Biochemical and Biophysical Research Communications 470 (2016) 575–578

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Vitamin D receptor signaling is required for heart development in zebrafish embryo

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ARTICLE INFO

Article history:
Received 11 January 2016
Accepted 16 January 2016
Available online 19 January 2016

Keywords:
Vitamin D
Vitamin D receptor (VDR)
Zebrafish
Heart development

ABSTRACT

Vitamin D has been found to be associated with cardiovascular diseases. However, the role of vitamin D in heart development during embryonic period is largely unknown. Vitamin D induces its genomic effects through its nuclear receptor, the vitamin D receptor (VDR). The present study investigated the role of VDR on heart development by antisense-mediated knockdown approaches in zebrafish model system. In zebrafish embryos, two distinct VDR genes (*vdra* and *vdrb*) have been identified. Knockdown of *vdra* has little effect on heart development, whereas disrupting *vdrb* gene causes various cardiac phenotypes, characterized by pericardial edema, slower heart rate and laterality defects. Depletion of both *vdra* and *vdrb* (*vdra/b*) produce additive, but not synergistic effects. To determine whether atrioventricular (AV) cardiomyocytes are properly organized in these embryos, the expression of *bmp4*, which marks the developing AV boundary at 48 h post-fertilization, was examined. Notably, *vdra/b*-deficient embryos display ectopic expression of *bmp4* towards the ventricle or throughout atrial and ventricular chambers. Taken together, these results suggest that VDR signaling plays an essential role in heart development.

1. Introduction

Traditionally, vitamin D has been a well-known vital nutrient for healthy bones and calcium balance. However, recent researches are revealing that vitamin D is associated with numerous outcomes including not only rickets or osteomalacia but also cancer, immunity disorders, cardiovascular disease, and problems in pregnancy, muscle function and aging [1]. To function in target tissues, 1α,25-dihydroxyvitamin D3 (calcitriol, 1α,25$(OH)_2D_3$), an active metabolite of vitamin D binds to the vitamin D receptor (VDR). The ligand-bound VDRs homodimerize or heterodimerize with many other binding partners such as retinoid X receptors (RXRs) [2]. These can act as transcription factors and regulate the expression of various genes across the genome [3]. Recently, it has become clear that VDRs are present in a wide variety of tissues [4], explaining the potential for numerous biological effects.

There has been an increasing number of reports that vitamin D is related with cardiovascular disorders, and VDRs are found in many cells of the cardiovascular system [5,6]. However, relatively little is known regarding the role of VDR signaling in heart development. A recent study in mice found that VDR subcellular distribution in classic VDR target tissues of embryos is different from that of adults [7], implying that VDR may have unique functions during embryonic development, yet the biological function of the VDR at an early stage of development has not been well-defined *in vivo*.

The zebrafish has been recognized as an excellent model to study heart development [8–10]. Rapid external development and optical transparency of the embryo allows visualization of heart beating and the blood flow within the heart chambers without any special experimental procedures [11]. Furthermore, many zebrafish mutants with strong cardiovascular defects can continue to develop without fully functioning hearts thanks to their ability to oxygenate through passive diffusion [12], permitting the analysis of embryos with severe cardiac defects [8,9,13]. In the present study, the role of VDRs on heart development is investigated using a loss-of-function approach in zebrafish model system. Although mammalian genomes have one VDR gene, the zebrafish genome, due to the teleost-specific whole genome duplication, contains 2 VDR genes (*vdra* and *vdrb*) [14–16]. The data presented here show that knockdown of *vdra* has little effect on heart development, whereas knockdown of *vdrb* results in both functional and structural heart defects. Because many of genes and molecular mechanisms of

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http://dx.doi.org/10.1016/j.bbrc.2016.01.103
0006-291X/

regulatory pathways in heart development are well-conserved between zebrafish and mammals including human [8,9], this study may provide new insights into the function of VDR signaling in vertebrate heart development.

2. Materials and methods

2.1. Zebrafish embryos and developmental conditions

Wild-type control embryos were derived from AB line (Eugene, OR). Embryos were developed in fish water at 28.5 °C under standard conditions, staged according to Kimmel et al. [17]. The Animal Use Protocols (AUPs) used in this work were approved by the Institutional Animal Care and Use Committee (IACUC) of the Texas A&M University. They are in accordance with the National Institutes of Health guide for the care and use of Laboratory animals.

2.2. Morpholino injection

Gene knockdown experiments was conducted as previously described [18]. Morpholino oligomers (MOs) were obtained from Gene Tools and diluted in Danieau solution [19]. To knockdown *vdra*, a translation blocker (5′- AACGGCACTATTTTCCGTAAGCATC-3′) [14] was used. To knockdown *vdrb*, a splice blocker (5′-TCCATCACTAGCAGACGAGGGAAGA-3′) targeted to the intron2-exon3 (I2E3) junction was used. For all *vdrb* knockdown experiments, embryos were coinjected with 5 ng of *p53* MO to prevent non-specific cell death as described [20,21].

2.3. Measurement of heart rate, statistics and whole mount in situ hybridization

The heart rate was calculated by counting the number of contractions over a 1-min period under a dissecting microscope at room temperature. Statistical significance was evaluated using student's *t*-tests. Whole mount *in situ* hybridization was carried out as previously described [22,23].

3. Results

3.1. General effects of vdr knockdown on heart development

To determine whether VDR signaling is involved in zebrafish heart development, antisense morpholino oligomers against *vdra* and *vdrb* were injected to knockdown their functions and cardiac phenotypes were analyzed. Although it has been reported knockdown of *vdra* results in physiological defects such as calcium uptake inhibition in zebrafish embryo [14], the gross morphology of *vdra* MO-injected embryos (morphants) was relatively normal in the present experiment (Fig. 1B), as compared with wild-type control (Fig. 1A). However, *vdrb* morphants displayed prominent pericardial edema by 4 days post-fertilization (dpf), with 100% penetrance (n = 30; Fig. 1C). Double-knockdown experiments were also conducted. Embryos deficient in both *vdra* and *vdrb* showed comparable phenotypes to those of *vdrb* morphants (Fig. 1D).

To test whether the hearts of *vdr* morphants are functionally normal, heartbeats were measured because variations in heartbeat can be related with hidden heart defect [11]. There was no statistically significant difference in heart rates between controls and *vdra* MO-injected embryos (Fig. 2). In contrast, knockdown of *vdrb* causes a markedly affected heart rate (89 ± 8 beats/min, n = 8), a 28% decrease relative to the control (124 ± 2 beats/min, n = 5) at 2 dpf (Fig. 2). Co-injection of *vdra* MO and *vdrb* MO showed a 22% reduction in the cardiac rate (97 ± 6 beats/min, n = 7) compared with control, which was not significantly different from that of *vdrb* morphants. Taken together, these data indicate that loss-of-*vdrb* leads to an alternation in the cardiac function and *vdra* MO-injection did not enhance the deficits seen in *vdrb* morphants.

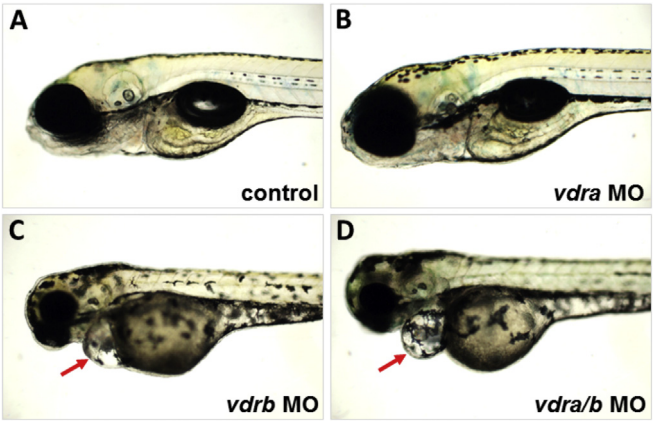

Fig. 1. Effects of VDR knockdown on zebrafish development. Images show lateral views of wild-type control (A), *vdra* morphant (B), *vdrb* morphant (C) and *vdra/b* double morphant zebrafish embryo at 4 days post fertilization (dpf). Red arrows indicate pericardial edema. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

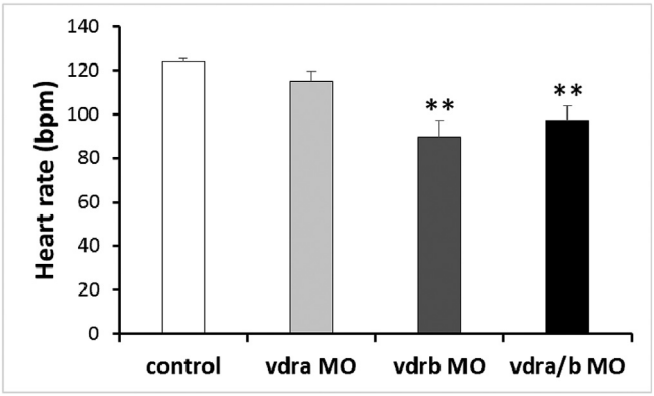

Fig. 2. The effect of VDR knockdown on heartbeat of embryos at 2 dpf. The average beats per minute (bpm) is shown. Data represent the mean ± SEM (n = 5 to 8 per each). Knockdown of vdrb significantly reduced the heart rate of these morphants. $^{**}p < 0.01$, comparison to wild-type control.

3.2. Loss of VDR results in heart laterality defects

During zebrafish development, asymmetrical positioning of primitive linear heart tube (the venous pole located at the anterior left and the arterial pole fixed at the mid-line) is observable by 1 dpf [8]. To more clearly understand the heart defect in *vdr* morphants at the linear heart tube stage, the expression of a cardiac marker *bmp4* was examined by *in situ* hybridization. At 24 h post fertilization (hpf), all of wild-type control embryos had a normal left jogging heart (Fig. 3A). Most of *vdra* morphants showed normal heart jogging, as did wild-type control embryos except that 9% (1 out of 11) of them had a right jogging heart (Fig. 3G). Intriguingly, knockdown of *vdrb* resulted in cardiac laterality defects. 30% of *vdrb* morphants displayed the right jogging heart (Fig. 3G). Injection of *vdra/b* double MOs led to further increase in the percentage of abnormal heart jogging to 45%, which include both right jogging (36%, Fig. 3C,G) and no jogging (9%, Fig. 3D,G). In other words, all three types of heart orientation were appeared in this group