Genome-wide transcriptomics analysis identifies sox7 and sox18 as specifically regulated by gata4 in cardiomyogenesis

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ABSTRACT

The transcription factors GATA4, GATA5 and GATA6 are important regulators of heart muscle differentiation (cardiomyogenesis), which function in a partially redundant manner. We identified genes specifically regulated by individual cardiogenic GATA factors in a genome-wide transcriptomics analysis. The genes regulated by gata4 are particularly interesting because GATA4 is able to induce differentiation of beating cardiomyocytes in Xenopus and in mammalian systems. Among the specifically gata4-regulated transcripts we identified two SoxF family members, sox7 and sox18. Experimental reinstatement of gata4 restores sox7 and sox18 expression, and loss of cardiomyocyte differentiation due to gata4 knockdown is partially restored by reinstating sox7 or sox18 expression, while (as previously reported) knockdown of sox7 or sox18 interferes with heart muscle formation.

In order to test for conservation in mammalian cardiomyogenesis, we confirmed in mouse embryonic stem cells (ESCs) undergoing cardiomyogenesis that knockdown of gata4 leads to reduced sox7 (and sox18) expression and that Gata4 is also uniquely capable of promptly inducing sox7 expression. Taken together, we identify an important and conserved gene regulatory axis from gata4 to the SoxF paralogs sox7 and sox18 and further to heart muscle cell differentiation.

1. Introduction

Heart development and particularly heart muscle differentiation (cardiomyogenesis) is controlled by an intricate Gene Regulatory Network (GRN). This GRN involves prominent members of specific transcription factor gene families, such as the Nkx2, Mef2, Tbx and Gata gene families (Harvey and Rosenthal, 1999; Olson and Srivastava, 1996). Among these important transcription factors, GATA4, GATA5 and GATA6 are identified as the cardiogenic gata transcription factors (e.g. Peterkin et al., 2005). GATA4 is particularly important as a potent driver of cardiomyogenesis. In fact, carefully stage-controlled experimental activation of ectopically expressed GATA4 alone is sufficient to induce differentiation of functionally beating cardiomyocyte tissue from pluripotent stem-cell-like Xenopus animal cap explants (Afouda et al., 2008; Latinkic et al., 2003, see also below, e.g. Fig. 3). Experimentally forced expression of Gata4 in mouse together with Tbx5 and the chromatin remodelling protein Baf60c can reprogram somatic mesoderm into heart muscle cells (Takeuchi and Bruneau, 2009); and combined expression of Gata4 with Tbx5 and Mef2c was reported to convert cultured fibroblastic cells into a cardiac lineage (Ieda et al., 2010).

Gata4 but also its paralogs Gata5 and Gata6 are required for normal heart formation in mammals (reviewed by Nemer, 2008) and human congenital cardiomyopathies are linked to mutations in the GATA4 gene, including valve and septal defects (Garg et al., 2003; Rajagopala et al., 2007). However, Gata4, Gata5 and Gata6 have redundant functions as demonstrated by studies in mice embryos: Gata4 and Gata6 double mutants have complete acardia (Zhao et al., 2008), compound Gata4/Gata5 mutants present severe cardiac defects (Singh et al., 2010) and compounds Gata4/Gata5 as well as Gata5/Gata6 mutants die embryonically or perinatally due to severe cardiac defects (Laforest and Nemer, 2011). Single Gata4 or single Gata6 both have milder phenotypes, though lack of Gata5 in mice leads to bicuspid aortic valve formation (Laforest et al., 2011), asserting its importance for mammalian heart formation.

There is a significant degree of conservation of the molecular pathways involved in vertebrate heart formation; studies in zebrafish have identified that, in addition to cardiia bifida, gata5 mutants (also known as faust) show loss of cardiomyocytes (Holtzinger and Evans, 2007; Reiter et al., 1999). Studies in both zebrafish and Xenopus laevis have confirmed that gata5 and gata6 have indeed redundant functions in cardiac progenitor specification (Haworth et al., 2008; Holtzinger and Evans, 2007; Peterkin et al., 2007).
Taken together, these observations confirm that the cardiogenic Gata factors represent genes with related function. In fact they derive through whole-genome duplications during the early vertebrate evolution (Dehal and Boore, 2005; Gillis et al., 2009; Ohno et al., 1968) from the same invertebrate ancestral gene (Ragkousi et al., 2011). This evolutionary origin reinforces the need to address questions about redundancy and unique functions of these cardiogenic gata transcription factors, since these genes may have partitioned an originally shared function (subfunctionalisation) or some of them may have acquired new specific functions (neofunctionalisation). Deciphering the genetic programme controlled by gata factors is therefore challenging, yet understanding the redundant and non-redundant specific function will be of great benefit for our understanding of heart development. We had previously uncovered some different requirements of cardiogenic gata factors for Xenopus cardiomyogenesis (Afouda and Hoppler, 2011). A more detailed understanding of the regulatory circuitry controlled by individual cardiogenic gata factors will shed more light on the complex GRN that drive cardiomyogenesis.

Fig. 1. Activin-induced cardiac explants as cardiogenic assay to screen for genes specifically regulated by cardiogenic gata genes. Activin-injected animal caps from myl2-GFP reporter Xenopus laevis transgenic line were cultured until stage 45 and photographed in bright field (A) or with GFP filter (B) allowing visualisation of myl2-expressing cells throughout the beating explant. Total RNA was extracted from stage 32 wild-type Xenopus laevis explants injected with either Activin (Act) alone or together with gata4, 5 or 6 morpholinos (MOs) for real-time RT-PCR monitoring of myl2 (C) or tnni3 (D) expression. Following validation, samples were subjected to high-throughput sequencing where (E) represents schematic pipeline of RNA-seq bioinformatics analysis and (F, H) Venn diagrams of numbers of genes that are differentially expressed compared to Activin-injected control with at least a two-fold reduction in expression and an adjusted p value of < 0.05. (G, I) Heatmaps of fold changes for differentially expressed genes (increased or decreased expression without any fold-change threshold applied) highlighting sub-clusters that are specifically regulated by gata4. Abbreviations: control, uninjected explant; G4MO, G5MO, G6MO and 456MOs representing respectively gata4, gata5, gata6 and all three combined morpholinos. Genes in sub-clusters 1 and 2 are listed in Fig. S1B, C.
Here, we sought to increase our knowledge of the roles of these factors in cardiomyogenesis (and consequently our knowledge of the cardiogenic GRN) by identifying their respective transcriptionally regulated genes on a genome-wide scale through RNA-seq analysis. We have used our established cardiogenic assay (Afouda, 2012; Afouda and Hoppler, 2009) of Xenopus laevis stem-cell-like explants combined with gene knockdown to identify genes that are differentially affected by each of the cardiogenic gata genes. By taking advantage of the recent release of X. laevis genome assemblies and annotations (Xenbase.org: (Karpinka et al., 2015)) we have identified and then validated sox7 and sox18 as genes specifically regulated by gata4 in cardiomyogenesis. Our genome-wide transcriptomics analysis therefore identifies within the GRN for cardiomyogenesis a conserved gene regulatory axis from gata4 to the SoxF paralog genes sox7 and sox18 and further to heart muscle cell differentiation. Our identification of genes that are differentially regulated by each of cardiogenic gata factors also provides a platform for future investigations that will further contribute to elucidating of molecular pathways and the GRN underpinning embryonic cardiomyogenesis.

2. Results

2.1. Xenopus stem-cell-like explants represent a reliable experimental model system for analysis of cardiogenic differentiation

Xenopus animal cap cells represent a pluripotent stem-cell-like tissue (Buitrago-Delgado et al., 2015) that can be induced to differentiate into various cell lineages by the addition of active inducer protein into the culture medium (Asashima et al., 2009) or by prior injection of inducer mRNA (Afouda, 2012; Warkman and Krieg, 2007; Weber et al., 2000). We have developed a reliable cardiomyogenesis assay consisting of injecting a low amount of Activin mRNA into animal cap embryos (subsequently called cardiac explants, Afouda and Hoppler, 2009; Afouda et al., 2008). A myl2–green fluorescence (GFP) reporter line (myl2 is encoding myosin light chain 2, MLC2), which faithfully recapitulates the expression of this marker in cardiomyocytes (Latinkic et al., 2004), was used to show that explants from such embryos not only differentiate into rhythmically beating tissues and further allowed lineage tracing of cardiomyocytes and panmyocardial expression of myl2 (Fig. 1A, B and suppl movie 1). The observed widespread expression of myl2 throughout each explant, and functional beating of essentially the entire explant implies that most of this tissue undergoes cardiogenic differentiation. This result demonstrates that our experimentally accessible cardiogenic model system is ideal for investigating the specific functions of cardiogenic gata factors in relative isolation from their other functions in other embryonic tissues. These advantages combined with the abundant amount of material available from each explant make this assay ideal for high-throughput sequencing approaches.

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2.2. Transcriptomics screen for gata4-, gata5- or gata6-regulated genes

We used this Xenopus stem-cell-like explant system to investigate at a genome-wide scale which genes are specifically regulated by different cardiogenic gata factors during cardiomyogenesis. Previously validated Morpholinos (MO) designed to knock down (inhibit) gata4, gata5, or gata6 expression were used (Afouda et al., 2005; Haworth et al., 2008; Peterkin et al., 2007). The efficacy of the gata MOs was further confirmed by observing altered inactive transcript isoforms in samples where splice MOs were injected (Fig. S1A). We prepared cultures of control cardiac explants, and cardiogenic explants with single gata4, gata5, or gata6 knockdown and with a triple gata4, 5, and 6 compound knockdown. We collected mRNA samples at stage 32 for subsequent RNA-seq analysis. Before sequencing, collected samples were validated for cardiac differentiation using quantitative RT-PCR (Taq man assay, Peterkin et al., 2003) by monitoring expression of terminal differentiation markers such as myl2 and tnni3 (encoding cardiac Troponin I, TnIc) (Fig. 1C, D). As expected, we observed reduced expression of these genes compared to control samples in single gata knockdowns, and a stronger reduction in the triple gata knockdown samples. Samples of mRNA from at least three validated independent biological experiments each were used for high-throughput sequencing (see M & M). Sequenced transcripts were identified by mapping to genes in the genome (X. laevis genome, version 9.1, Session et al., 2016), and counted to reveal expression differences between experimental samples [Fig. 1E and Materials and Methods; (Kim et al., 2015; Liao et al., 2014; Love et al., 2014)]. Threshold limits of two-fold difference in expression levels and statistical significance of 95% were chosen to assemble lists of differentially expressed genes (Fig. 1F, H; and Table 1 sheet 1 and Table 2 sheets 1 and 2 in Afouda et al., 2017). The chosen criteria were validated by confirmation that, as expected, expression of known cardiac differentiation markers, such as myl2 and tnni3, were identified as strongly reduced.

Using the above-mentioned bioinformatics strategy and criteria for the transcriptome-wide analysis to identify gata-dependent genes we found that expression of 1480, 1556 and 980 genes are decreased by either single gata4, gata5 or gata6 knockdown, respectively (Fig. 1F). Among these, 361 are specifically reduced by only the gata4 knockdown, 547 specifically by the gata5, 165 specifically by the gata6 single knockdown, while 529 genes are shared as they are reduced by either gata4, gata5, or gata6 knockdown (Fig. 1F; and Table 1 in Afouda et al., 2017). Overall 2834 genes are affected by the triple gata4, gata5 and gata6 knockdown, of which 962 are only reduced in this triple knockdown (Table 2 in Afouda et al., 2017). This genome-wide analysis of the requirements for gene regulation of the cardiogenic gata transcription factor genes gata4, gata5 and gata6 confirms that they have shared, redundant but also gene-specific functions during cardiomyogenesis.

2.3. sox7 and sox18 are among genes specifically regulated by gata4 during early cardiomyogenesis

Because of the potency of gata4 to promote cardiomyogenesis (see above) we started here to focus on genes specifically affected by the gata4 knockdown. We proceeded with analysing gene ontology (GO) terms associated with gata4-regulated genes (Fig. 1F; and see Afouda et al., 2017, Table 3 sheet 3 for all 1480 genes affected by gata4 knockdown and Table 3 sheet 4 for the 361 genes specifically affected by the gata4 knockdown). Among the GO terms associated with genes requiring gata4 functions are the GO terms “regulation of transcription” and “DNA binding” (see Table 3 in Afouda et al., 2017), suggesting that the gata4 gene is required during cardiomyogenesis for regulating expression of other DNA-binding transcription factor genes. Among those specifically reduced by lack of gata4 we discovered two paralogs of the SoxF family, sox7 and sox18 (see Afouda et al., 2017, Table S3 sheets 1 and 2, highlighted in red). Interestingly, SoxF family members have been associated with cardiovascular development in different vertebrate species (reviewed by Francois et al., 2010), and in Xenopus laevis sox7 and sox18 are essential for heart development (Zhang et al., 2005b). We therefore decided to pursue this potential regulatory axis involving gata4 and SoxF genes in cardiomyogenesis. In order to test such a link further unsupervised clustering of our transcriptome data was carried out (Fig. 1G, I). Again sox7 and sox18 were contained in sub-clusters that are specifically affected by the gata4 knockdown (Fig. 1G, I and Fig. S1B, C). Together, our transcriptomics analysis identify sox7 and sox18 as genes specifically regulated by gata4 during early cardiomyogenesis.
2.4. Temporal progression from gata4 to sox7 and sox18 expression during cardiomyogenesis

The above data suggest that sox7 and sox18 expression in early cardiomyogenesis is downstream of gata4 function and therefore predicted to be after gata4 expression in development. Cardiogenic explants were collected at various time points of development and gene expression analysed by RT-PCR. In mouse, Mesp1 has been identified as an early marker for cardiovascular progenitors and more importantly as a master regulator of cardiomyogenesis (Bondue and Blanpain, 2010; Bondue et al., 2008; David et al., 2008). In Xenopus, mespa has been shown to be the functional homologue of mammalian Mesp1 (Kriegmair et al., 2013). We found mespa and its paralog mespb induced in cardiogenic explants from gastrulation onwards (Fig. 2). gata4 as well as isl1 (marker for undifferentiated but committed cardiac precursors in this context, Pandur et al., 2013) are induced early, mostly preceding expression of sox7 and sox18, followed by expression of structural and differentiation markers myl2 and tnni3 (Fig. 2).

To test the requirement for sox7 and sox18, we experimentally knocked down their expression with previously validated MOs (Zhang et al., 2005b). sox7 or sox18 knockdown caused no loss of early marker gene expression (mespa, mespb, gata4 and Isl1) but had a strong effect later on expression of differentiation markers (myl2 and tnni3) (Fig. 2). These observations further confirm that the explants recapitulate the cardiogenic development and place sox7 and sox18 temporally downstream of gata4 and functionally upstream of cardiomyocyte differentiation.

2.5. sox7 and sox18 are required downstream in gata4-induced cardiomyogenesis

In order to test for the ability of gata4 to induce sox7 and sox18 expression during cardiomyogenesis we used the previously established cardiogenic assay in animal cap explants driven by a hormone inducible gata4 (Afouda et al., 2008; Latinkic et al., 2003). All three cardiogenic gata factors are capable of inducing heart differentiation marker gene expression in this assay, but clearly to different extents, with only gata4 being able to drive differentiation into functionally beating cardiomyocytes (Afouda and Hoppler, 2011). We found that these differences are correlated with our finding that only gata4 is able to induce sox7 and sox18 expression in this assay (Fig. 3A). This result suggests that induction of beating cardiomyocytes by gata4 might involve these two paralogs of the SoxF family.
We therefore addressed the question whether these SoxF genes are required for gata4 to induce functional cardiomyocyte differentiation (see also (Zhang et al., 2005b)). To this end, we knocked down sox7 and sox18 in gata4-induced cardiomyogenesis and monitored expression of the cardiac differentiation markers myl2 and tnni3 (Fig. 3B, C). sox7 or sox18 knockdown causes substantially reduced cardiac differentiation as monitored by reduced expression of marker genes (Fig. 3B, C). Importantly we had made certain that the exogenously over-
expressed protein is efficiently synthesised in the presence of the MOs providing evidence that the observed effect is due to the intrinsic activity of GATA4 and its regulatory relationship with sox7 and sox18 (Fig. S1D). Our results show that sox7 and sox18 function is required downstream of gata4 for induction of functional cardiomyocyte differentiation.

In our effort to establish the epistatic functional relationship of gata4 with sox7 and sox18 during cardiac differentiation we next reinstated gata factor gene expression in a temporally controlled manner in Activin-induced cardiac explants in which sox7 and sox18 function was knocked down. To this end we used the same hormone-inducible version of gata4 (as introduced above and previously described in Afouda et al., 2005; Afouda and Hoppler, 2011; Afouda et al., 2008).

Induction of cardiomyogenesis was severely affected by knockdown of either sox7 or sox18, as measured by expression of terminal differentiation markers myl2 and tnni3 (Fig. 3D, E); and cardiomyogenesis could not be restored by experimentally over-activating gata4 function. Our data confirm the requirements for both paralogs of SoxF family downstream of gata4 function during cardiomyocyte differentia-
tion, which raises the question about possible redundancy between these two factors.

To examine redundancy between sox7 and sox18 function, we simultaneously knocked down both in cardiogenic explants and proceeded with monitoring expression of cardiac differentiation markers (Fig. 3F, G), as well as functional differentiation (suppl movie 2 and Fig. S1E). Re-instating gata4 activity was unable to substantially recover expression of marker genes for cardiac differentiation (Fig. 3F, G). It is worth noting that neither sox7 nor sox18 knockdown affects their own or each other’s expression. Additionally, reduced cardiac differentiation in the sox7 knockdown (or sox18 knockdown, data not shown) can also not be recovered by overexpression of either gata5 or gata6 (Fig. 3H, I), confirming that neither gata5 nor gata6 function downstream of sox7 (or sox18) function. As the amounts of the gata proteins present after activation are comparable (Fig. S1F), we can be certain that the observed effects are due to the intrinsic potential of each of these GATA factors in these experimental assays.

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2.6. sox7 and sox18 can partially substitute for gata4 function during cardiomyogenesis

We wondered to what extent SoxF function was able to mediate gata4 function in cardiomyogenesis. In order to address this question we developed an experimental design that would allow us to re-instate sox7 or sox18 activity in a temporally controlled manner in a gata4 knockdown background. To this end we fused the sox7 and the sox18 coding sequence in frame with the ligand-binding domain of the human glucocorticoid receptor (GR) (see Materials and Methods for details). The obtained fusion construct called respectively sox7GR and sox18GR were tested in animal cap explants and found to induce expression of terminal cardiac differentiation markers myl2 and tnni3 (data not shown). Inhibition of gata4, as expected caused a clear reduction of cardiogenic marker gene expression (cf. Afouda et al., 2008); interestingly, marker gene expression is noticeably reinstated to some extent when either sox7 or sox18 is experimentally activated (Fig. 4A, B). As the efficiencies of the over-expressed sox7 or sox18 proteins are not affected by the MOs (Fig. S1G), we can conclude that sox7 or sox18 is able to induce expression of cardiac differentiation markers when there is insufficient gata4 function and that sox7 and sox18 are therefore able to mediate some gata4 function during cardiac differentiation.

2.7. The gata4-soxF regulatory axis mediates non-redundant functions of cardiogenic gata factors in early versus later cardiomyogenesis

The aim of this investigation was to identify specific functions for the cardiogenic gata genes in the GRN driving vertebrate cardiomyogenesis. We had previously discovered non-redundant functions for gata4 and gata5 (Afouda and Hoppler, 2011), briefly with gata4 required for earlier and gata5 for later stages of cardiomyogenesis. Our transcriptomics analysis here has now identified the SoxF genes sox7 and sox18 as specifically regulated by gata4 (Fig. 1) and the above experiments confirm a functional role for sox7 and sox18 downstream of gata4 during cardiomyogenesis (Figs. 2–4A, B). We therefore wondered to what extent sox7 and sox18 function fits with this concept of early versus late functions of cardiogenic gata factors during cardiomyogenesis and therefore particularly focussed here on gata4 and gata5. In addition, to avoid the supplied exogenous GATA mRNAs being targeted by the MOs we have here used the previously validated splice MOs to strictly target endogenous gata4 and gata5 (Afouda and Hoppler, 2011; Haworth et al., 2008). In the gata4 knockdown, as expected we observe strongly reduced expression of early cardiac markers such as tbx5 and of both sox7 and

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**Fig. 5.** sox7 and sox18 are required for endogenous cardiac differentiation in vivo. Semi-quantitative (A) and quantitative (B) RT-PCR analyses of cardiac differentiation (myl2 expression) in Xenopus stage 32 dorsal marginal zone (DMZ) explants injected with sox7 MO together with 200 pg of mRNAs encoding dexamethasone-inducible gata proteins, as indicated. Note considerable requirement of sox7 function for cardiac differentiation (myl2 expression) and the ability of experimentally activated gata4, 5 or 6 to cause only a partial recovery of cardiac differentiation in a sox7 knockdown (compare with Activin-induced cardiomyogenesis, see Fig. 3H and I). (C–G) Whole-mount in situ hybridisation analysis of tnni3 expression of stage 32 embryos which are either un.injected (C) or had been injected at the four-cell stage in the two dorsal blastomeres with sox7 MO (D), sox7 MO plus gata4GR mRNA (E), sox18 MO (F) or sox18 MO plus gata4GR mRNA (G). Dexamethasone was added at stage 13 to activate the inducible gata4 protein (in E and G). Note that embryos with depleted sox7 and sox18 have reduced tnni3 expression compared to wild type (C) ranging from weak to complete reduction (D and F), which cannot be recovered with activated gata4 proteins (E and G). (H) Quantified data of tnni3 expression in each condition.

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and presumably as a consequence of cardiac differentiation markers such as myl2; all of which could be fully recovered with experimentally reinstated gata4 function (Fig. 4C). In contrast inhibition of gata5, as expected only affects the later stage differentiation marker myl2 but neither sox7 nor sox18 expression; nor early stage cardiac markers (Fig. 4C). Interestingly reinstating gata5 activity could substantially reestablish the expression of the differentiation marker myl2. These observations confirm that gata5 function during cardiomyogenesis is sox7- and sox18-independent, in contrast to its paralog gata4.

We then decided to explore to what extent the other cardiogenic gata factors gata5 and gata6 could replace gata4 in the regulation of sox7 and sox18 during cardiomyogenesis. We conducted experiments in which gata4 function was knocked down while concomitantly reestablishing gata4; or activating overexpressed gata5 or gata6, instead. We discovered that in such an experimental setting, reduced expression of sox7 and sox18 as well as of the cardiac differentiation markers myl2 and tnni3 could be recovered, as expected, when gata4 was reinstated, either at stage 8 or stage 13; but experimentally activated gata5 or gata6 could only recover expression of differentiation markers but neither sox7 nor sox18 (Fig. 4D, E). Our data show that only gata4 regulates sox7 and sox18 during cardiomyogenesis and that the other cardiogenic gata factors gata5 and gata6 cannot compensate for gata4 in this assay and must therefore here function via sox7- and sox18-independent mechanisms.
2.8. sox7 and sox18 are required for heart muscle differentiation in the embryo

Activin-induced cardiac explants are good for studying cardiomyogenesis in relative isolation from neighbouring embryonic tissues and therefore for studying cardiogenic gata and SoxF function specifically in cardiogenic mesoderm. Dorsal marginal zone (DMZ) explants differentiate into heart tissue in the absence of added factors (Foley and Mercola, 2005; Schneider and Mercola, 2001), presumably because the cardiac precursors reside within this part of intact gastrulating Xenopus embryos (Afouda and Hoppler, 2009). These DMZ explants therefore represents a first step in putting the cardiogenic tissue back into the normal wider embryonic context. We conducted similar experiments using DMZs to those described above with Activin-induced cardiac explants (cf. Fig. 3H). DMZs in which sox7 is knocked down (or sox18, data not shown) have reduced cardiac differentiation marker gene expression as measured by semi-quantitative and quantitative RT-PCR (Fig. 5A, B). Interestingly when any one of the cardiogenic gata factors is activated we observe a noticeable recovery of cardiomyogenesis, which is different from what had been observed above in Activin-induced cardiogenic explants (cf. Fig. 3H). This observation suggests existence of endogenous tissues or factor(s) in DMZ explants that are not present within Activin-induced cardiac explants, which allow the paralogs gata5 and 6 to reinitiate cardiac differentiation even in a sox7 (or sox18) knock down.

As a further step for putting the cardiogenic tissue back into the normal embryonic context we studied a whole embryo sox7 knockdown or sox18 knockdown by targeting injection of sox7 or sox18 MOs into the prospective heart tissue of 4-cell stage Xenopus embryos (Afouda et al., 2008; Foley and Mercola, 2005; Martin et al., 2010; Nascone and Mercola, 1995; Sater and Jacobson, 1990). Although mixed phenotypes were observed, there was a reduction of expression of the myocardium differentiation marker tnni3 in the sox7 or the sox18 knockdown (Fig. 5C-H). Additionally we found no difference between those knockdown embryos and knockdown embryos in which gata4 activity has been experimentally activated (Fig. 5E, G). We therefore conclude that sox7 and sox18 are required for normal myocardium differentiation in a manner consistent with functioning downstream of gata4 during cardiac differentiation.

2.9. Sox7 regulation by Gata4 is conserved in mammalian cardiomyogenesis

Molecular pathways involved in cardiomyogenesis are generally well conserved among vertebrates (Bruneau, 2002; Zaffran and Frasch, 2002). In order to test for conservation in mammalian cardiomyogenesis of the proposed Gata4 to Sox7/Sox18 regulatory axis we used mouse Embryonic Stem Cells (mESCs) cultured as Embryonic Bodies (EBs) that spontaneously undergo cardiomyocyte differentiation (Boheler et al., 2002). We confirmed that Gata4 (Fig. 6A), the cardiomyocyte differentiation marker Tmnt2 (Fig. 6B), Sox7 (Fig. 6C) and Sox18 (Fig. 6D) are expressed in these EBs. In a Gata4 shRNA knockdown (Fig. 6A-D), expression of Sox7 and Sox18 are reduced (Fig. 6C, D), as is the expression of Tmnt2 (Fig. 6B). This confirmed the Gata4 requirement for normal Sox7 and Sox18 expression during cardiomyocyte differentiation. In order to test for the ability of cardiogenic Gata factors to regulate Sox7 and Sox18 expression, we used mESCs lines in which the expression of individual cardiogenic gata factors can be experimentally activated (iGata4, iGata5, iGata6, Turbendian et al., 2013). Experimental activation of Gata4 expression prompted a quick and dramatic induction of Sox7 expression (Fig. 6E). Other cardiogenic Gata factors were only able to cause a much more modest initial induction of Sox7 expression (iGata6, Fig. 6G) or only a delayed response (iGata5 and iGata6, Fig. 6F, G), suggesting indirect mechanisms. In contrast, experimental overexpression of Gata4 caused only a slight increase in Sox18 expression (Fig. 6H), whereas ectopic expression of Gata5 and Gata6 cause a greater increase in Sox18 expression (Fig. 6I, J). These findings suggest that regulation by Gata4 of the SoxF genes Sox7 and Sox18 is conserved in the context of mammalian cardiomyogenesis. However, in contrast to animal caps, we find that Sox18 is regulated by all three cardiogenic Gata factors in EBs (Fig. 6I-J).

3. Discussion

Wnt/β-catenin signalling can inhibit early cardiomyogenesis (e.g. Ueno et al., 2007), which we discovered is mediated by negative regulation of gata gene expression (Afouda and Hoppler, 2009; Afouda et al., 2008; Martin et al., 2010). Our subsequent studies had additionally discovered some specific functional requirements of different cardiogenic gata factor genes for cardiomyogenesis (Afouda and Hoppler, 2011). In this study we aimed to explore such gata gene-specific functions more comprehensively using genome-wide transcriptomics analysis, in order to widen our general understanding of the gene regulatory network (GRN) directing cardiomyogenesis. Using gene knockdown approaches we have identified genes that are specifically regulated by individual cardiogenic gata factor genes gata4, gata5 or gata6; as well as those regulated by either one of them, and those regulated by all three of them together.

We are here focussing only on some aspects of the wealth of information that was generated in our high-throughput sequencing experiments, especially on genes specifically regulated by gata4. Gene ontology (GO) analysis suggests that gata4 tends to regulate other transcription factor genes during cardiomyogenesis, among which we identified the SoxF subfamily genes sox7 and sox18 (Fig. 1). This requirement of specifically gata4 for sox7 and sox18 expression is confirmed in subsequent validation experiments (e.g. Fig. 4), which also demonstrate that re-instating gata4 is capable of recovering sox7 and to some extent sox18 expression. Mammalian Sox7 expression also requires Gata4 function in a mouse ESC model of cardiomyogenesis, where Gata4 is also uniquely capable of promptly inducing Sox7 expression (Fig. 6). The discovery that this SoxF gene is also downstream of Gata4 in our mouse ES cell model strongly validates our Xenopus data and furthermore demonstrates that molecular pathways involved in early vertebrate cardiomyogenesis tend to be conserved. We conclude that we have identified an important gene regulatory axis from gata4 to SoxF family of transcription factors in heart muscle differentiation.

This gata4-SoxF regulatory axis is, however, only part of a much wider gata gene-mediated GRN for cardiomyogenesis. Future experiments will be required to explore other gata4-regulated candidate genes identified by our transcriptomics analysis (and those regulated by gata5 and gata6, see Fig. 1; and Table 1 in Afouda et al. (2017)), and to determine whether the transcriptional regulation of sox7 and sox18 by GATA4 is direct or involves important yet unidentified intermediary factors. It is expected that gata4 function in cardiomyogenesis involves other downstream mediators, since the sox7 and sox18 knockdown phenotype is less severe then the gata4 knockdown (Fig. 2) and also since experimentally reinstating sox7 and sox18 function cannot fully recover cardiomyogenesis in a gata4 knockdown (Fig. 4). Identification of such key genes downstream of gata4 and of other cardiogenic gata genes promises to provide a better understanding of human cardiomyopathies resulting from GATA4 mutations (Garg et al., 2003; Rajagopal et al., 2007) and mouse phenotypes such as acardia in Gata4 and Gata6 double mutant embryos (Zhao et al., 2008).

An important role for SoxF genes for cardiovascular development is further supported by the cardiovascular failure of mice lacking Sox7 that leads to death of embryos at embryonic day 10.5 (Wat et al., 2012), by the importance of Sox7 in arterial specification (Hermkens et al., 2015) and by the essential functions of sox7 and sox18 in Xenopus cardiomyogenesis (Zhang et al., 2005b). Our data indicates possible redundancy between sox7 and sox18 as knockdown of both together...
leads to a more severe reduction of cardiac marker gene expression than either of them alone (Fig. 3) (see also Zhang et al., 2005b).

However, there are also SoxF-independent regulatory mechanisms expected to drive cardiomyogenesis. It is intriguing that although gata5 and gata6 are not able to reinitiate sox7 or sox18 expression in a gata4 knockdown, they can substantially reinitiate expression of cardiac differentiation markers (Fig. 4). This suggests sox7- and sox18-independent pathways downstream of gata5 and gata6 (Fig. 7). This confirms our and other previous studies (Afouda and Hoppler, 2011; Ieda et al., 2010) uncovering divergent functional roles of cardiogenic GATA factors during cardiomyogenesis. Our discovery of sox7 and sox18 as genes specifically regulated by gata4 not only increases our general understanding of cardiogenesis but also provides specifically insights into the gene regulatory mechanisms involved. Functional interactions with other regulators of cardiogenesis are likely, for instance, SOX proteins have previously been shown to interact with β-catenin (Kormish et al., 2010; Zorn et al., 1999), which can function as an inhibitor during early cardiogenesis (Afouda et al., 2008; Ueno et al., 2007). However, further studies have shown that while SOX7 physically interacts with β-catenin and functionally inhibits Wnt signalling pathway activity; a version of SOX7 that cannot interact with β-catenin is still able to induce cardiogenesis (Zhang et al., 2005b) suggesting that this aspect of Sox7 function in cardiogenesis is Wnt/β-catenin-independent. Since sox7 and sox18 have been reported to induce the expression of nodal4, nodal5, and nodal6 in cardiogenesis in Xenopus explants (Zhang et al., 2005a, 2005b) we include them in a model to depict the functional differences between gata paralogs during cardiomyogenesis (Fig. 7).

It is also the wider embryonic context with neighbouring tissues that provides further dependability and built-in redundancy to the gene regulatory mechanisms driving cardiomyogenesis. While studying cardiogenesis in relative isolation in Activin-induced cardiac explants was effective for uncovering the gata4-SoxF regulatory axis (Fig. 3), the wider embryonic context of DMZ explants suggests that there are additional pathways allowing all cardiogenic gata factors to relieve inhibition of cardiac differentiation in the sox7 knockdown (Fig. 5A, B). Future experiments will need to explore any role particularly for the endoderm: it is intriguing for instance that we observe a further loss of expression of cardiomyocyte differentiation markers in the sox7 knockdown (Fig. 3H, I), and of earlier cardiac progenitor markers such as mespa (data not shown), when cardiogenic GATA function is experimentally activated during early stages, which could be explained by the pro-endoderm-inducing activity of these GATA factors (Afouda et al., 2005). In support, we do observe a slight increase in the induction of the third SoxF paralog sox17α, a known endoderm marker (data not shown). A role of the endoderm for cardiomyogenesis in the wider embryonic context is of course consistent with the previously proposed requirements of signals from this layer for cardiac development (Foley et al., 2007; Foley and Mercola, 2005; Liu et al., 2014; Nascone and Mercola, 1995, 1996; Schneider and Mercola, 2001).

In conclusion, our results identify an important gene regulatory axis from gata4 to the SoxF paralogs sox7 and sox18 for heart muscle differentiation, which is conserved in wider embryonic contexts (DMZ and entire embryo, Fig. 5) and in mammalian cardiomyogenesis (Fig. 6). Our findings represent a further important advance in the molecular dissection of the regulatory mechanisms controlling heart formation. They pave the way for further investigations into elucidating GRNs that are involved downstream of gata4 and the other paralogs gata5 and gata6 in heart muscle development.

4. Materials and methods

4.1. Ethics statement

All Xenopus experiments were performed according to the University of Aberdeen’s Code of Practice on the Use of Animals in Research as well as the legal requirements of the Animals (Scientific Procedures) Act 1986 ( Licence PPL 60/4376) and the Home Office Code of Practice guidance.

4.2. Expression constructs, mRNA synthesis and morpholinos

Activin β B, gata4GR, gata5GR and gata6GR DNA constructs for mRNA synthesis have been described previously (Afouda et al., 2005, 2008; Afouda and Hoppler, 2011). Xenopus laevis sox18GR DNA construct was made by in frame fusion of its coding region to the region encoding the hormone-inducible domain of human glucocorticoid receptor in pSP64T-GR as previously described (Tada et al., 1997). To this end, a BamHI site was created at the end of the amplified coding sequence and inserted into BglII-digested vector. The following primer sequences were used to amplify Xenopus laevis sox18 (pDONR223Xsox18 a kind gift from Prof Aaron Zorn and Scott Rankin). Xsox18: 5′ GGA TCG GAT CCA CCA GGA TGC ATA GAT CTA GC 3′ for the forward primer and 5′ GAT CGG GAT CCA CCA GGA TGC ATA GAT CTA GC 3′ for the reverse primer (accession number NM_001088635). The Xenopus laevis sox7 fusion construct (Xsox7GR) was made from stage 32 cardiac explants cDNA synthesised according to a previously described protocol (Weber et al., 2000). To this end we’ve designed specific primers to sox7 sequence accession number NM_001085868 flanked by BglII sequence to amplify its cDNA. The following primers sequences were used: Xsox7: 5′ GGA TCA GAT CTA CCA GGA TGA TGA TGG GAT CC 3′ for the forward primer and 5′ GAT CCA GAT CTA CAA ACA CTA TAA CTG TGG 3′ for the reverse primer. The PCR product was BglII-digested and inserted...
in frame into pSP64T-GR vector as above. All the constructs were checked by restriction digestion and by sequencing. All fusion plasmids were Salt-linearized and in vitro transcribed with SP6 using mMESSAGE mMACHINE kits (Ambion) according to the manufacturer’s instruction. The following amounts of RNA were injected: 50 fg for Activin, 200–1000 pg for all other constructs per embryo (see Figures legends). Xenopus gata4 splice morpholin, gata5 splice morpholin (Haworth et al., 2008), gata6 morpholin (Peterkin et al., 2003), Xsox7 and Xsox18 (Zhang et al., 2005b) morpholinos have been previously described. The amounts of MOs injected per embryo are: 50 ng (gata4, 4MO), 8 ng (gata5, 5MO), 10 ng (gata6, 6MO), 30 ng for single (sox7, 7MO) and (sox18, 18MO) and 15 ng of each when combined.

4.3. Embryos and explants culture

Xenopus laevis embryos were obtained as previously described (Afouda et al., 2005). Embryos and explants culture as well as embryos injection were as described in Afouda et al. (2008) and Afouda and Hoppler (2011). Animal cap explants were excised as previously described (Afouda, 2012) and where applicable dexamethasone was added at final concentration of 10 µM (at either stage 8 or 13, see Figures legends) for activation of GR-fusion proteins. Live GFP beating embryos were imaged using Zeiss Axio Observer Z1 microscope with the Axiovision software and movies were taken using Leica M60 microscope mounted with Leica MC 170 HD camera.

Counts were averaged for each condition and then these averages were standardised to have the same mean across the different conditions. Similarity between each gene expression profile within each cluster was then computed with Euclidean distance and shown as heatmap (Fig. 1G, I). For gene ontology (GO) analyses, GO classes containing at least six genes were taken into consideration.

4.4. RNA extraction, RNA expression analysis and protein analysis

Whole mount in situ hybridisation (WISH) was conducted as previously described (Cau-Uitz et al., 2000). A digoxigenin-labelled RNA probe was prepared with T7 polymerase, using High Yield Megascript Kit (Ambion) from NotI-linearized plasmid template for tnni3 (Drysdale et al., 1994). The abundance of RNAs was determined using semi-quantitative Reverse Transcriptase PCR (RT-PCR) (Afouda and Hoppler, 2011) and real time quantitative Taq man assays using the comparative CT method. For this, at least three repeats were done for each experimental group and 20–30 cardiogenic explants were analysed for each condition (or 25–50 mESC Embryoid Bodies). For each experimental repeat measurements for each condition were conducted in triplicate and relative mRNA expression levels were calculated by normalisation to Xenopus odc or housekeeping gene Gapdh mRNAs. Raw data were analysed with the ΔΔCT method (Livak and Schmittgen, 2001). Data are mean ± SEM, n = 3, P ≤ 0.05. Data presented are representative of one experiment. Reactions were run on ABI 7700 Sequence Detector (Afouda et al., 2005; Peterkin et al., 2003). Sequences of primers and probes for quantitative Taq man were as described in (Peterkin et al., 2003). Sequence of primers used for semi-quantitative RT-PCR are as follow: gata4, myl2, tnni3 and odc (Afouda and Hoppler, 2011); Xenopus laevis mespa and mespb (Hitachi et al., 2009); Xenopus laevis Isl1 (Gessert and Kühl, 2009); sox17a (Afouda et al., 2005). For both sox7 and sox18 the primers were designed based on accession numbers mentioned above that are homoeologs of the ones identified in our RNA-seq results and are as follow: sox7 primers 5’ ATG ACT ACC CTG ATG GGA TCC TAC AGC 3’ for forward primer and 5’ AGA AAC ACT ATA ACT GTT GTA GTA CG 3’ for reverse primer and for sox18 5’ ATG CAT AGA TCT ACG TGC AGA G 3’ for forward primer and 5’ GCC AGT AAT ACA GGG GGT GTA GGA 3’ for reverse primer. Protein extraction and western blot analysis were as previously described (Afouda et al., 2005).

RNAs were extracted from ES cells and Embryoid Bodies using the Absolutely RNA Microprep Kit (Agilent) according to the manufacturer’s instructions. cDNAs were prepared as previously described (Afouda et al., 2005) and quantitative PCR was performed using LightCycler 480 Probes Master Mix from Roche with Universal Probe Library (UPL) and reaction run on LightCycler 480 machine.

4.5. RNA-seq experiments and analysis

At least 30 explants were used for RNA preparation with a previously described protocol (Afouda and Hoppler, 2011; Afouda et al., 2008). RNA quantity and quality were checked on electrophoretic agarose gel, a fraction of which was used for validation with gene expression analysis by quantitative RT-PCR (Afouda et al., 2005) to confirm expected increase or decrease of known control gene expression before RNA-seq sequencing. RNA was isolated from three independent biological replicates for each condition. Illumina TruSeq RNA libraries were constructed and sequenced on the Illumina HiSeq_2500 platform at the Earlham Institute, Norwich Research Park, Norwich, UK. 100 bp paired-end sequencing reads were aligned to the Xenopus laevis genome (version 9.1) using Hisat2 (Kim et al., 2015) and quantification was done using featureCounts (Liao et al., 2014). Differential expression analysis was performed using DESeq. 2 (Love et al., 2014) with an adjusted p value < 0.05. Differentially expressed genes were identified using a threshold of log2 fold change > 1 (for at least two times increased) or < −1 (for at least two times reduced) in comparison to Activin-induced Xenopus animal cap cardiac explant controls. Analyses of differentially expressed genes were performed using Partek genotypes Suite 6.6. Similarly hierarchical clustering was performed in Partek Genomic genomics Suite 6.6. Counts were averaged for each condition and then these averages were standardised to have the same mean across the different conditions. Similarity between each gene expression profile within each cluster was then computed with Euclidean distance and shown as heatmap (Fig. 1G, I). For gene ontology (GO) analyses, GO classes containing at least six genes were taken into consideration.

4.6. ES cell culture and differentiation

Inducible reporter Mesp1/Gata4 and iGata cell lines were obtained from Professors Cedric Blanpain (Université Libre de Bruxelles, Belgium, (Bondue and Blanpain, 2010))) and Todd Evans (Weill Cornell Medical College, USA, (Holtzinger et al., 2010; Turbendian et al., 2013)), respectively. Inducible Mesp1 cells were maintained and differentiated as previously described (Bondue and Blanpain, 2010; Bondue et al., 2008). The iGata4/5/6 cells were maintained and differentiated as previously described (Holtzinger et al., 2010; Turbendian et al., 2013), except that Embryoid Bodies were differentiated in hanging drop culture. shRNAs were obtained from Qiagen and stable transfected cell lines were generated according to manufacturer’s instructions.

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Competing interests

The authors declare no competing or financial interests. Author contributions

B.A.A performed the Xenopus experiments. A.T.L performed the ES
cell experiments. B.A.A and EdP performed the bioinformatic analyses. B.A.A. and S.H wrote most of the manuscript. A.T.L commented the entire manuscript and wrote part of it. B.A.A, A.T.L. and S.H designed and analysed experiments.Funding

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ydbio.2017.11.017.

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