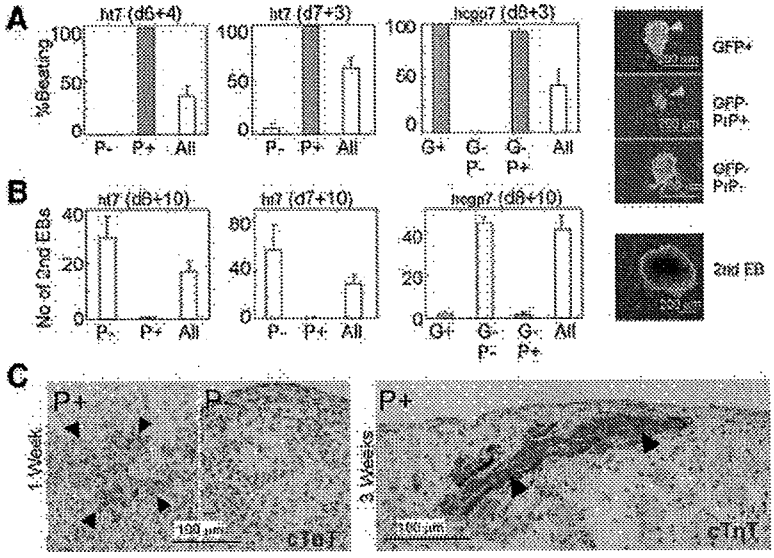


Figure 2. PrP specifically marks a cardiomyogenic population derived from ES cells. A, The cardiomyogenic potential of the PrP^+ fraction of post- and prebeating EBs. Cells were sorted on day 6, 7, or 8 (beating began on day 7), reaggregated, and cultured in Matrigel for 3 to 4 days. Beating foci (denoted with yellow arrowheads) were found in all reaggregates of PrP^+ cells. Error bars represent the standard error of the mean (n=3). B, Sorting using PrP depleted the undifferentiated cell population. Reaggregates of PrP^+ or PrP^- cells were grown in Matrigel for 10 days. Clearly visible colonies (secondary EBs) were scored. Error bars represent the SEMs (n=3). C, Transplantation and survival of PrP^+ cells in vivo. Reaggregates of PrP^+ cells were transplanted under the kidney capsule. Arrowheads indicate $cTnT^+$ cells.

found that GFP expression was not strong enough to allow us to capture all of the $Nkx2.5^+$ cells (see next section). Therefore, we simultaneously detected the surface expression of PrP and intracellular sarcomeric proteins. On day 6 or 7, all of the sarcomeric myosin heavy chain $(Myhc)^+$ cells that were derived from the $Nkx2.5^{+/+}$ ES cell (Figure 1B) or $Nkx2.5^{GFP/+}$ ES cell population (not shown) were PrP^+, suggesting that the nascent cardiomyocytes were PrP^+. Expression of PrP on $Myhc^+$ cells gradually started to decrease on day 8. Immunofluorescence in cytospin samples (Figure 1C) and cultured cells (Figure 1D) further confirmed that PrP can be used to enrich the $Myhc^+$ and GATA-binding protein 4 $(Gata4)^+$ cell populations.

PrP Specifically Identifies a Cardiomyogenic Cell Population Derived From ES Cells

To determine whether PrP can be used to enrich not only differentiated cardiomyocytes but also cardiac progenitors, we used reaggregation assays to assess the cardiomyogenic activities of cells sorted from pre- or postbeating EBs. PrP^+ cells sorted at day 6 (prebeating), day 7 (onset of beating), or day 8 (beating) generated beating reaggregates, whereas PrP^- cells did not (Figure 2A). In contrast, the PrP^- fraction gave rise to secondary EBs in Matrigel cultures, suggesting the presence of undifferentiated cells[21] that were not produced from the PrP^+ fraction (Figure 2B). We also found that GFP^-PrP^+ reaggregates derived from $Nkx2.5^{GFP/+}$ ES cells had beating foci. Consistent with this result, the GFP^-PrP^+ cells expressed low levels of mRNA encoding Nkx2.5 and other cardiac transcription factors (Figure 3A). Thus, enriching the cardiomyogenic fraction using the cardiogenic marker PrP yielded better results than when enrichment was performed using $Nkx2.5^{GFP/+}$ knock-in ES cells. Importantly, even without using genetically modified ES cells, we were able to purify the cardiogenic fraction before beating began.

To test whether PrP can be used to separate transplantable cells, we transplanted reaggregates from day 6 EBs under the kidney capsules of nude mice. Immunohistochemical analysis demonstrated that cardiac troponin $(cTn)T^+$ cells were present in the PrP^+ cell grafts, but not in the PrP^- grafts (Figure 2C). Tumor formation was frequently observed in unsorted cell grafts (data not shown) but not in PrP^+ grafts (41 transplanted aggregates). These results suggested that the PrP^+ cell population was able to generate cardiomyocytes and survive in vivo for at least 3 weeks.

PrP^+ Cells Differentiate From Primitive to Definitive Cardiomyocytes

Monolayer cultures of PrP^+ cells sorted from day 7 or day 8 EBs started to beat within 24 hours. After 5 days of culture, approximately one third of the cells spontaneously beat (data not shown). Single-cell recordings of membrane potentials from 16 of 18 quiescent cells revealed action-potential profiles similar to those of working (atrial or ventricular) cardiomyocytes (Figure 3A). The rest of the cells displayed sinus nodal cell-like spontaneous beating activity. Pharmacological studies using multielectrode arrays indicated that the cultured PrP^+ cells were responsive to isoproterenol and carbamylcholine, suggesting the presence of functional β adrenergic receptors and sinoatrial cardiomyocytes, respectively (Figure 3B). Immunofluorescence indicated that PrP^+ cells differentiated into $cTnI^+$ (a definitive marker of cardiomyocytes) cells. These were either myosin light chain 2v $(Mylc2v)^+$ or $Mylc2v^-$ (Figure 3C). A few atrial natriuretic peptide $(ANP)^+$ cells were also detected.

To examine the cell types quantitatively, we performed flow cytometry with atria and ventricles from mouse embryos as reference samples (Online Figure I, E). Among the atrial and ventricular cells from embryonic day (E)13.5 embryonic heart, 25% and 2% to 4% were ANP^+, respectively. Myosin light chain 2a (Mylc2a) was detected in both the atrial and ventricular cells, although higher levels were observed in atrial cells. Thus, neither ANP nor Mylc2a can be used to distinguish atrial cells from ventricular cells. In contrast, all embryonic ventricular cells were $Mylc2v^+$, whereas all atrial cells were $Mylc2v^-$. Approximately 50% of cultured PrP^+ cells were $Mylc2v^+$, although the expression level varied between cells (Figure 3D). Consistent with the immunofluo-

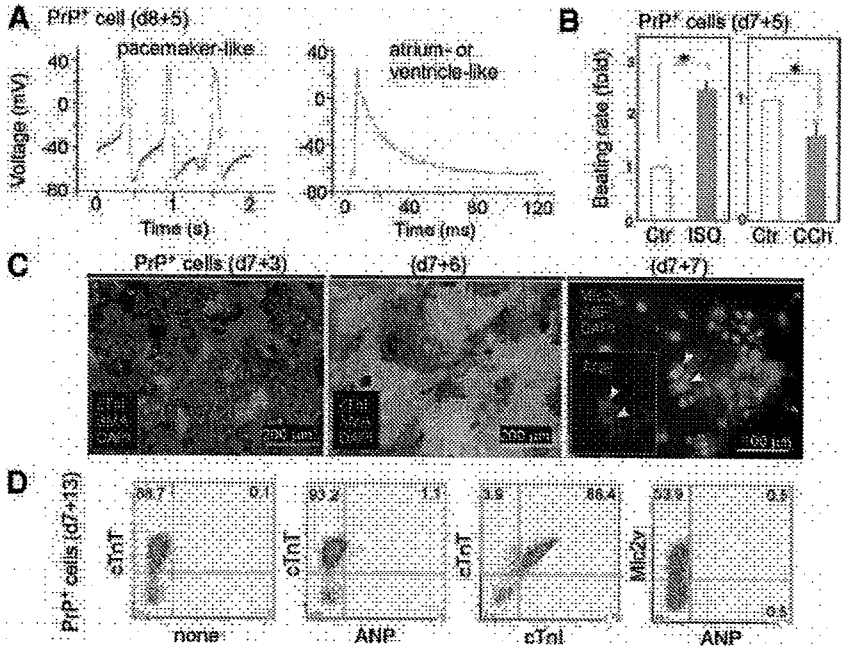


Figure 3. PrP$^+$ cells differentiate into cTnI$^+$ definitive cardiomyocytes with an atrial or a ventricular identity. A, Traces of the membrane potential recorded from a single PrP$^+$ cell. PrP$^+$ cells sorted on day 8 were cultured for 5 days. Approximately, one third of the cells spontaneously beat (data not shown). Sixteen of 18 quiescent cells exhibited a working myocardium-type (atrial or ventricle) action potential, whereas the rest exhibited a pacemaker-type action potential. Action potentials were elicited at 1 Hz. B, Pharmacological analysis of PrP$^+$ cells. PrP$^+$ cell aggregates were analyzed using multielectrode arrays and isoproterenol (ISO) (1 μmol/L) or carbamylcholine (CCh) (10 μmol/L). All aggregates were sensitive to CCh, suggesting that they contained atrial cells. Error bars represent the SE (n=9 independent samples for each group). *$P<0.05$ from a paired t test. C, Immunofluorescence analysis for cTnI (a definitive cardiomyocyte marker), Mylc2v (a ventricle marker), and ANP (a chamber myocardium marker). PrP$^+$ cells sorted on day 7 were cultured on gelatin-coated dishes for 1 week. The number of cTnI$^+$ cells increased during the culture. Mylc2v$^+$ cells were frequently observed, whereas the percentage of ANP$^+$ cells was low (1% to 2%). Arrows indicate cells that are ANP$^+$MLC2v$^+$, a profile characteristic of left ventricular cardiomyocytes. D, Flow cytometric analysis of PrP$^+$ cell cultures. Sorting on day 7 revealed that the cardiomyocytes were cTnT$^+$ but not cTnI$^+$ (data note shown). After culturing the cells for 13 days, 90% to 95% expressed both cTnT and cTnI, 50% to 60% expressed Mylc2v, and 1% expressed ANP. Control experiments using embryonic heart and PrP$^-$ fractions are shown in Online Figure III.

rescence results, only 1% to 2% of the cells were ANP$^+$. The PrP$^+$ cell fraction initially contained cells expressing Myhc and cTnT but not cTnI (data not shown).[20,22] Interestingly, after 10 to 13 days of culture, 90% of the cells differentiated into cTnI$^+$ cardiomyocytes. Collectively, these results suggested that PrP$^+$ cells differentiated from cTnI$^-$ to cTnI$^+$ cardiomyocytes with an atrial or a ventricular identity.

The PrP$^+$PDGFRα^+ Population Contains Myhc$^-$ Cardiomyogenic Progenitors

Although PrP expression was first detected at day 5, we were unable to enrich the cardiomyogenic population using this marker until day 6. To determine whether combination with other surface markers would allow better enrichment of the progenitor population at day 5, we examined Flk1, c-kit, and platelet-derived growth factor receptor (PDGFR)α as secondary surface markers. Flk1 is a marker of cardiovascular progenitors: common progenitors for cardiac, smooth muscle, and endocardial cells.[5] PrP$^+$ cells expressed a very low level of Flk1 on day 5 and even less on day 6 (Online Figure IV, A), suggesting that PrP$^+$ cells may have already diverged from the vascular lineage. c-kit, which is reported to be a cardiovascular stem cell marker in adult and embryonic heart,[8] was not expressed on PrP$^+$ cells. PDGFRα is widely expressed in mesoderm, including the cardiac lineage, although its expression is gradually downregulated in heart tube (elsewhere[23] and Online Figure VI). PrP expression was first observed on day 5 on PDGFRα^+ and PDGFRα^- cells (Figure 4A). Molecular analysis indicated that the PrP$^+$PDGFRα^+ cell fraction from day 5 EBs expressed cardiac marker genes, including *Nkx2.5*, *Tbx5*, and *Isl1*, whereas the PrP$^+$PDGFRα^- cell fraction did not (Figure 4B and 4C). Consistent with these data, only the PrP$^+$PDGFRα^+ cell fraction gave rise to beating reaggregates (Online Figure IV, D). These results suggested that double-positive PrP$^+$PDGFRα^+ cells, hereafter referred to as PRa cells, are the earliest known cardiomyogenic population.

PRa Cells Differentiate Into Cardiac Cells and Smooth Muscle Cells

To test the cardiomyogenic potential of PRa cells, we used PRa cells sorted from day 5.5 EBs, at which point Myhc was not expressed (data not shown), and directly differentiated the cells as 3D reaggregates (3D cultures) or as sparsely cultured 2D monolayers (2D cultures). The reaggregates developed into beating cardiomyocytes, whereas the monolayer cultures did not (Online Figure V, D). Reaggregate cultures expressed such cardiac-specific genes as *Myl7* (encoding Mylc2a), *Myh6* (encoding myosin heavy chain α), and *Tnni3* (encoding cTnI) (Figure 5A). In contrast, smooth muscle genes, including *Tagln* (encoding SM22α), *Acta2* (encoding smooth muscle actin, SMA), and *Myh11* (encoding smooth muscle myosin heavy chain [SMMyhc]), were expressed in the monolayer cultures. Interestingly, adding Wnt3a (wingless-related MMTV integration site 3A) to the reaggregates inhibited cardiac differentiation (Figure 3C). Wnt, however, did not increase the levels of smooth muscle markers, suggesting that it did not drive the progenitors to a smooth muscle fate. Immunostaining experiments revealed that aggregates expressed the cardiac muscle proteins Myhc and cTnI, whereas the

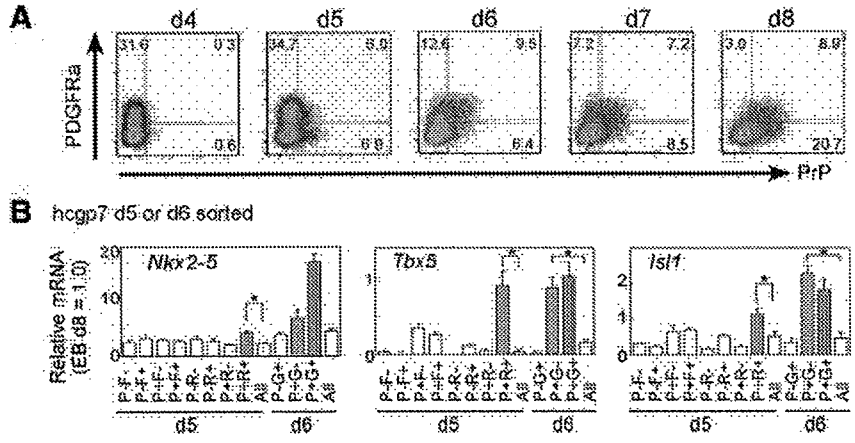


Figure 4. The PrP$^+$PDGFRα^+ (PRa) population contains bipotential cardiomyogenic progenitors that can differentiate into cardiomyocytes or smooth muscle cells. A, Flow cytometric analysis of EBs using PrP- and PDGFRα-specific antibodies. PrP$^+$ cells detected on day 5 were PDGFRα^+ or PGDFRα^-. The expression profiles of PDGFRα and PrP overlapped on day 6 and then gradually receded as differentiation proceeded. B, RT-PCR analysis of hcgp7-derived cells sorted using PrP (P), PDGFRα (R), Flk1 (F), and GFP (G). PrP$^+$ cells on day 5 were PDGFRα^+ or PDGFRα^- and Flk1$^{Low/-}$ (see Online Figure II). Compared with the unsorted fraction, Nkx2.5, Tbx5, and Isl1 were expressed at significantly higher levels in the P$^+$R$^+$ fraction (pink bars) and P$^+$G$^-$ fraction (blue) on day 5, as well as in the P$^+$G$^+$ fraction (green) on day 6. Error bars represent the SEM (n=3 independent samples for each group). *$P<0.05$ from an unpaired Student t test. Other marker genes are shown in Online Figure II.

monolayer cells expressed such smooth muscle proteins as SMA, SM22α, and calponin (Figure 5B). SMMyhc, a very specific marker of smooth muscle cells,[8] was also detected in the monolayer cells. These results suggested that the PRa cell fraction contained 2 different lineages and/or bipotential progenitors. To examine the latter possibility, we next cultured single cells isolated from days 5 to 5.5 EBs in methylcellulose (MEC). Typically, ≈100 colonies

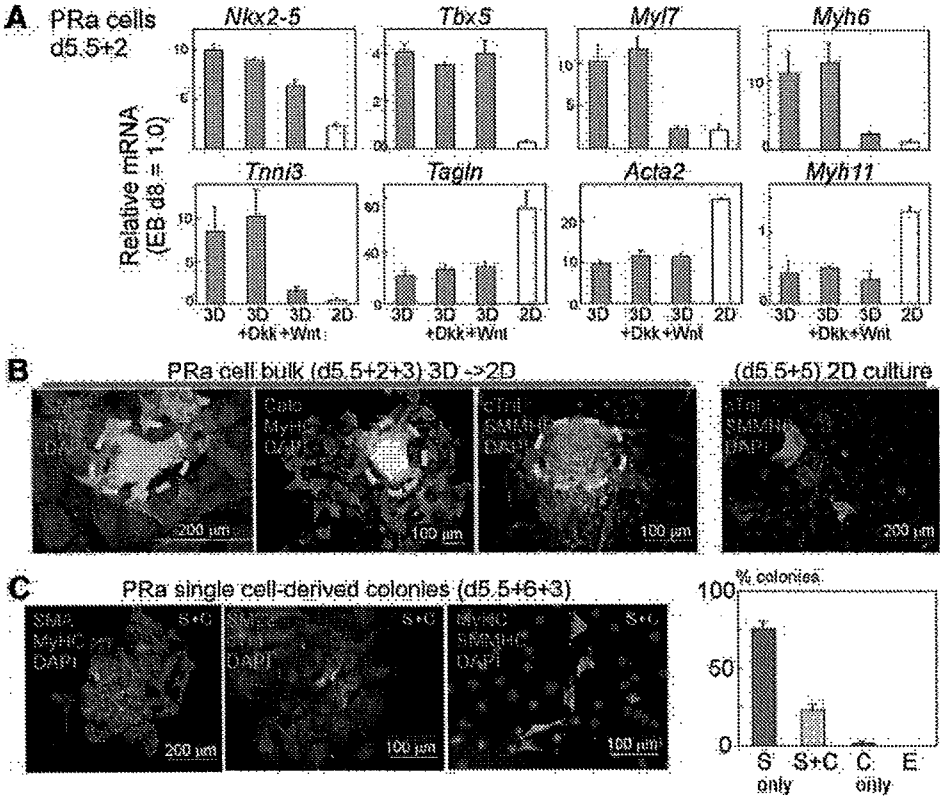


Figure 5. Developmental potential of PRa cells. A, Differentiation of reaggregated cultures of PRa cells. PRa cells sorted on day 5.5, at which point the cells were still negative for Myhc (Online Fig V), were allowed to differentiate as reaggregates (3D) or sparse monolayers (2D) with or without Wnt or the Wnt inhibitor Dkk1 (dickkopf homolog 1). After 2 days of culture, cardiac (*Myl7*, *Myh6*, *Tnni3*) and smooth muscle (*Tagln*, *Acta2*, *Myh11*) markers were analyzed in RT-PCRs. Error bars represent the SEMs (n=3 independent samples for each group). B, Immunostaining of 3D or 2D cultures of PRa cells for cardiac and smooth muscle marker proteins. PRa cells sorted on day 5.5 were aggregated in an ultralow binding plate for 2 days and then transferred to a gelatin plate for 3 days (3D→2D) or were cultured on only a gelatin plate for 5 days (2D). Beating aggregates (marked with the dotted lines) strongly expressed the cardiac proteins Myhc and cTnI. Monolayer cells expressed a number of smooth muscle cell markers, including SMA, SM22, calponin, and SMMyhc. C, Expansion and differentiation of single PRa cells. PRa cells sorted on day 5.5 were expanded in MEC at clonal density for 6 days and colonies derived from single cells were transferred to gelatin-coated multiwell plates for immunostaining. Colonies composed of SMA$^+$Myhc$^-$ smooth muscle cells only (S only), SMA$^+$Myhc$^-$ smooth muscle cells and SMA$^+$Myhc$^+$ cardiac muscle cells (S+C), Myhc$^+$SMA$^+$ cardiac muscle cells only (C only), or platelet/endothelial cell adhesion molecule–positive endothelial cells (E) were counted. Error bars represent the SEMs (n=5 independent samples for each group).

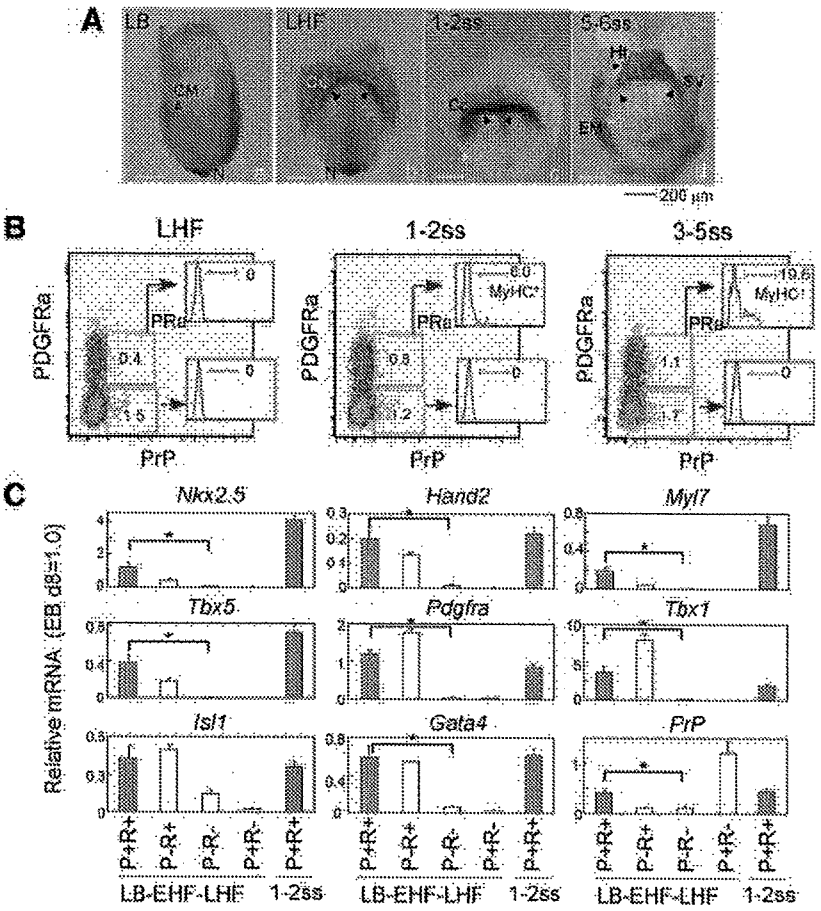


Figure 6. PrP is expressed before Myhc in mouse embryonic cardiac mesoderm. A, Whole-mount in situ hybridization analysis for *Prnp* mRNA (a through c) and immunohistochemical analysis for PrP protein (d). Note that *Prnp* mRNA was expressed in the cardiac mesoderm (CM) beginning at the late-bud stage (LB) and expression was maintained in the cardiac crescent (Cc) and heart tube (Ht) at the late headfold stage (LHF), 1 to 2 somite stage (1 to 2ss), and 3 to 4 somite stage (not shown). PrP was also expressed in the node (N). PrP protein was detected in the cardiac crescent at the late headfold stage (see Online Fig. VI). At the 5- to 6-somite stage, expression was also observed in the sinus venosus (Sv). Nonspecific staining was detected in extraembryonic membrane (EM). B, Flow cytometric analysis of surface and intracellular proteins. After staining the cell surfaces with anti-PrP and anti-PDGFRα antibodies, cells were fixed and permeabilized. Myhc$^+$ cells were first detected in 1- to 2-somite stage embryos and were exclusively observed in the PRa fraction. C, RT-PCR analysis of sorted cells from pools of late-bud to late headfold embryos (LB-EHF-LHF) or 1 to 2 somite embryos (1 to 2 ss). Note that the PRa cell fraction from presomite stages, before Myhc was expressed, was specifically enriched with cardiac markers. Error bars represent the SEMs (n=3 independent samples for each group).

were created from 5000 single-cell isolates (Online Figure III, C). Colony formation was inhibited by the addition of the Wnt inhibitor Dkk1 (dickkopf homolog 1) and promoted by Wnt3a or a glycogen synthase kinase-3β inhibitor, suggesting that the proliferation of PRa cells was dependent on canonical Wnt signaling. Individual colony analysis indicated that approximately 25% of Myhc$^-$ PRa cell–derived colonies expressed both smooth and cardiac muscle proteins (Figure 5C), whereas the rest of the colonies expressed only smooth muscle proteins. Endothelial cell differentiation, assessed based on platelet/endothelial cell adhesion molecule expression, was not observed. These results strongly suggested that the Myhc$^-$ PRa cells contained bipotential (cardiac and smooth muscle) progenitor cells.

Expression of PrP in Mouse Embryos

We next examined the spatial and temporal profiles of PRa cells in embryos. Transgenic mouse studies using β-galactosidase suggested that *Prnp* is expressed in embryonic heart at E8.5.[24] Endogenous expression of PrP mRNA and protein during embryonic early stages (E7 to E7.5) has not been adequately elucidated, however.[25,26] To examine the expression of PrP in early mouse embryos, we performed whole-mount in situ hybridizations and immunohistochemical analysis. During early developmental stages, we observed specific expression of *Prnp* mRNA in the cardiac mesoderm during late-bud stage (Figure 6A). We also detected PrP protein in the cardiac crescent at headfold stages and in the heart tube at somite stages (Online Figure VI). On the other hand, PDGFRα expression was observed widely in the mesoderm, including cardiac mesoderm, of late-bud to 1 to 2 somite stage embryos (Online Figure VI). PDGFRα expression gradually decreased in cardiomyocytes after they reorganized into the heart tube.

We next analyzed PrP expression in mouse embryos using flow cytometry. As expected from the immunohistochemical analysis, PrP$^+$ cells were either PRa cells or PrP$^+$PDGFRα^- cells from the E7 pool (primarily consisting of presomite stage mouse embryos) and the E8 pool (primarily consisting of somite stage mouse embryos) (Online Figure VII, A). As observed in ES cell–derived cells, little overlap between PrP and Flk1 expression was detected at these stages. Sorting using PrP clearly enriched the cardiac marker–expressing cells from the E8 pool (Online Figure VII, B), suggesting that PrP was a specific marker of cardiomyocytes at these stages. To determine whether the PRa population contained both Myhc$^+$ cardiomyocytes and Myhc$^-$ progenitor cells, flow cytometric analysis was performed with embryos at different developmental stages. Myhc$^+$ cells were detected at 1 to 2 somite stages but not at presomite stages. The nascent Myhc$^+$ cells were exclusively identified in the PRa cell

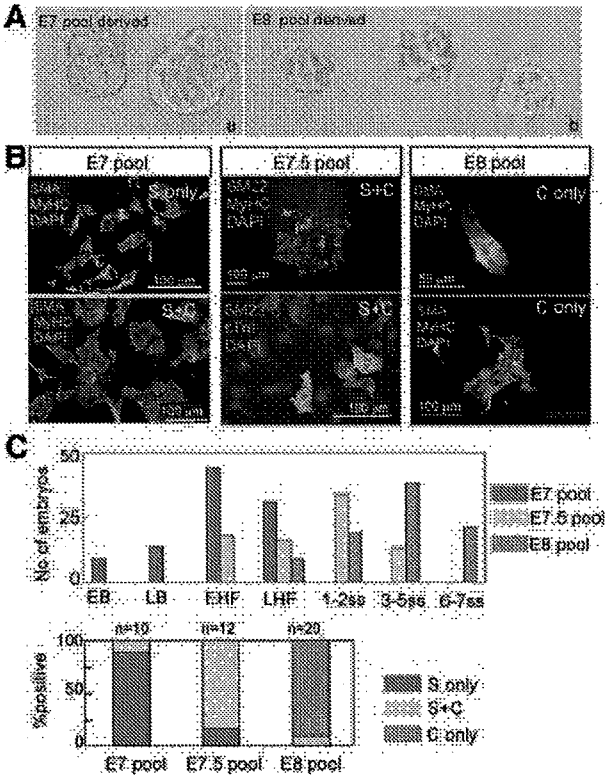


Figure 7. Analysis of single PRa cell–derived colonies from mouse embryos. A, Schematic of the experiment. B, Representative colonies from MEC cultures of E7 pool (a) and E8 pool (b) embryos. Individual colonies from MEC cultures derived from E7 pool, E7.5 pool, or E8 pool embryos were transferred to 96-well plates for immunofluorescence analysis. C, Percentages of the colony types based on immunofluorescence analysis. Embryonic stages in each pool used (top) and colony types derived from each pool (bottom) are shown. The numbers of colonies composed of SMA^+ cells (S only), SMA^+Myhc^+ cells and SMA^+ cells (S+C), or SMA^+Myhc^+ cells (C only) were counted. Eighteen of 20 colonies from the E8 pool contained only cardiomyocytes (C only) and exhibited spontaneous beating.

fraction (Figure 6B). PRa cells sorted from presomite stage embryos (a pool of late-bud to headfold stage embryos) expressed mRNA encoding Nkx2.5, Isl1, Tbx5, and other cardiac transcription factors (Figure 6C). $PrP^+PDGFR\alpha^-$ cells, probably from such extracardiac regions as node, did not express cardiac genes. These results suggested that the embryonic PRa population, similar to ES cell–derived PRa cells, contained $Myhc^+$ nascent cardiomyocytes as well as $Myhc^-$ cardiomyogenic progenitors.

Commitment Status of PRa Cells Derived From Mouse Embryos

We then cultured PRa cells from mouse embryos. Similar to cells from EBs, PRa cells from embryos formed colonies in MEC cultures (Figure 7A). Immunostaining analysis revealed that one of ten colonies derived from an E7 pool contained $Myhc^+$ cells (Figure 7B–C). The other colonies expressed the smooth muscle proteins SMA, SM22, and SMMyhc. PRa cells from the E7.5 pool frequently generated $Mhyc^+$ cardiomyocyte–containing colonies (10 of 12). These samples also contained smooth muscle colonies, suggesting from the presence of bipotential progenitors. PRa cells from the E8 pool generated small beating cardiac colonies. Such beating colonies exclusively contained cardiomyocytes, and not smooth muscle cells, suggesting that the isolated cells were committed to the cardiomyogenic lineage. These results suggested that PRa cells isolated from the cardiac mesoderm could differentiate into either cardiac or smooth muscle cells. Thus, PRa cells from mouse embryos and EBs may share common differentiation and proliferation potentials.

Discussion

In this study, we have demonstrated that the surface marker PrP can be used to enrich cardiomyocytes derived from ES cells. Several groups have examined methods to enrich cardiomyocytes using gravity or introduction of a selectable marker. Surface markers that allow cardiomyocytes to be selected directly, however, have not been extensively elucidated. Importantly, up to 90% of cells derived from PrP^+ cell cultures expressed cTnI (a marker for definitive cardiomyocytes). The other cells expressed SMA, suggesting that the cultures contained at least 2 different lineages. To improve the purity of the population, other markers or directional differentiation methods should be combined with PrP expression. We also showed that the cultured PrP^+ cells differentiated into both atrial and ventricle cardiomyocytes. Hcn4 protein, a specific marker for sinus node, was barely detectable. Because spontaneous pacemaker-like activity was detected in immature atrial or ventricular cardiomyocytes, PrP^+ cells may not have differentiated into mature sinus node cells, at least under our culture conditions. Cells expressing ANP, a marker of chamber myocardium,[27] were not found in significant numbers in the PrP^+ cell cultures, suggesting that the PrP^+ cells had not differentiated into chamber myocardium cells. Consistent with this idea, flow cytometric analysis of the expression patterns of Mylc2v and ANP produced results that were more similar to those observed for E9.5 heart than for E13.5 heart.

At earlier stages, PrP did not define a cardiomyogenic population. The $PrP^+PFGFR\alpha^+$ fraction (PRa cells) contained nascent cardiomyocytes and their progenitors. It would be interesting to understand the relationship between PRa cells and previously described cardiovascular progenitor/stem cells. Because PRa cells did not markedly express Flk1, they may have segregated from the endothelial lineage. Consistent with this idea, we did not observe endothelial cell differentiation from PRa cell–derived cells even in the OP9 culture system, which efficiently supported endothelial cell differentiation (Online Figure V). The proliferation and differentiation of PRa cells are regulated positively and negatively by Wnt signals, respectively. The presence of $Isl1^+$ cells in the PRa cell population (Online Figure IV, E) indicates that PRa cells represent an intermediate progenitor, located between $Isl1^+$ multipotential cells and committed cardiomyocytes. Other groups have described bipotential progenitors, such as $c\text{-}kit^+Nkx2.5^+$ cells and $Tbx18^+$ epicardial cells.[28,29] Because PRa cells did not display surface expression of c-kit,

they are likely not related to c-kit$^+$Nkx2.5$^+$ cells. Interestingly, we observed Tbx18 expression in PRa cell–derived 2D cultures but not in 3D cultures (data not shown). Recent studies showed that the Nkx2.5$^+$ lineage also diverges into Tbx18$^+$ epicardial cells.[30] Thus, it would be intriguing to know if PRa cells can differentiate into epicardial cells.

Embryonic localization in the cardiac crescent and their limited differentiation potential strongly suggest that PRa cells include progenitors from the primary heart field. Consistent with this idea, PRa cells sorted from presomite-stage embryos expressed Tbx5. On the other hand, PRa cells also include progenitors from the secondary heart field, because they also expressed Tbx1 and Isl1. Flow cytometric analysis further revealed that PRa cells were present in the outflow tract segment, which is derived from the secondary lineage (Online Figure VII, C). As in the early somite stages, outflow-derived PRa cells included Myhc$^+$ cells. Thus, PRa can serve as a pan-marker for cardiomyogenic progenitors independent of the heart field lineage. Interestingly, compared with embryo-derived PRa cells, EB-derived PRa cells expressed lower levels of Tbx1. This suggests that, under our culture conditions, primary heart lineage progenitors preferentially developed. However, it should be determined whether or not EB-derived PRa cells differentiated into outflow cardiomyocytes.

Based on molecular studies and in vitro cultures of isolated cells, we propose that PRa cells include a cardiomyogenic population, which can differentiate into cardiac or smooth muscle cells (Online Figure VIII). Considering their limited differentiation potential, PRa cells may be downstream of Flk1$^+$ cells, separate from the endothelial lineage. Immediate early progenitors may be in the Flk1$^+$PDGFRα^+ fraction, because this transient cell faction (observed on day 5) specifically expressed *Mesp1* (data not shown). Multipotent or pluripotent stem cells, including induced pluripotent stem cells, are expected to be a powerful tool for transplantation therapy and drug screening. Testing the ability of ES cell–derived cardiomyocytes to rescue in vivo heart function has been hampered by a lack of markers that allow scalable purification of the population without recourse to the genetic manipulations required to insert lineage- or stage-specific selectable markers. The mouse cell surface marker PrP defines a cardiogenic population in differentiating ES cells, enabling efficient isolation and enrichment of ES cell–derived cardiomyocytes.

Acknowledgments

We thank S. Suzuki, M. Honda, and other members of the Morisaki laboratory for technical assistance.

Sources of Funding

This work was supported, in part, by the National Institute of Biomedical Innovation (NIBIO); the Ministry of Health, Labour and Welfare; and the Ministry of Education, Culture, Sports, Science and Technology, Japan (K. H. and T. M.). M. D. S. is the British Heart Foundation Simon Marks Chair in Regenerative Cardiology.

Disclosures

None.

References

1. Brand T. Heart development: molecular insights into cardiac specification and early morphogenesis. *Dev Biol.* 2003;258:1–19.
2. Buckingham M, Meilhac S, Zaffran S. Building the mammalian heart from two sources of myocardial cells. *Nat Rev Genet.* 2005;6:826–835.
3. Laugwitz KL, Moretti A, Caron L, Nakano A, Chien KR. Islet1 cardiovascular progenitors: a single source for heart lineages? *Development.* 2008;135:193–205.
4. Hochgreb T, Linhares VL, Menezes DC, Sampaio AC, Yan CY, Cardoso WV, Rosenthal N, Xavier-Neto J. A caudorostral wave of RALDH2 conveys anteroposterior information to the cardiac field. *Development.* 2003;130:5363–5374.
5. Kattman SJ, Huber TL, Keller GM. Multipotent flk-1+ cardiovascular progenitor cells give rise to the cardiomyocyte, endothelial, and vascular smooth muscle lineages. *Dev Cell.* 2006;11:723–732.
6. Yamaguchi TP, Dumont DJ, Conlon RA, Breitman ML, Rossant J. flk-1, an flt-related receptor tyrosine kinase is an early marker for endothelial cell precursors. *Development.* 1993;118:489–498.
7. Moretti A, Caron L, Nakano A, Lam JT, Bernshausen A, Chen Y, Qyang Y, Bu L, Sasaki M, Martin-Puig S, Sun Y, Evans SM, Laugwitz KL, Chien KR. Multipotent embryonic isl1+ progenitor cells lead to cardiac, smooth muscle, and endothelial cell diversification. *Cell.* 2006;127:1151–1165.
8. Wu SM, Fujiwara Y, Cibulsky SM, Clapham DE, Lien CL, Schultheiss TM, Orkin SH. Developmental origin of a bipotential myocardial and smooth muscle cell precursor in the mammalian heart. *Cell.* 2006;127:1137–1150.
9. Kwon C, Arnold J, Hsiao EC, Taketo MM, Conklin BR, Srivastava D. Canonical Wnt signaling is a positive regulator of mammalian cardiac progenitors. *Proc Natl Acad Sci U S A.* 2007;104:10894–10899.
10. Naito AT, Shiojima I, Akazawa H, Hidaka K, Morisaki T, Kikuchi A, Komuro I. Developmental stage-specific biphasic roles of Wnt/beta-catenin signaling in cardiomyogenesis and hematopoiesis. *Proc Natl Acad Sci U S A.* 2006;103:19812–19817.
11. Nakamura T, Sano M, Songyang Z, Schneider MD. A Wnt- and beta-catenin-dependent pathway for mammalian cardiac myogenesis. *Proc Natl Acad Sci U S A.* 2003;100:5834–5839.
12. Ueno S, Weidinger G, Osugi T, Kohn AD, Golob JL, Pabon L, Reinecke H, Moon RT, Murry CE. Biphasic role for Wnt/beta-catenin signaling in cardiac specification in zebrafish and embryonic stem cells. *Proc Natl Acad Sci U S A.* 2007;104:9685–9690.
13. Laflamme MA, Chen KY, Naumova AV, Muskheli V, Fugate JA, Dupras SK, Reinecke H, Xu C, Hassanipour M, Police S, O'Sullivan C, Collins L, Chen Y, Minami E, Gill EA, Ueno S, Yuan C, Gold J, Murry CE. Cardiomyocytes derived from human embryonic stem cells in pro-survival factors enhance function of infarcted rat hearts. *Nat Biotechnol.* 2007;25:1015–1024.
14. van Laake LW, Passier R, Doevendans PA, Mummery CL. Human embryonic stem cell-derived cardiomyocytes and cardiac repair in rodents. *Circ Res.* 2008;102:1008–1010.
15. Nelson TJ, Faustino RS, Chiriac A, Crespo-Diaz R, Behfar A, Terzic A. CXCR4+/FLK-1+ biomarkers select a cardiopoietic lineage from embryonic stem cells. *Stem Cells.* 2008;26:1464–1473.
16. Liu Y, Asakura M, Inoue H, Nakamura T, Sano M, Niu Z, Chen M, Schwartz RJ, Schneider MD. Sox17 is essential for the specification of cardiac mesoderm in embryonic stem cells. *Proc Natl Acad Sci U S A.* 2007;104:3859–3864.
17. Terami H, Hidaka K, Shirai M, Narumiya H, Kuroyanagi T, Arai Y, Aburatani H, Morisaki T. Efficient capture of cardiogenesis-associated genes expressed in ES cells. *Biochem Biophys Res Commun.* 2007;355:47–53.
18. Linden R, Martins VR, Prado MA, Cammarota M, Izquierdo I, Brentani RR. Physiology of the prion protein. *Physiol Rev.* 2008;88:673–728.
19. Zhang CC, Steele AD, Lindquist S, Lodish HF. Prion protein is expressed on long-term repopulating hematopoietic stem cells and is important for their self-renewal. *Proc Natl Acad Sci U S A.* 2006;103:2184–2189.
20. Hidaka K, Lee JK, Kim HS, Ihm CH, Iio A, Ogawa M, Nishikawa S, Kodama I, Morisaki T. Chamber-specific differentiation of Nkx2.5-positive cardiac precursor cells from murine embryonic stem cells. *FASEB J.* 2003;17:740–742.
21. Kouskoff V, Lacaud G, Schwantz S, Fehling HJ, Keller G. Sequential development of hematopoietic and cardiac mesoderm during embryonic stem cell differentiation. *Proc Natl Acad Sci U S A.* 2005;102:13170–13175.

22. Westfall MV, Samuelson LC, Metzger JM. Troponin I isoform expression is developmentally regulated in differentiating embryonic stem cell-derived cardiac myocytes. *Dev Dyn.* 1996;206:24–38.
23. Prall OW, Menon MK, Solloway MJ, Watanabe Y, Zaffran S, Bajolle F, Biben C, McBride JJ, Robertson BR, Chaulet H, Stennard FA, Wise N, Schaft D, Wolstein O, Furtado MB, Shiratori H, Chien KR, Hamada H, Black BL, Saga Y, Robertson EJ, Buckingham ME, Harvey RP. An Nkx2–5/Bmp2/Smad1 negative feedback loop controls heart progenitor specification and proliferation. *Cell.* 2007;128:947–959.
24. Tremblay P, Bouzamondo-Bernstein E, Heinrich C, Prusiner SB, DeArmond SJ. Developmental expression of PrP in the post-implantation embryo. *Brain Res.* 2007;1139:60–67.
25. Bosque PJ, Ryou C, Telling G, Peretz D, Legname G, DeArmond SJ, Prusiner SB. Prions in skeletal muscle. *Proc Natl Acad Sci U S A.* 2002; 99:3812–3817.
26. Manson J, West JD, Thomson V, McBride P, Kaufman MH, Hope J. The prion protein gene: a role in mouse embryogenesis? *Development.* 1992;115:117–122.
27. Christoffels VM, Habets PE, Franco D, Campione M, de Jong F, Lamers WH, Bao ZZ, Palmer S, Biben C, Harvey RP, Moorman AF. Chamber formation and morphogenesis in the developing mammalian heart. *Dev Biol.* 2000;223:266–278.
28. Cai CL, Martin JC, Sun Y, Cui L, Wang L, Ouyang K, Yang L, Bu L, Liang X, Zhang X, Stallcup WB, Denton CP, McCulloch A, Chen J, Evans SM. A myocardial lineage derives from Tbx18 epicardial cells. *Nature.* 2008;454:104–108.
29. Christoffels VM, Grieskamp T, Norden J, Mommersteeg MT, Rudat C, Kispert A. Tbx18 and the fate of epicardial progenitors. *Nature.* 2009; 458:E8–E9.
30. van Wijk B, van den Berg G, Abu-Issa R, Barnett P, van der Velden S, Schmidt M, Ruijter JM, Kirby ML, Moorman AF, van den Hoff MJ. Epicardium and myocardium separate from a common precursor pool by crosstalk between bone morphogenetic protein- and fibroblast growth factor-signaling pathways. *Circ Res.* 2009;105:431–441.

Supplement Material

Materials and Methods

Flow cytometry.
Embryos and EBs were dissociated to single cells by treating them with collagenase type II (Sigma) for 10-12 min at 37°C, which was followed by treatment with Cell Dissociation Buffer (enzyme-free, Hanks'-based, Invitrogen) for 5-8 min. Cells were stained with allophycocyanin (APC)-, phycoerythrin (PE)-, or biotin-conjugated anti-PrP (SPI-Bio, mouse monoclonal, clone SAF83), anti-Flk1 (BD Bioscience, rat monoclonal, clone Avas 12α1), or anti-PDGFRα (eBioscience, rat monoclonal, clone APA5) antibodies. Biotin-conjugated antibodies were further stained with PE-Cy7-conjugated streptavidin. To detect surface and intracellular antigens simultaneously, live cells were first stained for surface antigens, and then fixed and permeabilized using Foxp3 staining buffer (eBioscience). Primary antibodies for intracellular antigens were detected with AlexaFluor 488- or AlexaFluor 647-conjugated anti-mouse or anti-rabbit IgG antibodies (Invitrogen). Analyses were carried out using a FACSCanto flow cytometer (Beckton Dickinson, San Jose, CA). Cell sorting was performed using a FACSAria cell sorter (Beckton Dickinson). Flow cytometric data were analyzed using FlowJo software (Treestar, San Carlos, CA).

Cultures of sorted cells.
To examine the cardiac differentiation of PrP^+ cells, sorted cells were plated at 5×10^4 cells/cm^2 in gelatin-coated plates containing GMEM medium supplemented with 10% FCS. To examine the cardiomyogenic potential of PRa cells, sorted cells were plated at 1×10^3 cells/well in low-cluster 96-well plates (Ultra Low Attachment, U-bottom, Costar 7007) to promote the formation of reaggregates or in gelatin-coated 96-well plates to promote the formation of monolayers. For long-term cultures (> Day 10), reaggregates were embedded and cultured in Matrigel (BD Bioscience). When indicated, Wnt3a (R&D Systems, 100 ng/ml) or Dkk1 (R&D Systems, 150 ng/ml) was added to the reaggregates. To form single cell–derived colonies, sorted cells were plated at a clonal density (500 cells/cm^2) in methylcellulose (MEC, R&D Systems) as described previously,[2] although DT4 conditioned medium was not added to the samples. Instead of 150 ng/ml Dkk1, 100 ng/ml Wnt3a, or 2.5 μM (2′Z,3′E)-6-bromoindirubin-3′-oxime (BIO, GSK3β inhibitor IX, Calbiochem) was added to the samples. Six days after plating, the colonies that formed in the MEC were individually transferred onto gelatin-coated 96-well plates and further cultured for immunofluorescence analysis.

RT-PCR.
RNA was extracted from different samples using Trizol Reagent (Invitrogen). cDNA was generated with SuperScript III (Invitrogen). Real-time PCRs were performed with SYBR Green ER qPCR SuperMix (Invitrogen) and the products were analyzed using an ABI PRISM 7900 sequence detection system (Applied Biosystems). Levels of GAPDH mRNA were determined using rodent GAPDH control reagents (Applied Biosystems) and used to normalize the cDNA levels of other genes. Levels of expression relative to uterus (for *Myh11*), Day-4 EBs (*Mesp1* and *T*), or Day-8 EBs (for the rest of the genes) are shown. The primers are listed in Online Table I. RT-PCR data with error bars in the figures are from at least three independent experiments, and are not a result of replicates of the PCRs.

Immunohistochemistry and *in situ* hybridization.
Immunohistochemistry and immunofluorescence analyses were carried out with the antibodies listed in Online Table II. To detect PrP in fixed samples, clone SAF32 was used instead of SAF83. Embryos or cultured cells were fixed in 4% paraformaldehyde at 4°C. Unlabeled primary antibodies were detected with secondary antibodies conjugated to AlexaFluor 488, AlexaFluor 546, or horseradish peroxidase (Nichirei). APC-conjugated anti-PECAM or Cy3-conjugated anti-SMA antibodies were also used. Whole-mount mouse immunohistochemistry was performed according to protocols from Dr. Andras Nagy's laboratory (http://www.mshri.on.ca/nagy/). Whole-mount *in situ* hybridization was performed as described previously. [3]

Patch clamp.
Membrane potentials were recorded in P-positive cells on a cover slip using the patch clamp technique in

the whole-cell configuration and an amplifier (Axopatch-1D, Axon Instruments, Burlingame, CA). The bath temperature was maintained at 37°C and cells were perfused with normal Tyrode's solution (143 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 0.25 mM $MgCl_2$, 0.25 mM NaH_2PO_4, 5 mM HEPES, and 5 mM glucose at pH 7.4). The pipette solution contained 80 mM KCl, 60 mM KOH, 40 mM aspartate, 5 mM HEPES, 10 mM EGTA, 5 mM MgATP, 5 mM sodium creatine phosphate, and 0.65 mM $CaCl_2$ (pH 7.3). Action potentials were elicited at 1.0 Hz. Data were analyzed using pClamp software (Clampfit, Axon Instruments).

Transplantation of sorted cells.
Reaggregates of PRa cells were labeled with DiI, mixed with Matrigel and injected under the kidney capsules of nude mice using a glass capillary tube. One week following transplantation, DiI-labeled cells were detected. Three weeks following transplantation, the kidneys were fixed overnight in 4% paraformaldehyde at 4°C and embedded in Optimal Cutting Temperature compound. After cryosectioning (7 μm), cardiomyocytes were detected with anti-cardiac troponin T antibodies.

Figure legends
Online Fig. I. A, RT-PCR analysis of EBs. Expression of PrP was observed 2 days before spontaneous beating began. Arrows indicate the onset of spontaneous beating. Representative results from two or three independent experiments are shown. **B**, Immunodetection of PrP protein in ES cell-derived cells. Note that most of the area that was positive for tropomyosin (green) also contained PrP (red).

Online Fig. II. A, Experiments using EB3 ES cells, which show different differentiation kinetics than those of ht7 cells (see Online Fig. IA). (a) Cytospin analysis of $Myhc^+$ cells after sorting on Day 7 (1 day after beating began). The PrP^+ cell fraction contained $Myhc^+$ cells, whereas the PrP^- cell fraction did not. (b) The PrP^+ cell fraction sorted on Day 5 (1 day before beating began) gave rise to beating reaggregates, whereas the PrP^- fraction did not. (c) Additional culturing of the PrP^- cell fraction produced secondary EB-like cells, suggesting the presence of undifferentiated cells. Values are the means ± SE from three independent experiments. **B**, RT-PCR analysis of hcgp7 cells ($Nkx2.5^{GFP/+}$ ES cell). Cells were sorted on Day 8 using PrP (P) and GFP (G). The GFP^-PrP^+ (G-P+) cell fraction expressed cardiac markers. Values are means ± SE from five independent experiments.

Online Fig. III. A, Flow cytometry analysis of intracellular proteins in embryonic heart–derived cells. Whole hearts dissected from E9.5 embryos, and atria and ventricles dissected from E13.5 embryos were dissociated using collagenase and dissociation buffer (Invitrogen). Cells were fixed, permeabilized, and stained with anti-cTnT, anti-cTnI, anti-Mylc2v, anti-Mylc2a, and anti-ANP antibodies. Note that ventricular cardiomyocytes were $Mylc2v^+$ and atrial cells were $Mylc2v^-$, suggesting that Mylc2v can be used to distinguish ventricular cells from atrial cells. ANP, a chamber myocardium marker, is preferentially expressed in atrial cardiomyocytes; the percentage of positive cells was similar to that observed in the immunofluorescence analysis of cultured cells (approximately 20%; data not shown). Mylc2a was expressed in both atria and ventricles, although higher expression levels were observed in atria. **B**, Flow cytometry analysis of PrP^- and PrP^+ cell cultures. Cells were sorted on Day 7 and cultured on gelatin-coated plates for 10 days. Note that PrP^- cells did not markedly differentiate into $cTnT^+$ cardiomyocytes. In PrP^+ cell cultures, $cTnT^-$ cells were SMA^+, suggesting that the noncardiomyocytes in the cultures were smooth muscle cells.

Online Fig. IV. A, Flow cytometry analysis of cell fractions sorted using PrP (P), PDGFRα (R), and Flk1 (F). **B**, RT-PCR analysis of cell fractions. Relative mRNA levels are shown (Day-4 EB = 1.0 for T and Mesp1; Day-8 EB = 1.0 for the rest of the genes). Expression levels of Nkx2.5, Tbx5, Isl1 (see Fig 3), Hand2, and Gata4 were enriched in the $PrP^+PDGFR\alpha^+$ (PRa) fraction (pink bars) on Day 5, whereas cardiac contractile protein markers were not expressed. Kdr (also known as Flk1), T (primitive streak marker), Mesp1 (mesoderm marker), Gata1 (hematopoietic cell marker), Tbx1 (second heart field marker), and Nfatc1 (endocardial cell marker) were not expressed in the PRa cell fraction. The P^-R^+ fraction may contain progenitors that are earlier in the developmental process than PRa cells, because Mesp1 expression was detected in this fraction. Error bars represent the standard errors of the mean (n = 3). **C**, The percentage of beating reaggregates of sorted cells. Cells were sorted on Day 5 and plated

in a 96-well ultra-low binding plate. Wells containing beating aggregates were counted. The PRa cell fraction frequently gave rise to beating aggregates. Error bars represent the standard errors of the mean (n = 5).

Online Fig. V. A, Schematic diagram of the culture methods for PRa cells. Sorted PRa cells were plated on OP9 stromal cells or in methylcellulose (MEC) at a clonal density to obtain colonies derived from single cells. Colonies grown in MEC were picked on Day 6 and plated on gelatin-coated plates. To differentiate cells directly, cells were cultured on gelatin-coated plates (2D cultures) or after reaggregation in ultra-low binding multiwell plates (3D cultures). **B**, Analysis of the ability of the OP9 or MEC culture system to support the undifferentiated state. PRa or P^-R^+ cell fractions were sorted and cultured on OP9 cells (b, c, d) or in MEC (a, e, f, g, h) for 8-9 days. After cell dissociation, cell surface and intracellular markers were analyzed using flow cytometry. We found that OP9 cells expressed PDGFRα (data not shown) and could be distinguished from ES cell–derived cells (pink open circles in b and c; the $PDGFR\alpha^+$ fraction was gated out in d). Whereas OP9 cells induced the differentiation of both endothelial ($CD31^+$, b) and smooth muscle cells (SMA^+, d), MEC did not (a, c, e), suggesting that MEC supported the undifferentiated state. When MEC colonies were cultured on gelatin-coated plates for 3 days (MEC → 2D), they started to differentiate into smooth muscle cells and cardiomyocytes (h). In contrast to P^-R^+ cells, the PRa cell fraction did not give rise to endothelial cells (d, g), suggesting that the PRa cell fraction did not contain multipotent cardiovascular progenitors. **C**, Colony formation from single PRa cells in MEC cultures depended on Wnt signaling. The colonies derived from Day-5.5 PRa cells were counted. **D**, Differentiation of Day-5.5 PRa cells after 2D or 3D cultures. Bars represent standard errors of the mean from three independent experiments.

Online Fig. VI. A, Immunohistochemical analysis of PrP protein. PrP was detected in cardiac crescent (Cc, black arrowhead) and node (N, yellow arrowhead) at the late headfold stage (LHF). Expression of PrP persisted in heart tube (Ht) from E8.5 to E10.5. PrP expression was detected in ganglia at E9.5 (yellow arrowheads). Background staining was observed in extraembryonic mesoderm (EM). **B**, Immunohistochemical analysis of PDGFRα. PDGFRα expression was observed in mesodermal tissues, including cardiac mesoderm (CM) at the early headfold stage (EHF) and in cardiac crescent (Cc) at E8.0. At E8.25, PDGFRα was expressed in the sinus venosus (SV, black arrowheads), whereas expression levels were downregulated in the heart tube (white arrowhead). **C**, Immunofluorescence analysis of PrP and PDGFRα. Overlapping expression was observed in cardiac crescent (Cc) at the late headfold stage (LHF) and in heart tube (Ht) at the 6-7 somite stage (6-7ss). **D**, *In situ* hybridization analysis of cardiac transcription factor mRNA at the early headfold stage. *Nkx2.5*, *Tbx5*, and *Isl1* mRNA was detected cardiac mesoderm (CM).

Online Fig. VII. A, Flow cytometry analysis of surface proteins on mouse embryo–derived cells. Mouse embryos were dissected and the extraembryonic region was removed. E8 pools (containing embryos from LHF to 7ss) were stained with antibodies specific for PrP, PDGFRα, and Flk1. **B**, RT-PCR analysis of sorted cells from E8 pool embryos. Note that the PrP^+ fraction was specifically enriched for cardiac markers. Representative results from three independent experiments are shown. **C**, Flow cytometry analysis of PrP, PDGFRα, and Myhc in the outflow tract (OFT) segment derived from E9.5 embryos. The OFT was dissected, dissociated, and analyzed using flow cytometry. Overlapping expression of PrP and PDGFRα was observed. Intracellular staining revealed that PRa cells included $Myhc^+$ cells.

Online Fig. VIII. Potential and committed cardiac progenitor cells based on ES cell studies. The earliest cardiomyogenic population was the PRa cells. PRa cells include $Myhc^+$ nascent cardiomyocytes as well as $Myhc^-$ bipotential progenitors that can differentiate into cardiac or smooth muscle cells. PrP can also be used to isolate nascent cardiomyocytes that differentiate into atrial or ventricular cardiomyocytes.

Online Table I. Primers used for real-time RT-PCRs

	Gene Symbol	Sense primers (5'→3')	Antisense primers (5'→3')
Early mesoderm	*T*	TCCCGAGACCCAGTTCATAG	GGTCGTTTCTTTCTTTGGCA
	Mesp1	GCGACATGCTGGCTCTTCTA	TGGTATCACTGCCGCCTCTTCC
	Kdr (Flk1)	GGCGGTGGTGACAGTATCTT	CTCGGTGATGTACACGATGC
	Pdgfra	CAACCACACTCAGACGGATG	CTCCCGTTATTGTGCAAGGT
Cardiac transcription factors	*Isl1*	TCATCCGAGTGTGGTTTCAA	CCATCATGTCTCTCCGGACT
	Tbx1	CGACAAGCTGAAACTGACCA	ACTGTCTTTTCGAGGGTCCA
	Tbx5	GGAGCCTGATTCCAAAGACA	TTCAGCCACAGTTCACGTTC
	Hand2	TCAAGGCGGAGATCAAGAAG	TGGTTTTCTTGTCGTTGCTG
	Hand1	GCCTACTTGATGGACGTGCT	GCGCCCTTTAATCCTCTTCT
	Nkx2-5	ACCCAGCCAAAGACCCTC	GACAGGTACCGCTGTTGCTT
	Gata4	TCTCACTATGGGCACAGCAG	GCGATGTCTGAGTGACAGGA
	Mef2c	ACTGGGAAACCCCAATCTTC	ATCAGACCGCCTGTGTTACC
	Tbx2	GGTCATCTGCTAGCCTCAGT	AAAGTGGGCATTGGGATT
	Tbx3	CCTTCCACCTCCAACAACAC	GCATGCTGTTCAAATTGAGG
	Tbx18	ACGAAATAGGCACCGAGATG	CCTGCCACCATCCACTTAGA
Endocardial cells	*Nfatc1*	TCATCCTGTCCAACACCAAA	TCACCCTGGTGTTCTTCCTC
Hematopoietic cells	*Gata1*	AGCATCAGCACTGGCCTACT	AGGCCCAGCTAGCATAAGGT
Cardiac muscle	*Prnp*	CTGAAGCATTCTGCCTTCCT	GCCGACATCAGTCCACATAG
	Myl2 (Mylc2v)	AAAGAGGCTCCAGGTCCAAT	CCTCTCTGCTTGTGTGGTCA
	Myl7 (Mylc2a)	TCAGCTGCATTGACCAGAAC	AAGACGGTGAAGTTGATGGG
	Tnni3 (cTnI)	CTGCCAACTACCGAGCCTAT	CTCGTTCCATCTCCTGCTTC
	Myh6	GAGATTTCTCCAACCCAG	TCTGACTTTCGGAGGTACT
	Hcn4	CTGGGGTCAACAAATTCTCC	ATCAGCAACAGCATCGTCAG
	Nppa	AGTGGACTAGGCTGCAACAGCTTC	ACACACCACAAGGGCTTAGGATC
Smooth muscle	*Myh11* (SMMyhc)	CAAATGAAGCCTCGTTTCCT	ATGAGGCCACAGAGAGCAAT
	Tagln (SM22a)	GATGGAACAGGTGGCTCAAT	TTTTGGTCACAGCCAAACTG
	Acta2 (SMA)	AGCCAGTCGCTGTCAGGAA	CTTACAGAGCCCAGAGCCAT

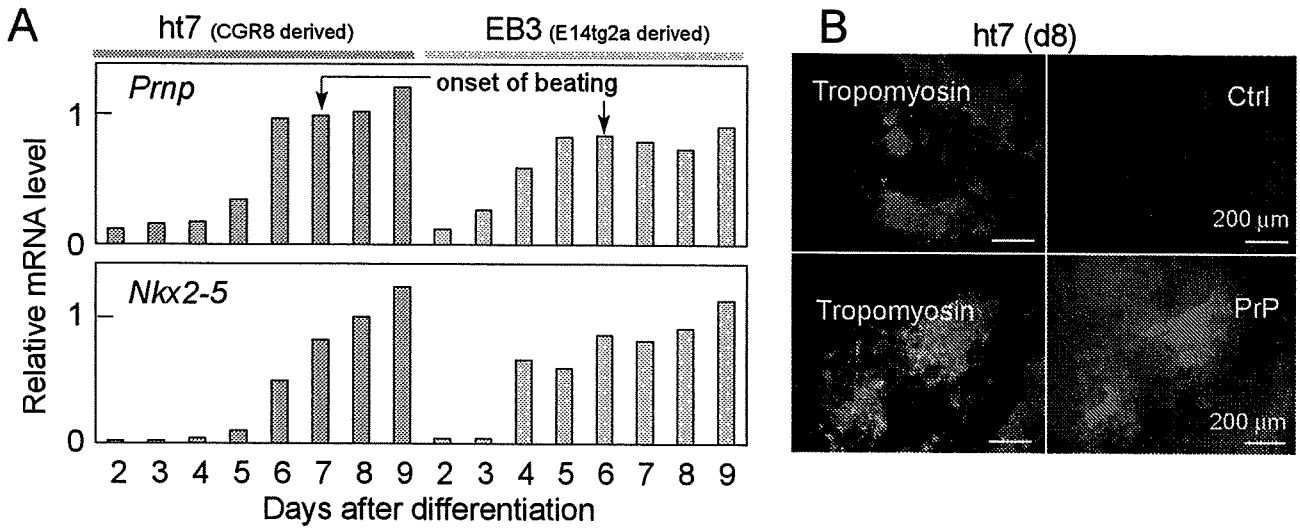
Online Table II. Antibodies used in this study

		Protein Name	Abbreviation used in this study	Antibody source (clone name)	Label
Surface staining of live cells		Prion protein	PrP	SPI-BIO, mouse monoclonal (SAF83)	Biotin APC
		PDGF Receptor α	PDGFRα	eBioscience, rat monoclonal, (APA5)	Biotin, PE, APC
		Flk1	Flk1	BD Bioscience, rat monoclonal, (Avas12α1)	PE
		c-kit	c-kit	eBioscience, rat monoclonal,	PE
		PECAM	CD31	eBioscience, rat monoclonal,	APC
Intracellular staining of fixed cells	Transcription factors	Gata4	Gata4	Santa Cruz, rabbit polyclonal	
		Islet-1	Isl1	Hybridoma Bank, mouse monoclonal (clone 39.4.D5)	
	Cardiac proteins	Prion protein	PrP	SPI-BIO, mouse monoclonal (SAF32)	
		Myosin heavy chain	Myhc	Hybridoma Bank (clone MF20)	
		Tropomyosin	Tm	Sigma, mouse monoclonal (clone CH1)	
		Cardiac troponin I	cTnI	DSMZ, mouse monoclonal (clone TI-1)	
		Cardiac troponin T	cTnT	Santa Cruz, goat polyclonal	
		Cardiac troponin T	cTnT	Abcam, mouse monoclonal (clone 13-11)	
		Atrial natriuretic peptide	ANP	Protos Biotech (rabbit polyclonal)	
		Myosin light chain 2v	Mylc2v	Alexis, mouse monoclonal (clone F109.3E1)	
		Myosin light chain 2a	Mylc2a	Sigma, rabbit polyclonal	
		Hyperpolarization activated cyclic nucleotide-gated potassium channel 4	HCN4	Chemicon, rabbit polyclonal	
	Smooth muscle proteins	Smooth muscle α actin	SMA	Sigma, mouse monoclonal (1A4)	
		SM22α	SM22	Abcam, goat polyclonal	
		Calponin	Calp	Abcam, rabbit monoclonal	

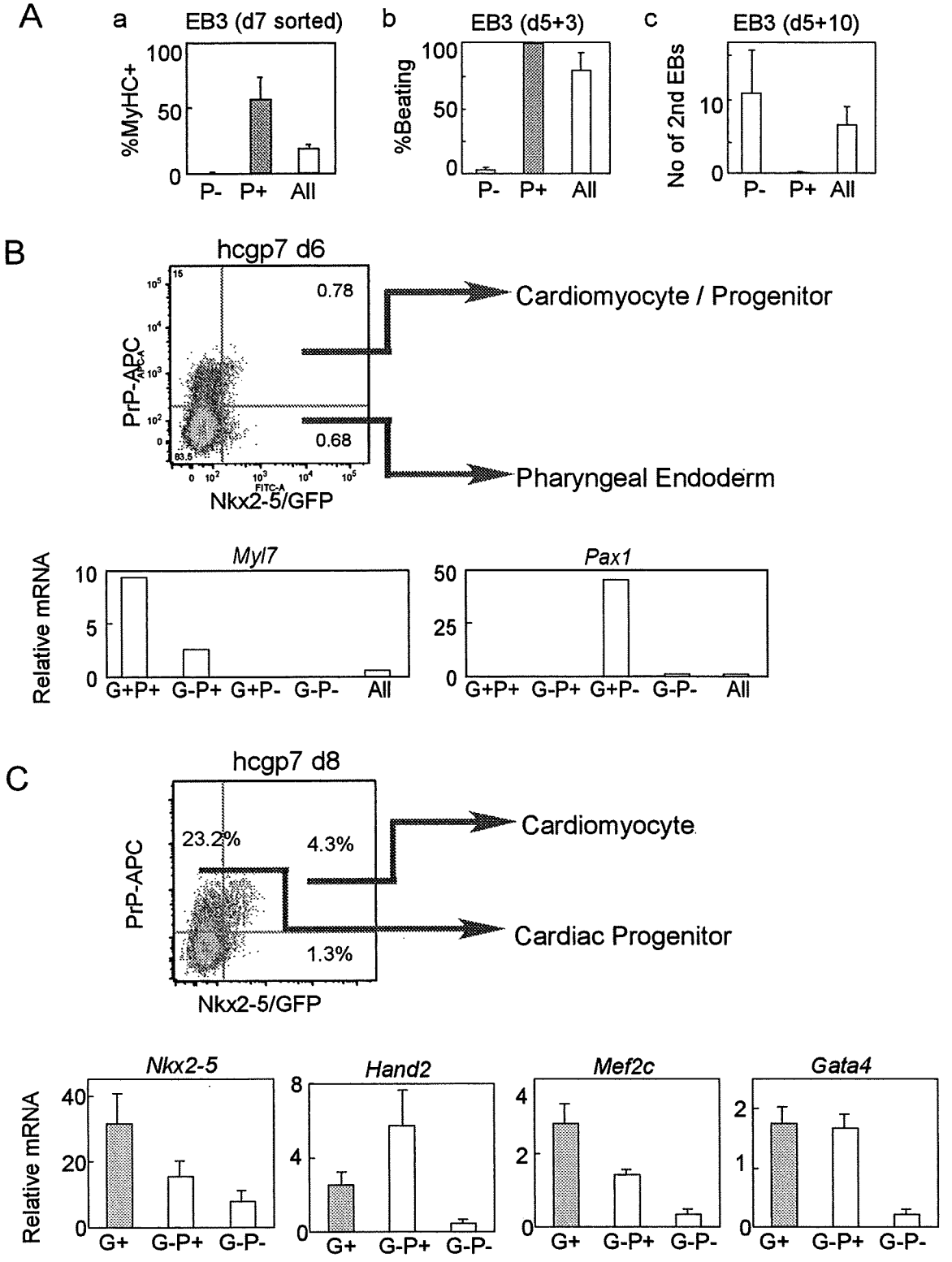
		Smooth muscle myosin heavy chain	SMMyhc	Biomedical Technologies, rabbit polyclonal	

References

1. Hidaka K, Lee JK, Kim HS, Ihm CH, Iio A, Ogawa M, Nishikawa S, Kodama I, Morisaki T. Chamber-specific differentiation of Nkx2.5-positive cardiac precursor cells from murine embryonic stem cells. *Faseb J.* 2003;17:740-742.
2. Kattman SJ, Huber TL, Keller GM. Multipotent flk-1+ cardiovascular progenitor cells give rise to the cardiomyocyte, endothelial, and vascular smooth muscle lineages. *Dev Cell.* 2006;11:723-732.
3. Wilkinson D. *In Situ Hybridization: A Practical Approach.* New York: Oxford University Press. 1992.
4. Cai CL, Liang X, Shi Y, Chu PH, Pfaff SL, Chen J, Evans S. Isl1 identifies a cardiac progenitor population that proliferates prior to differentiation and contributes a majority of cells to the heart. *Dev Cell.* 2003;5:877-889.
5. Prall OW, Menon MK, Solloway MJ, Watanabe Y, Zaffran S, Bajolle F, Biben C, McBride JJ, Robertson BR, Chaulet H, Stennard FA, Wise N, Schaft D, Wolstein O, Furtado MB, Shiratori H, Chien KR, Hamada H, Black BL, Saga Y, Robertson EJ, Buckingham ME, Harvey RP. An Nkx2-5/Bmp2/Smad1 negative feedback loop controls heart progenitor specification and proliferation. *Cell.* 2007;128:947-959.

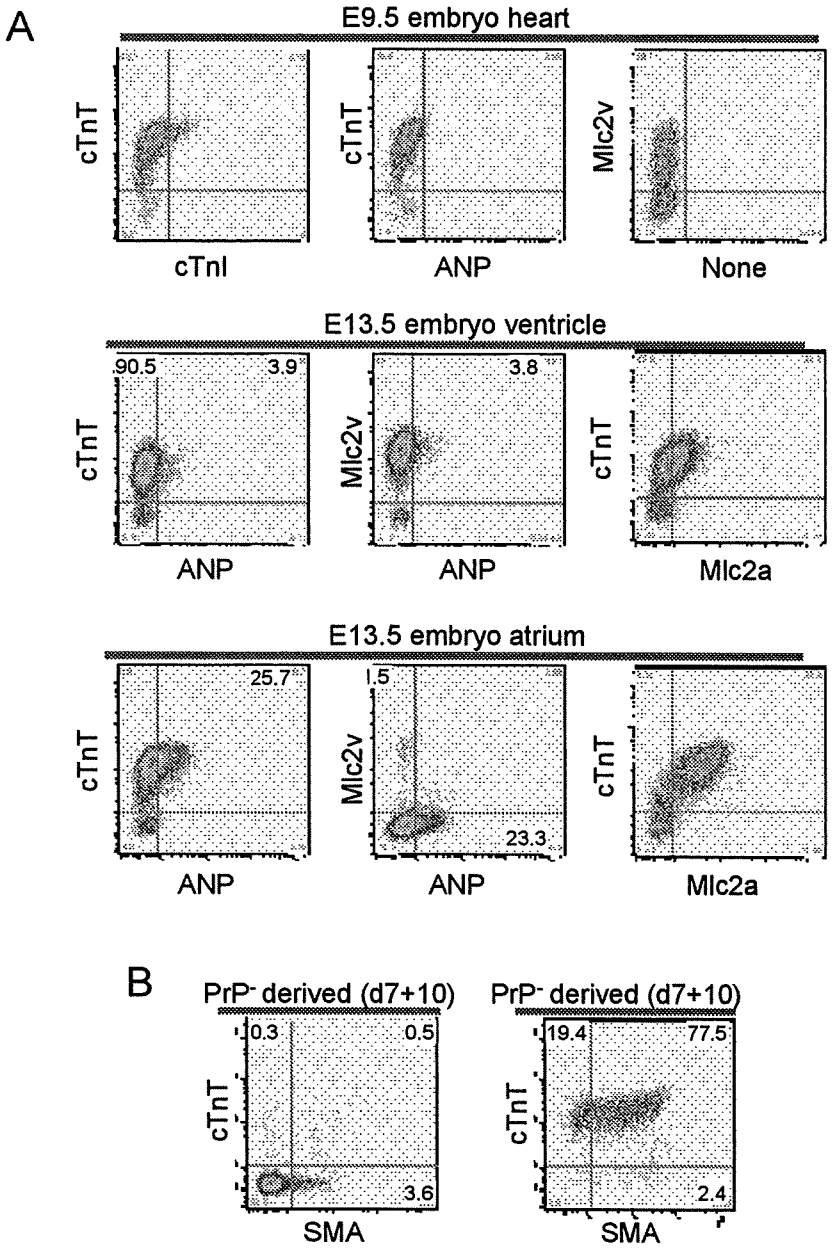


Online Fig. I. A, RT-PCR analysis of EBs. Expression of PrP was observed 2 days before spontaneous beating began. Arrows indicate the onset of spontaneous beating. Representative results from two or three independent experiments are shown. B, Immunodetection of PrP protein in ES cell-derived cells. Note that most of the area that was positive for tropomyosin (green) also contained PrP (red).



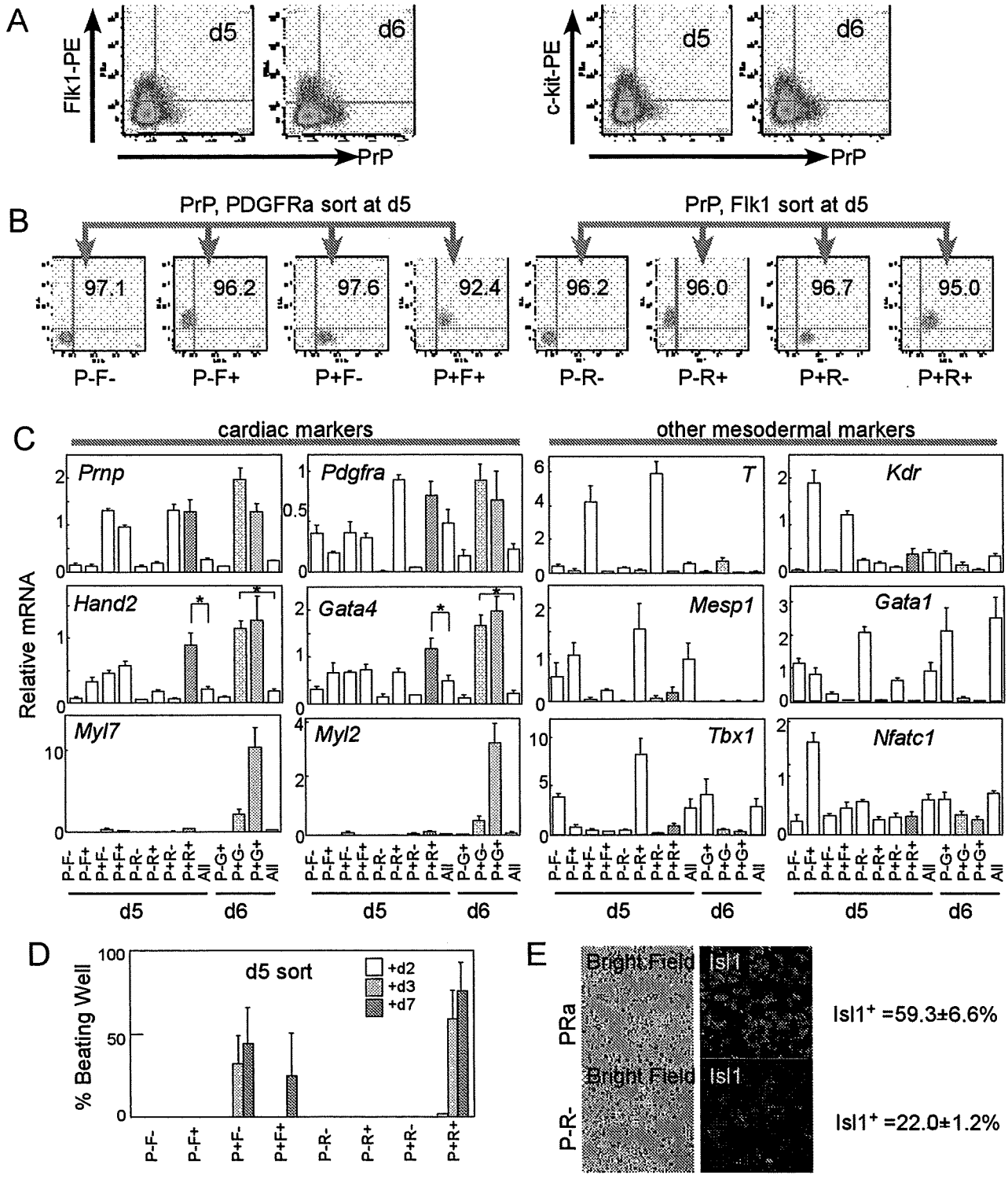
Online Fig. II. A, Experiments using EB3 ES cells, which show different differentiation kinetics than those of ht7 cells (see Fig. S1A). (a) Cytospin analysis of Myhc$^+$ cells after sorting on Day 7 (1 day after beating began). The PrP$^+$ cell fraction contained Myhc+ cells, whereas the PrP$^-$ cell fraction did not. (b) The PrP$^+$ cell fraction sorted on Day 5 (1 day before beating began) gave rise to beating reaggregates, whereas the PrP$^-$ fraction did not. (c) Additional culturing of the PrP$^-$ cell fraction produced secondary EB-like cells, suggesting the presence of undifferentiated cells. Values are the means ± SE from three independent experiments. B, RT-PCR analysis of hcgp7 cells (Nkx2.5$^{GFP/+}$ ES cell). Cells were sorted on Day 8 using PrP (P) and GFP (G). The GFP$^-$PrP$^+$ (G-P+) cell fraction expressed cardiac markers. Values are means ± SE from five independent experiments.

Downloaded from circres.ahajournals.org at National Cardiovascular Center on January 7, 2010



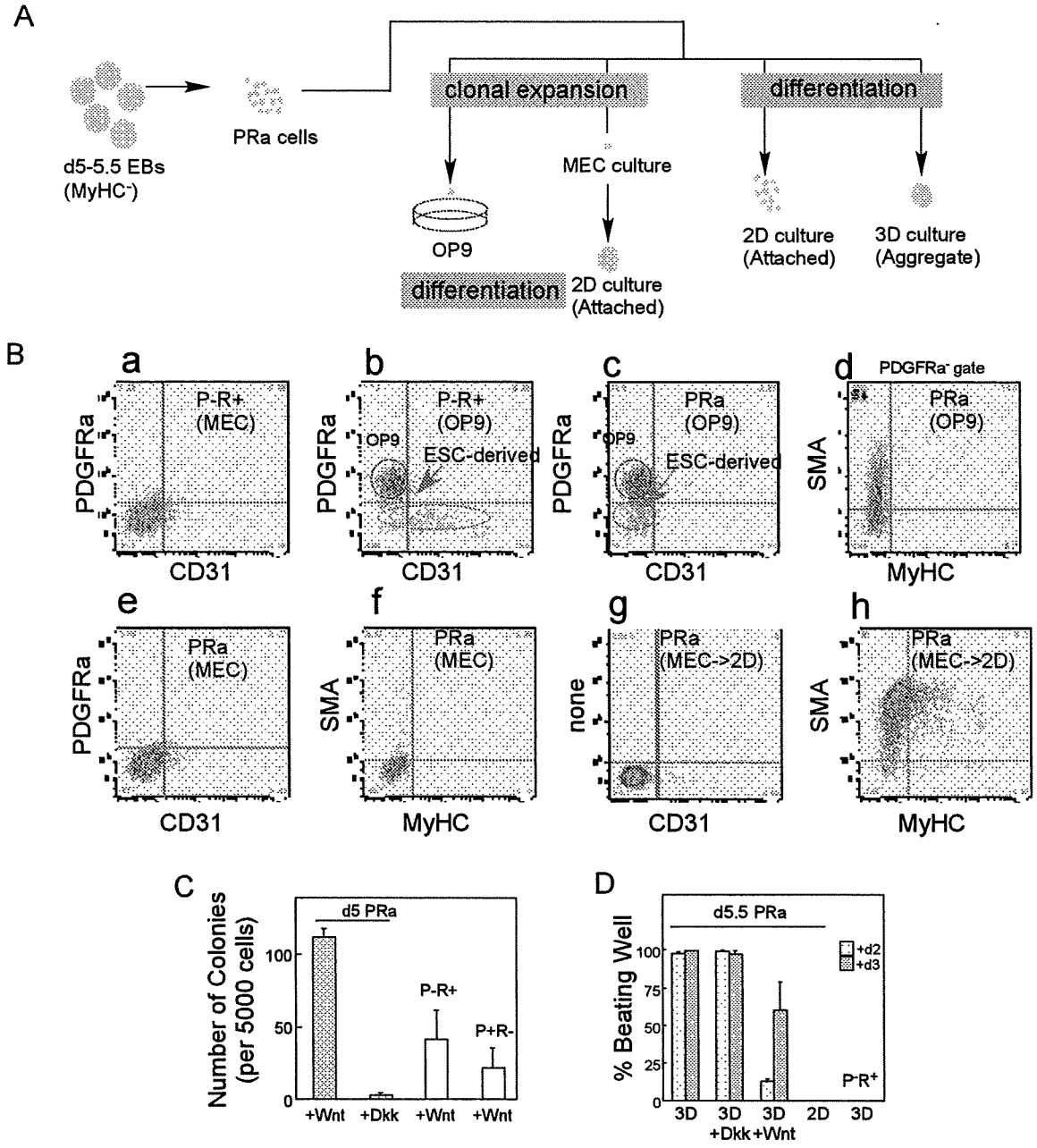
Online Fig.III. A, Flow cytometry analysis of intracellular proteins in embryonic heart-derived cells. Whole hearts dissected from E9.5 embryos, and atria and ventricles dissected from E13.5 embryos were dissociated using collagenase and dissociation buffer (Invitrogen). Cells were fixed, permeabilized, and labeled with anti-cTnT, anti-cTnI, anti-Mylc2v, anti-Mylc2a, and anti-ANP antibodies. Note that ventricular cardiomyocytes were Mylc2v$^+$ and atrial cells were Mylc2v$^-$, suggesting that Mylc2v can be used to distinguish ventricular cells from atrial cells. ANP, a chamber myocardium marker, is preferentially expressed in atrial cardiomyocytes; the percentage of positive cells was similar to that observed in the immunofluorescence analysis of cultured cells (approximately 20%; data not shown). Mylc2a was expressed in both atria and ventricles, although higher expression levels were observed in atria. B, Flow cytometry analysis of PrP$^-$ and PrP$^+$ cell cultures. Cells were sorted on Day 7 and cultured on gelatin-coated plates for 10 days. Note that PrP$^-$ cells did not markedly differentiate into cTnT$^+$ cardiomyocytes. In PrP$^+$ cell cultures, cTnT$^-$ cells were SMA$^+$, suggesting that the noncardiomyocytes in the cultures were smooth muscle cells.

Downloaded from circres.ahajournals.org at National Cardiovascular Center on January 7, 2010



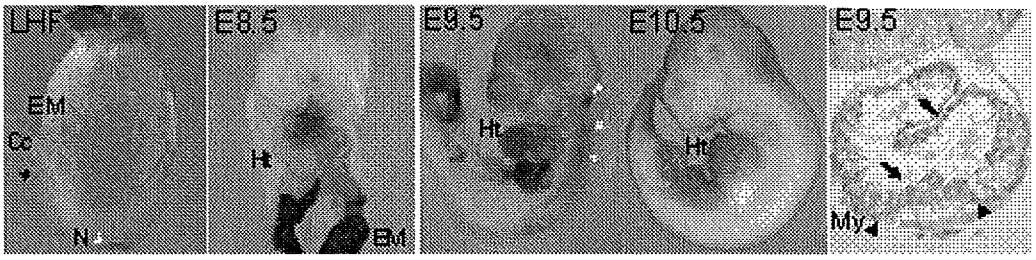
Online Fig. IV. A, Flow cytometry analysis of cell fractions sorted using PrP (P), PDGFRα (R), and Flk1 (F). B, RT-PCR analysis of cell fractions. Relative mRNA levels are shown (Day-4 EB = 1.0 for T and Mesp1; Day-8 EB = 1.0 for the rest of the genes). Expression levels of *Nkx2.5*, *Tbx5*, *Isl1* (see Fig 3), *Hand2*, and *Gata4* were enriched in the PrP$^+$PDGFRα$^+$ (PRa) fraction (pink bars) on Day 5, whereas cardiac contractile protein markers were not expressed. *Kdr* (also known as *Flk1*), T (primitive streak marker), *Mesp1* (mesoderm marker), *Gata1* (hematopoietic cell marker), and *Nfatc1* (endocardial cell marker) were not expressed in the PRa cell fraction. The P-R+ fraction may contain progenitors that are earlier in the developmental process than PRa cells, because *Mesp1* expression was detected in this fraction. Error bars represent the standard errors of the mean (n = 3). C, The percentage of beating reaggregates of sorted cells. Cells were sorted on Day 5 and plated in a 96-well ultra-low binding plate. Wells containing beating aggregates were counted. The PRa cell fraction frequently gave rise to beating aggregates. Error bars represent the standard errors of the mean (n = 5).

Downloaded from circres.ahajournals.org at National Cardiovascular Center on January 7, 2010

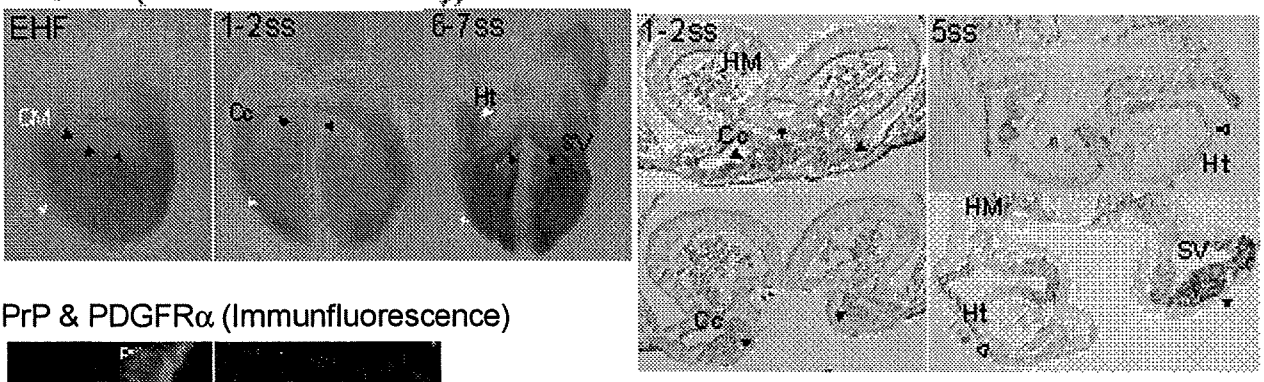


Online Fig. V. A, Schematic diagram of the culture methods for PRa cells. Sorted PRa cells were plated on OP9 stromal cells or in methylcellulose (MEC) at a clonal density to obtain colonies derived from single cells. Colonies grown in MEC were picked on Day 6 and plated on gelatin-coated plates. To differentiate cells directly, cells were cultured on gelatin-coated plates (2D cultures) or after reaggregation in ultra-low binding multiwell plates (3D cultures). B, Analysis of the ability of the OP9 or MEC culture system to support the undifferentiated state. PRa or P-R+ cell fractions were sorted and cultured on OP9 cells (b, c, d) or in MEC (a, e, f, g, h) for 8-9 days. After cell dissociation, cell surface and intracellular markers were analyzed using flow cytometry. We found that OP9 cells expressed PDGFRα (data not shown) and could be distinguished from ES cell-derived cells (pink open circles in b and c; the PDGFRα+ fraction was gated out in d). Whereas OP9 cells induced the differentiation of both endothelial ($CD31^+$, b) and smooth muscle cells (SMA^+, d), MEC did not (a, c, e), suggesting that MEC supported the undifferentiated state. When MEC colonies were cultured on gelatin-coated plates for 3 days (MEC → 2D), they started to differentiate into smooth muscle cells and cardiomyocytes (h). In contrast to P-R+ cells, the PRa cell fraction did not give rise to endothelial cells (d, g), suggesting that the PRa cell fraction did not contain multipotent cardiovascular progenitors. C, Colony formation from single PRa cells in MEC cultures depended on Wnt signaling. The colonies derived from Day-5.5 PRa cells were counted. D, Differentiation of Day-5.5 PRa cells after 2D or 3D cultures. Bars represent standard errors of the mean from three independent experiments.

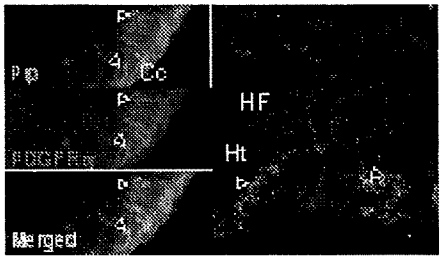
A PrP (Immunohistochemistry)



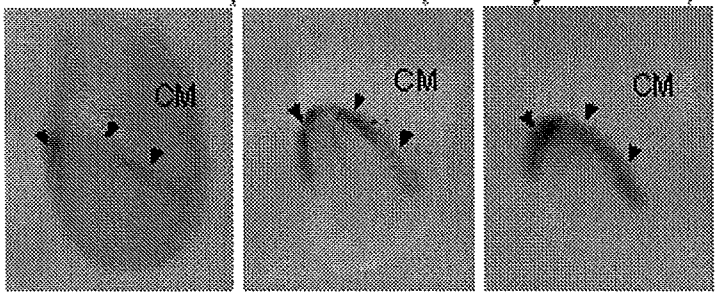
B PDGFRα (Immunohistochemistry)



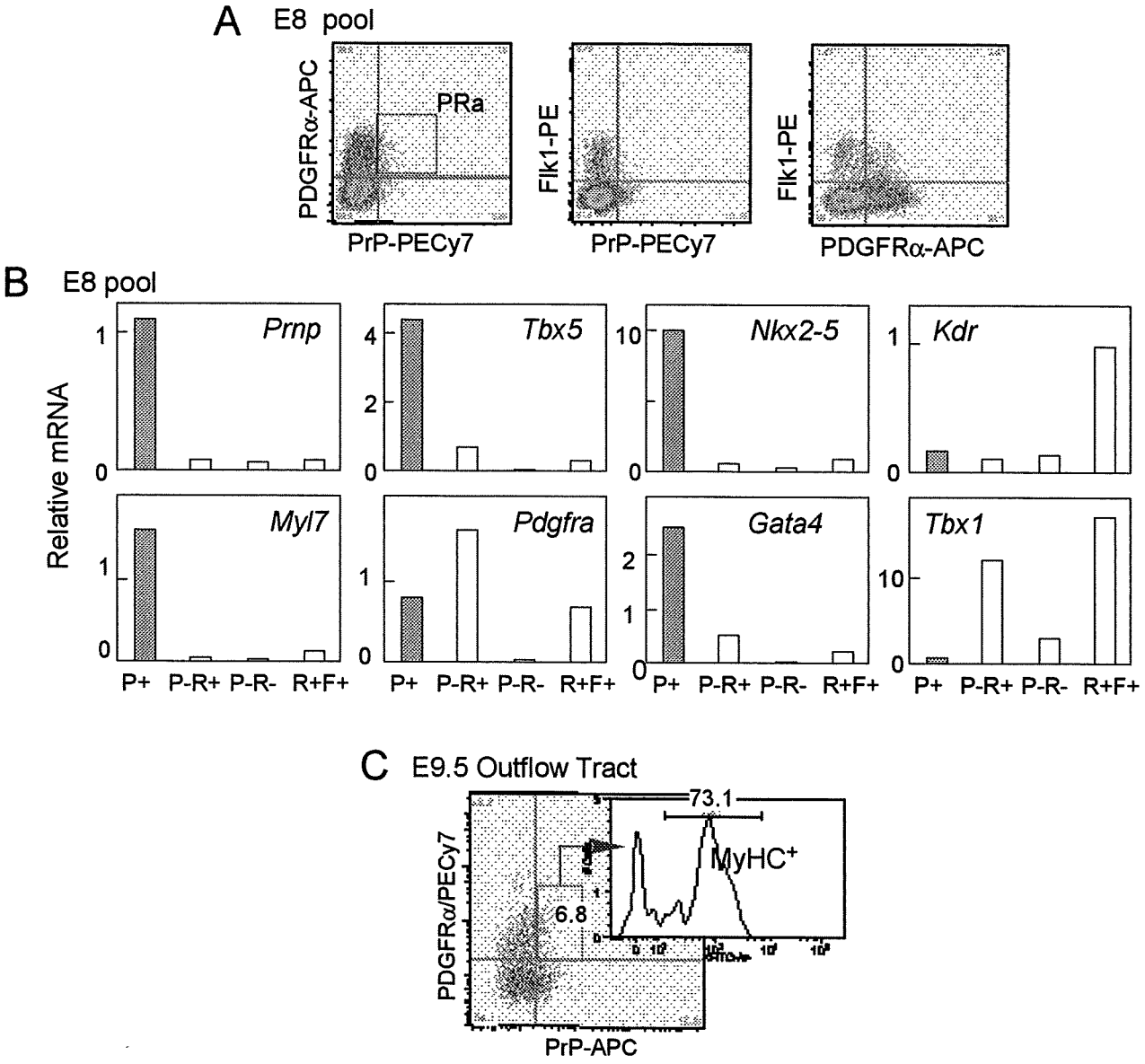
C PrP & PDGFRα (Immunfluorescence)



D Cardiac Transcription Factors (in situ hybridization)



Online Fig. VI. A, Immunohistochemical analysis of PrP protein. PrP was detected in cardiac crescent (Cc, black arrowhead) and node (N, yellow arrowhead) at the late headfold stage (LHF). Expression of PrP persisted in heart tube (Ht) from E8.5 to E10.5. PrP expression was detected in ganglia at E9.5 (yellow arrowheads). Background labeling was observed in extraembryonic mesoderm (EM). B, Immunohistochemical analysis of PDGFRα. PDGFRα expression was observed in mesodermal tissues, including cardiac mesoderm (CM) at the early headfold stage (EHF) and in cardiac crescent (Cc) at E8.0. At E8.25, PDGFRα was expressed in the sinus venosus (SV, black arrowheads), whereas expression levels were downregulated in the heart tube (white arrowhead). C, Immunofluorescence analysis of PrP and PDGFRα. Overlapping expression was observed in cardiac crescent (Cc) at the late headfold stage (LHF) and in heart tube (Ht) at the 6-7 somite stage (6-7ss). D, *In situ* hybridization analysis of cardiac transcription factor mRNA at the early headfold stage. *Nkx2.5*, *Tbx5*, and *Isl1* mRNA was detected cardiac mesoderm (CM).



Online Fig. VII. A, Flow cytometry analysis of surface proteins on mouse embryo-derived cells. Mouse embryos were dissected and the extraembryonic region was removed. E8 pools (containing embryos from LHF to 7 somite stage) were labeled with antibodies specific for PrP, PDGFRα, and Flk1. B, RT-PCR analysis of sorted cells from E8 pool embryos. Note that the PrP^+ fraction was specifically enriched for cardiac markers. Representative results from three independent experiments are shown. C, Flow cytometry analysis of PrP, PDGFRα, and Myhc in the outflow tract (OFT) segment derived from E9.5 embryos. The OFT was dissected, dissociated, and analyzed using flow cytometry. Overlapping expression of PrP and PDGFRα was observed. Intracellular staining revealed that PRa cells included $Myhc^+$ cells.