An *In Vivo* Map of *Bone Morphogenetic Protein 2* Post-transcriptional Repression in the Heart

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Abstract

The *Bmp2* 3'untranslated region (UTR) sequence bears a sequence conserved between mammals and fishes that can post-transcriptionally activate or repress protein synthesis. We developed a map of embryonic cells in the mouse where this potent *Bmp2* regulatory sequence functions by using a *lacZ* reporter transgene with a 3'UTR bearing two *loxP* sites flanking the ultra-conserved sequence. Cre-recombinase-mediated deletion of the ultra-conserved sequence caused strong ectopic expression in proepicardium, epicardium and epicardium-derived cells (EPDC) and in tissues with known epicardial contributions (coronary vessels and valves). Transient transfections of reporters in the epicardial/mesothelial cell (EMC) line confirmed this repression. Ectopic expression of the recombined transgene also occurred in the aorta, outlet septum, posterior cardiac plexus, cardiac and extra-cardiac nerves and neural ganglia. *Bmp2* is dynamically regulated in the developing heart. 3'UTR-mediated mechanisms that restrain BMP2 synthesis may be relevant to congenital heart and vasculature malformations and to adult diseases involving aberrant BMP2 synthesis.

Keywords

mRNA; post-transcriptional; gene regulation; transgenic mice; aorta; coronary vasculature; epicardial cells; neural crest

Introduction

Bone morphogenetic protein (BMP)2 is an essential morphogen involved in numerous developmental and physiological processes (Chen *et al.*, 2004; Hogan, 1996; Tsuji *et al.*,

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2006; Zhang and Bradley, 1996). In particular, BMP2 signaling is required for essential aspects of heart development, including myocardial differentiation and survival, the development of the atrioventricular (AV) canal and outflow tract (OFT), valves, and the atrioventricular conduction (AVC) system ((Kirby, 2007) and refs. therein).

As expected for a morphogen, precise local regulation of BMP2 levels is crucial (Raftery and Sutherland, 2003; Zhang and Bradley, 1996). Different concentrations of active BMP2 or length of exposure regulate different cardiac genes to varying degrees (Alsan and Schultheiss, 2002; Schlange *et al.*, 2000). A delicate balance between BMP2 signals and other signals such as FGF, control the behavior of proepicardial cells (Ishii *et al.*, 2010; Kruithof *et al.*, 2006; Schlueter *et al.*, 2006; Torlopp *et al.*, 2010; van Wijk *et al.*, 2009). That appropriate levels of BMP2 are essential for normal development was shown by transgenic studies where BMP2 levels were experimentally elevated (Blessing *et al.*, 1993) or reduced (Singh *et al.*, 2008). As discussed below, post-transcriptional gene regulation mechanisms can control protein levels with precision during embryogenesis.

We have studied many aspects of *Bmp2* gene regulation during the retinoic-acid (RA) induced differentiation of F9 embryonal carcinoma cells (Abrams *et al.*, 2004; Fritz *et al.*, 2006; Fritz *et al.*, 2004; Heller *et al.*, 1999; Hu *et al.*, 2006; Rogers, 1996; Rogers *et al.*, 1992). We have identified control regions that determine whether the *Bmp2* gene is on or off (*e.g.*, cell specific transcriptional enhancers) as well as elements that control the precise level of protein synthesis (*e.g.*, translation efficiency and mRNA stability). An ultra-conserved sequence (UCS) within the *Bmp2* 3'UTR is 73% identical over 265 nt. between mammals and fishes. Our bioinformatics comparisons to other highly conserved non-coding sequences established that this sequence ranks highly relative to other evolutionarily conserved sequences described to date (Abrams *et al.*, 2004; Fritz *et al.*, 2004). Our molecular analyses strongly suggests that this striking conservation reflects vital regulatory functions.

By analogy to numerous growth factors and cytokines (*e.g.*, TNFα, interleukins) that are rapidly and dramatically regulated by 3'UTR elements, the 3'UTR is the logical location for mechanisms that regulate the BMP2 morphogen as precisely and dynamically as required in the heart. Our *in vitro* biochemistry and cell culture studies (Devaney *et al.*, 2009; Fritz *et al.*, 2006; Fritz *et al.*, 2004; Jiang *et al.*, 2010) demonstrated that the ultra-conserved sequence controls protein synthesis at the post-transcriptional level and acts as an autonomously acting repressor in normal lung and mesenchymal cells (Jiang *et al.*, 2010; Kruithof *et al.*, 2010a). We corroborated this model in neonatal and adult lung, aorta, and coronary vasculature by the *in vivo* deletion of the ultra-conserved sequence from a *Bmp2*-driven *lacZ* transgene (Fritz *et al.*, 2006; Jiang *et al.*, 2010; Kruithof *et al.*, 2010a).

Previous *in vivo* studies were guided by studies in cell types that exhibited ultra-conserved sequence-mediated repression *in vitro*. However, in the transgenic mouse, we observed many additional cell types where transgene expression was repressed by the ultra-conserved sequence. As in normal lung and mesenchymal cells, the *Bmp2* gene may be transcribed, but BMP2 synthesis may be blocked at a post-transcriptional step. Because protein synthesis can be rapidly and precisely controlled by post-transcriptional means, such a mechanism would readily explain the dynamic patterns of *Bmp2* expression observed in embryos. Identifying the cells poised to synthesize BMP2 in response to developmental signals is essential. We now provide a detailed map of the embryonic cells in which the ultra-conserved sequence represses gene expression. These include precursor cells of the coronary vasculature during development, including the proepicardium, and epicardium-derived cells and other specific cell types of cardiovascular relevance, including cells of the valves, outlet septum, posterior cardiac plexus, cardiac and extra-cardiac nerves.

Methods

Animals

All animals were handled in accordance with the Guidelines for Care and Use of Experimental Animals and approved by the NJMS IACUC (protocols #04086, #00100). Procedures as described in (Kruithof *et al.*, 2010a).

Immunohistochemistry

Whole-mount tissues stained for βgal were embedded in paraffin and sectioned (6 mm). Sections were boiled for 20 minutes in 0.01M citrate buffer for antigen retrieval. For α-smooth muscle actin (α-SMA) staining, sections were blocked with 5% donkey serum in 0.1% Tween-20/PBS, incubated with primary antibodies against α-SMA (Dako North America, Inc., Carpinteria, CA), followed by Alexa-conjugated secondary antibody (Molecular Probes/Invitrogen, Carlsbad, CA) incubation to visualize the antibody binding. DAPI containing media (Vector) was used to visualize the nuclei. Because both DAPI and βgal staining are blue, we used NIS-Elements Microscope Imaging Software (Nikon) to convert the DAPI to green for better contrast with β-gal.

For neurofilament 160 (NF160) staining, sections were treated with 3% H_2O_2 to block endogenous peroxidase activity, blocked with 5% horse serum, and incubated with NF160 antibody (Sigma). The Vectastain Elite ABC Kit and Novared Substrate kit (Vector) were used to visualize antibody binding.

Tissue culture and transfections

The rat epicardial/mesothelial cell (EMC) line (Eid *et al.*, 1992) was grown in DMEM supplemented as described in (Wada *et al.*, 2003). Cells were transfected using FuGene6 Transfection Reagent (Roche, Indianapolis, IN) following the manufacturer's protocol. 24 hrs after transfection, cells were lysed with 1X Passive Lysis Buffer (Promega, Madison, WI) and luciferase activities were measured using the Luciferase Assay System (Promega, Madison, WI) following the manufacturer's protocol. CMVLUCBmp (pCβSLucmHCNSPvAcGH) containing the mouse (9,574 nt to 9,938 nt) ultra-conserved sequences inserted downstream of luciferase in CMVLUC (pCβSLuc) was described in (Devaney *et al.*, 2009).

Results

Transgenic reporter genes mimic endogenous Bmp2 expression patterns

To map where the ultra-conserved sequence functions *in vivo*, we used a transgenic mouse strain bearing a *lacZ* transgene driven by the mouse *Bmp2* promoter region, the entire *Bmp2* 3'UTR and 934 nt of downstream sequence (Fritz *et al.*, 2006). Two loxP sites flanking the ultra-conserved sequence in the reporter gene (Figure (Fig.) 1A) permitted its *in vivo* excision by Cre-recombinase (Jiang *et al.*, 2010; Kruithof *et al.*, 2010a). Supplemental Fig. 1 shows that the expression of the intact *Bmp2* reporter gene in several structures closely resembles published patterns of *Bmp2* expression at these stages. These representative images (Supplemental Fig. 1, n = 10 – 20 embryos) show expected embryonic patterns in pericardial mesoderm (Zhang and Bradley, 1996), interdigital cells of the developing paw (Laufer *et al.*, 1997; Salas-Vidal *et al.*, 2001), eye (Belecky-Adams and Adler, 2001; Dudley and Robertson, 1997; Furuta *et al.*, 1997), cartilage primordium (Lyons *et al.*, 1989), and trigeminal nerves (Jin *et al.*, 2001). Similar expression patterns also were observed in congenic mice backcrossed into the C57Bl/6 strain for 10 generations (not shown). The reporter gene was not expressed in some embryonic tissues that do express *Bmp2* such as the atrioventricular canal (AVC) (Abdelwahid *et al.*, 2001) and the condensing mesenchyme

and hypertrophic cartilage of the long bones (Lyons *et al.*, 1989). This was expected for this transgene, because other studies have identified extremely distant *Bmp2* regulatory regions (Chandler *et al.*, 2007). Thus several, although not all, BMP2-expressing tissues also express this reporter gene.

Deletion of the ultra-conserved region induces reporter gene expression in vivo

We queried the embryonic regions where the ultra-conserved region represses gene expression by deleting the ultra-conserved region in the *Bmp2* reporter gene using a ubiquitously expressed Cre-recombinase (Schwenk *et al.*, 1995). Deletion caused the βgal pattern to expand beyond the regions where the intact reporter gene was expressed (Fig. 1). For example, in the trigeminal nerves, βgal staining outlines the termini of the nerve network in mice with the intact transgene (no Cre, Fig. 1C). In the Cre-expressing mice, terminal expression appears intensified and the field of expression clearly extends back into the nerve cell bodies. βgal staining also occurred in the dorsal root ganglia of Cre-expressing mice (Fig. 1). Because BMP2 is an essential signal during the development of the heart and cardiovasculature, we carried out a detailed analysis of reporter gene expression in these tissues.

Ultra-conserved sequence-mediated repression in proepicardial, epicardial and coronary vascular cells in vivo and recapitulation of ultra-conserved sequence-mediated inhibition in cultured epicardial cells

Proepicardial cells have been extensively shown to differentiate into coronary endothelial, smooth muscle and fibroblastic cells both *in vivo* and *in vitro* (Dettman *et al.*, 1998; Grieskamp *et al.*, 2011; Olivey *et al.*, 2004; Reese *et al.*, 2002; Wessels and Perez-Pomares, 2004; Winter and Gittenberger-de Groot, 2007). A recent report suggests that coronary endothelium also forms from the outgrowth of the sinus venosus endothelium (Red-Horse *et al.*, 2010). Fig. 2 demonstrates that proepicardial (embryonic day E10.0; Fig. 2A), epicardial (Fig. 2B) and subepicardial cells (E11.5, E12.5; Fig. 2B, D) express the transgene specifically in Cre-expressing mice. In contrast, the epicardium of no Cre embryos showed little, if any, expression of the intact transgene, although the intact transgene was expressed in the pericardium (Fig. 2C). Expression in the epicardium appeared to decrease after birth (Fig. 2H). Immunohistochemical analyses using α-smooth muscle actin (SMA) and cell morphologies established that the ultra-conserved sequence repressed βgal synthesis in vascular epicardium-derived cells: a subset of coronary endothelial cells (EC), smooth muscle cells (SMC) and perivascular fibroblasts (PF) (Fig. 2F–G).

Previously, we first established that the ultra-conserved sequence repressed protein synthesis in specific cell types *in vitro* and then subsequently verified the phenomenon *in vivo* (Jiang *et al.*, 2010; Kruithof *et al.*, 2010a). Now, guided by the *in vivo* results, we describe a new *in vitro* model of *Bmp2* gene repression. The rat epicardial/mesothelial cell (EMC) line (Eid *et al.*, 1992; Wada *et al.*, 2003) has been used to map the epicardial control region derived from the chick GATA-5 gene (Burch, 2005; MacNeill *et al.*, 2000). Fig. 2E demonstrates that the ultra-conserved sequence significantly repressed reporter genes driven by a heterologous cytomegalovirus (CMV) promoter (compare CMVLuc to CMVLUCBmp) in the EMC line, thus corroborating the *in vivo* transgene repression in embryonic epicardial cells. In summary, we have demonstrated that the ultra-conserved sequence inhibits gene expression in cells of known epicardial origin. Furthermore, we have identified a new cell culture model of this repression.

Ultra-conserved sequence-mediated repression in cardiac cushions, valves, aorta, and pulmonary trunk

In the outflow tract (OFT), cushions are formed that will give rise to the outlet septum and the semilunar (aortic and pulmonary) valves (Kirby, 2007). The recombined *Bmp2* reporter transgene was expressed in the distal cushions (Fig. 3A; Supp. Fig. 2A) and outlet septum (Fig. 3B; Supp. Fig. 2B, C) of the OFT in +Cre embryos, whereas expression was absent in these tissues in no Cre embryos (Fig. 3C). In E18.5 +Cre embryos, transgene expression was observed in the developing aortic, but not the pulmonary, valve near the attachment to the vessel wall (Fig. 3G). After birth, both valves had blue cells near the attachment (N0.5 and N4.5, not shown). Subsequently in the adult, the number of blue cells in the valves was increased and they were located nearer the distal side of the leaflets (Fig. 3H).

The recombined *Bmp2* reporter gene was robustly expressed in all smooth muscle cells of both the ascending and descending aorta of +Cre mice in adult mice (Kruithof *et al.*, 2010a). Here we show that transgene expression in +Cre embryos was detectable in the aorta at E13.5 (Fig. 3D). In contrast, expression of the recombined *Bmp2* reporter gene was delayed in the pulmonary trunk until E15.5 (Fig. 3F).

In the region of the inflow tract (IFT), we observed expression of the recombined transgene in the dorsal mesenchymal protrusion (DMP) and the inferior atrioventricular (AV) cushions (Fig. 3I), which will contribute to the AV septum and septal leaflets of the AV valves (mitral and tricuspid valves). Expression also occurred in the dorsal mesocardium (DM; Figs. 2D, 3I), which connects the DMP to the dorsal body wall. Transgene expression was observed in the septal leaflets of the AV valves from E15.5 (Fig. 3J; Supp. Fig. 2D) and in the mural leaflets of the AV valves from E18.5 in +Cre embryos. This was predominantly in the proximal leaflet portion adjacent to the annulus fibrosus and at the attachment to the papillary muscle (Fig. 3K). At N13.5, expression of the recombined *Bmp2* transgene had expanded and was located mainly at the ventricular side of the AV valves (Fig. 3M). In all locations, expression was undetectable in littermates without the Cre gene (not shown).

The AV valves contain pigmented melanocytes from about E17.5 (Mjaatvedt *et al.*, 2005). Pigmented melanocytes developed in our mice, because the CMV-Cre parent was strain C57Bl/6. At E18.5, a subset of the βgal-positive cells in the septal leaflets of the AV valves also was pigmented (Fig. 3L). Together, these observations reveal spatial and temporal differences in the acquisition of the repression mechanism mediated by the *Bmp2* ultra-conserved sequence.

Ultra-conserved sequence-mediated repression in other cardiac and extra-cardiac tissues

From E15.5 onwards, βgal expression resulting from the recombined transgene was observed in the ventricular myocardium close to the apex of the heart, exclusively in Cre-positive mice. This expression remained after birth (Fig. 4A). Expanded expression of the recombined transgene lacking the ultra-conserved sequence was previously noted in the trigeminal nerves and dorsal root ganglia (Fig. 1). Staining for neurofilament 160 also colocalized with βgal in Cre-expressing embryos in neurons inside and outside the developing heart. These included the dorsal root ganglia (E11.5, Fig. 1C, D; 4B), vagal nerve (Fig. 4C), cardiac plexus (Fig. 4F), and nerves surrounding the venous pole and on the atrial wall (Fig. 4E, F). Neural expression was absent in littermates without the Cre gene (Fig. 4D and not shown).

Discussion

Gene proximal elements functioning in a subset of Bmp2-expressing tissues

The intact reporter bearing 3.9 kb of *Bmp2* non-coding sequence was expressed in several tissues that synthesize BMP2 (pericardial mesoderm, interdigital cells, eye, cartilage primordium, and trigeminal nerves (Belecky-Adams and Adler, 2001; Dudley and Robertson, 1997; Furuta *et al.*, 1997; Jin *et al.*, 2001; Laufer *et al.*, 1997; Lyons *et al.*, 1989; Salas-Vidal *et al.*, 2001; Zhang and Bradley, 1996). Thus elements that drive expression in these tissues map to the distal promoter region or within 2.2 kb of the stop codon. Extremely distant *Bmp2* regulatory regions explain incomplete recapitulation of the endogenous BMP2 pattern (Chandler *et al.*, 2007).

Two general repression patterns

In some embryonic regions, deletion of the UCS caused expansion of βgal into cells adjacent to regions that expressed the intact transgene. The trigeminal nerves illustrate this pattern (Fig. 1). In other cells, expression of the intact transgene was not detectable, but the recombined transgene was strongly expressed. Using biochemically tractable systems, we and others proved that the UCS functions post-transcriptionally in diverse cell types (Devaney *et al.*, 2009; Fritz *et al.*, 2006; Fritz *et al.*, 2004; Fukui *et al.*, 2006; Jiang *et al.*, 2010; Kruithof *et al.*, 2010b). The ectopic expression caused by removing the UCS revealed cells that can synthesize BMP2 quickly in response to local developmental stimuli, as typifies many potent cytokines and growth factors.

Potential lineage-specific activation of regulatory elements

After deletion of the UCS, *Bmp2* transgene expression was observed in proepicardium, epicardium, subepicardium, coronary endothelial cells, smooth muscle cells, and perivascular fibroblasts suggesting that epicardial lineage specific elements reside in the 3.1 kb of *Bmp2* DNA remaining in the recombined transgene. Prior to our study, only one other regulatory region that could activate transgene expression in epicardial cells *in vivo* and in the rat epicardial/mesothelial cell (EMC) line had been delineated; chick GATA-5 (MacNeill *et al.*, 2000). Elucidating gene regulatory processes in these essential cells requires characterizing these epicardial-specific control modules.

Both cardiac and extra-cardiac structures with known neural crest cell contributions (OFT cushions, outlet septum, mesenchymal inflow structures, AV valves, posterior cardiac plexus, cardiac and extra-cardiac nerves) expressed the recombined *Bmp2* transgene (Figs. 3, 4; (Brito and Kos, 2008; Hildreth *et al.*, 2008; Jiang *et al.*, 2000; Nakamura *et al.*, 2006). The versatile cranial neural crest cells provide both instructive signals and structural components to the heart, vasculature, and associated nerves. These cells may rapidly release BMP2 in response to local stimuli.

UCS-mediated repression varies in time and space

Although the recombined transgene was widely expressed, the expression was dynamic. Whereas virtually all +Cre epicardial cells were positive at E11.5, only a subset of the epicardium-derived cells were positive later. Such changeable expression is consistent with cells encountering different environments and suggests that local signals modulate the balance of positive and negative gene regulatory factors in these cells. For example, widespread expression of the recombined transgene in large coronary vessels and along the ventricular side of the mitral and tricuspid leaflets suggests that a hemodynamic component may activate the transgene (Kruithof *et al.*, 2007). Non-uniform labeling in all endothelial cells also may reflect multiple origins (Cai *et al.*, 2008; Red-Horse *et al.*, 2010; Zhou *et al.*, 2008).

Potential regulators

We have identified three cultured cell types that retain the unique and unexpected aspect of *Bmp2* repression observed *in vivo*. The UCS represses gene expression in mouse epicardial cells *in vivo* and in the rat epicardial/mesothelial cell (EMC) model of these cells (Fig. 2, (Eid *et al.*, 1992; Wada *et al.*, 2003)); in mesenchymal cells of the mouse aorta and coronary vasculature *in vivo* and in C3H10T½ mesenchymal cells (Fig. 3, (Devaney *et al.*, 2009; Kruithof *et al.*, 2010a)); and in mouse lung *in vivo* and in BEAS-2B immortalized bronchial epithelial cells (Jiang *et al.*, 2010). With these three tissue culture models of the repressive function seen in three *in vivo* tissues, we can identify the biochemical mechanisms that mediate repression.

Potential regulatory molecules include proteins; *e.g.,* HuR and nucleolin (Devaney *et al.*, 2009; Fritz *et al.*, 2006) and microRNAs (Jing *et al.*, 2005; Wu *et al.*, 2006; Zhao and Srivastava, 2007). Hybridization to conserved anti-sense miRNAs would explain some of the extraordinary sequence identity between vertebrate *Bmp2* 3'UTRs. Furthermore, Cre-mediated deletion of the UCS from the recombined *Bmp2* reporter gene would render the reporter mRNAs immune to microRNA-mediated repression. Over 40 microRNAs have been computationally predicted to interact with the *Bmp2* 3'UTR, a majority within the UCS (http://www.targetscan.org and http://pictar.bio.nyu.edu). Tests of microRNA function including mutations of putative binding sites are currently in progress. Understanding the molecules that sustain or release this braking mechanism is directly relevant to the etiology of congenital malformations of the heart and vasculature and to adult diseases involving abnormal BMP2 production.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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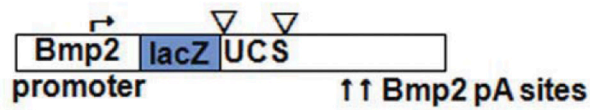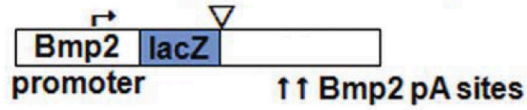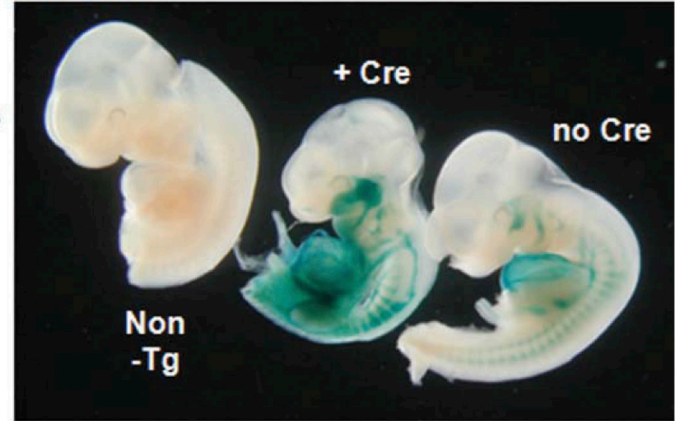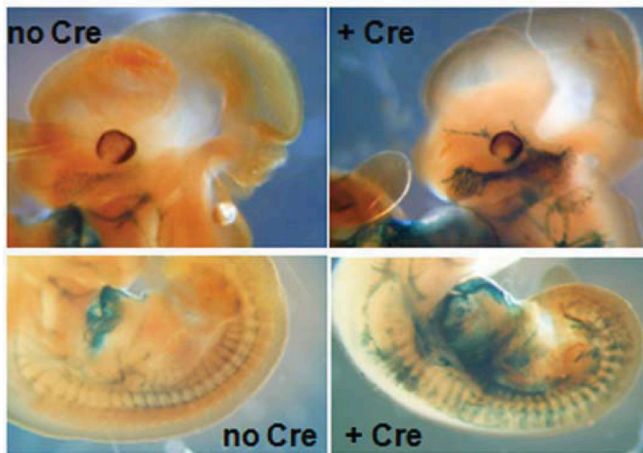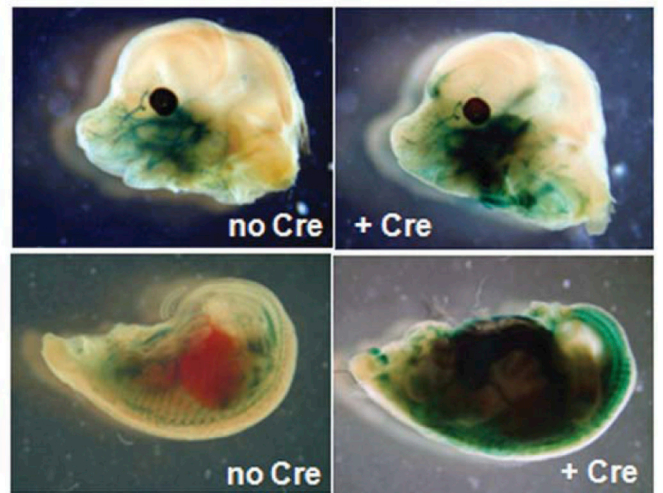
Fig. 1. Cre-mediated deletion of the ultra-conserved region activates reporter gene expression in embryos

A. Diagrams of a reporter transgene with the murine *Bmp2* promoter (⌐►, nt −1237 to 471) and 3'UTR regions (nt 9392 to 11604) flanking the *lacZ* gene. ∇ marks the *loxP* sites flanking the ultra-conserved sequence (UCS, nt 9392 to 10200). The approximate locations of the 2 natural *Bmp2* poly(A) signals at 10,332 and 10,619 (Fritz *et al.*, 2004) relative to the promoter (yielding 3'UTRs of ~870 and 1175 nt, respectively) are indicated by arrows (↑). **B – D.** Mice carrying the *Bmp2* transgene were mated with mice hemizygous for a Cre-recombinase gene driven by the ubiquitously expressed CMV promoter. Embryo littermates (ages indicated in panels) were whole mount stained for βgal (blue staining). The domains of βgal activity intensified and expanded in + Cre animals.
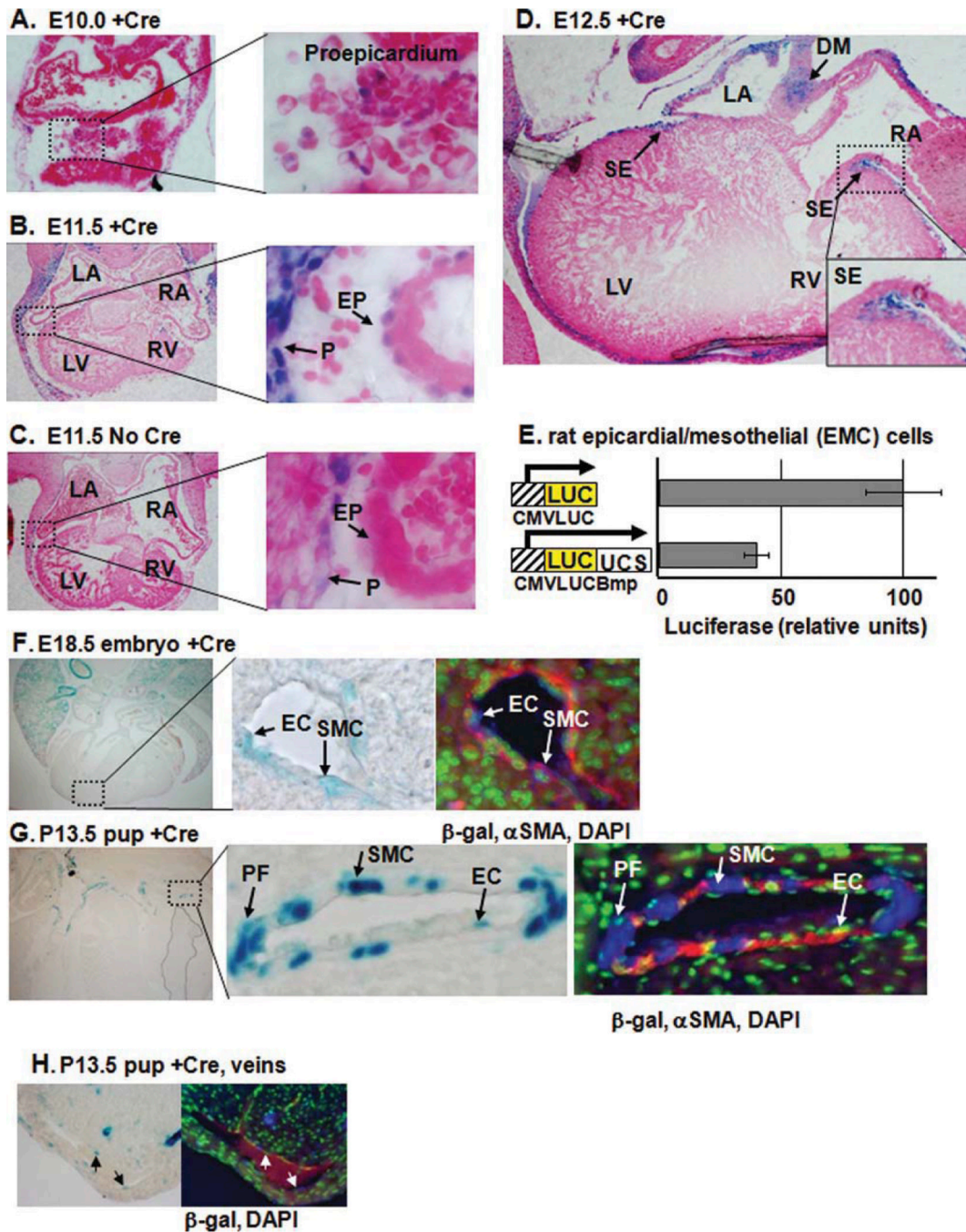
Fig. 2. Proepicardium and epicardium express the recombined reporter transgene in +Cre mice
Sections of whole-mount βgal-stained (**blue**) Cre-expressing embryos. **A.** E10.0 embryos
(+Cre) express the transgene in proepicardium. **B, C.** E11.5 embryos with both the
recombined (+Cre) and the intact (no Cre) transgenes contain βgal-positive cells in the
pericardial mesoderm. Only +Cre embryos also express the transgene in the epicardium
(**EP**). **D.** In E12.5 embryos (+Cre), transgene expression is also observed in subepicardial
(**SE**) cells and dorsal mesocardium (**DM**; also see Fig. 3). Left atrium (**LA**), right atrium
(**RA**), left ventricle (**LV**), right ventricle (**RV**), dorsal mesocardium (**DM**). **E.** Luciferase
activity generated in rat epicardial/mesothelial cells (EMC) after transfection with reporter

genes driven by the constitutive cytomegalovirus (**CMV**) promoter with (**CMVLUCBmp**) or without (**CMVLUC**) the mouse ultra-conserved sequence (**UCS**, nt 9574–10204 relative to the mouse distal promoter) inserted downstream of LUC. Relative reporter activity is shown ± SEM, n = 8. **F–H.** Sections were immuno-histochemically stained with an antibody against α smooth muscle actin (**α-SMA**, **red**). Some sections were counterstained with **DAPI** (**green**) to mark nuclei. The merged images illustrate colocalization of βgal activity with endothelial cells (**EC**) and α-SMA positive smooth muscle cells (**SMC**) and perivascular fibroblasts (**PF**). **F** and **G** show coronary arteries at E18.5 and P13.5, respectively. **H** shows a section of a coronary vein with βgal-positive endothelial cells at P13.5.
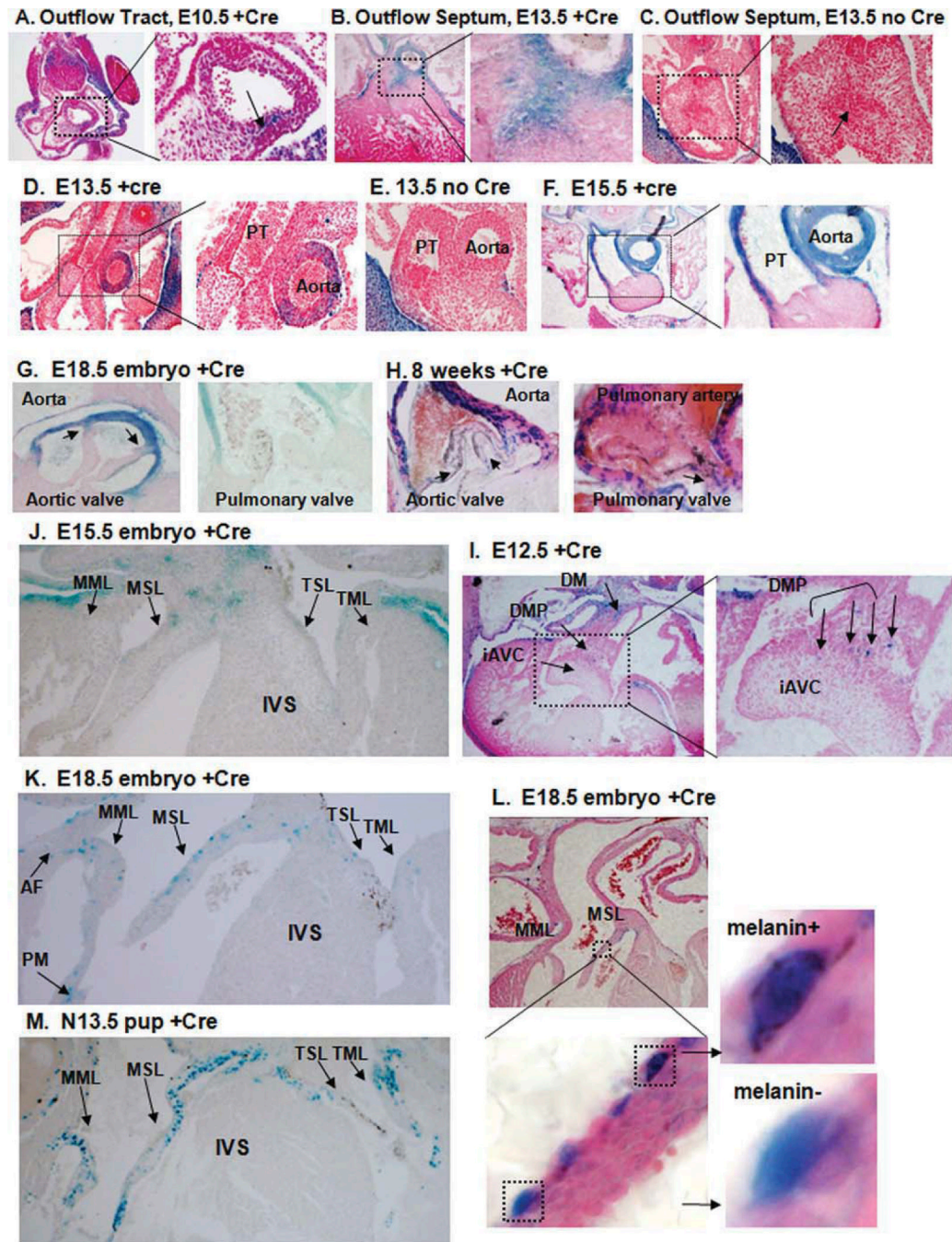
Fig. 3. Recombined Reporter Gene Expression in valve progenitors, valves, aorta and pulmonary trunk

Sections of whole-mount βgal-stained (**blue**) Cre-expressing embryos. **A, B.** βgal-positive cells were present in the distal outflow tract cushions at E10.5. (**A**), and in the outlet septum at E13.5 (**B**) **C**. βgal-stained cells were absent in the outlet septum of no Cre embryos. **D.** βgal-positive cells were present in the aorta, but not in the pulmonary trunk (**PT**) of Cre-expressing (+**Cre**) E13.5 embryos. **E.** No expression was observed in the aorta and pulmonary trunk of the no Cre mice. **F.** At E15.5, the reporter gene was strongly expressed in both the aorta and pulmonary trunk of Cre-expressing embryos. **G.** Before birth, recombined transgene expression was only detectable in the in the aortic, but not the

pulmonary, valve near the attachment to the vessel wall. **H.** After birth, blue cells were observed throughout both aortic and pulmonary valves. **I**. At E12.5, transgene expression is found in dorsal mesocardium (**DM**), dorsal mesenchymal protrusion (**DMP**) and inferior atrioventricular cushion (i**AVC**). **J–M**. βgal-positive cells were present at E15.5 in septal AV leaflets (**J**) and at E18.5 (**K**) also in the mural AV leaflets mainly near the attachment to the annulus fibrosus **(AF)** and papillary muscle **(PM). M.** At N13.5, βgal-positive cells were present mostly at ventricular side of the valves. Interventricular septum (**IVS**), mitral mural leaflet **(MML)**, mitral septal leaflet (**MSL**), tricuspid septal leaflet (**TSL**), and tricuspid mural leaflet (**TML**). **L.** At E18.5, melanin positive cells co-localized with βgal-stained nuclei (arrows and enlarged insets) in the MSL.
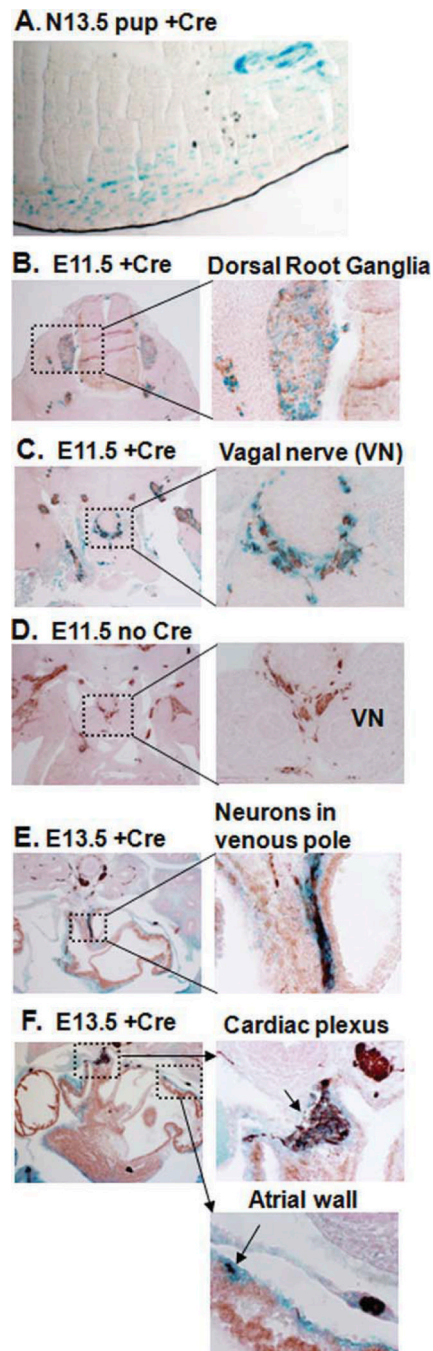
Fig. 4. Recombined Reporter Gene Expression in Other Tissues
A. βgal-stained (**blue**) cells were observed within the compact layer of the ventricle of Cre-expressing embryos (not shown) and pups. **B – F**. Sections of whole-mount βgal-stained embryos were immuno-histochemically stained with an antibody against NF160 (**brown**). Cre-expressing (+Cre) embryos expressed the transgene strongly in NF160-positive cells in (**B**) dorsal root ganglia, (**C**) vagal nerve (**VN**), (**E**) venous pole (**VP**), (**F**) cardiac plexus (**CP**), and atrial wall (**AW**). (**D**) Mice with the intact (no Cre) transgene showed little, if any, βgal-staining in these structures at all stages (E11.5 shown here; E14.5 not shown).