RESEARCH COMMUNICATION

Cooperative regulation in development by SMRT and FOXP1

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A critical aspect of mammalian development involves the actions of dedicated repressors/corepressors to prevent unregulated gene activation programs that would initiate specific cell determination events. While the role of NCoR/SMRT corepressors in nuclear receptor actions is well documented, we here report that a previously unrecognized functional interaction between SMRT and a forkhead protein, FOXP1, is required for cardiac growth and regulation of macrophage differentiation. Our studies demonstrate that SMRT and FOXP1 define a functional biological unit required to orchestrate specific programs critical for mammalian organogenesis, linking developmental roles of FOX to a specific corepressor.

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Although activation of transcription has long been recognized as an essential component of gene regulation during development, the critical role of transcriptional repression programs is apparent from the pluripotent stem cell to terminal differentiation events. Nuclear receptors, including retinoic acid and thyroid hormone receptors (RAR and T₃R), regulate development through both ligand-dependent activation and active repression by unliganded nuclear receptors (Glass and Rosenfeld 2000), and their ability to actively repress transcription in the absence of their cognate ligands is conferred by their interaction with SMRT or with the highly related corepressor NCoR (Chen and Evans 1995; Horlein et al. 1995). NCoR and SMRT also confer transcriptional repression to many additional members of the nuclear receptor superfamily, as well as on a variety of unrelated transcription factors, at least in part due to corecruitment of histone deacetylase proteins (HDACs) (Privalsky 2001; Jepsen and Rosenfeld 2002; Jones and Shi 2003).

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The forkhead family of transcription factors, which includes over a hundred genes in several species named for the forkhead-box (FOX) DNA-binding domain, has been characterized as both transcriptional activators and repressors (for review, see Wijchers et al. 2006). While all four FoxP family members function as transcriptional repressors, the mechanism of this repression remains largely uncharacterized, although FoxP3 has been shown to be capable of interacting with HDAC proteins (Li et al. 2007). Gene deletion studies have revealed that FOXP1 mutant mice have defects in cardiac morphogenesis, a thin ventricular myocardial compact zone, and lack of proper ventricular septation, which together result in embryonic death at embryonic day 14.5 (E14.5) (Wang et al. 2004). Interestingly, maintaining the proper balance of histone acetylation/deacetylation is critical for proper cardiac development and growth (Backs and Olson 2006; Montgomery et al. 2007), leading us to consider potential links between FOXP1 and recruitment of specific corepressor complexes.

Here we report that deletion of the gene encoding the corepressor *SMRT* resulted in specific developmental abnormalities including hypoplasia of the ventricular chambers of the heart and a defect in ventricular septation, accompanied by up-regulation of the CDK inhibitor *p21* (WAF-1/CIP-1/SDI-1), a phenotype analogous to that reported for gene deletion of *FOXP1*. We found that both SMRT and FOXP1 are recruited to the *p21* promoter, and that these proteins physically interact. *SMRT*^{+/-}/*FOXP1*^{+/-} double heterozygote mice exhibit a phenotype comparable with either single gene deletion, suggesting that, together, SMRT and FOXP1 regulate a program of gene repression essential to proper myocardial development. This SMRT/FOXP1 corepressor complex appears to mediate a more general strategy, as SMRT and FOXP1 proteins prove to be a component of *c-fms* regulation in monocytes, indicating that SMRT-mediated corepression may be a common mechanism by which FOXP1 and other FOX proteins regulate gene expression programs in development of target organs.

Results and Discussion

Characterization of *SMRT*^{-/-} cardiac defect

SMRT gene-deleted mice initially generated and analyzed for defects in neural development in Jepsen et al. (2007) were analyzed to delineate potential roles of SMRT independent of its actions on nuclear receptors. We observed that the majority of *SMRT*^{-/-} mice died by E16.5. Histological examination of E14.5 embryonic hearts revealed a hypoplastic ventricular wall and a defect in ventricular septation (Fig. 1A–D), detectable as early as E11.5 (Fig. 1E–H). The transformation of the ventricular chamber from the thin-walled state observed at E9.5 to the mature ventricle requires two distinct waves of proliferation that are accompanied by morphogenic changes, both beginning around E9.5–E10.5. The proliferating myocardium on the endocardial (inner) side of the ventricles becomes the trabecular layer while the proliferating myocardium on the epicardial (outer) side of the ventricles forms the compact zone. Further expansion of the compact zone allows for formation of the interventricular septum by cardiomyocytes. Genetic

Figure 1. Gene deletion of SMRT results in a severe heart defect. (A–H) Low-power (A,B,E,F) and high-power (C,D,G,H) photomicrographs of H&E-stained transverse cryo-sections of control and *SMRT*^{-/-} heart reveal thinned ventricular walls (open arrow) and a ventricular septum defect (filled arrow) in *SMRT*^{-/-} hearts as observed at E14.5 (A–D) and E11.5 (E–H). (I,J) High-power photomicrographs of E15.5 wild-type (I) or *SMRT*^{-/-} (J) hearts stained with an antibody specific for SMRT indicating expression in trabeculae (t), compact zone (cz), and epicardium (e).

mutations affecting compact zone formation cause embryonic lethality beginning around E14.5, consistent with the timing of death observed in *SMRT*^{-/-} mice (Fig. 2E). Radioactive in situ analysis using probes to cardiac α -actin, the ventricular-specific myosin light chain 2v (*MLC2v*) and atrial-specific myosin light chain 2a (*MLC2a*) detected no differences between wild-type or *SMRT*^{-/-} hearts at E14.5, suggesting that initial cardiac and chamber specification occurred normally (data not shown).

SMRT is required in cardiac myocardium

The mature heart originates mainly from four separate tissues (neural crest, myocardium, endocardium, epicardium). In addition to the myocardial cell themselves, signals from the epicardium to the myocardium play an

essential role in ventricular wall compact zone expansion (Kreidberg et al. 1993; Kwee et al. 1995; Yang et al. 1995; Chen et al. 2002; Pennisi et al. 2003), while neither endocardium nor neural crest has been implicated. The phenotype observed for *SMRT*^{-/-} embryos was strikingly similar to the heart phenotype reported previously for Retinoid X Receptor α (*RXR* α) gene-deleted mice (Kastner et al. 1994; Sucov et al. 1994) that has been reported to be independent of *RXR* α expression in myocytes (Chen et al. 1998; Tran and Sucov 1998; Subbarayan et al. 2000b) but to require *RXR* α in the epicardium (Chen et al. 2002). As SMRT has been reported to interact with *RXR* α to repress transcription (Ghosh et al. 2002), and as expression of SMRT protein was specifically detected in both epicardium and myocardium, as well as in trabeculae and endocardium (Fig. 1I,J), we sought to determine whether or not SMRT was required in a cell-autonomous manner.

To this end, we generated transgenic mice in which myocyte-specific expression of a dominant-negative SMRT protein was driven by the α -myosin heavy-chain (α -MHC) promoter (Subramaniam et al. 1991). The region of SMRT chosen as a dominant negative is based on previously published reports (Feng et al. 2001; Koide et al. 2001) and consists of the region C-terminal to the repressor domains (amino acids 1500 through the stop codon) (Supplemental Fig. 1A). Pronuclear injection of linearized HA-tagged α -MHC-DN-SMRT was performed, and litters were sacrificed at E13.5, E14.5, or E15.5 for transgenic founder analysis. Significant embryonic lethality was observed at both E14.5 and E15.5 (18% and 20% of all embryos recovered, respectively), and rates of positive transgene recovery also fell with embryonic age (Supplemental Fig. 1B), suggesting that the embryonic lethality was associated with expression of the DN-SMRT transgene. Indeed, expression of DN-SMRT in the myocardium using the α -MHC promoter resulted in a phenotype at E13.5 that was analogous to that of the *SMRT* gene-deleted mice in that the compact zone was reduced in thickness and there were ventricular septation defects (cf. Figs. 1A–D and 2A–D). As the α -MHC promoter drives expression specifically in cardiomyocytes (Subramaniam et al. 1991), these data suggest that the requirement for SMRT is cell-autonomous. To provide further evidence of the cell-autonomous role for SMRT, we tested whether transgenic re-expression of *SMRT* in mutant cardiac myocytes could rescue these defects (Supplemental Fig. 1A). Indeed, the *SMRT* gene deletion phenotype was rescued by the expression of full-length SMRT in myocardium as evidenced by increased survival at later embryonic ages (Fig. 2E). Additionally, α -MHC-SMRT-rescued *SMRT*^{-/-} embryos have an intact ventricular septum and a compact zone thickness that nears that of wild-type embryos (Fig. 2F,G). Survival after birth was precluded by the presence of a severe secondary palate defect that was not rescued by the cardiomyocyte-specific expression of SMRT (Jepsen et al. 2007). In contrast, overexpression of the non-cell-autonomous *RXR* α in *RXR* α ^{-/-} mice using the α -MHC promoter did not prevent either fetal lethality or the myocardial defects observed in these mice (Subbarayan et al. 2000a).

SMRT interacts with FOXP1

That the *SMRT*^{-/-} cardiac phenotype was cell-autonomous essentially precluded the hypothesis that SMRT

Figure 2. The requirement for SMRT in heart development is cell-autonomous. (A–D) Low-power (A,B) and high-power (C,D) photomicrographs of H&E-stained transverse cryo-sections from E13.5 wild-type (A,C) and transgenic (B,D) hearts expressing dominant-negative SMRT driven by α -MHC (α -MHC-DN-SMRT) revealed thinned ventricular walls (open arrow) and a ventricular septum defect (filled arrow) in transgenics. (E) Summary of live embryos recovered from SMRT^{-/-} mice crossed to α -MHC-FL-SMRT⁺/SMRT^{-/-} mice at various ages. (F,G) Low-power photomicrographs of H&E-stained transverse cryo-sections from E17.5 wild-type (F) or α -MHC-FL-SMRT⁺/SMRT^{-/-} (G) hearts.

was acting exclusively by repressing RXR α gene targets. We were, therefore, particularly intrigued by a report that gene deletion of *FOXP1* results in a myriad of heart defects, including a thinned compact zone and ventricular septal defect similar to what was observed for SMRT^{-/-} mice (Wang et al. 2004). As *FOXP1* has been characterized as a DNA-binding transcriptional repressor (Shu et al. 2001; Wang et al. 2003), we wanted to test the possibility that SMRT could act as a functional co-repressor with *FOXP1* in heart, such that ablation of either factor would produce a similar phenotype. Coimmunoprecipitation experiments revealed that *FOXP1* and SMRT interact both when overexpressed in HEK 293 cells (Fig. 3A) and in hearts dissected from E13.5 embryos (Fig. 3B). *FOXP1* could also interact with both full-length SMRT and the dominant-negative form of SMRT used to create the phenocopying transgene in GST-TNT assays (Fig. 3C). This is consistent with the idea that *in vivo*, overexpressed DN-SMRT interacts with *FOXP1* and potentially prevents recruitment of wild-type SMRT, thus phenocopying the cardiac defect observed in the absence of SMRT.

Several genes have been implicated in the proliferation and differentiation of myocytes during embryonic development. Quantitative RT-PCR (qRT-PCR) was used to compare expression of *p21*, *p27*, and *p57* between wild-type and SMRT^{-/-} hearts. While significant differences were not observed for *p27* or *p57*, *p21* mRNA levels were up-regulated in SMRT^{-/-} hearts (Fig. 3D), a finding also reported for *FOXP1*^{-/-} hearts (Wang et al. 2004). Expression of *FoxP1* itself was unchanged in SMRT^{-/-} hearts compared with wild type (Fig. 3D). *p21* has been identified as regulated directly by the FOX family of transcription factors, including *FoxG1* and *FoxO* in neuroepithelial and glioblastoma cells, through a conserved Fox consensus binding site at -1930 in the mouse *p21* promoter (Seoane et al. 2004). To determine whether *FOXP1* and SMRT were recruited to the *p21* promoter during heart development, we isolated E10.5 myocardium from wild-type embryos and performed chromatin immunoprecipitation (ChIP) assays using antibodies specific to *FOXP1* or SMRT. PCR amplification using oligonucleotides surrounding the Fox-binding site identified in the *p21* promoter revealed enrichment of both *FOXP1* and SMRT on this promoter, as well as dimethylated histone H3 Lys 9

(DimeH3K9) (Fig. 3E), a mark associated with repression of *p21* (Nishio and Walsh 2004; Duan et al. 2005). In contrast, the natriuretic peptide precursor type A (*ANF*) promoter region was assayed as a control and was positive only for DimeH3K9 (Fig. 3E). Increases in *p21* levels have been reported to be correlated with the exit from cell cycle that occurs in the neonatal heart and with differentiation of cultured cardiomyocytes (Flink et al. 1998; Koh et al. 1998). Thus, it is logical to think that up-regulation of *p21* might result in a block in cell proliferation that could account for the thinned myocardium observed in SMRT^{-/-} and *FOXP1*^{-/-} embryos. To evaluate changes in myocardial cell proliferation, we used antibodies to phospho-histone-H3 (PO4-H3), which labels cells undergoing mitosis. No differences in the number of PO4-H3-positive cells at E10.5, E11.5, E12.5, or E13.5 were observed, either in compact or trabecular zones, and we could find no evidence of increased apoptosis at these ages (data not shown). Interestingly, *FOXP1*^{-/-} embryos exhibited no change in PO4-H3 expression in the compact zone at E13.5 and an increase in PO4-H3 expression in the trabecular zone (Wang et al. 2004), suggesting that the etiology of this heart defect is complex.

SMRT/*FOXP1* double heterozygote mice mimic the cardiac defect observed in either single gene-deleted animal

To test whether there was a genetic interaction between SMRT and *FOXP1*, an embryonic stem (ES) cell clone with a retroviral gene trap insertion in the *FOXP1* gene (obtained from the Soriano Laboratory Gene Trap Resource at the Fred Hutchinson Cancer Research Center) was used to generate mice heterozygote for *FOXP1*. Heterozygote *FOXP1* mice were then interbred, and histological analysis confirmed that E14.5 *FOXP1*^{-/-} embryonic hearts had the same ventricular septation defect and thinned compact zone phenotype originally observed for *FOXP1*^{-/-} (Supplemental Fig. 1C,D; Wang et al. 2004). SMRT^{+/+} mice were then interbred with *FOXP1*^{+/+} animals to generate SMRT^{+/+}/*FOXP1*^{+/+} double heterozygotes. While SMRT^{+/+}, *FOXP1*^{+/+}, and wild-type animals were all recovered at near the 25% expected Mendelian rate, SMRT^{+/+}/*FOXP1*^{+/+} double heterozygotes were re-

Figure 3. SMRT and FOXP1 interact in the heart to regulate proper heart development. Coimmunoprecipitation of SMRT and FOXP1 in HEK 293 cells (A) and in E13.5 hearts (B). (C) Interaction of 35 S-radiolabeled wild-type SMRT or DN-SMRT (amino acids 1500 through the stop codon) with GST-FOXP1 fusion protein or GST protein alone. (D) qRT-PCR for p21, p27, p57, and FOXP1 on cDNA isolated from E10.5 wild-type or *SMRT*^{-/-} hearts ($\pm$ standard error of the mean, SEM; ** $P < 0.005$). (E) ChIP analysis on E10.5 hearts using specific anti-SMRT, anti-FOXP1, and anti-histone H3 dimethyl K9 antibodies and oligonucleotides surrounding the FOX-binding site of the p21 promoter. (F–H) Photomicrographs of H&E-stained transverse cryo-sections from *FOXP1*^{+/-} (F), *SMRT*^{-/-} (G), and *FOXP1*^{+/-}/*SMRT*^{+/-} (H) E14.5 embryos. Arrowhead indicates thinned ventricular wall. Arrow indicates ventricular septum defect. (Tx) Transfected; (UnTx) untransfected; (ANF) natriuretic peptide precursor type A.

covered at a rate of just 10%, suggesting embryonic lethality. Histological analysis of embryos generated from *FOXP1*^{+/-} crossed to *SMRT*^{+/-} at E14.5 revealed that ~50% of *FOXP1*^{+/-}/*SMRT*^{+/-} double heterozygote embryos had a thinned myocardium and ventricular septal defect similar to that observed for gene deletion of either factor alone (Fig. 3H). As such a phenotype was not observed for single heterozygote animals (Fig. 3F,G), this provides genetic evidence of a *SMRT*^{+/-}/*FOXP1*^{+/-} interaction in heart development.

SMRT mediates FOXP1 repression of *c-fms* in a monocyte cell line

To determine if SMRT can serve as a corepressor for FOXP1 in cells other than myocardium, we investigated the role of SMRT in regulating expression of *c-fms*, which encodes the colony-stimulating factor 1 receptor (Csf1r), a receptor–tyrosine kinase responsible for the differentiation and maturation of macrophages. Transcriptional repression of *c-fms* in undifferentiated THP-1 cells has been reported to be mediated by FOXP1, and overexpression of FOXP1 during the differentiation process delays induction of *c-fms* with the protein kinase C activator phorbol myristate acetate (PMA) (Shi et al. 2004). Lipofection of two distinct siRNAs specific for *SMRT* (Supplemental Fig. 1E) consistently increased expression of *c-fms* (Fig. 4A), suggesting that SMRT can act as a corepressor of FOXP1 in repression of *c-fms*. siRNA spe-

cific for *FOXP1* (Supplemental Fig. 1H) also resulted in a significant increase in *c-fms* expression (Fig. 4A). However, we note that the fold induction of *c-fms* with siRNA-mediated removal of SMRT or FOXP1 is not as great as that observed when THP-1 cells are treated with PMA (Supplemental Fig. S1F), suggesting that to fully activate *c-fms* gene transcription in this assay, a specific activation signal in addition to removal of a repressor complex may be required as has been demonstrated in the case of the RAR (Ogawa et al. 2004). Alternatively, as lipofection of control siRNA results in increased *c-fms* gene expression compared with nonlipofected cells (Supplemental Fig. S1G), it is possible that the *c-fms* induction observed with siRNAs specific for *SMRT* or *FOXP1* is dampened by the increased baseline of *c-fms* expression.

To determine whether FOXP1 and SMRT can directly repress *c-fms*, we performed ChIP assays using primers surrounding the previously identified FOXP1-binding sites in the *c-fms* promoter (Shi et al. 2004). Both FOXP1 and SMRT were detected as bound to the *c-fms* promoter in untreated cells, but not in cells treated with PMA (Fig. 4B–D), consistent with the fact that PMA induces *c-fms* expression (Supplemental Fig. 1F; Shi et al. 2004). To

Figure 4. SMRT and FOXP1 coregulate *c-fms* expression in THP-1 cells. (A) Effect of siRNAs specific to FOXP1 or SMRT on *c-fms* mRNA levels as measured by qRT-PCR ($\pm$ SEM; * $P < 0.01$, ** $P < 0.005$). (B,C) qPCR analysis of chromatin-immunoprecipitated SMRT or FOXP1 on untreated (undiff) and PMA-treated (diff) THP-1 cells using specific anti-SMRT and anti-FOXP1 antibodies and oligonucleotides surrounding the FOX-binding site of the *c-fms* promoter. (D) Agarose gel representation of ChIP analysis in B and C. (E,F) qPCR analysis of chromatin-immunoprecipitated SMRT or FOXP1 using oligonucleotides surrounding the FOX-binding site of the *c-fms* promoter performed on THP-1 cells lipofected with FOXP1 siRNA.

determine whether FOXP1 is itself responsible for recruitment of SMRT, we performed ChIP analysis in THP-1 cells following treatment with specific siRNA for *FOXP1* (Supplemental Fig. 1H). Indeed, neither FOXP1 nor SMRT was detected on the *c-fms* promoter in the absence of FOXP1 (Fig. 4E,F). This indicates that SMRT is recruited to *c-fms* promoter specifically through interactions with FOXP1. Together these data indicate that the function of the SMRT/FOXP1 unit extends to a second biological system, suggesting a more general mechanism whereby FOXP1 repression programs depend on SMRT corepression.

Transcriptional activation and repression mediated by sequence-specific DNA-binding factors underlie the binary decisions necessary for progression of cell determination events, and it is increasingly clear that cofactors, including NCoR and SMRT, are indispensable for proper development in a variety of developmental systems. Interestingly, despite their high homology, NCoR and SMRT appear to have nonoverlapping roles in many instances, with NCoR gene-deleted mice exhibiting defects in developmental progression of erythrocytes and thymocytes (Jepsen et al. 2000). Neural stem cells (NSCs) isolated from either gene-deleted mouse exhibit spontaneous differentiation into astrocytes, but only SMRT is required for repression of neuronal differentiation (Hermanson et al. 2002; Jepsen et al. 2007). Interestingly, in NSCs, we found that SMRT was required to repress an RAR-dependent differentiation program (Jepsen et al. 2007). Here we propose a novel role for SMRT in regulating heart development through a forkhead protein as SMRT is required to cell-autonomously regulate growth of the ventricular compact zone, distinct from the requirement for RXR α in epicardium (Chen et al. 2002). Indeed, as SMRT and FOXP1 both appear to be used for gene regulation in heart and macrophages, we can suggest that this is a general mechanism by which FOXP1, and perhaps other family members of this forkhead protein subclass, mediate transcription. Thus we have demonstrated that the previously unrecognized relationship between SMRT and a forkhead family member underlies multiple biological programs.

Materials and methods

Immunoprecipitation

HEK 293 cells were transfected with pCMX-SMRT (mouse) and pcDNA-FOXP1 (human) using Lipofectamine 2000 (Invitrogen). For immunoprecipitations from heart, the ventricles of ~50 hearts were dissected from E10.5 mouse embryos. Immunoprecipitations were performed as described previously (Heinzel et al. 1997) using anti-FOXP1 antibodies (Shi et al. 2004).

Protein interaction assays

Full-length or DN-SMRT were in vitro translated using the TNT Quick-Coupled Transcription/Translation System (Promega). A GST-FOXP1 fusion protein was generated from a pGEX vector containing human FOXP1 cDNA in frame with GST. GST-TNT interaction assays were performed essentially as described previously (Olson et al. 2006).

ChIP

ChIP was performed as described previously (Jepsen et al. 2000). Ten E10.5 dissected hearts or 10^7 THP-1 cells were used per antibody, using 1 μ g of the following antibodies: SMRT (Affinity Bioreagents, PA1-843); FOXP1 (AVIVA Systems Biology, ARP32564; and CeMines, AB/FOX330, pooled); dimethyl histone H3 K9 (Upstate Biotechnology, 07-441). See the Supplemental Material for the primers used.

FOXP1 gene-deleted mice

ES cell line S17-4A, which features insertion of a retroviral gene trap vector into the fifth intron of *FOXP1* (NT_039353), was kindly provided by Philippe Soriano and was used to generate *FOXP1* gene-deleted mice. Genotypes were determined by PCR analysis [see the Supplemental Material].

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