Glycogen synthase kinase 3α and 3β have distinct functions during cardiogenesis of zebrafish embryo

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Abstract

Background: Glycogen synthase kinase 3 (GSK3) encodes a serine/threonine protein kinase, is known to play roles in many biological processes. Two closely related GSK3 isoforms encoded by distinct genes: GSK3α (51 kDa) and GSK3β (47 kDa). In previously studies, most GSK3 inhibitors are not only inhibiting GSK3, but are also affecting many other kinases. In addition, because of highly similarity in amino acid sequence between GSK3α and GSK3β, making it difficult to identify an inhibitor that can be selective against GSK3α or GSK3β. Thus, it is relatively difficult to address the functions of GSK3 isoforms during embryogenesis. At this study, we attempt to specifically inhibit either GSK3α or GSK3β and uncover the isoform-specific roles that GSK3 plays during cardiogenesis.

Results: We blocked gsk3α and gsk3β translations by injection of morpholino antisense oligonucleotides (MO). Both gsk3α- and gsk3β-MO-injected embryos displayed similar morphological defects, with a thin, string-like shaped heart and pericardial edema at 72 hours post-fertilization. However, when detailed analysis of the gsk3α- and gsk3β-MO-induced heart defects, we found that the reduced number of cardiomyocytes in gsk3α morphants during the heart-ring stage was due to apoptosis. On the contrary, gsk3β morphants did not exhibit significant apoptosis in the cardiomyocytes, and the heart developed normally during the heart-ring stage. Later, however, the heart positioning was severely disrupted in gsk3β morphants. bmp4 expression in gsk3β morphants was up-regulated and disrupted the asymmetry pattern in the heart. The cardiac valve defects in gsk3β morphants were similar to those observed in axin1 and apcenc mutants, suggesting that GSK3β might play a role in cardiac valve development through the Wnt/β-catenin pathway. Finally, the phenotypes of gsk3α mutant embryos cannot be rescued by gsk3β mRNA, and vice versa, demonstrating that GSK3α and GSK3β are not functionally redundant.

Conclusion: We conclude that (1) GSK3α, but not GSK3β, is necessary in cardiomyocyte survival; (2) the GSK3β plays important roles in modulating the left-right asymmetry and affecting heart positioning; and (3) GSK3α and GSK3β play distinct roles during zebrafish cardiogenesis.
Background
Glucogen synthase kinase 3 (GSK3) encodes a multifunctional serine/threonine protein kinase, which is ubiquitously expressed in organisms ranging from yeasts to mammals [1–3]. GSK3 is, therefore, very important in the cellular signaling network. In addition to playing pivotal roles in the canonical Wnt and PI3K-PKB/AKT pathways, it has been shown to phosphorylate glucogen synthase, eLF2B, NPTF, c-jun, CyclinD1, NF-kB, as well as many others [4]. GSK3 is involved in many biological processes, including cell survival, tumorigenesis, and developmental patterning.

There are two closely related GSK3 isoforms encoded by distinct genes: GSK3α (51 kDa) and GSK3β (47 kDa) [5]. The difference in size is due to a glycine-rich extension at the N-terminus of GSK3α. GSK3α and GSK3β are highly homologous within their kinase domains [6]. Homologues of GSK3 isoforms from species as distant from each other as flies, zebrafishes and humans display over 90% sequence similarity within the kinase domain [7,8].

Despite that GSK3α and GSK3β share common substrates, their expression patterns, substrate preferences, regulation, and cellular functions are not identical [1,6,9,10]. In vitro study reveals that GSK3α and GSK3β are inactivated by phosphorylation of a specific N-terminal serine residue (Ser-21 in GSK3α; Ser-9 in GSK3β) catalyzed by either MAPKAP kinase-1/or p70S6k [11,12], whereas protein kinase C phosphorylates and partially inhibits GSK3β, but not GSK3α [13]. In humans, only GSK3α is deactivated by insulin during physiological conditions [14,15], whereas supraphysiological insulin injection in the rat leads to deactivation of both GSK3α and GSK3β [15,16]. Although differential regulations by the two isoforms of GSK3 were proposed, the exact roles of GSK3α and GSK3β and endogenous targets of such regulation remain to be investigated.

Several groups have identified small-molecule GSK3 inhibitors [17,18]. Most drugs bind to the ATP pocket of GSK3 and compete with ATP. However, these inhibitors are not only inhibiting GSK3, but are also affecting CDK kinase (2 and 5) and many other kinases. In addition, there appears to be only a single amino acid difference (Glu196 in GSK3α, Asp133 in GSK3β), making it difficult to identify an inhibitor that can be selective against GSK3α or GSK3β [19]. This finding is why it is difficult to analyze the exact roles of GSK3α and GSK3β in vitro and in vivo.

Recent years, numerous studies indicate that GSK3 negatively regulates cardiac hypertrophy [20–22]. Despite that GSK3β functions as a negative regulator of cardiac hypertrophy, GSK3 also plays an important role in regulating cardiac development. Transgenic mice over-expressing GSK3β in the heart have impairments of postnatal cardiomyocyte growth and abnormal cardiac contractile function [23]. In Xenopus, injection of gsk3β mRNA in embryos induces expression of Nkx2.5 and Tbx5 [24]. Oral treatment with lithium, a mood-stabilizing drug that is inhibitory for GSK3, in pregnant women showed a higher incidence of congenital heart defects in babies [25–27]. These findings prove that GSK3 might be involved in heart development. Unfortunately, disruption of the gsk3β gene in mice results in embryonic lethality caused by severe liver degeneration [9], and no report is available to demonstrate that cardiac defects are happened in GSK3β mutants. Thus, whether the roles of GSK3α and GSK3β in different species are conserved remain to be investigated. Moreover, the roles of GSK3 in cardiac development are still unclear. Also, whether GSK3α and GSK3β play similar roles in heart development is ambiguous.

We have previously identified two zebrafish homologues related to mammalian GSK3: zebrafish GSK3α and GSK3β [8]. In this report, we have taken advantage of the zebrafish system to address the distinct roles of GSK3α and GSK3β during heart development of zebrafish. Our findings suggest that, in zebrafish, GSK3α, but not GSK3β, is necessary in cardiomyocyte survival; whereas the GSK3β isoform plays important roles during zebrafish cardiogenesis, modulating the left-right asymmetry and affecting heart positioning.

Results
Knockdown of gsk3α and gsk3β display similar heart defects in the embryos
To address the role of GSK3 during zebrafish cardiogenesis, we designed gsk3α- and gsk3β-MO for specifically inhibiting the translation of gsk3α and gsk3β, respectively. When the protein lysate was extracted from gsk3α- and gsk3β-MO-injected embryos at 24 hours postfertilization (hpf), Western blot analysis was performed by using isoform-specific antibodies. Results showed that the protein levels of GSK3α and GSK3β were largely reduced in the protein extracts from gsk3α- and gsk3β-MO morphants, respectively (Fig. 1), suggesting that the MOs we designed in this study were isoform-specific.

Similar morphological defects of the heart were observed in gsk3α- and gsk3β-MO-injected zebrafish embryos at 72 hpf, such as a thin and string-like shape, pericardial edema, and blood pooling (Fig. 2F, G, I, J). These defects occurred initially in some 2 days postfertilization (dpf) morphants, and then were predominantly observed in most 3- and 4-dpf morphants. Although the heart defects were similar between gsk3α- and gsk3β-MO-injected zebrafish embryos, the defects of the gsk3α morphants were more severe than those of the gsk3β morphants (Fig.
Heart defects caused by gsk3α- and gsk3β-MO are induced differently

We investigated whether the MO-induced defects could be rescued by co-injecting synthetic gsk3α or gsk3β-mRNA with its corresponding MOs, and vice versa. Results showed that co-injection of gsk3α-MO with synthetic gsk3α-mRNA could effectively rescue the defects caused by the injection of gsk3α-MO alone (Table 1). Similarly, the gsk3β-MO-induced defects were rescued by injection of gsk3β-mRNA. However, the synthetic gsk3α-mRNA did not rescue the gsk3β-MO-induced phenotype, and vice versa (Table 1). This evidence clearly demonstrates that two isoforms of GSK3 are necessary for heart development, but the function of GSK3α and GSK3β is not redundant, suggesting that GSK3α and GSK3β play specific roles in cardiogenesis during zebrafish development.

We injected either gsk3α- or gsk3β-MO into embryos derived from the transgenic line Tg(cmlc2: gfp), in which the GFP is expressed specifically in heart, resulting in a good material to monitor cardiac development of zebrafish [29]. In the wild-type embryos, the heart precursor cells completed their in situ formation, elongated, and jogged to the left at 24 hpf; started looping at 30 hpf; and completed looping at 48 hpf [34]. However, we observed that heart development was retarded, failing to elongate at 24 hpf (Fig. 3B) and even ceasing at heart-cone stage without further morphogenesis to a heart tube at [30-36] hpf (Figs. 3E, H) in the gsk3α-MO-injected embryos. We observed defective hearts as stretched to a thin and string-like shape at 72 hpf (Fig. 3L). Nevertheless, unlike in gsk3α morphants, elongation of the heart tube in gsk3β morphants at 24 hpf was as normal as in wild-type zebrafish (Fig. 3C), but heart looping was incomplete from 30 to 36 hpf (Figs. 3F, I), resulting in a stretched heart at 72 hpf (Fig. 3M).

In addition, we have designed an experiment for using a standard negative control morpholino (MO) injection: 5'-CCTCTTACCTCAGTTACAATTTATA-3' (Gene Tools, USA). This oligo has no target, no significant biological activity. After 2 and 6 ng of this control MO were injected, no any defects were observed at 24 hpf. The morphology and development of heart appeared normally (see Additional file 1 and Figure 3N). These results reveal that the defects induced by the gsk3α- and gsk3β-MO are specific in this study.

Effect of GSK3 on the number of cardiomyocytes is isoform-specific

Compared to that of wild-type and gsk3β morphants, the GFP signals in cardiomyocytes of gsk3α morphants were greatly reduced (Fig. 3B). To investigate whether the reduced GSK3α level affects the cardiomyocyte number, we used a cardiomyocyte marker, cardiac myosin light chain 2 (cmlc2), to detect cells at heart-field and heart-cone stages. We found that the number of cmlc2-positive cells was greatly reduced in gsk3α morphants at both heart-field and heart-cone stages (Fig. 4B, E), indicating that the cardiomyocyte number was greatly reduced in the gsk3α morphants. These results suggest that the retarded heart development in gsk3α morphants is due to the
The morphological defects in gsk3α and gsk3β morphants. Wild-type embryos (A, E, H), gsk3α (B, C, F, I), and gsk3β (D, G, J) morphants. The 24 hpf gsk3α morphants have mild (C) to severe (B) defects in axis formation. At 72 hpf, both gsk3α and gsk3β morphants displayed pericardial edema (F, G, I, J) and an unlooped, stretched heart (I, J).
Results also clearly demonstrate that GSK3α play distinct roles during cardiogenesis. In head of controls (G) and especially in the head (Fig. 4H) but was limited in the embryos cytes, which results from apoptosis in the head. Apoptosis occurs in the head of gsk3α morphants was due to the reduced number of cardiomyocytes during early cardiogenesis. In contrast, gsk3β morphants displayed normal cmlc2 staining (Fig. 4C, F), indicating that cardiomyocyte number remains unchanged in gsk3β morphants. These results also clearly demonstrate that GSK3α and GSK3β play distinct roles during cardiogenesis.

Table 1: Morphological phenotypes of zebrafish embryos derived from fertilized eggs injected with different materials

| Injected materials | Concentration | Number of embryos surviving among number of injected eggs | Wild-type phenotype | Abnormal Heart Development |
|---------------------|---------------|-----------------------------------------------------------|---------------------|---------------------------|
| gsk3α-MO            | 0.5 ng        | 146/155 (94.2%)                                           | 85 (58.2%)          | 61 (41.8%)                |
| gsk3α-MO            | 1 ng          | 176/191 (92.1%)                                           | 61 (34.6%)          | 115 (65.4%)               |
| gsk3α-MO            | 2 ng          | 212/273 (77.7%)                                           | 25 (11.8%)          | 187 (88.2%)               |
| gsk3β-MO            | 2 ng          | 126/129 (97.7%)                                           | 88 (69.8%)          | 38 (30.2%)                |
| gsk3β-MO            | 4 ng          | 137/144 (95.1%)                                           | 69 (50.4%)          | 68 (49.6%)                |
| gsk3β-MO            | 6 ng          | 280/314 (91.1%)                                           | 36 (12.5%)          | 252 (87.5%)               |
| axin1-MO            | 6 ng          | 151/164 (92.1%)                                           | 27 (17.9%)          | 124 (82.1%)               |
| dsRed mRNA          | 100 pg        | 68/68 (98.8%)                                             | 83 (100%)           | 0 (0%)                    |
| Control MO          | 2 ng          | 105/108 (97.2%)                                           | 101 (96.2%)         | 4 (3.8%)                  |
| Control MO          | 6 ng          | 112/125 (89.6%)                                           | 103 (93.6%)         | 7 (6.4%)                  |
| gsk3α-mRNA, gsk3α-MO | 50 pg + 2 ng  | 59/69 (85.5%)                                             | 18 (30.5%)          | 41 (69.5%)                |
| gsk3α-mRNA, gsk3α-MO | 100 pg + 2 ng | 93/118 (78.8%)                                            | 51 (45.4%)          | 63 (54.6%)                |
| gsk3β-mRNA, gsk3α-MO | 50 pg + 2 ng  | 72/90 (80.0%)                                             | 9 (12.5%)           | 86 (87.5%)                |
| gsk3β-mRNA, gsk3β-MO | 100 pg + 2 ng | 91/134 (67.2%)                                            | 5 (5.5%)            | 86 (94.5%)                |
| gsk3β-mRNA, gsk3β-MO | 50 pg + 6 ng  | 73/77 (94.8%)                                             | 45 (61.6%)          | 28 (38.4%)                |
| gsk3β-mRNA, gsk3β-MO | 100 pg + 6 ng | 128/144 (89.0%)                                           | 96 (75.0%)          | 32 (25%)                  |
| gsk3α-mRNA, gsk3β-MO | 50 pg + 6 ng  | 83/109 (76.1%)                                            | 15 (18.1%)          | 68 (81.9%)                |
| gsk3α-mRNA, gsk3β-MO | 100 pg + 6 ng | 92/127 (72.4%)                                            | 8 (8.7%)            | 84 (91.3%)                |
| dsRed-mRNA, gsk3α-MO | 100 pg + 2 ng | 83/96 (86.5%)                                             | 7 (8.4%)            | 76 (91.6%)                |
| dsRed-mRNA, gsk3β-MO | 100 pg + 6 ng | 75/82 (91.4%)                                             | 13 (17.3%)          | 62 (82.7%)                |

Fertilized eggs were injected at the 1-cell stage, and then gsk3α morphants were observed at 36 to 48 hpf; gsk3β morphants were observed the heart positioning at 24 to 36 hpf. Results are from three independent experiments. dsRed mRNA: served as a negative control.

decreased number of cardiomyocytes during early cardiogenesis. In contrast, gsk3β morphants displayed normal cmlc2 staining (Fig. 4C, F), indicating that cardiomyocyte number remains unchanged in gsk3β morphants. These results also clearly demonstrate that GSK3α and GSK3β play distinct roles during cardiogenesis.

Apoptosis occurs in the head of gsk3α knockdown embryos

The pronounced degeneration in the head of gsk3α morphants at [18-30] hpf were also observed (Fig. 2C). To confirm whether the reduced cardiomyocyte number in gsk3α morphants was due to apoptosis, the embryos were analyzed by Terminal deoxynucleotidyl Transferase Biotin-dUTP Nick End Labeling (TUNEL) assay after MO injection. In wild-type embryos at 20 hpf, apoptosis was low (Fig. 4G). However, in gsk3α morphants at the same stage, apoptosis was pronounced throughout the axis, especially in the head (Fig. 4H) but was limited in the head of controls (G) and gsk3β morphants (I). Moreover, In gsk3α morphants, the GFP signal was very faint at 24 hpf (Fig. 4N). The apoptotic signals were co-localized with the heart-specific GFP signal, indicating that the reduced cardiomyocyte numbers was due to apoptosis in heart (Fig. 4O). Taken together, the heart defects in gsk3α morphants was due to the reduced number of cardiomyocytes, which results from apoptosis in the head.

GSK3β, but not GSK3α, is involved in the cardiac positioning

Although the heart of gsk3β-MO-injected embryos eventually becomes a string-like shape, we found that the cardiomyocyte development was not affected in the gsk3β morphants during early cardiac development, suggesting that GSK3β may play a unique role in cardiac morphogenesis. Whole-mount in situ hybridization of the cmlc2 probe at 36 hpf, outlining cardiac looping, was marked by a rightward bending in the ventricle in wild-type embryos (Fig. 5A). However, no looping was observed in gsk3β morphants (Fig. 5B–D). Upon detailed analysis of the early (jogging) and late (looping) stages of cardiac positioning in the gsk3β morphant heart (Table 2), we found that heart positioning was severely disrupted in gsk3β morphants and that the extent of the defect was proportional to the amount of gsk3β-MO we injected. The majority of gsk3β morphant hearts failed to jog (69.9%; 65/93). Moreover, this defect was frequently accompanied by no looping (45.3%) or L-looping (14.4%) of the heart tube, compared to wild-type, which has correct left-jogging (93.1%; 67/72) and D-looping (92.4%; 122/132). These results indicate that knockdown of GSK3β resulted in a severe disruption of jogging and looping of cardiac positioning. However, we found that the ventricle-specific marker vmhc and the atrium-specific marker amhcc were normally transcribed in gsk3β morphants (Fig. 5E, G vs. 5F, H), suggesting that GSK3β might not affect the cham-
The cardiac defects induced by the knockdown of zebrafish GSK3α and GSK3β. Anti-sense morpholino oligonucleotide (MO), which was designed to specifically inhibit the translation of either gsk3α-(gsk3α-MO) or gsk3β-mRNA (gsk3β-MO), was injected into one-celled stage embryos and the heart morphology was observed at the stage as indicated. The elongation of heart tube was normally developed at 24 hpf in the wild-type (A) and in the gsk3β morphants (C); whereas the heart of gsk3α morphant did not elongate to from a heart-tube (B). The wild-type (D) and gsk3β morphant’s heart (F) developed normally at 30 hpf, but the heart of gsk3α morphant was still retardant development at 30 hpf (E), and even ceased at heart-cone stage at 36 hpf (F). Compared to the wild-type (G), however, the heart positioning was abnormally in the gsk3β morphant at 36 hpf (I, J). Eventually, both gsk3α and gsk3β morphants displayed an unlooped and stretched heart (L, M). The heart morphology of embryos injected with the control MO was also observed at 72 hpf (N). a: atrium; v: ventricle.
The heart defects in gsk3α morphants were due to a reduced cardiomyocyte population size. Dorsal (A-I) and lateral (J-O) views of embryos stained by in situ hybridization (A-F) and TUNEL assay (G-L). Whole mount in situ hybridization staining with cmlc2 at 18 and 23 hpf received that gsk3α-MO causes a repressive influence on cardiomyocyte formation (B, E). The heart defect in gsk3α morphants was due to the reduction of cardiomyocyte population size. However, gsk3β morphants display normal cardiomyocyte formation (C, F) compared to wild-type embryos (A, D). TUNEL labeling was evident throughout the head of gsk3a-MO-injected embryos (H), especially in the head, but was limited in the head of controls (G) and gsk3β morphants (I). Compared to embryos derived from the transgenic line, Tg(cmlc2:EGFP), which has heart-specific GFP (K), we observed that the heart of gsk3α morphant did not elongate to form a heart-tube and the GFP signal was very faint at 24 hpf (N). Panels L and O are the merged images from J and K, and M and N, respectively. The apoptotic signals were co-localized with the heart-specific GFP signal, indicating that the reduced cardiomyocyte numbers was due to apoptosis in heart (O). h: heart.
involved in heart positioning. We also noted that the heart positioning in gsk3α morphants was delayed but that correct jogging (left-jog) and looping (D-loop) were observed at [36-48] hpf, indicating that GSK3α was not involved in heart positioning.

**GSK3β mediates bmp4 and lefty-1 in cardiac positioning and is required for left-right patterning**

Cardiac bmp4 is an integral component involved in the asymmetric signaling pathway and interprets left-right information for the zebrafish embryo heart [35]. The bmp4 transcripts became markedly asymmetric, with far more on the left side than on the right side of the heart at 20 hpf (Fig. 6A, B), just before jogging. This left-predominant asymmetric persists through the stages of jogging (25 hpf, Fig. 6C). However, the pattern of bmp4 expression in gsk3β morphants was symmetrical before jogging (Fig. 6D, E) and ectopic around the heart-tube stage at 25 hpf, thereby disrupting the pattern of left-pre-dominant asymmetry (Fig. 6H, I). Moreover, another asymmetric marker, lefty-1 [36], lost its expression domain in the left side of the midline in gsk3β morphants (Fig. 6C, F). We propose that GSK3β mediates bmp4 and lefty-1 in cardiac positioning.

**GSK3β modulates valve formation and heart position through Wnt/β-catenin signaling**

Many morphological defects of heart were found in the gsk3β-MO-injected zebrafish embryos. Moreover, when we used the valve markers bmp4 and versican to detect the gsk3β-MO-injected embryos at 60–72 hpf, we found that these valve markers were markedly up-regulated in the heart (Fig. 7A–D), suggesting that GSK3β might also be involved in the formation of cardiac valves. Thus, we used a two-photon fluorescence image to directly observe the valve formation of embryos derived from the transgenic zebrafish line Tg(cmlc2:GFP) [28]. The yellow color shown in our nonlinear microscopy image (valves and red blood cells) is corresponding to the image modality taken by the Third-Harmonics-Generation Microscopy. Valves were normally formed in the wild-type embryos (Fig. 7E), but valves of embryos injected with gsk3β-MO were totally absent (Fig. 7F).

Hurlstone et al [37] reported that cardiac valve formation is severely affected in zebrafish APC mutants (apcmcr). Furthermore, when axin1, another key component in the Wnt pathway, is knocked down, either a reduction or absence of heart positioning of the heart tube was frequently observed (see Additional file 2A–D). GSK3 is known to be important in the canonical Wnt pathway, and the defective valves and hearts in gsk3β-MO-injected embryos were identical to those observed in the apcmcr mutants and axin1 morphants, suggesting that GSK3β modulates cardiac development through Wnt/β-catenin signaling.

**Discussion**

GSK3β knockout mice display an embryonic lethal phenotype, indicating that GSK3α does not compensate for the loss of GSK3β [9]. Furthermore, the activity of GSK3α, but not GSK3β, is required for the production of amyloid-β in the brain of patients with Alzheimer’s disease [38]. All these findings indicate that there may be isoform-specific functions of GSK3, although they exhibit similar biochemical and substrate properties in vitro [7]. However, to clearly delineate the biological functions of these two GSK3 isoforms in vivo is difficult and little reported. In this report, we study the roles of GSK3α and GSK3β in the cardiogenesis of zebrafish using a loss-of-function approach. The loss of either GSK3α or GSK3β results in abnormal development of heart. Yet, the role that GSK3 plays in cardiogenesis is in an isoform-specific manner. GSK3α plays a role in the survival of cardiomyocytes, whereas GSK3β plays a role in heart left-right biased positioning, modulated through the Wnt/β-catenin signaling pathway.

**GSK3α and GSK3β play different roles during zebrafish embryogenesis**

Although, knockdown of gsk3α and gsk3β causes similar defective phenotypes, such as an unlooped and stretched heart, pericardial edema, blood pooling. We used gsk3α-MO and gsk3β-MO in the transgenic zebrafish line Tg(cmlc2:GFP), in which GFP is expressed in the myocardium specifically, to modulate and observe, in real-time, the different defective phenotypes. The hearts of gsk3α morphants failed to elongate at 24 hpf. We prove that the heart defects induced by the gsk3α-MO are due to a decreased number of cardiomyocytes. On the other hand, the gsk3β-MO-injected embryos develop normally before the onset of cardiac jogging. Defective heart positioning is observed after 26 hpf. Rescue experiments revealed that GSK 3α and GSK3β do not function redundantly. Taken together, we conclude that each isoform of GSK3 plays its own distinct role during cardiogenesis of zebrafish.

**GSK3α, but not GSK3β, is involved in apoptosis during early embryogenesis**

GSK3 plays an important role in the regulation of apoptosis/cell survival through the activation of caspase3 [39,41,42]. These findings support a role of GSK3β in controlling apoptosis. Many studies reporting the affect of GSK3β on apoptosis have been confirmed by using GSK3 inhibitors, including lithium, the first known inhibitor, and many synthetic ones [43-45]. However, these inhibitors have many effects on cells and are not isoform-specific. Thus, whether GSK3α and GSK3β function redundantly or distinctly on cell survival is still ambiguous. In our study, extensive apoptosis is observed
throughout the head region in the gsk3α morphants. On the other hand, only slight apoptosis is noticed in the gsk3β morphants, suggesting that GSK3α, but not GSK3β, is greatly involved in apoptosis during early embryogenesis. Moreover, embryos that are co-injected with gsk3α-MO and gsk3β mRNA do not show reduced apoptosis, suggesting that GSK3α and GSK3β do not function redundantly in cell survival.

GSK3α-mediated apoptosis may not be implicated in Wnt signaling

The Wnt signaling are involved in cell proliferation and in apoptosis [46-49]. On the other hand, PKB/Akt, a major regulator of GSK3, also triggers a network that regulates cell cycle progression through inactivation of GSK3β [50]. It has been shown that PKB/Akt promotes cell survival in cardiac myocytes [51,52]. In zebrafish, apc<sup>mut</sup> mutant’s hearts are morphologically normal during early cardiogenesis, but they fail to undergo looping morphogenesis [37]. Both apc<sup>mut</sup> and axin1 mutants (mbl) display cardiac defects that are similar to those of gsk3β morphants. However, no information is provided about apoptosis in apc<sup>mut</sup> and mbl mutants. In this report, we find that apoptosis occurs in the head of gsk3α morphants. In addition, the axin1-MO-injected embryos and the mbl mutant of zebrafish have defects of looping morphogenesis in the heart, which are similar to defects occurring in the gsk3β morphants but are unlike defects occurring in the gsk3α morphants (see Additional file 2). Therefore, we know that GSK3α may not mediate apoptosis implicated in Wnt.

Table 2: Knockdown GSK3β levels that disrupt normal cardiac jogging and looping

| gsk3α morphants were analyzed for heart positioning at 36 to 48 hpf; gsk3βmorphants were analyzed for heart positioning at 24 to 36 hpf. |
signaling because apoptotic signals do not increase in 

\textit{axin1} morphants (data not shown). It is worth studying which pathway is implicated in GSK3\(\alpha\)-mediated apoptosis.

\textbf{gsk3\(\beta\) regulates bmp4 during cardiac development through Wnt/\(\beta\)-catenin signaling}

The phenotypes of \textit{apc}\textsubscript{mcr} and \textit{mbl} mutants are similar to our results in that inhibition of GSK3\(\beta\) also causes unlooping heart tube, pericardial edema, and blood pooling [37]. In addition, valve development is totally lost in gsk3\(\beta\) morphants (Fig. 7), which is similar to that of \textit{apc}\textsubscript{mcr} mutants. Ectopic expression of \textit{bmp4} in the heart at 24–72 hpf and ectopic expression of \textit{versican} in the valve at 60–72 hpf are also observed in the \textit{apc}\textsubscript{mcr} mutant and in the gsk3\(\beta\) morphant (Figs. 7A–D). Moreover, the retention of \textit{bmp4} symmetry is associated with disordered jogging, and we observe that \textit{bmp4} retention occurred in the gsk3\(\beta\) morphant. In addition, \textit{bmp4} is downstream of Wnt/\(\beta\)-catenin signaling in several systems [53,54]. Therefore, it is reasonable to conclude that GSK3\(\beta\) might regulate zebrafish cardiac development by means of the canonical Wnt/\(\beta\)-catenin signaling pathway.

\textbf{GSK3\(\beta\) may be involved in the regulation of T-box genes during cardiogenesis}

Our study reveals that knockdown of gsk3\(\beta\) causes a string-like heart. This phenotype is similar to the \textit{heartstrings} mutant, caused by mutation of the \textit{tbx5} [55]. Patients with Holt-Oram syndrome, one of the autosomal dominant human “heart-hand” disorders, are caused by mutations of \textit{tbx5} [56]. Both loss and gain of \textit{tbx5} functions result in an absence of heart looping and an alteration in cardiac-specific genes [57,58]. In our study, we demonstrate that gsk3\(\beta\) morphants appear to have multiple heart defects, such as a non-looping or reversed looping heart, slower heart rate, and no blood circulation (Figs. 3, 4). In addition, after we probe with fin markers, we prove that the pectoral fin of the GSK3\(\beta\) morphant fails to differentiate (see Additional file 3). In chick, Tbx5 and Tbx4 trigger limb initiation through activation of the Wnt/Fgf signaling cascade [59]. Therefore, we propose that GSK3\(\beta\) and Tbx5 might be involved in the same regulatory mechanism during cardiogenesis.

\textbf{Zebrafish is an alternative, promising model animal to study GSK3-specific inhibitors in vivo}

GSK3 is a target of prominent drugs for treating many diseases, including Alzheimer’s disease and diabetes mellitus. Substrate-competitive inhibitors, which compete for the substrate binding site of the kinase, are more likely to be highly specific inhibitors. Several ATP-competitive inhibitors of GSK3 have also been defined [17,18]. However, the development of new drug not only requires the identification of the target, but also requires validation in an \textit{in vivo} system. Recently, Atilla-Gokcumen et al., [60] performed phenotypic experiments in zebrafish embryo which is served as an \textit{in vivo} experiment to analyse the functions of novel GSK3 inhibitor, organometallic reagent (R)-7. In this study, we clearly distinguish the morphological defects in zebrafish GSK3\(\alpha\)- and GSK3\(\beta\)-knockdown embryos. Therefore, these findings will surely provide new criteria for the \textit{in vivo} validation of potential isoform-specific inhibitors of GSK3.

\textbf{Different biological function of GSK3 isoform in species}

In this report, we have defined that GSK3\(\alpha\) and GSK3\(\beta\) play distinct roles during zebrafish cardiogenesis. Moreover, the defective valves and hearts in \textit{gsk3\(\beta\)}-MO-injected embryos were identical to those observed in the \textit{apc}\textsubscript{mcr} mutants and \textit{axin1} morphants, suggesting that GSK3\(\beta\) modulates cardiac development through Wnt/\(\beta\)-catenin signaling. In addition, GSK3 is a critical regulator of Wnt signaling mechanism, several recent studies have shown that the components of the Wnt signaling play an important role in heart development [3]. However, heart defects are not reported in the GSK3\(\beta\)-knockout mice. One of reasons is that mice GSK3\(\alpha\) might function redundantly to GSK3\(\beta\) during the heart development of mice. We also notice that the expression profiles of GSK3\(\beta\) in zebrafish and in \textit{Xenopus} are different: zebrafish gsk3\(\beta\) is weakly detected until 50–60% epiboly, but \textit{Xenopus} gsk3\(\beta\) is expressed strongly and constantly throughout embryogenesis [61,62]. Taken together, although GSK3 isoforms share highly conserved in their functional domain, the biological functions of GSK3 isoforms in different species are not identical.

\textbf{Conclusion}

Our data indicate that GSK3\(\alpha\) and GSK3\(\beta\) play distinct roles during zebrafish embryogenesis. GSK3\(\alpha\), but not GSK3\(\beta\), is necessary in cardiomyocyte survival; GSK3\(\beta\) plays an important role in regulating left-right biased heart positioning during the cardiogenesis in zebrafish. We also demonstrate that the cardiac valve defects observed in gsk3\(\beta\) morphants were similar to those observed in \textit{axin1} and \textit{apc}\textsubscript{mcr} mutants, suggesting that GSK3\(\beta\) modulates valve formation and heart position through Wnt/\(\beta\)-catenin signaling. Finally, the defects of gsk3\(\alpha\) morphant embryos cannot be rescued by gsk3\(\beta\) mRNA, and vice versa, demonstrating that GSK3\(\alpha\) and GSK3\(\beta\) are not functionally equivalent. Thus, we conclude that GSK3\(\alpha\) and GSK3\(\beta\) play distinct roles during zebrafish cardiogenesis.

\textbf{Methods}

\textbf{Zebrafish maintenance and observation}

The zebrafish AB strain, transgenic lines Tg(cmlc2:Hc-RFP) and Tg(cmlc2:GFP) were raised and staged as previously described (28–30). The heart formation were observed
Heart asymmetry was affected in gsk3β morphants. Normally, bmp4 transcripts accumulate predominantly on the left side of the heart tube at 20 hpf (A, B), and the left-predominant bmp4 asymmetry persists through the stages of jogging (G). However, in gsk3β morphants, the expression of bmp4 becomes symmetrical at 20 hpf (B, D). In gsk3β morphants, in which the heart fails to jog, bmp4 is more evenly distributed in the heart region (H, I). The left-sided lefty-1 domain was greatly reduced in gsk3β morphant hearts at 16 hpf (F). All are dorsal views. B, E are higher magnifications of A, D, respectively. Lines mark the midline. L, embryo left.

Figure 6
Heart asymmetry was affected in gsk3β morphants. Normally, bmp4 transcripts accumulate predominantly on the left side of the heart tube at 20 hpf (A, B), and the left-predominant bmp4 asymmetry persists through the stages of jogging (G). However, in gsk3β morphants, the expression of bmp4 becomes symmetrical at 20 hpf (B, D). In gsk3β morphants, in which the heart fails to jog, bmp4 is more evenly distributed in the heart region (H, I). The left-sided lefty-1 domain was greatly reduced in gsk3β morphant hearts at 16 hpf (F). All are dorsal views. B, E are higher magnifications of A, D, respectively. Lines mark the midline. L, embryo left.
**GSK3β modulates zebrafish cardiac valve formation.** Whole-mount *in situ* hybridization with *bmp4* and versican staining reveals that cardiac valve development was affected in *gsk3β* morphants. At 60–72 hpf, versican (A, B) and *bmp4* (C, D) expression was greatly up-regulated in *gsk3β* morphants. Tg(*cmlc2: Hc-RFP*) embryos were injected with gsk3β-MO and observed by *in vivo* two-photon fluorescence imaging of a live transgenic zebrafish heart at 100 hpf. The endocardial cells and blood are labeled yellow; the Hc-GFP-positive myocardial cells are labeled red. Valves are clearly observed in wild-type embryos (E; white arrows), but not in *gsk3β* morphants (F). b, blood cells; V, ventricle; A, atrium.
under a fluorescent stereomicroscope MZ FLIII (Leica) and two-photon fluorescence microscope and Third-Harmonic-Generation Microscopy [28].

**Knockdown microinjection of zebrafish embryos**
The following morpholino antisense oligonucleotides (MOs) were obtained from Gene Tools: gsk3α-MO, CCGTGCCTGCATTTGCGGGCA; gsk3β-MO, GTTCTGGGCCGACCGGACATTTTTC; axin1-MO, GCTAATGCCGTCATATCCTCGC; standard negative control-MO, CCTCTACCACGTACATTTATA. All MOs were prepared at a stock concentration of 1 mM and diluted to the desired concentration for microinjection into each embryo.

**Western blot**
The embryos were dechorionated and deyolked with two extra washing steps as described in Link et al. [31]. Deyolked samples were dissolved in 2 μl of 2 × sodium dodecyl sulfate (SDS) sample buffer per embryo and incubated for 5 min at 95°C. After full-speed centrifugation for 1 min in a microcentrifuge to remove insoluble particles, samples were loaded on a 12% SDS gel (seven embryos per lane). Antibodies used were anti-GSK3β (Santa Cruz, SC-7291, 1:750) and anti-α-tubulin (Sigma-Aldrich, T9026, 1:750).

**Whole-mount in situ hybridization**
Whole-mount in situ hybridization techniques have been described previously [32]. The probes were digoxigenin-labeled, after which we cloned their partial DNA fragments.

**mRNA preparation for the rescue experiment**
Capped mRNAs of gsk3α, gsk3β, and RFP were synthesized according to the protocol of the manufacturer (Epicentre). The resultant mRNAs were diluted to 44 ng/μl with distilled water. Approximately 2.3 nl was injected into one-cell stage embryos.

**Detection of apoptotic cell death**
The apoptosis assay was performed using The DeadEnd™ Colorimetric TUNEL System (Promega) and has been described previously [33].

**Authors’ contributions**
HCL designed and performed all the experiments, analyzed the data, and prepared the manuscript. JNT and WCC carried out the Western blot analysis; KYL, PYL and WYT took care of fish handling including morpholino injection. CCC and CKS performed the two-photon fluorescence microscope imaging; HJT was a P.I. of this project. All authors read and approved the final manuscript.

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**Additional material**

**Additional file 1**
Morphological phenotypes of zebrafish embryos derived from fertilized eggs injected with standard control morpholino. The standard negative control-MO has no target, no significant biological activity, and are commonly used in many studies (Hultman et al., 2007; Besser et al., 2007; Nixon et al., 2005). After 2 and 6 ng of this control MO were injected, no any defects were observed at 24 hpf. The morphology and development of heart appeared normally.

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**Additional file 2**
Similar cardiac defects in the axin1 and the gsk3β morphants. Axin1-MO or gsk3β-MO were microinjected to And observed under dissecting microscope by bright filed (A, C) or fluorescence (B, D). Incomplete looping of the heart tube was also observed in axin1 mutant heart.

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**Additional file 3**
Arrested pectoral fin bud induction in gsk3β morphants. At 72 hpf, wild-type pectoral fins elongate (A), but gsk3β morphants have still not developed fin buds (arrows; D). Whole mount in situ hybridization with shh and dlx2 staining reveal that the developed of fin bud were affected in gsk3β morphants. At 36 hpf, wild-type embryos continue shh (B) and dlx2 (C) expression in the developing bud mesenchyme, but in gsk3β morphants, the shh and dlx2 expression is greatly decreased.

Click here for file [http://www.biomedcentral.com/content/supplementary/1471-213X-7-93-S3.doc]
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