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Msx1^{CreERT2} Knock-in Allele: A Useful Tool to Target Embryonic and Adult Cardiac Valves

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Summary: Heart valve development begins with the endothelial-to-mesenchymal transition (EMT) of endocardial cells. Although lineage studies have demonstrated contributions from cardiac neural crest and epicardium to semilunar and atrioventricular (AV) valve formation, respectively, most valve mesenchyme derives from the endocardial EMT. Specific *Cre* mouse lines for fate-mapping analyses of valve endocardial cells are limited. *Msx1* displayed expression in AV canal endocardium and cushion mesenchyme between E9.5 and E11.5, when EMT is underway. Additionally, previous studies have demonstrated that deletion of *Msx1* and its paralog *Msx2* results in hypoplastic AV cushions and impaired endocardial signaling. A knock-in tamoxifen-inducible Cre line was recently generated (*Msx1*^{CreERT2}) and characterized during embryonic development and after birth, and was shown to recapitulate the endogenous *Msx1* expression pattern. Here, we further analyze this knock-in allele and track the Msx1-expressing cells and their descendants during cardiac development with a particular focus on their contribution to the valves and their precursors. Thus, *Msx1*^{CreERT2} mice represent a useful model for lineage tracing and conditional gene manipulation of endocardial and mesenchymal cushion cells essential to understand mechanisms of valve development and remodeling. genesis 53:337–345, 2015. © 2015 Wiley Periodicals, Inc.

Key words: Msx; mouse development; tamoxifen-inducible Cre; heart; lineage tracing; valvulogenesis

INTRODUCTION

The expression pattern of *Msx1* is well established: initially expressed in extra-embryonic tissues at E6.5 (Robert *et al*., 1989), it is present in the neural tube and the neural crest from early developmental stages onwards, in the mandibular arch and the mouth structures that emanate from it, in the developing limb bud, as well as in the branchial arches and the heart (Hill *et al*., 1989; Robert *et al*., 1989). *Msx1* and *Msx2* have well documented roles in epithelial-mesenchymal interactions during organ formation, and are both crucial effectors of the BMP cascade (Liu *et al*., 2005; Vainio *et al*., 1993) that display redundant functions during vertebrate development.

Cardiac cushion formation in the outflow tract (OFT) and atrioventricular canal (AVC) is a crucial step during valvulogenesis. The initially acellular, cardiac jelly-filled

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cushions become populated by endocardial cells that undergo endothelial-to-mesenchymal transition (EMT) and invade the underlying extracellular matrix (Hinton and Yutzey, 2011). These cells that now have mesenchymal properties will then proliferate and through complex and still poorly understood morphogenetic processes will lead to the enlargement, elongation, and remodeling of the forming valves.

Signals emanating primarily from the cushion myocardium are important for the initiation, progression, and control of EMT. One of the key regulators is myocardial Bmp2, that works in concert with endocardial Notch1 to promote EMT in presumptive valve tissue, via Snail1 activation (Luna-Zurita *et al*., 2010). Bmp2 also activates Tgfβ2, both in endocardium and myocardium.

Msx1 and *Msx2* are specifically activated in atrioventricular (AV) endocardium by Bmp2 during cardiac cushion EMT (Ma *et al*., 2005). Single $Msx1^{-/-}$ and $Msx2^{-/-}$ mutant mice exhibit normal valve formation, whereas double $Msx1^{-/-}$;$Msx2^{-/-}$ mutant mice have hypoplastic AV cushions with decreased endocardial activation (reduced expression of *Has2*, *NFATc1*, and *Notch1*) and impaired EMT, which in turn lead to short and deformed AV valves (Chen *et al*., 2008). *Msx1* and *Msx2* have overlapping expression patterns in a subpopulation of endocardial and mesenchymal cells during AV cushion morphogenesis, but *Msx2* is also strongly expressed in the AVC myocardium between E9.5 and E11.5. Overall, *Msx1* and *Msx2* are shown to be important in endocardial activation prior to EMT, induction of EMT and post-EMT valve remodeling (Chen *et al*., 2008).

The most widely used mouse line specifically labeling valvular or endocardial tissue is the Nfatc1-cre line. *Nfatc1* is a transcription factor strongly expressed in valve endocardial cells during EMT and valve elongation (de la Pompa *et al*., 1998; Ranger *et al*., 1998). There are two *Nfatc1-Cre* lines: A pan-endocardial line that labels all endothelium of the heart and endocardial and endocardial-derived mesenchymal cells within the valves, and a valve-specific endocardial line (driven by a valve-specific enhancer), which only labels endocardium and not endocardium-derived mesenchymal cells (Wu *et al*., 2011; Zhou *et al*., 2005). The first Nfatc1-cre line has the obvious disadvantage of being expressed throughout the endocardium (and not only in valve endocardium) and therefore cannot be used for valve-specific gene manipulations. Similarly, *Tie2-cre*-driven gene manipulation is widely used for valve targeting, but is pan endothelial and hematopoietic (Kisanuki *et al*., 2001). The second *Nfatc1-Cre* line only marks those cells within the endocardial cushions that do not undergo EMT but remain within the endocardium as a proliferative population, which will participate in valve leaflet elongation. Therefore, gene manipulation does not take place in the endocardium-derived mesenchymal population of the cardiac cushions (Wu *et al*., 2011).

The recently constructed tamoxifen inducible $Msx1^{CreERT2}$ knock-in mouse line has been described previously (Lallemand *et al*., 2013). The aim of this study was to further analyze the cardiac Cre activation domain of this mouse line with particular emphasis on the valves. We found that this inducible Cre line is a useful tool for lineage tracing or gene manipulation (activation or deletion) during cardiac cushion formation, EMT induction and progression as well as valve remodeling.

RESULTS AND DISCUSSION

Early Embryonic Expression of $Msx1^{creert2}$ in the Endocardial Cushions

Previous studies described expression of *Msx1* in a subpopulation of endocardial and cushion mesenchymal cells during the formation of outflow tract (OFT) and atrioventricular canal (AVC) cushions (Supporting Information Fig. S1), suggesting that *Msx1* is a good marker to track endocardial cells during EMT (Chen *et al*., 2008; Hill *et al*., 1989; Robert *et al*., 1989). In this regard, we decided to use the $Msx1^{CreERT2}$ knock-in mouse line to perform lineage tracing of Msx1-expressing cells and their descendants prior and during EMT. In this model, the Cre^{ERT2} cassette was inserted into the first exon of the *Msx1* coding sequence (Lallemand *et al*., 2013). To examine the recombination activity of tamoxifen-activated CreERT2 prior and during EMT, $Msx1^{CreERT2}$ mice were crossed with $Rosa26R^{LacZ}$ ($R26R^{LacZ}$) or $Gt(ROSA)26Sor^{tm4(ACTB-tdTomato,-EGFP)Luo}$ ($Rosa^{mTmG}$) reporter lines that express β-galactosidase or green fluorescent protein (GFP) upon Cre-dependent recombination. Tamoxifen (3mg) was administrated to pregnant females by intraperitoneal injections on 3 days at E7.5, E8.5, and E9.5, whereby endogenous *Msx1* transcription begins (Catron *et al*., 1996; Houzelstein *et al*., 1997). When embryos were recovered at E10.5 (24 h after the last injection), the X-gal staining was similar to the endogenous *Msx1* expression pattern at E10.5, as recently described (Supporting Information Fig. S2) (Lallemand *et al*., 2013). In the absence of tamoxifen treatment, *LacZ* expression was not detected at the same stage of $Msx1^{CreERT2}$ embryos (Supporting Information Fig. S3), confirming the tight regulatory control of CreERT2 activation.

When embryos were recovered at E9.5, after a single injection at E8.5, and treated with X-gal solution, very few cells (20 ± 7%, mean ± SD, $n = 3$ embryos) were β-galactosidase (β-gal)-positive within the endocardial cushions of $Msx1^{CreERT2}$; $R26R^{LacZ}$ embryos (Fig. 1a), suggesting that transcriptional activation of *Msx1* in

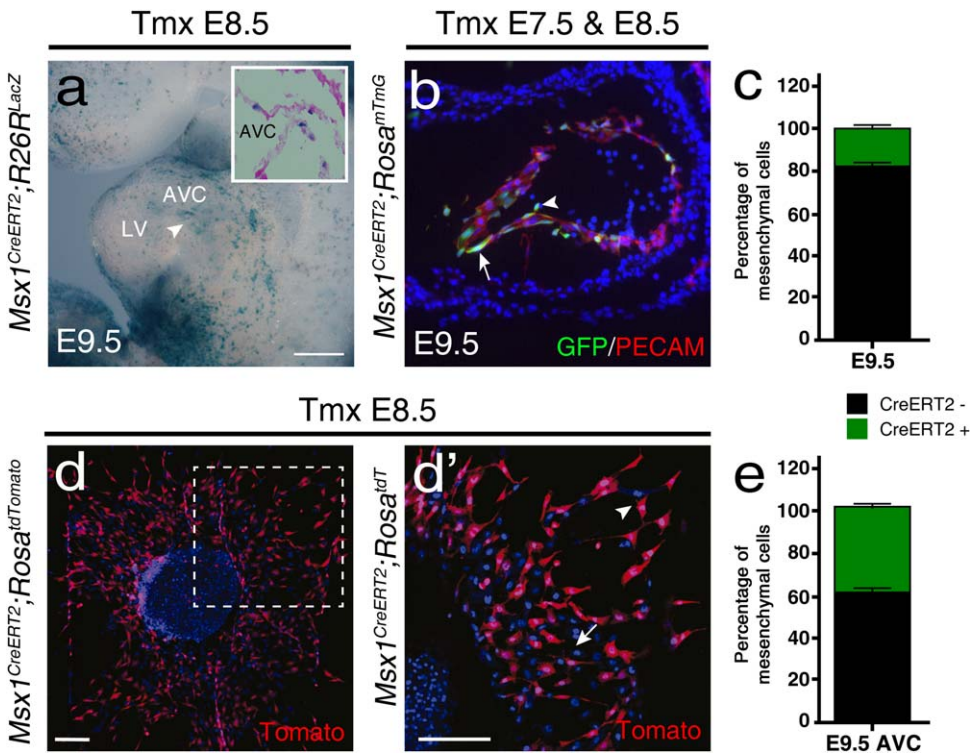


FIG. 1. Expression of *Msx1*^{CreERT2} allele in the cushions of the atrioventricular canal. Activation of CreERT2 in an Msx1-dependent manner between E7.5 and E9.5 marks a limited number of cells within the heart, localized very specifically in the endocardial cushions of the atrioventricular canal (AVC) and the outflow tract (OFT). (**a**) Single tamoxifen injection at E8.5 activates the Cre recombination of the *R26R*^{LacZ} reporter allele in 20 ± 7% (mean ± SD, n = 3 embryos) of cells among the cushion endocardial cell population as observed on E9.5 X-gal-stained *Msx1*^{CreERT2}*; Rosa*^{mTmG} heart. **(**Inset in a) Section of *Msx1*^{CreERT2}*; R26R*^{LacZ} embryos showing X-Gal-positive cells in the AVC. (**b,c**) Consecutive injections of tamoxifen in *Msx1*^{CreERT2}*; Rosa*^{mTmG} mice at E7.5 and E8.5 causes Cre recombination in 18 ± 5% (mean ± SD, n = 3 embryos) of cushion endocardial cells, as shown by co-localization of GFP (green) with PECAM (red) staining and the corresponding endocardial cell quantification. (**d, d', e**) Recombination in AVC explants of *Msx1*^{CreERT2}*; Rosa*^{tdTomato} embryos. Approximately 40 ± 3% of mesenchymal cells (mean ± SD, n = 6 AVC from two different litters) are labeled (arrowhead), and are clearly distinguishable from nonlabeled cells (arrow) in this setting. 140 μm Z-stack, inset (d) 40 μm Z-stack. Nuclei are marked with DAPI. AVC, atrioventricular canal; LV, left ventricle. Scale bars: 200 μm.

endocardial cells was limited at the time of injection. To examine the cell type expressing Cre we performed co-immunohistochemistry with PECAM, an endothelial/endocardial marker, on *Msx1*^{CreERT2}*; Rosa*^{mTmG} embryos, which received consecutive injections of tamoxifen at E7.5 and E8.5. A large majority of GFP-positive cells co-expressed PECAM (Fig. 1b,c), after two consecutive tamoxifen injections at E7,5 and E8.5, similar to the percentage achieved after one single injection at E9.5. Recombination efficiency was evaluated by counting GFP-positive cells divided by the total number of PECAM-positive cells. Approximately 18 ± 5% (mean ± SD, n = 3 embryos) of endocardial cells activated CreERT2 and were GFP-positive in the cushions (Fig. 1b,c). In order to quantify recombination within AVC endocardium-derived mesenchyme, we performed ex vivo AVC explant cultures from E9.5 *Msx1*^{CreERT2}; *Rosa*^{tdTomato} embryos 24 h after tamoxifen injection. Following fixation, direct imaging of the fluorescent reporter in the cellular outgrowth revealed that approximately 40 ± 3% (mean ± SD, n = 6 AVC from two different litters) of mesenchymal cells were tdTomato-positive (Fig. 1d,d',e). Thus, Cre activation in *Msx1*^{CreERT2} mice at E8.5 is sufficient to trace mainly the endocardial cell population that undergoes EMT. Of note, no recombined cells were observed within the epicardium of E13.5 and E14.5 embryos injected at E8.5 alone or E8.5 and E9.5 (Supporting Information Fig. S4), suggesting that contribution of the *Msx1*-Cre-lineage to the epicardium is absent or limited.

We further evaluated the efficiency of CreERT2 activation in the AVC and OFT during EMT of endocardial cells. In these experiments, *Msx1*^{CreERT2}*; R26R*^{LacZ} mice received single or multiple tamoxifen injections at different combinations between E7.5 and E9.5 (Fig. 2). When embryos were recovered at E10.5 from *Msx1*^{CreERT2}*; R26R*^{LacZ} females that received single (E9.5) or consecutive (E8.5 and E9.5) injections, X-gal staining revealed that recombined cells were located predominantly in the mesenchyme of AVC and OFT cushions (Fig. 2a,b, 2e-f), colocalizing largely but not completely with *Msx2*-expressing cells (Supporting

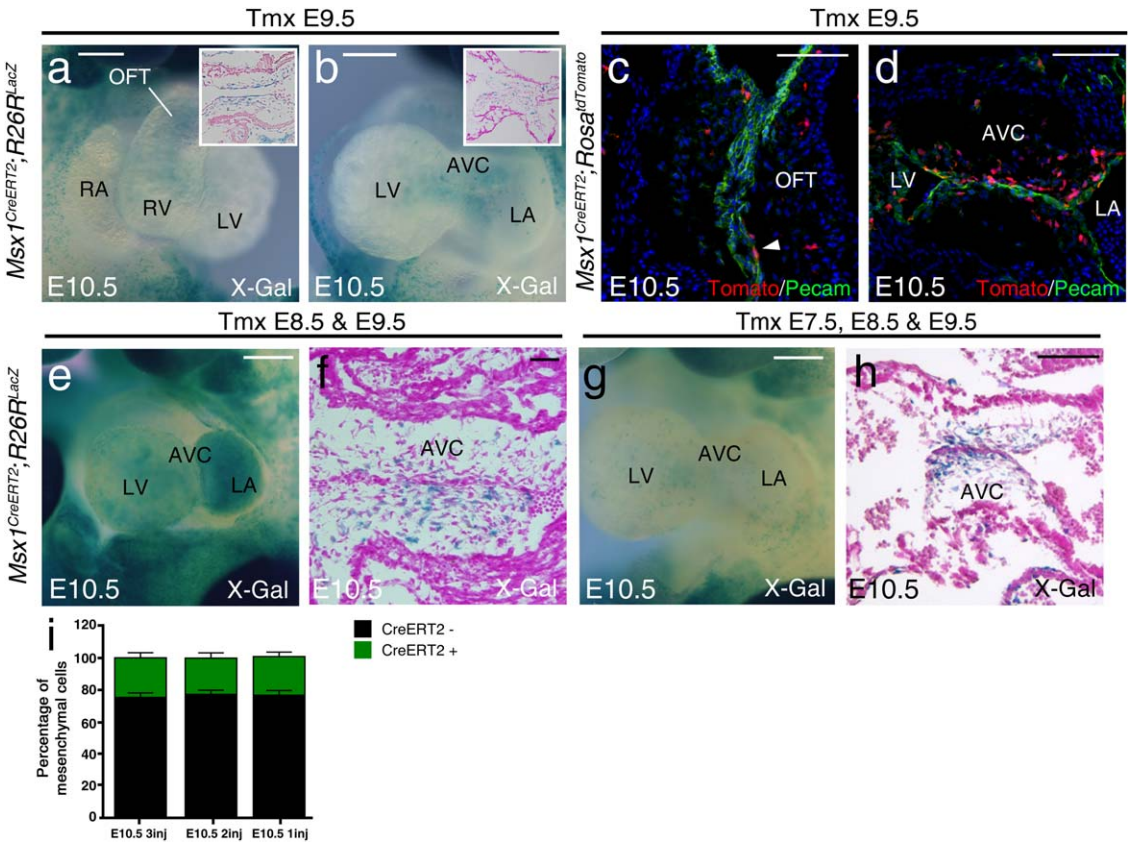


FIG. 2. *Msx1*^{CreERT2} recombination during endothelial-to-mesenchymal transition in the atrioventricular canal. (**a, b, e, g**) Whole-mount X-gal staining on *Msx1*^{CreERT2}*; R26R*^{LacZ} embryos recovered at E10.5. X-gal staining (**f, h**) and immunostaining (**c, d**) on sections of E10.5 embryos. Tamoxifen was injected at E9.5 (a–d), E8.5 and E9.5 (e, f) and E7.5, E8.5 and E9.5 (g, h); 23 ± 4% (single tamoxifen injection, b, d, i) and 24 ± 8% (two injections, f, i) of mesenchymal cells are β-gal-positive at E10.5. Cells that have expressed *Msx1* from E7.5 onwards end up in the cushion mesenchyme by E10.5 (25 ± 7% of mesenchymal cells, mean ± SD, *n* = 4 embryos, h, i). This means that the endocardial cells that are *Msx1*-positive between E8.5 and E9.5 eventually undergo EMT, detach from the endocardial layer and populate the cushion. (c, d) Injection of tamoxifen in *Msx1*^{CreERT2}*; Rosa*^{dtTomato} mice at E9.5 causes Cre recombination in a small number of mesenchymal cells in E10.5 OFT, whereas more mesenchymal cells expressing the Tomato-reporter (red) are observed in the AVC at E10.5. Endothelial cells are labeled by PECAM (green). Nuclei are marked with DAPI. LV: left ventricle; RV: right ventricle; AVC: atrioventricular canal; LA: left atrium; OFT, outflow tract; RA, right atrium. Scale bars: 200 μm (a, b, e, g), 100 μm (c, d, h), and 50 μm (f).

Information Fig. S5). This result was also observed by immunohistochemistry on *Msx1*^{CreERT2}; *Rosa*^{tdTomato} mice (Fig. 2c,d). At E10.5, AVC exhibited 23 ± 4% (single tamoxifen injection) and 24 ± 8% (two injections) of β-gal-positive cells (mean ± SD, $n = 3$ embryos) (Fig. 2i). However, when embryos were recovered from females which received three tamoxifen injections at E7.5, E8.5, and E9.5, quantification of β-gal-positive cells was not dramatically increased (25 ± 7% of mesenchymal cells, mean ± SD, $n = 4$ embryos) (Fig. 2g,h,i), suggesting that transcriptional activation of *Msx1* between E8.5 and E9.5 is sufficient to initiate recombination in endocardial cells that undergo EMT and then proliferate.

We next evaluated the contribution of *Msx1*-expressing cells to the developing valves after an early activation of the *Msx1*^{CreERT2} allele. X-gal staining revealed β-gal-positive cells in AV and semilunar valves, as observed at E13.5 (Fig. 3a,b), E17.5 (Fig. 3c,d), and P0 (Fig. 3e,f). More lacZ staining was consistently observed in atrioventricular compared with semilunar valves at this stage, probably due to the early activation of the CRE in the AVC than the OFT (Fig.2c,d). Leaky Cre activity was occasionally observed in some of the cells in the septum but the rest of the heart was negative for *Msx1*-expressing cells or their descendants. Taken together these results demonstrate that *Msx1*-expressing cells correspond to a subpopulation of endocardial cells that undergoes EMT and contributes to the formation of AV and semilunar valves.

Late CRE Activation

We next utilized *Msx1*^{CreERT2} mice to examine the activation pattern of the Cre when tamoxifen was injected at later stages and hence the contribution of *Msx1*-expressing cells to valve leaflet formation. In

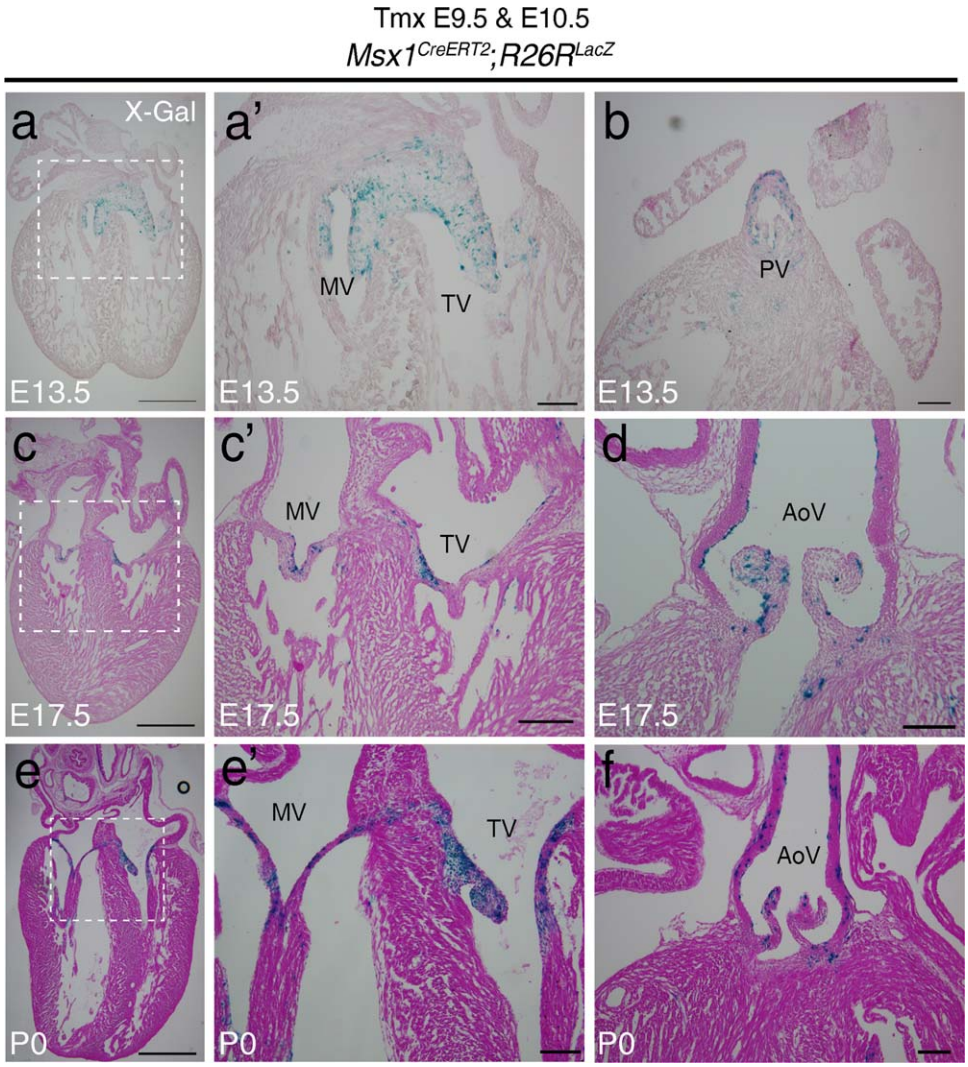


FIG. 3. Lineage tracing of *Msx1*^{CreERT2}-expressing cells in the heart at late embryonic (E13.5, E17.5) and neonatal (P0) stages. CreERT2 was activated by tamoxifen injections at E9.5 and E10.5. Embryos were recovered at E13.5 (**a, b**), E17.5 (**c, d**), and P0 (**e, f**). X-gal staining marks a large part of the valve leaflets. This suggests that the initial mesenchymal *Msx1*-positive cells proliferate during mid-gestation and expand within the valve leaflets. (**a', c', e'**) Higher magnifications of the zones delimited in panels a, c, and e, respectively. AoV, aortic valve; PV, pulmonary valve; MV, mitral valve; TV, tricuspid valve. Scale bars: Scale bars: 500 μm (a, c, e), 200 μm (c', e'), and 100 μm (a', b, d, f).

these experiments, we traced the *Msx1*-positive cells of both *Msx1*^{CreERT2}*; Rosa*^{mTmG} and *Msx1*^{CreERT2}*; R26R*^{LacZ} mice by consecutive tamoxifen injections at E14.5 and E15.5 or E16.5 and E17.5, respectively. Immunohistochemistry with an anti-GFP antibody marked exclusively mesenchymal cells (and not PECAM^{+} endocardial cells) in the AV and semilunar valves of *Msx1*^{CreERT2}*; Rosa*^{mTmG} hearts at E16.5 (Fig. 4a-d). We found that 23 ± 5% (mean ± SD, n = 3 embryos) of the AV valve mesenchyme and 30 ± 7% (mean ± SD, n = 3 embryos) of the semilunar valve mesenchyme were stained. The fact that no endocardial cells were stained at this stage supports the idea that after initial activation of endocardial cushion cells at E9.5 and their subsequent transition to mesenchymal cells, *Msx1* is activated mainly (if not exclusively) in the mesenchymal proliferating population. Similarly, injections at E16.5 and E17.5 caused CreERT2 activation very specifically in the valves just before birth (E18.0). The activation of the Cre at these stages seems weaker compared with earlier stages suggesting a progressively restricted pattern of lacZ-expressing cells (Fig. 4e-i). Interestingly, X-gal staining was mainly located at the distal region of the mitral and tricuspid valves (Fig. 4h,i). Since neural crest cells contribute to semilunar valve formation (Kirby, 2007), and that *Msx1* expression was observed in the dorsal region of the neural tube (Supporting Information Fig. S1 and S2) (Hill *et al.*, 1989; Robert *et al.*, 1989), we examined the implication of neural crest cells expressing *Msx1* to the formation of

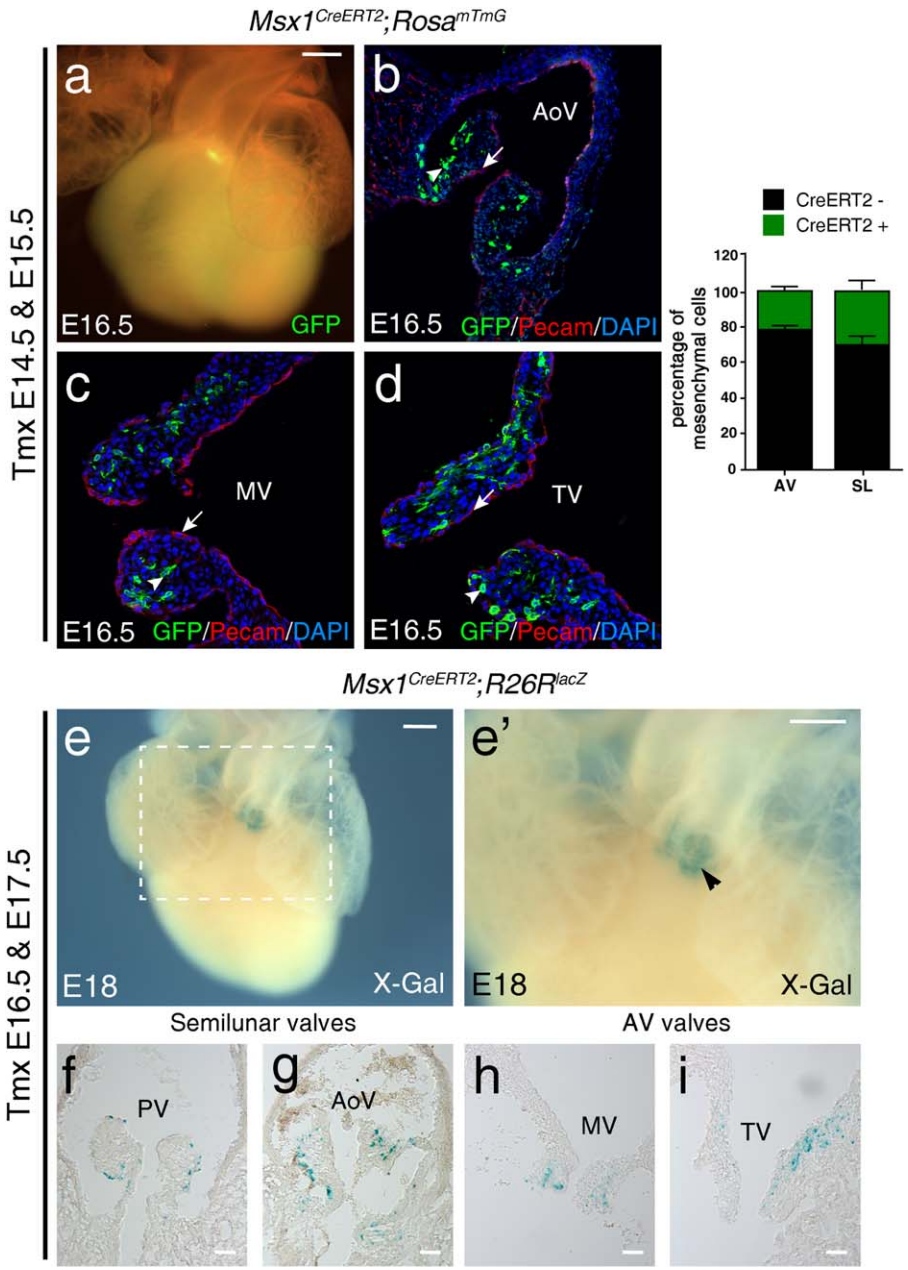


FIG. 4. Activity of *Msx1*^{CreERT2} in the heart at late embryonic stages. Late embryonic (E16.5 and E18.0) activity of cre after tamoxifen injections at E14.5 and E15.5 or E16.5 and E17.5, respectively. Late Msx1-dependent CreERT2 activation causes restricted recombination of cells within the cardiac valves. (**a**) Whole-mount image of an E16.5 *Msx1*^{CreERT2}*; Rosa*^{mTmG} heart injected with tamoxifen at E14.5 and E15.5. Not recombined cells are stained red with the membrane tomato reporter (mTomato), while Cre-recombined cells are stained green (GFP). (**b–d)** 10 μm cryosections of E16.5 *Msx1*^{CreERT2}*; Rosa*^{mTmG} hearts injected at E14.5 and E15.5, immunostained with anti-GFP (green) and anti-Pecam1 (red) antibodies, marking Msx1-expressing and endocardial cells, respectively. Not recombined endocardial cells are shown with arrows and recombined mesenchymal GFP^{+} cells with arrowheads. Nuclei are marked with DAPI. The graph shows quantification of mesenchymal cells at E16.5 after two consecutive injections at E14.5 and E15.5. 23 ± 5% and 30 ± 7% of mesenchymal cells (mean ± SD, *n* = 3 embryos) were CreERT2+ (GFP+) in the atrioventricular (AV) and semilunar (SL) valves, respectively. (**e**) Whole-mount image of an E18.0 *Msx1*^{CreERT2}*; Rosa*^{lacZ} heart injected at E16.5 and E17.5 (**é.** inset, magnification). Recombined cells are seen in blue after X-Gal β-galactosidase-dependent staining. 10 μm cryosections of the valves of this heart are shown in panels **f–i.** AoV, aortic valve; PV, pulmonary valve; MV, mitral valve; TV, tricuspid valve. Scale bars: 50 μm.

OFT endocardial cushions. To examine neural crest cells expressing Cre we performed co-immunohistochemistry with AP-2α, a neural crest marker, on *Msx1*^{CreERT2}*; Rosa*^{tdTomato} embryos. Frontal sections of E9.5 embryos, which received injections of tamoxifen at E7.5 and E8.5 revealed co-localization of AP-2α and Tomato-reporter

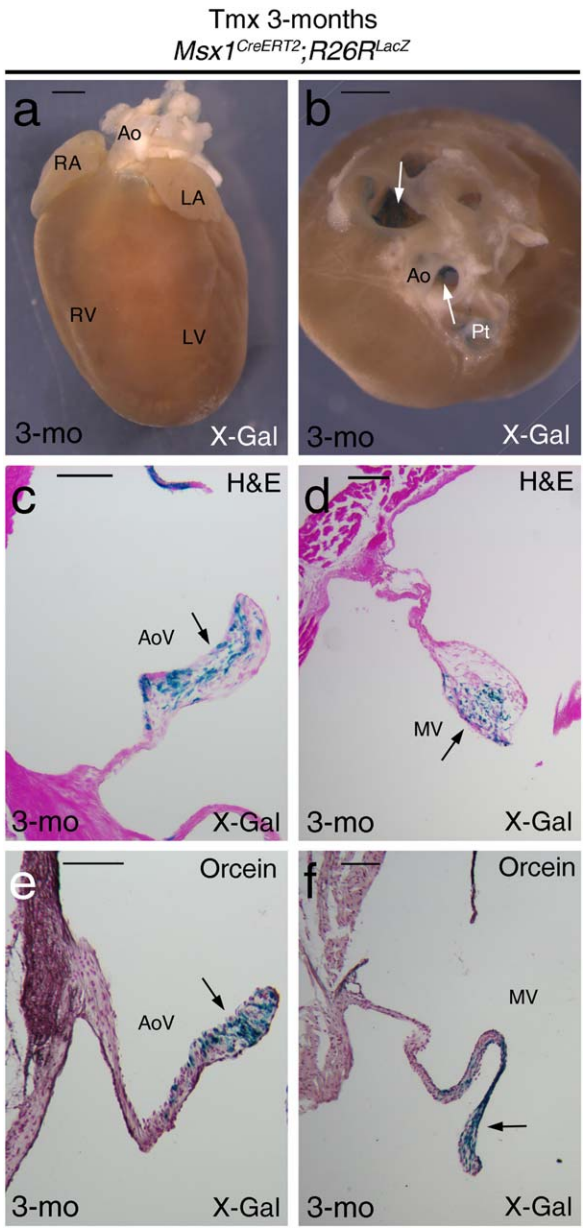


FIG. 5. *Msx1*^{CreERT2} recombination in the adult heart. Three-month-old *Msx1*^{CreERT2}*; R26R*^{LacZ} mice were injected with a single dose (3 mg) of tamoxifen. Mice were sacrificed 1 week after injection. Whole-mount images (**a, b**) and 10 μm sections (**c–f**) stained with hematoxylin and eosin (H&E, c, d) or orcein (e, f), of a 3-month-old heart reveal CreERT2 activity exclusively in the cardiac valves (arrows). Elastic fibers within the AV valves are marked with Orcein staining in e and f. Scale bars represent 1 mm (a, b) and 100 μm (c–f). RA, right atrium; LA, left atrium; RV, right ventricle; LV, left ventricle; Ao, aorta; Pt, pulmonary trunk; AoV, aortic valve; MV, mitral valve; TV, tricuspid valve.

gene in migrating neural crest cells located into branchial arches 1, 2, and 3 (Supporting Information Fig. S6). However, transverse sections of the caudal pharynx at E10.5 did not reveal *CreERT2* activation in neural crest cells entering the OFT (Supporting Information Fig. S6), suggesting that neural crest cells that express *Msx1* do not contribute to the OFT region that patterns semilunar valves. At E17.5, we observed co-localization of Tomato-reporter with alpha smooth muscle actin (α-SMA) of the ascending aorta from *Msx1*^{CreERT2}*; Rosa*^{tdTomato} fetuses (Supporting Information Fig. S6), suggesting a contribution of Msx1-expressing neural crest cells to smooth muscle cells of the great arteries.

Finally, we examined the activity of *Msx1*^{CreERT2} allele in the adult heart. For this purpose, *Msx1*^{CreERT2}; *R26R*^{LacZ} mice (n = 3) were injected with 3 mg of tamoxifen at 3 months of age. When hearts were recovered 7 days after injection, X-gal-positive cells were observed in the valve leaflets (Fig. 5). Although the localization of the cells is not restricted to a single area of the leaflets, there is a tendency for the lacZ-expressing cells to accumulate to the distal part of the valve leaflet. Our results confirmed that, after birth, the *Msx1*^{CreERT2} allele drives CreERT2 activity in valve leaflets.

In conclusion, here we have expanded the previous characterization of the *Msx1*^{CreERT2} line (Lallemand *et al.*, 2013) by describing its usefulness for lineage tracing and conditional gene manipulation of AV and semilunar valves. It should be noted though that the activation of the CRE, although specific within the heart, is quite broad throughout the embryo (Lallemand *et al.*, 2013) and this should be taken into account in future studies. Since *Msx1* is a marker for a subpopulation of mesenchymal cells in the AVC and OFT (Ma *et al.*, 2005), we hypothesize that adult valve interstitial cells derive from *Msx1*-expressing cells. These cells could be important to confer their biomechanical properties to the adult cardiac valves. Therefore, the *Msx1*^{CreERT2} allele will enable future investigation into potential existence of mesenchymal progenitor cells in adult mice.

METHODS

Mice

All mouse experiments were carried out using protocols approved by the "comité d'éthique pour l'expérimentation animale" and conformed to Directive 2010/63/EU of the European Parliament. *Msx1*^{CreERT2}*; Rosa*^{mTmG}, *Msx1*^{CreERT2}*; Rosa*^{LacZ}, and *Msx1*^{CreERT2}*; Rosa*^{tdTomato} transgenic male mice were used throughout the current study, crossed either with SWISS/CD1 or C57Bl/6J female mice (Muzumdar *et al.*, 2007; Soriano, 1999). Transgenic lines used in this study will be available to the research community upon request.

Tamoxifen Injections

Pregnant females were injected with 3 mg of tamoxifen (diluted in corn oil) intraperitoneally once or every 24 h for 2 to 3 consecutive days, depending on the required protocol.

Embryos were collected at the appropriate stage, fixed in 4% paraformaldehyde for 30 min at 15 to 25°C and processed either for Whole-mount X-gal staining or for embedding (in paraffin or in OCT). Embedded embryos were then microtome or cryostat sectioned at 10 μm.

X-Gal Staining on Sections or Whole-Mount

X-gal staining solution (400 μg/ml 5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside (Xgal) in dimethylformamide; 4 mM potassium ferrocyanide [K4Fe(CN)6] in 100 mM sodium phosphate buffer, pH 8.0; 4 mM potassium ferricyanide [K3Fe(CN)6] in 100 mM sodium phosphate buffer, pH 8.0; 2 mM magnesium chloride; 0.02% NP40; 1× PBS) was applied on paraffin sections, cryosections, or whole embryos for an overnight incubation at 37°C. Slides carrying sections were then counterstained with eosin for 5 min, dehydrated into xylene through short sequential incubations in increasing ethanol solutions and mounted with Eukitt mounting medium.

Immunohistochemistry on Paraffin Sections and Cryosections

Sections were deparaffinized in xylene (when necessary) and rehydrated into distilled water through a series of ethanol solutions of decreasing concentration. Tissue permeabilization was performed in PBS containing 0.1% Triton-X for 30 min at 15 to 25°C. Blocking of unspecific binding was achieved with a 2% inactivated horse serum-containing solution for several hours at 15 to 25°C. Primary antibody-containing PBS solutions were applied overnight at 4°C. The next day, after thorough washing, secondary antibody solution was applied for 2 h at 15 to 25°C, followed by more washes in PBS, incubation in DAPI-containing PBS for 10 min and mounting with Fluoromount.

AVC Explant Cultures

Gestating females were induced with a single dose of tamoxifen at E8.5. For AVC explant cultures, embryos were isolated at E9.5 and AVCs were dissected as previously described (Luna-Zurita *et al.*, 2010; Runyan and Markwald, 1983). Briefly, AVCs dissected, opened and placed endocardial side down on collagen type I gels (Collagen type I from rat tails—EMD Millipore, 10× DMEM—Invitrogen, NaOH 1M, H_20) overnight in a cell culture incubator. The following day, culture media (DMEM 10% fetal calf serum, Insulin-Transferrin-Selenium—SIGMA, P/S—Invitrogen) was added. Explants were fixed (4% PFA) following 48 h in culture and imaged using an SP5 confocal microscope (Leica).

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