A Study of the Regulatory Role of Retinoic Acid Receptor Gamma in Zebrafish Development

Htoo Aung Wai

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Aston University

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Retinoic acid (RA) is thought to signal through retinoic acid receptors (RARs), i.e. RARα, β, and γ to play important roles in embryonic development and tissue regeneration. In this thesis, the zebrafish (*Danio rario*) was used as a vertebrate model organism to examine the role of RARγ. Treatment of zebrafish embryos with a RARγ specific agonist reduced the axial length of developing embryos, associated with reduced somite number and loss of *hoxb13a* expression. There were no clear alterations in *hoxc11a* or *myoD* expression. Treatment with the RARγ agonist disrupted the formation of anterior structures of the head, the cranial bones and the anterior lateral line ganglia, associated with a loss of sox9 immunopositive cells in the same regions. Pectoral fin outgrowth was blocked by treatment with the RARγ agonist; however, this was not associated with loss of tbx5a immunopositive lateral plate cells and was reversed by wash out of the RARγ agonist or co-treatment with a RARγ antagonist. Regeneration of the transected caudal fin was also blocked by RARγ agonist treatment and restored by agonist washout or antagonist co-treatment; this phenotype was associated with a localised reduction in canonical Wnt signalling. Conversely, elevated canonical Wnt signalling after RARγ treatment was seen in other tissues, including ectopically in the notochord. Furthermore, some phenotypes seen in the RARγ treated embryos were present in mutant zebrafish embryos in which canonical Wnt signalling was constitutively increased. These data suggest that RARγ plays an essential role in maintaining neural crest and mesodermal stem/progenitor cells during normal embryonic development and tissue regeneration when the receptor is in its non-ligated state. In addition, this work has provided evidence that the activation status of RARγ may regulate *hoxb13a* gene expression and canonical Wnt signalling. Further research is required to confirm such novel regulatory roles.

Key words: Zebrafish, Retinoic Acid, Wnt Signalling, Regeneration, Stem/Progenitor Cells

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Chapter 1

Introduction

1.1. Retinoic Acid

Retinoic acid (RA) is an active metabolite derived from vitamin A. Its role in medicine and biology has been the subject of interest since the early age of ancient civilization. More and more discoveries about RA and its functions were made throughout the centuries of scientific research alongside with the evolution of scientific awareness and improved technology. RA functions have been involved in many essential biological processes during life, especially including developmental biology, stem cells and regeneration. Therefore, RA is considered one of the most important molecules in the biology, involved in the regulations of many tasks.

1.2. Discovery of RA

Retina was first discovered and named as retinol by a German mathematician and astronomer called Johannes Kepler (Finger 2001). The name of retinol, derived from its involvement in retinal biology, which is a part of the eye. In fact, retina came from the Latin word "*rete*" which means the net of eye.

The structure of vitamin A was first proposed by Paul Karrer, in 1952 (D'Ambrosio *et al.,* 2011), who also showed its roles in the retina, although the discovery of vitamin A was in the 1920s. There were several studies which ran from the 1930s to 1950s leading to the identification of different isoforms of vitamin A, but Dowling and Wald (1960) finally called one of the isoform as vitamin A acid, because it has the carboxylic acid group instead of hydroxyl group. Another paper published in the same year also mentioned the effects of hypervitaminosis A was due to vitamin A acid (Thompson and Pitt 1960). One year later, it was shown that vitamin A acid was oxidized enzymatically from vitamin A aldehyde (Futterman 1961). Therefore, multiple events from the studies of the retina and eye disease through to the discovery of vitamin A led to the discovery of RA.

1.3. Chemical structures and properties of RA

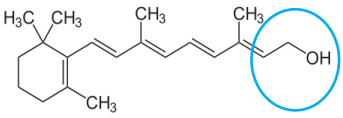
Vitamin A (retinol) and RA share similar chemical structures (Figure 1.1). Both have four isoprenoid units attached to the cyclic ring, although retinol has a hydroxyl group attached to the end of the polyunsaturated side chain whereas the carboxylic group is attached to RA. In fact, RA is not the only derivatives of retinol. The two other metabolites of retinol are *9-cis*-RA and *11-cis*-retinal, in which the roles of the former are less known while the roles of the latter is well known in vision are understood better (Blomhoff and Blomhoff 2006). Among the metabolites of RA, *all-trans*-RA is the most common form found in *in vivo* (Blomhoff and Blomhoff 2006). Therefore, in this thesis, the abbreviation RA has been used to refer to *all-trans*-RA rather than *9-cis*-retinoic acid.

In fact, all the retinol derivatives and retinol share similar chemical structures and carbon numbers, although individual derivatives have different groups attached to the side chain, as well as *trans* and *cis* isomeric forms. Therefore, the International Union of Pure and Applied Chemistry (IUPAC) designated the term " retinoid", which applies to all of chemical compounds consisting of four isoprenoid units joined in a head-to-tail manner between a monocyclic parent compound at one end and a functional terminal group at the other (Johnson 1994). It is generally assumed that the natural or synthetic compounds which share the similar chemical structures and biological functions are called "retinoid" by definition. However, some synthetic chemicals produce similar biological responses similar to the retinoid responses regardless to their different chemical structure. Therefore, the term "retinoid" nowadays refer to all the compounds which have similar chemical structures plus the synthetic compounds which produce the similar biological activities regardless to their structures (Johnson 1994).

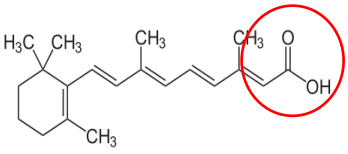
All retinoid are very sensitive to oxygen and light (Blomhoff and Blomhoff 2006). Moreover, the presence of a polyunsaturated chain provides hydrophobic properties to retinols (Curley 2012). Therefore, vitamin A itself is one of the fat soluble vitamins, alongside vitamins D, E and K. In *in vivo,* the *trans* form of RA is the most common form found in the body,

whereas *11-cis*-retinal is strictly found in the retina (Blomhoff and Blomhoff 2006). Therefore, the roles of atRA *in vivo* are well known compared to the *9-cis*-RA. However, a recent paper has reported that *9-cis*-RA was found in murine pancreatic cells although its roles in pancreas are still not clear (Kane *et al.,* 2010).

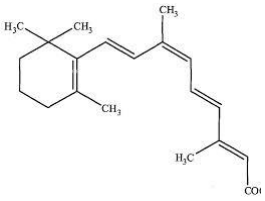
Retinol (Vitamin A)



All-trans-Retinoic Acid (AtRA)



9-cis-Retinoic Acid



11-cis-Retinal

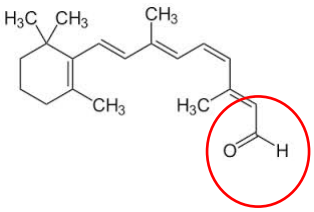


Figure 1.1. Structures of vitamin A and its derivatives, all-trans RA, 9-cis RA and 11-cis retinal

The chemical structures of vitamin A and its metabolites are explained. Vitamin A and all its derivatives are composed of three main chemical groups, a cyclic ring, a polyunsaturated chain and a terminal group. The different forms are decided by substituting the terminal group at the end of the polyunsaturated chain. For example, the hydroxyl group (blue circled) is attached to the vitamin A, retinol while the carboxylic acid and aldehyde groups (red circles) are attached to the RA and 11-*cis* retinal respectively. RA also exists as all-*trans* and 9-*cis* forms although the main active found in *in vivo* is all-*trans* form.

1.4. Sources of RA

RA is synthesized by a series of enzymatic reactions from vitamin A (Theodosiou *et al.*, 2010). Vitamin A cannot be synthesized within the animal bodies. Therefore, the only source of vitamin A is dietary. Although animals have to rely on the dietary vitamin A, plants and microorganisms have the ability to synthesize it by themselves. The precursors of vitamin A are known as carotenoids and are rich in yellow, orange, red and purple colour plants, fruits and vegetables (Fraser and Bramley 2004). The carotenoids are cleaved into vitamin A in the liver of animals (D'Ambrosio *et al.*, 2011). Therefore, the two main sources of vitamin A in the diet are carotenoid-rich vegetables and animal liver, which already has vitamin A (Blomhoff and Blomhoff 2006). A summary of dietary vitamin A intake and processing has been summarized in D'Ambrosio *et al.*, (2011). The structure of vitamin A consists of a β-ionone ring, a polyunsaturated side chain and a polar end group, making the molecule more hydrophobic and lipophilic (Figure 1.1) (Curley 2012). Dietary vitamin A, retinol, is absorbed by the enterocyte of the small intestine alongside other dietary fats. Retinol is then transformed into retinyl ester by lecithin:retinol acyltransferase (LRAT) in the intestinal enterocyte. Retinyl ester is packed into chylomicrons alongside other dietary fat and cholesterol and is secreted into the lymphatic system to transport them to the liver. The chylomicrons are picked up by the liver hepatocytes. The retinyl ester is hydrolysed back to retinol by the hepatic retinyl ester hydrolases (REHs) in the hepatocyte. Retinol is bound to retinol binding protein (RBP) and secreted into the bloodstream for peripheral distribution (Figure 1.2).

RA is an important morphogen for embryonic development and is involved in various developmental processes. Maternal retinol is transferred to the zygote because retinol cannot be synthesized within the zygote. The transfer process of retinol from the adult to the embryo is summarized in Rhinn and Dollé (2012). Retinol binding protein 4(RBP4) is the main protein bound to retinol in the mammalian blood stream, because the carrier proteins are required for the hydrophobic retinol distribution. In mammals, the retinol is transferred from mother to the embryo via the placenta. In oviparous species, in which a yolk sac is attached to the embryo, the

circulating retinol is accumulated from the blood stream into the yolk sac during vitellogenesis or yolk sac deposition. Therefore, the yolk sac serves as the main source of vitamin A for oviparous embryos. However, maternal RBP4 itself cannot cross the placenta or yolk sac. Therefore, retinol diffuses across the placenta and yolk sac membrane and is picked up by the zygotic RBP on the embryo side. At the target cells, the RBP bound retinol is taken by into the cytoplasm via STRA6 (stimulated by retinoic acid) transmembrane protein (Kawaguchi *et al.*, 2007).

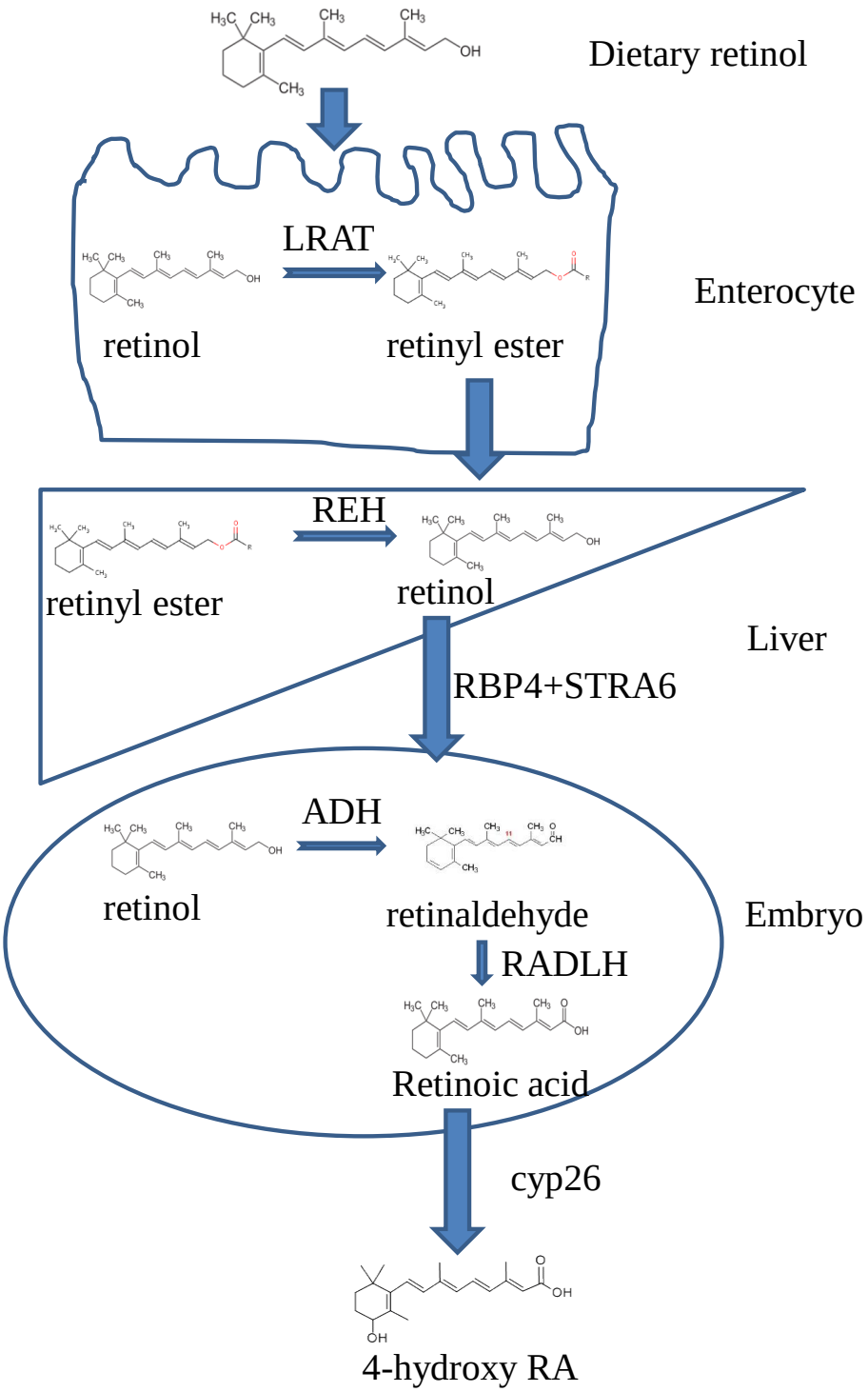

Dietary retinol
LRAT
retinol
retinyl ester
Enterocyte
REH
retinyl ester
retinol
Liver
RBP4+STRA6
ADH
retinol
retinaldehyde
Embryo
RADLH
Retinoic acid
cyp26
4-hydroxy RA

Figure 1.2. Flow diagram of RA metabolism including its source, transport, synthesis and degradation.

RA metabolism starts from the diet. Dietary vitamin A (retinol) is absorbed in the intestine where it is transformed into retinyl ester by lecithin:retinyl acyl-transferase (LRAT). Retinyl ester is transported to the liver via the bloodstream, where it is stored as retinol. Peripheral distribution is carried out by the retinol binding protein synthesized (RBP4) by liver. At the targeted cells, retinol is synthesized into RA by two step reactions, from retinol to retinaldehyde by alcohol dehydrogenase (ADH) and from retinaldehyde to RA by retinaldehyde dehydrogenase (RALDH). Excess RA is degraded by the cyp26 enzyme into 4-hydroxy RA which is non-active form.

1.5. RA Synthesis

Vitamin A is transformed into two main metabolites inside the body, known as *all-trans*-retinoic acid (RA) and *11-cis*-retinal (Blomhoff and Blomhoff 2006). *11-cis*-retinal plays in an important role in vision. It binds to the opsins of the retina which are light sensors which involve in the visual cycle (Kusakabe *et al.*, 2009). The active form of RA inside the body is all trans. RA is synthesized by two step reactions from retinol in the cytoplasm. The first step of retinoic acid biosynthesis is oxidation of retinol to retinaldehyde. The enzyme involves in this oxidation process is alcohol dehydrogenase (ADH) and the reaction is considered as the rate limiting step because it is slow reaction (Parés *et al.*, 2008). The product, retinaldehyde is reversible to retinol (Napoli 1986).

The second step reaction is irreversible conversion of retinaldehyde to RA by retinaldehyde dehydrogenase (RALDH) (Duester 2008). There are four RALDHs which are discovered in vertebrates named as RALDH1, RALDH2, RALDH3 and RALDH4 (Albalat and Cañestro 2009). The synthesized RA is metabolically active and can travel across the cell membrane. Therefore, RA acts as a paracrine morphogen. Its regulation needs to be controlled tightly and the RA degradation is performed by the enzymes of cytochrome P26 family (CYP26) (Petkovich 2001) (Figure 1.2).

1.6. Conventional RA signalling

RA is metabolically active and can function through its receptors at the targeted cells. Here, the term "conventional" is used for a particular well known RA signalling where it binds to the nuclear receptors which have high affinity to RA. These receptors are known as the retinoid acid receptors (RARs). RA can also bind to the non-conventional retinoic acid receptors (Theodosiou *et al.,* 2010). The RARs belong to the nuclear receptor family, which has two main domains, DNA binding domain and ligand binding domain (Petkovich 2001). In fact, RARs interact with another type of nuclear receptor known as retinoid x receptor (RXR). In the nucleus, RAR and RXR forms a heterodimer and binds to the specific DNA sequence known as retinoic acid response element (RARE) (Mangelsdorf *et al.,* 1990). There are three main RAR in vertebrates, known as α, β and γ (Lohnes *et al.,* 1994). The non-conventional RA signalling involves peroxisome proliferator-activated receptor gamma (PPARG) and retinoic acid receptor related orphan receptors (RORs) (Theodosiou *et al.,* 2010).

The details of RA signalling is explained in (Kam *et al.,* 2012). In RA responsive cells, the ligand (RA) is translocated from the cytoplasm to the nucleus after binding to the cellular retinoic acid binding protein (CRABP). In the nucleus, RA binds to RAR/RXR heterodimer which is sitting on the RARE site together with co-repressor. Binding ligand to the receptor complex leads to the conformational changes of RAR/RXP complex followed by the release of co-repressor and recruitment of co-activator resulting in the gene transcriptions (Figure 1.3). There are over 500 reported genes which are regulated by RA signalling and at least 127 genes are the candidates for direct targets (Blomhoff and Blomhoff 2006).

Figure 1.3.Conventional RA signalling

The picture is adapted from (Linney *et al.,* 2001) and the permission to reuse this figure is obtained from the publisher (License Number: 3636071077483) . At the ligand-free state, RAR/RXR heterodimer complex is bound to the specific DNA sequence known as retinoic acid response element (RARE) together with histone de-acetylase 3 (HDAC3) and co-repressor. However, the direct protein-protein interaction between RAR/RXR and HDAC3 has not been reported. Upon the ligand binding to the receptor, the conformational changes of RAR/RXR heterodimer allow the replacement of co-repressor by co-activator and HDAC3 by histone acetylase (HA), resulting in the RA targeted gene transcription.

1.7. Non-conventional RA signalling

RA can also bind to fatty acid binding protein 5, (FABP5). FABP5 is a family member of intracellular lipid binding protein (iLBP), which is the same family member as CRABP (Tan *et al.,* 2002). FABPs are generally known for their ability to bind the fatty acids in the cytoplasm and translocate FABP-fatty acid into the nucleus to bind the peroxisome proliferator activated proteins (PPARs) in adipocytes and keratinocytes, the similar process of RA-CRABP translocation and binding to RAR-RXR (Tan *et al.,* 2002). RA is shown to have a high binding capacity of FABP5, followed by the nuclear translocation of FABP5 and highly selective interaction with PPARβ/δ rather than other PPAR subtypes (Schug *et al.,* 2007). Therefore, non-conventional RA signalling via PPARs suggests the roles of RA signalling might be more complicated than before in the studies which are related to the adipogenesis and energy homeostasis. RA is also shown its binding affinity to the one of the RA receptor related orphan receptor, RORβ (Stehlin-Gaon *et al.,* 2003). RORs are orphanage from nuclear family member because of their monomer conformation while all the other nuclear receptor members exist as dimers (Jetten *et al.,* 2001).

All the RA signalling mentioned above occurs within in the nucleus. In fact, RA also has the extra-nuclear effects. Several studies have shown RA can rapidly and transiently activate the several kinase cascades (Al Tanoury *et al.,* 2013).

1.8. Structures of RARs

Three subtypes of RARs, α, β and γ, are encoded by the separated genes. Each subtype also has the two isoforms depending on their promoters, alternative splicing mechanism and variable N terminal regions. In *in vivo,* RAR/RXR heterodimer acts as signal transducer after binding to the RA ligand, in which RXRs also have three different subtypes α,β and γ (Germain *et al.,* 2006). Each of RARs has a variable N-terminal domain (NTD), a conserved DNA binding domain (DBD) and a conserved ligand binding domain (LBD) (Al Tanoury *et al.,* 2013) (Figure 1.4). DBD and LBD are connected by a flexible hinge peptide.

LDP is composed of 12 conserved α-helices and a β-turn, separated by loops and folded into three layers, helical sandwiches. Helix number 12 (H12) has a conformational flexibility depending on the ligand free or bound stage. In addition, H12 conformational change is also involved in the heterodimerization with RXR as well as the interaction with multiple coregulators. The ligand, RA, binding pocket is made up of hydrophobic residues from the helices H3, H5 and H11 plus the β hair pin s1-s2. Heterodimerization surface is mainly composed of helices H9 and H10.

DBD consists of a sequence specific DNA binding region, which has two zinc-nucleated modules and two α- helices. The DBD is made up of several highly conserved sequence elements, known as P,D,T and A boxes which are important for the dimerization as well as for the DNA sequence recognition.

Compared to the LBD and DBD, NTD is not conserved among the RARs, even among the isoforms. The structures of NTD are also naturally disordered according to the several biochemical and structural studies. Recent studies show NTDs have the regions which are reactive to the kinases and ubiquitin-ligases, suggesting the post-translational modification of NTD may play an important role for the specific functions of different RARs.

Figure 1.4. RAR Structure

The picture is adapted from (Al Tanoury *et al.*, 2013) and the permission to reuse this figure is obtained from the publisher (Reference Number- 240-283-6616). All the RARs have a conserved ligand binding domain (LBD), a conserved DNA binding domain (DBD), a hinge which connects LBD and DBD, and a variable N-terminal domain (NTD). The phosphorylation sites of the LBD and NTD are also shown. LBD possess a ligand binding pocket (red circle). DBD binds to the specific DNA sequence known as retinoic acid response element (RARE), which in turn acts as the transcriptional promoter for RA responsive genes.

1.9. Structure of PPARβ/δ

PPARs are also the member of nuclear receptor superfamily. Like RARs, PPARs forms the heterodimer with RXRs and also bind to RARE. There are three main subtypes of PPARs which are PPARα, PPARγ and PPARβ/δ. They are encoded by the separated genes but interestingly, they all share very similar structures to the RARs (Hihi *et al.*, 2002). All the PPARs are sensitive to the various fatty acids and fatty acid derivatives. Interestingly, PPARβ/δ has the binding capacity to RA although its binding affinity to RA is lower than those of RARs. The binding pocket of PPARβ/δ is considering ably larger than any other nuclear receptor which may explain its binding affinity to RA (Xu *et al.*, 1999).

1.10. Co-repressors, co-activators and co-regulators

In addition to the heterodimer formation with RXRs, RARs also interact with co-repressors, co-activators and other co-regulators for gene regulations. These accessory proteins generally bind to the H3 and H4 hydrophobic areas of LBD (Glass and Rosenfeld 2000). Recently, other regulator proteins which bind to the NTD have been identified (Al Tanoury *et al.,* 2013). Interestingly, RARβ and RARγ have poor interaction with co-repressors (Hauksdottir *et al.,* 2003).

In ligand-bound stage, RARs recruit the co-activators. The known co-activators for RARα in ligand-bound stage are p160 family of steroid receptor co-activators (SRC), which are SRC1, SRC2 and SRC3 (Lefebvre *et al.,* 1995).

In ligand free stage, RARs bind to the co-repressors. Nuclear receptor co-repressor (NCoR) and silencing mediator of retinoic acid and thyroid hormone (SMRT) are identified as the co-repressors of RARα (Al Tanoury *et al.,* 2013). Unlike ligand free stage, ligand bound stage RARs recruit many molecules as the co-activators. These molecules are "Mediator" subunit (Lefebvre *et al.,* 1995), the receptor interacting protein (RIP) (Hu *et al.,* 2004), the preferentially expressed antigen in melanoma (PRAME) (Epping *et al.,* 2005), the transcriptional intermediary factor-1α (TIF1α) (Le Douarin *et al.,* 1995)and thyroid receptor interacting protein-1 (TRIP1) (vom Baur *et al.,* 1996).

There are many other molecules bound to LBD of RARs as co-regulators such as cyclin H (Bour *et al.,* 2005) and CRABPII (Despouy *et al.,* 2003). The molecules found in the NTD of RARs are Acinus-S, a nuclear protein involved in the apoptotic chromosome condensation and mRNA processing, and HACE1, and HECT domain and ankyrin repeat-containing E3 – ubiquitin protein ligase (Vucetic *et al.,* 2008; Zhao *et al.,* 2009).

1.11. Retinoic acid response element (RARE)

RAR/RXR heterodimers bind to the specific sequence of DNA known as retinoic acid response element (RARE) and serves as the promoter of the target genes. RAREs are generally composed of two direct repeats (DR) of a core hexameric (A/G) G (G/T) TCA separated by 1,2 or 5 nucleotides and referred as DR1, DR2 and DR5 (Balmer and Blomhoff 2005). RAREs have been identified in the promoters of RA targets genes which have a variety of functions, such as transcription, cell signalling, development, neuronal functions and tumour suppression.

PPAR/RXR heterodimer also bind to the response element which has two direct repeats of a core hexameric motif with DR1 spacing and serves as a promoter for the genes involved in energy metabolism (Chandra *et al.*, 2008).

1.12. Roles of RA during development

RA is a critical regulator for cell proliferation, cell differentiation, body axis formaton and organogenesis for a developing embryo (Mark *et al.,* 2006). Different roles of RA have been reported in hindbrain formation, pharyngeal arches and lungs formation, pancreatic formation, limb formation and somitogenesis.

Experiments where excess RA is provided or RA is restricted in the developing embryo show changes in hindbrain pattern. Providing the excess vitamin A to the female pregnant rat causes changes in the head morphology of embryo including hindbrain pattern changes (Morriss 1972). On the other hand, normal hindbrain development requires RA. When Japanese quails are provided with a vitamin A restricted diet, their embryos show severely truncated hindbrains (Gale *et al.,* 1999). This hindbrain truncation can be rescued by injection of retinol into the egg, suggesting RA is needed for hindbrain formation (Gale *et al.,* 1999). Similar changes are also found in the other vertebrates as well. The RA synthesizing enzyme, *Raldh2 (-/-)* knock- out mice shows lack of segmentation and reduction in posterior hindbrain (Niederreither *et al.,* 2000). Zebrafish *raldh2* mutant also shows the changes in hindbrain pattern (Grandel *et al.,* 2002; Begemann *et al.,* 2001).

Pharyngeal arches are the derivatives of neural crest cells and theese arches differentiate into a variety of tissues and organs including the cranial cartilages, parathyroid glands, thyroid gland and thymus. RA plays an important role for the formation of these tissues and organs during development. *Raldh2(-/-)* mice embryo dies from the cardiac defects and further investigation shows loss of pharyngeal arches in the mutant embryos (Niederreither 2003). The genes affected in this mutant are *Hoxa1* and *Hoxb1*, which are the downstream targets of retinoic acid responding element (RARE) (Niederreither 2003).

Raldh2 (-/-) mutant mice shows a lack of primary lung bud induction and growth, the phenotype is also associated with increased level of TGF-beta signalling and decreased level of *Fgf10* expression (Chen *et al.,* 2010). Therefore, RA is crucial for lungs formation and growth.

RA is required for the induction of pancreatic primordia from endoderm by signalling from mesoderm. Zebrafish pancreatic mutant line shows mutations in *aldh1a2* gene, which codes the enzyme to transform retinol to retinaldehyde. The chemical treatment with *aldh1a2* inhibitor or morpholino blocking to *aldh1a2* also show the defect in pancreas formation, confirming the important roles of RA in pancreas formation (Alexa *et al.,* 2009). The similar pancreatic defect is also observed in the *Raldh2*(-/-) mice embryo (Molotkov *et al.*, 2005).

RA is also important for formation of forelimbs. Studies in zebrafish show RA necessary for induction of forelimb (Grandel and Brand 2011; Gibert *et al.,* 2006). However, the growth of forelimb is independent of RA signalling. Studies of forelimb development in murine embryo show the growing limb expresses *Cyp26b1* in the growing end of the limb and disruption of the gene results in outgrowth defect (Yashiro *et al.*, 2004; Probst *et al.,* 2011).

1.13. Functions of individual RAR during development

Although RA signalling is important for a variety of development, the roles of individual receptor are still not clear. Based on their pattern of expressions during development, the possible roles of individual RAR are summarized in (Dollé 2009) (Figure 1.6). The gene knock out experiments for individual receptor in mouse show RARα and β knock out embryos are viable and mild phenotypes (Li *et al.*, 1993; Ghyselinck *et al.*, 1997). However, RARγ knock out mice has severe growth defect including bone dysmorphogenesis (Subbarayan *et al.*, 1997). Therefore, it was generally thought RARγ has a very important role for normal embryonic development

Interestingly, the idea of "a binary ligand-dependent transcriptional repressor-activator paradigm for RAR function" has been proposed based on the *in vitro* studies (Bastien and Rochette-Egly 2004). The binary paradigm of RARs suggests the receptors are not only important for the gene transcriptions, the ligand free RARs are also necessary for the transcriptional repression for some biological process. Although the binary function model of RAR is widely found in *in vitro* studies, its role in *in vivo* was quite unclear. Using the *Xenopus* as a model organism for RAR repression function in development, Koide and colleagues (Koide *et al.*, 2001) showed the active repression of RAR was required for normal head formation. In RARγ knockout mice, the bone growth retardation is found together with dysmorphic epiphyseal plate (Williams *et al.*, 2009). It is also showed the defective osteogenesis found in the RARγ knock out mice was due to loss of the transcriptional repressive activities of RARγ. Therefore, these recent findings have changed the traditional ways of RAR signalling during development.

Figure 1.5. Summary table of RARs expressions in different tissues and organs during development

The table was adapted from (Dollé 2009) the permission to reuse this figure is obtained from the publisher. The data was essentially obtained from mouse embryo.

1.14. Using zebrafish as model of developmental biology

In recent years, the teleost fish *Danio rerio*, commonly known as zebrafish has become a new model organism of developmental and genetics study. Zebrafish produces relatively large embryos and has the ability to breed all year round in the right temperature. Importantly, the eggs are fertilized externally which make it easy to adjust the time point of fertilization as well as observation of the developing embryo. Moreover, zebrafish embryo has rapid embryonic development. The fertilized embryo reaches the organogenesis stage within 24 hours. Because of its transparency, it is also easy to inject fluorescent dye to track the cells and to create transgenic lines for tissues of interest or genes.

Zebrafish is also a model organism to create mutant lines. By treating the adult fish with mutagens, many mutant lines can be created by screening the embryos. Since the zebrafish genome has been sequenced, the interesting mutant lines can be further analysed to track down the gene responsible for the mutation. In the opposite way, the gene of interest can be knocked down by injection of morpholino antisense molecules and the embryonic phenotype can be analysed. However, morpholino efficiency reduces as the embryos grow further. Therefore, morpholino based gene knock down study is limited to early embryonic development. In very recent years, a method to selectively knock down targeted genes to create permanent mutant zebrafish line has been developed using zinc finger nuclease (ZFN), transcription activator like effector nuclease (TALEN) and cluster regularly interspaced short palindromic spaces (CRISPR) tools (Hisano *et al.,* 2013; Barrangou *et al.,* 2007; Hwang *et al.,* 2013).

Zebrafish has the similar mechanism of embryonic development to other vertebrates. Therefore, using the zebrafish mutant lines, many zebrafish disease models to human diseases have been identified. Zebrafish also plays an important model organism for drug screening. Drug screening and toxicity tests can be easily done by adding the drugs into the embryonic water and the developmental changes can be observed.

1.15. Zebrafish embryonic development summary

The details of zebrafish embryonic development were described in (Kimmel *et al.*, 1995) as follow. All the observations regarding to the zebrafish embryonic development were documented at 28.5 degree Celsius. In general, the stages of zebrafish embryonic development were divided into zygote period, cleavage period, blastula period, gastrula period, segmentation period, pharyngula period, hatching period and early larval period.

Zygote period (0-0.45hpf) - This period starts from the newly fertilization stage and ends in the cleavage stage. The period approximately lasts 40 minutes.

Cleavage period (0.45hpf-2.15hpf) - During the cleavage stage, the meroblastic cell divisions start to occur at every 15 minutes. A meroblastic pattern is defined as incomplete divisions of cells at the certain side of the fertilized egg in which the cytoplasm of divided cells are still interconnected. The total of six cells cycles are formed during the cleavage period resulting in the 64 newly divided cells lasting approximately two hours. During the cleavage cell division, the size of the cells becomes smaller after each cycle.

Blastula period (2.15hpf-5.15hpf) - After 64 cell division cycles in cleavage stage, the embryo enters into blastula period around 2hpf (hour post fertilization). The blastula period is characterized by three main changes which are mid blastula transition (MBT), the yolk syncytial layer (YSL) formation and epiboly formation. MBT is the transitional period in which the decline of maternal RNA level and rise in zygotic RNA level. During this transition, the rate of cell divisions becomes slower and the zygote transform into ball-shaped. A new layer of cells is also formed known as YSL. The cells at the animal pole become oblong and start to migrate to the vegetal pole.

Gastrula period (5.15-10h) - In brief, the main characteristic of gastrula period is major cell movements. The cells from animal pole where most of the cytoplasm and nucleus are present migrate to the vegetal pole where the most of yolk is present. During this movement, the three germ layers are formed known as ectoderm, mesoderm and endoderm. The embryo gradually transforms from the dome stage to tail bud stage where two buds can be seen in both poles of.

Segmentation period (10-24hpf) - Both buds from the head and tail regions go through the massive morphological transformation during segmentation period. The size of both head and tail buds became larger and the first somite is formed in the trunk region around 10hpf followed by waves of somites come from the tail bud which is now also known as pre-somitic mesoderm. At the same time, the cells in the head bud start to transform into the optic capsules, forebrain and midbrain. Segmentation period is completed after 30 somites are formed in clock and wavefront patterns.

Pharyngula period (24-48hpf) – During this period, the embryo goes through dramatic organogenesis. Eyes, heart, circulation, pectoral fin, pigments are formed during this period.

Hatching period (48-72hpf) – As the name suggest, the embryo hatches from the chorion just after 48hpf. The other obvious changes are pharyngeal arches formation and mouth formation.

Early larval period (after 3dpf) - Most of the morphogenesis are completed after 3dpf and the embryo starts to grow rapidly. The yolk sac is depleted after 5dpf and swimming bladder is formed. The larvae grow into sexually mature adult at 3 months old stage.

Figure 1.6. The stages of zebrafish embryonic development.

The picture was adapted from Kimmel et al 1995 with the copy right clearance from John Willey and sons publishers (License Number: 3620160782752)

1.16. RA signalling during zebrafish development

Like in other vertebrate embryos, RA plays an important role in zebrafish embryonic development and is involved in axis formation, somitogenesis and the formation of pharyngeal arches, hindbrain, pancreatic and pectoral fin. As RA is a morphogen (a molecule which is important for morphogenesis), the signalling needs to be tightly regulated. Therefore, as in the embryonic development of other vertebrate, RA signalling in zebrafish embryonic development involves the source of vitamin A, the RA synthesizing enzyme, the RA degrading enzyme and the RA receptors.

The source of vitamin A in zebafish embryo comes from yolk sac. The zebrafish oocyte derives from oogonial stem cell population and undergoes vitellogenesis during maturation. The oocyte starts to collect the yolk proteins including vitamin A which come from the blood stream of adult liver during meiotic prophase (Gilbert 2000). Although the transport route of retinol from the zebrafish yolk sac to the developing embryo has not been properly mapped, it can be predicted using the pattern of expressions of the proteins which carry and transport the retinol in the developing zebrafish. As in the mammalian system, the first protein of the transport chain is zebrafish RBP4 or rbp4 which collects the retinol from the yolk sac. There is no evidence of *rbp4* expression in zebrafish until somitogenesis. At 12hpf (hours post fertilization), *rbp4* expression is found in the yolk syncytial layer cells which lie between the yolk sac and the developing embryos (Li *et al.,* 2007), suggesting *rbp4* may be important for retinol transfer from the yolk to the developing embryo. Visualization of intrinsic RA in transgenic reporter zebrafish (Shimozono *et al.,* 2013) also shows the intrinsic RA is seen in 3 somite stage which is around 11-12hpf. The transmembrane protein, stra6, which import extracellular retinol into the cell cytoplasm, also expresses at 12hpf (Isken *et al.,* 2008). Interestingly, *rbp4* morpholino injected zebrafish embryos shows abnormal yolk sac retention.

Like other organisms, zebrafish embryos express two orthologous alcohol dehydrogenases (*adh*). Adh proteins are the catalytic enzymes which are involved in the

transformation of retinol (vitamin A) to retinaldehyde. They are *adh5* and *adh8. Adh8* has two isoforms in zebrafish, *adh8a* and *adh8b*. *adh5* expression starts ubiquitously at 50% epiboly stage (Thisse *et al.*, 2004). *Adh8b* expression starts during somitogenesis stage and is limited to the yolk syncytial layer cells (Thisse *et al.*, 2004). However, the *adh8a* expression patterns have not been reported.

As mentioned above, retinaldehyde dehydrogenase (Raldh) is the key enzyme in RA synthesis. Although there are three *raldhs,raldh2, raldh3* and *raldh4*, *raldh2* is assumed to be the most important gene for RA synthesis because the patterns of *raldh2* expression overlap with the patterns of RA reporter transgene expression (Perz-Edwards *et al.*, 2001). Whole mount *in situ* hybridization for zebrafish *raldh2* (Grandel *et al.*, 2002) shows that the expression starts at 30% epiboly stage (Figure 1.7). Strong expression is found during later epiboly stage. During somitogenesis stage, the expression is found in the developing somitic mesoderm rather than pre-somitic mesoderm (PSM) which is the stem cell zone. In 20 somite stage, the expression is found in pharyngeal arch primordium and the eyes. From 26hpf, the expression is also detected in the pectoral fin bud area but the expression disappears later on. From 36hpf, the expression is present in the brain although the expression in the other part of the embryo disappears.

The other two zebrafish *raldh* family members, *raldh3* and *raldh4* expression patterns are also been analysed (Liang *et al.*, 2008). These two genes express much later than the previous one. *raldh3* expression starts from 10hpf in the eye primordium until 19hpf. From 28hpf, the expression is limited to the ventral eye area and at 32hpf, the expression is also found in the otic vesicle. *raldh4* expression starts from 2dpf and its expression pattern is limited to the liver and intestine. Interestingly, there is no *raldh1* gene in zebafish which is orthologous to mammalian *RALDH1* (Liang *et al.*, 2008).

The synthesized RA functions through its nuclear receptors. There are three main retinoic acid receptors in mammals which are RARα, RARβ and RARγ. However, zebafish has

only two receptors, rar-alpha (rara) and rar-gamma (rarg). Being teleost (ray-finned fish species) and teleost genes are duplicated; both zebrafish receptors have two sub-types, which are raraa, rarab, rarga and rargb (Hale *et al.,* 2006; Waxman and Yelon 2007).

The tissues or cells which do not need RA signalling during development are prevented from the teratogenic effects of RA by synthesizing *cyp26*. There are three cyp26 genes in zebafish which are *cyp26a1, cyp26b1* and *cyp26c1*. *cyp26a1* expression starts from 50% to 75% epiboly during the gastrulation period (Dobbs-McAuliffe *et al.,* 2004; Kudoh *et al.,* 2002) (Figure 1.8). The other areas of *cyp26a1* expression include the tail bud during somitogenesis stage, ventral part of somites, growing pectoral fin, notochord, eye and pharyngeal arches. Comparative analysis of double *in situ* hybridization of *cyp26a1* and *raldh2* shows non-overlapping patterns of expression between the synthesizing and degrading enzymes (Dobbs-McAuliffe *et al.,* 2004*). cyp26b1* expression is also very similar to *cyp26a1* expression (Zhao *et al.,* 2005). However, its expression in the tailbud during somitogenesis is transient and the expression in the pectoral fin is restricted to the growing edge. *cyp26c1* expression is limited to the hindbrain during gastrulation period and found in the brain and the pectoral fin during the period of organogenesis (Gu *et al.,* 2005).

Figure 1.7. raldh2 expression during zebrafish embryonic development.

The picture was adapted from (Grandel *et al.*, 2002) and the permission to reuse this figure is obtained from the publisher. Whole-mount in situ hybridisations showing *raldh2* expression in the zebrafish embryo and larva at (A) 30% epiboly, (B-E) gastrula, during (F-I) somitogenesis, and (J-R) larval stages. (A) Marginal view, animal pole is upwards. (B, D) Animal view, dorsal is upwards. (C,G) Dorsal view, animal/anterior is upwards. (E) Sagittal section along the animal vegetal axis. (F) Lateral view, anterior is upwards. (H) Cross section perpendicular to anteroposterior axis. (H inset) Dorsolateral view, anterior is to the left. (I,J,L,P) Lateral view, anterior is to the left. (K,M,O,Q,R) Dorsal view, anterior is to the left. (N) Ventral view, anterior is to the left. anterior left, dorsal top; (D,F,H,J,L,N) dorsal view, anterior left; arrow indicates the developing eye region or the ventral retina of eye and the arrow head does the otic vesicle. (B) 14 hpf; (C–D) 19 hpf; (E–F) 24 hpf; (G–H) 36 hpf; (I–J) 2 dpf; (K–L) 3 dpf; (M–N) 5 dpf; (O) 7 dpf.

Figure 1.8. cyp26a1 expression during zebrafish embryonic development.

The picture was adapted from (Dobbs-McAuliffe *et al.*, 2004) according to the permission to reuse from the publisher (License Number-3636520286688). Analysis of *cyp26a1* expression in zebrafish during the first 48hpf of development using in situ hybridization. (A,C) dorsal right, anterior top. (B,D,E) dorsal forward, anterior top

(F,G, I–L) dorsal top, anterior left. (H) dorsal forward, anterior left. Expression of *cyp26a1* during epiboly (A,B) is found in the presumptive neural plate and around the blastoderm margin, but is excluded from the presumptive notochord region (B, black arrow). (C-E) *cyp26a1* expression in the presumptive neural tube condenses, forming two bands of expression corresponding to the presumptive forebrain (fb) and midbrain (mb). At bud stage (C,D) we also see a band of expression in the middle portion of the embryo (black asterisk). (C,F,G,I) *cyp26a1* is expressed in the tailbud throughout growth of the tail. By 24hpf (I) *cyp26a1* is down regulated in the trunk and tail. During somitogenesis *cyp26a1* is expressed in the ventral boundary of somites (G, white arrowheads), in the developing branchial arches (F,H,J, white arrow), and in the posterior notochord (I, black arrow). Post-somitogeneis expression of *cyp26a1*initiates in the neural retinal (K r, retina, l, lens), base of the pectoral fin (L, white asterisk), and cells in the hindbrain (L, black arrowhead).

1.17. Patterns comparison between RA synthesizing genes and RARs

When the patterns of *raldh2* expression and *rar* expressions in the developing zebrafish are compared, the results show that not all of the *rar* isoforms overlap with *raldh2* expression (Grandel et al., 2002; Waxman and Yelon 2007). At 30 to 40% epiboly stage, the weak expression of *raraa* is localized in the dorsal area of animal pole (Figure 1.9) while the *raldh2* expression at the same time point is limited in the ventral side of blastula. Although many tissues of developing zebrafish embryo express *raldh2* during early embryonic development, the question is whether this gene transcription expression is associated with protein translation and RA synthesis. Visualization of intrinsic RA shows (Shimozono *et al.,* 2013) the first wave of synthesized RA starts to be seen at 75% epiboly stage although the transcripts of *raldh2* are detected at 30% epiboly stage. Using the transgenic zebrafish which has the transgene of *cyp26a1:*eYFP (Li *et al.,* 2012), RA signalling is visualized using *cyp26a1* promoter which has a RARE sequence. This transgenic line also confirms the gene expression at 75% epiboly stage in the area of presumptive neural plate. Therefore, the regions of RA synthesis and RA signalling overlap at 75% epiboly stage. Interestingly, only *rarab* is expressed in the same region at 75% epiboly stage (Waxman and Yelon 2007). In tailbud stage, *raldh2* is express in the somites while *rarga* and *rargb* are strongly expressed in the pre-somitic mesoderm (PSM). During pectoral fin formation, no RA signalling or RA synthesis is reported in the growing pectoral fin. Non-overlapping patterns of expression are associated with *rarg* and *raldh2* while *rarg* expression patterns overlap with *cyp26a1* expression patterns in tailbud, pectoral fin and pharyngeal arches. Therefore, the differential expression patterns between *rarg* and *raldh2* suggest zebrafish *rarg* has little or no contact with RA.


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Figure 1.9. rar expressions during zebrafish embryonic development.

The picture was adapted from Waxman and Yelon 2006 with the permission to reuse from the publisher (License Number- 3636520796934). Comparison of zebrafish *rar* expression patterns from 40% epiboly through 24 hpf. ISH depicts expression patterns of *raraa*, *rarab*, *rarga*, and *rargb*. A–D: Lateral views of embryos at 40% epiboly, animal pole at the top, dorsal on the right. Arrow in A indicates dorsal expression. E–H: Lateral views at shield stage. I–L: Lateral views at 80% epiboly. Arrows in K indicate low expression around the margin. M–P: Lateral views at the tailbud stage, anterior at the top. Arrow in O indicates anterior mesendoderm expression. Arrowhead in P indicates higher anterior expression. Arrow in P indicates tailbud expression. Q–T: Lateral views at the 15-somite stage. U-X: Lateral views at 24 hpf.

1.18. Overlapping expressions patterns between zebrafish rarg and cyp26a1

For many years, RAR functions have been studied in cell proliferation, differentiation, developmental biology and cancers assuming RARs as transcriptional activators. The functional studies were mainly done by studying the specific receptor knock-out phenotypes. For example, RARα knock-out mice were used to study the loss of function study for RARα gene and no observable phenotype was found (Li *et al.,* 1993). RARβ knock-out mice had reduced weight and an ocular defect (Ghyselinck *et al.,* 1997). Both RARγ subtypes knock-out in mice shows loss of growth, cartilage defect and vertebrate malformation (Subbarayan *et al.,* 1997). Therefore, it was generally thought gene knock out studies were important to study RAR functions based on loss of RAR transcriptional activations. However, Williams and colleagues proposed an interesting function of RARγ as "ligand-less repressor function" based on their study in knock-out mice cartilage (Williams *et al.,* 2009). In their study, they found that the avascular zone of long bone cartilage has high level of RARγ expression and RARγ (-/-) mice show defective cartilage formation. They also suggested that avascular zone does not receive liver synthesized retinoid, suggesting that RARγ repressor roles are involved in normal bone growth. Therefore, this murine data suggests RARγ may have an important role in the absence of ligand. Koide and colleges also showed ligand free RAR transcriptional repression is required for the normal head formation in *Xenopus* (Koide *et al.,* 2001). A very recent paper also mentions ligand free RARγ is required for the normal body axis extension and treatment with RARγ specific agonist caused the loss of body axis extension (Janesick *et al.,* 2014). Therefore, co-expression patterns of *rarg* and *cyp26a1* in PSM and pharyngeal arches of zebrafish embryonic development also suggests *rarg* may also have functions in the absence of ligands.

1.19. Hypothesis

The main objective of the project is to reveal the importance of *rarg* during zebrafish embryonic development and tissue regeneration using a novel synthetic RARγ specific drugs that were previously developed for the treatment of the malignant conditions (Hughes *et al.*, 2006). Treating embryos with RARγ specific synthetic retinoids was performed to target the developmental expression of *rarga* and *rargb* in zebrafish. If zebrafish *rarg* plays an important role during its ligand-free state, then targeting the receptor with a specific ligand was expected to result in triggering transcriptional activation. Therefore, cells or tissues which need to have ligand-free *rarg* might be expected to have developmental changes as a result of receptor specific agonist treatment. In summary, the main tasks of the projects were:

- To examine the dose-dependent effects of RARγ agonist treatment on zebrafish embryonic development. To analyse which tissues or cells were affected by RARγ agonist treatment in order to identify which cells and signalling pathways were involved
- To examine which mechanisms underpinned this pathway

Chapter 2

Materials and Methods

2.1. Zebrafish Husbandary

Stocks of the wild-type AB strain of zebrafish were maintained in the fish unit of the Biomedical Research facility in Aston University according to UK Home Office guidelines. The unit had a 14/10 hours day/night cycle according to zebrafish husbandry guidelines (Kimmel *et al.,* 1995). For breeding, the sexually mature male and female zebrafish were selected from the group and transferred to a breeding tank. The breeding tank consists of two compartments for each male and female zebrafish which were separated by a divider. Breeding preparation was done in the late evening to minimize the stress to the zebrafish. In the following morning, the divider was removed from the tank to make the physical contact between the male and the female zebrafish. The embryos were collected using a tea strainer and transferred into petri dishes (SLS, Hessle, UK) of Hank's solution (Molekula, Dorset, UK) or E3 fish media (Wile *et al.,* 2009).

Full Strength Hank's Formula

0.137 M NaCl

5.4 mM KCl

0.25 mM $Na_2H\ PO_4$

0.44 mM $KH_2\ PO_4$

1.3 mM $CaCl_2$

1.0 mM $Mg\ SO_4$

4.2 mM $NaH\ CO_3$

E3 Buffer Formula

5 mM NaCl

0.17 mM KCl

0.33 mM $CaCl_2$

0.33 mM $MgSO_4$

Following breeding, zebrafish eggs were kept in an incubator at 28^{o}C in petri dishes in a 4ml solution of Hank's solution or E3 fish media. Experiments using transgenic reporter fish were performed at the National Institute of Genetics (NIG) in Mishima, Japan, with husbandry according to NIG Institutional guidelines (Westerfield, 2000). Two transgenic fish lines, SAGFF155A, hspGFFDMC28B, and hspGFF55B were generated by the Gal4-UAS method (Asakawa *et al.*, 2008). In the SAGFF155A and hspGFFDMC28B zebrafish, the gene trap and enhancer trap constructs are integrated within the hoxc11a and hoxb13a genes, respectively. Therefore, Gal4FF expression is likely to recapitulate that of the endogenous genes (Asakawa et al. 2008). In hspGFF55B, Gal4FF is expressed in the somite and heart. The HGn39D line was used to visualise the lateral line (Pujol-Martí *et al.*, 2012). The transgenic zebrafish reporter line, Tcf:mini-p line, was used to visualize canonical wnt signalling (Shimizu *et al.*, 2012), which was kindly provided by Dr Ishitani (Kyushu University). For Gal4-UAS breeding, either male or female adult zebrafish of the Gal4 line was crossed with UAS-GFP reporter zebrafish as pair-wise crossings in breeding tanks. The male and female zebrafish were separated using a barrier the evening before the actual day of breeding. The barrier was removed on the following morning to allow male and female mating and fertilised eggs were collected within 30-60 minutes of this using a strainer.

Neuronal specific *HuC*:rfp transgenic line, *apc (adenomatous polyposis coli)* mutant line and krt4p (keratin)-gal4:uas-dkk2 (dickov)-rfp transgenic zebrafish lines were kindly provided by Dr Hirinori Wada.

2.2. RARγ reagents: preparation and treatment protocols

The RARγ specific agonist and antagonist (Hughes *et al.*, 2006) were kept as 10mM stock solutions dissolved in 50% DMSO and 50% methanol. These stocks were diluted with 100% DMSO to give a working stock solution at 100μM according to previously published studies (Li *et al.*, 2010). These working stocks were then further diluted with Hank's solution or E3 media to the final concentrations used for the treatments of zebrafish embryos as given in Results. A control solution of a 50%:50% DMSO:methanol carrier alone stock further diluted into 100% DMSO and then into Hank's or embryo media was prepared using the exact dilutions that were used for the RARγ reagents. Following treatment of zebrafish embryos by immersion in E3 media supplemented with the RARγ reagents or carrier alone at 4 hours post fertilisation (hpf) (or at other times as indicated in Results), the treated and control embryos were incubated at 28^{o}C (Kimmel *et al.*, 1995). Zebrafish embryos were treated with the RARγ agonist at 4hpf because the agonist is not subject to degradation and *RARγ* expression is detected at the 40% epiboly stage, which is around 5hpf (Waxman and Yelon 2007).

atRA (all *trans* retinoic acid)(Sigma, St. Louis, USA) was also prepared in the same way to RARγ-specific agonist preparation. However, the 10mM atRA stocks were freshly prepared and not kept for more than two week because of its photosensitivity and degradation.

2.3. In situ hybridisation and immunolocalisation protocols

In situ hybridisation for *myoD* expression was performed according to previously published methods (Thisse and Thisse 2008). In details, zebrafish embryos treated with the RARγ agonist (at 4hpf) and control embryos were harvested at 22hpf. Chorions were removed either by pronase enzymatic digestion. 1% pronase (Sigma) solution was prepared in E3 media and warmed up to 28.5^{o}C. The embryos were placed in 100ml beaker and covered with minimal amount of solution. The warm pronase solution was added to the beaker and the digestion was monitored until the chorions were torn. The pronase solution was rapidly removed and replaced with fresh embryo media up to 100ml. When the embryos were settled at the bottom of the beaker, the embryo media was poured away without disturbing the embryos at the bottom. The washing steps were repeated three times. The embryos were then anaesthetized with 0.4% tricaine (Sigma) and fixed in 4% paraformaldehyde (PFA) (Sigma) at 4^{o}C overnight, before dehydrating in 100% methanol, also at 4^{o}C overnight. The embryos were then serially rehydrated through decreasing alcohols (100%, 70%, 50%, 25% methanol in phosphate buffer saline (PBS)) for five minutes each and finally to phosphate buffered saline (PBS) alone for 5 minutes four times. The rehydrated embryos were permeablelized with proteinase K (10µg/ml, Sigma) for 10 minutes. The digestion reaction was stopped by adding 4%PFA (Sigma) for 20 minutes. The samples were then washed with PBS four times.

In situ hybridisation was performed by incubating the embryos in hybridisation mix solution at 70^{o}C for 5 hours, prior to a further overnight incubation at 70^{o}C with the *myoD* probe (ZFIN:ZDB-GENE-980526-561).

Hybridization mix (HM) formula

-50% deionized formamide (Sigma)

-5xSSC (Sigma)

-0.1% Tween 20 (Sigma)

-50µg ml/1 of heparin (Sigma)

-500µg ml/l of RNase-free tRNA (Sigma) adjusted to pH 6.0 by adding citric acid (460 ml of 1M citric acid solution for 50 ml of HM).

Following a series of washes in sodium citrate pre-warmed solutions (HM mix alone, 75%HM mix + 25% 2xSSC, 50%HM mix + 50% 2xSSC, 25%HM mix +75% 2xSSC, 100% 2xSSC for 10 minutes each and 0.2xSSC for 30 minutes each at 70^{o}C)and then PBS and 0.2xSSC serially diluted solutions (75% 0.2xSSC+25%PBS, 50% 0.2xSSC+50%PBS, 25% 0.2xSSC+75%PBS and 100%PBS at room temperature for 10 minutes each) , the embryos were blocked with blocking buffer (1x PBT, 2% (vol/vol) sheep serum, 2 mg/ml of bovine serum albumin) for 3-4 hours at room temperature. Finally, the embryos were incubated overnight at 4^{o}C with anti-DIG-alkaline phosphatase antibody specific for the *myoD* probe (Invitrogen),

Anti-DIG-alkaline phosphatase was replaced and the embryos were washed with PBS for 15 minutes each at room temperature six times. The embryos were then washed with alkaline Tris buffer for 5 minutes each three times. Immunopositivity was revealed using a staining solution of Nitro Blue Tetrazolium (NBT, Sigma) and 5-Bromo 4-Chloro 3-Indolyl Phosphate (BCIP, Sigma). The stained embryos were then transferred to glycerol (Molekula) for microscopy and imaging.

Immunolocalisation was performed for the muscle marker, α-actinin, the neural crest marker, sox9, and the lateral plate mesoderm marker, tbx5, by adapting previously published methods (Codina *et al.,* 2010). In brief, RARγ agonist treated and control embryos were fixed in 4% PFA at 4^{o}C overnight at the time points indicated in Results, then washed repeatedly in PBS-Tween (PBS-T: 90%:10%) before an enzymatic digestion with collagenase (1mg/ml, Sigma) for 75 minutes at 37^{o}C. The embryos were then incubated with antibodies specific for α-actinin (1/100 dilution in PBS-T; Sigma) or sox9 (1/100 dilution in PBS-T, Clone ab76997, Abcam Ltd., Cambridge, UK) or tbx5 (Genetix, Irvene, USA) at 4 ^{o}C overnight. Embryos were then washed repeatedly in PBS-T prior to incubation with Alexa 594-conjugated anti-mouse antibodies for α-actinin or sox9 (1/250 dilution in PBS-T, Invitrogen Ltd, Carlsbad, USA) at 4^{o}C overnight, washing again in PBS-T, and then mounted in glycerol for microscopy and

image capture. For tbx5 immunolocalisation, embryos were similarly washed repeatedly in PBS-T after incubation with the primary antibodies, after which immunopositivity was revealed using biotin-anti rabbit secondary antibodies (1/400 in PBS-T; Vector Labs, Peterborough, UK) followed by further washes and incubation with streptavidin-linked FITC (1/50 in PBS-T; Vector Labs), adapting methods previously described (Johnson *et al.*, 2001).

2.4. Alizarin red staining for bone, Oil Red O staining for lipid particles and acridine orange staining for apoptosis

Alizarin red staining was performed to examine the presence of bone by adapting previously published methods (Li et al. 2010). In brief, RARγ agonist treated and control embryos were euthanized at 5 days post fertilisation (dpf) and fixed in 4% PFA at 4^{o}C overnight, then washed in PBS-T prior to staining in a solution of alizarin red (96% of 0.5% KOH:4% of 0.1% Alizarin Red S; Molekula) for 3 hours at room temperature. After staining, the embryos were washed and mounted in glycerol prior to microscopy and image capture.

Oil red o staining was also adapted from (Li *et al.*, 2010). The RARγ agonist treated and control embryos were euthanized at 5dpf and fixed in 4%PFA at overnight, then washed in PBS-T for 5 minutes. Then, the samples were stained with filtered 0.3% oil red o in 60% propan-2-ol in 2 hours. Thereafter, the samples were rinsed in PBST and mounted in glycerol for imaging.

Acridine orange staining protocol was adapted from zfin.org. The alive RARγ agonist treated and control embryos were incubated in 0.05% acridine orange solution for 30 minutes at 4^{o}C. The embryos were then washed with fresh embryo media for three times before mounting in 3% methyl cellulose and imaging under fluorescent microscope.

2.5. Image capture and analysis

Phase contrast or bright field digitised images were captured using either a Nikon DXM1200 camera attached to a Nikon SMZ745T stereomicroscope or a Leica DFX 300FX camera attached to a Leica MZ 16FA stereomicroscope or Jenoptik camera attached to Ceti inverted microscope. A Leica DFX 300FX camera, attached to a Leica MZ 16FA stereomicroscope, was also used to capture fluorescence images of the transgenic zebrafish embryos and of α actinin-immunostained embryos. Laser scanning confocal microscopy was performed with the Leica Microsystems DM6000B-SP57CS confocal system to generate z stacks of sox 9-and tbx5-immunostained zebrafish embryos. These were then converted into 3D projected images of the entire ventral-dorsal or lateral aspects. The projected images of sox9 immunostained zebrafish embryos were used to quantify the number of sox9 immunopositive cells present in the total head region that was anterior from the anterior aspect of the otic vesicle. Phase contrast digitised images were captured at low magnification (x 4 lens) and these images were analysed to determine the embryo length along the antero-posterior axis using Image J software.

2.6. Caudal Fin Transection

The AB strain or Tcf:mini-p transgenic zebrafish embryos at 2dpf or 3dpf, respectively, were anaesthetized with 0.4% Tricaine (Sigma) then placed on a glass slide and the caudal fin transected using a thin scalpel blade (SLS). The embryos were then transferred to fresh Hank's saline or E3 media in an incubator at 28.5^{o}C for those periods of fin re-growth indicated in results. The embryos were treated with the RARγ agonist (10nM) or carrier alone immediately following caudal fin transection. In some experiments, the AB strain embryos were treated with a RARγ specific antagonist at 3dpf (following RARγ agonist treatment at 2dpf) or subject to wash out of the RARγ agonist at 3dpf.

2.7. Statistical analysis

Data was evaluated for normal distribution using the D'Agostino and Pearson omnibus normality test. One way or two way ANOVAs were used to assess the relationships between treatment of zebrafish embryos with different concentrations of the RARγ agonist and fish length or the growth of treated versus control embryos over time, with post hoc analysis. Student paired t-tests or Mann Whitney *U* tests were used to examine differences between treatment versus control groups for hox gene expression or the prevalence of Sox9 immunopositive cells, according to whether the data was normally or not normally distributed, respectively. All statistical analysis was performed using GraphPad Prism software. Values were considered statistically significant, as indicated in Results, at $P \leq 0.05$ (*). Unless otherwise indicated all data have been presented as means ± standard deviations (SD) where a minimum of n=3 procedures were performed for all experiments.

Chapter 3

Phenotypic characterization of RARγ agonist treated zebrafish embryos

3.1. Introduction

The main question of this project is to investigate the importance of RARγ (zebrafish rarga and rargb) during zebrafish embryonic development. The gene expression patterns of zebrafish *raldh2* which is responsible for RA synthesis and zebrafish *rarg* expression patterns do not overlap during early zebrafish embryonic development (Grandel *et al.,* 2002; Waxman and Yelon 2007). The patterns of intrinsic RA synthesis using RA responsive reporter zebrafish line (Shimozono *et al.,* 2013) overlaps with the expression patterns of *cyp26a1:*eYFP transgenic reporter zebrafish line (Li *et al.,* 2012) in which RA signalling is visualized using *cyp26a1* promoter which has RARE sequence at 75% epiboly stage. Interestingly, the only *rarab* is expressed in the same region where RA is present at 75% epiboly stage (Waxman and Yelon 2007) and the chromatin immunoprecipitation results show zebrafish RARα binds to RARE (Li *et al.,* 2012). In tailbud stage, *raldh2* is expressed in the somites (Dobbs-McAuliffe *et al.,* 2004) while *rarga* and *rargb* are strongly expressed in the pre-somitic mesoderm (PSM) (Hale *et al.,* 2006; Waxman and Yelon 2007). During pectoral fin formation, both RA signalling and RA synthesis are reported to be present in the pectoral fin progenitor cells area of RA responsive reporter zebrafish line (Shimozono *et al.,* 2013) or *cyp26a1:*eYFP transgenic reporter zebrafish line (Li *et al.,* 2012). Therefore, non-overlapping patterns are found between *rarg* expression and *raldh2* expression while *rarg* expression patterns overlap with *cyp26a1* expression patterns in tailbud, distal edge of pectoral fin and pharyngeal arches. These non-overlapping expression patterns of *rarg* and *raldh2* suggest that *rarg* does not function during development to activate RA responsive genes. Instead, a lack of *rarg* dependent transcriptional activity may be important and *rarg* may play a key role in repressing gene expression in its non-ligated state.

The main objective of the project is to justify the importance of *rarg* during zebrafish embryonic development using a novel synthetic RARγ specific compound (Hughes *et al.,* 2006). Treating the zebrafish embryos with the RARγ agonist is expected to target the *rarga* and *rargb* in the developing zebrafish. If zebrafish *rarg* has the important role as ligand free receptor, targeting the receptor with specific ligand is expected to change the receptor

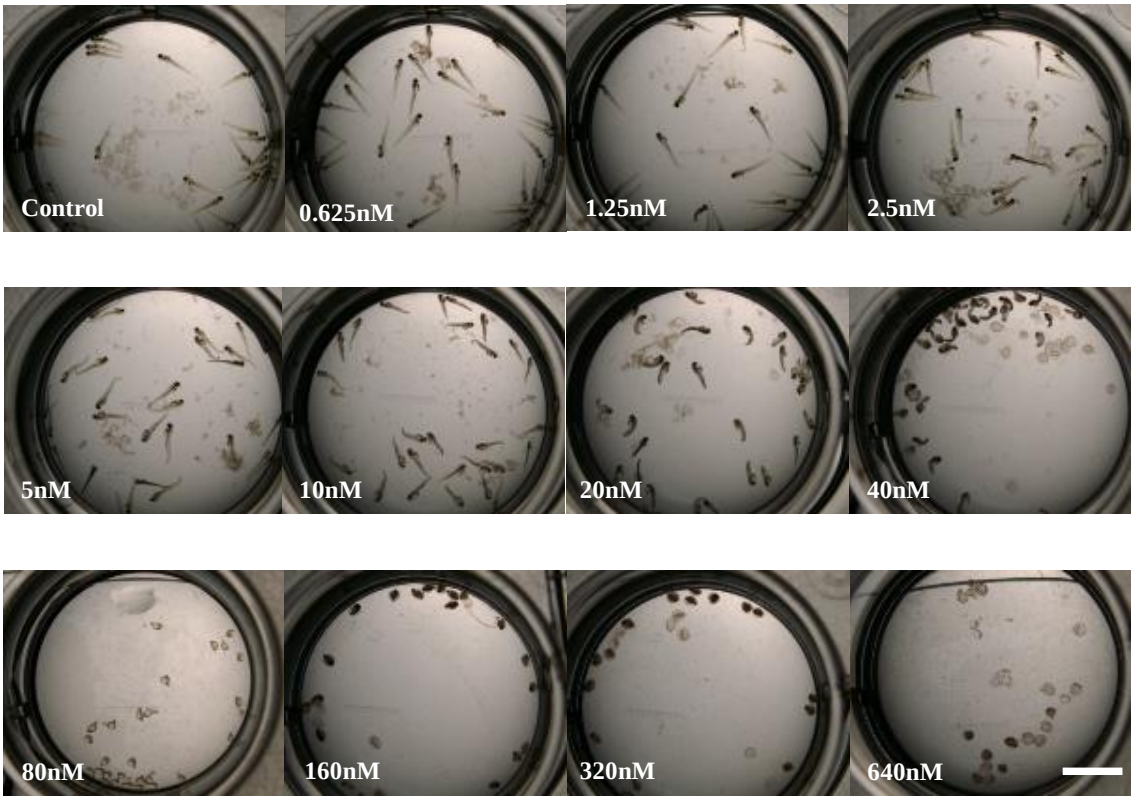
conformation resulting in triggering the transcriptional activation. Therefore, the tissues or cells which are needed to have the ligand free *rarg* for the transcriptional repression might have the changes as the result of receptor specific agonist treatment. The zebrafish embryos were initially treated with RARγ agonist at the dome stage, which is at 4hpf prior to the first expression of *rarg* in the epiblast at around 5.5hpf (Hale *et al.,* 2006; Waxman and Yelon 2007).

3.2. Treatment of zebrafish embryos with a RARγ agonist inhibited anterior-posterior growth.

Like RA treatment, the RARγ agonist treatment to the zebrafish embryos caused the embryonic truncation. Treatment of zebrafish embryos at 4 hpf with the RARγ agonist was associated with clear morphological differences during development, with the main difference being a reduced anterior-posterior axis length at 3dpf (Figure 3.1). The cardiac oedema was also present. The effect on reduced anterior-posterior axis formation in RARγ agonist treated zebrafish embryos was dose-dependent (Figure 3.2). The concentration ranged from sub-nano molar level (0.625nM) up to 5nM RARγ agonist treatment on zebrafish embryos had no obvious effects on anterior-posterior axis growth but the dramatically reduced anterior-posterior axis length was observed from 10nM according to the increasing concentration (Figure 3.2). However, the effects of RARγ agonist on zebrafish anterioranterior-posterior axis length reached the highest level when the concentration was more than 80nM. The zebrafish embryos treated with the RARγ agonist concentration more than 80nM also showed lack of hatching at 3dpf which physiologically happens between 48hpf to 72hpf (Kimmel *et al.,* 1995). The data analysis showed the half inhibitory concentration was 14.59nM (Figure 3.2). Therefore, 10nM concentration was chosen to use the agonist effect on embryonic development compared to 1nM or 100nM since the concentrations around 1nM produced the normal embryonic development and the concentrations around 100nM result in severe truncation and low survival rate.

Figure 3.1. Treatment of embryonic zebrafish with a RARγ agonist (10nM) at 4hpf was associated with a reduced anterior-posterior length at 3dpf.

A representative phase contrast microscopy picture showing the left lateral view of RARγ agonist (10nM) treated and control AB strain zebrafish embryos at 3 days post fertilisation (dpf). The RARγ agonist (10nM) treated embryo length was markedly shorter than the control. Cardiac oedema was also evident (arrowed). Scale bars = 250μm. (n=3 independent experiments)


Control
0.625nM
1.25nM
2.5nM
5nM
10nM
20nM
40nM
80nM
160nM
320nM
640nM

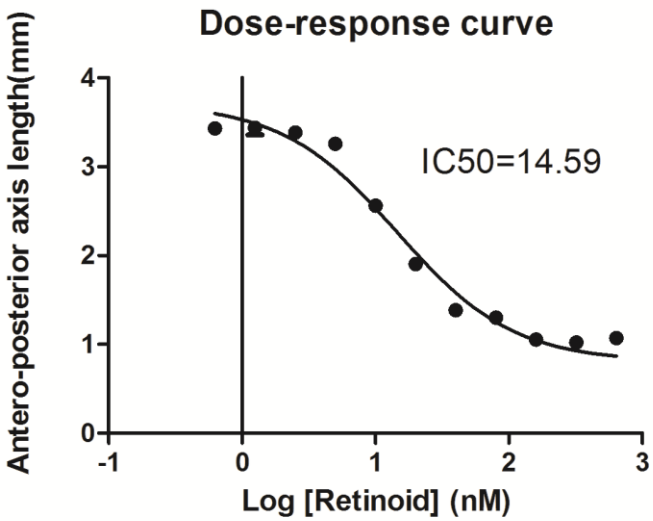

Dose-response curve
Antero-posterior axis length(mm)
IC50=14.59
Log [Retinoid] (nM)
0
1
2
3
4
-1
0
1
2
3

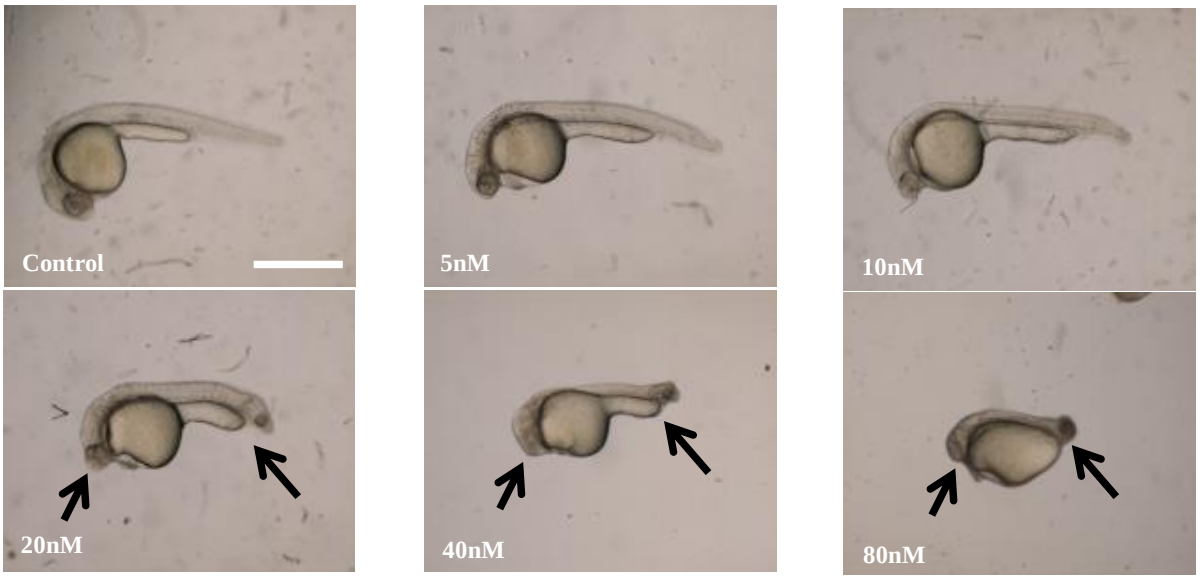
Figure 3.2. Treatment of embryonic zebrafish with a RARγ agonist (10nM) was associated with a dose-dependent decreased in anterior-posterior length at 3dpf.

Representative pictures are shown of the zebrafish embryos in the wells which were treated with different concentration of RARγ agonist (10nM) ranged from 0.625nM to 640nM.The sigmoid curve below shows the dose-response curve of the different concentration of RARγ agonist (10nM) plotted against the anterior-posterior axis length. The half inhibitory concentration, IC_{50} was 14.59nM. Treatment with of RARγ agonist (10nM) from sub-nanomolar (0.625nM) to 5nM concentrations to the zebrafish embryos at 4dpf did not cause the obvious changes to the anterior-posterior axis formation in embryonic zebrafish development at 3dpf. However, the dramatic changes in the RARγ agonist (10nM) treated zebrafish embryos at 3dpf was observed when the RARγ agonist (10nM) concentration ranged from 10nM and the shorter anterior-posterior length was reversely proportionate to the RARγ agonist (10nM) concentration up to 100nM,i.e, the higher the concentration of RARγ agonist, the shorter the anterior-posterior axis was. However, the effects were not different in the zebrafish embryos which were treated with RARγ agonist (10nM) at the concentration over 100nM. Scale bar = 5mm. (n=3 independent experiments)

3.3. Treatment of zebrafish embryos with the RARγ agonist might have affected somitogenesis.

Previous results show the effects of RARγ agonist treatment to the zebrafish embryos at 4hpf caused reduced anterior-posterior axis formation in the 3dpf embryos. Somites are the main building blocks of body axis formation and somitogenesis in zebrafish embryos completes at 24hpf (Stickney *et al.,* 2000). Therefore, there are two possibilities which caused the reduced anterior-posterior axis formation in RARγ agonist treated zebrafish embryos. The first possibility is RARγ agonist affected the somitogenesis, causing the reduced number of somites and reduced anterior-posterior axis. The second possibility is RARγ agonist did not affect the somitogenesis but inhibited further growth. To clarify the possible cause, the RARγ agonist treated embryos at different concentration were analysed at 1dpf, the time point in which zebrafish somitogenesis is completed (Stickney *et al., 2000)*

The results (Figure 3.3) show there was no significant difference in anterior-posterior length between control embryos and 5nM RARγ agonist treated embryos at 1dpf. However, the anterior-posterior length between the control embryos and the rest of RARγ agonist treated embryos (10nM, 20nM, 40nM and 80nM) were significantly different. Therefore, these evidences suggest RARγ agonist treatments affected the zebrafish somitogenesis. Moreover, two other changes were abnormal yolk sac formation and loss of anterior structures including eye formation in RARγ agonist treated zebrafish embryos (20nM, 40nM and 80nM) at 1dpf.


Control
5nM
10nM
20nM
40nM
80nM

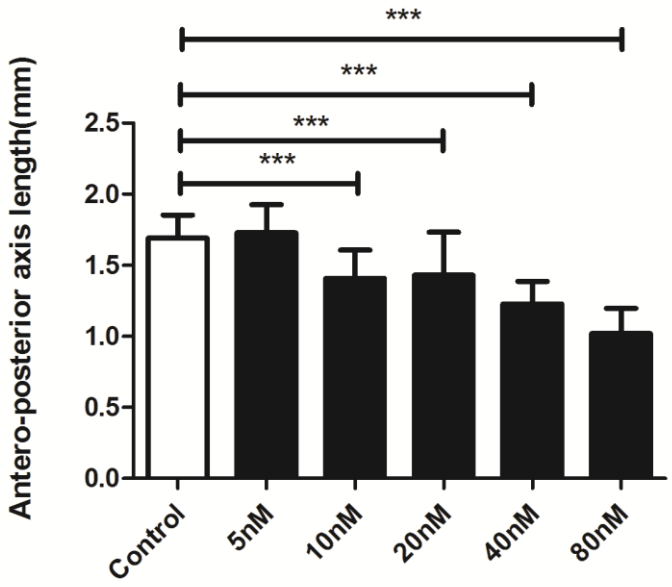

***
***
***
***
Antero-posterior axis length(mm)
2.5
2.0
1.5
1.0
0.5
0.0
Control
5nM
10nM
20nM
40nM
80nM

Figure 3.3. Treatment of embryonic zebrafish with a RARγ agonist (10nM) was associated with a reduced anterior-posterior axis length at 1dpf.

Representative pictures are shown of the left lateral views of the zebrafish embryos treated with the different concentration ranged from 5nM to 80nM at 1dpf. The zebrafish embryos which were treated with RARγ agonist (10nM) at the concentration of more than 10nM had defective eye structures as well as abnormal yolk sac extension (arrowed). The graph showed the anterior-posterior lengths between 5nM and control embryo were not significant although the rest of different concentration are significantly different to the control. The data are normal and unpaired t-test was used for significance test. Scale bars = 0.5mm. The data was evaluated for normal distribution using the D'Agostino and Pearson omnibus normality test. P value ≤ 0.001 (n=3 independent experiments)

3.4. The efficiency of RARγ agonist was lower than that of RA treatment to zebrafish embryonic development.

Since RARγ agonist is a synthetic retinoid for RARγ (Hughes *et al.,* 2006), the similarities between RARγ agonist and RA were also examined at different concentrations. Interestingly, RA treated zebrafish embryos had severe phenotypes such as shorter anterior-posterir axis length than that of RARγ agonist treated zebrafish embryo at the same concentration as well as loss of anterior head structures including eyes (Figure 3.4.). Moreover, RA treated embryos had bigger yolk sac than RARγ agonist treated zebrafish embryos at the same concentration. However, these phenotypes found in RA treated zebrafish embryos were also present in the RARγ agonist treated embryos only when the concentrations were higher than 10nM. Therefore, these findings suggest RA had higher efficiency than RARγ agonist and treatment with 10nM RARγ agonist may have activated the selective receptors.

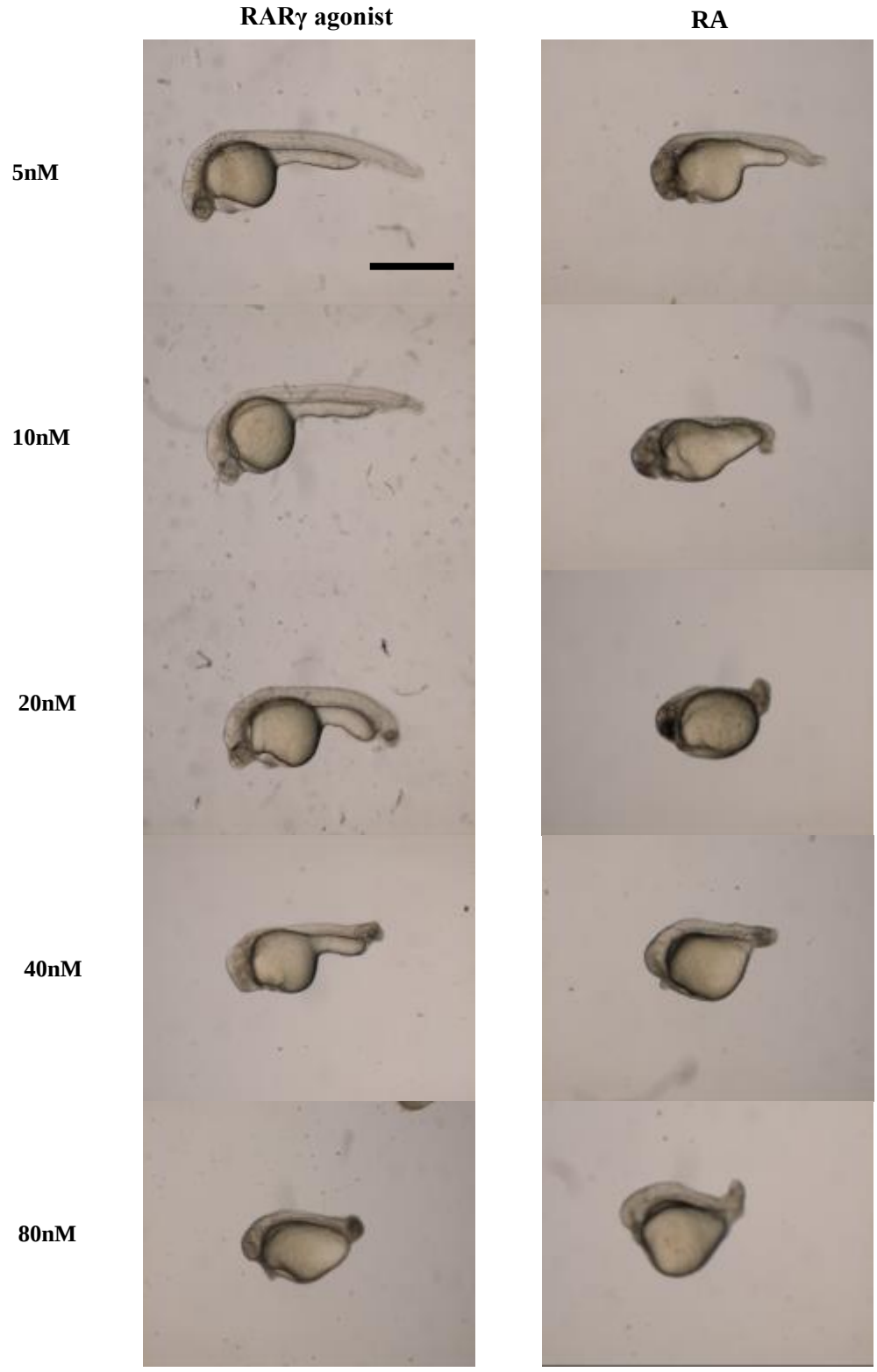

RARγ agonist
RA
5nM
10nM
20nM
40nM
80nM

Figure 3.4. The efficiency of RARγ agonist (10nM) treatment was different from that of RA treatment to zebrafish embryonic development at 1dpf.

Representative pictures are shown of the left lateral views of the embryos treated with the concentration ranged from 5nM to 80nM of RARγ agonist (10nM) and RA. At the same concentration, the severities of RARγ agonist (10nM) and RA on the zebrafish embryos were not the same. RA treated embryos had severely truncated body as well as malformed anterior head including eye formation even at 5nM. However, the malformed anterior structures in RARγ agonist (10nM) treated embryos were only found when the concentration was higher than 10nM. Scale bar=0.5mm (n=3 independent experiments)

3.5. The zebrafish chorion permeability did not interfere with the RARγ agonist efficiency.

Zebrafish chorion is a natural protective barrier for certain-sized molecules (Hagedorn 1998). Moreover, it has been reported that the interaction and permeability of chorion to the toxic chemicals (Henn and Braunbeck 2011). Thus, the natural properties of zebrafish egg chorion may provide the false interpretation of the experimental results in RARγ agonist treated zebrafish embryos. Therefore, the control experiments were done to investigate the difference in the RARγ agonist drug efficiency by removing the zebrafish chorion with pronase.

The zebrafish embryos were treated with the RARγ agonist (10nM) at 4hpf with or without chorion after treatment with pronase. The results showed there was no significant difference in anterior-posterior axis length at 3dpf between RARγ agonist treated chorion intact and chorion-removed embryos (Figure 3.5). Therefore, this result suggests there were no interference of zebrafish chorionic membrane on the interaction and permeability of RARγ agonist.

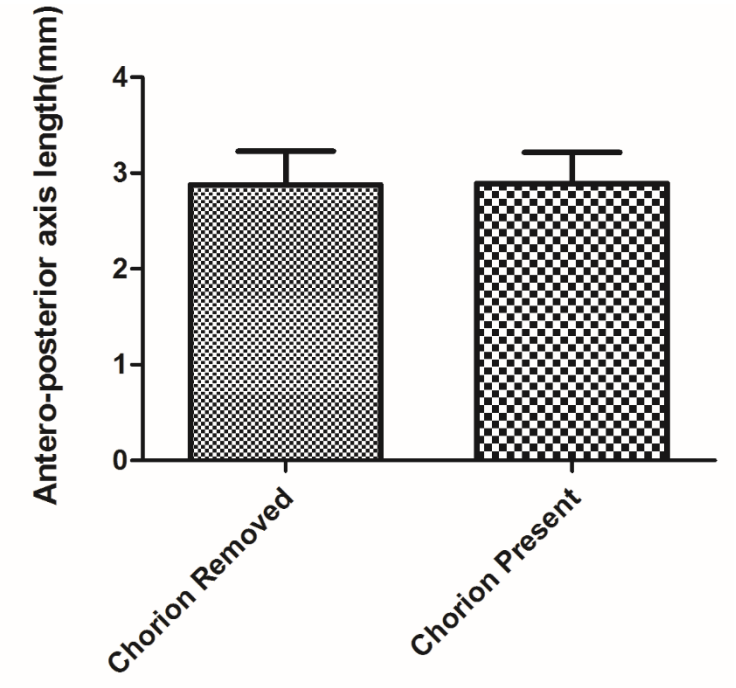


Figure 3.5. Zebrafish chorion permeability did not interfere with the RARγ agonist (10nM) efficiency.

The bar graph shows the comparison of the anterior-posterior axis length between RARγ agonist (10nM) treatment to the embryos in which chorions were removed by pronase treatment and to those which had intact chorion at 3dpf prior to the RARγ agonist (10nM) treatment. The results show there was no significant difference between chorion removed RARγ agonist (10nM) embryos and chorion intact RARγ agonist (10nM) treated embryos. The length of 23 chorion removed embryos and 33 chorion intact embryos were measured for this experiment.

3.6. Treatment of zebrafish embryos with the RARγ agonist caused the slow growth rate after 2dpf.

Treatment with RARγ agonist to the zebrafish embryos at 4hpf caused shorter anterior-posterior axis length at 1dpf compared to that of control embryos, suggesting the somitogenesis might have been affected by RARγ agonist in the treated embryos. However, it is still not clear whether the RARγ can inhibit the further growth of the treated zebrafish embryos. Therefore, the changes in the anterior-posterior length in RARγ agonist treated and control embryos were analysed from 1dpf to 5hpf.

The results showed there was a marked difference in the growth patterns between RARγ agonist treated and control embryos from 1dpf to 5dpf (Figure 3.6). From 1dpf to 2dpf, RARγ agonist treated and control embryos had a significant growth. However from 2dpf to 5dpf, the control embryos had the increased in the anterior-posterior axis length although the RARγ agonist treated embryos did not have significant increase in their anterior-posterior axis length. Therefore, this result suggests RARγ-agonist treatment caused the growth retardation.

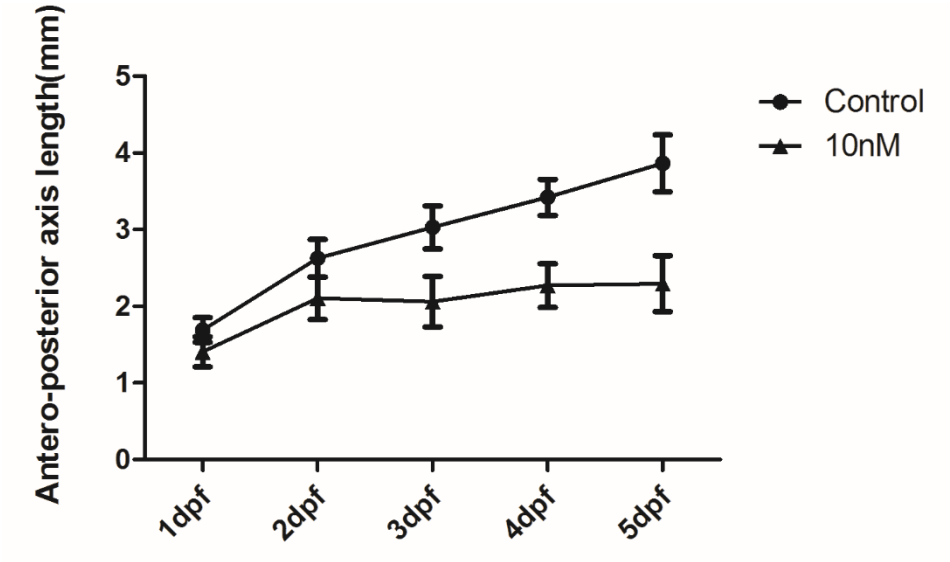


Figure 3.6. Treatment of embryonic zebrafish with a RARγ agonist (10nM) was associated with reduced growth rate.

A representative line graph id shown the changes in the anterior-posterior axis length of RARγ agonist (10nM) treated (10nM) and control embryos from 1dfp to 5dpf. The length of RARγ agonist (10nM) treated and control embryos show the gradual increase pattern from 1dpf to 2dpf. However, from 2dpf to 5dpf, the anterior-posterior axis length of RARγ agonist (10nM) treated embryos did not increase compared to the control embryos' growth. (n=3 independent experiments)

3.7. Treatment of zebrafish embryos with the RARγ agonist caused the progressive cardiac oedema.

RARγ agonist treated embryos were associated with visible cardiac oedema at 3dpf (Figure 3.1). Therefore, the cardiac oedema development in RARγ agonist treated embryos was further analysed. The beating heart in normal wild type embryos which are grown at optimal temperature is present at 42-48hpf (Kimmel *et al.*, 1995). Therefore, the progress of cardiac oedema in RARγ agonist treated embryos was analysed from 1dpf.

Since the heart formation in the developing zebrafish embryos is not complete until 42-48hpf (Kimmel *et al.*, 1995), both RARγ agonist treated and control embryos had no visible heart at 1dpf (Figure 3.7). However, RARγ agonist embryos had a small cardiac oedema at 2dpf while the control embryos had a visible normal heart at the same age. At 3dpf, the cardiac oedema was strictly localised to the heart area in the RARγ treated embryo at 3dpf. However, the oedema was spread posteriorly along ventral side of the body. The changes in the circulation were also found in RARγ treated embryos at 4dpf (Figure 3.8). The changes found in the RARγ agonist treated embryos at 4dpf were slower circulation, dilated and congested the common cardinal vein and the accumulated blood cells. The heart of RARγ agonist zebrafish embryos may not have fully functional stroke because the fluid retention in the cardiac oedema may have the pressure against the cardiac chamber probably resulting in cardiac induced hypoxic state. If the heart did not pump out the required amount of blood, the blood in the vein may have congested. Therefore, this circulation changes may be the evidences of the cardiac oedema progression.

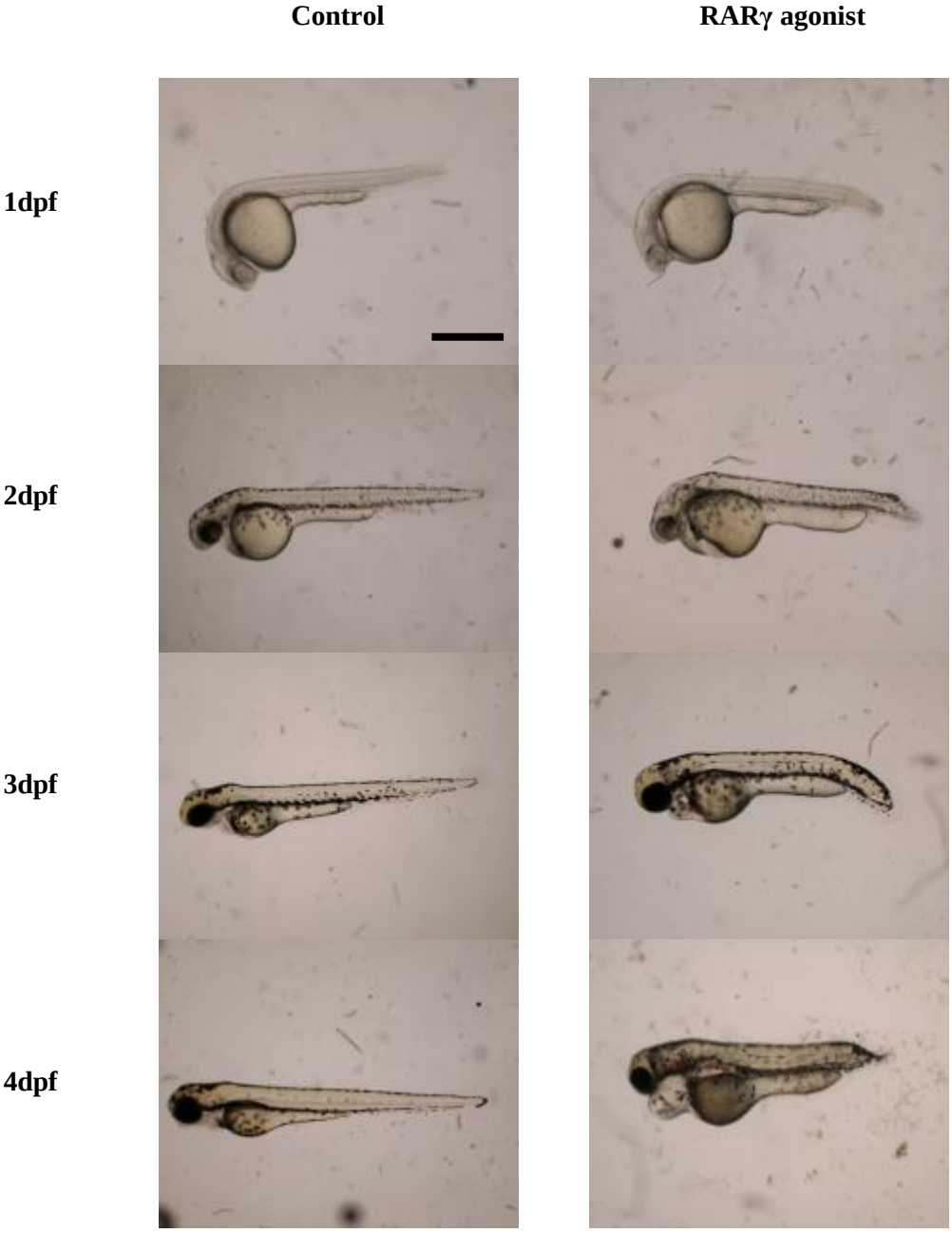

Control
RARγ agonist
1dpf
2dpf
3dpf
4dpf

Figure 3.7. Treatment of embryonic zebrafish with a RARγ agonist (10nM) was associated with progressive cardiac oedema throughout the development from 2dpf to 4dpf and bigger yolk sac.

The representative pictures show the progressive cardiac oedema in the RARγ agonist (10nM) treated embryos from 1dpf to 4dpf. Heart formation is not completed until 42hpf to 48hpf. Therefore, there was no obvious difference in the heart formation between RARγ agonist (10nM) treated embryos and control embryos at 1dpf. However, there was slight difference in the heart appearance between RARγ agonist (10nM) treated and control embryos, which is visible at 2dpf. However at 3dpf, a swelling is found in the heart area of RARγ agonist (10nM) treated embryos but not in control embryos. Moreover, the cardiac oedema was localized at the heart area at 3dpf but the oedema spread posteriorly to the yolk sac area at 4dpf in the RARγ agonist (10nM) treated embryos. Moreover, the yolk sac size of RARγ agonist (10nM) treated embryos was bigger than those of control embryos. Scale bar=250μm (n=3 independent experiments)

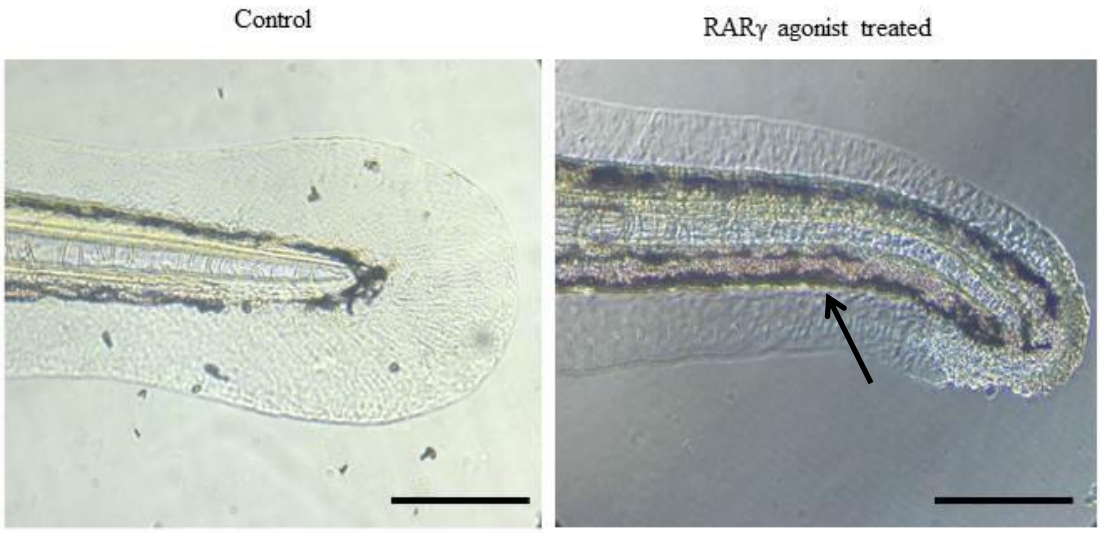


Figure 3.8. Treatment of embryonic zebrafish with a RARγ agonist (10nM) was associated with slow circulation in the common cardinal vein.

The representative pictures show the left lateral views of the tails in RARγ agonist (10nM) treated and control zebrafish embryos showed the evidences of slow circulation and accumulation of blood cells in the common cardinal vein of RARγ agonist (10nM) treated embryos at 4dpf. Therefore, the common cardinal vein in RARγ agonist (10nM) treated embryos was congested and dilated with the blood cells at 4dpf. Scale bar=250µm (n=3 independent experiments)

3.8. Treatment of zebrafish embryos with the RARγ agonist caused the loss of lipid molecules in the inter-somitic vessels.

The RARγ treated embryos showed the slow growth, congested yolk sac and the bigger yolk sac. The yolk sac is the source of nutrients for the zebrafish embryonic development until independent feeding at 5dpf (Kimmel *et al.*, 1995) and consists of lipid molecules and vitamin A. Therefore, the cardiac oedema, slow circulation and the bigger yolk sac in the RARγ treated embryos suggests RARγ treatment might have affected the lipid distribution in the RARγ treated embryos. Hence, the lipid distribution in RARγ agonist treated embryos were examined using oil red o staining which is a dye for neural lipids, triglyceride and some lipoproteins.

The oil red o staining results showed red coloured particles were present in the inter-somitic vessels in the trunk region of the control embryos at 5dpf while these particles were absent in the same area of RARγ agonist treated embryos at the same time point. Therefore, this evidence suggests treatment with RARγ agonist was associated with the lack of fat particles in the inter-somitic vessels (Figure 3.9). Lack of fat particles might be the secondary effects of cardiac oedema which failed to distribute the fat particles from the yolk sac to the inter-somitic vessels. Moreover, fat particle distribution failure might also be the reason to the bigger yolk sac in the RARγ agonist treated embryos as well as the slower embryonic growth.

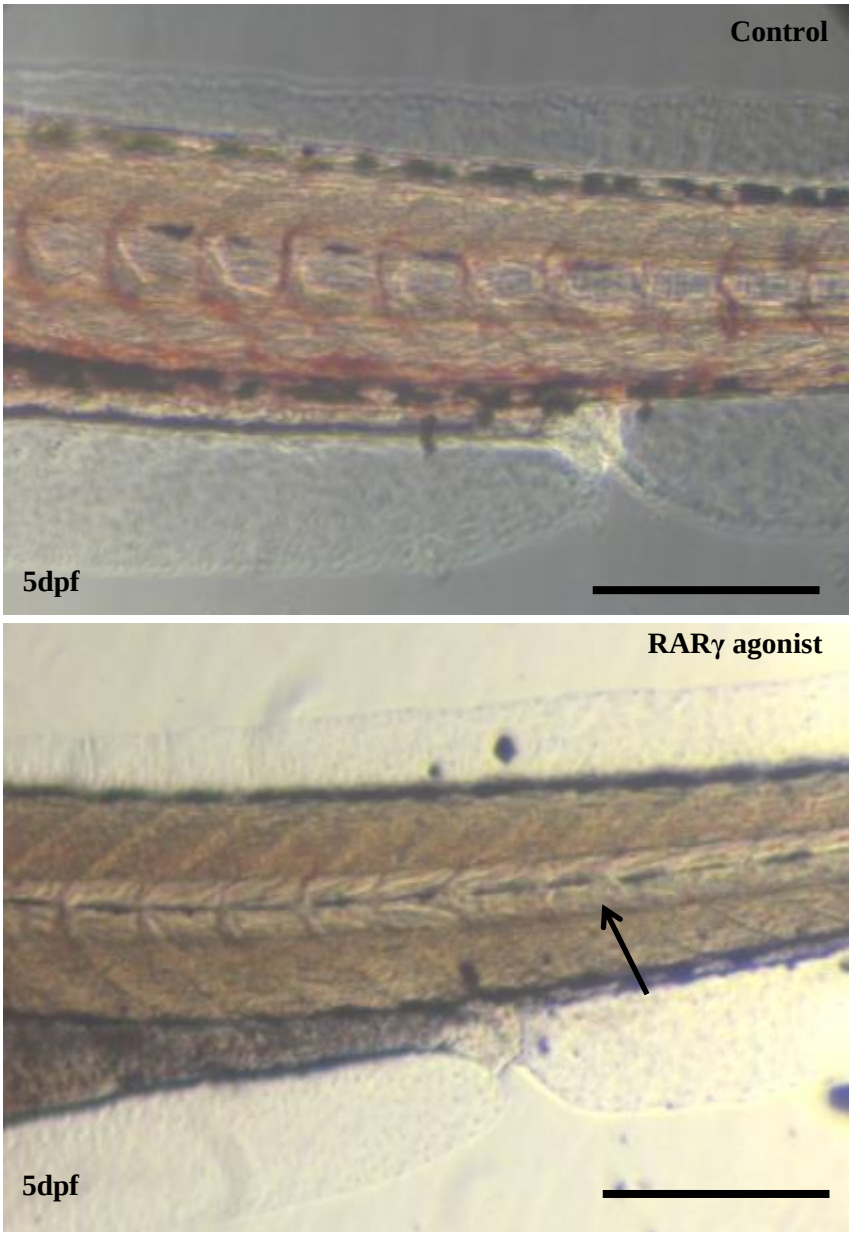


Figure 3.9. Treatment of embryonic zebrafish with a RARγ agonist (10nM) was associated with the loss of fat particles within the inter-somitic vessels.

The representative pictures show the left lateral views of the trunks in RARγ agonist (10nM) treated and control embryos at 5dpf. The lipid particles were stained with oil red o and visualized in the red colour. The representative results showed RARγ agonist (10nM) treated embryos had lack of red coloured lipid particles in the inter-somitic vessels while these red particles were present in the control embryos at 5dpf. . Scale bars = 250µm. (n=3 independent experiments)

3.9. Treatment of zebrafish embryos with the RARγ agonist caused the loss of caudal fin formation.

Another phenotype found in RARγ agonist (10nM) treated embryos was loss of caudal fin formation. Normal caudal fin formation in zebrafish embryos is found from 2dpf (Kimmel *et al.*, 1995). Therefore, there was no obvious changes in the caudal fins of RARγ agonist treated embryos at 3dpf. However, the caudal fin was clear visible in the control embryos at 3dpf while RARγ agonist treated embryos had loss of caudal fin (Figure 3.10). Normal fin mesenchyme can be found in the control embryos but this structure was not well developed in RARγ agonist treated embryos.

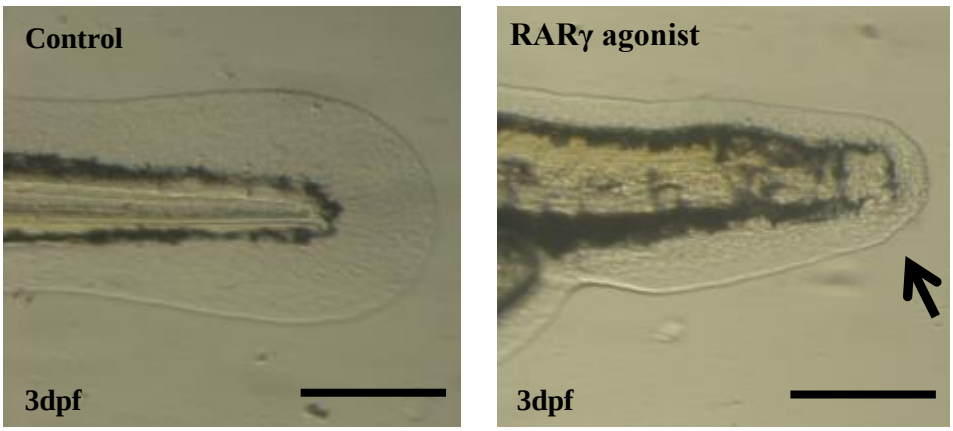


Figure 3.10. Treating embryonic zebrafish with RARγ agonist (10nM) was associated with the loss of caudal fin formation.

The representative pictures show left lateral view caudal fin of RARγ agonist (10nM) treated and control embryos at 3dpf. Wide and broad caudal fin mesenchyme was present in the control embryos while this mesenchyme was missing in RARγ agonist treated embryos. Arrow indicates the reduced and slightly kinked caudal fins that were observed in the RARγ agonist treated embryos. Scale bars = 250µm. (n=3 independent experiments)

3.10. Treatment of zebrafish embryos with the RARγ agonist caused the loss of pectoral fin formation.

Previous studies have reported that RA signalling is crucial for pectoral fin induction via *tbx5* gene expression which is the transcription factor (Begemann *et al.,* 2001; Grandel *et al.*, 2002). Pectoral fin outgrowth from the fin bud can be seen from 30hpf in normal zebrafish development (Kimmel *et al.,* 1995) and the full developed pectoral fin can be clearly seen at 3dpf.

The pectoral fin was absent in the RARγ agonist (10nM) treated embryos at 3dpf while the control embryo had a grown pectoral fin at the same stage (Figure 3.11). Although the pectoral fin was absent in the RARγ agonist treated embryos, there was a visible pectoral fin bud present in the RARγ agonist treated embryo. Therefore, the pectoral fin formation was lost in RARγ agonist treated embryos.

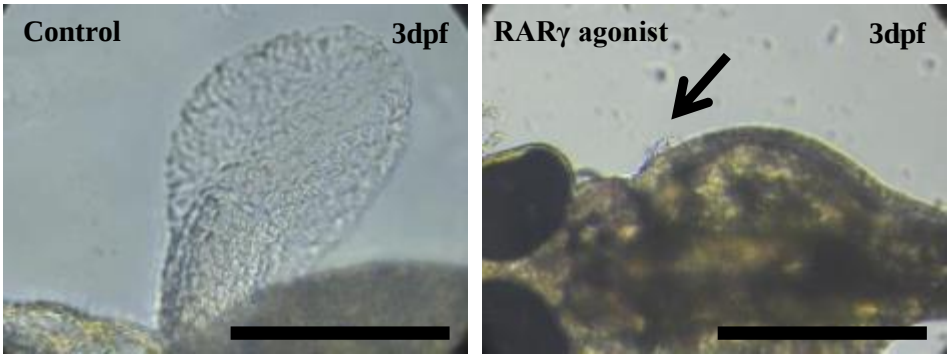


Figure 3.11. Treatment of embryonic zebrafish with the RARγ agonist (10nM) was associated with the loss of pectoral fin formation at 3dpf.

The representative pictures show the dorsal views of RARγ agonist(10nM) treated and control embryos at 3dpf in the pectoral fin area. The control embryo had well grown pectoral fin at 3dpf while the pectoral fin formation was absent in RARγ agonist (10nM) treated embryos at the same stage. However, the pectoral fin bud was present in RARγ agonist (10nM) treated embryos (arrowed). Scale bars = 250μm. (n=3 independent experiments)

3.11. Treatment of zebrafish embryos with the RARγ agonist caused the loss of pharyngeal arches.

RARγ agonist treated embryos were associated with changes in the head morphology including smaller eyes (Figure 3.12). In details, the head of RARγ agonist zebrafish embryos was smaller than that of the control embryo at 3dpf. Moreover, the shape of RARγ agonist treated embryos was round while that of the control embryos was rectangular in shape. RARγ agonist treated embryos also showed lack of pharyngeal arches inside the head whilst these structures were present in the control embryos at 3dpf. Pharyngeal arches are the supportive structures of the head. They are originated from the cranial neural crest and differentiate into the cranial cartilage and bone at later time point which is 5dpf. Therefore, this finding suggests RARγ agonist might have affected the cranial neural crest in RARγ agonist treated embryos.

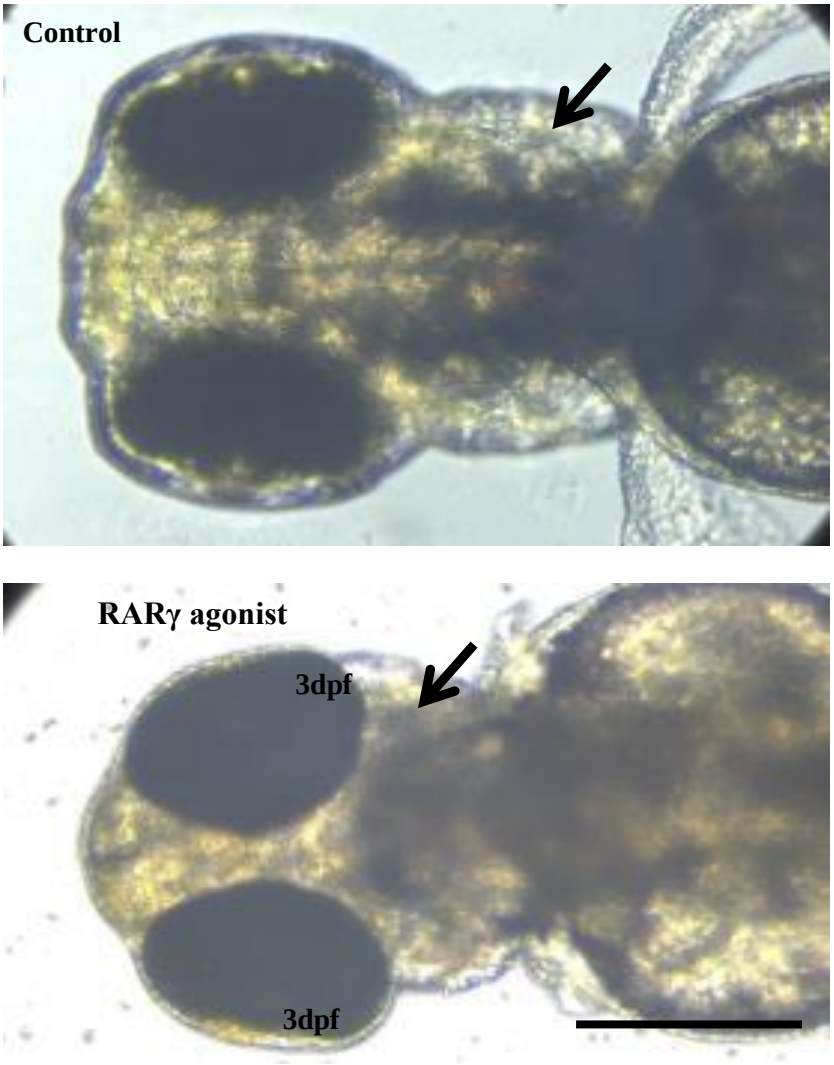


Figure 3.12. Treatment of embryonic zebrafish with RARγ was associated with the loss of pharyngeal arches at 3dpf and loss of cranial bones at 5dpf.

The representative pictures show the head dorsal views of RARγ agonist (10nM) treated and control embryos at 3dpf. The control embryos head was bigger than that of RARγ agonist embryo and rectangular in shape while the RARγ agonist treated head was round in shape. Moreover, RARγ agonist treated embryos had loss of pharyngeal arches (arrowed) while these arches were present in the control embryos at 3dpf (arrowed). Scale bars = 250µm. (n=3 independent experiments)

3.12. Discussion

The results show RARγ agonist treatment caused the pleiotropic effects on the zebrafish embryonic development when the embryos were treated with RARγ agonist at 4hpf. These effects included shorter anterior-posterior axis, slower growth rate, bigger yolk sac, cardiac oedema, venous dilation, lipid particle loss in the inter-somitic vessel, lack of caudal fin, pectoral fin and pharyngeal arches. Among them, shorter anterior-posterior axis in RARγ agonist treated embryos may be related to the cardiac oedema, bigger yolk sac, venous dilation and lipid particle loss in addition to the loss of somites. RARγ agonist treated embryos did not grow well after 2dpf. The timepoint is coincidently link to the formation of cardiac oedema at 2dpf which may result in heart failure and slow circulation. More importantly, the RARγ agonist treated embryos have very slow circulation at 4dpf and finally, the oil red o staining show no staining of fat indicating that the cascade sequences of cardiac oedema may be responsible for halting the growth of treated embryos beyond 2dpf. However, the length of the embryos have been significantly different in RARγ agonist treated and control embryos since 1dpf, there may be important changes happened before 1dpf.Therefore, cardiac oedema may be the primary effect of RARγ agonist on the heart formation which made the heart less fully functional resulting in the secondary effects followed. Changes in the head morphology and loss of pharyngeal arches in the RARγ agonist treated zebrafish embryos suggest the precursor cells, the cranial neural crest which involve in the zebrafish head skeletal structure might have been affected by the treatment.

RA has the teratogenic effects when the excessive level of RA is applied to the developing embryos. The pregnant women who were accidently exposed to the retinoid during their gestation period, gave birth to the babies which had the congenital defects including head and heart abnormalities depending on the time and period of exposure to the retinoid (Honein *et al.,* 2001). Patients who have the mutation in *CYP26,* the gene involved in the degradation of RA, genito-anal and lower limbs defect (Fukami *et al.,* 2010). The patients' plasma RA level is higher than normal range (Fukami *et al.,* 2010). These birth defects are known as posterior

regression syndrome (PRS). Interestingly, the parents of some PRS patients carry mutation in *HOXD13*, the gene which involve in posterior body part formation (Fukami *et al.,* 2010). The patients with *CYP26B1* mutation, have the craniofacial abnormalities (Laue *et al.,* 2011). Feeding retinoid to the pregnant mice also give the embryos with PRS including regressed tail formation and ano-rectal defect (Padmanabhan 1998). *Cyp26* mutant mice also has posterior regression including tail as well as shorter forelimbs (Sakai *et al.,* 2001).The zebrafish giraffe mutant (*gir),* which carry the mutation in *cyp26a1,* also shows the similar phenotypes to the RARγ agonist treated zebrafish embryos, which are shorter anterior-posterior axis, lack of pectoral fin, cardiac oedema, abnormal head morphology and venous dilation (Emoto *et al.,* 2005). Therefore, the phenotypes caused by RARγ agonist treatment to the zebrafish embryos are similar to those caused by RA in human, mice and zebrafish. Hence, these results confirm RARγ agonist worked through the similar mechanism of RA treatment.

RA treatment causes the changes in the Wnt and Hox genes expressions. Formation of posterior body part formation needs the continuous supply of cells from pre somitic mesoderm (PSM) and treatment with RA causes the down regulation of Wnt cytokine mRNA expression in mice embryos (Shum *et al.,* 1999). Moreover, RA treatment in mouse embryonic development causes the changes in Hox genes expression patterns (Kessel and Gruss 1991). In zebrafish, RA treatment interrupt the cell regulatory loop signalling in mesodermal progenitor niche (Martin and Kimelman 2010). *No tail (ntl),* which is orthologous to *Brachurary* in mammalian, is down-regulated at mRNA level after treatment with retinoic acid. Moreover, *no tail (ntl)* mutant or morpholino has shorter trunk and tail formation (Martin and Kimelman 2008).Therefore, treating the zebrafish embryos with the synthetic retinoid, RARγ agonist, which is supposed to be working through RAR also caused the embryonic teratogenic effects. *Hox* family is well known target of RA and their expression patterns are tightly regulated by the concentration of RA level. Therefore, treating with exogenic retinoic acid is related to increased or decreased expression of hox genes which are important for body axis specification (Koop *et*

al., 2010). Interestingly, overexpression of *hox5b* expression in the zebrafish embryo produced the similar phenotypes caused by RA (Waxman and Yelon 2009).

` Although there was no certainty that RARγ agonist specifically bound to the zebrafish *rarga* and *rargb*, the dramatic changes were found in the tissues or organs of RARγ agonist treated zebrafish embryos where *rarga* and *rargb* expressions were reported by *in-situ* hybridisation technique (Waxman and Yelon 2007). These tissues or organs involve the tail bud during somitogenesis stage, unlike to *raraa* and *rarab* which expressions are present mainly in the somites (Hale *et al.,* 2006). At 24hpf, the expression is found in the hindbrain, neural crest and tail. Although there was no obviosus change found in the brain in the RARγ agonist treated embryos, the treated embryos have the changes in the head morphology as well as the abnormal tail formation. Moreover, the pectoral fin bud which expresses the *rarg* at 48hpf was also affected by the RARγ agonist treatment. Morpholino gene knock down to *rarga* and *rargb* experiments also show the pharyngeal arches, pectoral fins and tail were affected(Linville *et al.,* 2009). Therefore, the treatment with RARγ agonist affected the tissues or organs where *rarg* express and are affected by morpholino knockdown. The most interesting in these findings is the tissues or organs where RA synthesizing enzyme is not present but the receptor is present. The distribution of retinoic is visualized in (Shimozono *et al.,* 2013). The transgenic zebrafish line which use RARE-YFP promoter for RA signalling, also confirms RAR/RXR mediated RA signalling does not overlap with the tissues and organs where zebrafish *rarga* and *rargb* (Waxman and Yelon 2007; Perz-Edwards *et al.,* 2001; Hale *et al.,* 2006). Therefore, these findings suggest zebrafish *rarga* and *rargb* need to be in quiescent state for normal embryonic development because they are normally present in retinoic acid free zone and co-localized with the RA degrading enzyme, *cyp26b1a* (Dobbs-McAuliffe et al. 2004).

Chapter 4

The involvement of regulatory pathways and stem/progenitor cells in the developmental phenotype following RARγ agonist treatment; somitogenesis, pectoral fin formation and the neural crest

4.1. Introduction

The results presented in Chapter 3 have shown that treatment with RARγ agonist to the zebrafish embryos was associated with a variety of phenotypic changes which included shorter antero-posterior axis, malformed caudal fin, and lack of pectoral fin and loss of pharyngeal arches. In this chapter, the possible causes which may have contributed to these changes were investigated, which involved the use of genetic analysis, immunostaining and transgenic zebrafish lines. Each of these investigations has been presented as subsections, including separate brief introductions to each of the studies performed, along with a description and discussion of all results shown. Data was presented in this way in order to demonstrate the progression of ideas and experiments.

4.2. Antero-posterior axis length and somite formation

To analyse the possible causes of the observed shorter antero-posterior body axis in RARγ agonist treated zebrafish embryo, somite formation was examined because the somite is the main building block for the trunk in the vertebrate and major contributor to overall embryo length (Hammett and Wallace 1928). Since the somite boundaries during the embryogenesis are not visually clear enough to reliably analyse the number, structure and patterns of somites present by simple light microscopy, somites were observed using an indicator transgenic zebrafish line (hspGFF55B), and following *in-situ* hybridization for the myogenic differentiation marker, *myo-D,* which has been used as a marker for muscle differentiation in somites (Davis *et al.,* 1989), and immunostaining for alpha actinin which is an actin binding protein found in the muscles.

Retinoic Acid and Somitogenesis

Somites are the paired segmented mesodermal blocks which form from the pre-somitic mesoderm (PSM) located at the most posterior region of the developing embryo during segmentation period. They are formed “rhythmic clock and wavefront” mechanism and are the precursors of dermatomes, myotomes and sclerotomes. Each of which later differentiates into skin, muscles and bones respectively.

The genes which control the somitogenesis mechanisms are the two antagonizing genes expressions within the PSM and outside the PSM. Theese oscillating genes are Wnt/Fgf and RA where Wnt/Fgf is mainly expressed in the PSM which RA is present in outside PSM. Interestingly, PSM is RA free zone and Wnt/Fgf induces *Cyp26b1* in this area to prevent RA (Figure 1.5).

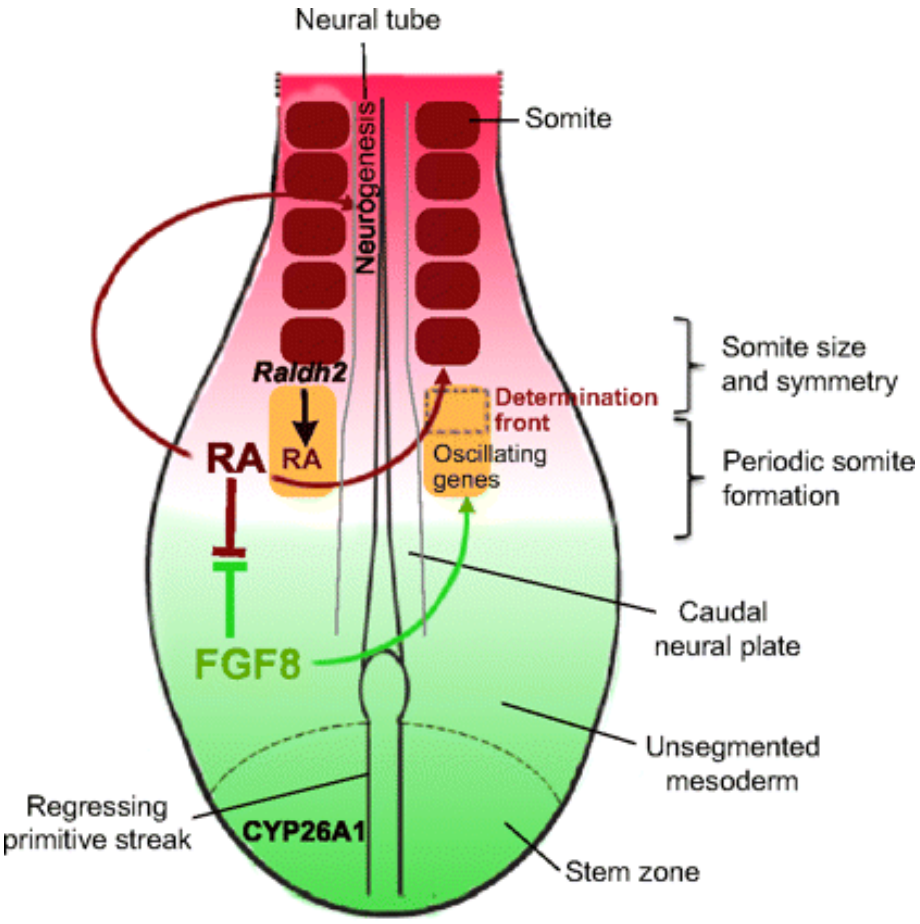


Figure 4.1. RA signalling during somitogenesis

The picture is adapted from (Rhinn and Dollé 2012) and the permission to reuse this figure is obtained from the publisher. During somitogenesis, RA is synthesized by Raldh2 which is expressed in the developing somites. However, RA is prevented in the pre-somitic mesoderm (PSM), the stem cell zone by expression Cyp26A1 which active RA into unactive 4-hydroxy RA. Fgf8 expression is also present in the PSM during somitogenesis.

Retinoic acid and Hox genes

Homeobox (Hox) genes are the evolutionarily conserved genes clusters which are expressed during segmentation periods of the developing embryos for the segmental identities. The hox genes families are normally important for anterior-posterior axis formation during development. Although it is not fully understood how the hox genes are regulated, it has been shown that hox genes are regulated by the endocrine factors such as RA, oestrogen and vitamin D.

Retinoic acid response elements (RARE) locations have been reported near 5' regions of Hoxa1, Hoxb1 and Hoxd4. And it has also been shown that RA directly regulate Hoxb1 gene. Moreover, the retinoic acid receptors (RARs) expression during development is also co-localized with Hox gene expression patterns during anteroposterior axis formation. In addition, the phenotypes of RAR knoc-out mices mimic Hox gene knock-out mice. Therefore, RA signalling plays as an important regulator for Hox gene expressions.

The hox genes family is a well-known target of RA signalling and key regulator controlling formation of the body plan and segmental identity (Koop *et al.*, 2010). Some hox genes are direct downstream targets of RA signalling (Pöpperl and Featherstone 1993; Huang *et al.*, 2002) and the RARE itself is located within the regulatory region of hox gene clusters (Zhang *et al.*, 2000). Moreover, knock-out of hox genes in mice can show a loss of normal vertebrae in the mutant mice, resulting in the changes of body axis (Wellik 2007). Therefore, the two available hox gene reporter transgenic lines from the zTrap library at NIG, Mishima (Kawakami *et al.*, 2010) were analysed for the patterns of gene expression after RARγ agonist treatment.

Therefore, in this chapter, the patterns of hox genes expression by RARα agonism were analysed using two hox gene transgenic zebrafish lines which have the

reporter GFP expression patterns in the tail regions. Hence, the changes in the GFP expression patterns of hox gene transgenic zebrafish embryos will recapitulate the effects of RARα agonism on somite formation and hox gene expression.

4.2.1 Treatment of the reporter fish line, hspGFF55B, with RARγ agonist was associated with reduction in somite number.

The transgenic fish line, hspGFF55B, from the zTrap library (Kawakami *et al.*, 2010) which has GFP expression both in somite and heart although the mapping for the insertion has not been done, this particular transgenic line has specific expression in somites and heart. The results of the RARγ agonist treatment on the transgenic line showed reduced number of somites in RARγ agonist treated embryo at 3dpf (Figure 4.1). This expression pattern permitted the observation of a reduced number of somites in the RARγ agonist treated reporter fish line at 3dpf compared with reporter fish treated with carrier alone (controls) (Figure 4.1). Hence, the number of somite present in RARγ agonist treated embryos at 3dpf was determined to be 5 fewer in treated embryos than the 30 somites seen in control embryo at the same stage, which is the normal number of somites formed in zebrafish during embryogenesis (Stickney *et al.*, 2000)

The reduced number of somite was further confirmed by α actinin antibody staining for myocytes in somite. Alpha-actinin is the protein which is important for z-band microfilament function and antibody staining to this protein has been used as the marker of functional myocyte for very long time (Sugita *et al.,* 1974). Since somite differentiates into myotome and sclerotome (Duband *et al.,* 1987), this antibody staining was used to visualize the number of somites formed in RARγ agonist treated and control embryos. The results show the agreement with the previous finding in RARγ agonist treated embryos which is lesser somite number than that of control embryos at 3dpf (Figure 4.2).

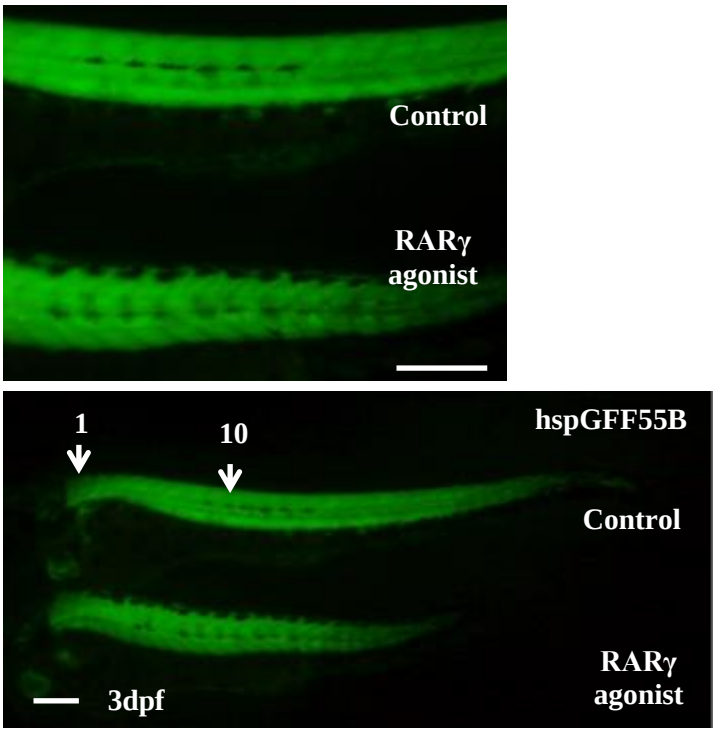


Figure 4.2. Treatment of embryonic zebrafish with a RARγ agonist (10nM) was associated with a reduction in somite formation.

A representative fluorescence microscopy picture showing the left lateral view of RARγ agonist (10nM) treated (10nM) and control hspGFF55B transgenic zebrafish embryos at 3dpf where the somites are indicated by GFP expression. There were 5 fewer somites present in the RARγ agonist (10nM) treated embryos (25 somites) compared to the control embryos (30 somites), but there was no discernible difference in the size of the individual somite present, as shown by counting from anterior somites 1-10. These differences were seen in very case of RARγ agonist (10nM) treated versus control embryos. Scale bars represent 250µm. (n=3 independent experiments)

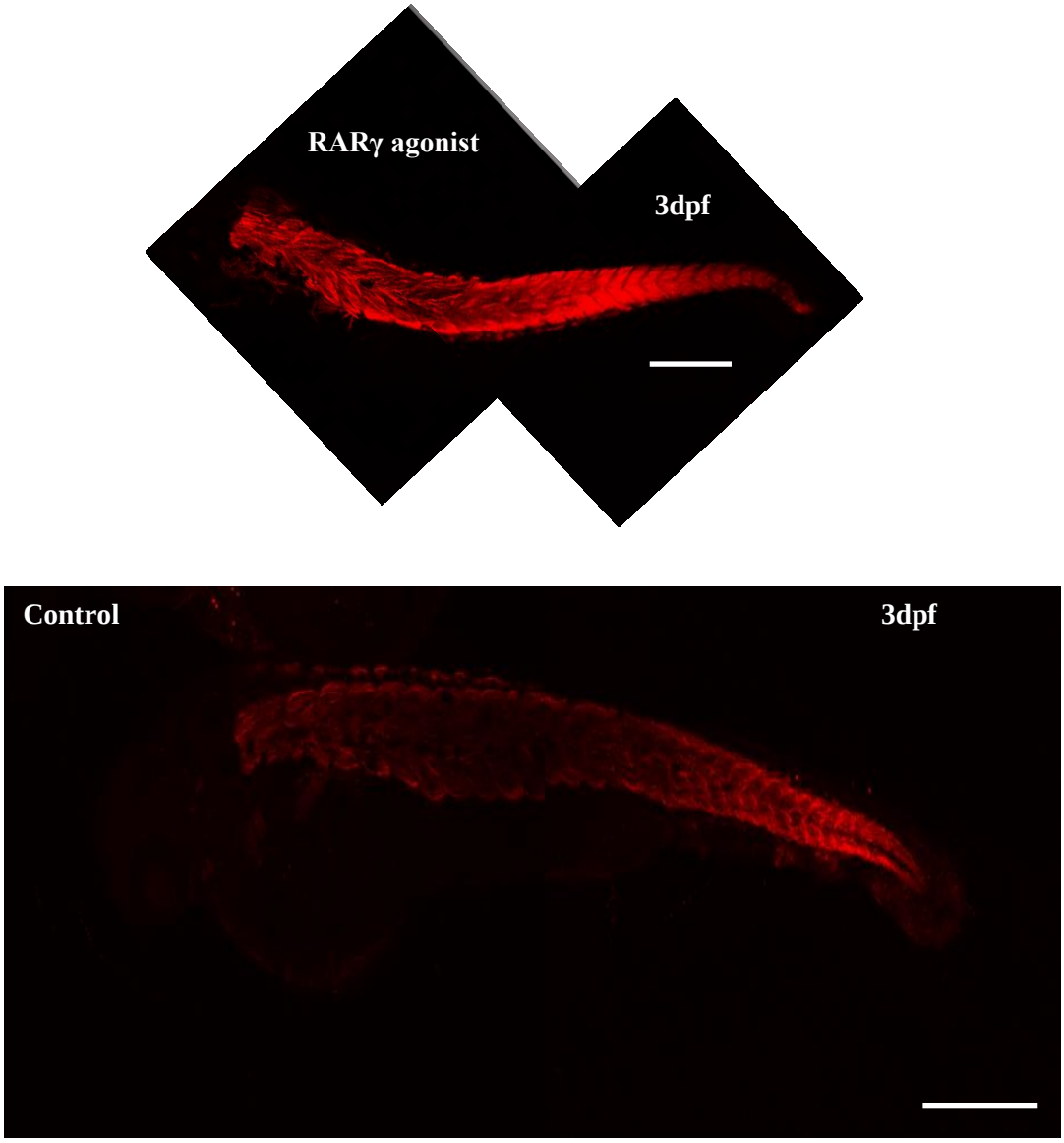


Figure 4.3. Treatment of embryonic zebrafish with a RARγ agonist (10nM) was associated with a reduction in somite formation confirmed by α actinin staining.

Representative fluorescence microscopy pictures of α actinin immunolocalisation in RARγ agonist (10nM) treated and control AB strain zebrafish embryos at 3dpf. No discernable differences in immunopositivity were seen. An identically reduced number of somites as that seen in the RARγ agonist (10nM) treated embryos immunostained for α actinin. Scale bars represent 250μm. Immunostaining for α actinin was performed in 14 control embryos and 14 RARγ agonist (10nM) treated embryos.

4.2.2. Treatment of embryonic zebrafish with RARγ agonist was associated with weaker *myo-D* expression at 22hpf.

To analyse the effects of RARγ agonist treatment on muscle differentiation patterns, the RARγ agonist treated and control embryos were stained for myo-D gene by *in-situ* hybridisation at 22hpf. The results showed the weaker expressions pattern of the staining in the RARγ agonist treated embryos which were harvested at 22hpf (Figure 4.3). The staining strength in the tails of both RARγ agonist treated and control embryos seems the same but the anterior part of treated embryos have weaker staining compared to that of control embryos.

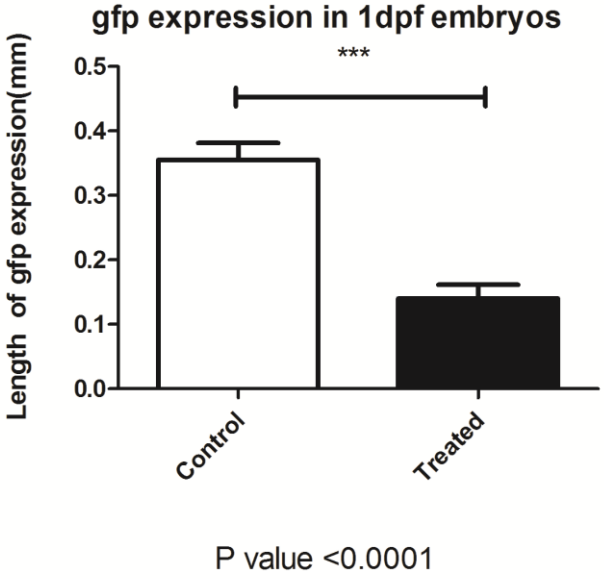
Figure 4.4. Treatment of embryonic zebrafish with a RARγ agonist (10nM) was associated with a reduction in myoD expression.

Representative bright field pictures of *in situ* hybridisation for *myoD* expression in the RARγ agonist (10nM) (10nM) treated and control AB strain zebrafish embryos at 22 hours post fertilisation (hpf), where a slightly reduced signal was observed in somitic *myoD* expression in the RARγ agonist (10nM) treated embryos. Scale bars represent 0.5mm. *myoD* was determined in 14 RARγ agonist (10nM) treated embryos versus 13 control embryos.

4.2.3. Treatment of embryonic zebrafish with RARγ agonist was associated with reduced GFP expression in *hoxc11a* transgenic line created by gene-trap method.

After observation of reduced number of somites in the RARγ agonist treated embryos than control embryos, the positions of missing somites were investigated using RARγ agonist treated *hoxc11a* transgenic line. Precise expression of hox-gene family during the body axis formation is necessary for normal antero-posterior axis in vertebrate (Wellik 2007). *Hoxc11* expression can be found in the posterior region of developing mice embryos (Hostikka and Capecchi 1998) and *Hoxc*-cluster homozygous knock–out mice shows transformation of caudal vertebra to lumbar vertebra resulting in loss of caudal vertebra (Suemori and Noguchi 2000). Here, our results also showed treatment with RARγ agonist to the *hoxc11a* gene trap line causes reduced expression of *hoxc11a* from 1dpf to 5dpf (Figure 4.4). In 1dpf, *hoxc11a* expression can be seen in the most posterior region of the developing embryo and the length of GFP expression in RARγ agonist treated embryo was significantly lower than that of control embryos. After hatching from the chorion at 2dpf, the area covered by the GFP expression in the posterior region is clearly visible. In the RARγ agonist treated embryo, the percentage of GFP expressing length to the whole body length never exceeded more than 30% from 2dpf to 5dpf while the GFP coverage in the control embryo was more than 40% at 2dpf and more than 50% from 3dpf to 5dpf. These results indicate *hoxc11a*-GFP expression moved from posterior to anterior end throughout the development in the control embryos whilst that anterior progression was halted in the RARγ agonist treated embryos.

A



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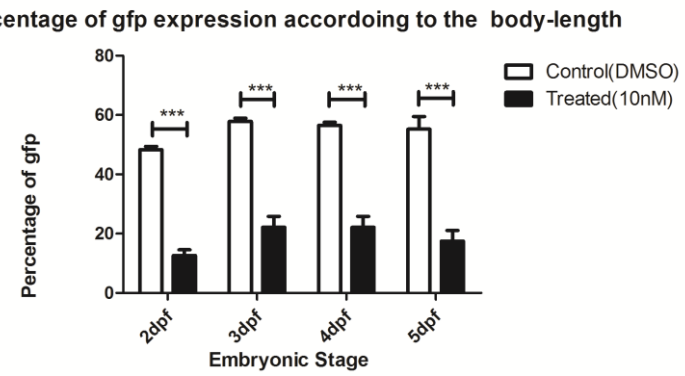
B



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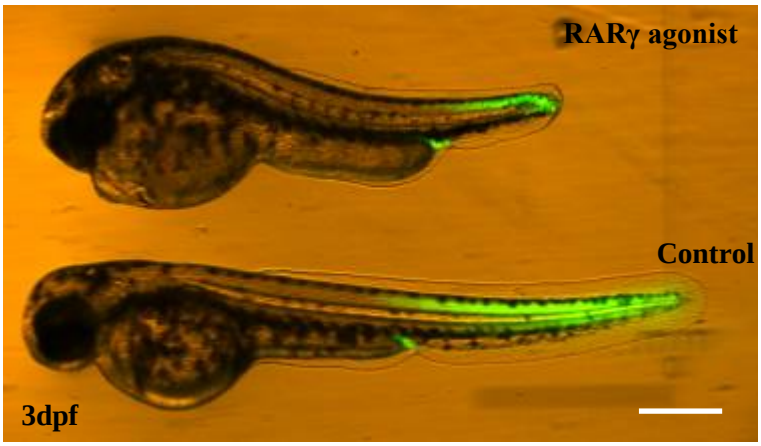

Figure 4.5. Differential patterns of hoxc11a expression in transgenic reporter zebrafish embryos demonstrated that the reduced axial length of RARγ agonist (10nM) treated embryos was associated with loss of their most posterior regions.

(A) A graph shows the difference in length of GFP expression between RARγ agonist (10nM) treated and control transgenic lines at 1dpf. The length of GFP expression in the tail region of RARγ agonist (10nM) was significantly reduced compared to the control. (B) A graph compares the percentage of the body length which had GFP between RARγ agonist (10nM) treated and control embryos from 2dpf to 5dpf. The percentage of GFP length did not significantly increase in the RARγ agonist (10nM) treated embryos compared to the control embryos throughout the development stage. (C) A representative fluorescence microscopy picture showing GFP expression in the hspGFF155A transgenic zebrafish reported line for *hoxc11a* expression at 3dpf, where a similar pattern of expression was seen in the RARγ agonist (10nM) treated and control embryos. Scale bar represents 1mm. (n=3 independent experiments)

4.2.4. Treatment of embryonic zebrafish with RARγ agonist was associated with the complete loss of gene expression in *hoxb13a* transgenic line.

Another hox gene line was also analysed to see the effects of hox gene in body axis formation in RARγ agonist treated embryos. The transgenic line was used from zTrap library (Kawakami *et al.,* 2010) created by enhancer trap line method. The GFP-sequence was inserted in *hoxb13a* of zebrafish genome. The GFP expression starts to be seen in the most caudal area of the embryos at 1dpf. In the mice embryos, the *hoxb13* expression can be in the tail-bud area around E9 stage (Zeltser *et al.,* 1996). Interestingly, the similar *hoxb13* expression is also found in the developing tail of axolotl as well as in the regenerating tail after amputation (Carlson *et al.,* 2001). Interestingly, there is no report of complete gene knock out for *hoxb13* but targeted mutations in mice hoxb13 gene causes overgrowth of tail vertebra and spinal cord (Economides *et al.,* 2003). The results of the RARγ agonist treatment on *hox13a* line showed the complete loss of GFP expression in RARγ agonist treated embryos in the tail and anal region from 1dpf to 5dpf (Figure 4.5).

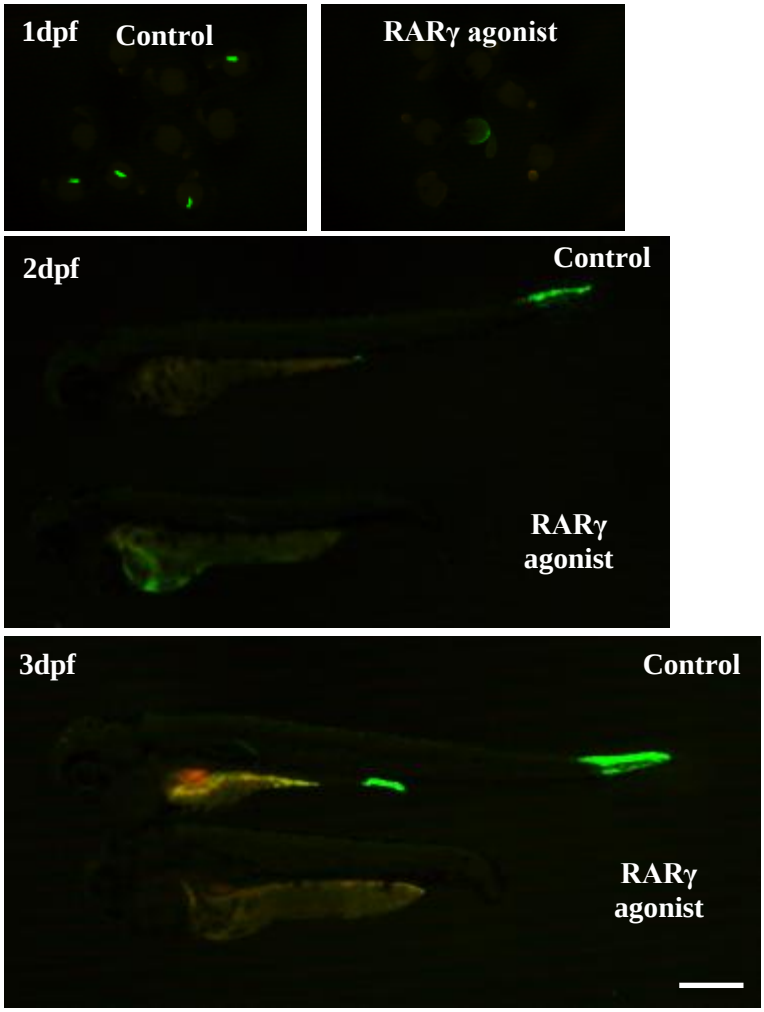

Figure 4.6. Differential patterns of hoxb13a expression in transgenic reporter zebrafish embryos demonstrated that the reduced axial length of RARγ agonist (10nM) treated embryos was associated with loss of their most posterior regions.

Representative fluorescence microscopy pictures showing GFP expression in the hspGFFDMC28B transgenic zebrafish reporter line for *hoxb13a* expression from 1dpf to 3dpf. In contrast to the control embryos, RARγ agonist (10nM) treated embryo exhibited a complete loss of GFP expression in the tail, as well as in the anal region. Scale bars represent 250μm.

4.2.5. *hoxb13a* GFP expression and caudal fin formation were rescued by wash out of the RARγ agonist or additional treatment with RARγ antagonist.

The previous results showed the complete loss of GFP expression in RARγ agonist treated *hoxb13a* transgenic reporter zebrafish embryos at 3dpf. Moreover, the data also suggested the loss of GFP expression was in the most posterior part of the embryos including the caudal fin plus loss of posterior tissues and somites. Zebrafish has been used as a good model for regeneration of various organs and tissues including the caudal fin (Kawakami *et al.,* 2004; Porcellini 2009). In addition, hox gene family are involved in caudal fin regeneration (Carlson *et al.,* 2001; Thummel *et al.,* 2007). Therefore, the follow-up experiments were done to test a hypothesis, if the loss of posterior tissues in RARγ agonist treated zebrafish embryos was mainly due to loss of *hoxb13a* effect by RARγ agonist treatment, the removal of RARγ agonist from the zebrafish embryos just before the time-point of *hoxb13a* expression should rescue the gene expression. Moreover, if *hoxb13a* expression in zebrafish is implicated in the caudal fin regeneration like in axolotl (Carlson *et al.,* 2001), the re-expression of *hoxb13a* should be associated with fin regeneration.

The results show the re-repression of *hoxb13a* was observed at 5dpf in the reporter transgenic zebrafish embryos when they were treated with RARγ agonist from 4hpf to 23hpf which is the time point that *hoxb13a* starts to express in the most posterior region of the tail, followed by the washing out of RARγ agonist, replaced with the control media or additionally treated with the RARγ antagonist (Figure 4.6). The GFP expression was not initially observed in the tail area until 5dpf. Initially, the GFP expression was observed in RARγ agonist washed out or RARγ antagonist co-added *hoxb13a* transgenic zebrafish embryos at 2dpf and 3dpf. The tiny GFP expression started to appear in the caudal area at 4dpf and finally, the clear expression of GFP was

observed at 5dpf. Therefore, the GFP re-expression of *hoxb13a* took approximately 3 days. The regenerated caudal fin was also present in the *hoxb13a* rescued transgenic zebrafish embryos (Figure 4.7). Therefore, the results showed the loss of GFP expression in the RARγ agonist treated *hoxb13a* transgenic embryos was rescuable by the removal of RARγ agonist and the time frame period to rescue *hoxb13a* expression including the caudal fin regeneration took approximately 3 days. Interestingly, the time frame for the re-expression of GFP and caudal fin regeneration coincides with the time to regenerate the zebrafish larval fin regeneration after amputation (Kawakami *et al.,* 2004). Therefore, the *hoxb13a* expression in the zebrafish caudal fin might have an important role for regeneration like in the axolotl (Carlson *et al.,* 2001).

The additional experiments related to the *hoxb13a* expression showed other interesting natures of the gene. The hypothesis was to test the repressive activity of RARγ agonist on the gene expression. The *hoxb13a* transgenic embryos were treated with RARγ agonist at 23hpf which is the time point that GFP expression started to appear in the tail. Interestingly, the GFP expression was observed in the RARγ agonist treated embryos at 2dpf although the caudal fin was not fully formed (Figure 4.8). Conversely, the transgenic embryos which were treated with RARγ agonist at 22hpf, one hour before the actual GFP expression appeared, did not show any GFP expression in the tail at 2dpf (Figure 4.9). Therefore, these data suggested the repressive activity of RARγ agonist targeted at the transcriptional level rather than the translational level of *hoxb13a* expression because treatment with RARγ agonist at 23hpf did not prevent the GFP expression at 2dpf.

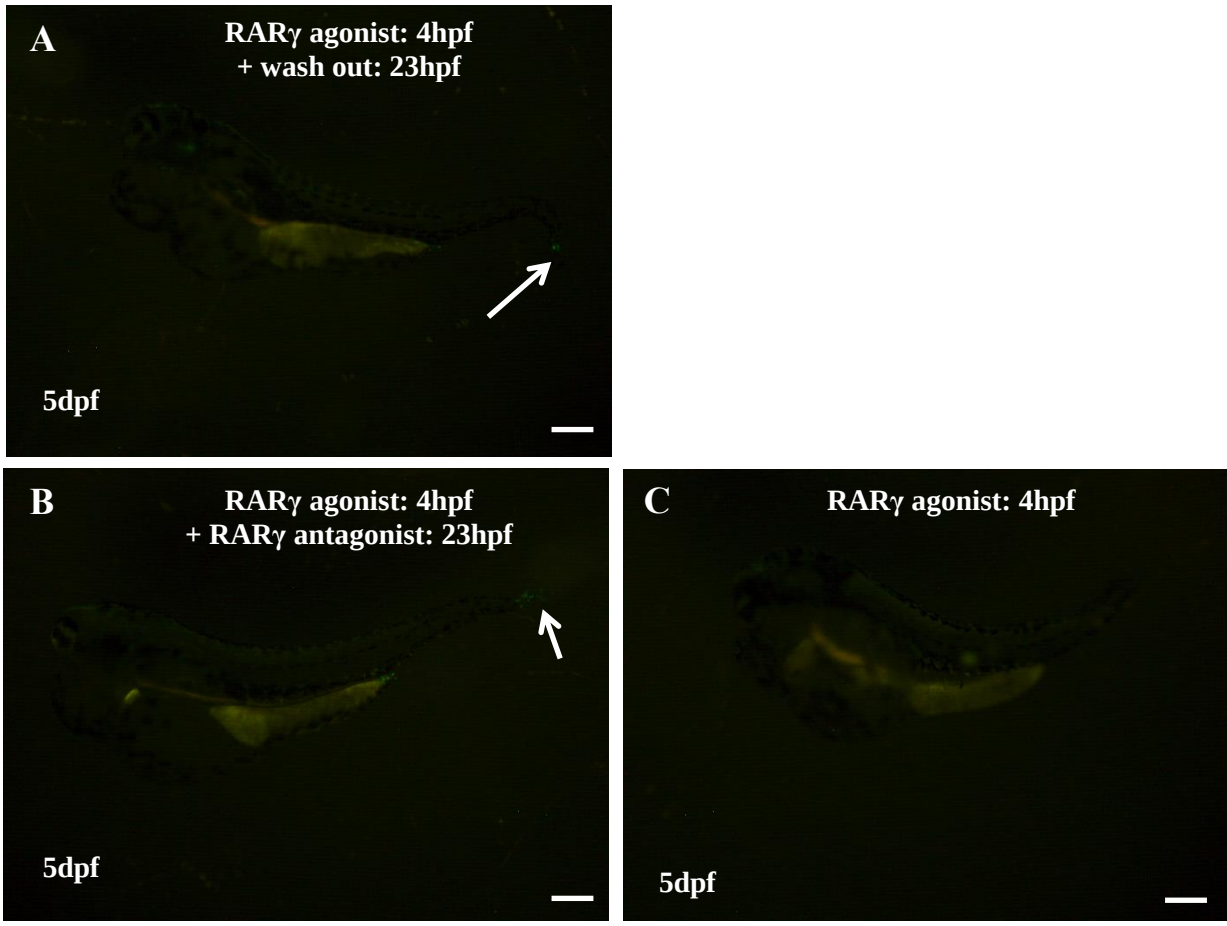

Figure 4.7. The effect of a RARγ agonist (10nM) on hoxb13a was reversed by agonist washout or co-treatment with a RARγ antagonist.

Representative fluorescent microscopic pictures showing GFP expression in the hspGFFDMC28B transgenic zebrafish reporter line for *hoxb13a* expression at 5dpf, in which the embryos were treated with RARγ agonist (10nM) at 4dpf and (A) washed out of RARγ agonist at 23hpf, (B) then co-treated with RARγ antagonist at 23hpf (C) no followed up modification. The arrows showed the GFP re-expression in the caudal fin area. Scale bars represent 250µm. (n=4 independent experiments)

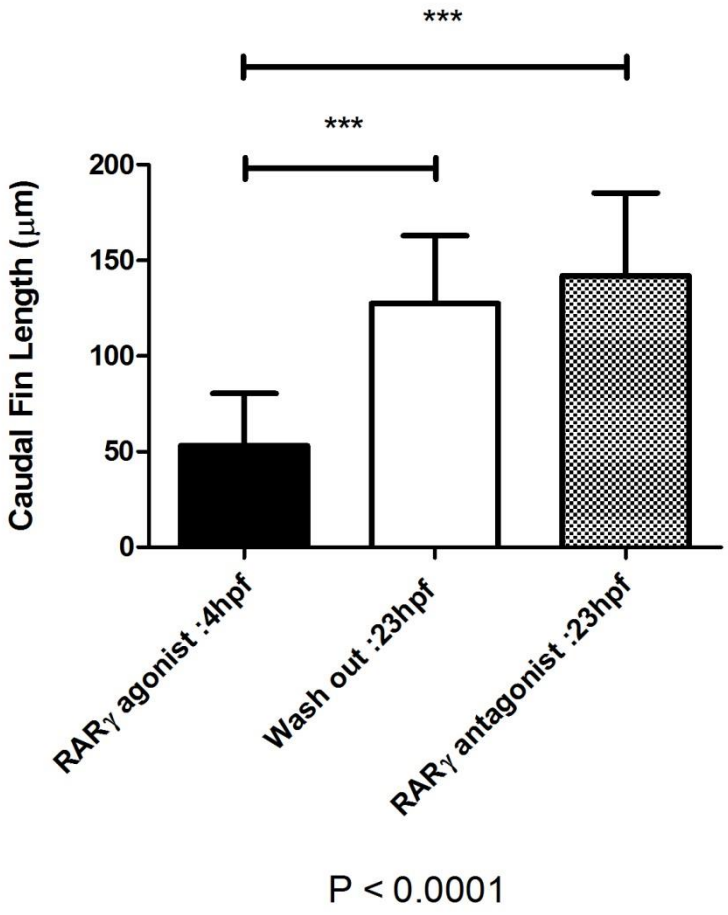

Figure 4.8. The effect of a RARγ agonist (10nM) on caudal fin outgrowth was reversed by agonist washout or co-treatment with a RARγ antagonist.

The graph shows the comparative length of caudal fin in the RARγ agonist (10nM) treated embryos versus the wash out or RARγ antagonist co-treated embryos at 5dpf. Data are shown as means +/- SD, pooled from 4 independent experiments.

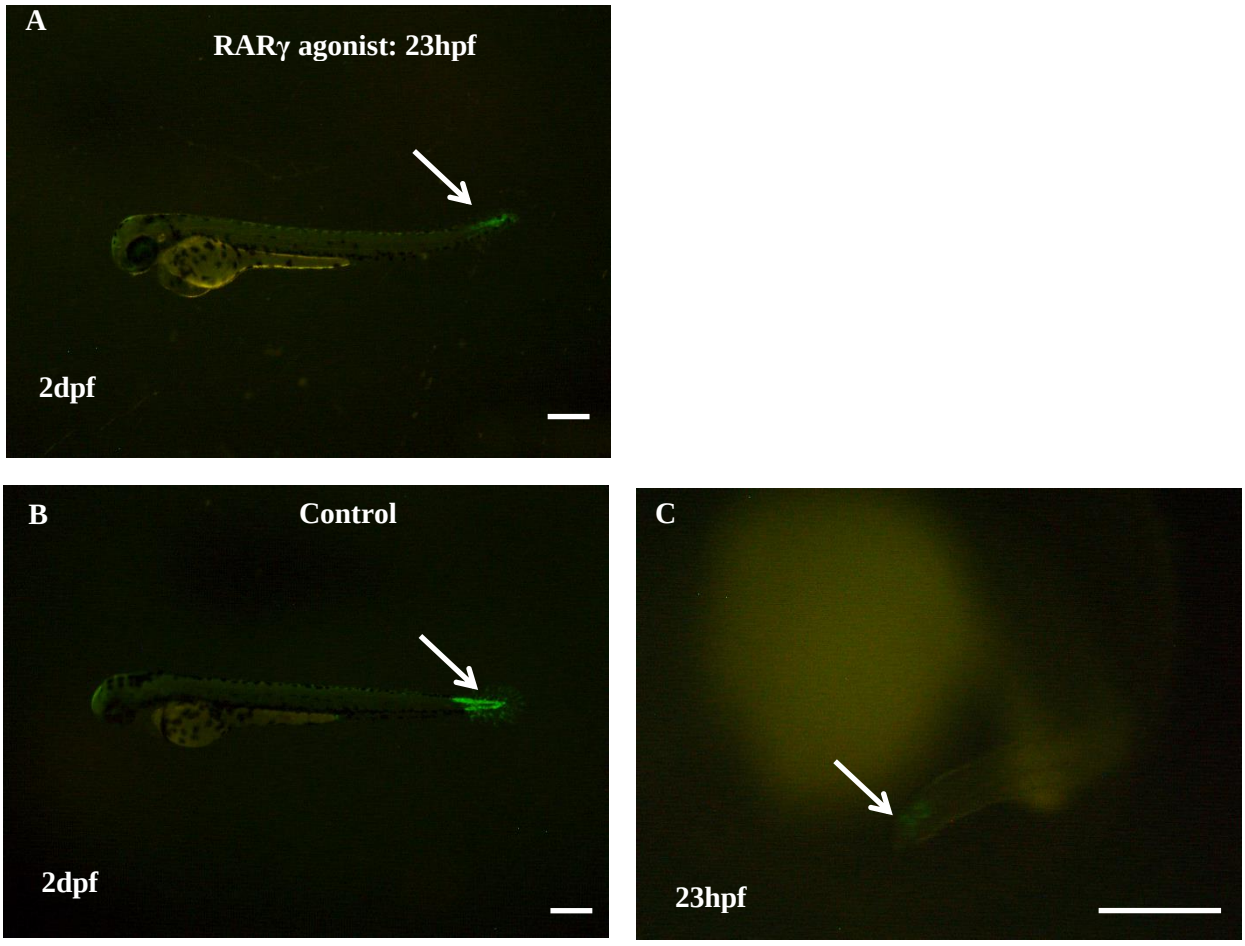

Figure 4.9. Treatment with a RARγ agonist (10nM) to the hoxb13a reporter transgenic line a23hpf failed to prevent the GFP expression at 2dpf.

Representative fluorescent microscopic pictures showing GFP expression in the hspGFFDMC28B transgenic zebrafish reporter line for *hoxb13a* expression in the caudal area (A) at 2dpf in which the embryos were treated with RARγ agonist (10nM) at 23hpf (B) at 2hpf in which the embryos were treated with control media at 23hpf and (C) at 23 hpf which was the first time point of GFP expression in the caudal area. The arrows showed the GFP expression in the caudal fin area. Scale bars represent 250µm. (n=2 independent experiments)

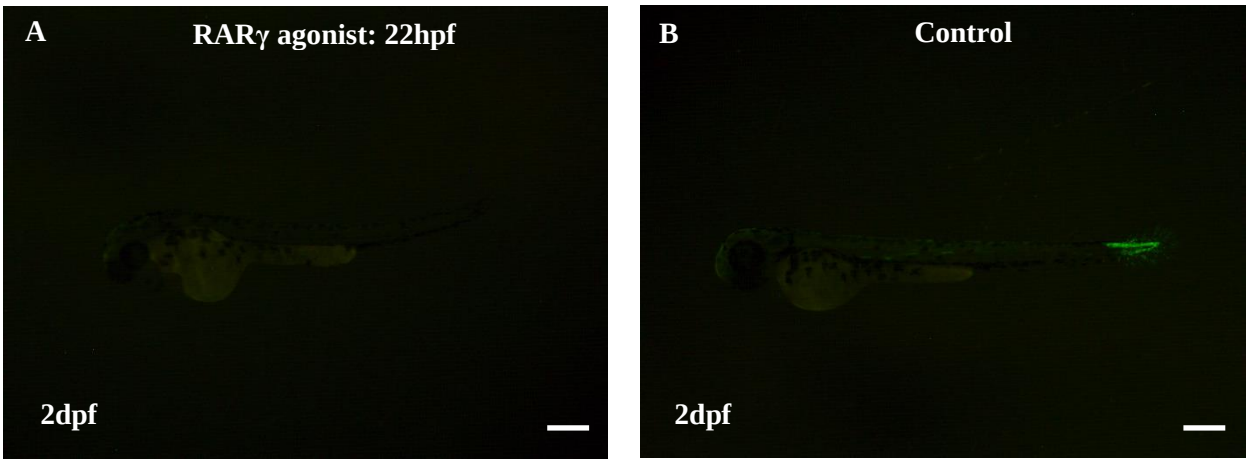

Figure 4.10. Treatment with a RARγ agonist (10nM) to the hoxb13a reporter transgenic reporter zebrafish embryos at 22hpf blocked the GFP expression at 2dpf.

Representative fluorescent microscopic pictures show the hspGFFDMC28B transgenic zebrafish embryos at 2dpf. The transgenic embryo which was treated with RARγ agonist (10nM) at 22hpf did not show any GFP expression in the tail region at 2dpf (A) while the control embryo showed the GFP expression in the tail region at 2dpf (B) (arrowed). Scale bars represent 250µm. (n=3 independent experiments)

4.2.5 Discussion

4.2.5.1 Loss of *hoxc11a* and *hoxb13a* indicated the missing caudal somites in RARγ agonist treated zebrafish embryos.

The findings above have simply driven to summarise hox-genes are responsible for the shorter body axis. However, it is not the conclusive answer because the loss of fat particles in RARγ agonist treated embryos has been reported in Chapter 3. The role of nutritional factors to the growth cannot be ignored and eventually, it is safer to conclude the short antero-posterior axis formation may be multi-factorial.

Short antero-posterior axis length in treatment is a common associated phenotype. All the accessible papers of drug toxicity on early zebrafish development published from 1997 until nowadays point out the shorter body axis regardless to the type of drugs. Therefore, it is impossible to rule out that the shortening of antero-posterior axis in this project is simply because of toxicity of the drug which may even have nothing to do with the receptor although the solution has the right pH.

However, there was a strong relationship between the changes of hox-gene expressions and the shorter antero-posterior axis in RARγ agonist treated zebrafish embryos. RARγ agonist treatment directly might have caused the reduced hox-gene expressions resulting in shorter antero-posterior axis. On the other hand, RARγ agonist might have interacted with another molecular signalling pathways resulting in loss of the posterior somite formation, and as the consequence, there are no somite to express hox genes. Nevertheless, both possibilities confirm the missing somite in RARγ agonist treated zebrafish embryos were from the caudal region.

Over 350 papers published about hox genes study in zebrafish show hox genes were impossible to visualize or clone unless somite are present because the segmentation patterns formation simply depends on somites. This information indicates the possibility about loss of *hoxc11a* and *hoxb13a* was due to lack of somite to express these genes. This hypothesis explains *hoxc11a* expression in the RARγ agonist treated embryos was never more than 30% of

total body length because there was no more somite forming at the most posterior region and loss of *hoxb13a* in the most posterior region of treated embryos was because of the somite which express *hoxb13a* never formed in the RARγ agonist treated embryos. Therefore, the loss hox gene expressions are secondary effects of somitogenesis which lead to investigate the signalling involved in somitogenesis which is wnt signalling (Gibb *et al.,* 2009; Martin and Kimelman 2008; Martin and Kimelman 2010).

However, it is difficult to ignore the facts that RARE (retinoic acid response element) is located in the regulatory region of hox gene clusters (Zhang *et al.,* 2000; Pöpperl and Featherstone 1993; Huang *et al.,* 2002) and the chemical used in this project itself has been reported for working through the retinoic acid receptor in mammalian cell line (Hughes *et al.,* 2006). More importantly, there is crucial evidence present in *hoxb13a* results (Figure 4.5) which shows there is an extra-somitic expression of hoxb13a gene in the anal fin area which is distant from the somite. This odd finding is purely contradicting the second hypothesis of somite are responsible for loss of gene expression. The *hoxb13a* expression in the protodeum is not ectopic or non-specific expression of the enhancer trap line because this hoxb13 gene expression pattern has been studied and its expression in hindgut and urogenital area of mice is also reported (Zeltser *et al.,* 1996). Interestingly, this protodeum is ectodermal derived, not mesodermal. Therefore, the hypothesis of somite-dependent hox gene expression is contradictory to this finding and it is also pointing loss of hox gene is direct effect of retinoic acid gamma agonist drug. These findings agree with the discussion of caudal regression syndrome symptoms and gene mutation found in human patients (Chapter 3). Therefore, the loss of hox genes may partially direct effect and partially indirect effect depending on the tissues or organs. However, this conclusion raises another question.

The experiments conducted in zebrafish so far show RA treated zebrafish has increased hox gene expressions and treatment with DEAB(retinoic acid inhibitor) shows loss of hox gene expressions according to the *in-situ* hybridisation techniques (Ishioka *et al.,* 2011; Maves and Kimmel 2005) and qtPCR results show mRNA of hox genes increase three to six folds after

treatment with RA (Oliveira *et al.,* 2013). These papers are clearly contradicting the results of *hoxc11a* and *hoxb13a* which have the reduced or loss of gene expression.

At last, the experiments on the re-expression of *hoxb13a* indicate the repressive activity of RARγ agonist on *hoxb13a* expression was temporary at transcriptional level and the gene expression can be restored after the removal of RARγ agonist. Moreover, the results also suggested the repressive activity of RARγ agonist on stem/progenitor cells which are responsible for the somite formation, was transient and the stem/progenitor cells niches were not permanently repressed. On the other hand, the results also indicated that the ligand free RARγ expression was important for the somite formation as well as in caudal fin formation. Interestingly, a very recently published paper (Janesick *et al.,* 2014) also showed the ligand free RARγ expression is required for vertebrate axis formation using *Xenopus* as model organism.

4.3. Introduction for loss of pectoral fin

Another phenotype found in the RARγ agonist treated zebrafish embryo is the loss of pectoral fin. As mentioned in the previous chapter, the treatment of RARγ agonist was associated with loss of pectoral fin at 3dpf. Therefore, in this chapter, the possible causes and mechanisms involved in the pectoral fin formation of RARγ agonist treated embryo is dissected. Since RARγ agonist is related to the retinoic acid signalling, the involvement of RA is first investigated. As mentioned in (Gibert *et al.,* 2006) paper, the pectoral fin formation starts with the *raldh1a2,* the gene responsible for the conversion of retinaldehyde to RA, expression in the somite region of 2 to 6. Localized expression of this enzyme synthesizes RA which can be visualized in the respective region (Shimozono *et al.,* 2013). The synthesized RA induces the *tbx5a* which is the transcription factor responsible for heart and pectoral fin formation. *Nls* (neckless) mutant zebrafish which has mutation in *raldh1a2* g (Grandel *et al.,* 2002) or RA synthesis inhibitor, DEAB (diethylaminobenzyldehyde), treated embryo show lack of pectoral fin formation including loss of *tbx5a* expression (Grandel and Brand 2011) as the result of failure to induce *tbx5a* by RA. Moreover, *tbx5a* mutation causes loss of pectoral fin formation in the mutant line called heart string in which *tbx5a* is mutated (Garrity *et al.,* 2002). Therefore, to find out why RARγ agonist treated embryos have loss of pectoral fin, the first gene to look at is *tbx5a* expression in the RARγ agonist treated embryos. It was hypothesized that the tbx5a gene expression might be down-regulated since high level of RA inhibits *raldh1a* expression by reducing the interaction of *raldh1* promoter binding and the proteins (Elizondo *et al.,* 2009). Therefore, the presence of *tbx5a* expression was analysed in RARγ agonist treated embryos which may be affected by down-regulation of *raldh1*a as the possible result of negative feedback mechanism of high level of RARγ agonist.

4.3.1. *tbx5a* expression was present in RARγ agonist treated embryos.

Zebrafish *tbx5a* expression is seen from 14hpf in the lateral late mesoderm and eyes to 4dpf in heart, eyes and pectoral fins according to *in-situ* hybridization report (Albalat *et al.,* 2010). The expression pattern found in lateral plate mesoderm which is common progenitor for heart and pectoral fins and become separated from 24hpf and at 27hpf, the expression can clearly be seen at three different tissues, eyes, heart and pectoral fin ridge which is about to become the fin bud (Albalat *et al.,* 2010). Therefore, the RARγ agonist treated and control embryos were fixed at 27hpf and stained for *tbx5a* expression. The results show both control and RARγ agonist treated embryos had the gene expression in heart and pectoral fin areas, but not in eyes (Figure 4.10).

The presence of *tbx5a* transcription factor was validated using non-immuno staining experiments. The hypothesis was to treat the embryos with RARγ agonist during the window periods which have *tbx5a* expression. In simple terms, if the transcription factor was not functioning or disrupted by the treatment, the fin formation will not form even if the RARγ agonist was removed after certain period of treatment. Again for the time point, the 27hpf was chosen again because it is the transition point where the lateral plate mesoderm expression *tbx5a* starts to form the pectoral bud (Albalat *et al.,* 2010). The results (Figure 4.11) show the confirmation of the presence of *tbx5a* in RARγ agonist treated embryos because the RARγ agonist treated embryos grew the pectoral fins back when the RARγ agonist media was replaced with control media or the same concentration of RARγ antagonist was added at 27hpf. This findings lead to assume the loss of pectoral fin in RARγ agonist treated embryos was nothing to do with *tbx5a* expression, and possibly the defects in outgrowth. To confirm this hypothesis, the results (Figure 4.11) show the wild type embryos could not grow the pectoral fins when they were treated with the RARγ agonist (10nM) at 27hpf.

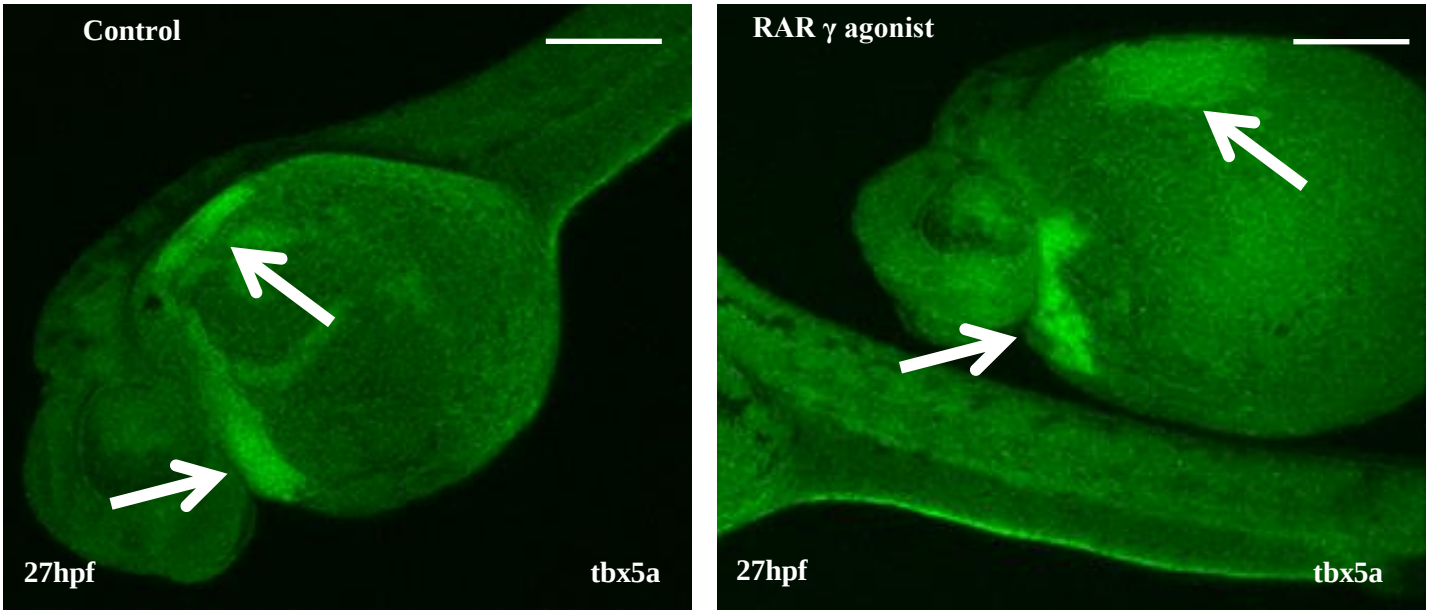

Figure 4.11. Treatment of zebrafish embryos with RARγ agonist (10nM) was associated with no evident changes in loss of tbx5a expression in the lateral plate mesoderm.

Representative confocal microscopy projected images of *z* stacks through the entire lateral perspective of AB strain zebrafish following tbx5a immunolocalisation. As shown, a similar distribution of tbx5a immunopositivity was seen in the lateral plate mesoderm and heart regions (both arrowed) at 27hpf in the RARγ agonist (10nM) (10nM) treated and control embryos following immunostaining in 10 control and 10 RARγ agonist (10nM) treated embryos. Scale bars represent 250µm.

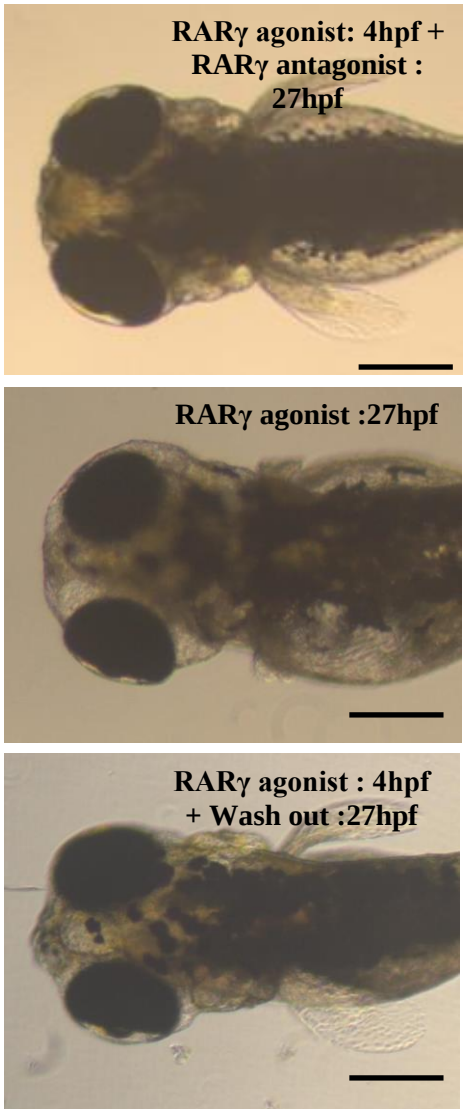

Figure 4.12. Treatment of zebrafish embryos with a RARγ agonist (10nM) was associated with a reversible block in pectoral fin outgrowth

Representative phase contrast microscopy pictures showing normal outgrowth of the pectoral fin in control AB strain embryos at 3dpf and the complete loss of pectoral fin outgrowth that was observed in RARγ agonist (10nM) treated embryos at the same time point. Pectoral fin outgrowth in AB strain embryos that had been treated with the RARγ agonist (10nM) at 4hpf was restored either by washing out the RARγ agonist (10nM) at 27hpf or adding an equal dose (10nM) of the RARγ antagonist at 27hpf. Conversely, pectoral fin outgrowth in control embryos was completely blocked when control media was replaced with the RARγ agonist (10nM) supplemented media at 27hpf. Scale bars represent 250µm. (n=3 independent experiments)

4.3.2. RA itself did not inhibit the pectoral fin outgrowth.

Since the early treatment of RA to the embryos at 4hpf resulted in embryonic truncation at 1dpf (Chapter 3), it was impossible to access whether the pectoral fins were affected by RA itself although there was no report that fin formation was affected by RA. Therefore, to confirm whether the inhibitory effect of RARγ agonist (10nM) was working through the gamma receptor, the embryos were treated with 10nM retinoic acid at 27hpf. The results (Figure 4.12) showed that the RA did not inhibit the pectoral fin outgrowth even the concentration was increased to 20nM. However, the fin length and orientation were not the same as control embryos of 3dpf.

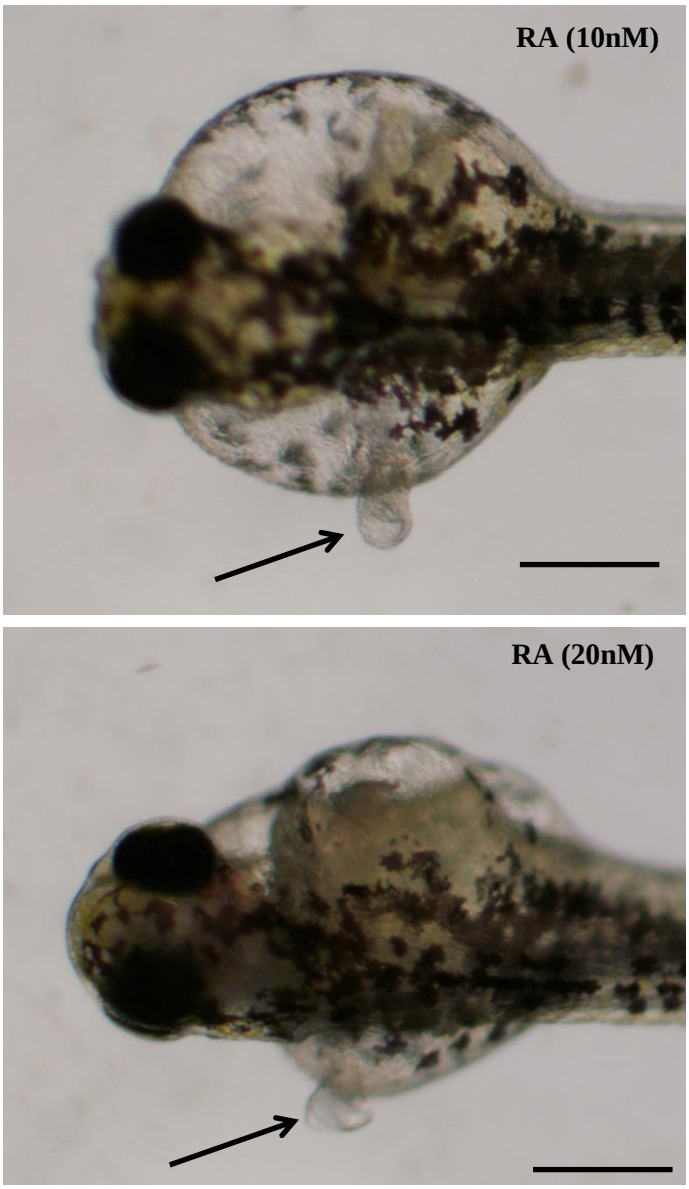

Figure 4.13. Treatment of zebrafish embryos with a RA was associated with no evidence for loss of pectoral fin.

Representative phase contrast microscopy pictures showing outgrowth of the pectoral fin in RA treated zebrafish embryos at 4dpf. Both zebrafish embryos which were treated with RA (10nM) and RA (20nM) at 27hpf had the pectoral fin outgrowth at 4dpf. Therefore, RA itself did not inhibit the pectoral fin outgrowth as seen in RARγ agonist treated zebrafish embryos following the observation in 30 RA treated embryos and 30 control embryos. Scale bars represent 250µm.

4.3.3. Discussion for pectoral fin.

Based on the results regarding to the pectoral fin formation in RARγ agonist treated embryo, there are three interesting points which need to be discussed. They are *tbx5a* expression pattern, reversible effects of RARγ agonist on pectoral fin outgrowth and the different effect of RA.

The first of the findings, *tbx5a* expression somehow shows it had not been affected and its existence was also confirmed by the wash-out experiments. However, this finding brings up another interesting thing for discussion which is not directly related to the RARγ agonist treatment on pectoral fin formation. Indeed, the immunostaining pattern showed lack of staining in the eyes which participation has been reported at the same time point by *in situ* hybridisation (Albalat *et al.,* 2010). This is rather interesting indication that non-overlapping finding between two different techniques, one detects the mRNA level and the other is for protein. In fact, this finding is more than a coincident because a zebrafish mutant line, known as "heart-string" which has premature mutation in *tbx5a* gene, shows lack of pectoral fin bud formation and severe heart oedema but, normal eye formation (Garrity *et al.,* 2002). In addition, translational blocking morpholino injection also indicates lack of pectoral fin, cardiac oedema but normal eye formation again(Garrity *et al.,* 2002), indicating the mRNA in the eyes are not translated into the protein. These findings indicate the different translational behaviours of *tbx5a.*

This pattern of gene expression does not only apply to the zebrafish, the 8 and 9 days old mice embryos also show the *tbx5* expression in the optic vesicle (Chapman *et al.,* 1996). Again, stage 14 chick embryos also show *tbx5* expression in the eye using *in-situ* hybridisation (Ohuchi *et al.,* 1998). The similar expression in the eye of Xenopus also has been reported (Horb and Thomsen 1999). *Tbx5* mutant mice also show the birth defect of heart and limb formation in both homo and heterozygous embryos, but no reported defect to eyes (Bruneau *et al.,* 2001). These findings are consistent with human *tbx5* mutation, Holt-Oram syndrome, presented by cardiac and hands defects, but not eyes (Muru *et al.,* 2011).

There is a small chance to argue about miRNA which is well-known post transcriptional regulatory mechanism, which may silence the mRNA in the eyes. Therefore, the gene expression in the eye may be the mRNAs which are about to be degraded by miRNA and the *in-situ* staining in the eyes are possibly the results of catching the tiny window period of mRNA. Nevertheless, this finding brings up a question which is far more important than ectopic expression pattern. *In-situ* technique has been using as a tool for genetics and developmental study since 1970s. In most scenarios where the antibodies are difficult to apply, especially in zebrafish, this technique has been using as the study for relative level of gene expression for decades. Therefore, the question is "how reliable is this technique as an indicator of gene expression if an organism itself has certain post-transcriptional mechanisms?" This finding also rings the bell for mRNA injection. RNA injection is widely used as rescue experiments for mutant embryos or morpholino injected embryos. How can the differences be told between 100% rescued embryos and partially rescued embryos?

Both *tbx5a* immunohistochemistry and wash-out experiments confirmed the RARγ agonist treatment did not interrupt with it and it also shows the interaction came from the down-stream genes of *tbx5a* and the whole effect is reversible. So, what are the down-stream genes and how? Using morphilonos and *in-situ* hybridization, *fgf8 and fgf10* are proposed as the downstream genes of *tbx5a* (Ng *et al.*, 2002). Another fgf family,*fgf24,* is also a proposed candidate for *tbx5a* downstream using a *fgf24* mutant line and *in-situ* hybridisation (Fischer 2003). Another non-*fgf* genes which seems to be downstream of *tbx5a* are *sall4* (Harvey and Logan 2006), *blimp-1* (Lee and Roy 2006), *prdm1* (Mercader *et al.*, 2006), beta-CaMK-II (Rothschild *et al.*, 2009) and *ndrg4* (Qu *et al.*, 2008). Therefore, RARγ agonist might have interacted one or more downstream genes of *tbx5a* mentioned above.

Another group of genes which is reported to be involved in pectoral fin formation is *hox* family. Regardless to whether this family is upstream or downstream of *tbx5a;* their expression is down-regulated by RARγ agonist treatment according to the previous findings on *hoxc11a* and *hoxb13a.* Using *in-situ* hybridization, *hoax* (Géraudie and Borday Birraux 2003;

Metscher *et al.,* n.d.), *hoxb* (Waxman *et al.,* 2008) and *hoxd* (Sakamoto *et al.,* 2009; Neumann *et al.,* 1999) are the genes expressed in the developing pectoral fins. Although there is no evidence that loss of any of hox genes mentioned above are responsible for loss of pectoral fin outgrowth, this group of gene expression in pectoral fin is an obvious clue for the relationship between hox genes and RARγ agonist treatment.

The finding of RA on pectoral fin is very different to the effects of RARγ agonist on pectoral fin. In fact, the arguable point about RA is the stability. Because of the structure of RA compound, it is very sensitive to light and temperature and oxidized. Although the plates were wrapped in foil, the temperature may have involved in oxidation of the RA resulting in reduced efficiency within hours after treatment. This no longer effective RA may not have prevented the pectoral fin outgrowth. Therefore, the difference in the chemical properties between RA and RARγ agonist may have resulted in opposite finding.

The dilemma in this project is the effects of RARγ agonist were expected to be the same as RA in most cases. Since there is no proof for the specificity of RARγ agonist, it is not very difficult to be surprised for the opposite effects between RA and RARγ agonist. So far, there is no paper showing the inhibitory effect of RA on pectoral fin outgrowth. In fact, RA itself has been used to rescue the pectoral fin formation in pectoral fin mutant embryos (Begemann *et al.,* 2001; Grandel and Brand 2011). More interestingly, apc morphant embryos which have the pectoral fin buds that fails to growth can be rescued by adding of RA (Nadauld *et al.,* 2004). These evidences indicate RA has promotional effect on pectoral fin formation rather than inhibitory effect. Even so, there is still an arguable point which is either RA or RARγ agonist may have other effects which do not involve the conventional ligand-receptor response.

Therefore, what about a mutant zebrafish which intrinsic RA is always high? Interestingly, a zebrafish mutant line called *giraffe,* which has RA degrading enzyme *cyp26a1*mutation, shows smaller pectoral fin outgrowth (Emoto *et al.,* 2005). Therefore,

pectoral fin formation in RA treated zebrafish might be the results of quick degradation of RA due to temperature and oxidation.

4.4. Introduction for loss of cranial structures

Another phenotype of RARγ agonist treated zebrafish embryos is loss of pharyngeal arches, which suggests the cells differentiate into pharyngeal arches, might not have formed. The pharyngeal arches originate from cranial neural crest (Kague *et al.*, 2012). Since the cranial neural crest can differentiates into other cranial structures such as cranial bones, cartilages and nerves (Dutton *et al.*, 2001; Rodrigues *et al.*, 2012), the cranial bone formation and lateral line ganglions were examined whether they were affected by RARγ agonist treatment. In addition, the changes in neural crest cell formation between RARγ agonist treated and a control embryo was also examined.

4.4.1. Treatment with RARγ agonist was associated with loss of almost all cranial bones.

The bone staining results of both calcein and alizarin red show lack of cranial bones in RARγ agonist treated embryos at 5dpf. Interestingly, all the neural crest derived bones were not lost. According to lineage tracing experiment on cranial neural crest, all the named bones mentioned in the results are neural crest derived including cleithrum (Kague *et al.*, 2012). Therefore, all the neural crest derived bones except ceratobranchial5 bone and cleithrum were lost in RARγ agonist treated embryo (Figure 4.13). Again, all the bones mentioned in the results are endochondrial bone except cleithrum which is dermal bone (Kague *et al.*, 2012). In the RARγ agonist treated embryos, only the most posterior part of ceratobranchial5 bone which is adjacent to cleithrum bone, was still present.

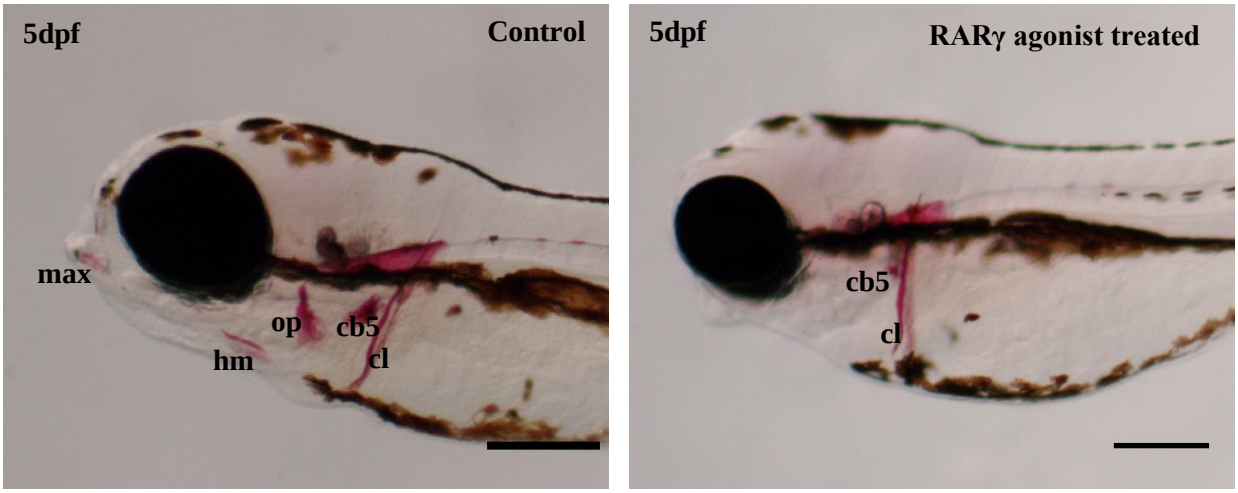

Figure 4.13. Treatment of zebrafish embryos with a RARγ agonist (10nM) was associated with loss of cranial bones.

Representative confocal and fluorescent microscopy pictures showing dorsal view calcein bone staining in RARγ agonist (10nM) treated and control AB strain embryos at 5dpf. Representative bright field microscopy pictures of Alizarin Red S stained AB strain embryos showing that all of the cranial bones were absent in RARγ agonist (10nM) treated embryos at 5dpf except for the most posterior part of the cerebrobranchial 5 bone and the cleithrum bones. Scale bars represent 250µm. (n=3 independent experiments) max=maxillary bone, hm= hyomandibular bone, op=opercle bone, cb5=cerebrobranchial bone 5 and cl= cleithrum bone.

4.4.2. Treatment with RARγ agonist was associated with loss of anterior lateral line ganglions.

The previous findings on the head morphology suggested the neural crests were affected. Therefore, in this experiment, the RARγ agonist was used to treat on the transgenic line from zTrap library (Kawakami *et al.,* 2010) which has the GFP-expression in lateral line and its ganglions. The gene mapping shows the HGn39D sequence was inserted within a locus coding for contactin-associated protein-like2 (Cntnap2b) and the protein is expressed in the lateral line and its ganglions (Pujol-Martí *et al.,* 2012). Lateral line system is found in aquatic animals, is a part of peripheral sensory nervous system and derived from neural crest (Collazo *et al.,* 1994). Therefore, the hypothesis was the lateral system will be affected if the neural crest cells were disrupted by RARγ agonist treatment.

The results of rarg-agonist to HGn39D show loss of anterior lateral line ganglion in RARγ agonist treated embryos (Figure 4.14). At 1dpf, RARγ agonist treated and control embryos had visible posterior lateral line ganglions. Small GFP expression in anterior lateral line ganglion in control embryos was also found at 1dpf. However, in the RARγ agonist treated embryos, the anterior lateral line ganglion was not present until 5dpf although they had the lateral line and posterior ganglion. Therefore, this results show loss of anterior lateral line ganglion in RARγ agonist treated embryos.

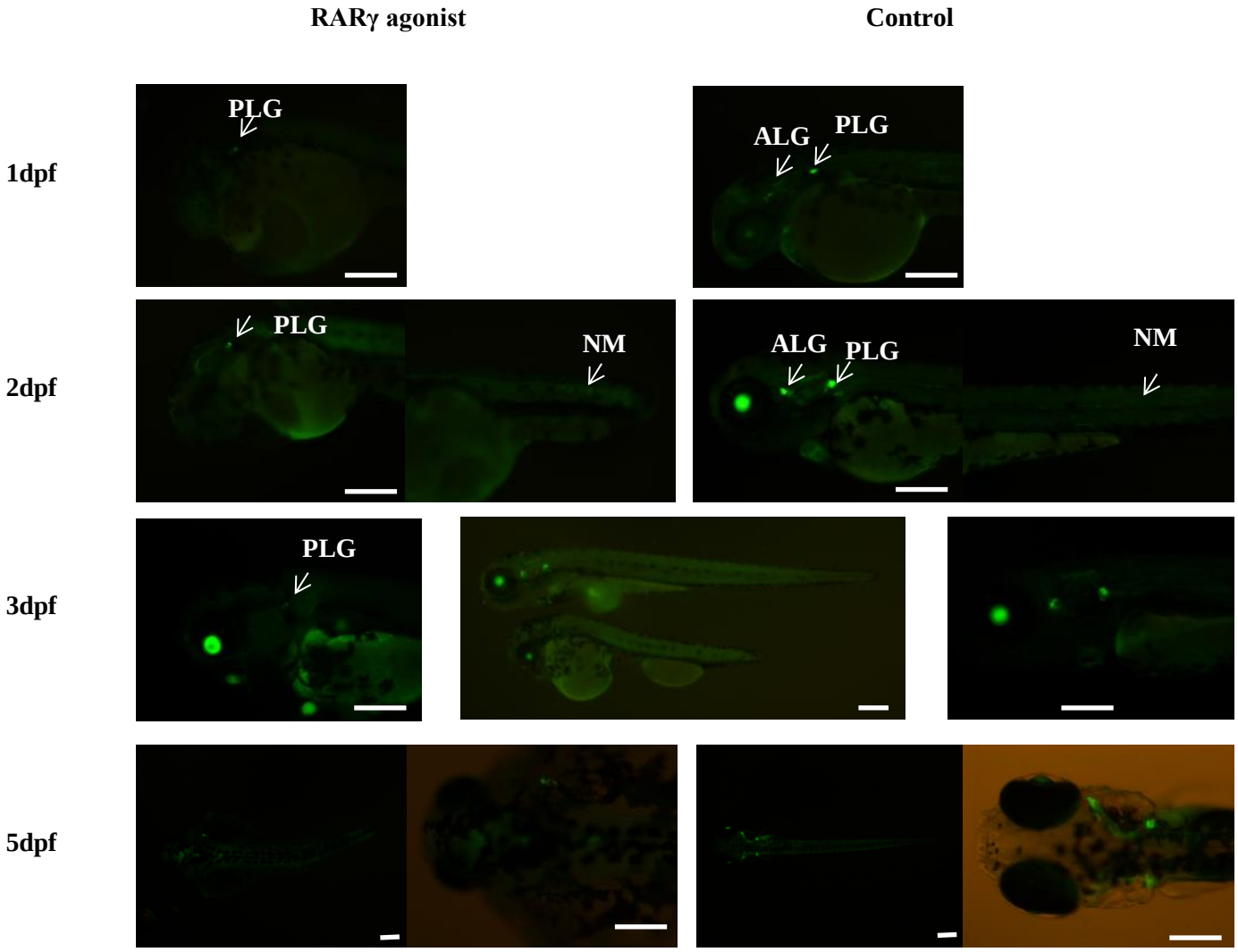

Figure 4.15. Treatment of zebrafish embryos with a RARγ agonist (10nM) was associated with loss of anterior lateral line ganglions.

Representative fluorescent microscopy pictures of the transgenic zebrafish HGn39D reporter line, demonstrating the specific loss of GFP signal in the anterior lateral line ganglia (arrowed) of RARγ agonist (10nM) treated embryos only at from 1dpf to 5dpf, but not in the posterior lateral line ganglia PLG (arrowed) or the lateral line (arrowed) itself, all of which were evident in control embryos at the same stage. Scale bars represent 250µm. (n=3 independent experiments) ALG= anterior laterline ganglion, PLG= posterior lateraline ganglion, NM= neuromast

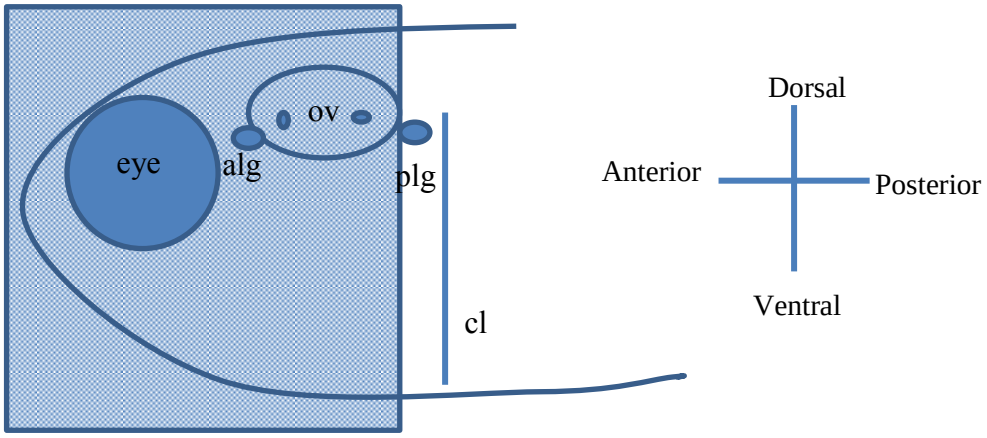

Figure 4.16. Treatment of zebrafish embryos with a RARγ agonist (10nM) was associated with loss of anterior cranial structures which were anterior to the posterior end of otic vesicle.

A diagram demonstrates the area of cranial structures which were affected by RARγ agonist (10nM) treatment. The highlighted area shows the areas which were anterior to the posterior margin of otic vesicle and the structures in this area were affected by RARγ agonist (10nM) treatment. These structures include cranial bones, anterior lateral line ganglions and eyes.

4.4.3. Lateral line function was still present in RARγ agonist treated embryos.

The previous results show the anterior lateral line ganglions were diminished in RARγ agonist treated embryos but they still had the posterior and the rest of lateral line. One of the functions of lateral line system is burst swimming response or escape response where the lateral line mediates very fast response to sudden mechanical stimuli (McHenry *et al.*, 2009). Therefore, the functional experiment for lateral line system was checked using touch- response test. Both the RARγ agonist treated and control embryos showed the response to the stimuli although the treated embryos had shorter period of swimming response time (Figure 4.16). This result shows the RARγ agonist treated embryos still had functional lateral line system although the anterior lateral line ganglion was diminished.

Control **RARγ agonist**

Figure 4.17. Treatment of zebrafish embryos with a RARγ agonist (10nM) was associated with no evidence of loss of swimming response.

Representative videos show the swimming response of control and RARγ agonist (10nM) treated embryos at 2dpf. The embryos were stimulated by a hair at the trunk region to see the burst swimming response and RARγ agonist (10nM) treated and control embryos showed the positive response although the RARγ agonist (10nM) treated embryos had weaker responding period. RARγ agonist (10nM) treated and control embryos had burst swimming response at 2dpf. (n=3 independent experiments)

4.4.4. Treatment with RARγ agonist was associated with reduced sox9 expression at 60hpf.

The final jigsaw part of these series of experiments is to find out whether the cranial neural crests were actually affected by RARγ agonist treatment. To check the pattern of expression in RARγ agonist treated embryos, *sox9* antibody was used as a marker for neural crest (Li *et al.,* 2002). *Sox9* expression can be found in neural crest cells, otic vesicle and pharyngeal arches (Rau *et al.,* 2006). Since both rarg receptors in zebrafish have different time point of expression and *sox9* expression starts to be seen in neural crest from early segmentation period (Li *et al.,* 2002), the neural crest formation in RARγ agonist treated zebrafish was analyzed at different time points to identify the possible receptor subtype affected by the treatment. The *rarga* expression starts at 24hpf (Hale *et al.,* 2006) and the *rargb* expression can be seen as early as 10-somite stage (Waxman and Yelon 2007). Therefore, the time point 22hpf was chosen to analyse the pattern of neural crest expression in the treated embryos because if the RARγ agonist was working through the *rargb*, there may be changes in neural crest expression pattern in the treated embryos at 22hpf which is the time point just before *rarga* starts to express at 24hpf.

The immunostaining results show there was no difference in the expression pattern of neural crest at 22hpf in RARγ agonist treated and control embryos (Figure 4.17). The next time point chosen to check the pattern of neural crest expression is 60hpf because the previous results show the neural crest derivatives in RARγ agonist treated embryos were already lost at 3dpf (after 72hpf). The staining results show the RARγ agonist treated embryos did not have the staining whereas the control embryos had the staining in the neural crest cells, pharyngeal arches and otic vesicle (Figure 4.17). The next experiment was to narrow down the time point and the staining from

48hpf time point showed there was no much difference between treated and control embryos.

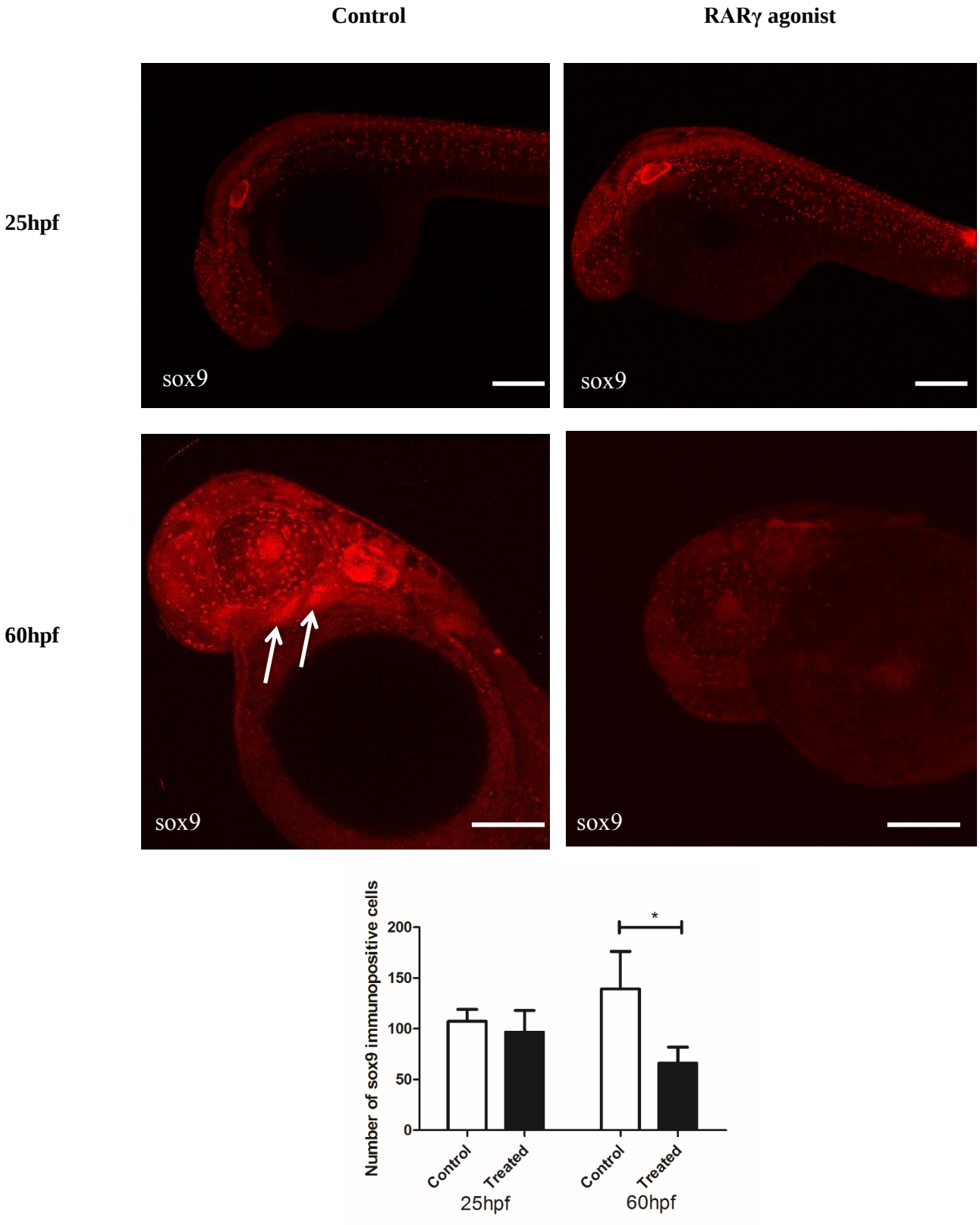
Control
RARγ agonist
25hpf
60hpf
sox9
sox9
sox9
sox9
Number of sox9 immunopositive cells
200
150
100
50
0
*
Control
Treated
Control
Treated
25hpf
60hpf

Figure 4.18. Treatment of zebrafish embryos with a RARγ agonist (10nM) was associated with loss of sox9 immunopositive cranial neural crest stem/progenitor cells.

Representative confocal microscopy projected pictures of *z* stacks through the entire lateral perspective of AB strain zebrafish following sox9 immunolocalisation are shown. A similar number of sox9 immunopositive pictures were seen in RARγ agonist (10nM) and control embryos at 25hpf. However, reduced numbers of sox9 immunopositive cells were observed in the RARγ agonist treated embryos at 60hpf compared to the control embryos ($p \leq 0.05$; Mann Whitney U test). Sox9 immunopositivity was observed in the developing pharyngeal arches (pa) was observed in control embryos only at 60hpf (arrowed), but were not seen in RARγ agonist (10nM) treated embryos. Scale bars represent 250µm. Data are shown as mean +/-SD, pooled from following immunostaining and score of 3 control and 6 RARγ agonist (10nM) treated embryos.

4.4.5. The acridine orange staining showed no difference in RARγ agonist treated and control embryos.

Sox9 immunostaining shows loss of staining in RARγ agonist treated embryos at 60hpf. Therefore, the next question to answer is "what happened to the neural crest in RARγ agonist treated embryos?" In previous results, it was suggested the loss of neural crest occurred between 48hpf and 60hpf. Therefore, the easiest and cheapest way to start to find out was to check the cell apoptosis using acridine orange. However, the results show there was no difference between rarg-agonist treated and control embryos at 2dpf (Figure 4.18).

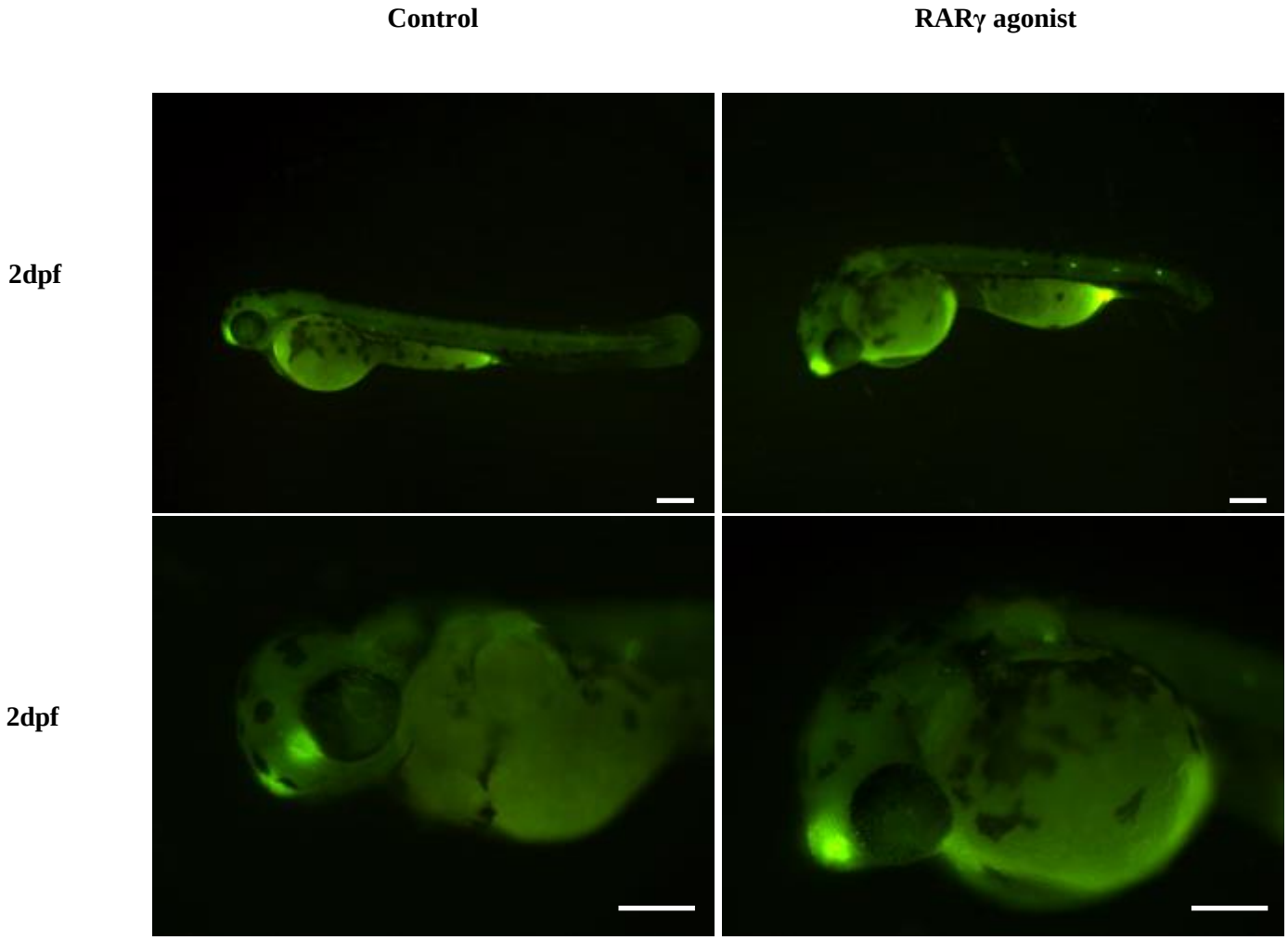

Figure 4.19. Treatment of zebrafish embryos with a RARγ agonist (10nM) was associated with no evidence of apoptosis in the head area at 2dpf.

The representative pictures show acridine orange staining in RARγ agonist (10nM) treated and control embryos at 2dpf. There was no evidence of staining in the cranial neural crest area of RARγ agonist (10nM) treated and control embryos at 2dpf. Scale bars represent 250μm. (n=2 independent experiments)

4.4.6. Discussion for neural crest

These findings confirm the loss of cranial bones had contributed to the changes in the head morphology and loss of pharyngeal arches in the RARγ agonist treated embryos. Rather interesting finding in this experiment was the pattern of cranial bone loss. Although almost all the neural crest derived bones were lost in the treated embryos, there were still intact cleithrum bone and part of ceratobranchial bone, both are neural crest origins (Kague *et al.,* 2012). In terms of ossification, ceratobranchial5 bone is endochondrial and cleithrum is dermal (Kague *et al.,* 2012). Therefore, the results show being neural crest origin or non-neural crest origin and being dermal or endochondrial ossifications do not play a role in these patterns of bone loss in RARγ agonist treated embryos. The most interesting finding in this result is all the neural crest derived bones anterior to the cleithrum bone were vanished apart from a very small part of ceratobranchial5. Therefore, it may suggest all the cranial neural crest cells before cleithrum bone were affected RARγ agonist treatment. This result also suggests there are possibilities that other neural crest derived organs may also have affected by the RARγ agonist treatment as well.

Loss of anterior lateral line ganglion in RARγ agonist treated reinforces another clue for loss of neural crest. However, it seems the effect was limited to the anterior ganglion while the posterior and the lateral line itself seemed intact. Therefore, this suggests loss of the anterior lateral line ganglion in the treated embryos is neither due to the inhibitory effects on cntnap2a protein nor the whole neural crest contribution to the lateral line formation; it is simply due to the disruptive effect to the area which involves the anterior lateral line ganglion.

Combination of loss of jaw bones and loss of anterior lateral line ganglion in RARγ agonist treated embryos bring rather interesting findings. Cleithrum bone is only paired vertical bone in zebrafish head (Eames *et al.,* 2013) which borders with the posterior margin of otic vesicle. The anterior and posterior lateral line ganglions are located in the most anterior margin and the most posterior margin of otic vesicle respectively (Pujol-Martí *et al.,* 2012). Therefore, imaginary overlapping of alizarin red staining and lateral line ganglions transgenic results in

5dpf treated embryos clearly suggest the affected areas is limited to the most posterior region of otic vesicle (Figure 4.8).

The touch response tests showed RARγ agonist treated embryos still had the functioning lateral line system although they had the absence of anterior lateral line ganglion. However, they had weaker duration of response compared to the control embryos which may not be related to the functionality of the lateral line system. There are many possible causes which are not directly related to the lateral line system. The treated embryos had cardiac oedema which may cause poor circulatory effect. The oil-red-o staining from previous results chapter also shows lack of fat droplets within the trunk which the fat from the yolk sac is essential source of energy for the embryo before they can independently catch the food at 5dpf. Finally, the RARγ agonist treated embryos do not have the appendages involved in swimming, which are the pectoral, caudal fin and fewer numbers of somite.

The results from *sox9* antibody staining show loss of *sox9* positive cells including neural crest were found in the RARγ agonist treated embryos. The pattern of antibody staining confirm the right staining pattern of sox9 expression according to the in-situ hybridization pictures (Li *et al.*, 2002; Chiang *et al.*, 2001). However, the results were far from conclusive because the experiment itself was only one repeat. Another issue of antibody staining in zebrafish is reliability. In fact, one of the weak points of zebrafish study is antibody staining. Most of the antibody staining used on zebrafish was slices rather than whole mount. In reality, transparency and advanced transgenic techniques favours the tide of zebrafish genetic and proteomic study towards the zebrafish transgenesis rather than antibody methods. And also, unlike the *in-situ* hybridization technique which has critical protocol paper (Thisse and Thisse 2008), there is no detailed protocol paper for zebrafish antibody staining. Moreover, this staining result is not like *tbx5a* which had other ways to prove its existence and not like *alpha-actinin*, which was confirmed by the somite transgenic zebrafish.

Nevertheless, there are still the interesting points to discuss. The results were suggesting loss of *sox9* positive cells in RARγ agonist treated embryos and there was no sign of apoptosis. But, it is still difficult to pinpoint where and when they were lost. More importantly, there are other markers involved in neural crest formation, migration and differentiation. Therefore, loss of sox9 expression alone may not be the indicator of loss of neural crest. On the other hand, there was no sign of cell death. These all suggestions bring the possibility that the neural crest cells in RARγ agonist treated embryos simply switched off sox9 expression. Both *sox9a* morpholino knock-down and *sox9a* mutant zebfish embryos show lack of cranial skeletal structures with intact clithrum bone suggesting *sox9a* in zebrafish functions mainly for neural crest differentiation, not formation and migration (Yan *et al.*, 2002). *Sox9b* morpholino knock-down zebrafish embryos also show the similar patterns to sox9a mutant zebrafish (Yan *et al.*, 2005). However, the fate of neural crests is not still clear in these embryos. There are numerous papers suggesting the role of RA in neural crest. But, it will be endless discussion to bring up RA effects on neural crest since there is no evidence of RARγ agonist was working through its receptor yet. Therefore, the best way to track down the fate of neural crest in RARγ agonist treated zebrafish is to use a neural crest transgenic line which has the permanent expression. A novel *sox-10* transgenic line created using Cre-lox system shows the traceable permanent staining on neural crest and its derivatives (Rodrigues *et al.*, 2012). Therefore, using the transgenic line for RARγ agonist treatment will provide the crucial evidence for the fate of neural crest in RARγ agonist treated zebrafish.

4.5. Summary

This chapter explains the possible causes of three RARγ agonist treatment effects which were shorter antero-posterior axis, lack of pectoral fin and loss of pharyngeal arches. Antero-posterior body axis was caused by reduced number of somite which was associated with changes in *hoxc11a* and *hoxb13a* expressions. The loss of pectoral fin was due to reversible inhibitory effects of RARγ agonist on fin outgrowth. Loss of pharyngeal arches was due to changes in neural crest formation which may not have gone for apoptosis.

Chapter 5

An investigation of the relationship between RARγ and canonical *Wnt* signalling in zebrafish embryos

5.1. Introduction

The possible mechanisms involved in the phenotypic abnormalities caused by RARγ agonist were investigated in the previous chapter. Changes in the expression of the regulatory genes or proteins that are involved in the formation of tissues affected by RARγ agonist such as *hoxc11a, hoxb13a, myoD, sox9* and *tbx5a* were identified. For example, there were observed changes in *hox* genes and sox9 protein in association with disrupted formation or development of somites and neural crest derivatives, respectively, while *tbx5a* expression, which regulates pectoral fin development (Grandel *et al.*, 2009) was not affected.

Although the RARγ agonist appeared to differentially affect (or not) different signalling pathways that are involved in the formation of the affected tissues, these tissues all have one interesting common signalling pathway that regulates their development, which is the Wnt pathway (Martin and Kimelman 2010; Sun *et al.*, 2008). In fact, the Wnt pathway also is known to have a relationship with RA signalling. For example, in zebrafish it has been shown that treatment with RA causes a down-regulation of *Wnt3a* and *ntl* (Orthologous to *Brachyuary* in mice) (Martin and Kimelman 2010). Therefore, it is very important to examine the potential involvement of Wnt signalling in the RARγ agonist effects that were previously shown.

Excess RA has been known for its teratogenic effects on posterior body truncation by disturbing *ntl* which is important for posterior body tissue formation (Martin and Kimelman 2010). Other investigations in mice have shown that such truncation after RA treatment is accompanied by a down-regulation of *Wnt3a*, apoptosis of posterior mesodermal cells, and diversion of these mesodermal tail bud progenitors into neurones (Shum *et al.*, 1999). Wnt signalling is also involved in neural crest cell differentiation into pharyngeal arches. *Wnt3a* morpholino injected zebrafish embryos show loss of pharyngeal arches and cranial cartilages (Sun *et al.*, 2008), whilst *Wnt9b* morphant zebrafish embryos similarly have a loss of these same tissues (Curtin *et al.*, 2011). Finally, treatment with morpholinos to block expression of the cell surface receptor protein involved in canonical Wnt signalling pathway, *frizzle b(fzb)*

also shows a loss of pharyngeal arches and cranial cartilages (Kamel *et al.*, 2013). Therefore, it is clear that Wnt signalling is required for cranial morphogenesis in zebrafish embryos and also that the effects of disrupting Wnt signalling are similar to the effects of treating zebrafish embryos with the RARγ agonist. These evidences indicate that the RARγ agonist may have repressed Wnt signalling which resulted in a loss of pharyngeal arches and cranial bones. Wnt signalling also has been implicated in pectoral fin formation and outgrowth (Grandel and Brand 2011; Sakamoto *et al.*, 2009; Gibert *et al.*, 2006).

Therefore, it is possible to hypothesise that down-regulation of *Wnt* signalling in RARγ agonist treated embryos may be the main molecular mechanism giving rise to the phenotypes caused by RARγ agonist treatment. To study this possibility, the effects of the RARγ agonist were examined using a Wnt/beta-catenin signalling reporter transgenic zebrafish line. The zebrafish line was generated by Shimizu *et al.*, 2012. In detail, the line was made by the insertion of a plasmid which contains six Tcf/lef binding sites, a mini-p (minimal artificial promoter) to prevent activation of other genes, and a coding sequence for d2EGFP (destabilized enhanced GFP version 2), which has a rapid protein turnover rate, and is reported as a sensitive indicator of canonical Wnt signalling (Shimizu *et al.*, 2012). The plasmid was delivered by the *tol2* transposase-mediated transgenesis method (Kawakami *et al.*, 2004). The reporter fish line embryos were treated with the RARγ agonist at 10nM and 4 hpf and changes in canonical Wnt signalling were observed using fluorescence microscopy.

Canonical Wnt signalling includes three major steps for signal transduction and gene transcription which are (1) surface receptor activation (2) inhibition of beta-catenin destruction complex and (3) activation of a Wnt-specific nuclear receptor complex (Saito-Diaz *et al.*, 2013). The Wnt cytokine binds to the cell surface receptors, frizzled and lipoprotein related protein (LRP5/6). Beta-catenin is continuously synthesized in the cytoplasm and destructed by APC, GSK-3, Axin, and CK1a complex. When the Wnt cytokine forms a complex with frizzled and LRP5/6, it recruits dishevelled protein which disrupts the destruction complex for beta-catenin resulting in increased cytoplasmic beta-catenin level. When the amount of beta-catenin level in

the cytoplasm reaches almost doubled of original amount, the translocation to the nucleus occurs and binds to Tcf/lef proteins which sit on specific DNA sequences accompanied by co-repressor protein. Beta-catenin replaces the co-repressor protein, Groucho, and transcribes the Wnt-regulated genes (Figure 5.1).

Figure 5.1. Canonical Wnt signalling.

The diagrammatic illustration of canonical Wnt signalling which involves the cell surface receptor activation, cytoplasmic protein translocation and nuclear gene transcriptions. The picture was adapted from (Saito-Diaz *et al.*, 2013) and the permission to reuse this figure is obtained from the publisher.

5.2. Canonical Wnt signalling in response to RARγ agonist treatment during somitogenesis.

Canonical Wnt signalling in the transgenic reporter (Tcf:minip) zebrafish embryos was visualized from the stage when the pre-somitic mesoderm (PSM) initially formed segmented somites through to when all somites have formed, which is from 10hpf-24hpf (Kimmel *et al.*, 1995; Stickney *et al.*, 2000; Shimozono *et al.*, 2013). GFP expression indicating canonical Wnt signalling was readily observed in the control zebrafish embryos at the 7-8 somites stage, including positivity in the PSM and formed somites. In contrast, although GFP expression was present in the RARγ agonist treated embryos at the same time point (12hpf), the levels of expression appeared reduced as well as showing a disorganised and diffuse pattern of positivity that did not match the somite patterns seen in the control fish (Figure 5.2). In contrast, at 1dpf, increased GFP positivity was seen in the control embryos at the presumptive tail edge while RARγ agonist treated embryos had loss of GFP expression in the tail area. In addition, strong ectopic GFP expression was also seen in the hatching glands of the RARγ agonist treated embryos as well as in the presumptive notochord area at the same time point. The patterns of canonical Wnt signalling seemed the same in the RARγ agonist and control embryos in the ventral and medial finfold, the otic vesicle, the pectoral fin bud, the lateral line primordium and at the mid-hindbrain boundary. These patterns of GFP expression were consistent, (data pooled from 3 independent experiments).

Hence, canonical Wnt signalling was transiently down-regulated in the PSM of the developing embryo during somitogenesis stage in association with RARγ agonist treatment, followed by the complete loss in the tail area at 1dpf. Therefore, these evidences suggested canonical Wnt signalling in RARγ agonist treated zebrafish embryos was not completely down-regulated as mentioned in Martin and Kimelman 2010 in which complete loss of *Wnt* mRNA was observed in the pre somitic mesoderm area following RA treatment. In addition, RARγ agonist may also have ectopic expression effects on hatching gland and notochord.

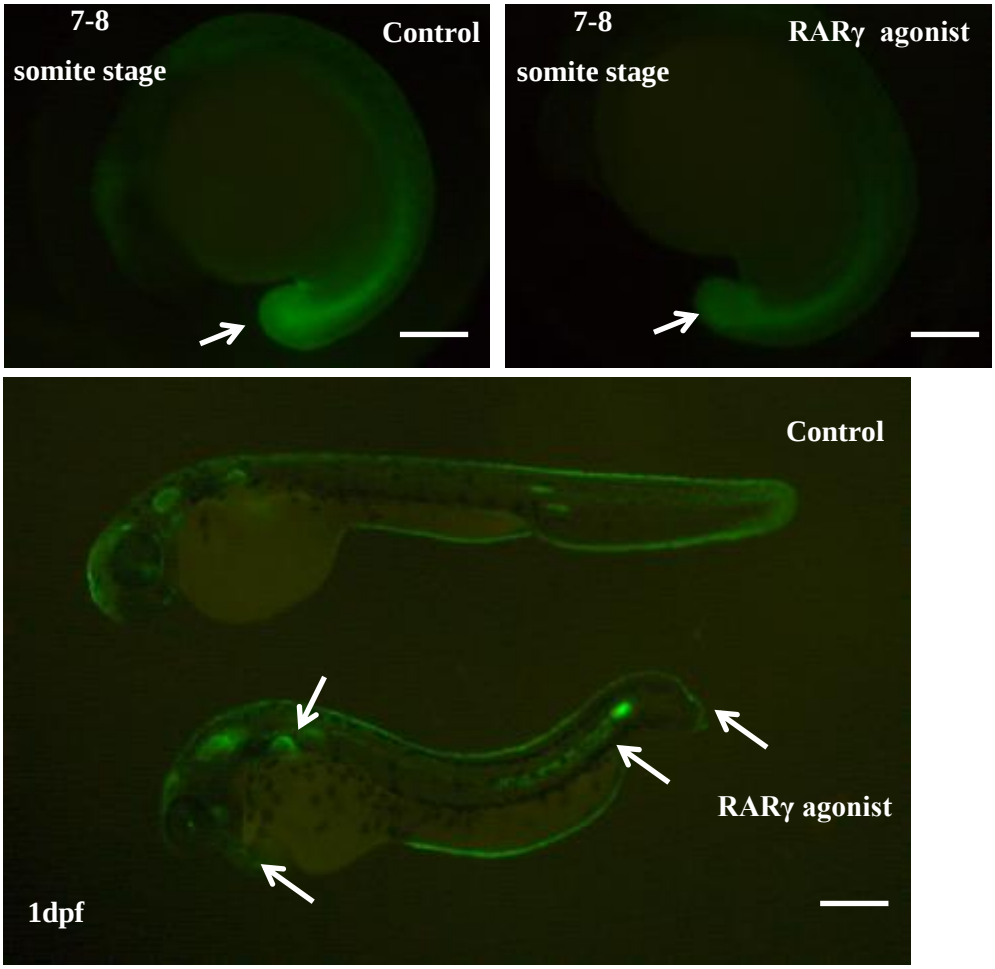

Figure 5.2. Treatment of zebrafish embryos with RARγ agonist (10nM) (10nM) was associated with no evidence of canonical Wnt signalling down-regulation.

Representative pictures of left lateral views of treated and control Tcf:minip canonical Wnt signalling reporter line are shown at 7-8 somite stage and 1hpf. The activity of Tcf:minip which reports GFP expression during somitogenesis, canonical Wnt signalling was partially down-regulated in the pre-somitic mesoderm (arrowed) and developing somites in the RARγ agonist (10nM) (10nM)(10nM)treated embryos compared to the control embryos. At 1hpf, loss of canonical Wnt signalling expression was observed at the end of tail (arrowed) of RARγ agonist (10nM) (10nM)treated embryos compared to the control embryo. GFP expression was also found in the hatching gland (arrowed) as well as presumptive notochord (arrowed) of the RARγ agonist (10nM) (10nM)treated embryos. The scale bar represents 250µm. n=3 independent experiments

5.3. Treatment of zebrafish embryos with the RARγ agonist was associated with a temporal increase in canonical Wnt signalling in the posterior notochord and pectoral fin bud at 2dpf.

At the end of the pharyngula period (48hpf) (Kimmel *et al.*, 1995), the most obvious GFP expression was found in the RARγ agonist treated embryos. This was most markedly seen in a tubular shape which ran along the antero-posterior axis and was located at the posterior part of the trunk (Figures 5.3). In addition, strong GFP positivity was also seen in the pectoral fin bud and occasionally in the ventral fin fold. Canonical Wnt signalling was examined in a double transgenic embryo fish line using the Tcf;minip fish line crossed with the HuC-RFP line, where the HuC promoter drives RFP expression in neurons (Park *et al.*, 2000). In these double transgenic report zebrafish embryos, enhanced GFP positivity was seen in the RARγ agonist treated embryos in a location that was ventral to the evident RFP positivity seen in the neural tube (Figure 5.3, lower panels). Enhanced GFP positivity was also seen in the pectoral fin bud, although not in the caudal fin. Hence, it appeared that treatment with the RARγ agonist was consistently associated with increased canonical Wnt signalling in the posterior notochord and the pectoral fin bud. The differential patterns of expression described for the notochord and pectoral fin at 2dpf were consistent in RARγ agonist treated and control Tcf;minip zebrafish embryos, (data pooled from 3 independent experiments). Similarly, the patterns of expression seen in the double transgenic zebrafish that showed notochordal and pectoral fin bud canonical Wnt signalling were consistent, (data from two independent experiments).

Canonical Wnt signalling was increased in several locations at 3dpf in control Tcf;minip zebrafish embryos, including the brain, pharyngeal arches, liver, pectoral fin, ventral fin fold and caudal fin. In contrast, the level of GFP expression in the RARγ agonist treated canonical Wnt signalling reporter line was decreased at 3dpf compared to the control embryos at all of these locations, except for the notochord and remaining fin bud (Figure 5.4). GFP expression in both the RARγ agonist treated and control embryos became weaker at 4dpf and invisible at 5dpf. These patterns of expression were consistently seen in all of the RARγ agonist treated embryos from 3dpf-5dpf.

Taken together, these data indicate that canonical Wnt signalling appeared to be increased prematurely in some tissues following RARγ agonist treatment, i.e. The pectoral fin bud at 2dpf, as well as being aberrantly induced in the notochord from late 1dpf.

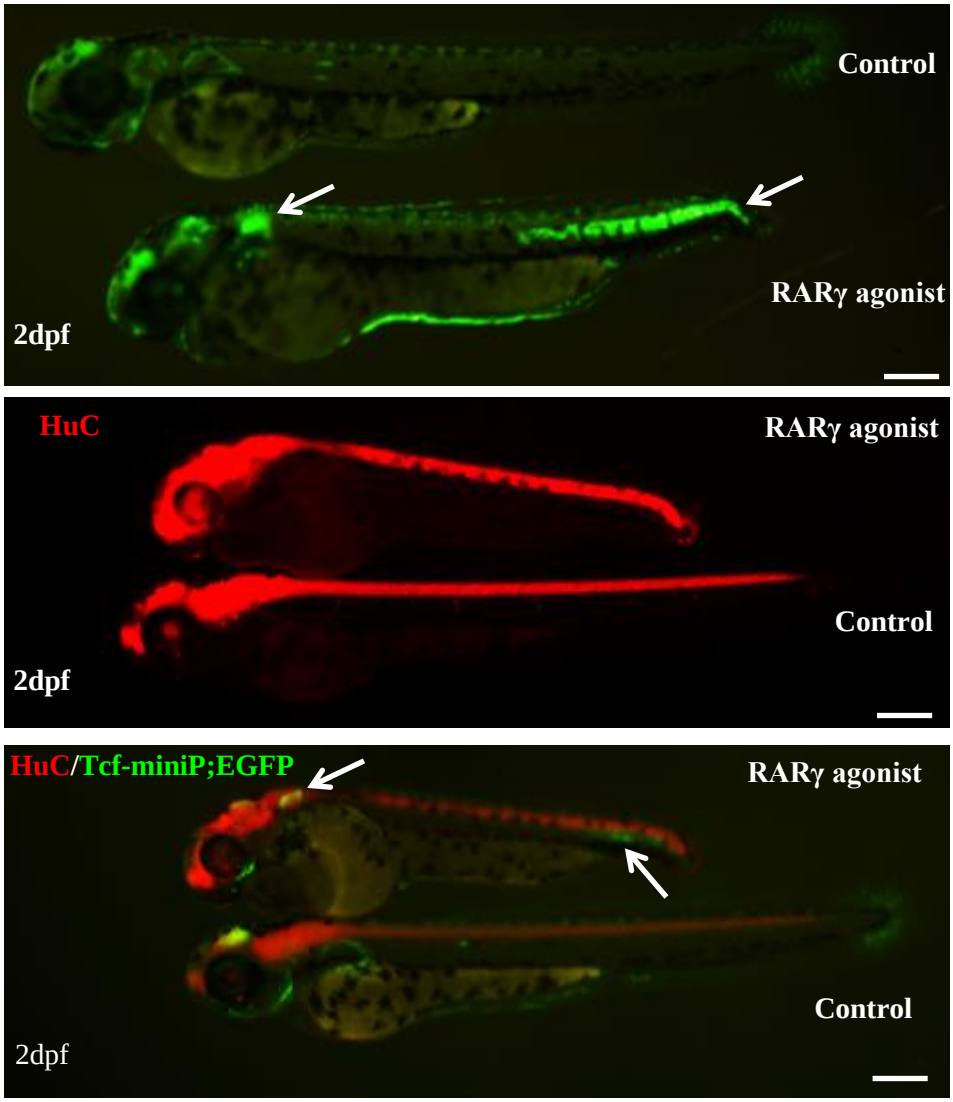

Figure 5.3. Treatment of zebrafish embryos with RARγ agonist (10nM)was associated with up-regulation of canonical Wnt signalling in the posterior end of the notochord.

Representative fluorescent microscopic pictures show the left lateral views of RARγ agonist (10nM)treated and control Tcf:minip canonical Wnt signalling reporter line embryos at 2dpf. At 2dpf, the strong expression of canonical Wnt signalling was observed in the posterior notochord of RARγ agonist (10nM)treated embryo (arrowed) and not in the posterior notochord of the control embryos. The double transgenic embryos of HuC-RFP, which has RFP expression in the neurons, and Tcf:minip showed the canonical Wnt signalling in the notochord of RARγ agonist (10nM) treated embryos at 2dpf (arrowed). The central nervous system and the neural tube in

both control and RARγ agonist (10nM) treated embryos expressed RFP. The scale bars represent 250μm. (n=2 independent experiments)

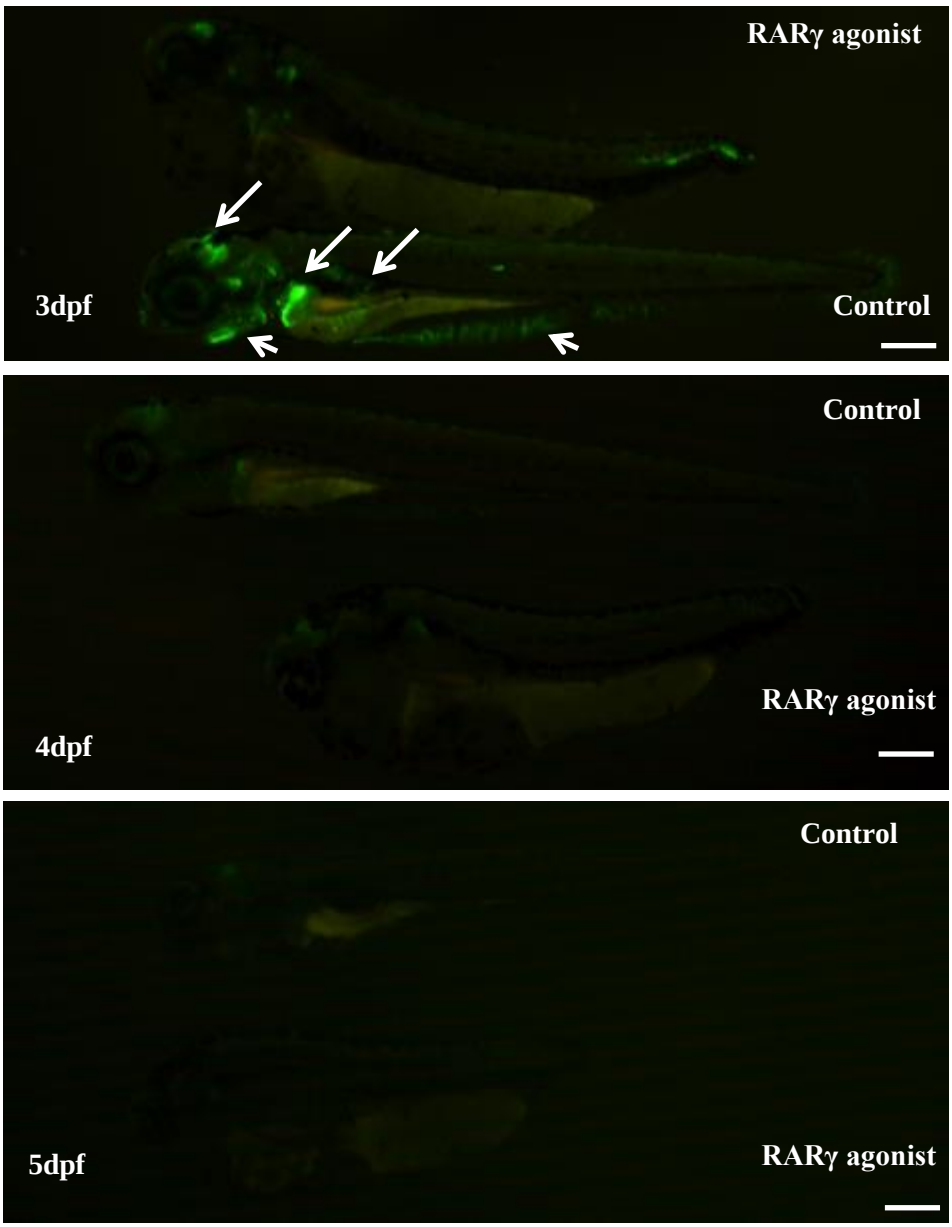

Figure 5.4. Treatment of zebrafish embryos with RARγ agonist (10nM) was associated with regression of canonical Wnt signalling from 3dpf.

Representative fluorescent pictures show the left lateral views of RARγ agonist (10nM) treated and control Tcf:minip canonical Wnt signalling reporter line embryos from 3dpf to 5dpf. The brightest pattern of GFP expression in RARγ agonist (10nM) treated embryos was observed in notochord, pectoral fin, brain and ventral fin-fold (arrowed) at 2dpf but the expression became weaker from 3dpf. In contrast, the GFP expression of the control embryos in brain, pectoral fin, ventral fin-fold, pharyngeal arches and liver became stronger at 3dpf (arrowed) while these expressions in the RARγ agonist (10nM) treated embryos became weaker at 3dpf. However,

both RARγ agonist (10nM) treated and control embryos showed very weak expression from 4dpf to 5dpf. The scale bars represent 250μm. (n=3 independent experiments)

5.4. The apc$^{-/-}$ mutant zebrafish embryos exhibit a similar phenotype to RARγ agonist (10nM) treated wild type zebrafish embryos.

The previous findings suggested that the phenotypes of RARγ agonist (10nM) treated zebrafish embryos were very likely to be associated with premature up-regulation of canonical Wnt signalling. To compare the phenotypic similarities between RARγ agonist (10nM) treated embryos and canonical Wnt signalling up-regulated embryos, the characteristics of *apc*$^{-/-}$ mutant zebrafish were analysed. The *apc*$^{-/-}$ mutant zebrafish (Hurlstone *et al.*, 2013) carries the mutation in *apc* (adenomatous polyposis coli) resulting in defective beta-catenin degradation mechanism and permanent activation of canonical Wnt signalling.

The heterozygous zebrafish line that carries a mutation for the *apc* gene has a normal phenotype. After crossing parental heterozygous zebrafish carrying this *apc* mutation, there were a number of similarities between the phenotype seen and the resultant *apc*$^{-/-}$ mutant embryos, in which canonical Wnt signalling is constitutively on (Hurlstone *et al.*, 2013), and the RARγ agonist (10nM) treated zebrafish embryos. In particular, the *apc*$^{-/-}$ mutant zebrafish embryos had a malformed head and showed lack of pectoral fin formation at 3dpf (Figure 5.4). The *apc*$^{-/-}$ mutant embryos also had a shorter body axis, a malformed caudal fin, cardiac oedema and smaller eyes, as seen in lateral views (Figure 5.5; bottom panel), compared with the *apc*$^{+/-}$ siblings. Interestingly, although the caudal fin formation appeared to be abnormal in *apc*$^{-/-}$ embryos, the caudal fin formation was not completely diminished (arrowed in Figure 5.5) as in RARγ agonist (10nM) treated embryos at 3dpf.

Taken together, the similarity between the RARγ agonist (10nM) treated embryos and the *apc*$^{-/-}$ mutant embryos, as well as the increased and premature canonical Wnt signalling seen in some locations after RARγ agonist (10nM) treatment, suggests that RARγ may have increased Wnt signalling to result in the phenotypes seen. However, the truncated caudal fin formation in RARγ agonist (10nM) treated embryos may not be related to the premature up-regulation of canonical Wnt signalling.

Figure 5.5. Characterization of apc homozygous mutant zebrafish embryo at 3dpf

Representative pictures show the phenotypic differences between *apc*$^{+/-}$ and *apc*$^{-/-}$ embryos at 3dpf. The dorsal views show the changes in head morphology and the lack of pectoral fins in *apc*$^{-/-}$ embryos. The left lateral views show shorter body axis, smaller eyes, cardiac oedema and abnormal caudal fin formation. The scale bars represent 250µm. (n=3 independent experiments)

5.5. Treatment with the RARγ agonist (10nM) failed to rescue fin fold formation in krt4p/dkk2-rfp transgenic zebrafish.

The previous data suggested RARγ agonist (10nM) treatment was linked to the premature up-regulation of canonical Wnt signalling. To investigate whether the RARγ agonist was working by affecting intracellular or extracellular elements of the Wnt signalling pathway, a transgenic zebrafish line was used in which dickkopf related protein (*Dkk*) was expressed in the skin and fin folds under the control of the krt4p promoter (Wada *et al.,* 2013). Dkk works as a negative regulator of canonical Wnt signalling by degrading the LRP5/6 co-receptor for Wnt (Saito-Diaz *et al.,* 2013). In this Gal4: UAS transgenic system line of krt4p/dkk2-rfp embryos, the Gal4 protein was driven by keratin 4 promoter which is predominately expressed in the finfold and skin. Therefore, *dkk-rfp* is expressed in the skin and fin folds, which can be seen through the presence of rfp. However, in these embryos fin formation is inhibited because canonical Wnt signalling is required and this is inhibited by Dkk. The hypothesis, therefore, was that if the RARγ agonist increased canonical Wnt signalling through altering intracellular elements of this signalling pathway, rather than affecting extracellular elements (e.g. by directly increased *Wnt* expression), then treatment of

Fin fold formation is normally observed in wild type embryo at 2dpf. However, fin fold formation was not seen in krt4p/dkk2-rfp embryos at this same time point (Figure 5.6). The phenotype of the RARγ agonist (10nM) treated krt4p/dkk2-rfp embryos was similar to that of wild type RARγ agonist (10nM) treated embryos, in that there was truncation of the antero-posterior body axis. In addition, although RFP expression was seen in the skin of these embryos, indicating *Dkk* expression, there was no fin fold formation in the RARγ agonist (10nM) treated embryos at 2dpf (Figure 5.6). This indicated that RARγ agonist (10nM) was not increasing Wnt signalling through changing elements of the pathways that were downstream of Dkk/LRP but from extracellularly instead.

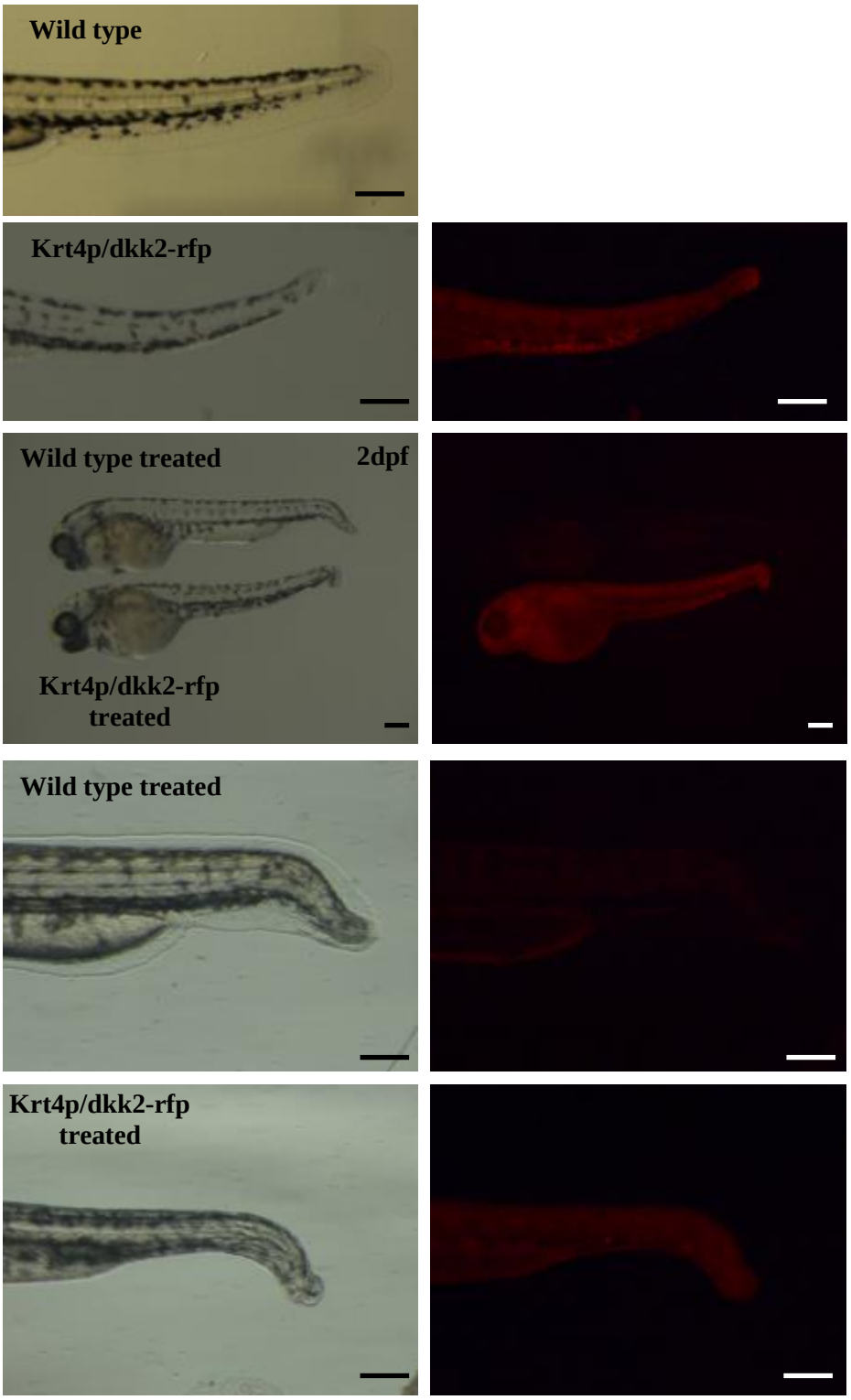

Figure 5.6. Treatment of zebrafish embryos with RARγ agonist (10nM) was associated with failure to rescue the fin fold formation in krt4p/dkk2-rfp transgenic embryo

Representative pictures show the posterior left lateral views of wild type, krt4p/dkk2 embryos and RARγ agonist (10nM) treated krt4p/dkk2 embryos at 2dpf. Normal fin-fold formation was found in wild type zebrafish embryos at 2dpf. However, fin-fold formation was inhibited in

krt4p/dkk2 embryos at 2dpf. RARγ agonist (10nM) treated krt4p/dkk2 embryos also did not have the fin-fold formation. The scale bars represent 250μm. (n=3 independent experiments)

5.6. RARγ and canonical Wnt signalling following caudal fin transection

The RARγ agonist has been shown to affect the development of tissues derived from stem/progenitors cell pools where RARγ is expressed, i.e. the neural crest, lateral plate mesoderm and presomitic mesoderm (Hale *et al.,* 2006; Waxman and Yelon 2007; Martin and Kimelman 2008). This was associated with altered canonical Wnt signalling. However, the treatment with RARγ agonist was at 4hpf stage in the development of the zebrafish embryos (4hpf throughout), which means that phenotypic effects seen may have been due to downstream events rather than direct effects on the RARγ expressing stem/progenitor cell pools. In addition, a recent study has reported that RA may influence gene expression without necessarily engaging with RAR by triggering intracellular kinase signalling pathways (Gudas, 2013).

To try to address the possibility of the RARγ agonist having non-RARγ specific activity on cells that were not from stem/progenitor pools, experiments were performed to examine the effects of the agonist on caudal fin regeneration following transection. This was done in wild type zebrafish embryos and in the Tcf;minip reporter zebrafish line because it is known that RARγ is up-regulated in the blastemal cells specifically during regeneration (White *et al.,* 1994). In addition, it is also known that *Wnt* expression is up-regulated during the regenerative process (Tal *et al.,* 2010). Although the involvement of canonical Wnt signalling in larval fin regeneration has not been reported, caudal fin regeneration was examined during embryonic development because this is rapid, i.e., it occurs within 2-3 days of transection (Kawakami, 2004; Yoshinari and Kawakami, 2011).

5.7. Treatment of zebrafish embryos with RARγ agonist was associated with a reversible inhibition of caudal fin regeneration and changes in canonical Wnt signalling.

The zebrafish larval caudal fin was transected at 2dpf and treated with 10nM RARγ agonist versus carrier alone (control). The larval caudal fin of the control embryos regenerated following transection to grow beyond the original line of transection. In contrast, the transected fins of RARγ agonist treated embryos did not regenerate such that at 5dpf they had not re-grown beyond the line of transection (Figure 5.7 top panels). Moreover, treatment with the RARγ agonist was also associated with a different distribution of melanocytes in the tail area. In the control embryos, melanocytes were absent from the dorsal area beneath the notochord, which is from where mesodermal progenitors arise during caudal fin patterning (Hadzhiev *et al.*, 2007) whereas melanocytes were seen to completely surround the notochord in the RARγ agonist treated embryos such that no gap was observed. This difference in the regeneration of the caudal fin and differential pattern of melanocyte distribution was consistent.

The inhibitory effects of the RARγ agonist on caudal fin regeneration were reversible. When the caudal fins were amputated and treated with the RARγ agonist (10nM) at 2dpf, but then subsequently were either co-treated with RARγ antagonist (10nM) at 3dpf or the media containing the RARγ agonist was removed and replaced with control media (wash out), there was increased fin regeneration beyond the site of transection (Figure 5.7 bottom panels). These observations were also completely consistent, with fin regeneration re-initiated in RARγ antagonist treated embryos and wash out embryos.

The caudal fins of Tcf;minip zebrafish embryos were transected at 3dpf rather than 2dpf (as was done in the wild type embryos) because GFP positivity is not seen at this stage of development (see Figure 5.3). However, caudal fin regeneration was also seen in these embryos at 4-5dpf, i.e. within 1 to 2 days post transection. In addition, increased GFP positivity was seen in the regenerating fins. This indicated that canonical Wnt signalling was associated with caudal fin regeneration in larvae. In contrast, there was no evidence of increased GFP positivity in the

transected caudal tips of the embryos when they were treated with the RARγ agonist (Figure 5.8). Hence, the inhibition of caudal fin regeneration may be linked to a lack of canonical Wnt signalling. However, increased GFP positivity was seen in the RARγ agonist treated embryos in the notochord. This differential pattern of GFP expression was consistent, being seen in all RARγ agonist treated and all control embryos. Therefore, these evidences suggest the effects of RARγ agonist on canonical Wnt signalling might be different depending on the developmental or regeneration process.

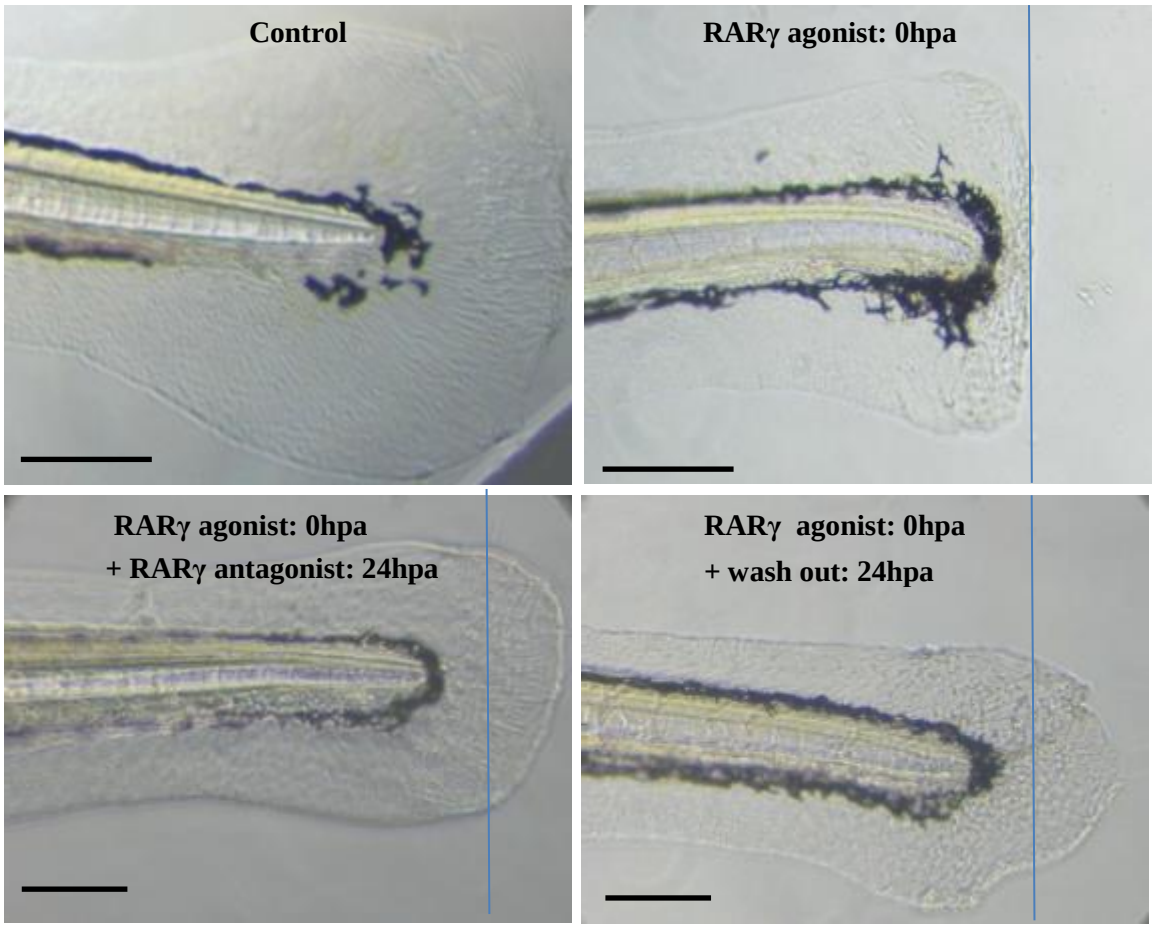

Figure 5.7. Treatment of zebrafish embryos with the RARγ agonist (10nM) was associated with reversible block in larval caudal fin regeneration.

Representative phase contrast microscopy pictures show the caudal fin regeneration in AB wild type zebrafish embryo at 5dpf after the caudal fin transection at 2dpf. The caudal fin was regenerated in the control embryo following the trasncetion at 2dpf (top left). However, the caudal fin regeneration was blocked in RARγ agonist (10nM) treated embryo at 5dpf (top right). Regeneration was restored in RARγ agonist (10nM) treated embryos at 5dpf after either washing out the RARγ agonist (10nM) at 24 hour post transection (bottom right) or adding an equal dose (10nM) of the RARγ antagonist at 24 hour post transection (bottom left). The scale bars represent 250μm. (n=3 independent experiments)

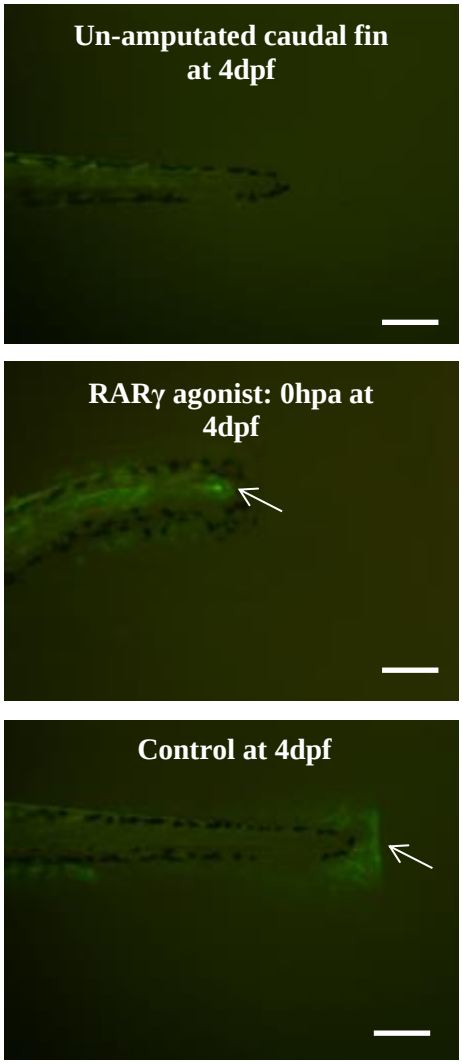

Figure 5.8. Treatment of zebrafish embryos with RARγ agonist (10nM) was associated with loss of canonical Wnt signalling in regenerating fin but up-regulation in the notochord.

Representative fluorescence microscopy pictures of canonical Wnt signalling in the transgenic zebrafish Tcf:mini-P reporter line. A low level of GFP expression to indicate canonical Wnt signalling was seen in the caudal tail of non transected zebrafish embryos at 4dpf (top). In contrast, there was a marked up-regulation in GFP expression demonstrating increased canonical Wnt signalling in the regenerating caudal fins of control embryos at the same time point, i.e. at 4dpf and 1 day after transection (white arrows) (bottom). No GFP-evident canonical Wnt signalling was seen in the transected fins of the RARγ agonist (10nM) treated embryos at 4dfp and 1 day after transection. These embryos did not regenerate transected caudal fins. However, increased canonical Wnt signalling was seen in the notochord (white arrow) (middle). Scale bars represent 250µm. (n=3 independent experiments)

5.8. Discussion

The results of RARγ agonist treatment to the canonical Wnt signalling reporter transgenic zebrafish line generally showed that the premature up-regulation of GFP expression in the notochord was associated with RARγ agonist treatment (Figure 5.2, Figure 5.3 and Figure 5.4). The up-regulation of GFP expression in the notochord of RARγ agonist treated embryos suggests the effects might be directly activated by RARγ agonist. The patterns of GFP expression also showed the up-regulation occurred from 1dpf to 2dpf followed by regression after 3dpf. Interestingly, the regression pattern in the notochord of the RARγ agonist treated embryos happened from anterior to posterior which may be the effects of agonist diffusion. The most posterior part of the RARγ agonist treated embryos might still have had more contact with the diffused agonists because of the wedged shape embryo in which the anterior part of the embryo was thicker than the posterior part of the embryo suggesting that agonist diffusion was more accessible to the notochord in the posterior part of the embryo. Another interesting finding was lack of GFP expression in the pharyngeal arches of RARγ agonist treated embryos from 2dpf to 3dpf (Figure 5.4). This finding agreed with the sox9 antibody staining results from chapter 4 which showed lack of sox9 positive neural crest staining in the treated embryos at 60hpf. In general, all the canonical Wnt signalling expression areas in the RARγ agonist treated embryos were weaker than those of control embryos apart from the ectopic expression in notochord. However, the expression in both RARγ agonist treated and control embryos became weaker in 4dpf and the weakest in 5dpf.

These findings suggested the ectopic expression in the notochord may be the direct effects of RARγ agonist on canonical Wnt signalling. Canonical Wnt signalling may play the important roles in the zebrafish development before 4dpf reaching the highest level of expression at 3dpf. However, the canonical Wnt signalling in the RARγ agonist treated embryos showed the premature peak at 2dpf and early regression at 3dpf. Therefore, canonical Wnt up-regulation caused by RARγ agonist could possibly be the main driving force which caused the phenotypes reported in Chapter 3.

Previous results showed the canonical Wnt signalling started from the most posterior part of the tail in the RARγ agonist treated embryos suggesting it might be the direct effects of RARγ agonist on canonical Wnt signalling. Moreover, the pattern of canonical Wnt signalling was the strongest in the most posterior region and the weakeest in the anterior region. The thickness of the zebrafish body is the thinnest in the most posterior region suggesting the penetration of RARγ agonist may play a role in the intensity of Wnt signalling and the level of expression may be concentration dependent.

Characterization of *apc*$^{-/-}$ mutant zebrafish embryos showed the similar phenotypes between RARγ agonist treated embryos and *apc*$^{-/-}$ mutant embryos at 3dpf. The morpholino knock down of the apc gene in zebrafish embryos also showed the same phenotypes as *apc*$^{-/-}$ mutant embryos (Nadauld et al. 2004). Therefore, the results of *apc*$^{-/-}$ mutant zebrafish embryos were consistent with RARγ agnist treatment of zebrafish causing the abnormal phenotypes by up-regulating of canonical Wnt signalling.

In the next experiment, the possible mechanism in which RARγ agonist interacted with the canonical Wnt signalling was further investigated. Since the retinoid is a lipophilic molecule which can interact with a cytoplasmic receptor (Rhinn and Dollé 2012), the experiment was set up to investigate whether RARγ agonist activated intracellular canonical Wnt signalling or extracellular pathways. The results showed RARγ agonist failed to rescue the finfold formation when dkk was activated in the finfold area to interrupt the canonical Wnt signalling at the cell membrane level. Therefore, it suggested the RARγ agonist might be activating the canonical Wnt signalling extracellularly.

The results from this Chapter so far agree with the finding of cross talk between RA signalling and canonical Wnt signalling in mouse chrodrocyte cell culture (Yasuhara *et al.*, 2010). These *in vitro* results show canonical Wnt signalling can be activated by RA treatment without Wnt cytokine in mouse chondrocytes. This activity is inhibited by co-treatment of dkk protein suggesting RA activation needs the intact surface receptor proteins. Interestingly, the

canonical Wnt signalling in these chondrocytes was inhibited by over-expression of RAR gamma receptor, not alpha and beta, suggesting that the release of the repressor activity of RAR gamma receptor on canonical Wnt signalling may be what is important. Their experiments also reported that ligand free RAR gamma receptor induces the dissociation of beta catenin from Tcf protein by binding to the beta catenin itself which may explain why the RAR gamma overexpression inhibited canonical Wnt signalling. Therefore, these *in vitro* findings support the results of canonincal Wnt signalling in RARγ agonist treated embryos.

Although the results suggested RARγ agonist needed the LRP membrane receptors to activate the canonical Wnt signalling, it is still not clear how it actually worked. The results from mouse chondrocyte (Yasuhara *et al.*, 2010) suggested that the repressor role of RAR gamma was inhibition of beta-catenin association to Tcf. Therefore, in our experiments, treating with RARγ agonist may have switched off the repressor role of the RARγ receptor favouring more beta-catenin binding to Tcf. If it was true, the RARγ agonist should have been able to activate the Wnt signalling in dkk expressing transgenic line. Therefore, it suggests activation of canonical Wnt signalling by RARγ agonist was not solely dependent on the repressor activity of RARγ; it also needed the role of membrane receptors. Hence, the cross talk between Wnt signalling and RA signalling may involve more than one mechanism. Another interesting question is why RARγ agonist needed the intact membrane LRP5/6 co-receptor although the RARγ agonist/retinoid itself is intracellular ligand (Rhinn and Dollé 2012). Therefore, it is interesting to know whether RARγ agonist itself can bind to the receptor or increase Wnt production. The level of Wnt cytokine expression in RARγ agonist treated embryos needs to be investigated. Recent studies show RA can activate non-genomic signalling pathways by interacting directly with kinases and cell-surface receptor (Al Tanoury *et al.*, 2013) although there is no report of interaction between retinoid and frizzled/LRP5/6 receptors.

In summary, the results showed the canonical Wnt signalling involvement in RARγ agonist treated embryos suggesting the up-regulation of canonical Wnt signalling played an important role for the phenotypes caused by RARγ agonist treatment. The results also suggested

the importance of RARγ agonist as a repressor to inhibit the beta-catenin and the mysterious role of RARγ agonist on the cell surface receptors for Wnt is yet to be investigated.

The caudal fin regeneration results showed interesting similarities between RARγ agonist on fin regeneration and pectoral fin formation. The growth of both tissues was reversibly inhibited by RARγ agonist. Therefore, RARγ agonist may be anti-proliferative/regenerative on these tissue outgrowths. However, it is still not yet clear how RARγ agonist affected the blastema. Although zebrafish RAR gamma is expressed at regenerating adult caudal fin (White et al. 1994) and at the tailbud during somitogenesis (Waxman & Yelon 2007; Hale et al. 2006), it is still not known whether RARγ is expressed during larval caudal fin regeneration. Nevertheless, these results suggested there was a possibility that RARγ may have interrupted the pluripotent cells in the regenerating caudal fin preventing the outgrowth required for regeneration.

The results of RARγ agonist on canonical Wnt signalling of regenerating larval caudal fin showed the paradoxical effects of RARγ agonist on canonical Wnt signalling during embryonic development. However, the opposite effects, the up-regulation found in the adjacent tissue, the notochord, versus the down-regulation found in the regeneration caudal fin suggesting the interactions between RARγ agonist and canonical Wnt signalling may not be the same during embryonic development and the regeneration process.

Chapter 6

Discussion

6.1. RARγ agonist and stem/progenitor cells

This study was performed to examine the potential role of RARγ in the regulation of embryonic development and tissue regeneration using wild type and reporter transgenic zebrafish as model systems. It was demonstrated that treatment of embryonic zebrafish with a RARγ specific agonist had profound developmental consequences, including decreased anterior-posterior growth, inhibition of cranial bone and neural tissue formation, and loss of growth of the pectoral and caudal fins, as well as abrogation of caudal fin regeneration following fin transection. It was also noted that RARγ agonist treated fish exhibited cardiac oedema. These findings strongly suggest that RARγ activity must be tightly regulated during normal embryonic development and tissue regeneration.

The tissues that were affected by treatment with the RARγ agonist derived from those stem/progenitor cell populations in which zebrafish *rarg* expression is restricted during early embryonic development, i.e., in the cranial neural crest, in the lateral plate mesoderm, and in the pre-somitic mesoderm of the tail bud (Hale *et al.*, 2006; Waxman and Yelon, 2007). In addition, the tissues affected by the RARγ agonist also mirror those that are similarly affected by morpholino gene knock down of *rarga* and *rargb*, namely the pharyngeal arches, pectoral and caudal fins (Linville *et al.*, 2009). Furthermore, this phenotype is similar to the effects of RA treatment on embryonic development (Shum *et al.*, 1999). Taken together, this suggests that the agonist used in the current study was specific for RARγ in zebrafish, as has been conclusively demonstrated in mammalian reporter cell lines (Hughes *et al.*, 2006).

The mechanisms involved in the developmental changes observed have been examined using a combination of transgenic reporter fish lines, *in situ* hybridisation and immunohistology for several important genes and appropriate stem/progenitor cell markers. The shorter body axis formation seen in RARγ agonist treated embryos was associated with decreased somite formation, as revealed in hspGFF55B transgenic embryos, *in situ* hybridisation of *myoD* expression and α actinin immunolocalisation. Analysis of the *hoxc11a* and *hoxb13a* transgenic

reporter fish lines demonstrated that the shorter body axis was largely the result of loss of the most posterior region of the embryos. Precise expression of the *hox* gene family is necessary for normal anterior-posterior axis formation in vertebrates (Wellik 2007). *Hoxc11* is developmentally expressed in the posterior region of the mouse embryo (Hostikka and Capecchi, 1998) and homozygous knock-out of the *hoxc*-cluster results in loss of caudal vertebrae (Suemori and Noguchi 2000; Wellik *et al.,* 2007). In mice, *hoxb13* expression occurs in the tail bud area around E9 (Zeltser *et al.,* 1996). *Hoxb13* expression is also found in the developing tail of axolotl and re-expressed in the regenerating tail after transection (Carlson *et al.,* 2001). These expression patterns suggest that *hoxb13* plays an important role in the development and growth or regrowth of the tail. Conversely, a heterozygous knock out of *hoxb13* in mice was shown to cause overgrowth of the tail (Economides *et al.,* 2003). The observation of a complete loss of *hoxb13a* expression in RARγ agonist treated zebrafish supports the hypothesis that this gene is essential for the formation of the posterior regions of developing embryos and may be regulated by RARγ. Extra-somitic expression of *hoxb13a* was also seen in the anal fin area of the control embryos, which was completely lost in the RARγ agonist treated embryos. Other researchers have similarly reported *Hoxb13* expression in the hindgut and urogenital area of mice (Zeltser *et al.,* 1996). Hence, the observation further suggests that treatment with the RARγ agonist targeted *hoxb13a* expression. Interestingly, *hoxb13a* expression was rescued by the removal of RARγ agonist or co-treatment of RARγ antagonist suggesting the loss of *hoxb13a* expression was transient. Moreover, it was also shown that re-expression of *hoxb13a* was also associated with regeneration of the caudal fin following transection. However, it remains a possibility that *hoxb13a* expression may simply be absent from the RARγ agonist treated embryos, because the tissues in which *hoxb13a* is normally expressed during development, including the anal area were not formed. A loss of *hox* gene expression in response to treatment with a RAR agonist is certainly paradoxical as RARE is located within the regulatory region of *hox* gene clusters (Zhang *et al.,* 2000), which are normally up-regulated in response to RA (Pöpperl and Featherstone 1993; Huang *et al.,* 2002;

Oliveira *et al.*, 2013). Such a paradox may be resolved if RARγ does not require RA or indeed does not function through RARE to regulate target gene expression.

The marked morphological changes seen in the head of the RARγ agonist treated embryos were associated with loss of cranial bones and the anterior lateral line ganglia as revealed by alizarin red staining and the HGn39D transgenic fish line, respectively. These tissues form from the neural crest, as well as the placode for the lateral line (Collazo *et al.*, 1994). This suggests that RARγ agonist treatment may have targeted neural crest stem/progenitor cells, which are known to express RARγ (Hale *et al.*, 2006; Waxman and Yelon, 2007). The fact that there were significantly fewer sox9 immunopositive cells in the cranial region of the RARγ agonist treated embryos might be considered to support this hypothesis. However, this difference was only seen in later time points, i.e. at 60hpf but not at 25hpf, even though RARγ is expressed at the earlier time point. Furthermore, even at 60hpf, sox9 immunopositive cells were still observed in the cranium. Therefore, it is also possible that fewer sox9 immunopositive cells were prevalent within the anterior cranial regions of the RARγ agonist treated embryos simply because the anterior tissues did not form. Further research is required to examine whether the loss of sox9 neural crest stem/progenitor cells following treatment with the RARγ agonist is causal to the loss of anterior cranial tissues observed. However, it is noteworthy that *sox9a* or *sox9b* morpholino knock-down and *sox9a* mutant zebrafish embryos were shown to lack cranial skeletal structures (Yan *et al., 2002*; Yan *et al.*, 2005). These developmental defects were associated with *sox9* aberrant neural crest stem/progenitor cell differentiation, rather than any changes in neural crest formation or cell migration. Similarly, it was concluded that treatment with the RARγ agonist did not markedly affect the formation of neural crest stem/progenitor cells, as depicted by sox9 immunopositive cells at 25hpf, but may well have influenced their survival, proliferation, migration or differentiation thereafter, to adversely affect the development of cranial tissues.

Although almost all of the neural crest derived cranial bones were absent or greatly decreased in the RARγ agonist treated embryos, there were still fully intact cleithrum bones,

which also are of neural crest origin (Kague *et al.,* 2012). There is no clear explanation for this difference. However, it is interesting that cleithrum bones undergo a process of dermal ossification, unlike other affected cranial bones that undergo endochondral ossification (Kague *et al.,* 2012; Eames *et al.,* 2013).

An additional major phenotype seen following treatment with the RARγ agonist was the loss of pectoral fin outgrowth, despite formation of the fin bud. Other studies have reported that the interaction of RA signalling with expression of the *tbx5a* transcription factor is essential for pectoral fin formation. Increased *raldh1a2* expression and its localized synthesis of RA in the region of somites 2-6, where *tbx5a* expression is induced in response to RA, is required for formation of the pectoral fin (Gibert *et al.,* 2006). Both the *nls* (neckless) mutant zebrafish, which carries a mutation in *raldh1a2* (Grandel *et al.,* 2002), and embryonic zebrafish that have been treated with the RA synthesis inhibitor, diethylaminobenzyldehyde (DEAB), do not express *tbx5a* and do not form pectoral fins (Grandel and Brand 2011). Moreover, loss of function *tbx5a* mutations block pectoral fin formation, as well as causing heart defects (Garrity *et al.,* 2002). Zebrafish *tbx5a* expression can be seen from 14hpf in the lateral late mesoderm in the common stem/progenitor cell population for the development of the heart and pectoral fins, which separate from 24-27hpf (Albalat *et al.,* 2010). Because the pectoral fins did not form in RARγ agonist treated embryos in this study, *tbx5* was considered a potential target gene for RARγ agonism. However, it was also found that there were no marked differences in the presence of tbx5a-immunopositive stem/progenitor cells in the RARγ agonist treated or control embryos, suggesting that *tbx5a* expression was unaffected. Furthermore, RARγ agonist wash out experiments or co-treatment with a RARγ specific antagonist (as well as the agonist) completely abrogated the block of pectoral fin formation, demonstrating that *tbx5a* was functional. Finally, treatment with the RARγ agonist at 27hpf, which was confirmed as when *tbx5a* was present in the lateral plate mesoderm, was also found to completely block pectoral fin outgrowth. Therefore, it can be concluded that the effect of RARγ agonist treatment on pectoral fin outgrowth was independent of *tbx5a*. Potential targets that lie downstream of *tbx5a* activity

include *fgf* family genes, i.e., *fgf8, fgf10* and *fgf24* (Ng *et al.,* 2002; Fischer 2003), as well as *sall4* (Harvey and Logan, 2006), *blimp-1* (Lee and Roy, 2006), *prdm1* (Mercader *et al.,* 2006), beta-CaMK-II (Rothschild *et al.,* 2009) and *ndrg4* (Qu *et al.,* 2008). In addition, *hox* gene expression was also associated with pectoral fin formation, including *hoxa* (Géraudie and Borday Birraux 2003), *hoxb* (Waxman *et al.,* 2008) and *hoxd* (Sakamoto *et al.,* 2009; Neumann *et al.,* 1999). Further study will elucidate if RARγ agonist treatment alters the expression of each of these genes, but given our finding that *hoxb13a* was completely inhibited by such treatment, this family is a clear target.

Lastly, it was tested whether the RARγ agonist affected tissue regeneration as well as development using transection of the caudal fin as a model. This was because RARγ is expressed in the distal edges of blastemal cells at least during adult zebrafish caudal fin regeneration (White *et al.,* 1994). Similar to the findings with pectoral fin outgrowth, it was found that RARγ agonist treatment was associated with a complete, but reversible block on caudal fin regeneration. Canonical Wnt signalling is known to play a major role in caudal fin regeneration (Tal *et al.,* 2010). Therefore, we investigated whether this pathway was affected by treatment with the RARγ agonist using the transgenic reporter line, Tcf:mini-P (Shimizu *et al.,* 2012). As it was hypothesised, the block in caudal fin regeneration following RARγ agonist treatment was associated with an observed reduction in Wnt signalling, suggesting that the Wnt pathway is a target for RARγ. However, similarly to our observations of sox9 immunopositive cells in the cranial neural crest, there is also the possibility that Wnt signalling was not seen following transection of the caudal fin and treatment with the RARγ agonist because the regenerating tissues did not form. Therefore, further study is required to determine whether the evident loss of Wnt signalling following RARγ agonist treatment is causal to the lack of a regenerative response.

The results show that the tissues which express *rarg* in zebrafish were affected by the RARγ agonist. These *rarg* expressing tissues are the pectoral fin, the neural crest and the tail-bud (Hale *et al.,* 2006; Waxman and Yelon 2007). Interestingly in zebrafish, the time-points of

rarga and *rargb* expressions in these tissues are coincided with the time-points in which the stem/progenitor cells are formed in the respective tissues. For example, localized high level expression of *rargb* is reported in the tail-bud area at 10hpf (Waxman and Yelon 2007). In zebrafish development, 10hpf stage is the time-point that somitogenesis starts (Kimmel *et al.*, 1995). During somitogenesis, zebrafish tail-bud serves as a stem/progenitor cells zone known as pre-somitic mesoderm which constantly produces the somites (Rhinn and Dollé 2012; Stickney *et al.*, 2000). Both *rarga* and *rargb* expression are found in the tail-bud area until the 15-somite stage but *rargb* expression persists until 24hpf (Waxman and Yelon 2007), which is the time point of somitogenesis completion (Stickney *et al.*, 2000). At 24hpf, both *rarga* and *rargb* expression are found in the lateral plate mesodermal regions of somites 2 to 6 (Waxman and Yelon 2007). Lateral plate mesoderm at 24hpf in these areas is where the heart and pectoral progenitor cells are accumulated (Gibert *et al.*, 2006; Grandel *et al.*, 2002). Moreover, *rargb* expression is also found in the areas that form the pharyngeal arches from cranial neural crest at 24hpf (Waxman and Yelon 2007), and again, neural crest cells are multi-potent stem/progenitor cells (Rodrigues *et al.*, 2012; Dutton *et al.*, 2001). Therefore, the expression of *rarga* and *rargb* appears to be related to the presence of stem/progenitor cells.

Another interesting fact is these *rarga* and *rargb* expressing tissues are not located within the intrinsic range of the presence of RA. A transgenic zebrafish line which provides evidence of the intrinsic distribution of RA showed a complete lack of RA in the tail-bud area during somitogenesis (Shimozono *et al.*, 2013). In fact, the RA synthesizing enzyme (*raldh2*) is not expressed in the tail-bud area during the somitogenesis stage (Grandel *et al.*, 2002). Moreover, the presomitic mesoderm during somitogenesis is protected from RA by expression *cyp26a1*, the RA degrading enzyme (Dobbs-McAuliffe *et al.*, 2004). Similar patterns of *raldh2* and *cyp26a1* expression are found in pectoral fin formation. *Raldh2* expression in the intermediate mesoderm of somite numbers 2 to 6 is found at the 12-13 somite stages, to induce the *tbx5a* for cardiac and pectoral fin progenitor cells (Grandel and Brand 2011). However, when the pectoral fin bud is formed and starts to grow from 27pf (Albalat *et al.*, 2010), the fin

growth from lateral plate mesoderm in the proximal to distal direction results in the growing part of pectoral fin receiving the lowest level of RA (Grandel and Brand 2011). In fact, it has been suggested that the growing pectoral fin expresses *cyp26a1* from 24hpf to prevent the effects of RA (Dobbs-McAuliffe *et al.,* 2004). At the same time, *rarga* expression is found in the lateral plate mesoderm from 24hpf to the formation of the pectoral fin at 48hpf (Hale *et al.,* 2006). The expression patterns of *rarga* and *rargb* in the cranial neural crest are also similar to the previous tissues. The strong expression pattern of *rarga* is observed in the pharyngeal neural crest at 48hpf (Hale *et al.,* 2006). Although *raldh2* expression is found in the zebrafish head area from 30hpf to 48hpf, it is not expressed in the pharyngeal arches where *cyp26a1* is heavily expressed (Dobbs-McAuliffe *et al.,* 2004). Therefore, the data above suggests the *rarg* in zebrafish may need protection from the influence of RA in regulating gene expression by co-expressing *cyp26a*.

The possible answer to this paradox is that the RA receptors can work as co-repressors of gene expression activity and this has been shown in mice (Koide *et al.,* 2001; Williams *et al.,* 2009). However, it has been reported that zebrafish *rara* may not have co-repressor activity (Waxman and Yelon 2011). Therefore, the findings of this study are clearly contradicting the previous finding regarding by Waxman and Yelon (2011), as they also suggest the necessity of *rarg* as repressor. There are two ways to prove the repressor activity of RARγ is important. First, if a tissue or cell needs a receptor as a repressor, this tissue or cell function will be disrupted when an agonist switches its receptor to become that of a transcriptional activator. Here, the results showed the tissues which expressed *rarg* were disrupted by agonism suggesting it has repressor activity. Secondly, if the receptor is needed as a transcriptional repressor, the mutant or morphant zebrafish of this gene will have phenotypes associated with the tissues or cells that express receptor. There is no current analysis on *rarg* zebrafish mutant lines, but *rarg* morpholinos knock down zebrafish morphant show effects on pectoral fin outgrowth, pharyngeal arches and the tail (Linville *et al.,* 2009). The combination of results presented in this thesis and the morpholinos results suggests the *rarg* may have a role in

repressing gene expression. Another interesting question is the main repressive function of *rarg*. Although its exact function is not still clear, it is heavily involved in stem cell maintenance. RARγ (-/-) murine embryonic stem cells lose gene expression in a number of genes, including *cyp26a1,* which prevents RA signalling (Kashyap *et al.,* 2013). Furthermore, the rate of RARγ overexpression in murine somatic cells used for induced pluripotency experiments was directly related to the rate and efficiency of reprogramming the cells into pluripotent stem cells (Wang *et al.,* 2011). Here, the results of this study showed that the stem cells niches were affected by RARγ agonism, such as pre-somitic mesoderm (Rhinn and Dollé 2012), neural crest (Rodrigues *et al.,* 2012) and the forming pectoral fin, which also has shown its involvement in regenerative capacity (Yano *et al.,* 2012). The caudal fin regeneration results also showed repressor activity on blastemal cells, because *rarg* is one of the genes network involved in the regenerating blastema of caudal fin (White *et al.,* 1994), while there is *raldh2* expression in the proximal area of the caudal fin rather than the blastema itself.

The last issue to solve is to determine if the effects caused by RARγ agonist treatment were purely due to disruptive effects on repressive activity alone or combination of repressive activity disruption and transcriptional activation. In other words, can the RARγ agonist activate the transcription by binding RAR/RXR complex? The conventional RA signalling pathway involves the formation of the RAR/RXR complex and transcriptional activation via RARE (Rhinn and Dollé 2012). Using the transgenic zebrafish line (RARE:YFP), which drives the reporter gene using RARE (Perz-Edwards *et al.,* 2001), has revealed a variety of areas where intrinsic RA signalling occurs throughout the zebrafish development. Interestingly, all the tissues in which the presence of RA signalling was detected, from the 18 somite stages through to 3dpf, do not include the tissues in which *rarg* is expressed, i.e. the pectoral fin, tail bud and pharyngeal neural crests. More importantly, treating this transgenic line with RA does not induce the reporter gene expression in the tailbud, pectoral fin and pharyngeal areas, suggesting that the RARγ/RXR complex may not have transcriptional activator role in zebrafish. Therefore, the effects caused by RARγ agonist treatment to zebrafish were very likely to be due

to disruptive effects on gene repressive activity, rather than transcriptional activation. However, it may not be completely concluded that the effects of RARγ agonist are solely driven through effects on the RAR/RXR, because RA can also trigger non-genomic effects by kinase cascade activation (Al Tanoury *et al.*, 2013).

6.2. Specificity Issue

The is an unresolved question in this project regarding the specificity of the RARγ agonist for zebrafish rarg. Although human and zebrafish RARs share similar homology (Hale *et al.,* 2006; Waxman and Yelon 2007), the specifity of the RARγ agonist was proven in a mammalian cell line (Hughes *et al.*, 2006). This means that the agonist may have the off-target affects in zebrafish cells. Moreover, zebrafish has two *rarg* isotypes, which are rarga and rargb (Waxman and Yelon 2007). Therefore, even if the agonist is specific for the receptor, it is important to know whether both receptors are responsive to the RARγ agonist or either of them.

The simplest and easiest way to investigate the specificity of the RARγ agonist is the experiment in which the rarg receptors have been disrupted, followed by RARγ agonist treatment. Morpholino is a common method to block gene translation into protein, but the use of morpholino injections can trigger the p53 pathway and induce apoptosis, plus most morpholinos have toxic off-target effects (Bedell *et al.,* 2011). Therefore, co-injection with p53-morpholinos has become a common practice to prevent morpholino-induced apoptosis (Bedell *et al.,* 2011). However, RA signalling itself is implicated in the apoptotic pathway (Noy 2010). Therefore, the experiment of using morpholino injections to block *rarg* expression, with co-injection of p53 morpholinos, followed by a RARγ agonist treatment may not give a straightforward answer on specificity. In fact, morpholino alone injections for *rar* give a lethal phenotype, and co-injection with p53 morpholino does provide viable embryos (Linville *et al.*, 2009). However, morpholinos to *rarga* and *rargb* show loss of pharyngeal arches, pectoral fin outgrowth and defective caudal fin (Linville *et al.,* 2009). Therefore, these morphants have tissue losses, which were supposed to be then be affected by RAR gamma agonism if such an experiment were to proceed. Hence, it is impossible to use *rarg* morpholinos to then examine RARγ agonist specificity.

Another possible model for specificity testing is using mutant zebrafish lines, which carry mutations in *rarg*. Using forward genetics screening using ENU (*N*-ethyl *N*-nitrosourea),

the Sanger Institute has been developing the zebrafish library of mutant zebrafish lines for every single zebrafish gene (Dooley *et al.*, 2013). The project has identified a mutant line of *rarga*. The mutation is a non-sense mutation and the detail can be found on the Sanger Institute website (http://www.sanger.ac.uk/sanger/Zebrafish_Zmpgene/ENSDARG00000034117). Therefore, this mutant zebrafish line is a useful candidate for examination of the RARγ agonist specificity. The mutant line is currently heterozygous. Phenotyping of the homozygous embryos is also an interesting study to compare with the morpholino-injected phenotype (Linville *et al.*, 2009). However, the current mutant line is in F1 stage and has not yet been released for investigation.

An alternative method to random mutagenesis for forward screening mutagenesis is to use reverse genetics. TALENs (Tal effector nucleases) have become powerful tools for gene editing (Chen *et al.*, 2013). These nucleases can bind to specific DNA sequences, induce double stranded DNA breaks, resulting in insertional and deletional mutations. The TALENs technique has been shown to be 10 times more mutagenic than ZFN (Zinc Finger Nucleases), but the efficiency of TALENs is negatively proportionate to the CpG islands percentage in the target DNA sequences (Chen *et al.*, 2013). Therefore, the same efficiency of TALENs mutagenesis cannot be found in *rarga* and *rargb* because the amount of CpG island content in *rarga* and *rargb* may not be equal. Using the free online CpG island finder software provided by University of Southern California, zebrafish *rarga* CpG islands in genomic DNA was 501bp out of 128483bp, but there was no CpG island in zebrafish *rargb*, suggesting higher success rate of TALEN mutagenesis can be achieved for *rarga* than *rargb* gene edits.

The breakthrough discovery of bacterial acquired immune system has opened a new chapter of genomic engineering. After viral or bacterial challenge, most bacteria and archaea integrates the foreign DNA spacer fragments, then they produce cluster regulatory interspaced short palindromic repeats (CRISPR) driven by CRISPR associated system (CAS) to resist the phage challenge (Barrangou *et al.*, 2007). This CRISPR mechanism causes double strand DNA breaks and it has been used as a powerful tool for genome editing for cell lines and model

organisms including zebrafish (Hisano *et al.*, 2013). In zebrafish, it has been shown the genome editing produced by CRISPR and Cas9 system can also transmit to the germ lines with an efficiency as high as 100%, providing a good perspective of permanent mutant lines generation (Hwang *et al.*, 2013). More interestingly, this technique can not only knock-out the expression of genes of interest in zebrafish, but also knock-in genes of desire at the same time by co-injection (Chang *et al.*, 2013). Therefore, using these target specific gene editing tools, not only can the RARγ agonist specificity problem can be solved, but also the whole new level of RA signalling study can be achieved.

The specificity test can be achieved by targeted genome edition on ligand binding domains of zebrafish *rarga* and *rargb*. By doing so, the genomic DNA which encodes the exons containing the ligand binding domains of *rarga* or *rargb* will be removed by double strand break. Therefore, the ligands will no longer bind to the modified receptors which will still have the genomic DNA encoding DNA binding domains and co-repressor binding domains. Treating these *rarga* or *rargb* with RARγ agonist will not only provide the specificity of this agonist but will also show the possible non-genomic effects of agonist. In fact, this genome editing tools can also bring the other aspects of RA receptor function rather than specificity. By mutating the DNA sequence of *rarga* or *rargb* which encodes the DNA Binding Domain (DBD), the study will further explore the receptor's involvement in its repressor role probably by mimicking the effects caused by *rarga* and *rargb* morpholinos injected embryos. Further studies can follow for the roles of co-repressor binding, RXR-binding and co-activator recruiting.

Although the induced mutation techniques can improve the specificity of RARγ agonist on zebrafish *rarg*, these techniques can still not prove the actual interaction between the ligand and the receptor complex. Therefore, immuno-precipitation method can provide the important ligand-receptor interaction information. If the ligand (RA) binds to the *rarg* and causes the transcriptional activation, the immuno-precipitation results should be able to show the difference between the receptor-complex composition before the RARγ agonist treatment and after treatment. For example, the receptor complex retrieved from *rarg* expressing tissues

before treatment should show the protein complex of rarg/rxr and co-repressor. However, the protein complex should show rarg/rxr and co-activator (probably other transcriptional activation protein complex) in the immuno-precipitation after the treatment if the RARγ agonist is involved in transcriptional activation.

Another interesting technique for specificity is to visualize the interaction between the ligand and the receptor using GEPRAs (Genetically Encoded Probes for RA). The technique uses the modified version of RAR which is tagged with GFP and YFP. In ligand free stage, the receptor emits the green colour and the yellow colour is emitted when the ligand is bound to the receptor. This is the protocol used for visualization of intrinsic RA in zebrafish which is already published (Shimozono *et al.*, 2013). Therefore, using the genetically modified *rarga* and *rargb* tagged with GFP and YFP transgenic lines may provide the evidence for interaction between the RARγ agonist and the receptor.

Another important question in this project is whether *rarga* and *rargb* are transcriptionally activated by RARγ agonist. The canonical RA signalling involves ligand binding to the receptor, RAR/RXRhetero dimer and RARE (Linney *et al.*, n.d.). Therefore, using the transgenic line which has RARE:YFP transgenic zebrafish (Perz-Edwards *et al.*, 2001) can provide the crucial information transcriptional activation.

6.3. Conclusion

Treatment of zebrafish embryos with a RARγ agonist showed disruptive effects on normal development because the *rarga* and *rargb* were presumably needed in generepressor roles rather than in a transcriptional activator role. Treating with RARγ agonist may have disrupted the *rarg* repressor functions which are needed for normal development. The results suggested zebrafish either *rarga* or *rargb* or both may have important roles in stem/progenitor cells.

Chapter 7

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Appendix

Publication attached.