Comparative Analysis Reveals Distinct and Overlapping Functions of Mef2c and Mef2d during Cardiogenesis in Xenopus laevis

Yanchun Guo¹,²*, Susanne J. Kühl¹,², Astrid S. Pfister¹, Wiebke Cizelsky¹,², Stephanie Denk¹, Laura Beer-Molz¹, Michael Kühl¹*

¹Institute of Biochemistry and Molecular Biology, Ulm University, Ulm, Germany, ²International Graduate School of Molecular Medicine Ulm, Ulm University, Ulm, Germany

Abstract

The family of vertebrate Mef2 transcription factors is comprised of four members named Mef2a, Mef2b, Mef2c, and Mef2d. These transcription factors are regulators of the myogenic programs with crucial roles in development of skeletal, cardiac, and smooth muscle cells. Mef2a and Mef2c are essential for cardiac development in mice. In Xenopus, mef2c and mef2d but not mef2a were recently shown to be expressed during cardiogenesis. We here investigated the function of Mef2c and Mef2d during Xenopus laevis cardiogenesis. Knocking down either gene by corresponding antisense morpholino oligonucleotides led to profound heart defects including morphological abnormalities, pericardial edema, and brachycardia. Marker gene expression analyses and rescue experiments revealed that (i) both genes are required for proper cardiac gene expression, (ii) Mef2d can compensate for the loss of Mef2c but not vice versa, and (iii) the γ domain of Mef2c is required for early cardiac development. Taken together, our data provide novel insights into the function of Mef2 during cardiogenesis, highlight evolutionary differences between species and might have an impact on attempts of direct reprogramming.

Introduction

In the mouse, the heart arises from two populations of cells referred to as the first and second heart field, respectively [1–3]. Cells of the first heart field (FHF) localize in the cardiac crescent and later form the linear heart tube that grows by continuous addition of cells from the venous as well as the arterial pole. These newly added cardiac cells originate from the so-called second heart field (SHF) that is characterized by the expression of transcription factors Islet-1 (Isl1) and T-box factor 1 (Tbx1) [4]. From lineage labeling studies, it is known that cells of the SHF contribute to most of the right ventricle and the outflow tract and contribute to the aortic arch, the endocardium, the ventricular and atrial septum as well as to the atrial myocardium whereas the other parts of the heart are mainly derived from cells of the FHF [5]. Data gained from work in mouse ES cells suggest that the FHF and the SHF are established from a common cardiac progenitor cell (CPC) population.

Cardiac development in Xenopus starts at the onset of gastrulation with the induction of two bilaterally located cardiac progenitor cell populations on either side of the Spemann Organizer [6,7]. During gastrulation, these cells migrate towards the anterior-ventral side of the embryo and fuse to a single common CPC population located adjacent and posterior to the cement gland anlage [8]. During further development, this CPC population becomes more and more heterogenic, so that at stage 24, two populations of cells can be distinguished by differential gene expression. Lineage labeling experiments have revealed that these two separate cell populations correlate to the first and the second heart field in mice, respectively. At this stage, cells of the Xenopus FHF already express genes indicating cardiac differentiation such as cardiac troponin (tnnt3) and myosin heavy chain (myh6). Upon further development, these cells will later contribute to forming the single ventricle, the two atria and the inflow tract [8]. Cells of the SHF are localized anterior to the first heart field adjacent to the cement gland and express isl1 and tbx1 as well as bone morphogenetic protein 4 (bmp4). These cells have been shown to develop into the outflow tract [8].

Mef2 (myocyte enhancer factor 2) proteins belong to the MADS (MCM1, agamous, deficient, serum response factor) family of transcription factors. Members of the Mef2 family contain a common MADS-box and a Mef2-type domain at their N-termini. The MADS-box serves as a minimal DNA-binding domain that requires the adjacent Mef2-type domain for dimerization and high-affinity DNA binding [9–11]. These proteins also contain a transactivation domain at the C-terminus which is involved in the regulation of transcriptional activity. The four vertebrate Mef2 genes - referred to as Mef2a, b, c and d - are expressed in precursors of the three muscle lineages as well as in neurons [10]. The vertebrate Mef2 gene products share about
Results

me2c and me2d are expressed in the developing heart in *Xenopus laevis*

To initiate this study we first analyzed available sequence information on me2 genes in *Xenopus laevis* and *Xenopus tropicalis*. Comparing the synteny of the me2a, me2c, and me2d genes in the human and the mouse genomes with the corresponding homologs in *Xenopus* confirmed that the available genomic sequences and EST clones code for the corresponding homologs in *Xenopus* (Fig. 1 A, C and D, Table S1) and that the current annotations of these me2 family members in *Xenopus* are correct. For me2b, no EST sequences were reported so far and no homolog has been identified in the *Xenopus tropicalis* genome. Therefore, we analyzed the genomic region of the *Xenopus tropicalis* genome where me2b should be localized in more detail. In humans, Me2b is localized on chromosome 19 and the neighboring genes are well known. This synteny is well conserved between the human and the mouse genomes (Fig. 1 B, Table S1). Using this information, we were able to identify one scaffold that should contain the genomic information for *Xenopus me2b*. Whereas the orthologs of the me2b flanking genes could be identified on this scaffold, no sequences coding for me2b were found. Interestingly, a region spanning about 277 kb was inverted in comparison to the human and mouse genomes. One DNA break point required for this inversion covers the region where me2b should be located. This observation and the lack of any me2b EST suggest that the gene coding for me2b was lost in *Xenopus* during evolution.

RT-PCR experiments revealed that *Xenopus* me2c and me2d transcripts are maternally supplied and expressed during the entire embryogenesis (Figure S1 A, B). We next confirmed the spatiotemporal expression pattern of me2c and me2d during *Xenopus laevis* cardiac development as previously published [8,13]. *Xenopus* me2c transcripts were faintly detected in the common cardiac progenitor cell (CPC) population at stage 20 whereas me2d transcripts were strongly detected in CPCs (Figure S1 C, L). During further development, me2c and me2d are continuously expressed in cardiac tissue (Figure S1 D–M, K–T). At stages 28 and later, both genes are expressed in the first heart field (FHF) region (Figure S1 F–K, O–T), whereas only me2c is expressed in the more lateral region of the second heart field (SHF) (Figure S1 G, red arrowhead). Transverse sections revealed that me2c is expressed in the myocardium (arrowhead) as well as in the endocardium (arrow) during tailbud stages (Figure S1 h), while stronger me2d expression is restricted to the myocardium at this time point (Figure S1 q, arrow). At stage 36, expression of both, me2c and me2d, was observed in the myocardium (Figure S1 j, s, arrows).

Loss of either Me2c or Me2d results in cardiac malformations

For loss of function studies we relied on morpholino oligonucleotide (MO) based antisense strategies. To test the binding efficiency and functionality of the MOs used, the MO binding sites of *Xenopus* me2c (here denoted as xmef2c), and *Xenopus* me2d (xmef2d) were cloned into the pG2s+ expression vector in front of and in frame with the GFP gene. The RNAs of these constructs were bilaterally co-injected with either Control MO or Me2c MO or Me2d MO into two-cell stage embryos. GFP expression was monitored at stage 20 (Figure S2). Co-injection of the xmef2c-GFP fusion construct together with Me2c MO blocked the translation of GFP, whereas co-injection of a Control MO had no effect on the expression. Similar, co-injection of the xmef2d-GFP fusion construct together with Me2d MO also blocked the translation of GFP. In order to rescue the observed phenotypes seen after
Figure 1. Synteny analyses of Mef2a, b, c, and d. A. Synteny analysis of mef2a. Schematic overview comparing the mef2a gene in Homo sapiens (chromosome 15), Mus musculus (chromosome 7) and Xenopus tropicalis (scaffold_3, Xenbase G-Browse). B. Synteny analysis of mef2b. Schematic overview comparing the mef2b gene in Homo sapiens (chromosome 19), Mus musculus (chromosome 8) and the mef2b neighboring genes in Xenopus tropicalis (scaffold_1, Xenbase G-Browse). C. Synteny analysis of mef2c. Schematic overview comparing the mef2c gene in Homo sapiens (chromosome 5), Mus musculus (chromosome 13) and Xenopus tropicalis (scaffold_1, Xenbase G-Browse). D. Synteny analysis of mef2d. Schematic overview comparing the mef2d gene in Homo sapiens (chromosome 1), Mus musculus (chromosome 3) and Xenopus tropicalis (scaffold_27, Xenbase
knocking down either Mef2c or Mef2d, we relied on RNA coding for murine Mef2c or human MEF2D that were not targeted by either MO due to sequence differences in the 5' UTR (mMef2c) or due to introduced silent point mutations in the coding sequence (hMEF2D). Again, the efficiency was tested as described above. The expression of mMef2c-GFP or hMEF2D-GFP was not blocked by Mef2c MO. The Mef2d MO did not have an inhibitory effect on hMEF2D-GFP or mMef2c-GFP (Figure S2). Taken together, these results support the idea that (I) both MOs specifically block the translation of the corresponding Xenopus mef2 gene, (II) Mef2c MO neither inhibits mMef2c nor hMEF2D translation and (III) the Mef2d MO does not interfere with mMef2c or hMEF2D expression. In conclusion, RNA coding for murine Mef2c and human MEF2D can be used as gene specific rescue constructs.

To interfere with Mef2c or Mef2d function during heart formation, either Mef2c or Mef2d MOs were injected bilaterally into both dorso-vegetal blastomeres of eight-cell stage Xenopus embryos to target cardiac tissue [27]. In all experiments, 0.5 ng GFP RNA was co-injected as a lineage tracer and to identify correctly injected embryos. Mef2c and Mef2d morphant embryos revealed abnormalities during cardiogenesis whereas Control MO-injected siblings showed a normal cardiac development (Fig. 2 A–D). Embryos depleted of Mef2c or Mef2d developed pericardial edema and malformed hearts and additionally showed a reduced heart rate at stage 42 (Fig. 2 A, B). To take a closer look at morphant hearts, we isolated the hearts of fixed Xenopus embryos. We observed that the heart tube was formed and the looping process was initiated in both Mef2c and Mef2d morphant embryos. However, during later development, defects during the looping process as well as smaller cardiac chambers were observed in Mef2c and Mef2d MO injected embryos (Figure 2 A–D). These results indicate that both members of the Mef2 family are required for cardiogenesis in Xenopus, and are particularly important for cardiac morphogenesis and heart looping.

**Mef2c or Mef2d are required for cardiac gene expression in the common cardiac progenitor cell population**

Mef2 transcription factors have been shown to be important regulators of cardiac differentiation [14,16,17]. To pinpoint the time point of when a loss of either Mef2c factor affects cardiogenesis, we analyzed the expression of different cardiac marker genes at different time points of cardiogenesis. For this purpose, we injected Mef2c or Mef2d MO unilaterally into one dorso-vegetal blastomere of eight-cell stage Xenopus embryos and analyzed marker gene expression at different stages by whole mount in situ hybridization. In this experimental setting, the uninjected side served as an internal control. The injected side was identified by co-injecting GFP RNA as a lineage tracer. We started our analysis at stage 20 when the CPC population is located on the ventral side of the embryo [8]. At this stage, the expression of tbx20 was found to be down-regulated on the injected side of Mef2c or Mef2d-depleted embryos as compared to the uninjected side and Control MO-injected siblings (Fig. 3). Loss of Mef2d but not Mef2c reduced tbx1 expression at this stage. The expression of isl1 and ntx2-5 however was not affected in either Mef2c or Mef2d deficient embryos (Fig. 3). This observation indicates that both Mef2c and Mef2d are required for proper expression of cardiac genes in the Xenopus CPC population.

At stage 28, when the CPC population has split into the FHF and SHF [8], both Mef2c MO and Mef2d MO-injected embryos revealed reduced expression of FHF marker genes such as tbx20 and gata6b as well as SHF marker genes such as tbx1 and isl1. Marker genes indicating terminal differentiation of cardiomyocytes, namely acta1, myh6 and tnni3, were also down-regulated upon loss of either gene (Fig. 4).

The γ domain of Mef2c is required for embryonic cardiogenesis

While cloning the *Xenopus laevis* mef2c gene from heart enriched explants, we isolated two different splice versions of this gene that differ in either the presence or absence of the γ domain, mef2cγ- and mef2cγ+, respectively. qPCR analyses with primer pairs specifically recognizing either splice variant revealed that both isoforms of Mef2c are expressed in heart tissue enriched explants cut at stages 24, 28, and 32 (Fig. 5 A). Please note that cardiac explants cannot be cut at stage 20 of development as the cardiac tissue at this stage is a thin layer of cells underlying the ectoderm in the anterior region of the embryo in close proximity to other sites of Mef2 expression. Interestingly, mef2cγ- was expressed at higher levels on RNA level than the mef2cγ+ variant. This is in agreement with previous data showing that mef2cγ- is the predominantly expressed variant during murine embryogenesis and the only isoform that was found to be expressed in the murine adult heart [12]. Moreover, also mef2d could be detected by qPCR experiments (Fig. 6A).

We next aimed to show the specificity of the observed phenotypes. To generate a mMef2cγ- construct, we deleted this domain in the mMef2cγ+ construct. Western Blot experiments using a Mef2c antibody showed that both variants of mMef2c are expressed at similar levels upon RNA injection (Fig. 5 B). The same holds true for the injected hMEF2D RNA which was detected by a specific MEF2D antibody (Fig. 6B). For rescue experiments, we co-injected in a first set of experiments Mef2c MO together with RNA coding for murine Mef2cγ+ and examined expression of myh6 and tnni3 at stage 28. This co-injection led to a significant rescue of marker gene expression (Fig. 5 C, D). Intriguingly, the mMef2cγ- version failed to rescue the observed changes in gene expression upon Mef2c knock down (Fig. 5 E, F). These data indicate for the first time that the γ domain of Mef2c has an important role during early cardiogenesis in Xenopus. The restored expression of myh6 and tnni3 was also observed in embryos co-injected with the Mef2d MO together with hMEF2D mRNA (Fig. 6 C, D).

**Mef2d can compensate for the loss of mef2c but not vice versa**

Given the similarities of the phenotypes observed upon depletion of either Mef2c or Mef2d but also taking into account the differences in the effects of either MO on gene expression in the CPC population, we aimed to further characterize the interplay of both factors. First, we injected low doses of Mef2c and Mef2d MO either alone or in combination into both dorso-vegetal blastomeres of eight-cell stage embryos (Fig. 7 A, B). Interestingly, whereas neither a low dose of Mef2c MO nor Mef2d MO resulted in a phenotype, the co-injection of both MOs at this low dose together resulted in a robust phenotype in a more than additive manner indicating a synergistic activity of both transcription factors. We furthermore performed cross-rescue experiments by co-injecting Mef2c MO together with hMEF2D or Mef2d MO...
with mMef2c or mMef2c RNA and analyzed marker gene expression at stages 20 and 28. By co-injecting the Mef2c MO together with hMEF2D, the expression of tbx20 at stage 20 and bmp4, myh6 and tnni3 at stage 28 was significantly restored (Fig. 7 C, D). However, co-injection of Mef2d MO with mMef2c failed to rescue the observed phenotypes independent of the mMef2c splice variant used (Fig. 7 E–H). These data clearly indicate that Mef2d can compensate for a loss of Mef2c but not vice versa.

Overexpression of mMef2c+ or hMEF2D but not mMef2c− accelerates the onset of cardiac differentiation

Finally, we performed gain of function experiments by injecting RNA coding for either Mef2 variant unilaterally into eight-cell stage embryos. In a first set of experiments we examined the expression of tnni3 at stage 24 when expression of this gene commences. Injection of mMef2c+ or hMEF2D RNA resulted in a slight increase of expression on the injected side. This was not observed in case of RNA coding for mMef2c− (Fig. 8 A, B). In contrast, we did not see any significant differences between the injected and the un.injected side when analyzing tnni3 expression at stage 28 (Fig. 8 C, D). Taken together, these data indicate that overexpression of mMef2c+ or hMEF2D but not mMef2c− rather accelerates the onset of cardiac differentiation than resulting in ectopic cardiomyocyte formation.

Discussion

Members of the Mef2 family of transcription factors are critical regulators of cardiac development in different species. However, no functional studies on mef2 genes during cardiogenesis have been performed in Xenopus laevis so far. A detailed synteny analysis as well as a comprehensive comparison of cDNA sequences confirmed the correct assignment of mef2a, c, and d homologs in Xenopus. This correct assignment is certainly important for the interpretation of the cross species rescue experiments performed in this study. Surprisingly, we were not able to identify any genomic sequence or any EST coding for mef2b in Xenopus and it is very likely that mef2b does not exist in Xenopus. It is possible, however,
that due to the lack of sequence information available in current public sequence databases, the assembly of the corresponding scaffold might not be complete yet. We argue, however, that as long as no \textit{mef2b} ortholog is identified in \textit{Xenopus}, it is likely that this gene was lost during evolution in this species.

In mice, Mef2a and Mef2c are functionally important for cardiac development as assessed by the phenotypes in genetically modified mice that lack either of these genes [17,18]. Knock out mice of either \textit{Mef2a} or \textit{Mef2c} die during embryogenesis due to problems in cardiac development. In contrast, Mef2d null mice develop normally until birth. Interestingly, \textit{mef2a} expression was recently found not to be present during cardiac development in \textit{Xenopus} [13], whereas \textit{mef2c} and \textit{mef2d} were robustly expressed during cardiogenesis [8]. Our functional analyses performed in this study indicate that indeed both members are needed for proper heart formation in \textit{Xenopus}. Moreover, based on our results, we speculate that in \textit{Xenopus}, Mef2d has taken over the function that Mef2a has in mice. A more detailed analysis of marker genes of Mef2a-deficient mice might shed more light onto this evolutionary difference. In addition, the identification of genomic regions bound by Mef2a in mice and Mef2d in \textit{Xenopus} might help to verify or falsify this hypothesis.

Of interest is also the observation that loss of Mef2c can be rescued by an overexpression of Mef2d but not \textit{vice versa}. Mef2 transcription factors have been shown to form homo- and heterodimers [9,10]. It is therefore very likely that Mef2c/Mef2d and Mef2d/Mef2d dimers or Mef2d monomers are functionally important during early cardiac development in \textit{Xenopus}. In this scenario, Mef2c/Mef2c dimers, or Mef2c monomers, would not be similarly efficient in transcription. Our findings therefore

Figure 3. Loss of Mef2c or Mef2d affects the cardiac progenitor cell population. Mef2c or Mef2d MO (10 ng) was unilaterally injected and expression of cardiac marker genes was monitored at stage 20. \textbf{A}, \textit{tbx20} expression was down-regulated in Mef2c MO- or Mef2d MO-injected embryos (arrowheads). In addition, Mef2d- but not Mef2c-depleted embryos showed reduced \textit{tbx1} expression (arrowhead). Expression of \textit{isl1} as well as \textit{bmp4} remained unchanged upon loss of Mef2c or Mef2d. Anterior views of embryos are shown. \textbf{B}, Quantitative presentation of observed phenotypes in A is given. N: number of examined embryos; n: number of independent experiments; *, p<0.05. doi:10.1371/journal.pone.0087294.g003
should foster more detailed analysis of this issue not only in *Xenopus* but in mouse as well.

Although Mef2a and Mef2c knock out mice have a cardiac phenotype, the role of both genes in regulating cardiac gene expression in the CPC population has not been reported and also the expression of Mef2 proteins in the early embryo has not yet been described. However, Mef2c has been shown to be expressed in early differentiating murine ES cell cultures suggesting a possible role for Mef2c in the CPC population [28]. In line with these findings is the observation that the use of a dominant negative Mef2 protein that blocks the activity of all four members of the Mef2 family in mice inhibits cardiac differentiation in P19 cells [29]. These data clearly indicate a functional redundancy of Mef2 members in mice. Interestingly, the loss of Mef2c is additionally accompanied by an up-regulation of Mef2b. Our data shown here clearly indicate that the loss of either Mef2c or Mef2d results in early defects during *Xenopus* cardiac differentiation thus suggesting that this functional redundancy is not so prominent in *Xenopus*. This statement is also supported by the observation of a more than additive phenotype after simultaneously knocking down both Mef2c and Mef2d, which thereby implies that these two transcription factors are acting synergistically rather than redundantly. The apparent lack of Mef2b in *Xenopus* might contribute to this disparate situation as well.

Transcriptional targets of Mef2 factors that have been identified in different species include alpha cardiac myosin heavy chain, alpha cardiac actin and cardiac troponin I [30–32]. Notably, the expression of these genes was down-regulated in our experiments after knocking down the *mef2* genes (see Fig. 4, myh6, actc1 and tnni3) suggesting that their regulation by Mef2 factors is also

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**Figure 4. Cardiac differentiation is affected upon loss of Mef2c or Mef2d.** A. At stage 28, depletion of Mef2c or Mef2d led to a reduced expression of cardiac markers including *isl1, bmp4, nkn2.5, tbx1, tbx20, gata6-b, myh6, actc1, and tnni3* (arrowheads). Ventral views of embryos are shown. B. Quantitative presentation of the observed phenotype in A is given. N = number of examined embryos; n = number of independent experiments; *, p<0.05; **, p<0.01.

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conserved in Xenopus. In the case of myh6, a recent study identified a conserved Mef2 binding site in its promoter region [33]. Altogether, the loss and down-regulation of marker gene expression in our morphant embryos is clearly in line with what previous studies have shown. Of particular interest as a target of Mef2 factors is Nkx2-5 [29]. Interestingly, we have not observed any down-regulation of nkx2-5 expression in the CPC population at stage 20, but we have seen a down-regulation at the stage 28. This might suggest that nkx2-5 is a target gene of Mef2 proteins only at later stages of development or this observation might point to diverse functions of Mef2 proteins as discussed in the next section.

![Image](image_url)

**Figure 5. Specificity of phenotype observed upon knock down of Mef2c.** A. mef2c- and mef2c+ isoforms are expressed on RNA level in heart tissue enriched explants at stages 24, 28, and 32 as revealed by qPCR. Expression is shown relative to gapdh. B. mMef2c- and mMef2c+ are expressed on protein level upon RNA injection into Xenopus embryo at comparable levels. β-Tubulin served as loading control. Note that the Mef2c antibody used does not recognize endogenous Xenopus Mef2c protein. The asterisk indicates unspecific background. C–F. Mef2c MO was unilaterally injected together with GFP, mMef2c+ or mMef2c- RNA as indicated. C. Expression of the cardiac marker genes myh6 and tnni3 was monitored at stage 20 or stage 28. Black arrowheads indicate reduced marker gene expression, white arrowheads highlight the rescued situation. Ventral views of embryos are shown. D, F. Quantitative presentations are shown. N: number of examined embryos; n: number of independent experiments; st: stage; **, p≤0.01. doi:10.1371/journal.pone.0087294.g005

The γ domain of mMef2c has been shown to function as a transcriptional repressor [12]. Our rescue experiments suggest that Mef2cγ+ but not Mef2cγ- is required for early cardiogenesis. This finding is unexpected because we found mef2γ- to be expressed higher on RNA level than the mef2γ+ isoform. Also in adult muscle and during skeletal myogenesis the γ version has been described to be predominantly expressed and to be of functional relevance [12]. Of note, we cannot exclude other functions for the mef2γ- variant that were not tested in our study. Interestingly, overexpression of a Mef2 engrailed repressor fusion protein (MEF2/EnR) in P19 cells results in an activation of Nkx2-5 during early phases of cardiogenesis whereas this construct inhibits

![Image](image_url)

**Figure 6. Specificity of phenotype observed upon knock down of Mef2d.** A. mef2d is expressed on RNA level in heart tissue enriched explants at stages 24, 28, and 32 as revealed by qPCR. Expression is shown relative to gapdh. B. hMEF2D is expressed on protein level upon RNA injection into Xenopus embryo. β-Tubulin served as loading control. Note the Mef2d antibody used does not recognize endogenous Xenopus Mef2d protein. C. Mef2d MO was unilaterally injected together with GFP or hMEF2D as indicated. Expression of cardiac marker genes myh6 and tnni3 was monitored at stage 28. Black arrowheads indicate reduced marker gene expression, white arrowheads highlight the rescued situation. D. A quantitative presentation of results is given. N: number of examined embryos; n: number of independent experiments; **, p≤0.01. doi:10.1371/journal.pone.0087294.g006
Figure 7. Cooperation of Mef2c and Mef2d in *Xenopus*. A, B. The injection of 7 ng Mef2c MO or 7 ng Mef2d MO (in both cases 3.5 ng per blastomere) did not result in cardiac defects. The co-injection of 7 ng Mef2c together with 7 ng Mef2d MO led to a significant increase of the phenotype. The dotted black lines indicate the heart. C, D. Mef2c MO was unilaterally injected along with RNA coding for hMEF2D. Expression of cardiac marker genes was monitored at stages 20 (tbx20; anterior views) or stage 28 (myh6, tnni3; ventral views), respectively. E, F. Mef2d MO was unilaterally injected along with mMef2c c+ RNA. Expression of cardiac marker genes was monitored at stages 20 (tbx1, tbx20; anterior views) or...
cardiogenesis at later time points \cite{29}. This is in line with our findings obtained here and implies that different splice variants of Mef2 transcription factors might be expressed during different phases of cardiac development. This has not been analyzed, at least to our knowledge, in a time and cell type specific manner during vertebrate cardiogenesis. The functional differences of two of these splice variants in our study indicate the requirement of additional expression and functional studies in the future to complete our understanding of Mef2 function during cardiogenesis. The \( \gamma \) domain has also been shown to be subject to regulation by phosphorylation \cite{12} suggesting that this type of regulation occurs during early cardiac development adding another layer of complexity to Mef2 function during this time period. The fact that human MEF2D that also harbors a sequence similar to the \( \gamma \) domain of Mef2c is able to rescue the Mef2c MO phenotype might trigger further experiments to analyze this short stretch of amino acids in MEF2D in more detail. Finally, the discovery that Mef2d is involved in \textit{Xenopus} cardiogenesis may also be useful as another model suitable to gain further insights into the function of Mef2d during cardiac remodeling in mice, particularly in the reactivation of a fetal gene expression program.

Of note, our experiments have additional implications as Mef2c was used as one of three components in direct reprogramming experiments to generate cardiomyocytes out of cardiac fibroblasts \cite{25}. Our findings should therefore also foster research that examines different Mef2 splice variants and their potential roles and functions in this setting with the aim to increase the efficiency of reprogramming.

### Materials and Methods

#### Xenopus embryos

\textit{Xenopus laevis} embryos were obtained by \textit{in vitro} fertilization, cultured and staged according to Nieuwkoop and Faber \cite{34}. All procedures were performed according to the German animal use and care law (Tierschutzgesetz) and approved by the German state administration Baden-Wurttemberg (Regierungspräsidium Tübingen).

#### Cloning

The \( \text{mMef2c} \) and \( \text{hMEF2D} \) constructs were purchased from ImaGenes GmbH and the open reading frames were subcloned into pCS2\( ^{+} \) \cite{35}. To obtain a rescue construct which is not targeted by the Mef2d MO, we inserted two additional silent point mutations in the \( D_{59}^{9} \) UTR of \( \text{hMEF2D} \) by mutagenesis using the QuickChange II Site-Directed Mutagenesis Kit (Stratagene). All constructs were verified by sequencing. The \( \text{mMef2c}^{-} \) deletion construct was generated by inverse PCR using the \( \text{mMef2c}^{+}/\text{pCS2}^{+} \) rescue construct as template and the proof reading Phusion DNA polymerase (Finnzyme) followed by re-ligation. The primers used for the inverse PCR were: for: 5\'-GAC CGT ACC ACC ACC CCT TCG A-3\'; rev: 5\'-GCT GAG GCT TTG AGT AGA AGG CAG G-3\'. For cloning full length \( \text{mef2c} \) from heart enriched stage 28 (\( \text{bmp4}, \text{myh6}, \text{tnni3}; \) ventral views), respectively. G, H. Mef2d MO was unilaterally injected along with \( \text{mMef2c}^{\gamma} \) RNA. Expression of cardiac marker genes was monitored at stage 28 (ventral view). In all cases, black arrowheads indicate reduced marker gene expression, white arrowheads highlight the rescued expression. B, D, F, H. Quantitative presentations of the experiments shown in C, E, G are shown. N: number of examined embryos; n: number of independent experiments; *, \( p<0.05; **, p<0.01. \) doi:10.1371/journal.pone.0087294.g007

![Figure 8. Gain of function analyzes reveals an earlier onset of cardiac differentiation.](https://example-image-url.com)

\[\text{WT} \quad \text{GFP} \quad \text{mMef2c}^{+} \quad \text{mMef2c}^{-} \quad \text{hMEF2D}\]

A

\[\% \text{ embryos with enhanced tnni3 expression}\]

B

\[\% \text{ embryos with enhanced tnni2 expression}\]

C

\[\% \text{ embryos with enhanced tnni3 expression}\]

D

Figure 8. Gain of function analyzes reveals an earlier onset of cardiac differentiation. A, C. RNA coding for \( \text{mMef2c}^{\gamma+}, \text{mMef2c}^{\gamma-} \) or \( \text{hMEF2D} \) was injected unilaterally into the dorsal-vegetal blastomere at eight cell stage and \( \text{tnni3} \) expression was monitored at stages 24 (A) or 28 (C). Ventral views of embryos are shown. B, D. The percentage of embryos with enhanced expression of \( \text{tnni3} \) on the injected side is given. N: number of examined embryos; n: number of independent experiments; **, \( p<0.01. \) doi:10.1371/journal.pone.0087294.g008
explains the following primers were used: Mef2c cloning for: 5′-GGT GGA GCA AGG AAA AT-3′, Mef2c cloning rev: 5′-GGT ATA AGC ACA CAC CTA GTG CA-3′.

Morpholino oligonucleotide (MO), MO specificity tests and RNA injections

All MOs were purchased from Gene Tools, LLC, OR, USA, resuspended in DEPC-H2O and stored as aliquots at −20°C. Mef2cMO: 5′-CCA TAG TCC CCG TTT TTC TGT CTT C-3′; Mef2dMO: 5′-AAT CTG GAT CTT TTT TCT GCC CAT G-3′. For knock down approaches, we injected the MOs (10 ng) into both dorso-vegetal blastomeres of eight-cell embryos to target the presumptive heart region [27]. For lineage labeling and to identify the injected side, we co-injected 0.5 ng GFP RNA. Only correctly injected embryos were considered for the experiments. For control injection experiments, the standard Control MO of Gene Tools was used. Control MO: 5′-CCT CTT ACC TCA GTT ACA ATT TAT A-3′. For rescue experiments, mRNA (0.1 ng) was injected together with MO. The binding specificity of MOs was tested in vivo were cloned in frame with and in front of the GFP open reading frame in pCS2+. The indicated RNA and MO were co-injected bilaterally into two-cell stage embryos and GFP translation was monitored at stage 20 using a fluorescence microscope.

Whole Mount in situ Hybridization (WMISH)

Probes for mef2c and mef2d were used as previously described [8]. Digoxigenin-labelled antisense RNA probes of analyzed genes were synthesized by restriction digestion and subsequent transcription with Sp6 or T7 RNA polymerase (Roche). To analyze marker gene expression at stages 20 and 28, MOs (10 ng) were unilaterally injected in one dorso-vegetal blastomere together with 0.5 ng GFP RNA. The un.injected side served as internal control. Wild type or injected embryos were fixed at +4°C in MEMFA (0.1 M MOPS, pH 7.4, 2 mM EGTA, 1 mM MgSO4, and 4% formaldehyde) at indicated stages. WMISH experiments were performed according to a standard protocol as previously described [36,37]. BM-Purple (Roche) was used for staining. Stained embryos were then bleached with 30% H2O2. For sections, stained embryos were embedded in gelatine/alumhine overnight at +4°C, sectioned using a vibratome with a thickness of 25 μm, coverslipped, and imaged with an Olympus BX60 microscope.

RNA isolation, RT-PCR and qPCR

Total RNA was isolated from whole Xenopus embryos at different stages with peqGOLD RNAPure (peqLab) according to the manufacturer’s protocol. To analyze mef2c variants in Xenopus cardiac tissue, the anterior-ventral part of stage 24, 28, and 32 of wild type embryos (heart-enriched regions) posterior to cement gland were dissected and total RNA was isolated using peqGOLD RNAPure (peqLab). cDNA was synthesized using random hexamers and the SuperScript II reverse transcriptase (Invitrogen). PCR was performed with the Phire Hot Start II DNA Polymerase (Thermo scientific). Primers for amplification were: gapdh_for: 5′-GCC GTG TAT GTG GAA TCT-3′; gapdh_rev: 5′-AAG TTT TCG TTT ATG ACC TTT GC-3′; H4_for: 5′-CGG GAT AAC ATT CAG GGT ATC ACT-3′; H4_rev: 5′-ATC CAT GGC GGT AAC TCT CTT CCT-3′; Mef2c_for: 5′-AGT CGG CAG GGA CTA CTT ATG-3′; Mef2c_rev: 5′-TCA CCT GTC GGT TAG GTT CA-3′; Mef2d_for: 5′-GGC GGT TAA ATC TCG GGA AG-3′; Xeno8c_rev: 5′-CGG TGT CAC TTT GCC TAT-3′; Quantitative PCR was performed on RNA isolated from heart-enriched regions using SYBR Green Master Mix (Fermentas) and a Roche Light Cycler 1.5. gapdh was used as housekeeping gene. Each sample was analyzed in triplicate. Primer pairs used were: Xenopus gapdh for: 5′-GCC GTG TAT GTG GAA TCT-3′; Xenopus gapdh rev: 5′-AAG TTT TCG TTT ATG ACC TTT GC-3′; Xenopus mef2c_for: 5′-CCG CAA ATG TGG CTT CAC TCC CAT G-3′; Xenopus mef2c_rev: 5′-GGAGGAGAACAGGTTTCTGACTTT-3′; Xenopus mef2c_for: 5′-TGTCCTAGTCTACTGGCTTGCGGAC-3′; Xenopus mef2c_rev: 5′-TGATTACGGTCTCCAGCTGCGTGGAG-3′; Xenopus mef2d_for: 5′-AGA CCT GGC ATC CCT CTC TA-3′; Xenopus mef2d_rev: 5′-TTG CCG TTG GTT ATG TTG TT-3′.

Western Blot

30 embryos injected with RNA were homogenized in 300 μl lysis buffer(20 mM Tris/ HCl, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 1 mM PMSF) and incubated on ice for 5 min. Protein samples were cleared at 13,000 rpm at 4°C for 5 min. For removal of lipids, the supernatant was mixed with 50 μl Freon followed by centrifugation at 13,000 rpm at 4°C for 5 min. The upper protein containing phase was collected. Concentration of protein samples was determined by Bradford assay with BSA as standard. Western blotting was performed according to standard procedures and proteins were visualized using a Li-COR ODYSSEY Imager. Primary antibodies were purchased from Abcam (rabbit polyclonal MeF2C, directed against residues 450 to the C-terminus of human MEF2C), Santa Cruz (mouse monoclonal anti Mef2D, H11, epitope mapping within an internal region of MEF-2D of human origin) and Serotec (γ-Tubulin, clone YL1/2). Secondary antibodies used were IRDye conjugates from Li-COR.

Synteny analysis

For synteny analysis, genomic structure and chromosomal organization of Mef2a, b, c and d in human, mouse and X. tropicalis were compared using NCBI and Xenbase G Browse.

Statistics

Data were obtained from at least three independent experiments and analyzed with statistical program GraphPad Prism. The number of embryos (N) and the number of independent experiments (n) performed for each experiment is indicated in the corresponding figures. For rescue experiments, embryos of the same batch were evaluated upon injection either with either MO or MO along with the RNA of interest. The nonparametric Mann-Whitney rank sum test was used to determine statistical differences. A p value of ≤0.05 was considered to be significant.

Supporting Information

Figure S1 Spatio-temporal expression of mef2c and mef2d in Xenopus. A. Temporal expression of mef2c. mef2c is maternally supplied. Mef2c embryonic expression starts at stage 9 and increases until stage 40. gapdh was used as loading control. – RT serves as negative control. B. Temporal expression of mef2d. mef2d is maternally supplied. Mef2d embryonic expression starts at stage 12. H4 was used as loading control. – RT serves as negative control. C-j. Spatial expression of mef2c. C. Anterior view with the dorsal side to the top. c. Sagittal section. D, F, H, J. Ventral views with anterior to the right. E, G, I, K. Dorsal views with anterior to the dorsal. Black arrowheads indicate the expression in the FHF, the red arrowhead highlights mef2c transcripts at the lateral sides of the SHF. C. Parasagittal section. d, f, h, j. Transverse sections. Black arrowheads indicate cardiac expression; the arrowhead in
shows mef2c expression in the endocardium, the black arrow in the myocardium. L–s. Spatial expression of mef2d. L. Anterior view with the dorsal side to the top. I. Sagittal section. M, O, Q, S. Lateral views with anterior to the right. N, P, R, T. Ventral views with anterior to the top. m, o, q, s. Transverse sections. White arrows indicate mef2d expression in cardiac progenitor cells. The white arrowhead indicates cardiac cells with low mef2d expression. Black arrows indicate mef2d expression in the myocardium, black arrowheads show mef2c expression in the first heart field (FHF). Sc. stage (TIF).

Figure S2 In vivo MO specificity test. Two-cell stage embryos were bilaterally injected and GFP fluorescence was monitored at stage 20. MO binding sites of Xenopus, mouse and human are indicated. Red letters indicate different bases in the MO binding sites, green letters indicate the ATG start codon. Upper panels show the light view, lower panels provide the fluorescent view. A. GFP fluorescence was observed upon injection of mef2l-GFP together with Control MO but not with Mef2l MO. Neither mchMEF2D-GFP nor hMEF2D-GFP were targeted by Mef2c MO. B. GFP expression was observed after the injection of Control MO. Co-injection of xenmef2d-GFP and Mef2d MO led to an inhibition of GFP expression. Neither the expression of hMEF2D-GFP nor mchMEF2D-GFP was influenced by Mef2d MO. (TIF)

Table S1 Gene abbreviations used in Figure 1. (PDF)

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Author Contributions

Conceived and designed the experiments: SJK MK. Performed the experiments: YG SJK ASP WC SD LBM. Analyzed the data: YG SJK ASP MK. Wrote the paper: YG SJK MK.

References