Expression pattern of zcchc24 during early Xenopus development

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ABSTRACT We report the expression pattern of a novel Xenopus laevis gene, zcchc24, which encodes a protein containing two zinc finger domains from the zf-CCHC and zf-3CxxC superfamilies. This protein shares >84% amino acid identity with its vertebrate homologues. During X. laevis embryonic development, zcchc24 is expressed at gastrula stages in the dorsal mesoderm, including the cardiac precursors region. During neurula stages, zcchc24 is expressed as two stripes in the dorsal region, more precisely, in the somitogenic mesoderm until the cardiac mesoderm. At early tailbud stages, zcchc24 continues to be expressed in these regions, but starts to be expressed in the migrating neural crest. Later, this gene is expressed in the head, branchial arches, heart and somites. The zinc finger domains present in Zcchc24 protein and its dynamic gene expression pattern suggest that Zcchc24 might be involved in the regulation of heart, somites and of branchial arch formation/patterning, namely in the regulation of apoptosis.

KEY WORDS: zcchc24, zinc finger, heart development, somitogenic mesoderm, neural crest
To analyze the potential function of *X. laevis* zcchc24 during early frog embryo development, we examined its expression by whole-mount in situ hybridization (WISH).

zcchc24 transcripts were first detected at midblastula stage, in both dorsal and ventral marginal zones of the embryos (Fig. 2 A,A'), showing a ring around the marginal zone or presumptive mesoderm. Then, at the onset of gastrulation, zcchc24 expression was observed in the dorsal mesoderm of both involuting marginal zone (IMZ), a region immediately above the dorsal blastopore lip, and non-involuting marginal zone (NIMZ), which is the region on the top of IMZ (Fig. 2 B,B').

During gastrulation, and more specifically at stage (st) 11-12, zcchc24 is expressed in the dorsal mesoderm, in the somitogenic mesoderm, excluding the dorsal midline (Fig. 2 C,C',E,E'). *myoD*, whose expression has been described in the same region, was used as a marker of somitogenic mesoderm (Fig. 2 D,F). This gene is involved in the formation of somites, and the knock-down of the MyoD disrupts the correct alignment of muscle fibers (Maguire et al., 2012). At this stage, zcchc24 is also expressed in two lateral mesoderm stripes around the blastopore that culminate in the dorsal side of the embryo (Fig. 2 C,E). Interestingly, these two lateral mesodermal stripes are correlated with the region in which the heart is originated. As a matter of fact, it has been suggested that, during gastrulation, the precardiac mesoderm migrates in two bilateral heart field located in the anterior lateral mesoderm (Sater and Jacobson, 1989).

From mid (st 11) to the end of gastrulation/beginning of neurulation (st 13), zcchc24 expression seems to follow the migration of involuting mesoderm along the antero-posterior axis (Fig. 3A). During neurulation, zcchc24 expression is detected in the anterior and somitogenic mesoderm (Fig. 3 B-B''-E), which later will give rise to lateral and neural plate, and the somites, respectively. At these stages, the expression of zcchc24 decreases progressively from the posterior to the anterior part of the embryo (Fig. 3 B,E). When compared with the early cardiac lineage marker *nkx2.5*, it is possible to observe that anteriorly zcchc24 expression is adjacent to the cardiac mesoderm, while more posteriorly, zcchc24 is expressed in the paraxial mesoderm. (Fig. 3 C,D)

Afterwards, at early tailbud stages, zcchc24 expression is detected in the unsegmented somitogenic mesoderm and in the somites (Fig. 4 A,B). In contrast, *nkx2.5* is predominantly expressed in the differentiating cardiac muscle (Fig. 4C). In addition, when we compare the expression of zcchc24 with the expression of *twi*, a neural crest marker, we observe that zcchc24 transcripts are in the migrating neural crest cells (Fig. 4 B,D,E).

At later tailbud stages, zcchc24 is expressed in the differentiating cardiac muscle, in the presumptive heart region and in the head, excluding the cleft between the branchial arches and cement gland (Fig. 4 F,G). A transverse section of a st 32 embryo showed...
that zcchc24 transcripts are clearly detected in heart region, more precisely, in endocardium, myocardium and pericardium, and in branchial arch mesenchyme (Fig. 4G'). The expression of zcchc24 (Fig. 4G) in the heart is similar to the expression of nkx2.5 in this region (Fig. 4 H,I). More, when zcchc24 and twi expressions are compared, it becomes obvious that both genes are expressed in the branchial arches (Fig. 4 J,K).

According to our data, the expression pattern of Xenopus zcchc24 is in part similar to the expression pattern of its chick homolog. Here, we show that, early in development, Xenopus zcchc24 is expressed in the dorsal mesoderm, in which the cardiac mesoderm is initially formed, and later is expressed in the head, heart and somites, like its chick homolog at Hamburger and Hamilton stage 10 (HH10; Fig. 5). This suggests that these homologs might have a role in heart formation, both in Xenopus and chick. Moreover, it demonstrates that zcchc24 expression pattern was conserved during evolution.

Several zinc finger proteins with a role in heart development display the animal hemisphere to the top. (B,B') In the beginning of gastrulation (st 10.5), zcchc24 expression is restricted to the dorsal mesoderm in both involuting and non-involuting marginal zone. (C,C', E,E') At late gastrula (st 11 and 12), zcchc24 transcripts are detected in two lateral mesoderm stripes around the blastopore that culminate in the dorsal mesoderm, the somitogenic mesoderm, but is excluded from the midline. (D,F) myoD is expressed in the somitogenic mesoderm. (A', B') Hemisections of (A,B), respectively. (C', E') Transverse sections of st 11 and st 12 embryos, respectively, with dorsal side displayed to the top. (B,C) Vegetal views. (D,E,F) Dorsal views. Dashed lines delimitate the blastopore. An, animal; Vg, vegetal; D, dorsal; V, ventral; dbl, dorsal blastopore lip; sm, somitogenic mesoderm.

Fig. 2. zcchc24 expression from blastula to gastrula stages. Whole mount in situ hybridization using DIG labelled zcchc24 (A-C, E,E') or fluorescein labelled myoD (D,F) was performed on embryos from blastula to gastrula stages. (A,A') At blastula stages, zcchc24 is expressed as a ring around the marginal zone in both dorsal and ventral sides. The embryos display the animal hemisphere to the top. (B,B’) In the beginning of gastrulation (st 10.5), zcchc24 expression is restricted to the dorsal mesoderm in both involuting and non-involuting marginal zone. (C,C’, E,E’) At late gastrula (st 11 and 12), zcchc24 transcripts are detected in two lateral mesoderm stripes around the blastopore that culminate in the dorsal mesoderm, the somitogenic mesoderm, but is excluded from the midline. (D,F) myoD is expressed in the somitogenic mesoderm. (A’, B’) Hemisections of (A,B), respectively. (C’, E’) Transverse sections of st 11 and st 12 embryos, respectively, with dorsal side displayed to the top. (B,C) Vegetal views. (D,E,F) Dorsal views. Dashed lines delimitate the blastopore. An, animal; Vg, vegetal; D, dorsal; V, ventral; dbl, dorsal blastopore lip; sm, somitogenic mesoderm.

Fig. 3. zcchc24 expression during neurula stages. Whole mount in situ hybridization using DIG labelled zcchc24 (A-B”, E) or fluorescein labelled nkx2.5 (D) and double whole mount in situ hybridization with DIG labelled zcchc24 and fluorescein labelled nkx2.5 (C) were performed on embryos at neurula stages. (A) At late gastrula/early neurula (st 13), zcchc24 is expressed as two stripes in the dorsal side of the embryo along the antero-posterior axis but is excluded from the notochord. (B-B’). (E) During neurula stages, zcchc24 is expressed in anterior and somitogenic mesoderm, decreasing the expression from the posterior to the anterior region. Comparison between the expression of zcchc24 and nkx2.5 (B,C,D) shows that the most anterior expression of zcchc24 is adjacent to the cardiac mesoderm. (A,B, C-E) Embryos in a dorsal view. (B,B”) Transversal sections of st 15 embryos with the dorsal region displayed to the top. Red arrows indicate the heart precursor region. A, anterior; P, posterior; n, notochord; sm, somitogenic mesoderm.
have been described. The GATA zinc finger-containing transcription factors are a family of proteins that have been implicated in regulation of gene expression in the heart development (Haworth et al., 2008). It was demonstrated in zebrafish that GATA5 is necessary for the production of the correct number of myocardial precursors and for the correct expression of several cardiac genes including nkk2.5. Indeed, the overexpression of GATA5 induces contractile heart-like tissue (Reiter et al., 1999). GATA4 is another GATA family zinc finger that is also implicated in heart and liver development. It was shown that the knock-down of this transcript affects heart and liver primordia following their specification (Haworth et al., 2008, Holtzinger and Evans, 2005). In addition, after heart specification, GATA4 interacts with another GATA family member, GATA6, during its action in the development of heart in mouse, *Xenopus* and zebrafish (Holtzinger and Evans, 2005, Peterkin et al., 2003, Zhao et al., 2005). Therefore, since *zcchc24* is expressed in heart precursor cells and later in the heart (Fig. 6), like GATA family genes, it is tempting to extrapolate a role for *zcchc24* in the formation of the heart.

Nevertheless, *zcchc24* is also highly expressed in somitogenic mesoderm and later in the somites. Sev-
eral zinc fingers proteins like Gli, Gli3 and Gli4 members of the Hedgehog (Shh) signaling pathway have been implicated in somite formation. Gli-type proteins function as transcriptional repressors that respond to Shh signals, and control the expression of Shh-responsive genes such as myf5, the muscle master regulator (Hui and Angers, 2011). Therefore, we think that Zcchc24 might have a putative role in the regulation of the proper somite segmentation could not be excluded.

The expression of zcchc24 in the migrating neural crest and later in the branchial arches suggests that zcchc24 might have a function during the development and/or migration of this tissue. Curiously, several zinc finger proteins of the Snail family have been associated to the neural crest formation (del Barrio and Nieto, 2002, Nieto et al., 1994). For example, it was reported that slug and snail, two members of this family, are important for neural crest specification and migration. In Xenopus embryos or animal caps, the overexpression of snail is able to induce the expression of slug among other neural crest markers such as foxD3, twi and ets1. On the other hand, slug is not able to induce these neural crest markers, however, gain-of-function studies performed in chick showed that slug overexpression increases cranial neural crest production, and its loss-of-function inhibits neural crest migration (Aybar et al., 2003, del Barrio and Nieto, 2002, Nieto et al., 1994).

Taken together, our results showed that zcchc24 is expressed mainly in three different precursors/structures: cardiac precursors/heart, somitogenic mesoderm/somites, and neural crest/branchial arches. Curiously, several proteins of the zf-CCHC superfamily of zinc finger containing proteins were described to have a role on cell death inhibition. Moreover, the proper formation of the heart, branchial arches and somites requires none or a low level of apoptosis. High levels of apoptosis in these three structures have been reported to be responsible for defects (Graham et al., 1996, Kang and Izumo, 2000, Sanders and Parker, 2001). These observations indicate that Zcchc24 might be particularly important for the regulation of migration and/or apoptosis. Nevertheless, further genetic and biochemical analysis must be performed to clarify the role of zcchc24 during X. laevis embryonic development.

Materials and Methods

Xenopus embryo manipulations

Xenopus eggs were obtained from females injected with 300 IU of human chorionic gonadotropin (Sigma) and were fertilized in vitro. Eggs were dejellied with 2% cysteine hydrochloride, pH 8.0.

Embryos were grown in 0.1X MBS-H (1X MBS-H = 86 mM NaCl, 1 mM KCl, 2.4 mM NaHCO3, 0.62 mM MgSO4, 0.41 mM CaCl2, 0.33 mM Ca(NO3)2, 10 mM HEPES, pH 7.4, 10 μg/mL streptomycin sulphate and 10 μg/mL penicillin) and staged according to Nieuwkoop and Faber (1967).

Cloning of partial coding sequence of zcchc24

Since the cloning sequence (CDS) of zcchc24 was not available in stock centers, to obtain it, a partial coding sequence was isolated by RT-PCR. With this purpose, total RNA from stage 20 of Xenopus laevis embryos was isolated using trizol reagent according to the manufacturer’s instruction. To perform the RT-PCR, first strand cDNA was synthesized using oligoDT hexamers as primers and zcchc24 CDS was amplified using a specific pair of primers (Forward 5’-CCATCCACTCCAGCTATCTGAGCA-3’; Reverse 5´-TTACTGAACACGGGGCAGTAGATGCG-3’). The PCR product was cloned into pGEM®-T Easy and sequenced to confirm for correct DNA sequence.

Whole mount in situ hybridization and histology

Single and double whole mount in situ hybridization and anti-sense probes preparation was carried out as previously described (Belo et al., 1997). To generate the fluorescein labelled nkk2.5, myoD and twi anti-sense RNA probes, plasmids containing fragments of these genes were linearized using XbaI, HindIII and EcoRI, respectively, and transcribed using T7 RNA polymerase. To synthesize zcchc24 DIG labelled probe, a plasmid containing zcchc24 fragment was linearized using Sall enzyme and transcribed using T7 RNA polymerase. Probes were purified using quick Spin Mini RNA columns (Roche). Hybridized RNAs were detected with alkaline phosphatase conjugated anti-DIG antibodies (Roche) and with alkaline phosphatase conjugated anti-fluorescein antibodies (Roche) and developed with BM purple (Roche) or BCIP (Roche). Stained embryos were bleached by illumination in 1% H2O2, 4% formamide and 0.5X SSC pH7.0. Embryos were photographed under bright light using a MicroPublisher 5.0 RTV camera coupled with a Leica MZ16FA stereo microscope or in a Zeiss Stereolux V12 Stereomicroscope coupled with an Axioskop MRC.

For histology, after in situ hybridization, the Xenopus embryos were fixed overnight at 4°C in 4% PFA, embedded in paraffin, and sectioned in a Zeiss microtome. Sections were observed in a Zeiss Z2 microscope and photographed with a Zeiss AxioCam ICc3 camera.

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