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Generation of a *Cre* Knock-in into the *Myocardin* locus to mark early cardiac and smooth muscle cell lineages

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Abstract

The molecular events that control cell fate determination in cardiac and smooth muscle lineages remain elusive. *Myocardin* is an important transcription co-factor that regulates cell proliferation, differentiation and development of the cardiovascular system. Here, we describe the construction and analysis of a dual *Cre* and Enhanced Green Fluorescent Protein (*EGFP*) knock-in mouse line in the *Myocardin* locus (*Myocd^{KI}*). We report that the *Myocd^{KI}* allele expresses the *Cre* enzyme and the *EGFP* in a manner that recapitulates endogenous *Myocardin* expression patterns. We show that *Myocardin* expression marks the earliest cardiac and smooth muscle lineages. Furthermore, this genetic model allows for the identification of a cardiac cell population which maintains both *Myocardin* and *Isl-1* expression, in E7.75 - E8.0 embryos, highlighting the contributions and merge of the first and second heart fields during cardiogenesis. Therefore, the *Myocd^{KI}* allele is a unique tool for studying cardiovascular development and lineage-specific gene manipulation.

Keywords

Myocardin; *Cre*; *EGFP*; heart; smooth muscle

The heart is the first functional organ of vertebrate animals, which evolves from a peristaltic linear tube to a complex four chamber pump (Moorman *et al.*, 2003; Virágh and Challice, 1973). As the heart develops the concomitant vasculature tree forms, grows and branches out from large vessels to tiny capillaries directing the blood flow and carrying nutrients and oxygen within (Baldwin, 1996). Vasculature inner pressure is generated by the heartbeat, and its magnitude is greater the closer a vessel is to the heart. Thus, to protect vessels against possible pressure damage, a smooth muscle sheath confers elasticity to aid in vessel stretching and strength to avoid vessel collapse (Owens *et al.*, 2004). Smooth muscle is not only found sheathing vessels, but also around or within organs which expand as a result of positive pressure, such as the trachea, lungs, bladder, lymphatic vessels, uterus, etc (Owens *et al.*, 2004). Interestingly, early cardiac tube gene expression resembles that of smooth muscle, expressing *SM22 α* , and *SM α -actin* genes along with cardiac-specific genes (Li *et al.*, 1996; Owens *et al.*, 2004). However, the molecular mechanisms that control cardiac and

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smooth muscle gene expression and cardiac and smooth muscle lineage specification are not fully understood.

Myocardin was identified through an *in silico* screening of a cardiac specific Expressed Sequenced Tagged (EST)-library (Wang *et al.*, 2001) and was characterized as a Serum Response Factor (SRF) transcription co-factor. Myocardin physically interacts with SRF and stabilizes a multimeric complex in which SRF ultimately binds to at least two SRF response elements located within the promoter/enhancers of many cardiac and smooth muscle genes. Thus, Myocardin expression and activity is necessary to induce or to enhance the expression of cardiac and smooth muscle genes such as Atrial Natriuretic Factor (ANF), Transgelin (Tlgn, SM22 α), smoothelins, etc. (Wang *et al.*, 2001; Wang *et al.*, 2004; Wang *et al.*, 2003). *Myocardin* mRNA expression was revealed to be restricted to cardiac progenitor tissue at early stages of development, while also being expressed in the developing and adult smooth muscle tissues present in different organs. *Myocardin* mRNA expression pattern during embryogenesis suggested its important role during cardiac and smooth muscle development (Wang *et al.*, 2001; Wang *et al.*, 2004; Wang *et al.*, 2003). Indeed, *Myocardin* homozygous mutation results in embryonic lethality at around embryonic day (E)9.5 displaying cardiac malformations and lack of smooth muscle formation (Li *et al.*, 2003). On the other hand, conditional cardiac ablation of *Myocardin* at late stages of development results in a post-natal cardiac physiology imbalance, an increase of fibrotic tissue and an increase of cell death leading to cardiac enlargement (Huang *et al.*, 2009). Conversely, *Myocardin* over-expression in primary human mesenchymal stem cells or human vascular smooth muscle cells results in a reduction of cell proliferation and a forced expression of cardiac and smooth muscle molecular markers (Chen *et al.*, 2011; Tang *et al.*, 2008; van Tuyn *et al.*, 2005; Wang *et al.*, 2003; Wystub *et al.*, 2013). Further, *Myocardin* over-expression induces cardiac hypertrophy in neonatal rat cardiomyocytes as well as in transgenic mice (Wystub *et al.*, 2013; Xing *et al.*, 2006).

As noted above, the mRNA expression of *Myocardin* was revealed to be restricted to cardiac forming tissues from E7.5. Later on, it is also expressed in the smooth muscle cells from E9.5 at the time of vasculature formation during mouse embryogenesis (Wang *et al.*, 2001). *Myocardin* expression is regulated by several transcription factors and signaling regulators in a complex genetic network. More specifically the homeodomain protein Nkx2-5 (Ueyama *et al.*, 2003), the MADS box transcription factor Mef2c, the transcription enhancer factor Tead1, the forkhead box binding protein Foxo4, the nuclear factor of activated T cells Nfatc3, as well as Myocardin itself, have been shown to interact with specific cis-acting elements, present within a 10Kbp promoter/enhancer DNA fragment upstream of the *Myocardin* transcriptional start site, to regulate the transcription of the *Myocardin* gene (Creemers *et al.*, 2006; Liu *et al.*, 2014; Wang *et al.*, 2010). Additionally, the transcription factor Smad3, a transforming growth factor TGF- β signal transducer, has been shown to repress *Myocardin* expression in the early stages of smooth muscle differentiation by sequestering the phosphoinositol-3 (PI3) kinase-activated Nkx2-5 (Xie *et al.*, 2011). Together, these studies suggest that the combination of different factors at a given time results in cardiac and/or smooth muscle specific *Myocardin* expression (Creemers *et al.*, 2006).

In this regard, we decided to knock-in the Cre enzyme and the Enhanced Green Fluorescent Protein (EGFP) reporters into the *Myocardin* locus in order to facilitate the investigation of *Myocardin* expression and function. In this model the first exon of the *Myocardin* coding sequence was replaced with a *Cre-IRES2-EGFP-bGHpA* cassette followed by an *Frt*-flanked *Neomycin* resistance gene (Fig. 1A). The final construct, named *Myocd-KI* was linearized, purified and electroporated into 129SJ1;C57BL/6 hybrid mouse embryonic (ES) stem cells. Targeted ES cells were screened by PCR and Southern blot (Fig. 1B–C). Seven clones were identified to have the correct homologous recombination and one was used for blastocyst injection. Six male chimera mice were obtained, all lead to germline transmission and viable heterozygous mutant offspring.

Analysis of EGFP expression under UV light at different stages of mouse embryonic development shows a recapitulation of *Myocardin* expression pattern. EGFP expression is observed from early stages of development and is restricted to the cardiac crescent at E7.5, and to cardiac tissue throughout embryogenesis in *Myocd^{KI/+}* mice (Fig. 1D, Supplementary Fig. 1). Also, a faint EGFP expression is observed in the forming somites from E8.0 until E11.5; however, this faint expression is no longer visible in embryos beyond E11.5, indicating a transient expression of *Myocardin* in this region (Fig. 1D). Nevertheless, upon dissection, EGFP expression in adult tissues was readily observed in the heart as well as in tissues which contain smooth muscle cells such as the bladder, intestines, veins, arteries and other large blood vessels (Fig. 1E, F). Homozygosity of the *Myocd^{KI}* allele results in embryonic lethality showing cardiac edema, a sign of cardiac dysfunction, and overall developmental delay (Fig. 2A). This phenotype is consistent with what has been reported from an independent *Myocardin* mutant mouse line (Li *et al.*, 2003). Interestingly, cardiac EGFP expression is maintained in *Myocd^{KI/KI}* embryos indicating that the *Myocardin* promoter is still active even during cardiac abnormalities at this developmental stage. Further, RNA was isolated from E9.5 embryonic hearts and used in qPCR assays; results show that *Myocardin* expression is abolished in *Myocd^{KI/KI}* embryos when compared to wild type controls (Fig. 2B). Taken together, these results confirm that our knock-in strategy results in a true *Myocardin* null mutation.

In order to confirm the efficacy of the Cre enzyme present in our genetic model, lineage tracing analysis was performed utilizing the R26R LacZ conditional reporter mouse line. Indeed, LacZ expression recapitulates *Myocardin* expression in double heterozygous embryos (*Myocd^{KI/+}; R26R^{+/+}*) (Fig. 3A–E). In particular, LacZ expression is observed in the developing heart from early stages of development, similar to that of EGFP expression (Fig. 3A). Stronger LacZ staining was detected in the ventricles of the developing heart when compared to that of the atria (Fig. 3B, C). Additionally, LacZ positive signal was observed in the somites and midbrain of E9.5 and E10.5 embryos, indicating the transient expression of *Myocardin* in these tissues (Fig. 3A). At E13.5, LacZ expression is observed to extend to the limbs and at E15.5 LacZ staining appears to be restricted to the dorsal side of embryo (Fig. 3A). However, closer inspection of the internal organs shows LacZ expression in the smooth muscle layers of other organs such as the lung and intestines, in addition to the heart (Figs. 3C–E, Supplementary Fig. 2); and LacZ staining is clearly detected in the smooth muscle layer of bronchioles in the developing lung (Fig. 3E).

In order to confirm the above observations, we utilized another independent conditional reporter mouse line, the Tomato/EGFP (mTmG) in which expression of EGFP (mG) is achieved conditionally by a Cre-induced excision of an upstream *loxP*-flanked actively expressed red fluorescent tdTomato gene (mT). Indeed, the defined expression domains of *Myocardin*, such as cardiac, somite and midbrain were further confirmed in double heterozygote *Myocd*^{KI/+}; *mTmG*^{m/+} mice (Fig. 4A). Additionally, EGFP expression was also observed in skeletal muscle, foot pad, tongue, bladder, lung and other smooth muscle containing tissues (Fig. 4B, C; Supplementary Figure 3). As a result, lineage tracing analyses reveal a larger and broader *Myocardin* expression domain and pattern when compared to earlier in situ hybridization reports (Wang *et al.*, 2001). Together, our results are in accord with other reports obtained with a similarly engineered Cre knock-in mouse line (Long *et al.*, 2007).

The formation of the embryonic heart is a very dynamic process with the involvement of at least two cardiac progenitor cell populations termed first (primary) and second heart fields (Vincent *et al.*, 2010). These cardiac fields, which display distinct molecular signatures, give rise to different cardiac regions of the mature heart (Cai *et al.*, 2003; Lyons *et al.*, 1995; Meilhac *et al.*, 2004; Tanaka *et al.*, 1999). Indeed, the first heart field is molecularly primed by the expression of the homeodomain transcription factor Nkx2-5 that regulates a downstream cascade of molecular signals to warrant the formation of the left ventricle and parts of both atria (Lyons *et al.*, 1995; Tanaka *et al.*, 1999). The second heart field was characterized by the specific and restricted expression of the Lim/homeodomain transcription factor Isl-1 which warrants the formation of the right ventricle, outflow tract and portions of both atria and inflow tract (Cai *et al.*, 2003). Both *Nkx2-5* and *Isl-1* genes are expressed at the earliest stages of cardiac development; however, the expression of *Isl-1* becomes restricted to the dorsal side and to the cranial and caudal poles of the forming tubular heart (Cai *et al.*, 2003; Zhuang *et al.*, 2013). Even though *Nkx2-5* is one of the earliest markers of cardiac lineage, its expression domain is not restricted to the primary heart field as its expression domain expands to almost the entire myocardium at later developmental stages (Espinoza-Lewis *et al.*, 2011; Kasahara *et al.*, 1998).

It has proven challenging to find a cardiac molecular marker that is unique to the first heart field. It was only until very recently that the Hyperpolarization-activated cation-gated channel 4 (*Hcn4*), a specific sinoatrial node and pacemaker molecular marker (Garcia-Frigola *et al.*, 2003; Stieber *et al.*, 2003), was described as a unique first heart field molecular marker (Liang *et al.*, 2013; Später *et al.*, 2013). However, the scarcity of first heart field molecular markers continues. Thus, we asked if *Myocardin* could also be a first heart field marker. We obtained *Myocd*^{KI/+}; *R26R*^{+/+} embryos from E7.5 up to E8.0 and subjected them to X-gal staining, which recapitulates the expression of *Myocardin*, followed by Isl-1 immunostaining. Whole mount staining reveals that LacZ (*Myocardin*) expression and Isl-1 protein expression domains are mostly mutually exclusive (Fig. 5A). Indeed, transversal sections in these embryos show that *Myocardin* and Isl-1 expression domains are complementary; indicating that *Myocardin* is not expressed in the second heart field. However, there is significant expression overlapping at the edges of both genes expression domains (E8.0) (Fig. 5A–C, arrowheads). Regions of overlapping are located in both the primordial outflow and inflow tract. These results indicate that there is a cell population that

still maintains Isl-1 protein expression, and indicate that the switch from cardiac progenitor cell to a differentiated cardiomyocyte is a progressive event and not immediate. Prior reports addressing this issue utilize Hcn4, Isl-1, and MLC2a as markers for cardiac progenitors of the first heart field, second heart field and differentiated cardiomyocytes, respectively. Fluorescent *in situ* hybridizations show that the expression patterns of Hcn4 and Isl-1 do not overlap with that of MLC2a and are clearly discernible indicating a clear cellular phenotype difference (Cai *et al.*, 2003; Liang *et al.*, 2013; Später *et al.*, 2013). Interestingly, our studies suggest that myocardin expression marks first heart field during early embryogenesis. Additionally, *Myocardin* expression highlights a cell population which also transiently expresses Isl-1, indicating a transitional phenotype between progenitor and differentiated cell and the merge of both first and second heart fields.

Taken together, here we describe the generation and characterization of a Myocardin-Cre-EGFP knock-in mouse model as a highly useful biological and genetic tool. This mouse model recapitulates *Myocardin* expression and is a true null allele; additionally, the inclusion of both the *Cre* as well as the *EGFP* genes provides a dual tool for further cell fate and lineage tracing analysis and conditional inactivation of target genes. Gene inactivation and cellular analyses are also possible even at the earliest stages of cardiac development with high accuracy and could be performed in parallel and at the same time due to the duality of the model. Finally, the presence of the EGFP gene in this model makes it useful for possible further screening experimentation to elucidate the complex regulation of cardiac and smooth muscle cell fate specification and differentiation.

Materials and Methods

Generation of the *Myocd*^{KI/+} knock-in mouse line and other mouse strains

A BAC (RP24213H11, BAC library RP24, C57BL/6 Male, Children's Hospital Oakland Research Institute Oakland, CA) containing the *Myocardin* locus was purchased from used as a template to amplify by PCR a long 6.5Kbp upstream fragment and a short 4.7Kbp downstream fragment, relative to the *Myocardin* gene first exon. The fragments were inserted into the TOPO-pCRII vector containing a *Cre-IRES2-EGFP-bGHpA* cassette followed by an *Frt*-flanked *Neomycin* resistant gene and a Thymidine Kinase (*TK*) negative selection gene. The *Cre-IRES2-EGFP-bGHpA* cassette contains the DNA sequences for the Cre enzyme, the Internal Ribosome Entry, the Enhanced Fluorescence Protein and the bovine Growth Hormone polyadenylation signal (a specialized termination sequence present in several cloning vectors including the pcDNA3.1 family of vectors). The Final *Myocd*-KI construct was linearized with *NdeI* restriction endonuclease, purified by Phenol:Chloroform:IAA (25:24:1) and electroporated into 129SJ1;C57BL/6 hybrid mouse stem cells. Stem cells were cultured under G418 conditions. Surviving cell clones were further screened by PCR and southern blot. Seven clones were identified as having the correct genomic homologous recombination and one clone was selected for blastocyst injection. Six male and one female chimera were obtained. All males were fertile and produced *Myocd*^{KI/+} offspring, all displaying expected myocardin expression pattern. One mouse line was kept for further analysis and experimentation. This mouse line is available upon request.

The ROSA26R conditional LacZ (B6.129S4-*Gt(ROSA)26Sor^{tm1Sor}/J*) (hitherto termed R26R) and the ROSA26mT/mG (*Gt(ROSA)26Sor^{tm4}(ACTB-tdTomato,-EGFP)Luo/J*) (hitherto termed mTmG) reporter mouse lines were obtained from The Jackson Laboratory.

X-gal staining

Tissues and mouse embryos at different developmental stages were dissected in PBS, fixed in 4% Paraformaldehyde (PFA) in PBS at various times at room temperature or at 4°C overnight. They were rinsed in X-gal washing buffer (PBS, 2mM magnesium chloride, 0.02% NP-40, 0.01% deoxycholate) 3 times, 15 min each at room temperature or on ice. Specimens were incubated in X-gal staining solution (5–16mM ferricyanide, 5–16mM ferrocyanide, 2mM MgCl₂, 1mg/ml X-gal in PBS) for various times at room temperature or at 4°C overnight.

Immunostaining

Early stage embryos were dissected and rinsed in PBS at room temperature, fixed in 4% PFA for 2h at room temperature or at 4°C overnight. Embryos were rinsed in PBS 3 times, 15 min each in PBS, dehydrated in a series of methanol, post-fixed in a Methanol:DMSO (4:1) solution at 4°C overnight, bleached with a Methanol:DMSO:H₂O₂ (4:1:1) solution for 5h at room temperature, rinsed in methanol and rehydrated to PBS. Embryos were incubated with primary antibody (mouse anti Isl-1, DHSB 39.4D5c, 1:500) and secondary antibody (HRP-conjugated goat anti-mouse IgG2b, 1:200) in PBSMT (PBS, 2% non-fat dried milk, 0.5% triton X-100) for 48h and 24h respectively at 4°C. Rinsing with PBTx (PBS pH 7.2, 2% BSA, 0.5% Triton X-100) was performed 3 times, 1h each at room temperature prior to incubation in DBA (100ng/ml in PBS pH7.2) for 30min at room temperature followed by color reaction solution (DBA, H₂O₂ 1:150). Color reaction was observed under microscope and stopped by the addition of 3 volumes of distilled water. Embryos were finally rinsed in distilled water, fixed in 4%PFA in PBS, and treated for paraffin embedding.

X-gal staining followed by immunostaining

Early stage embryos were dissected in PBS at room temperature, fixed in 4% PFA 15 min on ice, rinsed in PBS containing 2mM MgCl₂ 3 times, 10 min each on ice. Embryos were then incubated in X-gal staining solution (PBS, 2mM MgCl₂, 5mM ferricyanide, 5mM ferrocyanide, 1mg/ml X-gal) 1h at 37°C. When X-gal staining is deemed complete embryos were washed in PBS 3 times, 10min each at room temperature and further fixed in 4% PFA for 2h at room temperature or at 4°C overnight. Immunostaining was performed as described above.

Southern Blot

For Southern blot 20µg of genomic DNA (gDNA) were digested with the respective restriction endonuclease and run in a 0.8% agarose gel overnight. The gel was depurinated (0.25N HCl, 15min at room temperature), denatured (0.5N NaOH, 15min at room temperature), neutralized (0.65M Tris base, 1M NaCl) and rinsed in distilled water. Transfer of gDNA to a nitrocellulose membrane was performed by capillary action in a 10XSSC bath overnight at room temperature. Transferred gDNA was cross linked under UV light in a

cross linker oven. The membrane was prehybridized (20XSSPE, 10% PEG-8000, 7% SDS) at 65°C for 1h. Hybridization was carried with a radioactive probe added to fresh warmed prehybridization solution and incubated overnight at 65°C. Washes were performed at 65°C and film exposure was carried at -80°C for a week.

Radioactive probe labeling

Probes were amplified from genomic DNA by PCR, labeled following manufacturer's instructions (AMERSHAM Rediprime labeling kit II), and purified following manufacturer's instructions (Illustra NICK columns, Sephadex G-50).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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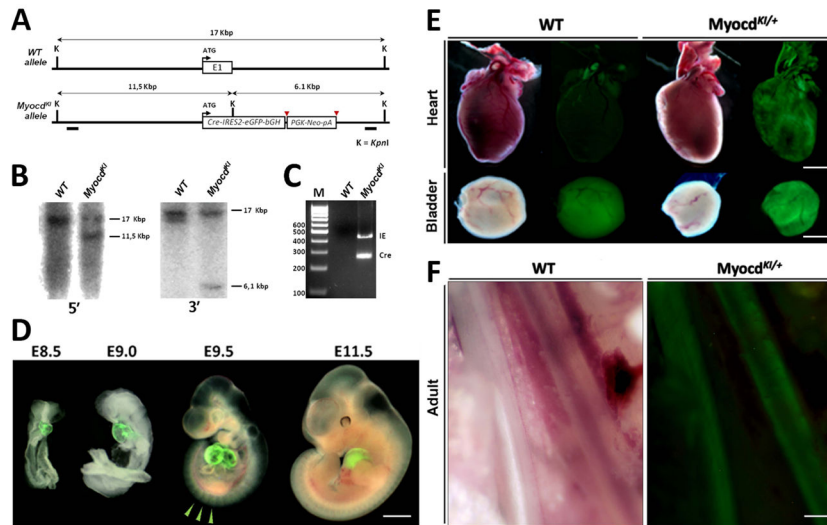


Figure 1. Generation of *Myocardin^{KI}* allele

A) Diagram depicting the genomic structure of *Myocardin* wild type (WT) and mutant (*Myocd^{KI}*) alleles. *Myocardin* mutant allele contains a *Cre-IRES2-EGFP-bGHpA* cassette followed by an *Frt*-flanked positive selection *Neomycin* cassette which replaces the first Exon (E1) of the gene reading frame. B) Southern blots with 5' and 3' probes (black boxes in genomic locus) showing the specificity of the genomic recombination. C) PCR genotyping indicates the presence of the *Cre-IRES2-EGFP-bGHpA* cassette only in DNA obtained from mutant samples. D) Cell fate determination by observation of EGFP expression from early stages of embryonic development. EGFP is detected in developing hearts. EGFP is also observed in somites (arrowheads) (Scale bar, 1mm). E) EGFP expression tissues such as the heart and the bladder. Note the EGFP signal in the bladder of WT mice is an auto-fluorescence signal due to urine content (Scale bar; hearts, 2mm; bladders, 1mm). F) EGFP expression in postnatal day 2 (P2) tissues in tissues containing smooth muscle such as the Esophagus (E) and Dorsal Aorta (DAo) (Scale bar, 100µm).

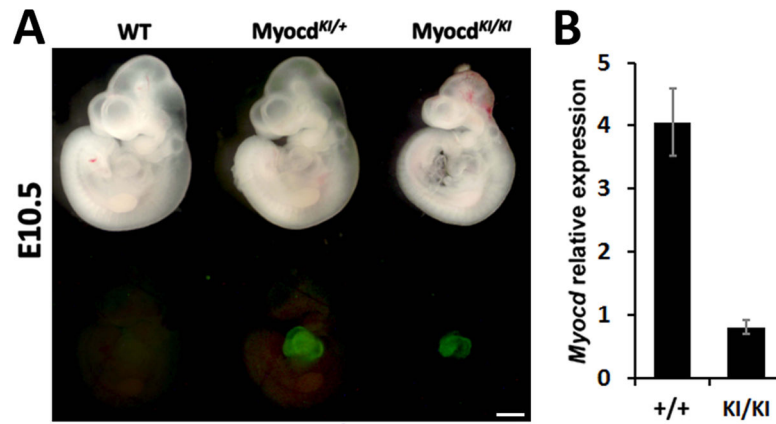


Figure 2. *Myocd*^{KI} allele is a true *Myocd* null allele

A) EGFP expression in cardiac tissue of E10.5 *Myocd* wild type (WT), heterozygote (*Myocd*^{KI/+}) and null mutant (*Myocd*^{KI/KI}) embryos. The *Myocd*^{KI/KI} embryo shows cardiac defects and embryonic development delay (Scale bar, 1mm). B) *Myocardin* relative RNA expression levels by qPCR in *Myocd* wild type (WT) and null mutant (*Myocd*^{KI/KI}) hearts.

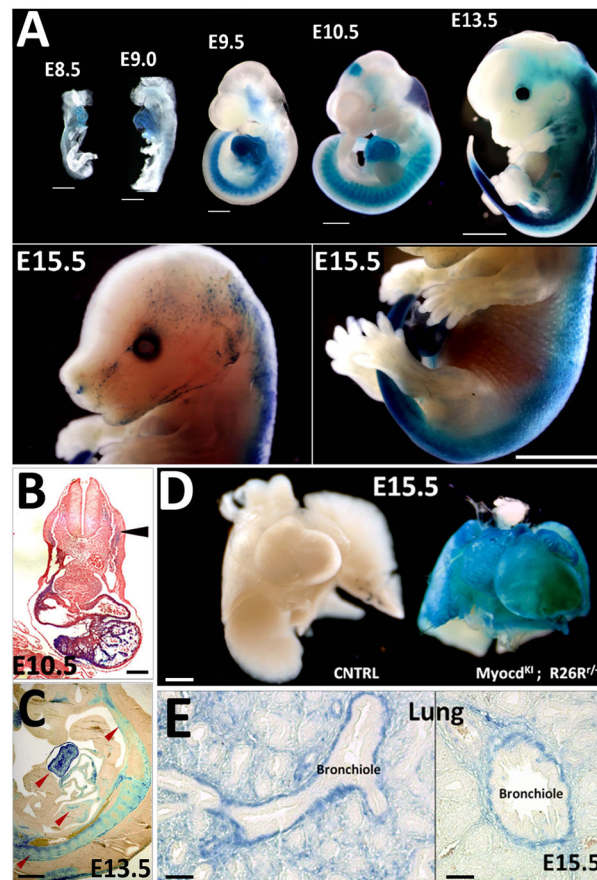


Figure 3. Myocardin expression pattern in *Myocd*^{KI/+}; *R26R*^{r/+} reporter mice

A) X-gal staining at different stages of developing mouse embryos. LacZ expression is observed in the developing heart, somites and mid-brain. Note that LacZ expression expands to proximal limb internal structures (from E13.5) (Scale bars, 250 μ m). B) Transversal section of an E10.5 embryo showing cardiac specific expression of LacZ, somite expression is low compared to the heart (arrowhead) (Scale bar, 500 μ m). C) X-gal staining on a sagittal section of an E13.5 embryo reveals LacZ expression in the heart, somite derivatives and forming vasculature (arrowhead) (Scale bar, 500 μ m). D) LacZ staining in an E15.5 *Myocd*^{KI/+}; *R26R*^{r/+} embryo heart and lung. A wild type littermate embryo was used as a control (Scale bar, 500 μ m). E) Cross sections of a *Myocd*^{KI/+}; *R26R*^{r/+} E15.5 embryo lung after X-gal staining showing LacZ expression in the smooth muscle layer surrounding the forming bronchioles (Scale bars, 125 μ m).

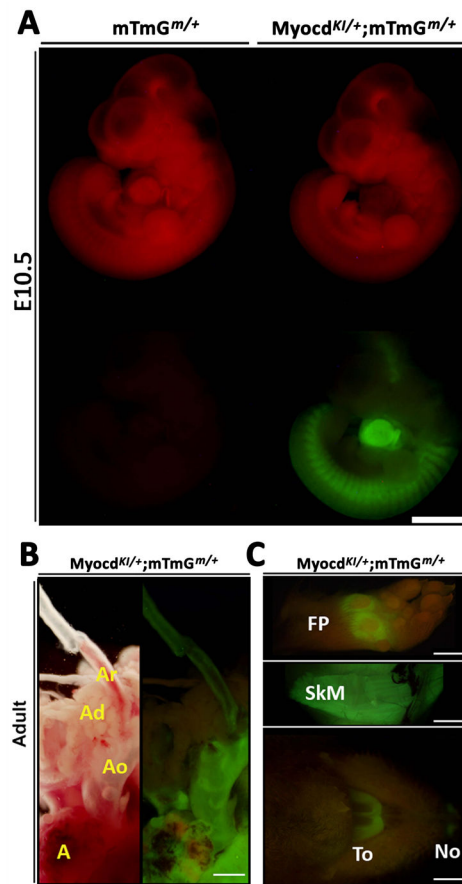


Figure 4. EGFP expression pattern in a *Myocd^{KI/+};mTmG^{m/+}* double heterozygote E10.5 embryo**
 A) EGFP expression in the heart, somites and mid-brain of a *Myocd^{KI/+};**mTmG^{m/+}* embryo. Notice that red fluorescence is diminished in regions in which EGFP is expressed. A *mTmG^{m/+}* single heterozygote embryo was included as a control (Scale bar, 1mm). B) EGFP expression in a 2 month old adult *Myocd^{KI/+};**mTmG^{m/+}* mouse cardiovascular tissues such as the Atrium (A), Aorta (Ao), and in the left common carotid aortic branch (Ar), while EGFP is not observed in Adipose tissue (Ad) of (Scale bar, 2mm). C) EGFP expression in the foot pad (FP), skeletal muscle (SkM), tongue (To) and nostrils (No) of a 2 month old adult *Myocd^{KI/+};**mTmG^{m/+}* mouse (Scale bar, 4mm).

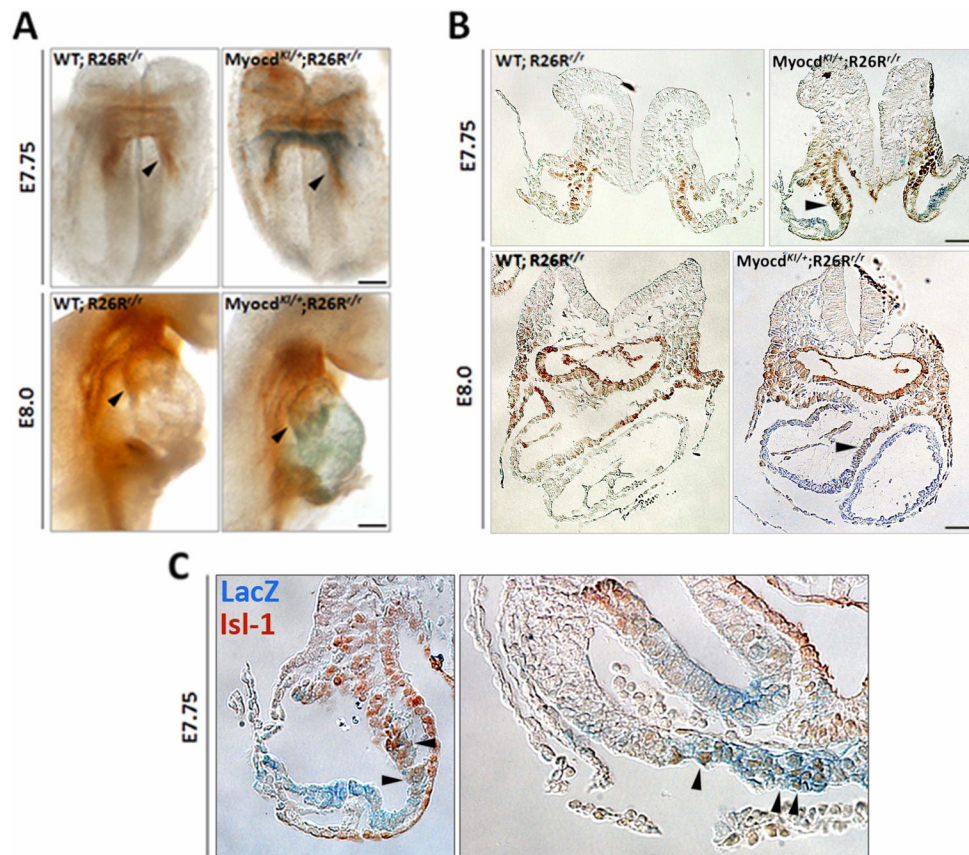


Figure 5. *Myocardin* is expressed in the first heart field

A) Whole mount X-gal staining and Isl-1 immunostaining at early developmental staged embryos (E7.75 and E8.0) of *Myocd*^{KI/+}; *R26R*^{+/+} mice. At E7.75 LacZ expression appears ventral to that of Isl-1 staining (arrowheads) in the primordial forming heart. At E8.0 LacZ expression is present in the lineal heart tube while Isl-1 expression remains dorsal to that of LacZ and at the poles of the forming tubular heart (arrowheads). WT; *R26R*^{+/+} were included as controls. B) Transversal sections of E7.75 and E8.0 embryos stained for LacZ followed by Isl-1 immunostaining showing the different expression domains. Arrowheads point to a cell population which seems to express both markers, LacZ and Isl-1. C) Higher magnification pictures of the transversal sections of the E7.75 *Myocd*^{KI/+}; *R26R*^{+/+} embryo depicted in A) and B). Images correspond to the horns and to the medial arc of the forming cardiac tube respectively. Arrowheads point to cells and regions where double staining is observed (Scale bars, 100 μ m.).