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Comparative gene expression analysis and fate mapping studies suggest an early segregation of cardiogenic lineages in *Xenopus laevis*

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ABSTRACT

Retrospective clonal analysis in mice suggested that the vertebrate heart develops from two sources of cells called first and second lineages, respectively. Cells of the first lineage enter the linear heart tube and initiate terminal differentiation earlier than cells of the second lineage. It is thought that both heart lineages arise from a common progenitor cell population prior to the cardiac crescent stage (E7.5 of mouse development). The timing of segregation of different lineages as well as the molecular mechanisms underlying this process is not yet known. Furthermore, gene expression data for those lineages are very limited. Here we provide the first comparative study of cardiac marker gene expression during *Xenopus laevis* embryogenesis complemented by single cell RT-PCR analysis. In addition we provide fate mapping data of cardiac progenitor cells at different stages of development. Our analysis indicates an early segregation of cardiac lineages and a fairly complex heterogeneity of gene expression in the cardiac progenitor cells. Furthermore, this study sets a reference for all further studies analyzing cardiac development in *X. laevis*.

Introduction

Current models of cardiac development suggest that cardiomyocytes develop from two sources of cells (Buckingham et al., 2005; Laugwitz et al., 2008; Srivastava, 2006). In mice, cells of the cardiac crescent located in the anterior lateral mesoderm will form the linear heart tube. Later, cells of the initially formed heart tube will contribute to the left ventricle and most of both atria (Kelly et al., 2001). This linear heart tube continues to grow during development by the addition of cells to both the cranial and caudal poles (Buckingham et al., 2005; Kelly and Buckingham, 2002; Stalsberg and DeHaan, 1969; Viragh and Challice, 1973) giving rise to the outflow tract (OFT), most of the right ventricle and the sinus venosus (inflow tract, IFT).Three separate studies identified the origin of those cells added at the arterial pole of the heart tube and later forming the OFT in chicken (Mjaatvedt et al., 2001; Waldo et al., 2001) and mice (Kelly et al., 2001).

In chicken, Waldo et al. (2001) identified a population of cells located in the splanchnic mesoderm underlying the caudal pharynx to later contribute to the OFT, in particular the distal OFT. This population of cells was named secondary heart field in contrast to the term primary heart field describing cells destined to form the heart tube. Mjaatvedt et al. (2001) identified a population of cells including the more cranial pharyngeal mesoderm which extends into the pharyngeal arches and contributes to OFT formation. This population of cells was called the anterior heart field in chicken. Due to technical differences, these reports (Mjaatvedt et al., 2001; Waldo et al., 2001) differed in the description of the precise location of this second source of progenitor cells as well as their contribution to the OFT region (Buckingham et al., 2005). Cells of the anterior heart field were shown to contribute rather to the proximal OFT (Conus arteriosus) whereas cells of the secondary heart field contributed to more the distal OFT (Truncus arteriosus) (Buckingham et al., 2005; Mjaatvedt et al., 2001; Waldo et al., 2001). More recently, it has been demonstrated that also in chicken the right ventricle is derived at least in part from the anterior/secondary heart field (Rana et al., 2007).

In mice, cells contributing to the arterial pole of the heart are characterized by the expression of FGF10 (Kelly et al., 2001). Using a lacZ transgene under the control of the FGF10 regulatory elements, Kelly et al. could show that labeled cells of the pharyngeal mesoderm expressing FGF10 contribute to the OFT. Furthermore, FGF10 positive cells were also found to contribute to the right ventricle of the mature heart (Zaffran et al., 2004). The expression of FGF10 allowed mapping of these cells at the crescent stage to be medial to the cardiac crescent. Later, other marker genes were characterized to describe cells of the anterior heart field or the secondary heart field such as Tbx1 and FoxH1 (Hu et al., 2004; von Both et al., 2004; Xu et al., 2004; Yamagishi et al., 2003).

Studies on the transcription factor Islet1 (Isl1) in mice extended this concept of different populations of cells contributing to the heart as cells expressing Isl1 at E7.5 do not only contribute to the arterial pole of the heart tube, but also to its venous pole (Cai et al., 2003). The term second heart field (SHF) was coined to describe the early location and later contribution of those cells to the heart (Buckingham et al., 2005; Laugwitz et al., 2008). In particular, Isl1 positive cells

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contribute to the OFT, most of the right ventricle, both atria and the IFT. Cardiogenic cells of the first heart field (FHF) in contrast are negative for Isl1 at E7.5, will form the left ventricle and will also contribute to both atria and the inflow tract (Abu-Issa and Kirby, 2007; Cai et al., 2003; Laugwitz et al., 2008). Cells of the FHF already started to terminally differentiate at this stage of development as indicated by the expression of myosin light chain (MLC). Within the SHF subdomains of gene expression can be identified as an Isl1-cre driven reporter labels the whole second heart field (Cai et al., 2003) whereas a cre reporter driven by some regulatory elements of the Mef2C gene only labels progenitors of the OFT and the right ventricle (Verzi et al., 2005) and thus an anterior subpopulation of the SHF.

The concepts presented above use the term "field" mainly to describe different populations of cells characterized by differential gene expression, and therefore fields are territories of gene expression. The concept of an embryonic "field" is, however, more widely used in developmental biology. Today, the term "field" is used to describe "a group of cells provided with self-organizing and self-regulating properties whose position and fate are specified with respect to the same set of boundaries thus forming a discrete unit of embryonic development and thereby will give rise to a particular organ" (Gilbert et al., 1996; Ingham and Martinez Arias, 1992). From this perspective, the use of the terms "first" and "second heart field", respectively, describing a territory of gene expression only are not necessarily in agreement with the meaning of an embryonic field which includes also some functional properties. Therefore others have argued that there is rather one heart field that is subdivided by e.g. external signals thereby generating territories of differential gene expression and subsequent different cellular behaviour within this single field (Abu-Issa and Kirby, 2007; Moorman et al., 2007).

This notion is also supported by the idea that both, FHF and SHF, originate from a common progenitor cell population (Buckingham et al., 2005; Meilhac et al., 2004) that splits into two lineages prior to the cardiac crescent stage. In this concept, both fields are not unbridgeable populations of cells but are derivatives of a common cardiac progenitor cell population. This led to a model in which all cardiac precursor cells initially express Isl1 which than is turned off in those cells starting terminal differentiation (FHF). In contrast, Isl1 is active for a longer time period in those cells that are added to the different poles of the heart later, thus representing the SHF (Laugwitz et al., 2008). Consistently, Isl1 was also found to be expressed earlier during development in cells later forming the FHF (Prall et al., 2007; Sun et al., 2007). In chicken, Isl1 seems to be also expressed in all cells of the bilateral heart fields before fusion at the ventral midline (Yuan and Schoenwolf, 2000).Thus, the first lineage as determined by retrospective clonal analysis represents cells of the FHF and is Isl1 negative at E7.5. The second lineage is still Isl1 positive at E7.5 and represents the SHF (Cai et al., 2003) which also contains those subpopulations of cells that were earlier called secondary heart field or anterior heart field as discussed above (Kelly, 2005). Consistently, it is thought that these lineages differ with respect to timing of terminal differentiation with cells of the first lineage starting differentiation earlier than those of the second lineage. In our view, this concept of a common cardiac progenitor cell population with defined boundaries and different lineages within this population described by the expression of certain marker genes would be in better agreement with the more general use of the term "field" in embryology. In line with this argumentation we here use the term field only to describe territories of gene expression and to compare our data to those in other publications using this term.

Data concerning the different cardiogenic lineages in *Xenopus* are not yet available. We here provide a first systematic and comparative expression analysis of cardiac marker gene expression during *Xenopus laevis* development complemented by single cell RT-PCR data and DiI fate mapping studies. We thereby establish a model in which the early cardiac progenitor cell population can be described by the expression of Isl1, Nkx2.5, Tbx1, and GATA6b as early as stage 13. Later, these progenitor cells exhibit an unexpected heterogeneity in gene expression likely representing the different lineages discussed earlier (Kelly, 2005). Based on our finding, we support the idea launched by others that the cardiac progenitor cells are rather defining one single heart field that is later on subdivided into smaller areas with differential gene expression representing different lineages (Abu-Issa and Kirby, 2007; Abu-Issa et al., 2004; Buckingham et al., 2005; Meilhac et al., 2004; Moorman et al., 2007).

Materials and methods

Xenopus embryos

X. laevis embryos were obtained by *in vitro* fertilization, staged and analyzed by *in situ* hybridization as described (Gessert et al., 2008).

In situ hybridization

Probes for *in situ* hybridization were used as: Flk1 (Cleaver et al., 1997), FoxH1 (Howell et al., 2002), FoxC1 (Koster et al., 1998), FoxC2 (El-Hodiri et al., 2001), DM-GRASP (Gessert et al., 2008), Ror2 (Hikasa et al., 2002), Wnt11-R (Gessert et al., 2008), MHCα (Gessert et al., 2008), GATA4 and GATA5 (Jiang and Evans, 1996), GATA6b (Gove et al., 1997), Tbx20 (Brown et al., 2003), TnIc (Drysdale et al., 1994), and Xmsr (Devic et al., 1996). The cActin probe was provided by P. Krieg, the N-Cadherin probe by D. Wedlich. Probes for Dkk1, eHand, dHand, Mef2c, Mef2d, Tbx1, and Tbx5 were newly cloned. For cloning novel probes, the total RNA of *Xenopus* embryos at stages 26 and 31 was isolated utilizing the Gentra Purescript™ RNA isolation kit (Biozym). Subsequently the cDNAs were synthesized using Superscript II RNase H^- reverse transcriptase (Invitrogen) and random primers. For the amplification, the Phusion High Fidelity DNA polymerase (Biometra) or PfuUltra II DNA polymerase (Stratagene) with proof reading activity was used. All amplification products were cloned into the pSC-B vector (Stratagene). The primers for amplification were: Dkk1_l: 5′-GAC AGT CGG AGC CGG CGC TGC-3′, Dkk1_r: 5′-TTA GTG TCT TTG GCA AGT GTG AAG-3′; dHand_l: 5′-GCC GAC TGC ACC CAT GAA AGC-3′, dHand_r: 5′-TCT GGA ATG TTT CTC TTC GGA-3′; eHand_l: 5′-CTT TGC CTG GAT GAA GCT TGC-3′, eHand_r: 5′-CTC TCG TCC TCT TAT TGC AGA-3′; Mef2c_l: 5′-GAG GGA GGT AAG AAA ACG ACA CCT-3′, Mef2c_r: 5′-TCA TGT TGC CCA TCC TTC AGA GAG-3′; Mef2d_l: 5′-GAG AAG CAG CTC TCG GCC CTG GTA-3′, Mef2d_r: 5′-TTA TGT TAC CCA TGC GTC AAG TCG-3′; Tbx1_l: 5′-GCA CTG CCA CAG CAG CAC TAG-3′, Tbx1_r: 5′-TAT AGC CTC CTG CTT CAG ATG-3′; Tbx5_l: 5′-GTG CCA CTG CCT GAA GTG CAG-3′, Tbx5_r: 5′-TTA GCT GTT TTC ATT CCA GTC TGG-3′. Sizes of amplification products were: Dkk1: 0.881 kb; dHand: 1.041 kb; eHand: 0.981 kb; Mef2c: 1.737 kb; Mef2d: 1.732 kb; Tbx1: 1.644 kb; Tbx5: 1.694 kb. For sectioning, the embryos were first equilibrated in gelatine/BSA solution overnight at 4 °C. The embryos were embedded in gelatine/BSA and sectioned with the thickness of 20–30 μm using a vibratome.

Single cell RT-PCR

For single cell RT-PCR, we dissected the cardiac region of stage 24 embryos with fine forceps. After removing the pigmented epidermal layer, we separated the cells in CMFM (Calcium–Magnesium-free medium; 88 mM NaCl; 1 mM KCl; 2.4 mM $NaHCO_3$; 1 mM EDTA; 7.5 mM Tris–HCl pH 7.3). Then, we collected single cells of the mesodermal layer. For cell lysis, we used 8 μl of a lysis buffer for one single cell (1× One Step RT-PCR buffer (QIAGEN); 0.5% NP-40; 8 U RNase inhibitor (Invitrogen)) with a subsequent incubation for 10 min at room temperature. After a DNase I step, we performed a PCR reaction with the Single Cell RT-PCR Kit (QIAGEN) following the

protocol of the company. Sequences of the used primer are: GAPDH_l: 5′-GCC GTG TAT GTG GAA TCT-3′; GAPDH_r: 5′-AAG TTG TCG TTG ATG ACC TTT GC-3′; Isl1_l: 5′-AGC GCC TGA TTT CTC TGT GT-3′; Ils1_r: 5′-GTC TGC CCG ACA GAA GAG TC-3′; Nkx2.5_l: 5′-GAG CTA CAG TTG GGT GTG TGT GGT-3′; Nkx2.5_r: 5′-GTG AAG CGA CTA GGT ATG TGT TCA-3′; Tbx1_l: 5′-ACA AGT CCA CCA GGA ACA GG-3′; Tbx1_r: 5′-GGC CTA TCA GAA CCA CAG GA-3′; Tbx5_l: 5′-AAT GTG GAC AGA TGT TGG AAG G-3′; Tbx5_r: 5′-AGC ACA CTG AGT AAT ACA CTG C-3′. To exclude the possibility of endodermal cell contaminations, we performed a control PCR with Shh primers: Shh_l: 5′-GGT TGA CCG CGG CCC ATC TAC-3′; Shh_r: 5′-AGG CGA ATA AGC TCC AGT GTC C-3′. The fragment lengths vary between 200 and 400 bp.

DiI labeling for fate mapping studies

Labeling of single cells or a population of a small number of cells were done using DiI (1,1′-Dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate) from Sigma Aldrich. The final labeling solution contained 3 mg DiI in 1 ml absolute EtOH and embryos were labeled with a small volume by delivery through a microinjection needle. The ethanol itself had no effect on the development of the embryos including the heart. For documentation purposes, we took pictures of all embryos immediately after injection using a fluorescence microscope (Olympus). At stage 43, we took pictures of all labeled embryos from the outside. Subsequently, hearts were dissected, documented again and analyzed for labeled regions.

Results and discussion

Reference points of cardiac development in X. laevis

In this study we provide the first systematic and comparative analysis of the spatio-temporal expression of important cardiac marker genes during *X. laevis* development. To compare our data to those obtained in other species, it is useful to define reference points during *X. laevis* cardiac development as judged by the expression of key marker genes such as Isl1 or morphological features.

In mice, the so-called FHF and SHF are defined by the expression of Nkx2.5 and Isl1 at E7.5 (Abu-Issa and Kirby, 2007; Laugwitz et al., 2008). At this point, the SHF is Isl1 positive and located more medially to the FHF. Later during development at embryonic day 8.0, the SHF is more anterior and dorso-laterally to the FHF. It is noteworthy to mention that cells within the FHF at this stage already start with the expression of terminal differentiation markers such as MLC (Cai et al., 2003). Formation of the linear heart tube by cells of the FHF has not yet started. We have recently suggested that this situation (cardiac crescent stage, E7.5–7.75 in mice) resembles the situation of a stage 24 *X. laevis* embryo (Brade et al., 2007; Gessert et al., 2008). At this stage, Nkx2.5 exhibits a broad expression domain in the ventral mesoderm thereby marking the equivalents of FHF and SHF, whereas Isl1 is only expressed in the anterior part of the Nkx2.5 expressing domain, the SHF (Brade et al., 2007). In addition, BMP4, which is also thought to label the SHF field in mice (Cai et al., 2003; Winnier et al., 1995), is also expressed in the Isl1 positive territory (Fig. 2A) (Gessert et al., 2008). Also at stage 24, we detect the first signs of terminal differentiation with the onset of cardiac Troponin I (TnIc) expression (Fig. 3B). Note, that this detection of TnIc expression at stage 24 is earlier than previously described (Drysdale et al., 1994). Also, formation of the heart tube has not started at this stage of development in *X. laevis*.

At stages 29–31 of *X. laevis* development the expression domains of both genes, Nkx2.5 and Isl1, have become nearly separated as Nkx2.5 is expressed in the FHF derived heart tube that forms during these stage (Brade et al., 2007) (Fig. 2A). These cells do not express Isl1. We therefore suggest, that the stages 29–31 of *X. laevis* development resemble E8.0 of mouse development, with respect to cardiogenesis.

Expression analyses of marker genes at earlier stages of cardiac development in mice are rare. However, data from mouse embryonic stem cells suggest the existence of a common precursor cell for cardiomyocytes, endothelial cells and smooth muscle cells (Laugwitz et al., 2008; Moretti et al., 2006; Wu et al., 2006). In agreement with this hypothesis, Isl1 and Nkx2.5 have a common expression domain at earlier stages in *X. laevis* (Brade et al., 2007; Gessert et al., 2008). Marker gene analysis at stage 15/16 and 20 of *Xenopus* development indicate that the cardiac progenitor cells (CPCs) that are positive for Isl1 and Nkx2.5 abut the cement gland posteriorly (Fig. 2A). To further characterize these cell populations and territories of gene expression we here provide a detailed analysis of additional cardiac marker genes at these reference points of cardiac development.

The cardiac progenitor cells at stage 12/13

Specification of CPCs occurs at the onset of gastrulation of *X. laevis* embryos on either side of the Spemann organizer. Later during gastrulation these cells are thought to migrate anteriorly and ventrally (Sater and Jacobson, 1989; Sater and Jacobson, 1990). We therefore started our analysis of cardiac marker gene expression at late gastrulation stages. At the end of gastrulation at stage 13, the expression of Isl1, Nkx2.5, GATA6b and Tbx1 is detected in the anterior region of the embryo (Fig. 1). Sections of those embryos confirm the expression of these genes in the mesodermal layer, thus likely representing the CPC population (Fig. 1). Furthermore, expression of FGF8b can be visualized in the anterior region of the

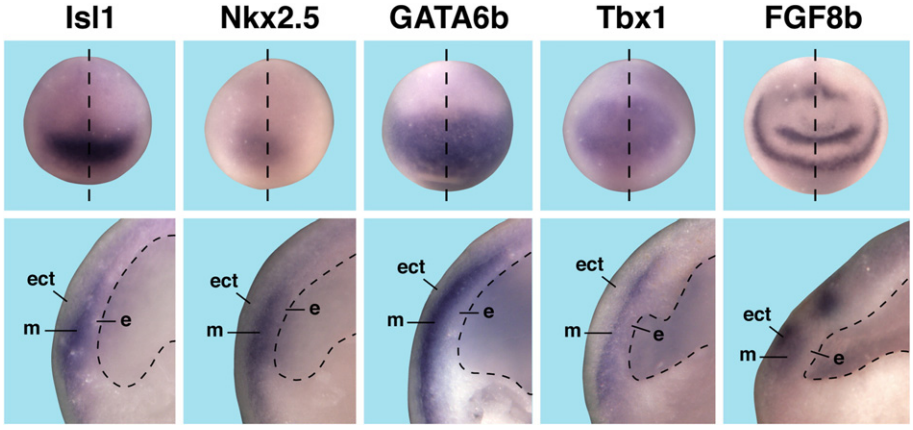

Fig. 1. Whole mount *in situ* hybridization of *Xenopus laevis* embryos at stage 13. The expression of Isl1, Nkx2.5, GATA6b, Tbx1, and FGF8b is given. Anterior views (upper row) of embryos are shown. Dashed lines indicate the level of sagittal sections shown in the lower row. e, endoderm; ect, ectoderm; m, mesoderm.

embryo, mainly in the neuroectoderm, but with a faint expression in the mesodermal layer overlapping with the region of Isl1, Nkx2.5, GATA6b and Tbx1 expression (Fig. 1). In addition we detected a weaker expression of Isl1, Nkx2.5, GATA6b and Tbx1 in the anterior region of the embryo already at stage 12 (data not shown). These findings demonstrate that at the end of gastrulation CPCs are already located in the anterior ventral mesoderm.

The cardiac progenitor cells at stage 15/16

At stage 15/16 we detected the following genes in CPCs posterior to the cement gland: robustly Isl1, Nkx2.5, GATA6b, Tbx1, BMP2 and 4, and weakly GATA4, GATA5, Flk1, Tbx20, Dkk1, and the non-canonical Wnt co-receptor Ror2, while dHand has a punctate expression pattern (Figs. 2 and 3).

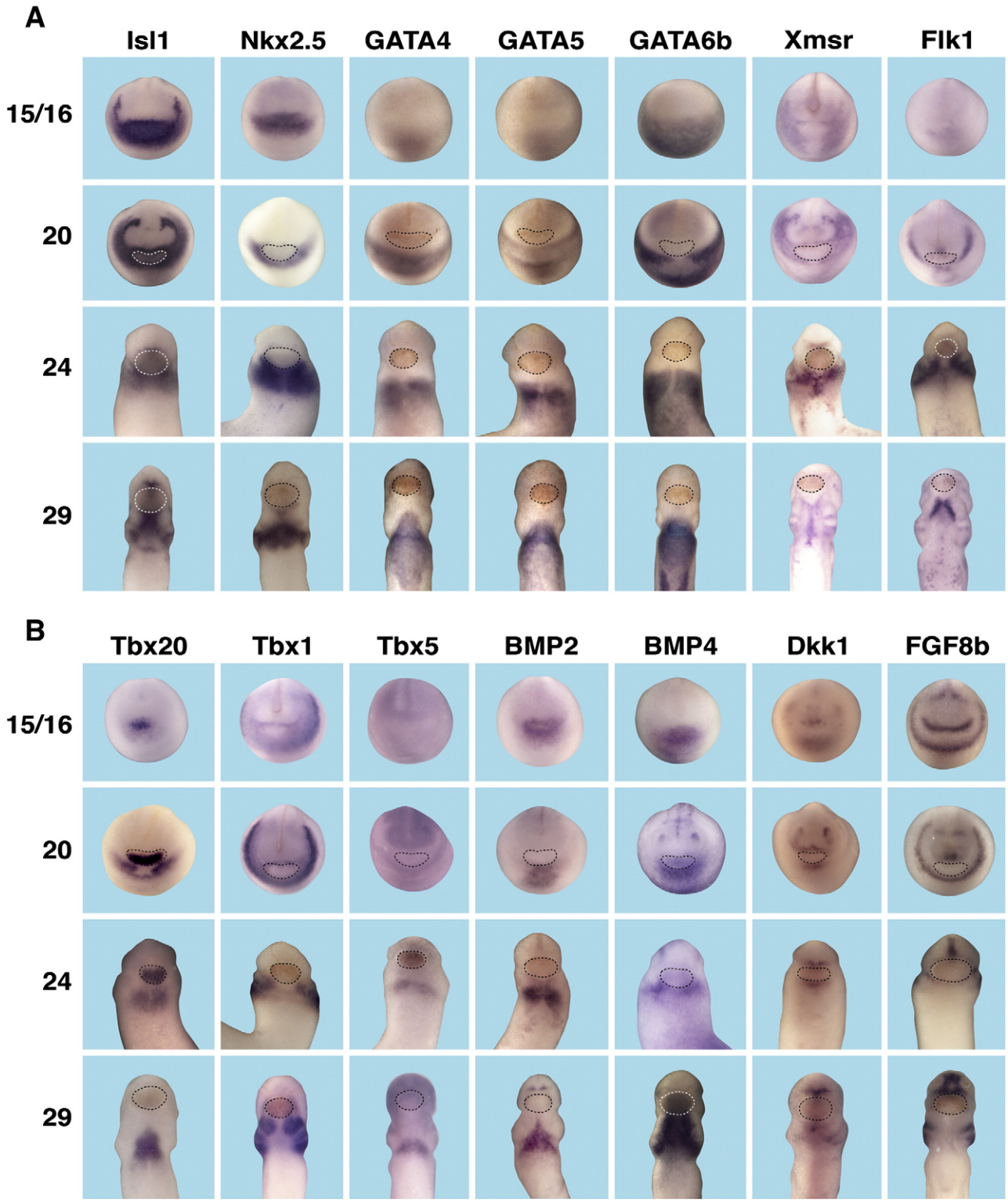

Fig. 2. (A) Cardiac expression of Isl1, Nkx2.5, GATA4, GATA5, GATA6b, Xmsr and Flk1 in *Xenopus* embryos at stages 15/16, 20, 24 and 29 as indicated. (B) Cardiac expression of Tbx20, Tbx1, Tbx5, BMP2, BMP4, Dkk1, and FGF8b at different developmental stages of *Xenopus laevis* as indicated. For embryos at stages 15/16 and 20 anterior views are presented, whereas embryos at stage 24 and 29 are shown from the ventral side, anterior is to the top. Dotted lines indicate the cement gland.

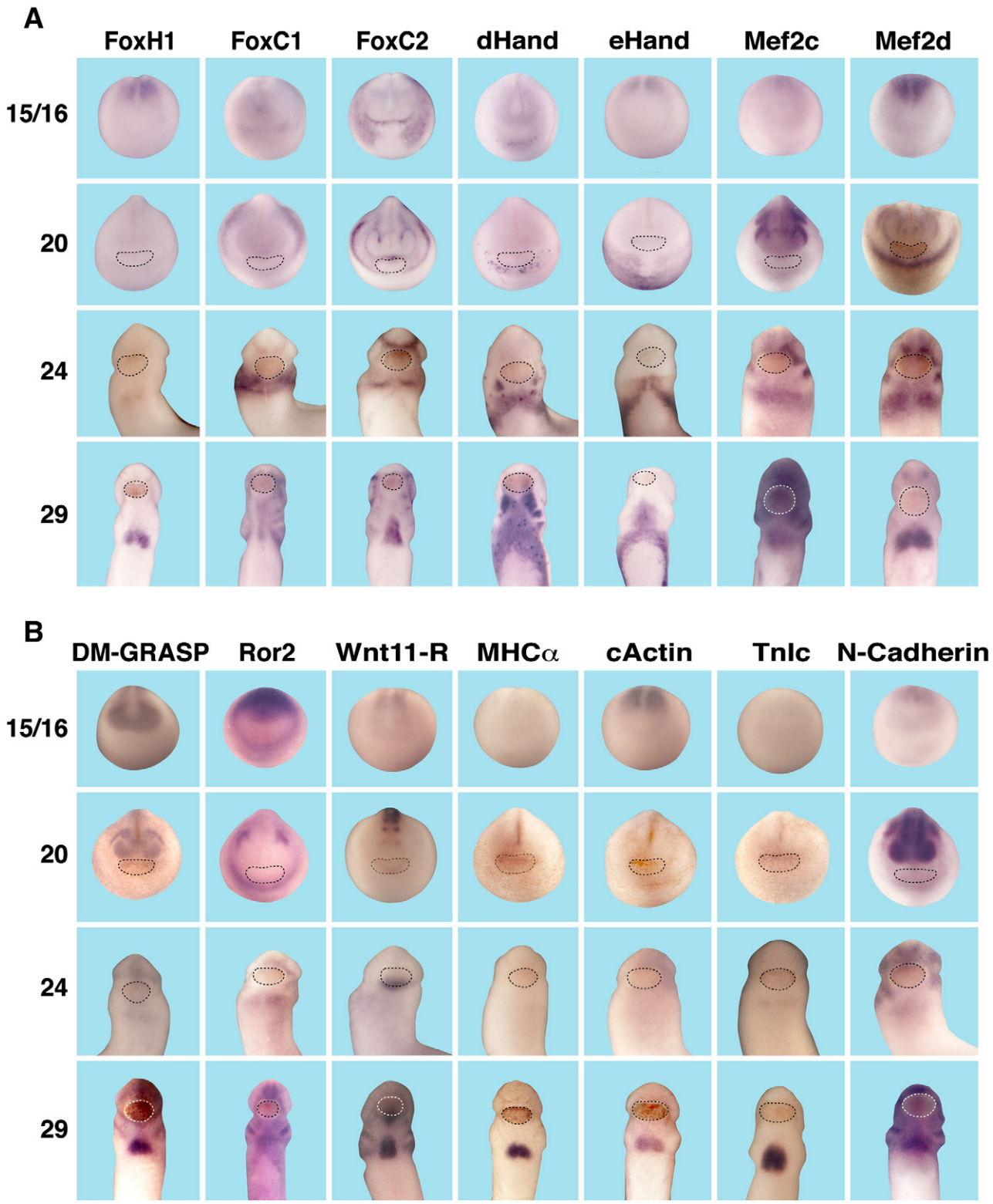

Fig. 3. (A) Cardiac expression pattern of FoxH1, FoxC1, FoxC2, dHand, eHand, Mef2c and Mef2d during *Xenopus laevis*. (B) Expression of the cardiac differentiation marker genes DM-GRASP, MHCα, cActin, Tnlc, N-Cadherin as well as Wnt11-R and Ror2 during cardiac development. For stages 15/16 and 20 anterior views are shown, stages 24 and 29 are from the ventral side, anterior is to the top. Dotted lines indicate the cement gland.

At this stage, Tbx1 – which is required for SHF development in mice (Hu et al., 2004; Xu et al., 2004; Yamagishi et al., 2003) – is expressed in a broad crescent covering an area larger but clearly overlapping with the expression domain of Nkx2.5 (Fig. 2). Overlapping with the expression of Nkx2.5 and Isl1 is also a faint expression of the endothelial marker gene Flk1 which becomes more prominent at stage 20 of development (Fig. 2A). This expression of Flk1 in early CPCs can also be found in mouse embryos at E7.5 (Yamaguchi et al., 1993) and in early differentiating murine ES cells (Kattman et al., 2006; Laugwitz et al., 2008; Moretti et al., 2006; Wu et al., 2006). Our observation therefore argues in favor of the recently suggested multipotent $Nkx2.5^{+}/Isl\text{-}1^{+}/Flk1^{+}$

cardiovascular progenitor cell (Laugwitz et al., 2008; Moretti et al., 2006; Wu et al., 2006). In mouse embryos, this triple positive cardiac precursor cell has been described not earlier than E8.25 which is considerably later in cardiac development than stage 15/16 in *Xenopus*. We would therefore argue that this triple positive cell is established significantly earlier in development as described before.

Another endothelial marker gene, Xmsr (Devic et al., 1996; Inui et al., 2006) is also expressed at this stage but shows a gap of expression at the ventral midline (Fig. 2A). However, both vascular marker genes, Xmsr and Flk1 do not have identical expression patterns at this early stage of development (Fig. 2A) as also observed in mouse at later stages of development (Devic et al., 1999). FoxC2 is also clearly expressed in the cardiac region with a ventral gap (Fig. 3A), an observation previously not described (El-Hodiri et al., 2001). Another cardiac marker gene, Tbx20, can be detected weakly in a diffuse spot likely representing the early cement gland (Fig. 2B).

Taken together, these findings suggest that the CPC pool might already be diverse at this early stage of development suggesting that the earliest sign of separating different clonal lineages might occur at this stage of development.

The cardiac progenitor cells at stage 20

The heterogeneity becomes even more evident at stage 20. Whereas Isl1, Nkx2.5, and Tbx1 are expressed immediately adjacent to the cement gland, the zinc-finger transcription factors GATA4, 5 and 6b exhibit a gap of expression to the cement gland (Fig. 2A). Whereas Nkx2.5 and Isl1 are still expressed in a crescent, GATA6b clearly exhibits a gap of expression at the ventral midline. It should be noted, that some earlier studies also detected this ventral gap in expression (Gove et al., 1997) but did not comment on this likely because the authors thought that the two lateral populations of cells represent the CPCs before fusion at the ventral midline. Based on the continuous expression of Isl1 and Nkx2.5 at the same stage of development we would rather argue that these lateral expression domains of GATA6b represent subpopulations of cells within the CPCs. Additionally we suggest that the fusion of the two heart fields happens earlier in *Xenopus* development because of the Nkx2.5 and Isl1 expression at stage 13 and 15/16 at the ventral midline (Figs. 1 and 2A). The related zinc-finger transcription factors GATA4 and GATA5, in contrast, do not show the gap of expression at the ventral midline (Fig. 2A). Thus, although the different GATA factors show similar expression patterns during cardiac development they are not entirely identical (Jiang and Evans, 1996). A similar gap of expression can be found in case of Tbx20. Tbx20 has in addition a more posterior but continuous expression domain (Fig. 2B). Interestingly, Dkk1 and Flk1 are expressed in the gap of Tbx20 expression in a small expression domain (Fig. 2). In contrast BMP2 exhibits a larger expression domain at the ventral midline (Fig. 2B). Note that, although Dkk1 has been suggested to function in cardiac development (Schneider and Mercola, 2001), no *in situ* data for the expression of this gene during early cardiac development in *Xenopus* have been available so far. Xmsr is still not expressed at the ventral midline (Fig. 2A). This gap at the ventral midline is also visible for eHand which in addition shows a lack of expression adjacent to the cement gland (Fig. 3A). We also provide a more detailed description of Mef2c and Mef2d expression during *Xenopus* cardiogenesis (Fig. 3A) (Chambers et al., 1992). Mef2c is weakly expressed at stage 20, whereas Mef2d transcripts can easily be visualized.

Based on these data we suggest that the CPC population at stage 20 can be subdivided into different subpopulations defined by differential gene expression. An anterior located population of cells expressing Nkx2.5, Isl1, and Tbx1 covering a horseshoe like domain (Fig. 4A). An Isl1/Tbx1 positive cell population likely extends more dorsally than the Nkx2.5 domain. Similarly, the GATA factors define a crescent shaped region more posterior to this first population of cells. Tbx20 defines a subpopulation of cells within the Nkx2.5 expression domain as it exhibits a gap of expression at the ventral midline. This midline population of cells is positive for Dkk1, BMP2 and Flk1. Although the technique of whole mount *in situ* hybridization has some limits with respect to spatial resolution and allows only limited conclusions about gene expression on a single cell level, our here provided data nevertheless indicate that already at stage 20 the *Xenopus* cardiac progenitor cell pool consists of several subpopulations characterized by differential gene expression.

First and second heart lineages at stage 24

The subdivision into first and second heart field lineages in the mouse is based on the expression of Isl1 (Cai et al., 2003). Whereas Nkx2.5 is expressed in a broad expression domain covering both, FHF and SHF, Isl1 is expressed only in the anterior part of the Nkx2.5 domain marking the SHF (Brade et al., 2007; Gessert et al., 2008) (Fig. 2A). GATA4, 5, and 6b are expressed in the FHF only (Fig. 2A). Similar, Tbx5, BMP2, FoxH1, Mef2c are expressed in the FHF (Figs. 2B and 3A). Tbx20 expression is retracting towards cells of the FHF. Note that we do not detect an expression of FoxH1 in the SHF which is in contrast to the mouse (von Both et al., 2004). FoxH1 knock-out mice lack of second lineage derived structures like the OFT and the right ventricle. Notably, *Xenopus* has only one ventricle as discussed in more detail below. Mef2d is enriched in the FHF but also shows a weaker staining in the SHF (Fig. 3A). In contrast, BMP4 and FoxC1 are expressed in the SHF (Figs. 2B and 3A). This resembles the situation in the mouse

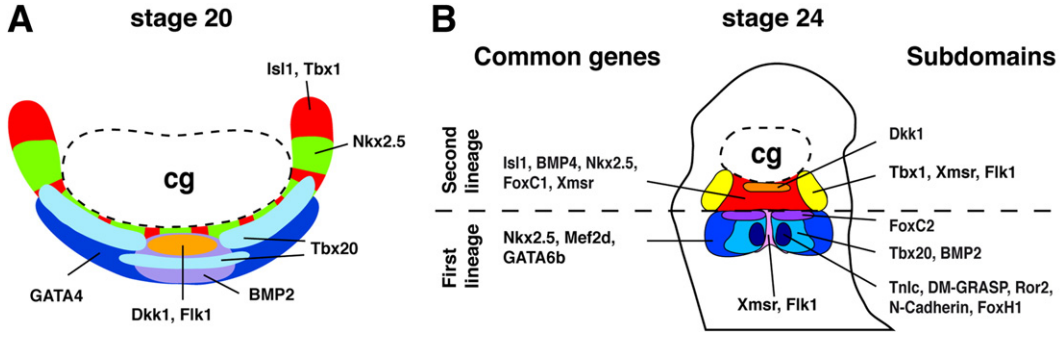

Fig. 4. Cardiac marker gene expression in *Xenopus laevis* at stage 20 and stage 24. (A) Schematic drawing of the cardiogenic region of *Xenopus* embryo at stage 20, anterior view, dorsal is to the top. Gene expression domains are color-coded as indicated. (B) Schematic picture of a *Xenopus* embryo at stage 24. A ventral view is shown, anterior is to the top. Genes expressed in the anterior region of the heart are Isl1, BMP4, Nkx2.5, FoxC1 and Xmsr (red). Nkx2.5, Mef2d and GATA6b are expressed in the FHF (blue). The anterior cardiac region can be subdivided in a Dkk1 expression (orange) and a Tbx1, Xmsr and Flk1 expression domain (yellow). The posterior heart region can be subdivided in a Tbx20 and BMP2 expressing domain (light blue) and more centrally, a domain expressing terminal differentiation marker genes (dark blue). Xmsr and Flk1 (pink) are expressed in the ventral midline of the FHF and FoxC2 between the anterior and posterior cardiac region (purple). The dashed line in A and B indicates the cement gland (cg).

where Isl1 and FoxC1 are co-expressed at E7.5 in the SHF (Seo and Kume, 2006). Also, BMP4 and Isl1 are co-expressed in the mouse (Seo and Kume, 2006). A weak BMP2 expression can be observed in the ventral midline of the SHF in *Xenopus*. In mouse, Cai et al. (2003) also described a partial overlapping expression of BMP2 with Isl1. Dkk1 is still expressed in a minor expression domain at the ventral midline of the SHF (Fig. 2B). At stage 24, Xmsr and Flk1 are also found in the region of the SHF with Flk1 likely defining the lateral borders of this territory (Fig. 2A). Also in mouse Flk1 expression was described in the SHF at stage 8.25 (Moretti et al., 2006). Both genes can be visualized also in the ventral midline of the FHF. Note the gap of expression at the ventral midline for some cardiac marker genes such as Nkx2.5 and GATA6b at this stage. This region has been indicated to harbour myeloid precursor cells as identified by Xpox2 expression (Smith et al., 2002). At stage 20, Xpox2 is expressed immediately posterior to the expression domain of Nkx2.5 and it is tempting to speculate that Xpox2 positive cells are located posterior to Nkx2.5 but in between the two lateral expression domains of GATA6b at this stage. In line with this issue is the recent discussion of a common genetic control of myeloid and cardiac lineage development in zebrafish (Peterkin et al., 2009).

The data obtained by whole mount *in situ* hybridization suggest that some genes are co-expressed in the same area of the embryo as indicated in Fig. 4B. The limited resolution of this method, however, does not allow to conclude whether these genes are co-expressed in individual cells. In an attempt to answer this question we isolated single cells of the cardiogenic mesoderm of *X. laevis* embryos at stage 24 and investigated the expression of four genes on a cellular level by RT-PCR: Nkx2.5, Isl1, Tbx1, and Tbx5 (Fig. 5). All cells tested were negative for the endodermal marker sonic hedgehog (Shh) indicating that the isolated cells are indeed of mesodermal origin and are not contaminations from the endoderm. Furthermore, a DNA contamination can be excluded, as in all shown samples at least one marker gene is negative although all primers were functional (Fig. 5B). Several signatures of gene expression could be identified repeatedly as indicated in Fig. 5A. In particular, we were able to identify $Isl1^{+}/Nkx2.5^{+}/Tbx1^{-}/Tbx5^{-}$ (signature 1) as well as $Isl1^{+}/Nkx2.5^{+}/Tbx1^{+}/Tbx5^{-}$ (signature 4) cells that represent the more anterior located cell populations (indicated in yellow and red in Fig. 4B). Furthermore, we were able to show the presence of $Isl1^{-}/Nkx2.5^{+}/Tbx1^{-}/Tbx5^{+}$ (signature 6) and $Isl1^{-}/Nkx2.5^{+}/Tbx1^{-}/Tbx5^{-}$ (signature 7) cells likely representing the more posterior located cells of the FHF (indicated in blue shades of color in Fig. 4B) and that will be the first cells contributing to formation of the heart tube later on. Other identified signatures (2, 3, 5) might represent intermediates between these populations or could indicate an even more complex picture of cardiogenic precursor cells. We cannot make any statement about the relative contribution of cells with distinct signatures within the cardiogenic area as this would require the detailed analysis of all cardiac precursors cells of an embryo. In summary, these data clearly indicate that the key signatures of gene expression as indicated in Fig. 4B can be traced back onto a cellular level.

Formation of the linear heart tube: stages 29–31

At these stages, cells of the FHF form the linear heart tube. During this process, the separation of first and second cardiac lineages as monitored by gene expression becomes more evident. The expression domains of Isl1 and Nkx2.5 are now separated into two populations of cells. The Isl1 positive cells are located more anteriorly than the Nkx2.5 expressing cells. Note the more posterior fine stripe of Ils1 expression that might represent cells of the forming IFT. Similar, the GATA factors can be detected in the region of the forming heart tube. Xmsr is expressed at the ventral midline in the forming endocardial cells (see below). Flk1 is highly expressed in two stripes of cells within the SHF (all Fig. 2A).

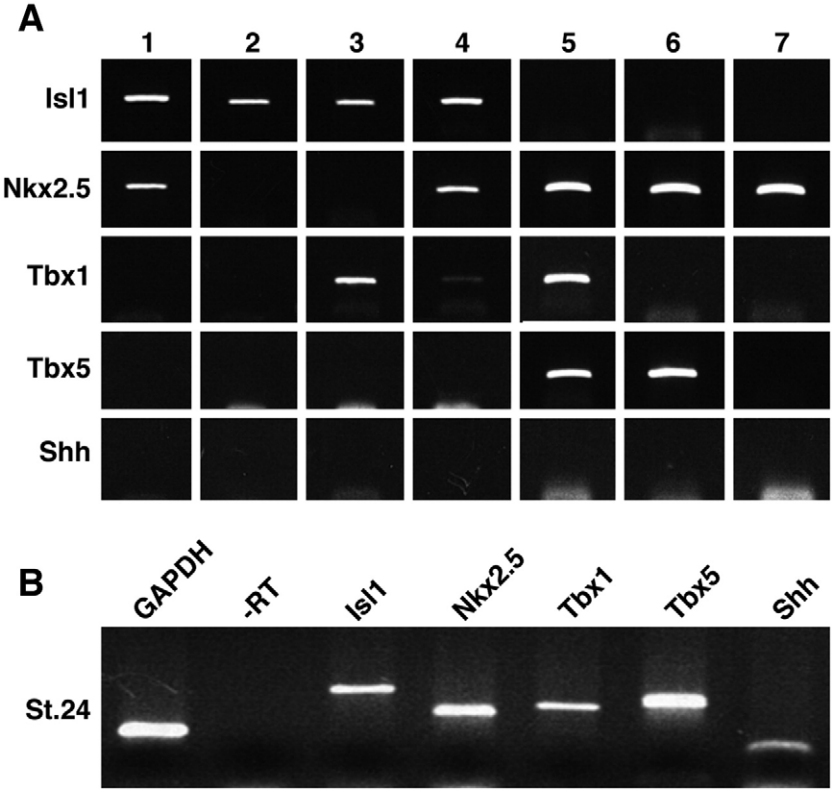

Fig. 5. Signatures of gene expression of cardiac precursor cells in *Xenopus laevis* at stage 24 in single cells. (A) Different signatures (1–7) could be identified repeatedly by single cell RT-PCR using cells from the cardiogenic region. All cells are negative for shh excluding an endodermal cell contamination as well as a DNA contamination. (B) Control RT-PCR with whole embryos indicating the functionality of all primer pairs used. GAPDH served as a positive control. −RT: negative control.

Tbx5 and Tbx20 expression at this stage is exclusively in the FHF, whereas Tbx1 is expressed in the pharyngeal arches that are part of the SHF. Whereas BMP2 is enriched in the FHF, BMP4 is strongly expressed in both populations of cells. Dkk1 is still faintly expressed in a subdomain of the SHF (all Fig. 2B).

At this stage, FoxH1 and FoxC2 are expressed in the FHF and FoxC1 expression can be detected at the ventral midline of both populations of cells. dHand and eHand can both be detected in the FHF region. Both also show an expression more posterior, likely the region of the forming sinus venosus. dHand in addition labels the pharyngeal arches that contribute to the SHF. Mef2d is expressed in FHF cells only, whereas Mef2c can be detected in both, FHF and SHF (all Fig. 3A). Dodou et al. also described the Mef2c expression in second (anterior) and first heart field in mouse (Dodou et al., 2004).

The expression of terminal differentiation markers, that started to be expressed faintly at stage 24, now is very robust in cells of the FHF. Our analysis reveals the expression of MHCα, cActin, TnIc, N-Cadherin, and DM-GRASP in these cells. Also, Wnt11-R and the non-canonical Wnt co-receptor Ror2 are expressed in the FHF. Both, however, can also be found in the pharyngeal arches (all Fig. 3B).

Marker gene expression at stage 31

To complete our analysis, we examined the expression of all marker genes at stage 31 shortly before linear heart tube closure. For this stage, we also performed an analysis in serial cross sections and provide sections on three different levels (Figs. 6 and 7) for each gene. The first level of sections (A) corresponds to the anterior region of the developing heart where the SHF is located. The second section is provided on the level of the forming linear heart tube that is in the process of closing (B). On this level the SHF is located dorsally to the heart tube (Figs. 8A, B). The third level of sections (C) is at the posterior end of the heart. Note, that the heart tube is not formed yet in this region that later on will contribute to the IFT.

In the SHF on level A the following genes were found to be robustly expressed: Isl1, Flk1 (Fig. 6A), Tbx1, BMP4 (both Fig. 6B) and dHand (Fig. 7A). GATA6b, Dkk1 and Wnt11-R can be detected in the overlying endoderm (Figs. 6A and 7B). Both, Dkk1 and Wnt11-R are secreted proteins and therefore could have an influence on the SHF. In mouse Dkk1 is expressed in the invaginating endoderm (Monaghan et al., 1999) for which an influence on heart development has been shown. Also for FGF8b, we could identify an expression in the pharyngeal endoderm, only (Fig. 6B).

Fig. 6. (A) Serial transverse sections indicate the expression of Isl1, Nkx2.5, GATA4, GATA5, GATA6b, Xmsr and Flk1 in *Xenopus laevis* embryos at stage 30/31. (B) Spatial expression of Tbx20, Tbx1, Tbx5, BMP2, BMP4, Dkk1 and FGF8b at stage 30/31. The upper row provides a lateral view. Dashed lines depict the level of sections A, B and C as indicated. Level A corresponds to the anterior region of the heart. Level B is at the level of the closing heart tube. Level C is at the posterior end of the heart where the IFT forms later in development.

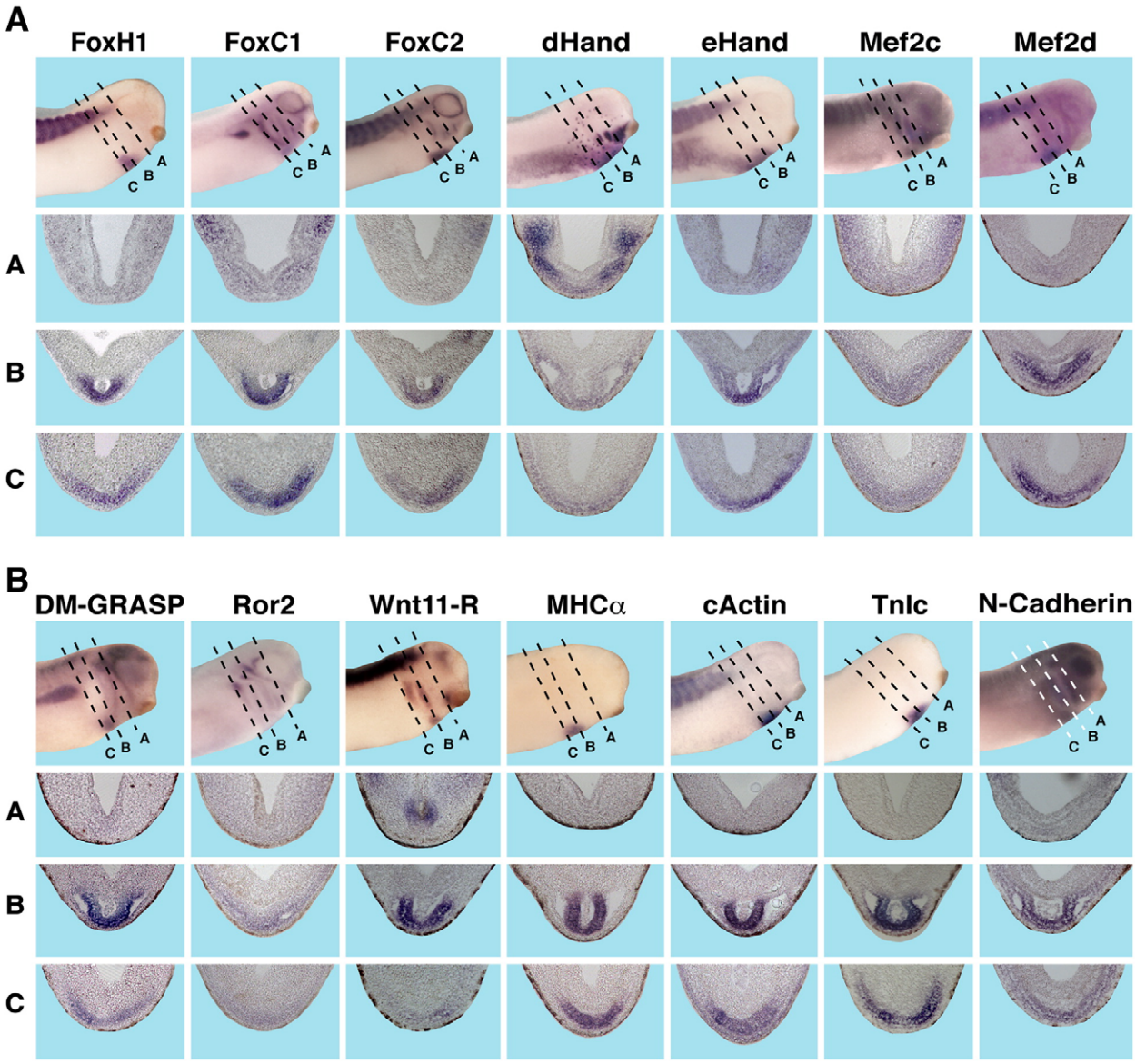

Fig. 7. (A) Expression of FoxH1, FoxC1, FoxC2, dHand, eHand, Mef2c and Mef2d at stage 30/31 in *Xenopus laevis* embryos. (B) Spatial expression pattern of DM-GRASP, Ror2, Wnt11-R, MHCα, cActin, cTnIc and N-Cadherin at stage 30/31. Embryos in the upper raw are shown from the lateral side. The dashed lines indicate the levels A (anterior part of the heart), B (closing linear heart tube) and C (IFT forming tissue) of transverse sections.

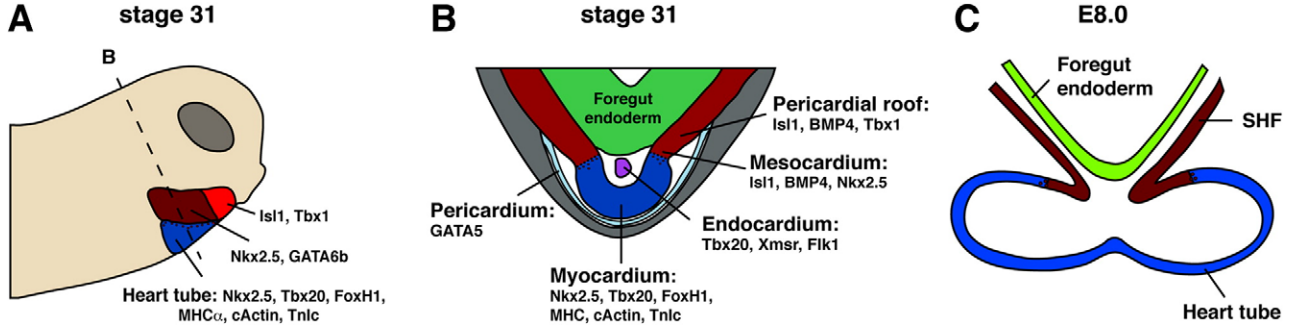

Fig. 8. Comparison of cardiac development in *Xenopus* (A, B) and mouse (C). (A) Schematic illustration of a *Xenopus* embryo at stage 31. Anterior is to the right. Cells expressing Isl1 and Tbx1 are depicted in red and dark red (additional expression of Nkx2.5 and GAGA6b). The heart tube expressing Nkx2.5, Tbx20, FoxH1, MHCα, cActin and TnIc is shown in blue. The dashed line indicates the sectional level shown in B. (B) A section through the embryo shown in panel A. The heart forming tissue can be subdivided into the myocardium (blue, first lineage), the endocardium (purple), the pericardium (light blue), the mesocardium and pericardial roof (dark red, second lineage). The foregut endoderm is highlighted in green. Genes expressed in the different tissues are listed. (C) Schematic drawing of section through a mouse embryo at E8.0. The closing heart tube is highlighted in blue, the SHF in dark red and the foregut endoderm in green. Dorsal is to the top.

In the region where the heart tube is closing (level B) different cardiac tissues can be distinguished: the myocardium, the endocardium, the pericardium, the mesocardium and the pericardial roof which is part of the SHF (see also Fig. 8B). Genes expressed in the *myocardium* at this stage are: Nkx2.5, GATA4, GATA5, GATA6b (Fig. 6A), Tbx20, slightly Tbx5, BMP2, BMP4 (Fig. 6B), FoxH1, FoxC1/2, eHand, slightly Mef2c, Mef2d (Fig. 7A), DM-GRASP, slightly Ror2, Wnt11-R, MHCα, cActin, TnIc and N-Cadherin (Fig. 7B). eHand shows a specific expression in the right myocardial wall as previously described by others (Mohun et al., 2000). In the *mesocardium* Isl1,

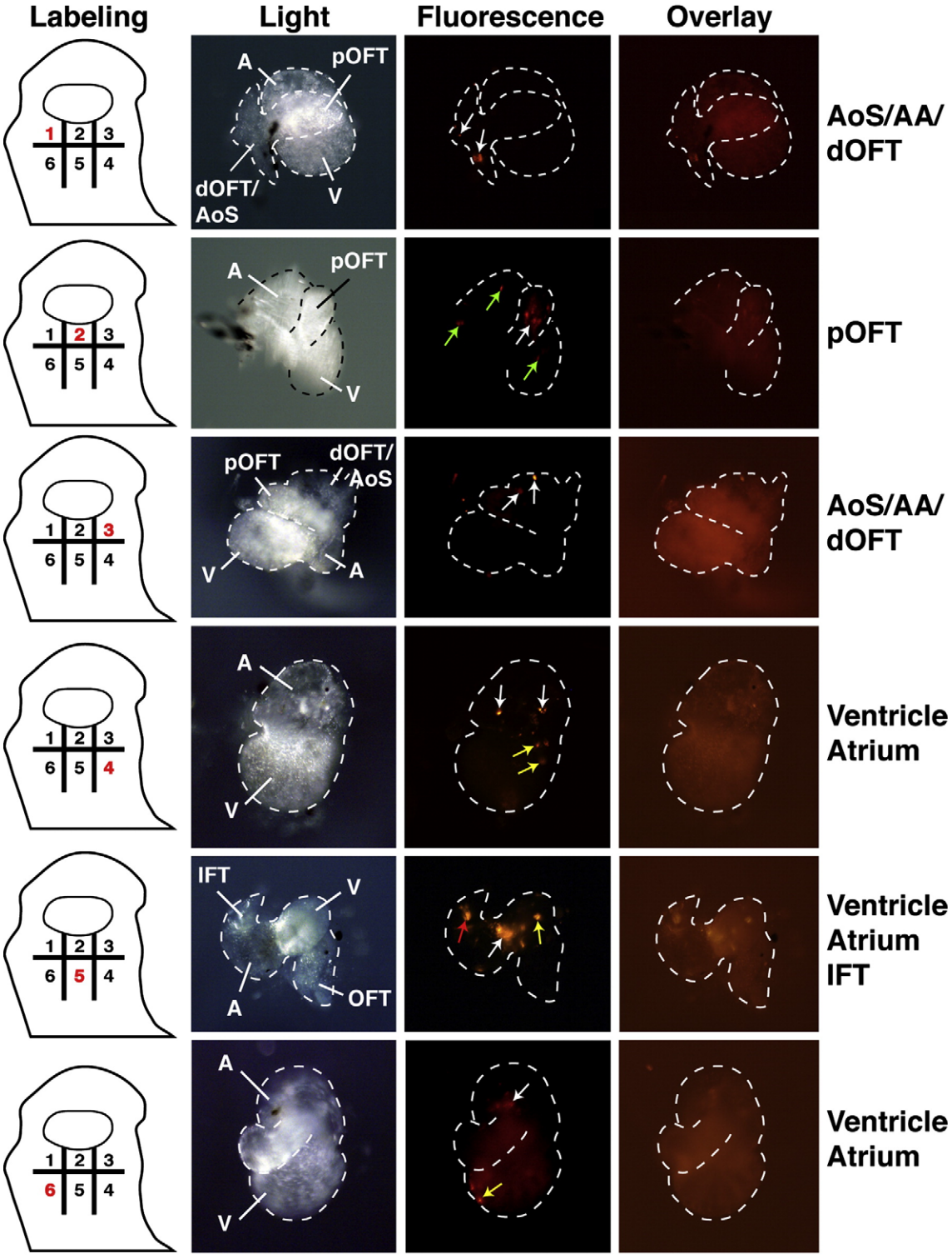

Fig. 9. Fate mapping of cardiac precursor cells at stage 24. At stage 24, DiI was used to label six defined regions of the cardiogenic region of *Xenopus* embryos (schematic illustrations on the left side, labeled regions are highlighted in red). At stage 42/43, hearts were isolated and analyzed for DiI fluorescence. Upon labeling of regions 1 and 3, DiI positive cells could be identified in the aortic sac (AoS) that connects the distal outflow tract (dOFT) to the aortic arche arteries (AA) (white arrows). Cells of region 2 contribute predominately to the proximal OFT (pOFT, white arrow). Some DiI positive cells are also presented in the aortic arches (green arrows, out of focus). Labeling in region 4 and 6 results in DiI fluorescence in the atrium (white arrows) and the ventricle (yellow arrows) as indicated. Cells of region 5 contribute to the ventricle (yellow arrow), atrium (white arrow) and IFT (red arrow). Abbreviations: A = atrium; AA = Aortic arches; IFT = inflow tract; OFT = outflow tract; V = ventricle.

Nkx2.5, GATA4, GATA5, GATA6b (Fig. 6A), BMP4 (Fig. 6B), Mef2d (Fig. 7A) and DM-GRASP (Fig. 7B) are expressed. The *pericardial roof* is characterized by the expression of Isl1, GATA5, GATA6b (Fig. 6A) and Tbx1, BMP4 (Fig. 6B), Mef2d (Fig. 7A) and DM-GRASP (Fig. 7B). Cells from both, the mesocardium as well as the pericardial roof, also contribute to the mature heart as previously indicated (Dunwoodie, 2007). In the *endocardium* which later on connects to the embryonic vascular system the endothelial marker genes Xmsr and Flk1 as well as GATA5 (Fig. 6A) and Tbx20 (Fig. 6B) can be observed.

In the posterior end of the heart (level C) where later on the IFT is forming, the following genes can be detected: Nkx2.5, GATA4, 5, 6B (Fig. 6A), Tbx20, Tbx5, BMP2, 4 (Fig. 6B), FoxH1, FoxC1/2, eHand, slightly Mef2c, Mef2d (Fig. 7A), DM-GRASP, slightly Ror2, Wnt11-R, MHCα, cActin, Tnlc and N-Cadherin (Fig. 7B). In mouse, Tbx5 also shows a graded expression with the highest levels at the posterior end of the heart tube where the sinus venosus is forming (Bruneau et al., 1999). Based on these findings we suggest a model for *Xenopus* cardiogenesis similar to those proposed for the mouse at E8 (Buckingham et al., 2005) with cells of the second lineage (SHF) laying anterior and dorsally to the heart tube (Fig. 8).

Fate mapping studies of cardiogenic precursor cells in X. laevis

The data provided so far are static gene expression data but do not allow to conclude to which regions of the mature heart these cells later on contribute to. For this purpose we subdivided the cardiogenic region of *Xenopus* embryos at stage 24 into six domains roughly representing the different domains of gene expression (Figs. 4B, 9 and Suppl. Fig. 1). DiI was then used to label a small group of cells within each domain that were later on followed during development. Labeling cells in region 1, 2, or 3, later on resulted in labeled cells populating the outflow tract at stage 43 (Fig. 9 and Table 1). Cells of the anterior medial region 2 contribute to the OFT in the majority of analyzed embryos. Interestingly, cells of the more lateral regions 1 and 3 mainly resulted in labeled cells in the aortic sac (AoS) and the aortic arch arteries (AA). In some cases, cells could also be found in the more distal part of the OFT. Note, however, that the transition from the distal outflow tract to the aortic sac is difficult to detect in those explants. These data suggest that cells of the regions 1–3 resemble the anterior or secondary heart field in chicken and mice that are also contributing to the OFT regions (Kelly et al., 2001; Mjaatvedt et al., 2001; Waldo et al., 2001). Furthermore, together with our marker gene analysis and single cell RT-PCR data these results argue for a heterogeneity of the secondary or anterior heart field regions. The OFT tract forms Tbx1$^+$ as well as Tbx1$^-$ negative cells. A similar finding was reported in the mouse (Huynh et al., 2007). Interestingly, in most cases cells of these regions also significantly contribute to the head musculature (Suppl. Fig. 2). This is consistent with data in the mouse showing the contribution of Isl1$^+$ precursor cells to this particular cell type (Harel et al., 2009; Nathan et al., 2008; Sambasivan et al., 2009).

Table 1
Fate mapping of cardiogenic mesoderm in *Xenopus laevis* embryos of stage 24.

Region	*N*	Distal. OFT/ AoS/AA (%)	Prox. OFT (%)	Ventrikel (%)	Atrium (%)	IFT (%)
1	13	92.3	7.7	0	0	0
2	11	0	90.9	9.1	0	0
3	7	85.7	14.3	0	0	0
4	11	0	18.2	90.9	81.9	0*
5	23	8.7	8.7	69.6	52.2	34.8*
6	11	0	18.2	63.6	63.6	9.1*

DiI fate mapping of regions 1–6 of *Xenopus laevis* embryos. *N* = number of experiments. Percentages for labeled regions at stage 42/43 are given. Major derivatives are highlighted in yellow. *The percentages for the IFT region are likely underestimated as the IFT is difficult to prepare and was lost in some of the explants. OFT = outflow tract; IFT = inflow tract, AA = aortic arche arteries. AoS = aortic sac.

In contrast, labeling of regions 4–6 resulted in labeled cells in the ventricle, the atrium and the IFT (Fig. 9 and Table 1). Note that the division of atria in *Xenopus* starts at stage 44 and is completed at stage 46 and requires histological analyses for differentiation (Kolker et al., 2000; Mohun et al., 2000).

Only in rare cases, labeling of cells in the more anterior regions (1–3) resulted in labeled cells in the ventricle, and vice versa, only in a few cases labeling of the more posterior region resulted in the detection of marked cells in the OFT region. The total percentage of "cross labeling" is 12% of all examined experiments (Table 1). This might also be due to fact that more than one cell is labeled by this approach. In summary these fate mapping studies at stage 24 clearly indicate two subpopulations of cardiogenic precursor cells: a first lineage that contributes to the ventricle, the atria, and the inflow tract, and a second lineage contributing to the OFT. Together with the single cell PCR data provided in Fig. 5, it becomes evident that those two main subpopulations (first and second lineage) can even be subdivided into smaller populations characterized by differential gene expression.

Furthermore, these data shed some light onto the identity of the single ventricle in the *Xenopus* heart. In mice, Isl1 positive cells are added to the arterial pole of the heart and give rise to the right ventricle as well as the outflow tract (Cai et al., 2003) and it was suggested that the interventricular septum is determined by the border between the primary heart field (forming the heart tube) and the anterior heart field (Zaffran et al., 2004). In *Xenopus* we find that Isl1 positive cells located in the anterior part of the cardiogenic area at stage 24 only contribute to the OFT but not to the ventricle. Furthermore, in mice, Tbx5 is expressed in the FHF and subsequently highest levels of expression can be found in the sinoatrial region of the linear heart tube (Bruneau et al., 1999). Loss of Tbx5 results in left ventricular defects in mice (Bruneau et al., 2001), while in Tbx5 deficient *Xenopus* embryos, the ventricle as well as the atria are affected (Brown et al., 2005). In *Xenopus*, our data suggest that Tbx5 positive cells are located in the more posterior part of the cardiogenic area and lineage labeling identified their likely contribution to the ventricle. Furthermore, we find eHand to be expressed in the more posterior region of the cardiogenic mesoderm that contributes to the ventricle (see Figs. 3 and 7). Consistently, in mice eHand is expressed specifically in the left ventricle (Firulli et al., 1998). In contrast, dHand does not show a chamber specific expression in *X. laevis* (Figs. 3 and 7), whereas in mice dHand is a marker gene for the right ventricle (Srivastava et al., 1997). Taken together, these findings strongly support the notion that *Xenopus* possess a left type of ventricle.

We next investigated whether these two lineages originate from a common precursor cell population and labeled cardiogenic cells in *Xenopus* earlier than stage 24. As the spatial distances of potentially different cell population at stage 20 were to small, we have just labeled small regions of cells in the cardiogenic region of stage 20 and stage 13 embryos and raised the question of whether cells of both lineages identified at stage 24 were labeled, e.g. whether labeling could result in descendants in the OFT as well as the ventricle and the atrium. This was indeed observed in 36% ($n = 33$) when labeling was done at stage 20, and in 67% ($n = 18$) when labeling was performed at stage 13 (Fig. 10). Although this method likely does not result in labeling of individual cells but small cell populations, these data nevertheless clearly indicate that the different cardiogenic lineages in *X. laevis* originate from a common precursor cell population identifiable as early as stage 13.

Conclusion

In summary, we provide a detailed and comparative expression analysis of cardiac marker genes during *X. laevis* development. This also allows a comparison of gene expression between *X. laevis* and the mouse (see Table 2). Despite some minor differences, the expression of cardiac marker genes in cells of the first and second lineages has

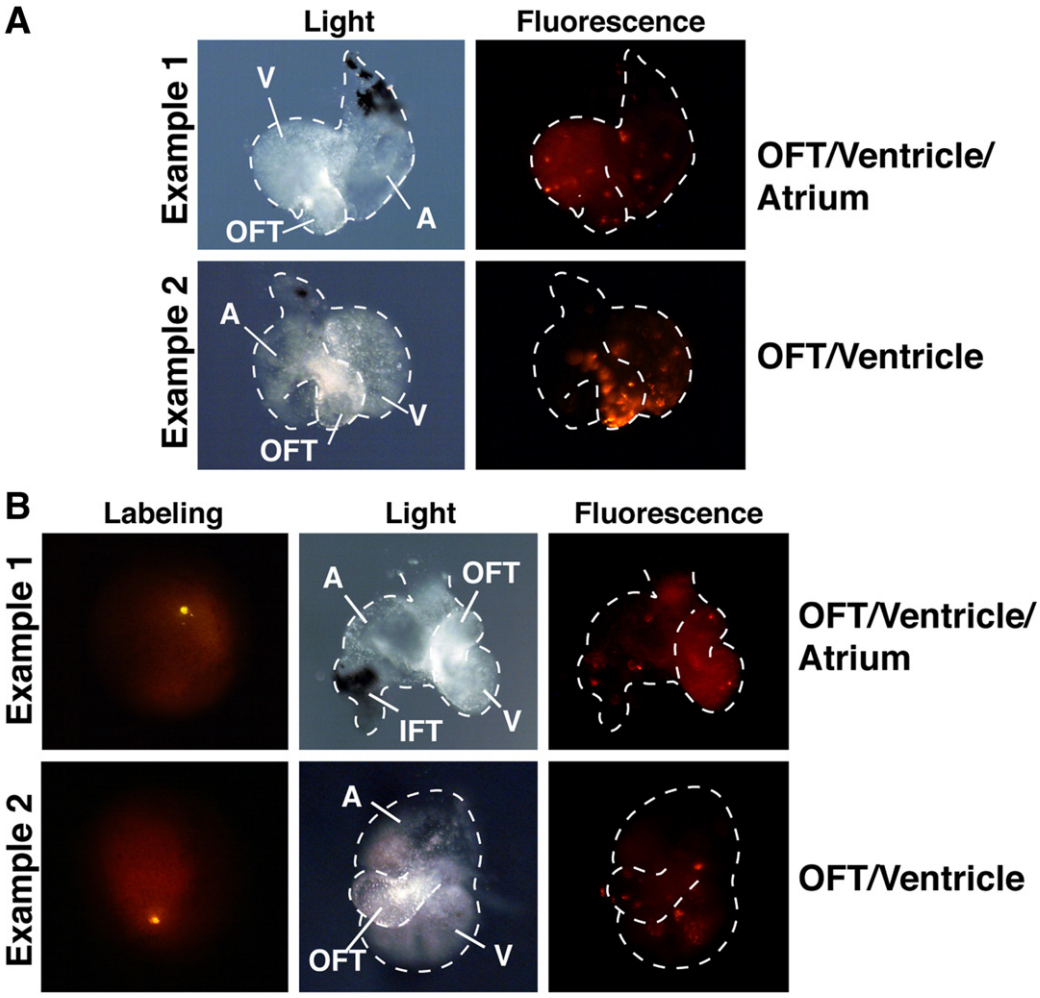

Fig. 10. Fate mapping of cardiac precursor cells at stages 20 and 13. DiI labeling was performed in stage 20 (A) or 13 (B) embryos in the cardiogenic region. Two examples for the distribution of labeled cells are shown for both stages of labeling. Additionally in B, the distribution of DiI immediately after the labeling is shown (left side). Abbreviations: A = atrium; IFT = inflow tract; OFT = outflow tract; V = ventricle.

Table 2
A comparison of gene expression of heart marker genes in mouse and *Xenopus laevis* (St. 24) in the first and second heart lineages and their derivates.

Gene	First lineage		Second lineage		References (mouse)
	Mouse	*Xenopus*	Mouse	*Xenopus*	
Ils1	No	No	Yes	Yes	Cai et al. (2003)
Nkx2.5	Yes	Yes	Yes	Yes	Lints et al. (1993); Prall et al. (2007); Stanley et al. (2002)
GATA4	Yes	Yes	No	No	Heikinheimo et al. (1994); Zhao et al. (2008)
GATA5	Yes	Yes	No	No	MacNeill et al. (2000)
GATA6b	Yes	Yes	Yes	No	Zhao et al. (2008)
Xmsr	n.d.	Yes	n.d.	Yes	Devic et al. (1999)
Flk1	Yes	Yes	Yes	Yes	Devic et al. (1999); Moretti et al. (2006); Yamaguchi et al. (1993)
Tbx20	Yes	Yes	Yes	Yes	Kraus et al. (2001); Singh et al. (2005); Stennard et al. (2003)
Tbx1	No	No	Yes	Yes	Hu et al. (2004); Xu et al. (2004)
Tbx5	Yes	Yes	No	No	Bruneau et al. (2001); Takeuchi et al. (2003)
BMP2	Yes	Yes	Yes	Yes	Cai et al. (2003); Winnier et al. (1995)
BMP4	No	No	Yes	Yes	Cai et al. (2003); Winnier et al. (1995)
Dkk1	n.d.	No	n.d.	Yes	David et al. (2008); Monaghan et al. (1999)
FoxH1	Yes	Yes	Yes	No	von Both et al. (2004); Weisberg et al. (1998)
FoxC1	No	No	Yes	Yes	Hiemisch et al. (1998); Kume et al. (2001); Seo and Kume (2006)
FoxC2	No	Yes	Yes	No	Kume et al. (2001); Seo and Kume (2006)
dHand (Hand2)	Yes	Yes	Yes	Yes	Biben and Harvey (1997); Srivastava et al. (1997); Thomas et al. (1998)
eHand (Hand1)	Yes	Yes	No	No	Biben and Harvey (1997); Srivastava et al. (1997); Thomas et al. (1998)
Mef2c	Yes	Yes	Yes	No	Dodou et al. (2004); Edmondson et al. (1994); Lin et al. (1997); Prall et al. (2007)
Mef2d	Yes	Yes	n.d.	Yes	Edmondson et al. (1994)
DM-GRASP	Yes	Yes	No	No	Hirata et al. (2006)
Ror2	Yes	Yes	No	No	Al-Shawi et al. (2001); Matsuda et al. (2001); Takeuchi et al. (2000)
xWnt11-R mWnt-11	Yes	Yes	No[a]	No	Kispert et al. (1996); Singh et al. (2005)

For *Xenopus* references see this paper and references therein.
[a] mWnt-11 is expressed in the SHF-derived OFT later during development.

been conserved during evolution. Both populations of cells are thought to derive from a common precursor cell (Meilhac et al., 2004). In *Xenopus*, this population of cells is positive for Isl1, Nkx2.5, GATA6b, and Tbx1. Fate mapping studies indicated that this population of cells contributes to all parts of the heart. As outlined before, we consider stage 24 of *Xenopus* embryogenesis to be equivalent to E7.5 of mouse embryogenesis. Earlier expression analyses of marker genes during mouse embryogenesis are rarely available. Our data here indicate that the CPC population is heterogeneous to a much earlier time point of development suggesting an early segregation of different lineages with distinct genetic programs (Fig. 4 and Suppl. Fig. 3).

At stage 24, Isl1 positive cells in the anterior cardiac region likely represent a population of cells comparable to the anterior or secondary heart field in mice and chicken and later contribute to the OFT region as well. We could not unambiguously demonstrate a contribution of Isl1 cells to the inflow tract region in *Xenopus* in this study. However, we recently demonstrated that a loss of Isl1 function results in deficits in all regions of the heart suggesting a contribution of Isl1 positive cells also to the IFT in *Xenopus* (Brade et al., 2007). This is further supported by the finding of an $Isl1^+$ cell population at stage 29 in the region where the IFT is forming (Fig. 2). The definite answer to this question will be available once we will have generated transgenic reporter lines in *Xenopus*. At this point, however, it is very likely that in *Xenopus* as well as in mice and chicken, the mechanism of extending the heart tube is very similar. This is in contrast to a recent study in zebrafish showing that Isl1 positive precursor cells only contribute to the IFT region (de Pater et al., 2009).

Based on our data we would like to suggest that the heart develops from a single cardiac field which is patterned within its borders as it has been also suggested by others (Abu-Issa and Kirby, 2008; Abu-Issa et al., 2004; Moorman et al., 2007). Furthermore, our data indicate that *X. laevis* is a suitable model organism to subsequently study the molecular mechanisms underlying segregation of different cardiac lineages by use of transgenic *Xenopus* embryos.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ydbio.2009.07.037.

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