Sox17 is essential for the specification of cardiac mesoderm in embryonic stem cells

Yu Liu*, Masanori Asakura*†, Hironori Inoue*‡, Teruya Nakamura*§, Motoaki Sano*¶, Zhiyv Niu*‖, Michelle Chen**, Robert J. Schwartz*‖, and Michael D. Schneider*††

*Center for Cardiovascular Development, Baylor College of Medicine, Houston, TX 77030; and **Agilent Technologies, Santa Clara, CA 95051

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Early steps for cardiac specification are problematic for the study of mammalian embryos, which has favored using pluripotent cells that recapitulate cardiac myogenesis. Furthermore, circuits governing cardiac specification have relevance to the application of ES cells and other cells for heart repair. In mouse teratocarcinoma cells, canonical Wnts that inhibit heart formation in avian or amphibian embryos and explants activate cardiogenesis, paradoxically. Here, we show that the Wnt/β-catenin pathway also is essential for cardiac myogenesis to occur in ES cells, acting at a gastrulation-like stage, mediating mesoderm formation and patterning (two prerequisites for cardiac myogenesis itself). Among genes associated temporally with this step was *Sox17*, encoding an endodermal HMG-box transcription factor. Using lentiviral vectors for RNA interference in differentiating ES cells, an essential role for *Sox17* was proven in cardiac muscle cell formation. *Sox17* short-hairpin RNA suppresses cardiac myogenesis selectively, acting subsequent to mesoderm formation yet before induction of *Mesp1* and *Mesp2*, a pair of related basic helix–loop–helix transcription factors that together are indispensable for creating heart mesoderm. *Sox17* short-hairpin RNA blocks cardiac myogenesis non-cell autonomously and impairs the induction of Hex, a homeodomain transcription factor that is known to be required for the production of endoderm-derived heart-inducing factors.

cardiac myogenesis | differentiation | β-catenin | heart | Wnt

Stem cells elicit intense interest on dual, complementary grounds. As fundamental science, pluripotent cells with a capacity to enter many or all lineages provide access into pathways governing cell fate, including transcription factor networks and extracellular cues that activate them. As applied science, the use of adult and ES cells in potential therapies arouses the imagination of clinicians, basic researchers, and the lay public alike. Although barriers to successful implementation are many, a cell-based approach to myocyte regeneration is highly cogent for cardiac disease (1, 2). Muscle cell death from acute ischemic injury is the most prevalent adult heart disorder; heart failure entails ongoing apoptosis; adult ventricular muscle cells characteristically do not reenter the cell cycle; and newly found progenitor cells in adult hearts are insufficient, on their own, to execute adequate self-repair. Although effects of grafted cells apart from myocyte creation might also be beneficial, the need for myocyte replacement once cell death has ensued reinforces the logic of dissecting cardiogenesis in pluripotent cells more systematically. Genome-wide expression profiling provides one route to such information.

Peptide growth factors instrumental to cardiac myogenesis include members of the bone morphogenetic protein (BMP) and Wnt families (3, 4). BMPs are the most commonly substantiated inductive signal, corresponding to an instruction supplied by endoderm during embryogenesis. In gastrulating frog and chick embryos and postgastrulation mesodermal explants, the "canonical" glycogen synthase kinase-3β/β-catenin/T cell factor-dependent Wnt pathway antagonizes cardiac myogenesis (3, 4). Conversely, a "noncanonical" Wnt, Wnt11, promotes cardiomyocyte formation in these systems and mouse embryonal carcinoma cells (5). Surprisingly, the canonical Wnt pathway was also essential in the latter, for cardiomyocyte formation (6). Three explanations might reconcile this seeming dichotomy. Embryonal carcinoma cells, although an informative model for cardiogenesis (7–9), are tumor-derived and potentially anomalous; phylogenetic differences could matter; or, canonical Wnts might drive cardiomyocyte formation by an early developmental event in pluripotent cells, preceding the stage at which they inhibit heart formation in chick and frog embryos.

Here, we tested whether the canonical Wnt/β-catenin pathway was essential for cardiac muscle specification in mouse ES cells, which are of openly greater relevance than embryonal carcinomas in fidelity to normal development and utility for heart repair. Cardiac myogenesis depended stringently on canonical Wnt signals, acting in an early interval controlling mesoderm formation, a presumptive requirement for cardiogenesis per se. Associated with this gastrulation-like intermediary state and contingent on canonical Wnts was the endodermal transcription factor Sox17 (10–12). Sox17 was indispensable for cardiac myogenesis in this system, shown by RNA interference. Sox17 short-hairpin RNA (shRNA) did not impair mesendoderm formation yet suppressed the induction of Mesp1 and Mesp2, transcription factors that together are pivotal to cardiac specification in primitive mesoderm (13).

Results

The Canonical Wnt Pathway Drives an Early Step Toward Cardiac Myogenesis in ES Cells. First, we examined mouse ES cells for the operation of Wnt- and BMP-dependent cardiogenic pathways [supporting information (SI) Fig. 5]. Noggin and soluble Frizzled 8 (sFz8) prevented the expression of genes for cardiogenic transcription factors (Nkx2–5, GATA-4) and cardiac structural proteins [α myosin heavy chains (αMyHC), Ryr2], induction of sarcomeric MyHC protein, and formation of beating colonies.

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Abbreviations: BMP, bone morphogenetic protein; Dkk, dickkopf; EB, embryoid body; MyHC, myosin heavy chain; QRT-PCR, quantitative RT-PCR; shRNA, short-hairpin RNA; sFz8, soluble Frizzled 8; BIO, 6-bromoindirubin-3′-oxime.

†Present address: National Cardiovascular Center, Osaka 565-8565, Japan.

‡Present address: Kagoshima University, Kagoshima 899-8520, Japan.

§Present address: University of Utah, Salt Lake City, UT 84132.

¶Present address: Keio University School of Medicine, Tokyo 160-8582, Japan.

‖Present address: Institute for Biosciences and Technology, Texas A&M University Health Science Center, Houston, TX 77030.

††To whom correspondence should be addressed. E-mail: michaels@bcm.tmc.edu.

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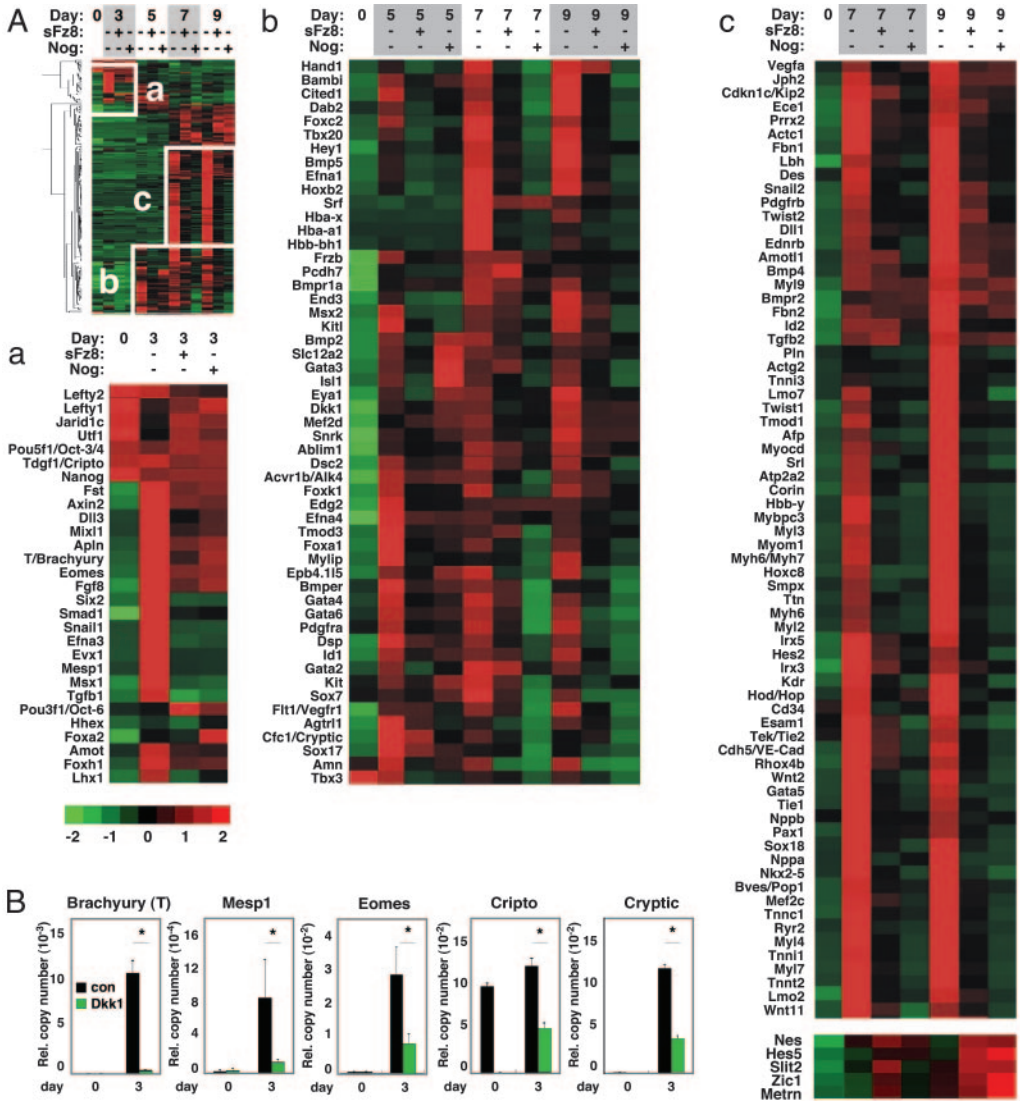

Fig. 1. Microarray analysis of Wnt- and BMP-dependent genes in differentiating ES cells identifies multiple early targets. (*A*) Partial cluster analysis of genes fulfilling the criteria of developmental regulation plus modulation by both sFz8 and Noggin at ≥1 days. Genes shown were chosen from the significantly inhibited GeneOntology and GenMAPP clusters, Entrez Gene entries, PubMed, and Mouse Genome Informatics database. The neurogenesis cluster shown for comparison in *c*, below, is taken from the developmentally up-regulated genes whose induction was enhanced by sFz8 and Noggin. $n = 4$ for 0–3 d; $n = 2$ for 5–9 d. (*B*) QRT-PCR confirmation of selected day three findings as contingent on canonical Wnts.

Next, to perturb the Wnt/glycogen synthase kinase-3β (GSK-3β)/β-catenin pathway selectively, we used dickkopf 1 (Dkk1) [a high-affinity ligand for the coreceptor LRP6 (14)] and 6-bromoindirubin-3′-oxime [BIO, an inhibitor of GSK-3β (15)]. Dkk1 suppressed *Nkx2–5* and α-*MyHC* in both cell types. Conversely, BIO bypassed the inhibition by sFz8 (SI Figs. 6 and 7). BIO had no effect on cardiac myogenesis in the absence of sFz8, when given on days 0–4 (not shown).

In pluripotent systems, canonical Wnts might promote cardiogenesis itself or a prerequisite prior step like mesoderm formation. Indeed, transient treatment of ES cells with Dkk1 during days 0–3 was especially effective at blocking cardiac myogenesis (SI Fig. 6 *G* and *H*). Thus, canonical Wnts impinge on cardiac myogenesis through an early step in the development of ES cells from an initially undifferentiated ground state.

Expression Profiling of Wnt- and BMP-Dependent Genes Identifies Mesoderm Transcriptional Factors Among the Early Targets. To canvas potential Wnt- and BMP-dependent mechanisms for cardiac myogenesis in pluripotent cells, we obtained genome-wide expression profiles (Fig. 1; *SI Materials and Methods*). Key findings were substantiated by quantitative RT-PCR (QRT-PCR) or Western blotting (SI Fig. 5*E*; data not shown) and were retested by using Dkk1 (Fig. 1*B*). In ES cells at 7–9 d, the impact of sFz8 and Noggin on cardiac transcription factors and structural genes was readily evident (Fig. 1*Ac*). Fewer than one gene in 40 fulfilled the criteria used. Significantly repressed GeneOntology clusters (fulfilling the criteria of developmental regulation, Wnt dependence, and BMP dependence) included muscle development, heart development, striated muscle thick filament, Z disk, and angiogenesis ($P \leq 0.0008$). Others bearing on cardiac cell fate and function included transcription factor activity and cytoskeleton ($P \leq 0.0001$). Analogously, the only significant GenMAPP clusters at 7–9 d were striated muscle contraction and smooth muscle contraction ($P \leq 0.0002$). Affected cardiogenic genes included *Nkx2.5*, *Mef2c*, *Mef2d*, *Gata4*, *Gata5*, *Gata6*, *Tbx20*, *Hand1*, *Hod/Hop*, *Irx3*, *Irx5*, and *Isl1*. Affected cardiac structural genes included those for sarcomeric proteins (*Actc1*, *Mybpc3*, *Myh6*, *Myl2*, *Myl3*, *Myl4*, *Myl7*, *Myom1*,

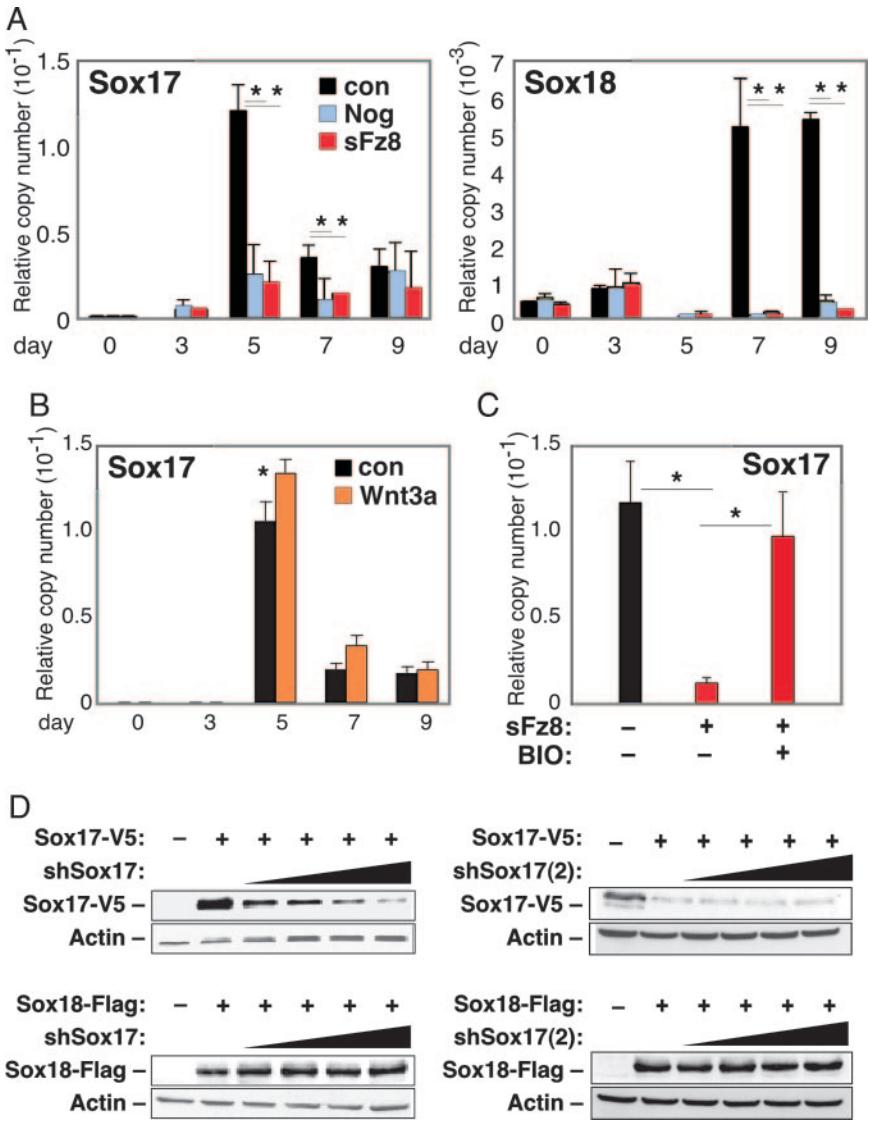

Fig. 2. Regulation of *Sox17* in differentiating ES cells by the canonical Wnt pathway. (*A–C*) QRT-PCR. $n \geq 3$; *, $P < 0.05$ vs. control cells. (*A*) sFz8 and Noggin suppress *Sox17* in differentiating ES cells. (*B*) Wnt3a up-regulates *Sox17*. (*C*) BIO rescues *Sox17* from repression by sFz8. (*D*) Immunoblotting for epitope-tagged Sox17 and Sox18, showing efficacy and specificity of Sox17 shRNAs.

Tmod1, *Tnnc1*, *Tnni1*, *Tnni3*, *Tnnt2*, and *Ttn*), cytoskeletal proteins (*Des*, *Smpx*), junctional proteins (*Dsp*, *Jph1*), and calcium homeostasis (*Atp2a2*, *Pln*, *Ryr2*, and *Srl*). Genes for vasculogenesis (*Bves*, *Cdh5*, *Flt1*, *Hey1*, *Kdr*, *Myocd*, *Pdgfra*, *Pdgfrb*, *Tie1*, *Tie2*, and *Vegfa*) and hematopoiesis (*Gata2*, *Hba-a1*, *Hba-x*, *Hbb-bh1*, *Hbb-y*, and *Lmo2*) also were inhibited. Conversely, neurogenesis and brain development GeneOntology clusters were up-regulated by sFz8 and Noggin ($P \leq 0.0003$; Fig. 1*Ac*). Thus, inhibition was selective for cardiomyocytes and closely related mesoderm derivatives.

At 3 and 5 d, TGF-β signaling was the only GenMAPP cluster impaired by sFz8 and Noggin ($P = 0.001$ and 0.00002, respectively), including *Bambi*, *Bmp5*, *Bmper*, *Fst*, *Smad1*, *Tgfb1*, and *Foxh1*, a nuclear target of Nodal required for the anterior heart field (16) (Fig. 1*A a* and *b*). Repressed GeneOntology clusters were development ($P = 0.00004$ and 0.001), transcription factor activity ($P = 0.0004$ and 0.00006), other synonymous groups, and heart development ($P = 0.0009$ at 5 d).

Notably, on day 3, sFz8 and Noggin disrupted the induction of many transcription factors that are required for mesoderm formation or mesoderm patterning (*T/Brachyury*, *Eomes*, *Evx1*, *Lhx1*, *Mesp1*, *Mixl1*, and *Snail1*) (17–20) (Fig. 1*Aa* and SI Fig. 5*E*). Mesp1 functions subsequent to T as the earliest molecular marker of cardiac precursor cells that migrate through the primitive streak and is essential along with Mesp2 for cardiac myogenesis in committed mesoderm cells (13). Other affected genes with known roles in cardiac myogenesis included *Fgf8* (21) and *Tdgf1*/*Cripto* (22). Similar results were obtained in P19Cl6 cells (SI Fig. 7).

An Essential Role for *Sox17* in Cardiac Mesoderm Specification. In prioritizing among early targets whose importance in cardiac myocyte creation is unknown or ambiguous, we were attracted to *Sox17*. *Sox17* has established roles in endoderm development (10–12) yet also is enriched in the cardiac crescent (23), cardiac side population cells (24), and differentiating Sca-1^+ cardiac progenitor cells (not shown). In differentiating ES cells, transient induction of *Sox17*, greatest on day 5, contrasts with the late onset of *Sox18* and small developmental fluctuations in *Sox7*, the other F group *Sox* genes (Fig. 2*A*; not shown). Confirming our microarray findings by QRT-PCR, *Sox17* was suppressed by Noggin and sFz8. Implicating canonical Wnts, the block by sFz8 was overcome by BIO, and Wnt3a augmented *Sox17* induction (Fig. 2 *B* and *C*).

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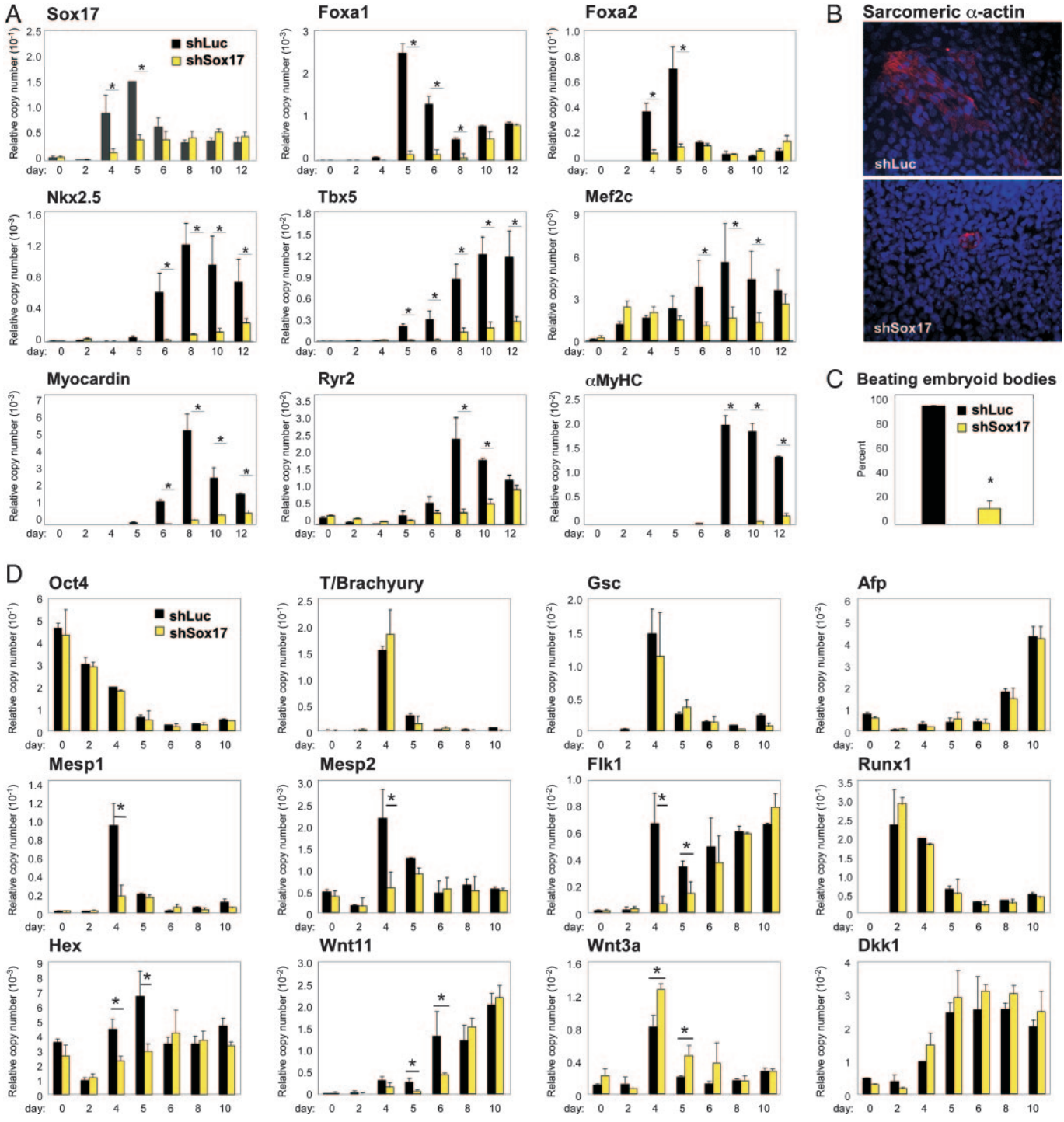

Fig. 3. An essential role for *Sox17* in cardiac specification. (*A–C*) Suppression of cardiac myogenesis by *Sox17* shRNA, shown by using QRT-PCR (*A*), immunostaining (*B*), and the prevalence of beating EBs (*C*). (*D*) QRT-PCR showing *Sox17* acts downstream of mesendoderm formation but upstream from *Mesp1* and *Mesp2*. $n \geq 3$; *, $P < 0.05$ vs. control cells.

We created two independent lentiviral vectors that suppress *Sox17* selectively (Fig. 2*D*). shRNAs were coexpressed along with enhanced GFP, infected ES cells were flow-sorted (SI Fig. 8 *A* and *B*), and the upper 50% of eGFP$^+$ cells were collected for functional studies (Fig. 3). Control cells were transduced with virus expressing enhanced GFP plus shRNA against firefly luciferase. Suppression of endogenous *Sox17* was confirmed, along with the direct *Sox17* targets *Foxa1* and *Foxa2* (11), and four of the five tested cardiogenic transcription factor genes (*Nkx2.5*, *Tbx5*, *Mef2c*, and *Myocd*). The exception was *Gata4* (not shown), which may signify a differing hierarchical relationship in the cardiomyocyte lineage, or *Sox17*-independent induction in a different cell type like endoderm. Cardiac structural genes (*α-MyHC*, *Ryr2*), sarcomeric protein staining, and the prevalence of beating embryoid bodies (EBs) were all suppressed, to nearly the same degree as by sFz8 or Noggin (Fig. 3 *A–C*; compare with SI Fig. 5). Identical results were obtained with

the second *Sox17* shRNA (SI Fig. 8*C*; not shown). Cardiac myogenesis was not inhibited by shRNAs against two other endoderm-expressed genes, *Pdx1* and *Afp* (SI Fig. 8*D*).

Next, we examined at which step *Sox17* functions (Fig. 3*D*). *Sox17* shRNA did not impair down-regulation of *Oct4* (a master regulator of pluripotency), induction of *T* and *Gsc* (indicators of mesendoderm formation), or the hematopoietic lineage marker *Runx1*. By contrast, *Sox17* shRNA suppressed both *Mesp1* and *Mesp2* (Fig. 3*D*). Thus, under these conditions, a *Sox17*-dependent pathway plays a selective role in mesoderm patterning. By contrast to *Runx1*, *Sox17* shRNA did suppress the hematopoietic/endothelial lineage marker *Flk1* at 4–5 d; interestingly, $Flk1^+/CXCR4^+/VE\text{-}Cadherin^-$ ES cells generate cardiac myocytes in culture (25), and fate-mapping has shown $Flk1^+$ cells to be cardiac muscle progenitors in mice (26).

***Sox17* Controls a Non-Cell-Autonomous Pathway for Cardiac Myogenesis in ES Cells.** Consistent with *Foxa1* and *Foxa2* being direct targets of *Sox17* in *Xenopus* endoderm (11), *Sox17* shRNA significantly suppressed both, as mentioned (Fig. 3*A*). By contrast, *Sox17* shRNA did not alter several other endodermal markers including *Afp* (Fig. 3*D*), *Gata4*, and *Sox7* (not shown). Together, this suggests that *Sox17* mediates a specific pathway or subset of endoderm, rather than panendoderm. An endodermal gene of known importance to heart formation is *Hex*, which regulates paracrine signals for cardiac myogenesis, depends on the canonical Wnt pathway, and mediates cardiac development in *Xenopus* and mice (27, 28). Notably, *Hex* induction at 4–5 d was markedly attenuated by *Sox17* shRNA, to the levels in undifferentiated cells (Fig. 3*D*).

To determine whether *Sox17* functions in ES cells primarily through cell-autonomous or non-cell-autonomous effects, wild-type cells were aggregated at various ratios (6:1 to 1:6) with cells harboring *Sox17* shRNA (SI Fig. 8*E*). At 6 d, the two cell populations were flow-sorted by using the GFP reporter and assayed separately for cardiogenic transcription factors (Fig. 4*A*). At each concentration, cocultured ES cells expressed *Nkx2.5* and *Tbx5* at levels distinct from those predicted for a cell-autonomous mechanism. An excess of wild-type cells completely rescued suppression of *Nkx2.5* and *Tbx5* in cells deficient for Sox17. Conversely, even equal numbers of cells containing Sox17 shRNA were sufficient to suppress *Nkx2.5* and *Tbx5* in wild-type cells. Thus, the impact of *Sox17* on cardiac myogenesis in ES cells is best explained by non-cell-autonomous mechanisms.

Sox17 Mediates Inactivation of the Canonical Wnt Pathway in Differentiating ES Cells. Of the HMG box proteins, the Sox family relates most closely to T cell factor/Lef, the nuclear targets for β-catenin (29). Mouse Sox17 bound to *in vitro*-translated β-catenin, by means of the latter's armadillo domains, as shown for *Xenopus* Xsox17β (30) (Fig. 4*B*). Several Sox proteins interfere with canonical Wnt signaling (31–33), but it is unknown whether this is true for mammalian Sox17. Indeed, mouse Sox17 interfered with the transcriptional function of constitutively active β-catenin in 293T cells (Fig. 4*C*).

Consistent with this inhibitory effect, in ES cells *Sox17* shRNA augmented the transcription of a T cell factor reporter gene ≈5-fold at 4.5–5 d, normally the time of *Sox17* induction, with no effect beforehand (SI Fig. 8 *F–I*). This concurs with the observed down-regulation of *Hex*, a β-catenin-inhibited gene (27) by *Sox17* shRNA and suggests *Sox17* might be instrumental to the normal inactivation of canonical Wnt signals in EBs after mesendoderm formation.

Discussion

Extracellular signals for cardiomyocyte specification, along with their intracellular effectors, provide fundamental insights into the cardiac fate and translational clues to augment cardiomyocyte creation. An obligatory role for canonical Wnts was substantiated in mouse ES cells, restricted to the first days of differentiation, with

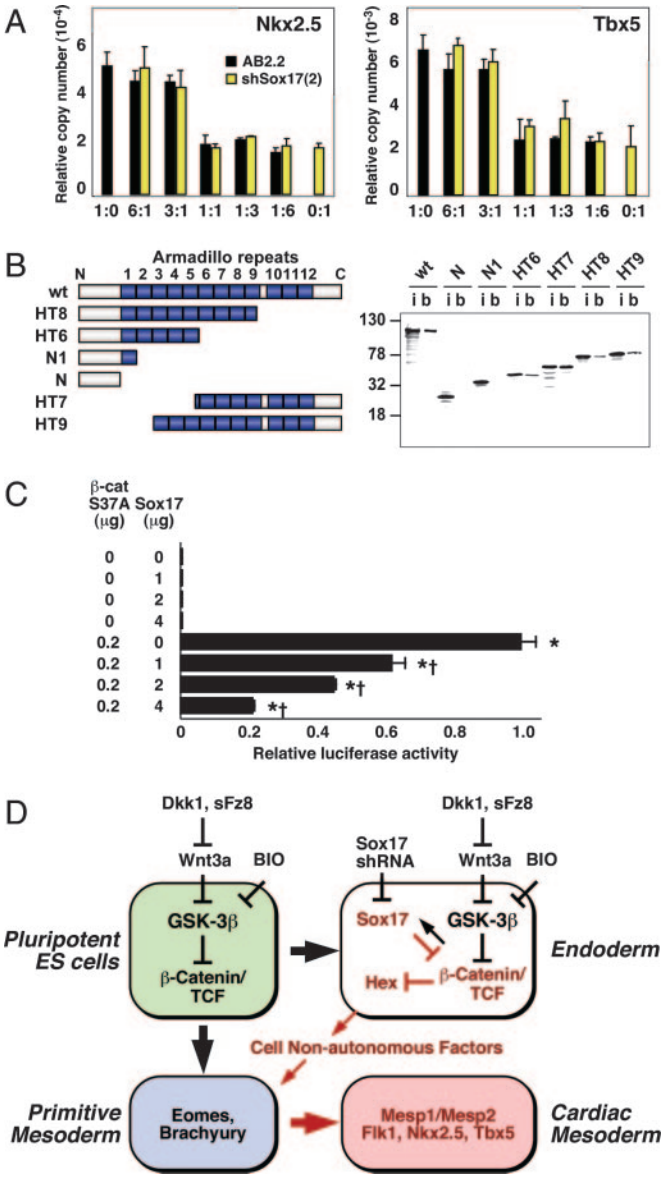

Fig. 4. Sox17 controls a non-cell-autonomous pathway for cardiac myogenesis in differentiating ES cells. (*A*) Wild-type and knockdown cells were cocultured as EBs, flow-separated, and analyzed by QRT-PCR. (*B*) Autoradiogram showing immobilized Sox17 binds [^{35}S]β-catenin, by means of the armadillo repeats. (*C*) Sox17 inhibits β-catenin-dependent transcription, shown by transient cotransfection of constitutive active β-catenin, Sox17, and TOPFLASH in 293T cells. $n \geq 3$; *, $P < 0.05$ vs. TOPFLASH alone; †, $P < 0.05$ vs. TOPFLASH plus β-catenin S37A. (*D*) Provisional model.

genes for gastrulation, mesoderm formation, and mesoderm patterning among the most obvious early responses. Our findings concur with other evidence for canonical Wnts and endodermal signals as mediating mesoderm formation and patterning in differentiating ES cells (19, 20, 34, 35). More importantly for the present report, early targets were identified by microarray profiling, and on this basis we investigated *Sox17*. Twenty Sox transcription factors exist in mice, with diverse functions in development (36). *Sox17* is known best as an endoderm marker and is required for definitive gut endoderm in mice and other species (10, 12, 37, 38). Here, two independent shRNAs against *Sox17* suppressed cardiac myogenesis, a previously unseen function of the gene.

The action of Sox17 was preferential for mesodermal patterning not mesendoderm formation, blocking both Mesp1 and Mesp2 but

neither T nor Gsc. Impaired expression of Hex and Wnt11 in Sox17-deficient cells, plus the cell mixing study, all support the inference that Sox17 functions here in a circuit for endoderm-derived signals driving cardiac myogenesis by primitive mesoderm (Fig. 4*D*). The direct target of Sox17 could be the signal itself, the number of signal-emitting endoderm-specified cells, or a regulator of the signal [as suggested by effects on Dab2 (Fig. 1*Ab*) and its target Hex (27, 39)]. The possibility that Sox17 functions cell-autonomously in mesoderm-specified cells is not supported by our data thus far.

Curiously, Sox17-null mice have no reported cardiac phenotype (38). Possibly, cardiac differentiation was insufficiently examined: only Gata4 was tested, and we found Gata4 to be independent of Sox17. Also, factors contributing to early cardiac development are highly redundant. In the embryo, cardiac myogenesis might draw on a more complete ensemble of signals and mediators than in a reductionist model: if so, embryonic development might be less vulnerable than EBs to loss of Sox17. Beyond its impact on fundamental knowledge of ES cell differentiation, dissecting the Sox17-dependent pathway for cardiac mesoderm specification may have applied significance, if used to help drive ES cells to a cardiac fate. Translational implications may hold importance, even if ES cells depart from *in utero* development in one or more ways.

Methods

Cell Culture. AB2.2 cells were differentiated by EB formation (40). EBs were collected on day 5, except where otherwise specified, and plated on 0.1% gelatin-coated dishes. P19Cl6 cells were differentiated by using 1% dimethyl sulfoxide (6).

Microarray Analyses. Over the 2-year course of the studies, differing chipsets and platforms were available, but for internal consistency a single technology was used in each set of comparisons. Samples were compared by using Affymetrix (Santa Clara, CA) MG 430 2.0 arrays for ES cells and Affymetrix MG U74Av2 arrays for P19Cl6 cells ± sFz8. Fluorescence intensities were captured with an Affymetrix GeneArray 2500 Scanner. Samples from P19Cl6 cells ± Noggin were compared by two-color hybridization by using Agilent (Palo Alto, CA) 22K mouse 60-mer arrays and an Agilent dual laser scanner. Expression data were analyzed by using dChip2004 (41) and GeneSpring 7 (Agilent). Differences were defined as developmental regulation (>2× vs. day 0), regulation by both sFz8 and Noggin (>1.2× vs. control cells), and absolute change >100.

Lentiviral Vectors. pLL3.7 was from L. Van Parijs (Massachusetts Institute of Technology, Boston, MA). Sox17 shRNA and Sox17 shRNA-2 target the sequences 5′-gcaggtgaagcgcatgaag-3′ (nt 1513–31) and 5′-gcacggaattcgaacagta-3′ (nt 2178–96), respectively, which lie 3′ to the conserved F group domain and have no significant similarity to other Sox family transcripts. For transduction, freshly trypsin-dissociated AB2.2 cells were mixed with lentivirus at a multiplicity of infection of 100, by using 8 μg/ml polybrene (Sigma, St. Louis, MO). Three days later, the upper 50% of EGFP$^+$ cells were isolated (Beckman–Coulter Altra, Fullerton, CA) and subjected to EB culture as above.

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