Cardiac differentiation in *Xenopus* is initiated by *mespa*

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Received 24 May 2012; revised 26 October 2012; accepted 28 November 2012; online publish-ahead-of-print 12 December 2012

Time for primary review: 33 days

Aims

Future cardiac repair strategies will require a profound understanding of the principles underlying cardiovascular differentiation. Owing to its extracorporal and rapid development, *Xenopus* laevis provides an ideal experimental system to address these issues in vivo. Whereas mammalian MesP1 is currently regarded as the earliest marker for the cardiovascular system, several MesP1-related factors from *Xenopus*—mespa, mespb, and mespo—have been assigned only to somitogenesis so far. We, therefore, analysed these genes comparatively for potential contributions to cardiogenesis.

Methods and results

RNA in situ hybridizations revealed a novel anterior expression domain exclusively occupied by mespa during gastrulation, which precedes the prospective heart field. Correspondingly, when overexpressed mespa most strongly induced cardiac markers in vivo as well as ex vivo. Transference to murine embryonic stem (ES) cells and subsequent FACS analyses for Flk-1 and Troponin I confirmed the high potential of mespa as a cardiac inducer. In vivo, Morpholino-based knockdown of mespa protein led to a dramatic loss of pro-cardiac and sarcomeric markers, which could be rescued either by mespa itself or human MesP1, but neither by mespb nor mespo. Epistatic analysis positioned mespa upstream of mespo and mespb, and revealed positive autoregulation for mespa at the time of its induction.

Conclusions

Our findings contribute to the understanding of conserved events initiating vertebrate cardiogenesis. We identify mespa as functional amphibian homologue of mammalian MesP1. These results will enable the dissection of cardiac specification from the very beginning in the highly versatile *Xenopus* system.

Keywords

Cardiac induction • mespa • mespb • mespo • *Xenopus*

1. Introduction

Future cardiac cell therapy requires a profound understanding of the principles underlying cardiovascular differentiation during vertebrate embryogenesis is mandatory. The *Xenopus* system has been exploited very successfully to investigate cell specification during early development. Key features of the cardiac regulatory blueprint are conserved, making *Xenopus* an attractive system to study molecular aspects of congenital heart diseases.¹ Owing to its extracorporal development, *Xenopus* provides a versatile and convenient platform to perform gain and loss of function approaches via microinjections. Thereby, molecular reagents can be administered either globally or targeted to specific areas based on detailed fate map information.²,³ As a pumping heart is formed in *Xenopus* tadpoles within 42 h of development, rapid experimentation is feasible in this model system.

In mammals the heart is formed from a common cardiac progenitor pool, which splits into first and second heart field lineages that form distinct heart compartments.⁴,⁵ In *Xenopus* cardiac progenitor cells arise during early gastrula stages as a pair of cellular patches representing dorsolateral mesoderm.³ Factors uniquely demarcating the earliest cardiac precursors are unknown in lower vertebrates. Cellular movements during gastrulation result in dorso-anterior translocation of these patches and subsequently in their ventral migration during neurula stages, which is accompanied by the induction of early

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cardiac transcriptional regulators. The heart progenitors converge anteromedially, thereby forming the equivalent of the mammalian cardiac crescent. Based on single-cell gene expression analysis and lineage tracing, these early progenitors combine features of both primary and secondary heart fields. Subsequently, a primitive linear tube is formed at the ventral midline from bilateral pre-cardiac patches of mesodermal cells expressing heart field markers, before looping, and remodelling of the heart tube takes place until tadpole stages. While coordinated heart contractions are established at the early tadpole stage, cardiac structural proteins are expressed already at the mid-tadbud stage.

We and others have recently characterized the early cardiovascular regulator MesP1, a bHLH transcription factor that is expressed very early in nascent mesoderm and essential for cardiogenesis. In contrast, the closely related MesP2 protein controls somitogenesis. This work revealed MesP1 as being the first factor sufficient to induce cardiovasculogenesis when ectopically overexpressed in differentiating embryonic stem (ES) cells. MesP1 binds in the vicinity of many cardiogenic regulatory genes and has been shown to activate directly transcription of the Wnt-inhibitor dkk1. Thus, MesP1 is acting at the top of a gene network promoting cardiac differentiation by cell-autonomous and non-autonomous mechanisms.

The high evolutionary conservation of MesP1-driven cardiovascular induction becomes evident from the fact that the chordate Ciona requires a mesp-related factor for normal cardiogenesis. Several mesp-related genes exist also in Xenopus and Zebrafish; however, no cardiogenic activity has been demonstrated for any of them yet. In this study, we have systematically analysed Xenopus mesp-related genes with respect to their potential involvement in cardiogenesis.

2. Methods

2.1 Xenopus laevis experiments

Animal work has been conducted in accordance with the Directive 2010/63/EU of the European Parliament; experimental use of Xenopus embryos has been licenced by the Government of Oberbayern (Projekt/AK ROB: 55.2.1.54-2532.6-3-11). Embryos were obtained by in vitro fertilization, cultured in 0.1× Modified Barth’s Saline as described, and staged according to the Xenopus normal table. Details on microinjection, Morpholino oligonucleotides (MO), and lineage tracing can be found in the Supplementary material online.

2.2 Plasmid construction and in vitro mRNA synthesis

The coding regions of Xenopus mesp-related cDNAs, specified in Supplementary material online, Figure S1B, were subcloned via PCR into the pRES-EGFP-2 vector for overexpression studies in Xenopus and ES cells. For details, please refer to Supplementary material online. For microinjections, capped mRNAs were generated in vitro as described.

2.3 RNA in situ hybridization

Whole-mount RNA in situ hybridizations were performed as described with antisense, digoxigenin-labelled RNA probes. Hybridized probes were detected with alkaline phosphatase-conjugated secondary antibodies (Roche) by NBT/BCIP staining. For further details, see Supplementary material online.

2.4 RNA extraction and real-time RT–PCR

Total cellular RNA was purified from ES cells and subjected to qRT–PCR analysis for monitoring relative gene expression levels as described.

Factors of changes in relative mRNA expression levels were calculated by normalization to histone H4 mRNA. Further details are provided in Supplementary material online.

2.5 ES cell culture and flow cytometry

Electroporation and isolation of stable clones using the murine ES cell line GSES-1 were performed as described. For further details on propagation, differentiation, and FACS analysis, please refer to the Supplementary material online.

2.6. Statistical analysis

Molecular phenotypes were validated using the two-tailed Fisher’s exact test. Significance of gene expression analysis in differentiating ES cells was analysed by the two-tailed Student’s t-test.

3. Results

3.1 The mesp-related bHLH gene family of X. laevis

The unique activity of human MesP1 to induce ectopic cardiogenesis in Xenopus embryos prompted us to search for an endogenous MesP1-related bHLH protein in Xenopus. Several candidates were identified based on sequence similarity to mammalian MesP1/MesP2 (see Supplementary material online, Figure S1). Since X. laevis has an allotetraploid genome, we found these candidates to be derived from non-allelic gene pairs. Two of these pairs we refer to as mespalpha/mespab and mespbalpha/mespbbeta, respectively. They have been studied extensively in the process of somitogenesis. The protein mesp have is related to mammalian pMesogenin1, but it shows also similarity with mammalian MesP and Xenopus mespab (Supplementary material online, Figure S1). Like the latter proteins, it has been implicated in somitogenesis. Mespalpha/mespb constitutethe third mesp-related gene pair in Xenopus.

As shown in Supplementary material online, Figure S1, the degree of sequence divergence between mammalian and Xenopus mesp-related proteins precludes the assignment of syngenic relationships. In previous studies, functional interference with mespb and mespo disrupted somitogenesis, with no reported effects on the heart. In contrast, the biological activities of mesp have not been investigated yet. Therefore, we undertook a systematic analysis to identify cardiogenic gene(s) functionally equivalent to mammalian MesP1 in Xenopus.

3.2 Expression of mespa implies a role in cardiac progenitor specification

Side by side in situ hybridization of sibling embryos from late blastula (NF9) to early neurula stages (NF13.5) revealed that the different mesp-related genes were consecutively activated. First, mespa mRNA appeared at early gastrula (NF10.25) in the preinvoluting mesoderm, when also mespo signals came up, although weaker than mespa (Supplementary material online, Figure S2A and F). This suggested a delay in mespo induction compared with mespa, which was confirmed by qRT–PCR (data not shown; see also gene expression levels for mespa and mespo on http://xenbase.org/common/). Expression of both genes persists during involution of the mesoderm (Supplementary material online, Figure S2B’ and G’). In contrast, mespb mRNA could not be detected before late gastrula (NF12.5–13), when it forms stripes in paraxial mesoderm (Supplementary material online, Figure S2N and O). These observations are in agreement with previous reports.
3.3 Mespa-related genes trigger ectopic cardiac differentiation in Xenopus embryos

In addition, we observed a novel transient expression domain for mespa in the anterior of late gastrulae (NF12.5; Figure 1). Mespa is expressed in two stripes of cells along the forming notochord and in two anterior wings, located at or close to the anterior front of the involuted mesoderm (Figure 1A and B and Supplementary material online, Figure S2D). At the lateral edges of these anterior wings, we detected a novel mespa positive cell population that extends in a salt and pepper pattern towards the anterior midline (Figure 1C). This anterior expression of mespa persists until late gastrula in the prospective domain of the cardiogenic region, in which dkk1, as well as isl1 and nkx2.5, mRNAs become detectable at about the same time (Figure 1E and F). Therefore, mespa is the first gene, whose expression bridges in Xenopus the temporal and spatial gap between cardiac induction in preinvoluting dorsolateral mesoderm, migratory cardiac precursors in prospective head mesoderm, and formation of the cardiac crescent.2,24

Figure 1 Mespa mRNA demarcates anterior cardiac progenitors at the late gastrula. (A–D) Whole mount RNA in situ hybridization for mespa at the late gastrula (NF12.5). Images display a representative embryo photographed from different angles to illustrate details of mespa expression. Scale bar: 1000 μm. (A) Posterior view, dorsal to the top—note: (i) periblastoporal staining in posterior mesoderm (arrowhead), (ii) longitudinal stripes next to notochord (asterisk), (iii) two anterior wings in involuted mesoderm (arrows). (B) The oblique dorsal view showing mespa positive cells anterolateral to the wings (black ovals). (C) The lateral view of the right side. Oval encircles a patch of mespa positive cells apparently emanating from the right wing towards the ventral midline. (D) Anterior view, dorsal to the top—salt and pepper pattern of mespa positive cells merging in anteriorventral position. White oval marks the cardiogenic region, which at this stage starts to express (E) dkk1 and the heart field markers (F) isl1 and nkx2.5.

In most cases, DNA-born overexpression of mespa-related genes did not enlarge or perturb the endogenous heart. This might have to do with the fact that injected plasmids stay episomal in Xenopus, however, were not observed for mespa. Thus, while all tested mesp-related factors can principally induce cardiac markers in Xenopus, mespa and hMesP1 stand out in both quantitative and qualitative terms.

Figure 2B–G markers exclusively in the heart (Figure 2B and C). Notably, ~10% of embryos injected with mespa or hMesP1 (but not with mespb and mespa) contained intense nkx3 staining in distinct hypomorphic tissue structures, comparable in size to the endogenous heart (Figure 2L and M). These structures were found both separated from and in contact with the endogenous heart. Areas with ectopic beating activity as reported for human MesP1 overexpressed in Xenopus, however, were not observed for mespa. Thus, while all tested mesp-related factors can principally induce cardiac markers in Xenopus, mespa and hMesP1 stand out in both quantitative and qualitative terms.

About 90% (P < 0.0001) of either mespa or hMesP1 injected embryo halves showed a strong ectopic expression of nkx2.5 and nkx3 in cell clusters (Figure 2D–G). Mespb and mespa induced smaller clusters at lower frequency (Figure 2H–K). Ectopic heart marker expression occurred in the anterior of the embryos and was never observed near the tailbud, where mespo is continuously expressed.18 This may be due to activities antagonizing cardiac differentiation in the posterior, for instance a higher level of canonical Wnt signal.25 Wild-type and GFP-injected embryos expressed cardiac markers exclusively in the heart (Figure 2B and C). Notably, ~10% of embryos injected with mespa or hMesP1 (but not with mespb and mespa) contained intense nkx3 staining in distinct hypomorphic tissue structures, comparable in size to the endogenous heart (Figure 2L and M). These structures were found both separated from and in contact with the endogenous heart. Areas with ectopic beating activity as reported for human MesP1 overexpressed in Xenopus, however, were not observed for mespa. Thus, while all tested mesp-related factors can principally induce cardiac markers in Xenopus, mespa and hMesP1 stand out in both quantitative and qualitative terms.

In most cases, DNA-born overexpression of mespa-related genes did not enlarge or perturb the endogenous heart. This might have to do with the fact that injected plasmids stay episomal in Xenopus and are activated only in mosaic fashion after zygotic genome activation at mid-gastrula. Therefore, we also overexpressed these factors by targeting mRNA microinjections into single dorsovegetal blastomeres.
3.4 Mespa and mespo induce cardiogenesis in naïve animal cap explants

Mammalian MesP1 biases ES cells towards the cardiovascular lineage. A rapid test for such a function is provided by the *Xenopus* animal cap assay. Embryos were bilaterally injected into the animal pole with 1 ng of the respective mRNAs at two-cell stage and cultured until late blastula (NF9), when the blastocoeol roof was explanted (Figure 3A). At this stage, the explants (‘animal caps’) contain pluripotent cells. Subsequently, they were tested by in situ hybridization for early heart field markers (dkk1 and isl1), definitive cardiac markers (tbx20 and tnni3), and for actc1 (Cardiac Actin) that is expressed embryonically in both skeletal and cardiac muscle.

As shown in Figure 3B, GFP-injected explants did neither express dkk1 nor muscle markers, confirming the absence of mesodermal contamination. However, they contained always some isl1+ cells, which we consider as a base level. Indeed, isl1 mRNA is expressed in the anterior ectoderm (Supplementary material online, Figure S11). Both dkk1 and isl1 were superinduced most prominently by mespa and mespo (P ≤ 0.001), but much less frequently by mespb or myoD (P ≤ 0.05). Only mespa- and mespo-induced definitive heart markers. Either protein activated tbx20 transcription in small clusters or isolated cells, while mespo overexpression induced larger tnni3-positive territories. In contrast, mespb failed to activate definitive cardiac markers in animal caps, although it induced weakly ectopic tnni3 expression in the embryo (Supplementary material online, Figure S3K). Actc1 was strongly up-regulated in explants injected with the skeletogenic regulatory factor myoD as reported. However, the myoD caps never expressed definitive cardiac markers. The mespo- and mespo-injected explants also abundantly expressed actc1, beside tbx20 and tnni3. This differential response to bHLH protein overexpression suggests that the explants distinguish precisely between skeletogenic (myoD), cardiogenic (mespa/mespo), and somitogenic (mespb) activities of bHLH proteins. Together these findings indicate that mespa and mespo can promote the cardiac fate in uncommitted embryonic cells.

3.5 Cardiogenic activities of mesp-related proteins in murine ES cells

To further compare the cardiogenic potential of mesp-related genes, we transfected the above-mentioned constructs into murine ES cells. Whereas we successfully generated normally appearing clones with mespo and mespo, we were unable to propagate cells transfected with mespb (Supplementary material online, Figure S4). FACS analysis for mespa and mespo-transfected cells revealed a strong decrease in EGFP expression during ES cell differentiation (Supplementary material online, Figure S5), in agreement with our previous observations.

We performed FACS analyses for Flk-1, the earliest surface protein demarcating lateral plate mesoderm. Flk-1 positive cells were not observed in the undifferentiated state (Figure 4A). Compared with
control cells, mespa overexpression led to a strong increase (three- to four-fold) in Flk-1+ cells during differentiation; a weaker increase was observed in mespo expressing cells. This increase is comparable with our previous results with hMesP1.8 Likewise, cells expressing Troponin I were highly increased by mespa, and moderately by mespo. The cardiogenic activity of mespa was confirmed by qRT–PCR assays for Flk-1, GATA-4, and MLC-2v (Figure 4B–D). Thus, also in the heterologous ES cell system, mespa and to a lesser extent mespo enforce cardiogenesis.

3.6 Mespa is required for cardiac gene expression

For loss-of-function analysis, we designed specific, translation-blocking antisense MOs, targeting sequences conserved between the non-allelic gene pairs (Supplementary material online, Figure S6A). These MOs inhibited efficiently their target mRNAs (Supplementary material online, Figure S6B). We injected 10 ng of each MO together with fluorescent dextrans into dorsovegetal blastomeres that contribute largely to the heart (Figure 5A). By in situ hybridization, we assessed the expression of dkk1 (early neurula/NF15) and the cardiac markers tbx5 and tbx20 (tailbud stage/NF24) (Figure 5B).

Uninjected and control MO (CoMO)-injected embryos displayed a symmetric expression of these markers in cardiac crescent and precardiac patches prior to heart tube formation. Dkk1 expression was strongly and frequently down-regulated in mespa morphants. The mespo MO had much less an effect on dkk1, whereas mespb and control morphants were comparable. Similarly, both T-box transcription factors were down-regulated in mespa morphants, showing a reduced or even absent stain in the injected side. The mespo MO reduced tbx20 expression only mildly in some embryos, but not tbx5. Knockdown of mespb had no discernable effect. In addition, nkker2.5 was not inhibited by any of the mesp-related MOs, even at higher MO doses or pairwise coinjections (data not shown). We also investigated the actc1 gene, which is transcribed in skeletal and cardiac myocytes. Its mRNA was reduced in the heart of mespa morphants (Supplementary material online, Figure S7). Because the mespb and mespo MOs had no or little effect on cardiac gene expression, we...
Figure 4 Xenopus mespa and mespo proteins promote cardiomyogenesis in murine ES cells. (A) Comparative FACS analysis for Flk-1 or Troponin I (TnI) in mespa-transfected, mespo-transfected, or untransfected (control) GSES-1 cells. Undifferentiated (Day 0) ES cells reveal no significant Flk-1 expression. Compared with the control, appearance of Flk-1 positive cells was approximately four-fold increased in mespa clones vs. approximately two-fold increased in mespo clones at Day 6 of differentiation, and approximately three-fold increased in mespa clones vs. ~1.5-fold increased in mespo clones at Day 18 of differentiation. Troponin I expression was monitored at Day 18 of differentiation. Compared with the control, TnI+ cells are increased approximately three-fold in mespa clones, but less than two-fold in mespo clones. (B–D) qRT–PCR analysis at Day 10 of differentiation. Relative to control cells, mespa clones show a strong increase in mRNA levels for (B) Flk-1 (approximately four to eight-fold), (C) GATA-4 (~8–17-fold), and (D) MLC-2v (~10–17-fold). Data are mean ± SEM; n = 6; *p ≤ 0.05; **p ≤ 0.01; ***p ≤ 0.005.
ascertained their in vivo blocking efficiency by injecting them into ventral mesoderm and monitoring their effects on somitogenesis. In agreement with previous results,20 both MOs inhibited pre-somitic expression of the notch ligand delta-like 1 (dll1, Supplementary material online, Figure S8).

The injected dorsovegetal blastomeres contribute both to the heart and to signalling centres that control embryonic patterning (i.e. Nieuwkoop center, Spemann’s organizer). Injections into this territory may cause phenotypes by unspecific perturbation of these centres. Therefore, we performed two additional control experiments. First, as a negative control we injected a MO against ngnr1, a Xenopus proneural bHLH protein.31 As expected, its effect on the heart was as negligible as the standard CoMO (Supplementary material online, Figure S9). Secondly, we coinjected mespa MO with hMesP1 mRNA, which largely rescued tbx5 and tbx20 expression (Supplementary material online, Figure S9). Also tnni3 mRNA, which was strongly down-regulated in mespa morphants, was partially restored by hMesP1 mRNA (Supplementary material online, Figure S10). Therefore, cardiogenesis clearly depends on mespa.

To extent the analysis of the requirements for mespa to earlier stages of cardiogenesis, we injected the mespa MO into one blastomere at the two-cell stage and investigated isl1 expression at early

**Figure 5** Mespa is required for cardiac differentiation. (A) Embryos were unilaterally injected with 10 ng of Morpholino (MO) into one dorsovegetal blastomere at eight-cell stage and analysed by in situ hybridization at the indicated time points for the respective cardiac markers. (B) dkk1—anterior view, right-side injected (dashed line demarcates the midline); tbx5—side views, anterior left, left-side injected; tbx20—ventral view, anterior left, right-side injected. WT—uninjected control; CoMO—standardized control MO. Scale bars: 500 μm. (C) Distribution of dkk1, tbx5, and tbx20 phenotypes; x-axis, total number of embryos/condition; n = 3 experiments.
neurula (Supplementary material online, Figure S11). The vast majority of these embryos was deficient for isl1 mRNA on the injected side (72%, $P \leq 0.001$). Parasagittal sections revealed that the absence of isl1 is coupled to a migratory defect of the involuted mesoderm, which stays behind on the morphant side. This finding indicates a role for mespa in the formation of all cardiac precursor cells.

### 3.7 Mespb and mespo cannot compensate for mespa

The murine MesP1/MesP2 genes can partly compensate for each other. We were wondering, whether this might be the case for the mesp-related genes as well. Therefore, we investigated, whether mesp-related proteins rescue tbx20 expression in mespa morphants (Figure 6). Coinjection of a MO-insensitive mespa variant mRNA restored the tbx20 stain on the injected side (Figure 6B, C, and G), comparable with hMesP1. In contrast, neither mespb nor mespo could achieve this (Figure 6D–F, H and I). Interestingly, mespo, although failing to activate tbx20 expression within the heart, induced it ectopically in the vicinity (Figure 6E–F). We conclude that mespa induction in dorsolateral mesoderm is required for cardiogenesis due to unique structural characters lacking in mespb/mespo proteins.

### 3.8 Epistasis of Xenopus mesp-related genes

Although our analysis suggested a sequential activation of mesp-related genes (Supplementary material online, Figure S2), other studies reported coinduction of mespa and mespb at late blastula by RT–PCR. To unravel the epistatic relationships among mesp-related genes, we injected embryos with mesp-related MOs and monitored mespa and mespo expression at early gastrula (Supplementary material online, Figure S12). As expected from its temporal expression profile, the mespb MO did not impact transcription of mespa or mespo genes, similar as the ngnr1 MO (Supplementary material online, Figure S11A and B). In contrast, knockdown of mespa strongly reduced mespo mRNA levels and also its own transcription (Supplementary material online, Figure S11A and B). We validated the specificity of this phenotype by coinjecting hMesP1 mRNA, which restored both mespa and mespo mRNA levels (Supplementary material online, Figure S11C and D). We conclude that mespa lies upstream of mespo and stimulates its own transcription via a positive feedback loop.

### 4. Discussion

We have identified here mespa as the sole functional Xenopus homologue of mammalian MesP1. This claim rests on three independent lines of arguments discussed below.

First, among the three mesp-related genes, only mespa is expressed at the right time and place to control specification, migration, and dif-
ferentiation of cardiac progenitors. RNA in situ hybridization analyses and epistasis experiments identified mespa as the earliest induced member of this family. Collectively, our analysis rules out a function for mespb or mespo in Xenopus cardiogenesis, and reveals that mespa is downstream of mespa during cardiac induction at the gastrula. As the mesodermal mantle involutes and moves anteriorly, mespa mRNA is maintained in two mediolateral wings at or near the leading edge, corresponding to the head mesoderm of Xenopus. Most notably, we found mespa to be transcribed in a domain that extends from the lateral edges of these cranial stripes to the ventroanterior midline, probably representing migrating cardiac progenitors. Around this time, heart field markers, such as isl1 and nklx2.5 (Figure 1), are induced in this location, and soon form the Xenopus equivalent of the cardiac crescent. An overlapping expression between mesp-related factors and pro-cardiac markers has not been described before in vertebrates, possibly due to the transient expression of mesp-related factors. This novel expression domain in Xenopus suggests that mespa stimulates cardiac crescent formation by direct activation of downstream target genes, as has been shown in ES cells. This conclusion is also supported by the results of mespa overexpression in animal caps. However, the initial isl1 and nklx2.5 domains appear more condensed than the punctate pattern of mespa in this region. Therefore, it seems likely that mespa stimulates their transcription through both cell-autonomous and non-autonomous mechanisms. A candidate for a non-autonomous signal is the validated mammalian MesP1 target gene dkk1. We have shown here that (i) dkk1 is co-expressed with mespa in punctate staining in the anterior of the embryo, (ii) dkk1 mRNA is induced by mespa overexpression in animal caps, and (iii) endogenous dkk1 mRNA is extinguished in the cardiac crescent of mespa morphant embryos. Our results are in full agreement with the reported cardiac function of dkk1 in Xenopus. A second source of support for the claim that mespa represents the functional homologue of mammalian MesP1 is derived from gain of function experiments. Although overexpression of any mesp-related family member resulted in some ectopic expression of cardiogenic regulators and/or differentiation markers, mespa stands out consistently as the strongest cardiogenic inducer. It produced ectopic cardiogenic areas of the largest size and with the highest frequency, comparable with hMesP1. It was also the only family member that could induce abundant tnni3 expression in naive animal caps. Other cardiac regulators, such as nklx2.5 or isl1, lack comparative activity. Although mespb could not be stably expressed in undifferentiated mouse ES cells, mespa promoted the induction of cardiogenic mesoderm with higher efficiency than mespo, and at least as efficient as hMesP1. This is remarkable, given that mespa and hMesP1 share only ~30% of sequence identity. A detailed analysis of mespa’s cardiogenic potential in both Xenopus and ES cells may aid the identification of structural domains associated with cardio-specific activity of MesP1.

The final argument for mespa being the functional MesP1 homologue derives from its unique loss of function phenotype. Although mespb and mespo knockdown revealed very little impact on heart marker gene expression, the mespo MO displayed strong defects that could be traced back to the preinvoluting dorsolateral mesoderm. Owing to interference with its autocatalytic loop, such morphants showed reduced transcription of mespa, suggesting that this protein is already active at the time when cardiac specification occurs in Xenopus. Subsequently, heart-specific transcription in the pre-cardiac patches was severely impaired in mespa morphants for both regulatory (dkk1, isl1, tnni3, actc1) and sarcomeric (tnni3, actc1) marker genes. These defects were rescued by hMesP1 and mespa, but not by mespb or mespo. In contrast, MO-mediated knockdown of other cardiac regulatory factors is known to result mostly in phenotypes during late differentiation of the embryonic heart. Xenopus tnni3 and tbnx5 morphant embryos express relatively normal levels of cardiac differentiation markers until the heart tube stage, whereas subsequent morphogenesis of the heart is perturbed. Nicx2.5 depleted zebralife show defects in heart looping, similar to Nicx2.5 morphant mice. The depletion of several GATA factors from the cardiogenic region reduced tnni3 only at late tailbud stages and reduced the size of the heart anlage. An exception to this is isl1—morphant embryos form smaller hearts with disturbed looping, but in addition, both early (tbnx20, nicx2.5) and late (tnni3, actc1) markers are downregulated already in the pre-cardiac patches. While the morphant phenotypes are similar for isl1 and mespa, overexpression of isl1 is not sufficient to induce ectopic cardiac differentiation—a hallmark activity of mespa as we have shown here. Furthermore, our gain and loss of function analysis (Figures 3 and Supplementary material online, Figure S11) indicates that isl1 is genetically downstream of mespa. The identification of mespa as sole functional homologue of mammalian MesP1 will provide unique opportunities to investigate the individual contributions and epistatic relationships within the cardiogenic network.

Supplementary material
Supplementary material is available at Cardiovascular Research online.

Acknowledgements

We thank Christiane Groß, Barbara Hölscher, Barbara Markieton, and Edith Mentele for technical assistance; Dario Nicetto for comments on the manuscript; Kris Henningfeld for the ngnr1 MO, and Frank Conlon, Kris Kintner, Paul Krieg and Michael Kühl for Xenopus probes.

Conflict of interest: none declared.

Funding

This work was supported by the FoFoLe Program of the LMU Munich (M23/2009 to M.K. and R.R.) and the Deutsche Forschungsgemeinschaft (DA 1296/2-1 to R.D., and FR 705/14-2 to W.F.).

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