Regulated Expression and Role of c-Myb in the Cardiovascular-Directed Differentiation of Mouse Embryonic Stem Cells

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Rationale: c-myb null (knockout) embryonic stem cells (ESC) can differentiate into cardiomyocytes but not contractile smooth muscle cells (SMC) in embryoid bodies (EB).

Objective: To define the role of c-Myb in SMC differentiation from ESC.

Methods and Results: In wild-type (WT) EB, high c-Myb levels on days 0–2 of differentiation undergo ubiquitin-mediated proteosomal degradation on days 2.5–3, resurging on days 4–6, without changing c-myb mRNA levels. Activin-A and bone morphogenetic protein 4–induced cardiovascular progenitors were isolated by FACS for expression of vascular endothelial growth factor receptor (VEGFR2) and platelet-derived growth factor receptor (PDGFRα). By day 3.75, hematopoiesis-capable VEGFR2+/PDGFRα+ cells were fewer, whereas cardio-myocyte-directed VEGFR2+/PDGFRα+ cells did not differ in abundance in knockout versus WT EB. Importantly, highest and lowest levels of c-Myb were observed in VEGFR2+ and VEGFR2+/PDGFRα+ cells, respectively. Proteosome inhibitor MG132 and lentiviruses enabling inducible expression or knockdown of c-myb were used to regulate c-Myb in WT and knockout EB. These experiments showed that c-Myb promotes expression of VEGFR2 over PDGFRα, with chromatin immunoprecipitation and promoter-reporter assays defining specific c-Myb-responsive binding sites in the VEGFR2 promoter. Next, FACS-sorted VEGFR2+ cells expressed highest and lowest levels of SMC- and fibroblast-specific markers, respectively, at days 7–14 after retinoic acid (RA) as compared with VEGFR2+/PDGFRα+ cells. By contrast, VEGFR2+/PDGFRα+ cells cultured without RA beat spontaneously, like cardiomyocytes between days 7 and 14, and expressed cardiac troponin. Notably, RA was required to more fully differentiate SMC from VEGFR2+ cells and completely blocked differentiation of cardiomyocytes from VEGFR2+/PDGFRα+ cells.

Conclusions: c-Myb is tightly regulated by proteosomal degradation during cardiovascular-directed differentiation of ESC, expanding early-stage VEGFR2+ progenitors capable of RA–responsive SMC formation.

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Key Words: cardiac progenitor cells ■ embryonic stem cells ■ smooth muscle differentiation ■ transcription factors

Studies examining atherosclerosis and vascular remodeling have suggested that the differentiation of smooth muscle cell (SMC) progenitors contributes to disease progression.1 As such, the molecular basis of SMC differentiation is of therapeutic relevance. Unlike cardiac and skeletal muscle cells, SMCs are derived from diverse populations of precursor cells during embryonic development. For instance, within the vascular system, coronary artery SMCs are derived from proepicardial cells, whereas SMCs of the aortic arch and thoracic aorta originate partly from neural crest.2 Furthermore, the diverse origins of SMCs may underlie their distinct structural and functional properties2 and possibly the differential expression of contractile proteins observed in SMCs from various tissues.3,4

Both in vitro and in vivo studies have shown that embryonic stem cells (ESC) and ESC-derived progenitors have the capacity to spontaneously differentiate to smooth muscle–like cells.5–7 However, little is known regarding the specific factors that promote embryonic and pluripotent stem cells to differentiate into SMC lineage(s) or their maturation into contractile forms. We reveal a critical role for the proto-oncogene c-myb during ESC differentiation to a specific progenitor population primed for a SMC fate.
c-Myb, the encoded gene product of the proto-oncogene c-myb, is a nuclear transcription factor known to regulate hematopoiesis. c-Myb is also involved in maintaining stem cells in colonic crypts and in the neural crest niche of the adult brain. Moreover, c-Myb has been implicated in SMC differentiation from neural crest and from ESCs. In the latter, we showed that c-myb knockout (KO) mouse ESCs failed to express SMC-specific genes and to differentiate into contractile SMC in embryoid bodies (EB) in vitro, making reduced contributions to SMC-containing tissues in chimeric embryos and adult mice in vivo. By contrast, c-myb KO ESCs appeared to differentiate into contractile cardiomyocytes with greater efficiency than wild-type (WT) ESCs, suggesting that c-Myb may play a role in lineage specification during cardiovascular development. However, direct evidence of this, or of the downstream mechanism(s) mediating this, were lacking.

Previous studies in mouse embryos and ESC differentiation models have indicated that cardiomyocytes, endothelial cells, and vascular SMCs (as well as hematopoietic cells) can develop from progenitor(s) expressing vascular endothelial growth factor receptor type-2 (VEGFR2), also known as the Fetal liver kinase-1 (Flk-1), and kinase insert domain protein receptor (Kdr). Of interest, EB derived from ESC null for VEGFR2 have limited formation of cells expressing smooth muscle actin (SMA+), by virtue of decreased expression of Pim-1 kinase, a modulator of DNA binding sites on c-Myb. Platelet-derived growth factor receptor alpha (PDGFRα) is also involved in several embryonic processes. Of relevance to cardiovascular development, mice null for Pdgfra display abnormalities in heart, pericardium, systemic hemorrhage, and cranial neural crest apoptosis. Like VEGFR2, PDGFRα is required for specific stages of mesodermal and cardiovascular development, and EB-derived cells positive for both markers (VEGFR2+/PDGFRα+) have been identified as cardiomyocyte progenitors in mouse. By contrast, human EB-derived cells with high levels of VEGFR2 (and c-kit) expression cells were found to express markers of hematopoiesis (GATA1) and vascular cells (CD31, SMA), whereas cells with low levels of VEGFR2 (and absence of c-kit) were shown to have greater facility for cardiomyocyte formation. More recently, mouse and human ESC- and iPS-derived VEGFR2+/PDGFRα+ progenitors could be optimized for more efficient differentiation to cardiomyocytes by small changes in Activin (Act) and bone morphogenetic protein (BMP) signaling.

Given these data, we hypothesized that c-Myb may play a role in defining how VEGFR2- and/or PDGFRα-expressing progenitors emerge from ESC and/or how they subsequently differentiate into SMC. Our study reveals that c-Myb levels are critically regulated by proteasomal degradation during the earliest stages of mouse ESC differentiation and regulate, in turn, the proportion of VEGFR2+ cells that emerge in EBs after this time period. Supporting a direct transcriptional mechanism, chromatin immunoprecipitation (ChIP) assays on ESCs show contemporaneous binding of c-Myb to the VEGFR2 promoter, with WT and point-mutated promoter-reporter assays in HEK293 cells confirming c-Myb–dependent VEGFR2 promoter activity. We also show in a serum-free differentiation system that fluorescence activated cell sorting (FACS)-purified VEGFR2+ and VEGFR2+/PDGFRα+ populations have diametrically opposite levels of c-Myb expression and respond to cardiogenic (basic fibroblast growth factor) and vasculogenic (VEGF) growth factors with distinct RA-sensitive patterns of subsequent differentiation. Collectively, our results suggest a key role for c-Myb in the derivation of VEGFR2+ progenitors and their subsequent capacity to differentiate into the SMC lineage.

Methods

An expanded Methods section is available in the Data Supplement at http://circres.ahajournals.org.

ESC Culture

Cytogenetic studies confirmed early (<10) passage c-myb–KO ESCs of the CCE genetic background to harbor normal chromosomes (data not shown), and all studies used ESC of this stage. WT and KO CCE ESCs were maintained as previously described. c-myb–KO ESC containing tetracycline-inducible c-myb cDNA have been previously described.

Cell Lines

A mouse aorta-derived immortalized vascular SMC line (MOVAS: ATCC CRL-2797) and a mouse hemangiendothelioma cell line (EOMA: ATCC, CRL-2586) were cultured in DMEM supplemented with 10% FBS and antibiotics. Human erythroleukemia cells (K562: ATCC, CCL-243) were maintained in RPMI1640 supplemented with 10% FBS and antibiotics. K562 and/or G1/S-synchronized MOVAS were used as positive controls in Western blots.

ESC Differentiation Protocols

These are detailed in the Online Supplement.

FACS

EBs were trypsinized and allowed to incubate with allophycocyanin-conjugated anti-mouse VEGFR2 and phycoerythrin-conjugated anti-
mouse PDGFrα antibodies (eBioscience) at 4°C for 30 minutes. Cells were washed twice, and FACS was performed by ARIA-RITT and LSR-SC (Becton-Dickinson). Data were analyzed on FlowJo software (Ashland).

**Quantitative RT-PCR and PCR**

These analyses were performed on the ABI Prism 7700 Sequence Detection System (Applied Biosystems) as described and the Roche LightCycler 480 System as per the manufacturer. Primers used are listed in Online Table I.

In silico analyses, ChIP, c-myb and short hairpin RNA (shRNA) expression constructs, site-directed mutagenesis, promoter-reporter assays, and immunostaining are described in the Online Supplement.

The Western blot and ubiquitination assay are described in the Online Supplement.

**Inducible shRNA**

To generate mouse ESC with tetracycline-inducible c-myb or scrambled (SCR) shRNA, mouse ESCs were transduced with lentivirus containing tet-operon–driven c-myb or SCR shRNA and human EF1α promoter–driven tTA-IRES-GFP.

**Statistical Analyses**

Data are presented as mean±SEM. Statistical analyses were performed by Student unpaired t test. Significance was defined as P<0.05.

**Results**

**c-Myb Levels Are Rapidly Reduced by Protein Degradation in Early-Stage Embryoid Bodies**

To examine expression levels of c-Myb during cardiovascular differentiation, we first performed Western blotting on whole-cell extracts from mouse ESC-derived embryoid bodies (EB) grown in serum-conditioned (ie, spontaneous differentiation) medium. Levels of both the p75 and p89 splice variants of c-Myb were rapidly and dramatically downregulated on day 3 of EB differentiation but recovered on days 4–6 (Online Figure I, A). Importantly, this drop in c-Myb protein could not be attributed to diminished c-myb mRNA levels, which increased over this time period, both in serum-supplemented (Online Figure I, A) and serum-free media (Figure 1B). Rather, the ability of both the proteosome inhibitor MG132 (0, 0.1, and 1 μmol/L) and lactacystin (10 μmol/L) and harvested at day 3 for Western blot (C). Lysates from EBs treated with or without MG132 (0.1 μmol/L) were further coimmunoprecipitated with anti–c-Myb antibody, and ubiquitinated c-Myb bands were visualized by anti-ubiquitin and c-Myb antibodies, respectively. EBs null for c-myb were used as a negative control (D). Expression levels of c-Myb were rapidly and transiently decreased on day 3 of differentiation. This finding was not associated with decreased levels of c-myb mRNA and was prevented by inhibition of the proteosome (n=3, *P<0.01, ***P<0.001 versus day 2).

**c-Myb Expression Is Differentially Regulated in Specific Progenitor Populations**

To explore which specific cell types within EBs express c-Myb, we cultured naive day-2 EBs in serum-free cardiovascular-directed differentiation medium containing VEGF:Act A: BMP4 (5:4:1 ng/mL) and isolated specific cardiovascular progenitor populations on day 3.5, using flow cytometry for the cell surface markers VEGFR2 and PDGFrα (Figure 2A). FACS-sorted populations (>98% purity) subjected to quantitative (q)RT-PCR (Figure 2B and Online Figure II, B) and Western blot (Figure 2C and Online Figure II, C) analyses revealed that VEGFR2+/PDGFrα− (V+/P−) and VEGFR2+/PDGFrα+ (V+/P+) cells express c-Myb, whereas VEGFR2−/PDGFrα− (V−/P−) and VEGFR2−/PDGFrα+ (V−/P+) cells do not express c-Myb.
c-Myb Levels Regulate the Early Appearance of VEGFR2+ Progenitors

We began by examining the relative abundance of specific progenitor populations in EBs derived from WT versus c-myb KO ESC cultured in serum-free conditions with VEGF (5 ng/mL) but varying combinations of Act A and BMP4 to explore whether WT and KO ESC differ in their responses to these agents (Figure 3A). At all combinations, including the 4:1 ratio of Act A:BMP4, FACS analyses (n=3) revealed a significant defect in the ability of c-myb KO EBs to form V+/P− cells (P<0.01), with fewer KO-derived cells becoming V+/P+ and more remaining negative for both markers (V−/P−; P<0.05 for both comparisons) as compared with WT (Figure 3B). Kinetic FACS analyses on WT and KO EBs were also performed in spontaneous (serum-conditioned) differentiation media. Initial experiments excluded the possibility that the appearance of VEGFR2+ progenitors was delayed in c-myb KO EBs, confirming a true defect in the ability of these EBs to express VEGFR2 by day 4 (Online Table IV). We next analyzed even later stages of differentiation in WT and KO EBs, culturing them in identical serum-free but VEGF:Act A:BMP4-stimulated (5:4:1 ng/mL) conditions for an additional 2 days (days 4−6). Interestingly, under these nonphysiological conditions, KO EBs suddenly began to express large numbers of V+/P− and V+/P+ cells on day 5 (Online Figure III). By contrast, WT EBs under identical conditions had no such shift in FACS profiles and instead began to show visible expression of hemoglobin (red cells) by day 6, marking the onset of erythropoiesis, which KO EBs never manifest. Furthermore, whereas the V+/P+ cells that emerged late from c-myb KO EBs could differentiate into beating cardiomyocytes, the V+/P− progenitors that appeared at these later stages (days 5−6) could neither proliferate nor differentiate like their WT counterparts (data not shown). These results show that whereas c-myb KO progenitors are capable of somehow achieving late onset c-Myb-independent VEGFR2 expression, a V+/P− progenitor that lacks c-myb is essentially incapable of SMC differentiation.

Next, we manipulated c-Myb levels in WT ESC using lentiviruses encoding a doxycycline (Dox)-responsive transcriptional activator (tTA-ON), an EGFP reporter, and a tTA-dependent shRNA against c-myb or a tTA-responsive scrambled (SCR) control.21 As shown in Figure 4A and 4B, doxycycline (5 µg/mL) reduced levels of c-Myb expression by ~60% in shRNA-transduced (EGFP+) ESCs, having no effect on ESCs transduced with SCR. After sorting twice for EGFP+ expression (yielding ~99% purity), we exposed transduced ESCs to serum-free cardiovascular-differentiation media (VEGF:Act A:BMP 5:4:1 ng/mL) and performed FACS analyses for VEGFR2 and PDGFRα. Reduction of c-Myb expression by turning shRNA “ON” resulted in significant decreases in the proportion of V+/P− and V+/P+ progenitors as compared with shRNA OFF, SCR ON, and SCR OFF (Figure 4C). These data closely mirrored
those obtained with early-stage c-myb KO ESC, including the observed increases in V−/P+ and V−/P− cell populations produced by knocking down c-Myb (Figure 4C).

We then examined the effects of c-Myb “rescue” in early-stage c-myb−KO ESCs and of preventing c-Myb degradation in day-3 EBs derived from WT ESC. Using a doxycycline-regulated (tTA-OFF) system enabling c-Myb expression in KO ESCs,18 we observed a c-Myb–dependent increase in the appearance of V−/P− cells (Figure 5). Of interest, mild leakiness in this system appeared to result in some c-Myb expression even in the presence of Dox,18 with greater formation of V−/P− cells than that observed in untransfected early-stage c-myb KO cells (Figure 5C: 7.5% versus Figure 3B: 0.60% ± 0.04%, P < 0.004). Similarly, preventing the degradation of c-Myb in day-3 EBs with the proteasome inhibitor MG13218 also resulted in a significant increase in the proportion of V+/P− cells as compared with DMSO-treated controls (Figure 6). Taken together, these data show that regulated increases in c-Myb expression (immediately after proteosomal degradation of c-Myb) promote the generation of V−/P− progenitors from undifferentiated ESCs.

Regulated Binding of c-Myb to the VEGFR2 Promoter
To explore a possible mechanism underlying c-Myb–dependent expansion of the V+/P− progenitor population within early-stage EBs, we conducted an in silico analysis for
putative c-Myb–binding sites in the putative promoter regions of both VEGFR2 and PDGFRα, using MatInspector (Figure 7A). Although the VEGFR2 promoter was predicted to harbor 4 consensus c-Myb binding sites highly conserved between mouse and human, the PDGFRα promoter was not predicted to harbor any (data not shown). We next performed chromatin immunoprecipitation (ChIP) experiments with an anti–c-Myb antibody and sheared chromatin nuclear extracts from day-2 and day-3.75 EBs, a mouse vascular SMC (MOVAS) known to regulate c-Myb in a cell cycle–dependent manner,23 and a mouse endothelial cell line (EOMA) shown to express VEGFR2 but not c-Myb (Figure 7C). In extracts from day-2 EBs, minimal binding of c-Myb was observed on 1 of the 4 putative binding sites in the VEGFR2 promoter (Myb binding site [MBS]1; Figure 7B). By contrast, significantly greater binding of c-Myb was observed on 2 of the 4 putative binding sites in day-3.75 EBs (MBS1, MBS4; Figure 7B). ChIP on extracts from G1/S cell cycle–synchronized MOVAS cells showed significant binding of c-Myb to all 4 putative binding sites on the VEGFR2 promoter (Figure 7B), with corresponding increases in VEGFR2 expression (Figure 7C). As a negative control, anti–c-Myb ChIP on extracts from EOMA cells known to express high levels of VEGFR2 (but no detectable levels of c-myb) (Figure 7C) revealed no binding of c-Myb to the VEGFR2 promoter (Figure 7B). Of note, expression of both c-myb and VEGFR2 mRNAs were also examined in day-0 to day-4 EBs. These data confirmed differentiation-dependent increases in endogenous VEGFR2 (Figure 7C). We next examined the functional importance of MatInspector-predicted and ChIP-confirmed MBS in the VEGFR2 promoter with WT and point-mutated promoter-reporter assays in HEK293 cells cotransfected with c-Myb. These experiments revealed that c-Myb–dependent VEGFR2 promoter activity was critically dependent on MBS1 (Figure 7D). Importantly, no regulation of the VEGFR2 promoter was seen in HEK293 cells not transfected with c-Myb (data not shown). Together, these results suggest that the reexpression of c-Myb protein after day 3 of EB differentiation may mediate expansion of the V+P− cell population through direct transactivation of VEGFR2 expression by c-Myb.

VEGFR2+ Progenitors Can Be Differentiated Into Vascular SMCs

On the basis of our earlier finding that c-myb KO ESC were incapable of generating contractile SMCs within EBs5 and current data supporting a role for c-Myb in the formation of V+P− cells, we hypothesized that the latter may be a dominant progenitor of contractile SMCs. To test this, day-3.75 EBs were FACS-sorted into 4 distinct populations (V+/P−, V+/P+, V−/P−, V−/P+), further cultured in serum-free medium containing VEGF and basic fibroblast growth factor (5 ng/mL each) to support proliferation, and examine levels of protein expression and cell morphology (confocal immunofluorescence). Only cells derived from V+/P− progenitors expressed early SMC-specific markers at day 7 such as calponin, α-smooth muscle actin (ASMA), and SM22α in the absence of RA (Figure 8B). In the presence of RA, even the 2 PDGFRα+ populations (V+/P+; V−/P+) did not express these markers.
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In the absence of RA, a transient expression of the endothelial marker PECAM-1 was observed at day 7 in all 4 progenitor populations, which could be induced to express these same markers, whereas the V-/P− population could not (Figure 8B). However, by day 14 of RA treatment, only cells derived from V+/P− progenitors expressed high levels of the mature contractile SMC markers h-caldesmon and smoothelin. Similarly, only cells derived from V+/P− progenitors responded to RA by demonstrating reduced expression of the fibroblast marker DDR2 (Figure 8C, C). A significant increase in VEGFR2+/V+/P− cells was caused by c-Myb expression (n=4; **P=0.002) (C).

Finally, RA treatment more easily induced SMC morphology in cultures derived from the V+/P− progenitor than in cells derived from the other 3 populations, as confirmed by immunostaining for calponin and SM22α, ASMA (early SMC markers), and Smoothelin A/B (mature SMC marker) (Figure 8C).

**Discussion**

Previous studies have shown a critical role for c-Myb in bone marrow hematopoiesis. Mucenski et al suggested that c-myb−/− mice die in utero at ~ED15 from severe anemia attributed to a failure to develop adult-type erythropoiesis in the liver.24 Using inducible tissue-specific gene deletion to bypass embryonic lethality, Liue and Reddy subsequently confirmed that adult bone marrow lacking c-Myb could not repopulate lethally irradiated recipients, also demonstrating that hematopoietic stem cells (HSC) from c-myb−/− mice could not differentiate into granulocyte, erythroid, monocyte, B-lymphoid, or megakaryocyte lineages.25 Together, these studies leave no doubt that c-Myb is essential for the self-renewal and multilineage differentiation of HSC.

In addition, c-Myb was shown to play an important role in SMC proliferation,23 with inducible tissue-specific overexpression of a dominant-negative c-Myb (Myb-Engrailed) inhibiting SMC proliferation and arterial remodeling after carotid artery injury in vivo.26 Furthermore, Ca2+-dependent G1-to-S phase cell cycle transitions in SMC20 were shown to be facilitated by c-Myb through regulated expression of the inositol 1,4,5-trisphosphate receptor type-1 (IP3R1)27 and plasma membrane Ca2+-ATPase-mediated effects on intracellular Ca2+.19,28 Although a role for c-Myb in the differen-
tiation of contractile SMC and a potential role for this transcription factor in lineage specification between SMC and cardiomyocytes was also suggested by studies of c-myb−/− ESCs, a direct mechanism linking c-Myb with the formation of a (common) SMC (and hematopoietic) progenitor was not previously known.

In the current study, we have shown for the first time that c-Myb levels are tightly regulated in a pinpoint manner at key early stages of SMC progenitor formation through rapid ubiquitination and proteosomal degradation (Figure 1). A parallel process may be operational during embryonic hematopoiesis, as c-myb gene rescue experiments in primary cultures of the aorta-gonad-mesonephros region of c-myb KO embryos showed that retrovirus-mediated expression of c-Myb could only rescue the loss of hematopoietic progenitors during a narrow time window.

Supporting the physiological relevance of our findings, c-myb KO ESCs efficiently competed for participation in cardiogenesis but showed poor (but not zero) contribution to vascular and visceral SMC development in chimeric mice in vivo. The current study suggests that the small numbers of c-Myb protein without changes in c-myc or translational one. Of potential interest, Kanei-Ishii et al showed that overexpression of NLK blocked the ability of c-Myb to keep the myeloblastic cell line M1 in an undifferentiated state, speculating that Wnt-1–dependent downregulation of c-Myb may play other roles in development.

Expression of c-Myb was detected in EBs even before the emergence of blood or cardiovascular progenitors (days 0–2). Although its role during this particular stage of development was not the focus of our study, co-IP experiments in which c-Myb–bound proteins were identified by mass spectroscopy (data not shown) suggest that the transcriptional activity of c-Myb may be suppressed during this stage by its known negative regulator, TIF1β. Of interest, Seki et al have recently shown that phosphorylated TIF1β in day-0 mouse ESCs (and iPSCs) activates expression of Oct3/4, nanog, and c-myc, whereas at days 1–2, dephosphorylated TIF1β regulates those same transcription factors. Further experiments are needed to investigate whether a similar relationship exists between TIF1β and c-Myb. During subsequent mesodermal specification (∼day 3), we observed rapid transient loss of c-Myb protein without changes in c-myb mRNA levels. Our ability to prevent this loss of c-Myb with the proteosome inhibitor MG132 and the 20S/26S proteasome-specific inhibitor lactacystin, as well as our finding of increased poly-ubiquitination of c-Myb, further suggested a protein degradation mechanism rather than a transcriptional or translational one. Of potential interest, Kanie-Ishii et al showed that Wnt-1 signals induce Nemo-like kinase (NLK)-mediated phosphorylation and subsequent ubiquitination-dependent proteosomal degradation of c-Myb. They also showed that overexpression of NLK blocked the activity of c-Myb to keep the myeloblastic cell line M1 in an undifferentiated state, speculating that Wnt-1–dependent downregulation of c-Myb may play other roles in development.

We show that intrinsic c-myb mRNA and c-Myb protein levels distinguish specific FACs-purified cell populations. The highest and lowest levels of c-Myb expression were...
found in VEGFR2+/PDGFRα− (V+/P−) and VEGFR2-/PDGFRα+ (V−/P+) cells, respectively. Although these data alone do not establish cause or effect, our experiments with c-myb−/− ESCs, MG132, and inducible constructs enabling upregulation and downregulation of c-Myb consistently demonstrated that increasing c-Myb expression in a cardiovascular-directed ESC differentiation system has the effect of expanding the proportion of V+/P− cells. Indeed, the ability

Figure 8. Retinoic acid induced earliest expression of SMC markers in VEGFR2+ progenitors. The 4 VEGFR2/PDGFRα sorted populations from day 3.75 EBs grown in cardiovascular-directed serum-free medium were plated as monolayer cultures on gelatin-coated 96-well plates. Defined serum-free medium was changed every 2 days, and cultures were expanded to gelatin-coated 24-well plates. During differentiation, cells were cultured in the presence or absence of RA (10nmol/L) and harvested on days 7 and 14 for qRT-PCR or immunostaining. Expression levels of α-SMA (Acta2), calponin (Cnn1), SM22α (Tgln), smoothelin A/B (Smtn), and caldesmon (Cald1) were quantified by qRT-PCR on day 3.75 (after FACS but before differentiation) and day 7 with or without RA (±RA) (B) and day 14 with RA (C). Identically prepared cultures were also plated on gelatin-coated cover slips for immunostaining. On day 14, cells were fixed, permeabilized, and stained with Cnn1 (red), smoothelin A/B (green), SM22α (red), α-SMA (ASMA) (green), and DAPI (blue). Magnification bars=20 μm (D) (*P<0.05; **P<0.01; ***P<0.001; versus day 3.75).
of higher concentrations of BMP4 to increase the abundance of V+/P− cells observed by us and others may be particularly dependent on c-Myb, as the inability of c-myb−/− EBs to generate this population (Figure 3) was rescued by lentiviral constructs with both leaky basal and inducible levels of c-Myb (Figure 5). As the importance of BMP4 in hematopoietic commitment of ESC has been shown to involve an interplay between VEGF, TGFβ/1, and Act A, it is tempting to speculate that c-Myb may lie at the nexus of these observations.

We also define a temporally regulated pattern of binding between c-Myb and the VEGFR2 promoter during EB differentiation, with only 1 c-Myb-binding site (MBS1) weakly bound at day 2 and 2 sites (MBS1, MBS4) better bound at day 3.75 (Figure 7B). By contrast, c-Myb strongly bound all predicted sites on the VEGFR2 promoter in mature mouse aortic VSMCs (Figure 7B). We next used promoter-reporter assays to establish that point mutations in MBS1 (ΔMBS1) reduced c-Myb–dependent VEGFR2 promoter activity to a greater extent than point mutations in MBS4 (ΔMBS4) (Figure 7D). These data clearly implicate MBS1 in the transcriptional activation of VEGFR2 that occurs in EB between days 2 and 3.75.

Such findings, together with our FACS results in c-myb−/− EBs (Figure 3A), suggest that c-Myb regulates VEGFR2 gene expression in a context-dependent manner. c-Myb exclusively influenced the appearance of the V+/P− population, having little effect on the formation of V+/P+ cells, revealing that c-Myb is not required for VEGFR2 expression in cells expressing PDGFRα. Of note, our study has not excluded the possibility that c-Myb may influence expression of PDGFRα. Indeed, the effects of modulating c-Myb during the earliest stages of EB differentiation (Figure 3 through Figure 6) could also be interpreted to include c-Myb-responsive repression of PDGFRα. Because MatInspector (Professional Version 8.04) did not reveal conserved c-Myb–binding sites in the putative mouse and human PDGFRα promoters, we did not explore this possibility further.

Having previously shown that the contribution of c-myb−/− ESC to SMC formation in chimeric mouse embryos and adults was significantly diminished as compared with control WT ESCs, the current report defines a mechanism underlying this observation. We show that c-Myb levels regulate the formation of a specific progenitor capable of developing into an SMC that expresses mature contractile markers. Although mice lacking c-myb show no defect in early blood and blood vessel formation in the yolk sac, this only argues against an absolute necessity for c-Myb in VEGFR2 expression as it pertains to blood island formation in the yolk sac and does not refute our observation that c-Myb is involved in SMC differentiation from VEGFR2+ progenitors. Indeed, support for our conclusion can be distilled from other studies. c-Myb has been implicated in the expression of SMA in rat hepatic stellate cells and in TGFβ1-stimulated proliferation and collagen synthesis in neural crest–derived SMC. Furthermore, whereas VEGFR2+ cells derived from ESC were shown to differentiate into endothelial cells and “mural” cells, VEGFR2−/− ESC were incapable of forming functional endothelial cells or SMA-expressing SMC in EBs. Finally, HSC from c-myb−/− embryos were severely limited in their ability to form vascular networks in a para-aortic, splanchnopleural explant model in vitro. Finally, of particular novelty, this study identifies the V+/P− progenitor to be uniquely responsive to RA in the expression of early and late (mature) SMC markers. Although RA, acting through RA-related orphan receptors, was known to promote the differentiation of ESCs to neurons, and SMCs, the mechanisms by which retinoid signaling pathways contribute to lineage specification were not well known. Recently, Huang et al demonstrated that RA-induced NFκb signaling may underlie the expression of miR-10a, a critical HDAC4 regulator that participates in SMC differentiation from ESC. These authors did not parse (by FACS) which progenitor population within their culture was the source of resulting SMC. Our data suggest that the V+/P− progenitor may be a cell type in which the miR-10a pathway is involved, and how this intersects with c-myb and VEGFR2 should be the focus of future studies. Of note, the addition of RA in our system inhibited expression of the fibroblast maker Ddr2 only in the V+/P− population, having no such effect on the other 3 progenitors (V+/P+, V−/P+, and V−/P−). It is also worth noting that only the V+/P− progenitor is capable of expressing SMC-specific genes even in the absence of RA and that whereas the addition of RA could induce the expression of SMC markers in other progenitors (V−/P+, V−/P−, and V+/P+), only the V+/P− progenitor responded more fully to RA with loss of Ddr2. This key finding suggests that whereas cells other than V+/P− are also capable of expressing SMC markers (in the presence of RA), they may be forming a myofibroblast lineage rather than the clearly distinct SMCs that emerge from V+/P− progenitors. Once again, it is tempting to speculate that a specific relationship may exist between c-Myb, VEGFR2, and retinoid signaling pathways with respect to DDR2.

In conclusion, this study demonstrates that c-Myb expression is rapidly regulated by proteasomal degradation during the earliest stages of EB development (days 2–3.75), with its reexpression marking specific progenitor types, and required to transcriptionally activate the VEGFR2 promoter. This leads to the early formation of a VEGFR2+/PDGFRα− progenitor that subsequently yields DDR2-negative SMCs. This study provides direct evidence supporting the notion that progenitors required for hematopoieses can also contribute to SMC formation.

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Disclosures

None.
References


Novelty and Significance

What Is Known?

- The transcription factor c-Myb regulates self renewal and lineage specification of adult hematopoietic stem cells.
- c-Myb regulates cell cycle–dependent intracellular Ca\(^{2+}\) concentrations and proliferation of vascular smooth muscle cells (SMC) and therapies targeting c-Myb limit neointima formation after carotid artery injury.
- Embryonic stem cells lacking c-Myb fail to form contractile SMCs in embryoid bodies (EBs) and have limited ability to contribute to the SMC compartments of chimeric embryos and adults.

What New Information Does This Article Contribute?

- c-Myb undergoes ubiquitination and proteosomal degradation at a stage of EB differentiation that precedes the specification of cardiovascular lineages and distinguishes distinct cardiovascular progenitor populations thereafter.
- The binding of c-Myb to specific binding sites on the VEGFR2 promoter is temporally regulated during this process and required for transactivation of VEGFR2 expression.

- c-Myb expression levels regulate the early appearance of a VEGFR2+ progenitor uniquely capable of retinoic acid-sensitive differentiation into SMCs expressing mature contractile markers.

The molecular mechanisms underlying SMC differentiation and maturation are incompletely understood, despite the importance of these steps to both normal vascular development in the embryo and vascular pathology in the adult. We demonstrate the role played by c-Myb, a transcription factor already known to regulate self-renewal and lineage specification in hematopoietic stem cells, during the earliest stages of mouse ESC differentiation and cardiovascular lineage specification. We show that manipulating c-Myb levels can profoundly affect the abundance of VEGFR2+/PDGFR\(\alpha\) /DDR2-negative SMC progenitors that are uniquely sensitive to retinoic acid–directed, DDR2-negative SMC maturation. This study describes mechanisms that may be exploited to direct the regenerative potential of ESC, with specific reference to SMC, cardiomyocytes, and fibroblasts.
Regulated Expression and Role of c-Myb in the Cardiovascular-Directed Differentiation of Mouse Embryonic Stem Cells
Masayoshi Ishida, Omar El-Mounayri, Steven Kattman, Peter Zandstra, Hiroshi Sakamoto, Minetaro Ogawa, Gordon Keller and Mansoor Husain

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Supplementary Materials & Methods:

ESC culture. ESCs were cultured in DMEM, 10% FBS (Hyclone), 1 mM sodium pyruvate, 2 mM L-glutamine, 0.1 mM non-essential amino acids, 1% penicillin/streptomycin (Invitrogen), 0.1 mM 2-mercaptoethanol (Sigma) and recombinant leukemia inhibitory factor (Samuel Lunenfeld Research Institute, Toronto) on gelatin-coated dishes without feeder cells.

ESC differentiation protocols. Spontaneous differentiation of EB: ESCs were cultured in serum conditioned differentiation medium composed of IMDM (Gibco Invitrogen), 15% FBS (Hyclone), 5 μg/ml ascorbic acid (Sigma), 50 μg/ml transferrin (Roche), 4.5x10^4 M monothioglycerol (Sigma), and antibiotics (Invitrogen). ESCs were trypsinized, cells were counted, and cultured in a 10 cm bacterial grade culture dish at a density of 7.5x10^4 cells/ml in suspension to allow for EBs formation. To examine c-Myb expression by Western Blot and qRT-PCR, mouse EBs were harvested daily for 6 d or more frequently on d0, d2, d2.75, d3 and d3.25.

Serum-free differentiation of progenitors: ESCs were allowed to differentiate in serum-free differentiation (SFD) medium as previously described. On d2, EBs were trypsinized, cells were counted, and allowed to aggregate in the presence of human (h) Activin A (4ng/ml), hBMP4 (1ng/ml), and hVEGF (5ng/ml) (all from R&D Systems) for an additional 1-2 d. ESCs were treated with proteasome inhibitor MG132 (0.1μM; Sigma), or clasto-lactacystin β-Lactone (10μM, lactacystin, Calbiochem) between d2.5 and 3.75. Transgenic mouse ESCs with tetracycline-inducible c-myb, c-myb shRNA or SCR control were treated with doxycycline (1μg/ml) and effects on VEGFR2 and PDGFRα expression were examined by FACS.

Serum-free differentiation of progenitors into SMC: EBs at d3.75 were trypsinized and sorted for VEGFR2 and PDGFRα by FACS into distinct progenitor populations. These cells were cultured at 3x10^4 cells/well or 1x10^4 cells/well on gelatin-coated 24 or 96-well plates respectively in SFD medium supplemented with hVEGF and hbFGF (each 5ng/ml; R&D Systems) with or without all-trans-retinoic acid (RA, 10nM; Sigma). Media were changed every 2-3 d. RNA was extracted at d3.75 (post-FACS), d7 and d14 for qRT-PCR. For immunostaining, sorted progenitors were grown as above on gelatin-coated coverslips for 14 d.

FACS. EBs were trypsinized and allowed to incubate with allophycocyanin (APC)-conjugated anti-mouse VEGFR2 and phycoerythrin (PE)-conjugated anti-mouse PDGFRα antibodies (eBioscience) at 4°C for 30 min. Cells were washed twice and FACS was performed by ARIA-RITT and LSR-SC (Becton Dickinson). Data were analyzed on FlowJo software (Ashland).

Western blot. EBs were lysed in a mild buffer of 20 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1 mM EDTA, 0.5% NP-40, 10% glycerol supplemented with protease inhibitor cocktail (Roche), briefly sonicated, and centrifuged at 10^4 g for 10 min to obtain cleared cell lysates. These were run on 4-12% Tris-Glycine NuPAGE gels (Invitrogen) and transferred onto PVDF (Perkin-Elmer). Membranes were blocked with 5% skimmed milk in PBST at room temperature for 2 h and incubated with rabbit anti-c-Myb (M19, sc-516) or anti-GAPDH (sc-25778) antibodies (Santa Cruz Biotechnology) at 4°C overnight. Membranes were washed 3 X with TBST and incubated with HRP-conjugated anti-rabbit IgG (Pierce Biotechnology) in 5% skimmed milk in PBST. Enhanced chemiluminescence (Perkin-Elmer) was used to visualize the bands.

Ubquitination assay. Cell lysates (500μg each) at d2, d2.5, d3, and d3 after 16h treatment with MG132, were immunoprecipitated (IPed) overnight at 4°C with anti-c-Myb antibody (1μg each) and pulled down with protein G magnetic beads (Dynal, Invitrogen). IPed beads were eluted with...
2X LDS sample buffer (Invitrogen) at 80°C for 10 min followed by Western blot for ubiquitin (anti-Ub Ab, Rockland) and c-Myb to reveal poly-ubiquitylated c-Myb.

**RNA extraction and cDNA synthesis.** RNA was extracted from EBs or sorted cell populations and MOVAS/EOMA by PicoPure RNA Isolation Kit (Arcturus, ABI Biosystems). DNase I (Fermentas) treatment was performed within the purification column at room temperature for 30 min. Total RNA was then reversed-transcribed using SuperScript III First-Strand Synthesis System (Invitrogen).

**Inducible shRNA.** GFP+ cells were sorted at least twice using ARIA-RITT to reach >98% purity for every experiment to omit cells with transgene silencing. Stably-transduced ESC lines were treated with different concentrations of doxycycline (Sigma) and tested with Western blot for c-Myb to establish functionality of the system. These ESC lines were then cultured and differentiated in the presence or absence of doxycycline.

**In silico analyses.** Putative mouse VEGFR2 (flk-1) and human VEGFR2 (KDR, flk-1) promoter regions (upstream and downstream from 5'-UTR, total 2 kb) were retrieved from Ensemble Genome Browser (http://www.ensembl.org/), and analyzed for putative c-Myb consensus binding sites (MBS) with MatInspector (Genomatix, Germany).

**ChIP.** EBs on d2 and d3.75 were fixed with 1% formaldehyde at room temperature for 15 min. Fixation was stopped by 125 mM glycine. Fixed cells were lysed and sonicated to shear chromatin into 200-1000 bp fragments, which were then immunoprecipitated overnight at 4°C with rabbit anti-c-Myb antibody (3 μg, SC-517X, Santa Cruz Biotechnology) or control rabbit IgG (3 μg, Santa Cruz Biotechnology), and pulled down with protein G-conjugated magnetic beads (Dynal, Invitrogen). Immunoprecipitated chromatin was reverse-crosslinked, treated with RNase-A and proteinase-K, and further purified with PureLink PCR Micro Kit (Invitrogen). PCR reactions were performed with Taq polymerase (Sigma) using GeneAmp PCR System 9700 (Applied Biosystems) with the following conditions: 94°C 3 min, 94°C 30 sec, 57°C 30 sec, 72°C 30 sec for 35 cycles, and 72°C 5 min. Primers for each putative c-Myb binding site in the VEGFR2 promoter (MBS: 1–4) and non-MBS region (upstream from MBS1, see Fig. 7A) are provided in Supplemental Table II. All buffers were purchased from ChIP-IT Express Kit (Active Motif) and ChIP was performed by manufacturer’s protocol. MOVAS and EOMA cell lines were used as positive and negative controls, respectively.

**Constructs. Mouse c-Myb overexpression vector:** Mouse p75 c-myb cDNA (a gift from Dr. Kheul) with IRES-hygroycin resistant gene was subcloned into pCAGS. VEGFR2 promoter-LUC reporter constructs: The mouse VEGFR2 promoter region (-1000: +980; total 1980 bp) was amplified from mouse genomic DNA by PCR using high fidelity Phusion Hot Start polymerase (Fynzyme). The PCR product was then cloned into EcoRI and NcoI sites of a promoter-less pGL3 basic vector (Promega), and sequence was confirmed. Site-directed mutagenesis: To delete the VEGFR2 start codon (ATG) and perform further mutagenesis of MBS1 (ΔMBS1: AACCG to gggaG), MBS4 (ΔMBS4: CTGTT to aacTT) and MBS1&4 (combination of ΔMBS1 and ΔMBS4), site-directed mutagenesis was performed using primers shown in Supplemental Table III and Phusion Hot Start polymerase (Fynzyme) according to manufacture’s protocol. Parental wild-type template vectors were eliminated by digestion with DpnI (Fermentas) before transformation. After mutagenesis, each clone was confirmed by DNA sequencing.

**Reporter assay.** HEK293 cells were plated into 24-well plates and co-transfected with c-Myb overexpression or empty vectors (1.0 μg/well) as well as wild-type or mutant VEGFR2 promoter-
reporter constructs (0.5μg/well) and pRL-TK (0.02μg/well, Promega) to control for transfection efficiency by jetPRIME (Polyplus-transfection reagent) as per the manufacturer. After 24-48 h, cells were lysed in 1X Passive Lysis buffer provided by Dual-Glo Luciferase Assay System (Promega). At least 3 independent transfections were done in duplicate, and luciferase activities were measured by a luminometer. Firefly/renilla ratios were normalized as described (http://www.promega.com/resources/articles/pubhub/cellnotes/normalizing-genetic-reporter-assays/).

**Immunostaining.** Cells grown on gelatin-coated coverslips were rinsed with PBS, fixed with 4% paraformaldehyde in PBS for 30 min, permeabilized with 0.2% Triton-X for 10 min, and blocked with 3% FBS/0.2% Triton-X/PBS for 2 h at room temperature. Cells were incubated with rabbit anti-transgelin (**SM22α**, Protein Tech), calponin (Cnn1, ab46794, Abcam), and mouse anti-αSMA (Acta2, clone 1A4, Sigma), and smoothelin A/B (Smtn, clone C6G, Hycult biotech) antibodies overnight at 4°C. Cells were then washed 3 X with PBS and incubated with Alexa594-anti-rabbit IgG (Molecular Probe, Invitrogen) and Cy2-anti-mouse IgG (Jackson Laboratory) in 3% FBS in PBS for 1 h. Cover slips were washed 3 X with PBS and mounted with Anti-fade Reagent containing DAPI (Molecular Probe, Invitrogen). Staining was analyzed on a FV1000 confocal microscope (Olympus Canada).

**Supplemental Table I.** Primers used in qRT-PCR analyses.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primers (5’ to 3’)</th>
<th>Product (bp)</th>
</tr>
</thead>
</table>
| Acta2 | Forward: CGACATGGGAAAAAGATCTGGCA  
Reverse: CCATCTCCAGAGAGTCAGCCAC | 243          |
|       | Cnn1                                                                               | 234          |
|       | Forward: AGCAGGAGCTGAGAGAGTG  
Reverse: TGGCCCTAAAGATGTCGTG | 234          |
| SM22α | Forward: GACTGACATGTTCAGACTGTTGAC  
Reverse: CAGTTGGGATCTCCACGGTAGT | 129          |
|       | Cald1                                                                               | 101          |
|       | Forward: ATGGTAGAGGAGAAAACACCAGA  
Reverse: CCATCCCCCTTCTATTTTGAGACTC | 192          |
| Smtn A/B | Forward: GACCACACTGCTTCCAGGAGTG  
Reverse: GGCACCTTACCAGGAGTGCAAA | 92           |
|       | Ddr2                                                                               | 97           |
|       | Forward: GTTTTGAGGAGGCTCATCCTGTG  
Reverse: CACCAAGCAGGCTGGTG | 195          |
|       | c-myb                                                                               | 195          |
|       | Forward: GCTGAAGAAGCTTGGAGGAC  
Reverse: CAACGCTTCCGGACCATAATT | 257^         |
| Vegfr2 | Forward: GGTTCTCTTCAAGTGGCAGGATTTA  
Reverse: AGCACACAGGCAGAAAACCAGTGA | 103          |
|       | Gapdh                                                                              | 224          |
|       | Forward: ACTGTGGATGGGCCCTCTGG  
Reverse: TGACCCTTGCCACAGCCTTG | 113          |
| Gapdh | Forward: AACTTTGGCATTTGGGAAGG  
Reverse: CACATTGGGGGTAGGAACAC | 257          |

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Supplemental Table II. Primers used in c-Myb-ChIP-PCR analyses.

<table>
<thead>
<tr>
<th>Seq Name</th>
<th>Seq 5' to 3'</th>
<th>Product (bp)</th>
</tr>
</thead>
</table>
| MBS1     | Forward: GCCAACGCCTCTACTGTTTT  
          | Reverse: CTAGCAGCCACTGGGAGAGG  | 209 |
| MBS2     | Forward: ATGGGCTCTGAGACACTTGC  
          | Reverse: GGCCAAAGCACCATAAAAACA | 253 |
| MBS3     | Forward: TCCACTCCTGCCTACCTAGC  
          | Reverse: GCCGAGAGTGGGAAATAGAAG | 207 |
| MBS4     | Forward: TGAGGGAGGAAGGTGTGCT  
          | Reverse: GCTAGGTAGGCAGGAGGTGGA | 257 |
| Non-MBS (Control) | Forward: TTGGCTGAAAGGTGCTCT | 180 |
|          | Reverse: CCCATAGCAAGGAGATGCAG  |    |

Supplemental Table III. Forward primers used in site-directed mutagenesis. Small capitals were post-mutagenesis nucleotides and underlined are c-Myb consensus binding sites.

<table>
<thead>
<tr>
<th>Forward primer</th>
<th>Seq 5' to 3'</th>
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<tbody>
<tr>
<td>VEGFR2-DATG</td>
<td>GAGGTGCAGGaaaGAGAGCAAGG</td>
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<tr>
<td>∆MBS1</td>
<td>CAGGAGTTCCACggaGAAAATGCTTCC</td>
</tr>
<tr>
<td>∆MBS4</td>
<td>CACTCCGTGaanTTGCCACCACG</td>
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Supplemental Results:

Supplemental Table IV. FACS-defined proportion (% of 10,000 total cells) of serum-conditioned WT- and c-myb KO-EBs that express VEGFR2 at specific time points of differentiation.

<table>
<thead>
<tr>
<th>Day</th>
<th>WT</th>
<th>CV(%)</th>
<th>KO</th>
<th>CV(%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>8.82</td>
<td>33.05</td>
<td>2.13</td>
<td>69.99</td>
</tr>
<tr>
<td>3.125</td>
<td>14.47</td>
<td>20.14</td>
<td>5.52</td>
<td>27.01</td>
</tr>
<tr>
<td>3.25</td>
<td>10.75</td>
<td>27.11</td>
<td>5.46</td>
<td>27.30</td>
</tr>
<tr>
<td>3.5</td>
<td>10.47</td>
<td>27.84</td>
<td>4.29</td>
<td>34.75</td>
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<tr>
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<td>66.26</td>
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<tr>
<td>4</td>
<td>6.36</td>
<td>45.83</td>
<td>3.68</td>
<td>40.51</td>
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</tbody>
</table>

Supplemental Figure Legends:

Supplemental Figure I. Expression levels of c-Myb in early stage EBs are tightly regulated by protein degradation. ESCs were trypsinized and suspension-cultured in serum-conditioned (15% FBS) medium in the absence of LIF to form EBs. These were harvested daily to examine c-Myb expression levels by Western blot (A, N=3 independent experiments) and qRT-PCR (B, N=3 independent experiments). MOVAS (M), an immortalized mouse vascular SMC line, and K562 (K),
a human erythroleukemia cell line, were used as positive controls for c-Myb expression. (*P<0.01, **P<0.005 vs. d0)

Supplemental Figure II. c-Myb expression levels remain very low throughout subsequent cardiomyocyte differentiation. Day 3.25 EBs induced by ActA/BMP4/VEGF underwent FACS for expression of VEGFR2 and PDGFRα. V+P+ cells were plated into cardiac-directed differentiation medium (containing bFGF, DKK, and VEGF as described4) for 4 additional days until spontaneous cardiac-like beating was observed. Cells were harvested on indicated days (A) for qRT-PCR (B) and Western blot (C).

Supplemental Figure III. c-Myb-independent VEGFR2 expression at late stages. Wild-type (WT) ESCs and those null for c-myb (KO) were allowed to form EBs. At d2, EBs were differentiated in serum-free cardiovascular-directed medium containing VEGF (5 ng/ml), Activin A (4.0 ng/mL) and BMP4 (1.0 ng/mL). On d4-6, EBs were trypsinized, stained for VEGFR2 and PDGFRα, and analyzed by FACS. Representative FACS profiles (N=3 independent experiments) are shown.

Supplemental References:

A

Days of Differentiation

<table>
<thead>
<tr>
<th>Days of Differentiation</th>
<th>K</th>
<th>M</th>
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<tbody>
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<td></td>
</tr>
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<td>K562</td>
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<tr>
<td>MOVAS</td>
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</tbody>
</table>

Supplemental Figure I

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A

Supplemental Figure II

B

c-myb/gapdh ratio

C

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Supplemental Figure III

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