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Original Article

TBX1, a DiGeorge syndrome candidate gene, is inhibited by retinoic acid

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ABSTRACT Both retinoic acid (RA) and Tbx1 are definitively indispensable for the development of the pharyngeal arches. The defects produced by a loss of Tbx1 highly resemble those induced by hyper- and hypo- RA. Based on these similarities, the effects of RA on Tbx1 expression pattern were explored during pharyngeal arch development in zebrafish. Whole-mount *in situ* hybridization and real-time quantitative PCR were used. Zebrafish embryos were treated with $5x10^{-8}$mol/L and 10^{-7}mol/L RA at 12.5 hours post fertilization for 1.5 hours, respectively. Whole-mount *in situ* hybridization showed that Tbx1 was expressed in the cardiac region, pharyngeal arch and otic vesicle between 24 hpf and 72 hpf in zebrafish. Tbx1 expression was obviously reduced, even lost, in the pharyngeal arch and outflow tract in RA treated groups. Real-time quantitative PCR analysis showed that Tbx1 expression rose to a peak level at 36 hpf in wild type group. Repression of Tbx1 expression was most evident at 36 hpf, 24 hours after RA treatment. 10^{-7} mol/L RA caused a more severe effect on the Tbx1 expression level than $5x10^{-8}$mol/L RA.The results suggested that RA could produce an altered Tbx1 expression pattern in zebrafish. In addition, RA could repress Tbx1 expression in a dose-dependant manner.

KEY WORDS: *Tbx1, retinoic acid, zebrafish, pharyngeal arch, cardiac development*

Introduction

DiGeorge/velocardiofacial syndrome (DGS/ VCFS), the most common genetic deletion syndrome in humans, with an incidence of 1 in 4000 live births, is the second leading cause of congenital heart defects in children (Scambler, 2000). The majority of DGS/ VCFS is characterized by microdeletion of the long arm of human chromosome 22 (22q11.2). DiGeorge syndrome is believed to be the result of developmental defects involving the third and fourth pharyngeal arches (Lammerand Opitz, 1986), because the affected organs are developmentally originated from this region. Pharyngeal arches are transient bulges of the embryonic oropharyngeal region, giving rise to specific sets of adult tissue derivatives. The transcription factors expressed in the pharyngeal arch play critical roles in the development of the pharyngeal arch. Tbx1, one of the genes included in the del22q11 locus in human, appears to be one of the earliest genes involved in pharyngeal arch development (Piotrowski *et al.,* 2000). In humans, Tbx1 is implicated in the etiology of the DiGeorge syndrome. In mouse, Tbx1 is required for segmentation of the embryonic pharynx, formation of the caudal pharyngeal arches, arch arteries, growth, alignment and septation of the outflow tract (Vitelli *et al.,* 2002). Recently, mutational analyses have found that Tbx1 mutation in patients with a 'pharyngeal' phenotype is essentially identical to that associated with del22q11 (Yagi *et al.,* 2003). Homozygous null mutation of Tbx1 in mouse and zebrafish displayed the common DGS features, characterized by defects in the pharyngeal arches and associated structures (Vitelli *et al.,* 2002; Merscher *et al.,* 2001; Piotrowski *et al.,* 2003). As a result of all these studies, Tbx1 currently represents the most promising candidate gene for DGS/VCFS.

However, knowledge remains limited concerning the upstream regulation of Tbx1 during pharyngeal arch development. Previous studies have shown that sonic hedgehog (Shh) can regulate Tbx1 expression during pharyngeal arch development (Garg *et al.,* 2001; Wendling *et al.,* 2000), but there remain other potential candidates regulating Tbx1.

The formation of pharyngeal arches is critically dependent on

Abbreviations used in this paper: amhc, atrial myosin heavy chain; DGS/ VCFS, DiGeorge/velocardiofacial; FGF, fibroblast growth factor hpf, hours post fertilization; PA, pharyngeal arch; RA, retinoic acid; RTQ-PCR, real-time quantitative PCR; Shh, sonic hedgehog; vmhc, ventricular myosin heavy chain.

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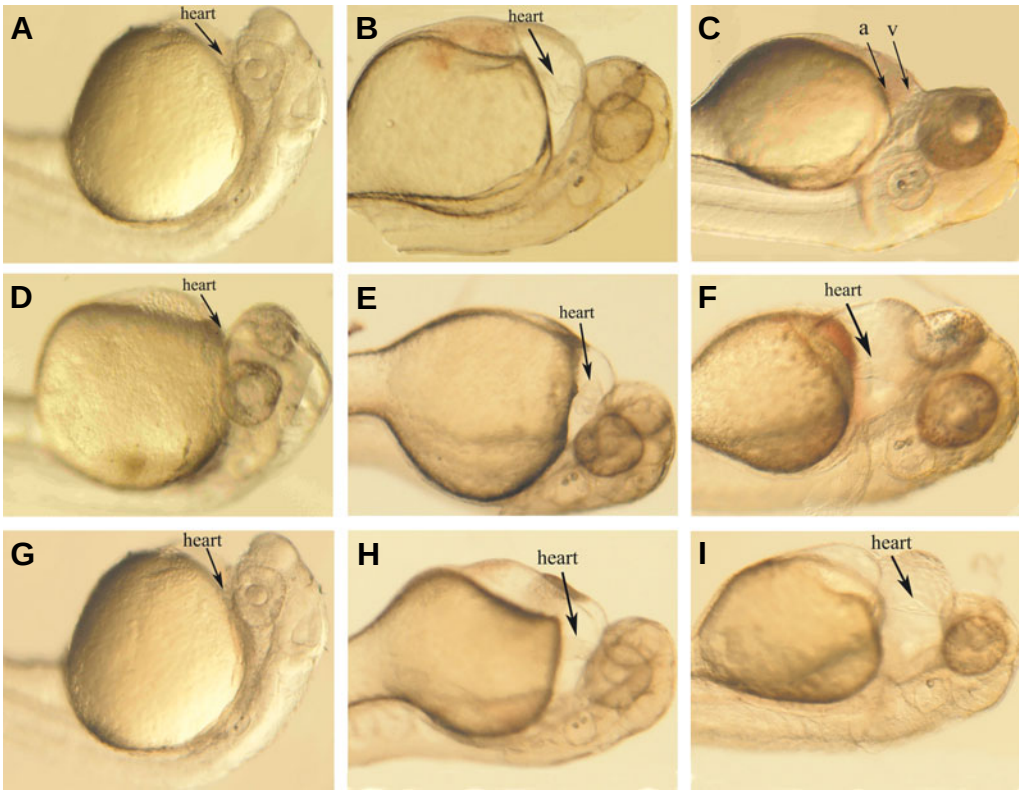


Fig. 1. RA causes ventricle and outflow tract deletion in zebrafish embryos. (A,B,C) *Lateral views of normal embryo at 24 hpf, 48 hpf and 72 hpf. From 24 hpf, the ventricle begins to turn to the right. At 72 hpf, the heart tube completes the D-loop; the ventricle lies to the ventral and right of the atrium.* **(D,E,F)** *Embryos were treated at 12.5 hpf with 5x10^{-8}mol/L RA for 1.5 h. (D) At 24 hpf, no obvious differences are apparent. (E) At 48 hpf, an incomplete D-loop and a reduced heart can be seen. (F) At 72 hpf, the heart is progressively deleted and circulation is slow.* **(G,H,I)** *Embryos were treated at 12.5 hpf with 10^{-7}mol/L RA for 1.5 h. Compared with 5x10^{-8}mol/L RA treatment, the heart is obviously smaller, with no D-loop and no circulation. The arrow points to the heart tube. Ventricle (V), atrium (A) and sinus venous (SV).*

retinoic acid (RA)(Mark *et al.,* 2004). RA, the active derivative of vitamin A, transduced by nuclear receptors, is involved in various developmental and homeostatic processes in the vertebrate. Its tissue distribution results from the balancing activities of RA-synthesizing enzymes RALDH (retinaldehyde dehydrogenases) and cyp26 (RA-catabolizing cytochrome P450 hydroxylases)(Duester, 2000; Reijntjes *et al.,* 2004). It is reported that human fetuses exposed to retinoids during gestation can phenotype DGS (Rosa *et al.,* 1986). Defects caused by excessive or insufficient RA in mouse recapitulate the typical phenotypes seen in human DGS/VCFS syndrome (Mendelssohn *et al.,* 1994; Ghyselinck *et al.,* 1997; Mark *et al.,* 1998; Niedereither *et al.,* 2003). Likewise, RALDH2 knockout and experimental manipulation of retinoid receptor in zebrafish also produce phenotypes of DGS (Begemann *et al.,* 2001; Lohnes *et al.,* 1994). These characteristic defects are all attributable to the loss of posterior pharyngeal arches just like DGS patients (Mulder *et al.,* 2000). These results all strongly demonstrated that RA signaling is definitively indispensable for the development of the pharyngeal arch.

Whether Tbx1 expression can be altered following RA treatment during pharyngeal arch development, disrupting caudal pharyngeal arches development, is a pivotal open question, although it has been rarely explored in the literature. The zebrafish, Danio rerio, as a major new genetic and embryological model system, offers numerous advantages for studying cardiac development, including the external fertilization, rapid development and optical clarity of its embryos (Thisse and Zon 2003; Fishman, 2001). In this paper, the effect of RA on the temporal-spatial expression pattern of Tbx1 was studied by RTQ-PCR and whole-mount *in situ* hybridization. Exploring the regulatory interactions between RA and Tbx1 will contribute to the understanding of the molecular pathways disrupted in DGS/ VCFS and also essential for understanding the genetic basis for the congenital heart disease involving the outflow tract.

Results

Aberrant phenotype of RA treated embryos with concentrations of 5x10^{-8}mol/L and 10^{-7}mol/L at 12.5 hpf

Zebrafish embryos were treated with 5x10-8mol/L and 10-7mol/L RA for 1.5h, starting at 12.5 hpf. Phenotypes of these embryos were analyzed and, no detectable effect on the overall body plan was found. However these normal looking embryos, as the animals develop, exhibited selective defects of the heart tube. 10-7mol/L RA had more pronounced effects on the development of heart tube than did 5x10-8mol/L (Fig. 1). The results *in situ* hybridization with vmhc and amhc showed that the heart is reduced in the ventricle and the outflow tract and enlarged in the atrium. In addition, circulation is inefficient or interrupted (Fig. 2).

Time course analysis of zebrafish tbx1 expression by real-time quantitative RT-PCR

The time course analysis of zebrafish tbx1 expression was performed with cDNA from embryos at different stages: 3–5 hpf, 12 hpf, 18 hpf, 24 hpf, 28 hpf, 36 hpf, 48 hpf, 60 hpf and 72 hpf. The results showed that the Tbx1 expression level is low at 3-5 hpf; the copy number is 7.64x10^3 molecules/μl. The Tbx1 expression remained at a low level prior to 18 hpf, with the copy number at 5.08x10^5 molecules/μl. From 18 hpf, levels were increased markedly, reaching high levels at 24 hpf and 28 hpf. The copy number rose to 1.62x10^7 and 2.58x10^7 molecules/μl at 24 hpf and 28 hpf

respectively, a nearly 50-fold increase compared to 18 hpf. The expression of Tbx1 was significantly increased at 24 hpf when compared to 18 hpf($p = 0.0053$). The value kept rising and reached a peak level at 36 hpf, with 1.26×10^8 molecules/μl, a nearly fourfold increase compared to 28 hpf ($p=0.0037$). From then the level began to drop slowly. The level drop to 11.6×10^7 molecules/μl and no significant difference was found between 48 hpf ($p = 0.037$) and 60 hpf. The copy number was 2.54×10^6 molecules/μl at 72 hpf, a prominent, nearly 10-fold decline relative to 60 hpf ($p=0.0025$) (Fig. 2).

Expression pattern of zebrafish Tbx1 *by whole-mount* in situ *hybridization*

To determine the expression pattern of Tbx1 in zebrafish, whole-mount *in situ* hybridization was performed. At 24 hpf, Tbx1 expression in the pharyngeal arch is faint, restricted to only part of the arches (Fig. 3A). At 36 hpf, the expressions in the pharyngeal arches become sharp, while the expression in the cardiac region is invisible (Fig. 3B). At 48 hpf, tbx1 begins to be upregulated in the outflow tract, bilaterally symmetric expression in pharyngeal arches spreads to most of arches and this persists throughout 72 hpf (Fig. 3 C,D). Subsequently, a few hours later (54h), when D-loop occurs, tbx1 expression can be seen not only in the outflow tract, but also in the ventricle and the atrium (Fig. 3 E,F). At 60 hpf, Tbx1 expression in the outflow tract is persistent and intense, as is the expression in the ventricle, atrium and sinus venous. In addition, at this stage, D-loop heart can be seen clearly (Fig. 3 G,H). Transcripts persist in these segments of the heart as looping is completed at 72 hpf, although, by that stage, cardiac expression is stronger in the outflow tract than elsewhere. Tbx1 expression in the otic vesicle begins to be up regulated from 48 hpf; tbx1 transcripts are dynamic in all three semicircular canals by 72 hpf (Fig. 3I).

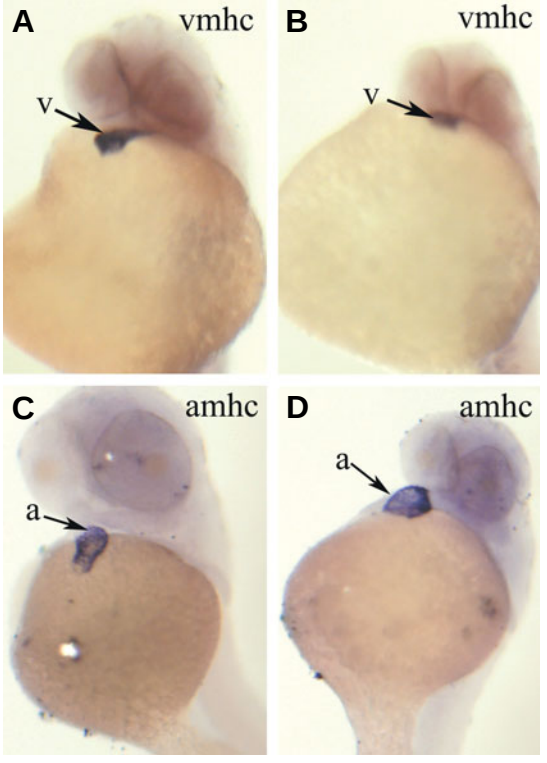


Fig. 2. Whole mount *in situ* hybridization showing *vmhc* and *amhc* expression in RA treated groups (B,D) and controls (A,C) at 48 hpf. *Zebrafish embryos were treated at 12.5 hpf with 10^{-7}mol/L RA and compared with untreated embryos (A,C) at same stages.* **(A,B)** *Lateral view at 48 hpf, anterior to the left. The* vmhc *expressing areas were reduced in treated embryos when compared to controls.* **(C,D)** *The* amhc *expressing areas were enlarged in treated embryos when compared to controls.*

Exogenous RA down-regulates Tbx1 *expression in zebrafish*

It is shown that the Tbx1 expression in the pharyngeal arches was down regulated in RA treated embryos. Tbx1 expression at 48 and 60 hpf is severely down regulated by whole-mount *in situ* hybridization in RA treated embryos. At 48 hpf, the expression difference in the pharyngeal arches become evident between RA treated groups and the controls. With the concentration of 5×10^{-8}mol/L RA, we observed that there is no distinct difference in the first and second pharyngeal arches between the treated and control embryos. Tbx1 expression was detected in the treated posterior pharyngeal arch regions; however, the expression was obviously reduced and not as sharp as in the controls. Increasing the concentration to 10^{-7}mol/L RA, the expression in both the anterior and posterior pharyngeal arches become faint, nearly lost in most of arches (Fig. 4 A,B,C). Likewise, the expression was also markedly decreased or lost in the outflow tract and ventricle in comparison to the control embryos. The reduced expression may be due to tissue deletion in the outflow tract and ventricle (Fig. 4 D,E,F).

To quantify the alteration of gene expression levels, real-time RT-PCR was conducted. It is shown that the levels of Tbx1 in RA treated group at 24 hpf and 28 hpf were only mildly lower than those in control embryos. However, the expression levels were significantly lower in RA treated groups at 36 hpf, 48 hpf and 60 hpf. The values were very similar in the treated groups at this three time points. At 36 hpf, the concentration of 5×10^{-8}mol/L RA repressed the Tbx1 level to 1.34×10^7 molecules/μl, nearly one fifth of the DMSO groups. The expression of Tbx1 was significantly decreased ($p=0.0078$) in 5×10^{-8}mol/L RA treated groups and 10^{-7}mol/

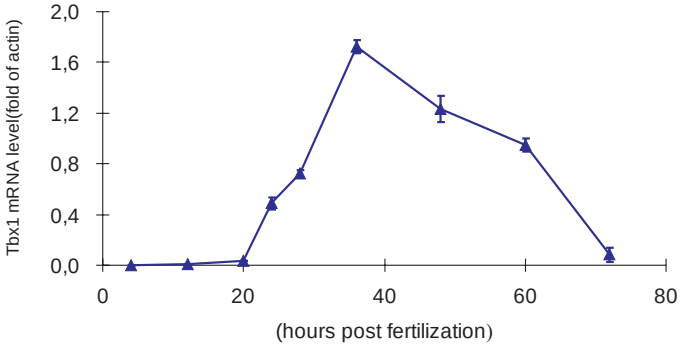
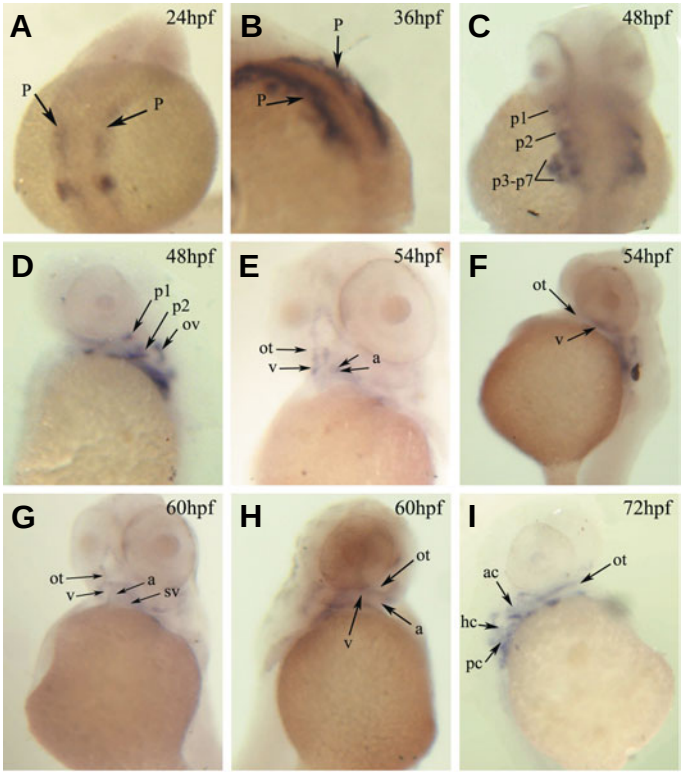


Fig. 3. RTQ-PCR analysis of *Tbx1* expression in Zebrafish at different stages. *The amount of* Tbx1 *transcripts is displayed as a relative value obtained by dividing the value for* Tbx1 *by the value for* β-actin *transcripts. Real-time quantitative PCR data were obtained from triplicate transwells and are representative of 3 independent experiments. Tht* Tbx1 *temporal expression trend changed rapidly with development procession.* Tbx1 *expression was low at 3-18 hpf. Beginning at 18 hpf, the* Tbx1 *expression level gradually upregulated and at 36 hpf, it reached its peak level, before falling off.*



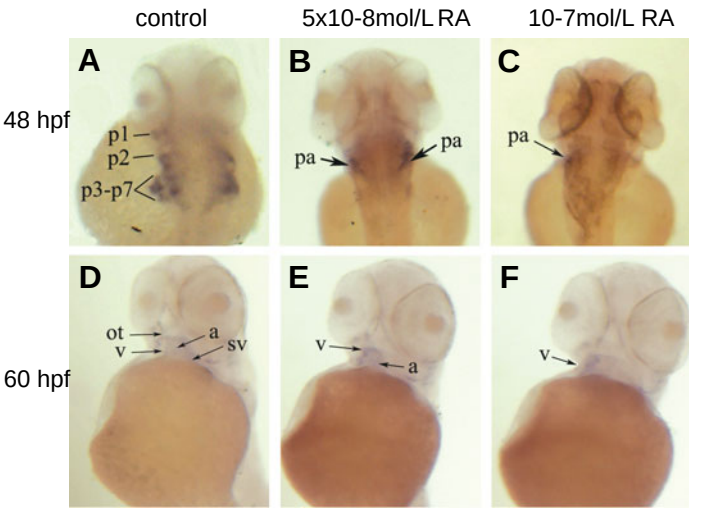


Fig. 4 (Left column). Whole mount RNA *in situ* hybridization showing *Tbx1* expression in wild type zebrafish embryos. (A) *Dorsal view at 24 hpf, anterior to the top.* Tbx1 *expression in the pharyngeal arch (p) is faint.* **(B)** *Dorsal view at 36 hpf, anterior to the left. From now,* Tbx1 *expression in the pharyngeal arch becomes intense.* **(C)** *Dorsal view at 48 hpf, anterior to the top. Transcripts are noted in the heart, the pharyngeal arch precursors (p) and the otic vesicle (ov). The heart and otic vehicle are out of the plane of focus. Expression has regionalized most of the pharyngeal arches and was strong.* **(D)** *Dorsal view at 48 hpf, anterior to the left. Expression in the otic vesicle can be seen clearly.* **(E,F)** *(E) is in ventral view and (F) is in left lateral view at 54 hpf. Expression can be clearly seen in the outflow tract (ot), ventricle (v) and the atrium (a). Pharyngeal arch and otic vesicle expression are out of the plane of focus.* **(G,H)** *(G) is in ventral view and (H) is in left lateral view at 60 hpf.* Tbx1 *expression in the outflow tract is persistently intense and expression in the ventricle, atrium and sinus venous (sv) is intense. The D-loop heart can be seen clearly.* **(I)** *Left lateral view at 72 hpf. Expression can be seen in all three semicircular canals (ac, anterior canal; hc, horizontal canal; pc, posterior canal).*

Fig. 5 (Right column). Decrease or loss of *Tbx1* expression following RA treatment. *Embryos were exposed for 1.5 h to 5x10^{-8}mol/L RA and 10^{-7}mol/L RA in 0.5% DMSO at 12.5 hpf, hybridization at 48 hpf (B,C) and 60 hpf (E,F). Controls at 48 hpf and 60 hpf(A, D) were incubated in 0.5% DMSO for the same periods of time. (A-C) Dorsal views at 48 hpf, anterior to the top.* **(A)** Tbx1 *expression in a control embryo has expanded to most of the pharyngeal arches.* **(B)** *Addition of 5x10^{-8}mol/L RA;* Tbx1 *expression in the anterior arches is strong, whereas expression in the posterior pharyngeal arches is significantly decreased.* **(C)** *Addition of 10^{-7}mol/L RA; expression in both the posterior and anterior arches is severely reduced or lost.* **(D)** Tbx1 *expression can be clearly seen in the ventricle, atrium, outflow tract and sinus venous at 60 hpf in controls.* **(E,F)** *Lateral view at 60 hpf, anterior to the left (right). Expression was markedly decreased or lost in the outflow tract and ventricle in comparison to control embryos. Reduced expression in the outflow tract and ventricle may be due to tissue deletion.*

L RA treated groups (p=0.0024) at 36 hpf when compared to DMSO controls. Concentration of 10^{-7}mol/L RA repressed Tbx1 levels further, a 8-fold decease compared with DMSO groups at 36 hpf. A Significant difference in Tbx1 levels was found at 72 hpf between 10^{-7}mol/L RA treated groups and DMSO controls (p=0.0038), but there was no obvious difference between 5x10^{-8}mol/L RA treated groups and DMSO controls at 72 hpf (p=0.0472)(Fig. 5). 10^{-7} mol/L RA causes a more severe effect on Tbx1 expression level than 5x10^{-8}mol/L RA. Copy numbers for embryo medium and DMSO treatment group are also calculated in parallel, showing that DMSO alone has no effect on Tbx1 expression.

Discussion

It is reported that RA is necessary for the initial formation of the pharyngeal arches and plays an indispensable roles in the specification of the endoderm. The retinoid-dependent signaling is regulated in a tissue-specific manner (Grandel *et al.,* 2002). The sensitivity of genes and tissues to retinoic acid correlates precisely with the time during which RA is synthesized (Grandel *et al.,* 2002). The previous study has shown that the critical time period in which RA signaling can cause endodermal fate transformation is near the end of gastrulation in zebrafish (Grandel *et al.,* 2002). Our results also showed that the embryos treated with RA during late gastrulation or early segmentation exhibit selective defects of abnormal ventricle and outflow tract developmentally derived from 3rd and 4th pharyngeal arches. These results indicated that RA plays an important role in the formation of the pharyngeal arches during a narrow developmental window corresponding to late gastrulation or early segmentation in zebrafish embryos.

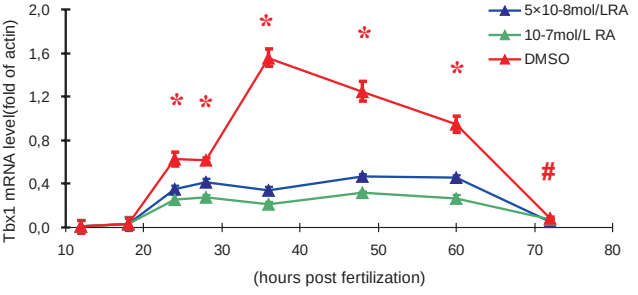
The defects induced by hyper- and hypo-RA exhibited a limited set of features that phenotype the human DGS syndrome (Begemann *et al.,* 2001; Walsh *et al.,* 2001; Lohnes *et al.,* 1994). Retinoic acid signaling controls the expression of numerous transcription factors in the pharyngeal arches. Disruption of retinoic acid signaling can cause abnormal gene expression in the pharyngeal endoderm and secondarily affects migration of neural

crest cells to the pharyngeal arches in mouse. Hoxa1 and Hoxb1 are RA target genes and are crucial for normal patterning of the pharyngeal endoderm (Marshall *et al.,* 1994; Huang *et al.,* 1998). Previous studies have shown that RA can regulate the expression of Hoxa1 and Hoxb1 during the specification of the pharyngeal endoderm. Pax1, Pax9, Fgf8 and Fgf3 expression patterns are all dramatically altered in panRAR (blocking the activity of all three RARs) antagonist treated mouse embryos (Wendling *et al.,* 2000). Tbx1 appears to be one of the earliest genes involved in this process as disruption of its function affects the segmentation of the endodermal pouches (Piotrowski and Nüsslein-Volhard, 2000), which is one of the earliest events in arch morphogenesis. Tbx1 is expressed in the pharyngeal endoderm and acts autonomously in the pharyngeal arch endoderm (Vitelli *et al.,* 2002). Our results show that Tbx1 expression levels mount to a peak value during development from 24 hpf to 48 hpf. In addition, the whole-mount *in situ* hybridization reveals that Tbx1 is expressed in the pharyngeal arches, pharyngeal pouches, cardiovascular structure and otic vesicle during early developmental stages. Tbx1 expression pattern is closely consistent with morphogenetic movements of the cardiac primordia and the pharyngeal arch development in zebrafish. The expression pattern also suggests that tbx1 may play critical roles in cardiovascular development. Over expression and haploinsufficieny of Tbx1 also produce similar DGS-like defects to those caused by RA treated models. The strong phenotypic reminiscence between RA treated DGS-like syndrome and Tbx1-/- mutants prompted us to investigate the effects of RA on the expression pattern of Tbx1.

Previous studies have shown that pharyngeal endoderm in RALDH2hypo mutant mouse displays a decreased expression of Tbx1. Similarly, perturbation of RA levels downregulate Tbx1 expression in the avian embryo (Roberts *et al.,* 2005). We performed a detailed analysis of the effects of RA on the expression pattern of Tbx1 during pharyngeal arch development. Our results demonstrate that the expression levels of Tbx1 were predominantly downregulated during the periods between 36 hpf and 60 hpf with administration of RA. Our results also suggest that exogenous RA can alter the spatial expression pattern of Tbx1 in zebrafish. Therefore, we speculate that there is a genetic link between RA signaling and Tbx1 during pharyngeal arch development in zebrafish. RA may regulate the expression of Tbx1 in a direct or indirect way. With administration of RA at 12.5 hpf, the repression of Tbx1 expression become apparent at 36 hpf, which raises the possibility that RA may not lead to the alteration of Tbx1 in a direct and specific way. A possible indirect mechanism might involve RA acting to alter the levels of other transcription factors, which secondarily alter the expression of Tbx1. The effect of retinoic acid signaling is mediated not only by the regulation of transcription factors, but also by Shh, which is expressed in discrete layers of the pharyngeal arches (Wendling *et al.,* 2000). Other results also suggested that Shh could regulate Tbx1 expression during pharyngeal arch development. Fgf8 has been shown to interact with Tbx1 genetically (Funke *et al.,* 2001; Vitelli *et al.,* 2002). Therefore, we speculate that RA may affect Tbx1 expression, through Shh during pharyngeal arch development. Roberts also proposed a direct RAR-mediated (retinoic acid receptor, RAR) effect upon Tbx1 control sequences or action through a direct effect upon other transcription regulators for Tbx1 (Roberts *et al.,* 2005). However, many questions remain concerning the mechanism by which Tbx1 controls PA development and the topic needs further investigation.

It is shown that addition of $5x10^{-8}$mol/L RA clearly repressed Tbx1 expression in the caudal PA, but the expression in the anterior arches is unaffected. 10^{-7} mol/L RA can repress Tbx1 expression both in caudal PAs and in anterior ones. RTQ-PCR results show that 10^{-7} mol/L RA cause a more severe effect on Tbx1 expression levels than $5x10^{-8}$mol/L RA. Analysis of the effect of different concentrations of RA on Tbx1 expression showed that RA regulated Tbx1 in a dose-dependant manner; in other words, the larger the dose of RA, the greater was the repression of Tbx1. The spatial alteration of Tbx1 mainly exists in the caudal pharyngeal arches not in the anterior arches, suggesting that the caudal pharyngeal arches are more sensitive to RA than the anterior ones. Therefore, we speculated that the formation of anterior pharyngeal arches may rely on RA receptors and RA controlled cellular and molecular mechanisms distinct from those involved in caudal arches

Despite the frequent occurrence of 22q11.2 deletions in DGS patients, DGS has long been recognized as an etiologically heterogeneous syndrome, which may involve other genetic loci, as well as epigenetic or environmental causes. The variability of the phenotype argues that epigenetic factors influence developmental events to determine the ultimate expression of the genetic abnormality (Vincent *et al.,* 1999). The supplements of more than 10,000 IU VitA daily during pregnancy closely associated with an increased risk of TGA (transverse grand artery, TGA) (Botto *et al.,*



Tbx1 expression level decreased by RA treatment in QRT-PCR

Fig. 6. Altered *Tbx1* expression in RA treated embryos. *Administration of $5x10^{-8}$mol/L RA and 10^{-7}mol/L RA at 12.5 hpf;* Tbx1 *expression was detected by RTQ-PCR in DMSO group and RA treated embryos. (See Materials and Methods for details of technique and data processing.) Real-time quantitative PCR data were obtained from triplicate transwells and are representative of 3 independent experiments. Values are the mean ± SE of three separate quantitative PCR (n = 3). The amount of* Tbx1 *transcripts is displayed as a relative value obtained by dividing the value for* Tbx1 *by the value for* β-actin *transcripts.* Tbx1 *expression levels at various points are analyzed as shown in Fig. 3. The temporal expression pattern for* Tbx1 *was considerably altered due to RA treatment. RA treated embryos exhibit down-regulation of* Tbx1 *expression at different developmental stages. A concentration of 10^{-7} mol/L RA repressed* Tbx1 *levels further compared with $5x10^{-8}$mol/L RA. * indicates that the expression of* Tbx1 *was significantly decreased ($p< 0.01$) in the RA treated group (including 10^{-7} mol/L RA and $5x10^{-8}$mol/L RA) when compared to controls. # indicates a significant difference in* Tbx1 *levels only between the 10^{-7} mol/L RA treated group and controls, but not between the $5x10^{-8}$mol/L RA treated group and controls.*

2001). Our data suggests that exogenous RA can produce an altered Tbx1 expression pattern; secondarily disrupt pharyngeal arch development. Therefore, the biologically available levels of RA must be precisely regulated in order to signal appropriately through retinoid receptors.

Materials and Methods

Zebrafish strains and maintenance

Wild-type (AB* strain) zebrafish stocks were obtained from the International Zebrafish Research Center. Embryos were obtained from natural spawning of wild-type adults. Zebrafish were raised and maintained under standard laboratory conditions at 28.5°C (Westerfield, 1995).In addition, morphological features were used to determine the stage of embryos, as described by Kimmel *et al.* (Kimmel *et al.,* 1995). The embryos older than 24 hours post fertilization (hpf) were incubated in 0.003% phenylthiourea to inhibit pigment formation.

RA treatment

All trans-RA was purchased from Sigma and a 10-2mol/L stock solution was made in dimethyl sulfoxide (DMSO). For treatment of embryos, the stock solution was dissolved in embryo medium at a final concentration of $5x10^{-8}$mol/L and 10^{-7}mol/L RA. The embryos were transferred to the RA solution for a period of 1.5h starting at 12.5 hpf. After treatment, embryos were washed extensively with tank water. The controls were immersed in 0.5%DMSO and embryo medium for the same periods.

Whole-mount in situ *hybridization*

Whole-mount *in situ* hybridization was performed essentially as described using digoxigenin antisense riboprobes constructed from linearized plasmids (Alexander *et al.,* 1998). Plasmids encoding zebrafish ventricular myosin heavy chain (vmhc) and atrial myosin heavy chain (amhc) were kindly provided by Tao Zhong (Vanderbilt University, The tbx1 RNA probe was synthesized as previously described (KOCHILAS L.K, 2003). The sense riboprobe was used as control. At various time points, following RA treatment, embryos were fixed in 4% paraformaldehyde/PBS at 4°C for 12-16 h, underwent serial dehydration through graded methanol solutions and stored at –20°C for future use. Embryos were treated with Proteinase K (10-25 μg/ml) for 5–20 min at room temperature depending on their developmental stages. Prehybridization was performed at 65°C for 2-5 h in prehybridization buffer containing 50% formamide, 5xSSC, 500 μg/ml yeast tRNA, citric acid, 0.1%Tween20 and 50 μg/ml heparin. Hybridization was performed in the same solution at 65°C over- night. After a series of washes, embryos were treated in blocking solution for 1-3 h and incubated with anti-digoxigenin antibody (Roche) over night at 4°C. NBT/BCIP color reaction was performed at room temperature. The control and experimental embryos were conducted in parallel.

RNA extraction and first strand cDNA synthesis

Total RNA was extracted from wild type and RA treated zebrafish embryos from different stages using the Trizol reagent (Invitrogen Technologies) and following the protocol described by the manufacturer. The final RNA pellets were resolved in 20 - 40 μl diethylpyrocarbonate-treated water. The purity of the RNA was high in all cases (A260/280 was between 1.8-2.0). The integrity of the RNA was checked by electrophoresis on a denaturing gel (agarose-formaldehyde-ethidium bromide) using 10 μg RNA per well and an rRNA standard (Sigma, Poole, Dorset, U.K.). No visible degradation was observed in any samples. Contaminating DNA was removed with DNase-1 (DNA-free, Ambion, Austin, TX) (4 units DNase/μg RNA) using the protocol described by the manufacturer. The RNA samples were diluted to 50 ng/μl and A260 was checked several times to ensure that the RNA concentrations of control and test samples were within 10% of each other. First-strand cDNA was reverse transcribed using oligo-dT primers and MMLV reverse transcriptase (Promega Technologies). The reverse transcriptase condition was comprised of 25°C 10 min, then 60 min at 37°C and 5 min at 95°C. The final volume was adjusted to 100 μl with RNase-free water. The cDNA was analyzed immediately or stored at -20°C for further use.

Real-time PCR

Real-time quantitative PCR was done using the ABI Prism 7000 Sequence Detection System (Perkin-Elmer Applied Biosystem, Inc). The sequence-specific primers were designed using Primer Express 2.0 software (Applied Biosystems, USA). Tbx1 forward: CCC TAT CCC TCA CCC AGC AT and Tbx1 reverse: TCG TCC GTC AGA AGC CAC TA. β-actin forward: GCC TGA CGG TCA GGT CAT CAC CAT CGG and β-actin reverse: CGC ACT TCA TGA TGG AGT TGA AGG TGG. Real-time PCR reactions were set up in duplicates for Tbx1 and β-actin at each time point. Amplification conditions were identical for all reactions and consisted of: 95°C 3min, then 45s at 95°C, annealing for 45s at 56°C and extension for 45s at by 40 cycles. PCR products were electrophoresed in 1.5% NA agarose gels (Amersham Pharmacia). Specific products were identified after ethidium-bromide staining under ultraviolet light, excised from the gel and extracted with QiaexII agarose gel extraction kit (Qiagen, Germantown, MD). The SYBR green method was used to quantify cDNA. Copy number in molecules per microliter was calculated using the equation (nanograms DNA $x10^{-9}$)/(length in base-pairs 660) x(6.022 $x10^{23}$). Copy number was diluted to 1 $x10^{11}$ molecules/μl and Tbx1 and β-actin PCR was performed as above, in duplicate on at least five serial dilutions for the Tbx1 and β-actin standard template, respectively, from 1 $x10^{8}$ to 10^{4} molecules/μl. Real-time PCR assays were done in triplicate in each run and the mean values were used for calculations of mRNA expression. β-actin was used as control to confirm similar amount of starting cDNA from all stages tested. The relative expression level of Tbx1 was computed with respect to the amount of β-actin in each sample, by dividing the amount of Tbx1 by the amount of β-actin to obtain a normalized target value. No-RT controls and water controls gave similar high threshold cycle values, demonstrating that contamination contributed to less than 0.1% of quantified product. Whole experiments were repeated three times starting from new and independent embryo collections. Figures thus show each value as the mean ± SE of three separate quantitative PCR (n = 3). Specificity of each reaction was controlled by melting curve analysis, which began at 50°C and increased to 95°C in 1°C increments.

Statistical Analysis

Statistical Analysis was performed on raw data using one-way analysis of variance (ANOVA). Significance was set at $P<0.01$.

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