodium acetate buffer (pH 5.2) was added to the linearised plasmid vial.
- In the same vial, 100µl of 100% ethanol was added and kept at -20°C for overnight precipitation.
- Centrifuged the vials at 8000rpm for 5min and washed the pellet with 200µl of 70% ethanol, centrifuged again, and then the pellet was collected.
- The pellet was dissolved in 10µl of nuclease free water, and the quantity was checked using Nano drop (Thermo Fisher, USA).
- The quality of linearised plasmid was checked by agarose gel electrophoresis.

The linearised plasmid was used for RNA probe synthesis

II) Probe synthesis

The DIG-labelled RNA probes were synthesised using DIG labelling mix (Roche, USA). The reaction mixture for probe synthesis was as follows:

Reagent	**Volume (µl)**
10x buffer	2
DIG-labelling mix	2
Linearised plasmid	2
RNA polymerase	1
RNAses inhibitor	0.50
Nuclease free water	12.5
Total	**20**

The reaction mixture was kept at 37°C for 2 hours, then 1µl of RNAses free DNAse was added in a vial and incubated for 15 more minutes. The reaction was stopped by adding 1µl of 0.2M EDTA (pH 8.0) to the reaction mixture.

Synthesised probes were further processed for precipitation and purification.
Precipitation of probes was done by LiCl precipitation method.

- 5µl of 4M LiCl and 125µl 100% ethanol was added. This mixture was kept at -20°C, overnight.
- For further precipitation of probes, the tubes were centrifuged for 10min at 1000g, the pellet was washed with 70% ethanol.
- Again, the probes were pellet down and resuspended in 75µl DEPC water, incubated at 65°C for 5min, then cooled on ice and processed for purification.

III) Probe purification

Purification of DIG-labelled probes was done by using Roche spin columns (Cat no. 11814427001) stored at 4°C. The spin columns had beads socked in the buffer and sealed with the lids at the top and bottom. The entire process of probe purification was done on ice. Lids from the top and bottom of the columns were removed and placed in a clean 1.5ml tube for centrifugation. The columns were centrifuged at 4000rpm for 5min to remove socking buffer and stack the beads. Elute was removed, and stacked columns were placed in a new 1.5ml tube. The resuspended probes were added to the column and centrifuged at 1000g for 10min at 15-20°C. The elute contained purified DIG-labelled RNA probes.

The quality and quantity of probes were checked and stored at -80°C until used.

IV) Hybridisation

The embryos were dissected in saline and fixed into 4% PFA in PBS for 1 hour at room temperature. Post-fixation embryos were rinsed in PBS-Hit (PBS+0.25% tween20) 3 times for 30min. Followed by methanol dehydration of the embryo using methanol series in PBS and lastly embryos were stored in 100% methanol at -20°C.

Embryos were rehydrated and washed with PBS-Hit for 5min, thrice. Proteinase K treatment was given to embryos for 5min on ice. Embryos were rinsed in PBS-Hit and refixed in 4% PFA with 0.2% glutaraldehyde. Post-fixation embryos were washed in PBS-Hit for 20min.

Reagents of prehybe (pre-hybridisation buffer)	**Volume (µl/ml)**
Formamide	25ml
SSC pH 5 (20X)	3.25ml
EDTA (0.5M)	0.5ml
Tween 20 (25%)	0.5ml
CHAPS (10%)	2.5ml
yeast RNA (20 mg/ml)	250µl
Heparin (50 mg/ml)	100µl
Total	**50ml** (Volume make up by deionised water)

The embryos were directly transferred to prehybe solution and kept at 65°C for 1 hour. Prehybe was replaced with probes (1µg/ml) diluted in prehybe solution and hybridised at 65°C overnight. Unbound probes were removed through multiple washing with wash buffer at 65°C. The washing step was further extended for 30min with MABT at room temperature.

Wash buffer reagents	**Volume (ml)**
Formamide	25
SSC pH 5 (20X)	2.5
Tween-20 (25%)	0.5
DEPC water	22
Total	50

Embryos were blocked with 2% BBR (Boehringer Blocking Reagent Catalogue # 10057177103, Roche) and 20% goat serum for 1 hour at room temperature. The blocking solution was replaced by antibody (1:2000) diluted in the blocking solution and kept at 4°C overnight. The next day, unbound antibodies were removed by washing embryos with MABT-Hit solution for 30min at room temperature. The washing step was extended overnight at 4°C.

Further, the hybridised embryos were washed in NTMT for 20min. The embryos were placed in BCIP-NBT solution to develop colour. Once the optimum colour developed

in the embryos they were washed in PBS-T and refixed. The images were taken using 50% glycerol in PBS for embryos of all stages.

Statistical Analysis

Raw data were processed and analysed to give group mean and standard error with significance. The linear data were analysed for statistical significance by unpaired Student's *t*-test followed by Bonferroni post-hoc test using GraphPad Prism 8.0 software (GraphPad Software Inc., USA). Image J software measured the fluorescence intensity for Images of IHC.

The specific methods are described in detail as a part of the chapters as required.

Table 2.1: List of Primers

Gene	Forward primer	Reverse primer	Accession no.
18S rRNA	GGCCGTTCTTAGTTGGTGGA	TCAATCTCGGGTGGCTGAAC	NR_003278.3
BMP4	TGGAAGAACGTGTCCATCGC	AGACTGGCATGGTGGCTCTC	NM_205237
BMP7	ATCTGCCTACAAAATTGGTTCTC	TACTCACAGCGCATTCTCACTT	NC_052551
CASP3	AAAGATGGACCACGCTCAGG	TGACAGTCCGGTATCTCGGT	NM_204725
CDH1	GAAGACAGCCAAGGGCCTG	TCTGGTACCCTACCCTCTTG	NM_001039258.2
CDH2	AGCCCAGGAGTTTGTAGTG	TTTGGTCCTTTTCTGAGGCCC	XM_025147080.1
DELTA	CCCAGTGAATGCAGGTGTCT	TTGCAGAGCACGGCTGAATA	NM_001397238.1
DKK1	GTGGCCCAGTAGGAAGCAAT	AAGTCGCCTCCTCACCTTT	AY049017.1
FGF8	GAGACCGACACCTTTGGGAG	TTGCCGTTACTCTTGCCGAT	NM_001012767.2
FGF9	GGTCACGGACTTGGACCATT	GCTGTCTACTCCTCGGATGC	NM_001397368.1
FOXD3	TTTCACACTGGACCATGCCC	CACCCCCGTGTTGGTTGTA	NM_204951.3
FZD5	AAGACCTTCGCCACCTTCTG	GGAGACGAAGAGGTAGCACG	NM_001305216.2
GATA4	GGCATGCCAACATCGAATTTTT	CTTGTCCGGGGTACTGTGAG	NM_001293106
GATA5	GGCAAAACCTCAACAGGGTC	CTTGTCCGGGGTACTGTGAG	NM_205421
GATA6	GCGCCCTACGACGGATCTC	GGGTGGTGGGCACGTAGAC	NM_205420
HAND2	AGCGGCGATGAGTCTTGTG	CAGCCGTGAAGTAGGGGTTC	NM_204966
MSX1	CTTACATAGGGCCGAGCCG	CAGGCACAGAACAGATCCCA	NM_205488.2
MSX2	ATATTCGCCTCCTCCAAGACA	CTGGAGAATTCGGCTCGCTC	NM_204559.2
MYOCD	GTCCCCCAACAGCCACTATC	CGTAGAGCCTTGCTGGGAGA	NM_001080715
OTX2	CAACTACGAACTCCGCACCA	ATTCGAGGATCCGGGTACCAT	NM_204520
PAX6	AACAGAAGGGCCAAGTGGAG	TGAAAGAGGAAACGGGGGTG	NM_001397301
PCNA	TGTTCCTCTCGTTGTGGAGT	TCCCAGTGCAGTTAAGAGCC	NM_204170
SHH	TGCTAGGGATCGGTGGATAG	ACAAGTCAGCCCAGAGGAGA	NM_204821
SIX3	CGGCTAAAAACAGGCTCCAG	CGTCTCGGCTCTTTCTGTCA	NM_204364
SNAIL1	CGAACGCACACTGGTGAAAA	CATACGGGAGAAAGTCCGGG	NM_205142
SOX2	TGGTCAAGACGGAATCCAGC	GATCATGTCCCGAAGGTCCC	NM_205188.3
TBX20	GATATGCCTACCACCGCTCC	CGTAGAGCCTTGCTGGGAGA	NM_204144
TGFB	TCGACTTCCGCAAGGATC	CCCGGGTTGTGTTGGTT	HE646744.1
TWIST	CGAAGCGTTCACGTCGTTAC	TAGCTGCAATTGGTCCCTCG	NM_204739.2
VIM	GACCAGCTGACCAACGA	GAGGCATTGTCAACATCC	NM_001048076.2
VSX2	AGCGTGATGGCTGAGTATGG	TTTTGTGCATCCCCAGGAGC	NM_204768.1
WNT11	GACCTGGGTATCGATGGGGA	GGCTTTCAAGACCTGTCTCC	NM_204784
WNT3A	TCGGAAACTCCCCTTTCAGC	TGCTCATCTTGCCTGGAG	NM_204675.2

Table 2.2: List of Antibodies

Primary antibody	Host	Dilution used	Catalogue no
β-catenin	Mouse	1:1000	C7207-100UL (Merk)
Cl.caspase3	Rabbits	1:1000	PC679 (Sigma)
COX2	Rabbit	1:1000	SAB4200576 (Sigma)
E-cadherin	Mouse	1:1000	MA5-12547 (Invitrogen)
Fgf10	Mouse	1:1000	PAB882Ra01 (Cloud Clone)
Fgf8	Rabbit	1:1000	PAC908Hu08 (Cloud Clone)
GAPDH	Mouse	1:1000	G8795-25UL (Merk)
MyoCD	Mouse	1:1000	M8948-200UL (Merk)
N-cadherin	Mouse	1:1000	MA1-91128 (Invitrogen)
Pax6	Mouse	1:1000	AMAB91372-100UL (Merk)
PCNA	Rabbit	1:1000	SAB2108448 (Sigma)
R-cadherin	Rabbit	1:500	MRCD5 (DSHB)
SHH	Mouse	1:500	MA5-17173 (Invitrogen)
SOX2	Rabbit	1:500	AB5603 (Merk)
FoxD3	Mouse	1:500	PA5-106539 (Invitrogen)

General chemical preparations:

- Phosphate Buffered Saline (PBS)

Reagent	Concentration
NaCl	137mM
KCl	2.7mM
Na_2HPO_4	10mM
NaH_2PO_4	1.8mM
pH 7.4	

- Tris Buffered Saline (TBS)

Reagent	Concentration
Tris base	50mM
NaCl	150mM
pH 7.5	

- Sample buffer (5X):

Reagent	Concentration
Tris base	250mM
SDS	10%
Glycerol	50%
Bromophenol blue	0.5%
β-Mercaptoethanol	Added freshly to make 100mM final conc.
pH 6.5	

- Tank buffer:

Reagent	Concentration
Tris base	25mM
Glycine	250mM
SDS	10%

- Gel stock (30%):

Reagent	**Concentration**
Acrylamide	29%
Bis-acrylamide	1%
Solution kept in the dark, overnight at room temperature and filtered before use	

- Resolving gel buffer (12%; 5ml):

Reagent	**Volume (ml)**
30% Gel stock	2
Double distilled Water	1.6
1.5 M Tris Cl (pH 8.8)	1.3
10% SDS	0.05
10% APS (freshly prepared)	0.05
TEMED	0.003
APS and TEMED were added just before pouring gel in PAGE assembly	

- Stacking gel buffer (4%; 3ml):

Reagent	**Volume (ml)**
30% Gel stock	0.4
Double distilled Water	1.8
1.5 M Tris Cl (pH 6.8)	0.75
10% SDS	0.03
10% APS (freshly prepared)	0.02
TEMED	0.002
APS and TEMED were added just before pouring gel in PAGE assembly	

Buffers for Western blot

- Semi-dry transfer buffer

Reagent	**Concentration**
Tris base	48 mM
Glycine	39 mM
pH 9.2 and stored at 4°C	
Methanol (freshly added)	10%

- Antibody dilution buffer

Reagent	**Concentration**
BSA in TBS-T	2%
Sodium Azide in TBST-T	0.02%

- Substrate solution:

Reagent	**Concentration**
Tris base	0.1M
NaCl	0.1M
$MgCl_2$	0.05M
pH 9.5	
BCIP	9.4mg/ml
NBT	18.75mg/ml
Stored in dark	