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Prominent Expression of *Lysyl Oxidase* During Mouse Embryonic Cardiovascular Development

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ABSTRACT

By mRNA differential display in mouse hearts, *lysyl oxidase* (*Lox*), a key enzyme catalyzing cross-links in elastin and collagens, was found to be up-regulated between embryonic days 11 (E11) and 13 (E13). This was confirmed by semiquantitative RT-PCR. We analyzed its spatio-temporal expression pattern by in situ hybridization in regard to the development of myocardial cells, endocardial cushion tissue, aortic arch vessels, and epicardium. Anat Rec Part A 270A:93–96, 2003. © 2003 Wiley-Liss, Inc.

Key words: differential display; extracellular matrix; aorta; heart development

Cardiac connective tissue induction, such as in cardiac valves and septa formation, is a critical step in the morphogenesis of the embryonic heart. The separation of the cardiac outflow tract and ventricular inflow occurs on embryonic days 11 (E11) and E13 in the mouse, respectively (Gittenberger-de Groot et al., 1995). After epithelial-mesenchymal transformation, the mesenchymal cells proliferate within the endocardial cushion tissue, produce extracellular matrix proteins, and eventually give rise to the dense connective tissue-like cardiac valves. The extracellular matrix is known to provide the embryonic cells and tissues with a proper microenvironment for optimum development, especially cell differentiation, organization, and migration (Campbell, 1995). In addition, the extracellular matrix may play an important role as functional properties of the developing myocardial change (Campbell, 1995).

To screen for genes that are important for cardiac connective tissue induction, we performed mRNA differential display (Liang and Pardee, 1992) with E11 and E13 mouse embryonic hearts. One of the differentially expressed genes is *lysyl oxidase* (*Lox*), a copper-dependent amine oxidase that plays a critical role in the biogenesis of connective tissue matrices by catalyzing lysine-derived cross-links in collagens and elastin (Smith-Mungo and Kagan, 1998). Although *Lox* activity is known to be regulated during embryonic development (Wu et al., 1992; Tchaparian et al., 2000), its role in embryonic heart development is poorly understood. In the present work we report that *Lox* is prominently expressed in the cardiovascular system during embryonic development.

C57Bl/6J mice (Jackson Laboratory) were used in this study. The morning a vaginal plug was found was designated as embryonic day 0 (E0). Fixation and permeabilization of the embryos was performed as described previously (Tsuda et al., 2001). The embryos were cryosectioned at 7 μm for in situ hybridization.

We utilized mRNA differential display, semiquantitative reverse transcription polymerase chain reaction (RT-PCR), and in situ hybridization in this study. Total RNA was isolated from E11, E13, and E15 fresh embryonic hearts (ToTALLY RNA™; Ambion, Austin, TX) and treated with DNase I (Message Clean™; Gene Hunter, Nashville, TN). Approximately 40, 16, and eight embryos were used to isolate total RNA for E11, E13, and E15 embryonic mouse heart RNA, respectively. The mRNA differential display was performed according to the proto-

Grant sponsor: National Institutes of Health; Grant number: GM55625.

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Received 28 July 2002; Accepted 9 October 2002
DOI 10.1002/ar.a.10002

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col recommended by the manufacturer (Gene Hunter). First strand cDNA reaction was performed with MMLV reverse transcriptase (Life Technologies, Rockville, MD) and oligo-dT primer from the same amount of total RNA (1 μg). Duplicates of the RT reaction products were amplified by PCR in the presence of [α-^{32}P]dCTP, and the PCR products were separated on a 6% polyacrylamide gel. The primers used in this display were H-T_{11}G (5′-AAGCTTTTTTTTTTTG-3′) and H-AP4 (5′-AAGCTTCT-CAACG-3′).

PCR amplifications for *Lox* and mouse *glyceraldehyde-3-phosphate* dehydrogenase (GAPDH) were performed with Taq polymerase for 22, 24, and 26 cycles, and for 16, 18, and 20 cycles, respectively, to ensure the amplification reactions would be in the linear range. For *Lox*, 24 cycles of amplification with the forward primer (5′-ATATAGGGGCGG-ATGTCAGAG-3′; GenBank accession number M65142, nucleotides 991–1010) and reverse primer (5′-CGAATG-TCACAGCGTACAAC-3′, nucleotides 1446–1427) were performed. For GAPDH, 18 cycles of amplification with the forward primer (5′-GATTGTTGCCATCAACGACC-3′) and reverse primer (5′-TCCACGACATACTCA GCACC-3′) were performed. PCR conditions were an initial denaturation at 94°C for 10 min, followed by 16–26 cycles of 94°C for 30 sec, 55°C for 30 sec, and 72°C for 1 min, and a final extension of 7 min.

In situ hybridization was performed with a 359 bp mouse *Lox* cDNA (nucleotides 1053–1411; GenBank M65142) labeled with α [^{35}S]-UTP (ICN, Costa Mesa, CA) following the a previously described protocol (Wawersik and Epstein, 2000; Tsuda et al., 2001). Sense probe was used as a negative control.

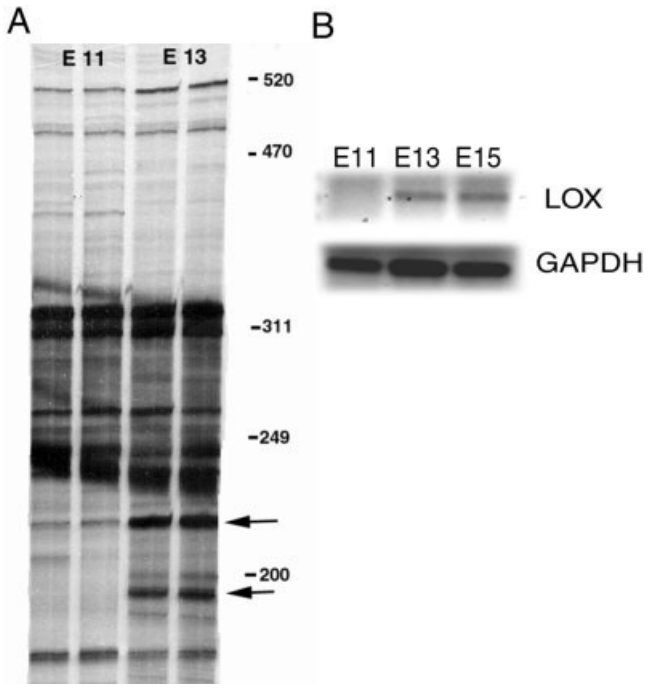

Fig. 1. **A:** mRNA differential display between total RNA from E11 and E13 mouse embryonic hearts. The arrows indicate two differentially expressed bands that are both up-regulated at E13. The cDNA sequences of both bands were 100% matched with two polymorphic mouse *lysyl oxidase* (*Lox*) transcripts, which utilize different polyadenylation sites. Numbers listed on the right side show the DNA size in nucleotides. **B:** Semiquantitative RT-PCR of *Lox* and GAPDH was performed with E11, E13, and E15 embryonic heart RNA. PCR products of the expected sizes were separated on a 1% agarose gel. *Lox* was barely seen at E11, but was up-regulated at E13 and E15. The identical results were obtained from two separate series of mouse embryonic heart RNA of E11, E13, and E15.

RESULTS AND DISCUSSION

Lox Is Up-Regulated at E13 in Mouse Embryonic Heart

We performed an mRNA differential display to search for the genes responsible for morphologic changes (i.e., induction and development of endocardial cushion tissue in the atrioventricular canal) in the mouse embryonic heart between E11 and E13. Two of the differentially expressed transcripts were found to be the polymorphic transcripts for *Lox* (Fig. 1A). The up-regulation of *Lox* mRNA in the embryonic heart from E11 to E13 and E15 was confirmed by semiquantitative RT-PCR analysis (Fig. 1B). Because total RNA was collected exclusively from embryonic hearts without aortic arch vessels, our finding suggests that *Lox* is up-regulated within the heart proper at E13 and E15. *Lox* expression has previously been shown to increase during development of embryonic chick aorta (Wu et al., 1992). We, therefore, investigated its involvement in the morphogenesis of the endocardial cushion tissue during cardiac development, and its expression pattern in the mouse embryo.

Lox Gene Is Expressed in Accordance With the Development of Myocardium, Endocardial Cushion Tissue, Aortic Arch Vessels, and Epicardium

In situ hybridization of E11.5 mouse embryos showed that *Lox* is weakly expressed in the myocardium of the tubular heart, but is not seen in mesenchymal cells in the endocardial cushion tissue (Fig. 2B and C). By E11.5, the outflow tract has usually completed septation, whereas the atrioventricular canal remains as a single orifice. The ventricular myocardium begins to show trabeculation, but is still a thin, two-layer structure. These myocardial cells are the predominant source of *Lox* (Fig. 2D). At this time, *Lox* is not expressed in the proximal aortic arch vessels (Fig. 2B). At E13.5, *Lox* expression becomes not only more prominent, but also quite distinct from the expression at E11.5. *Lox* expression is particularly strong in the media of aortic arch vessels (Fig. 2E and J). In the outflow tract, the prominent expression of *Lox* is seen on the vascular side (Fig. 2G and H) and not on the myocardial side, which appears to be remarkably different from the previous expression pattern at E11.5. The prominent expression in the media of aortic arch vessels suggests that *Lox* may be important in organizing ECM assembly during vascular smooth muscle cell proliferation and differentiation. Low to moderate expression is also observed in the mesenchymal cells in the endocardial cushion tissue of the outflow tract (Fig. 2G and H) and the atrioventricular canal (Fig. 2I). The expression in the myocardial cells, however, is no longer discernable. At E14.5, *Lox* is also seen in the epicardium and, to a lesser extent, in the compact zone of ventricular myocardium (Fig. 2K). Epicardial cells undergo epithelial–mesenchymal transformation and give rise to coronary vascular cells (Vrancken Peeters et al.,

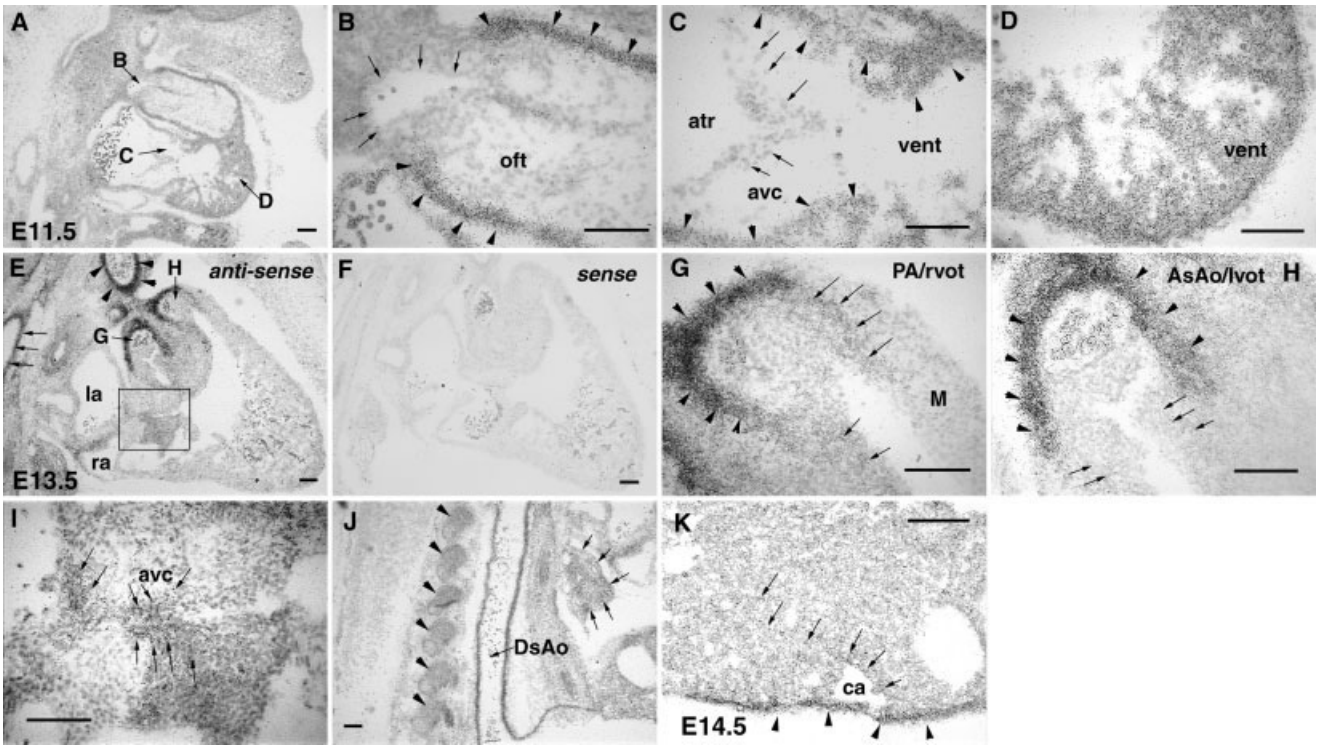

Fig. 2. In situ hybridization of *Lox* in E11.5 (**A–D**), E 13.5 (**E–J**), and E14.5 (**K**) mouse embryos. A: Low magnification of E11.5 mouse embryo shows that the overall expression of *Lox* transcript is not remarkable. B: Higher magnification of the cardiac outflow tract in panel A. *Lox* expression is restricted to the myocardial layer (arrowheads). Note that there is no up-regulation of *Lox* around the aortic arch vessel (arrows). At this stage, mesenchymal cells in the outflow cushion do not express *Lox*. oft, outflow tract. C: Higher magnification of the atrioventricular canal (avc) cushion tissue in panel A. *Lox* expression is predominantly seen in the myocardial layer (arrowheads). Again, mesenchymal cells (arrows) have not increased in number and do not express *Lox*. Most of the avc cushion tissue is occupied with acellular ECM. atr, atrium; vent, ventricle. D: Higher magnification of the trabeculated myocardium in the ventricle (vent). *Lox* expression is predominantly seen in the myocardial cell layer. E–J: *Lox* mRNA expression in E13.5. E: Low magnification of an embryonic heart in a sagittal section. *Lox* transcript is predominantly localized at the wall of aortic arch vessels (G and H). la, left atrium; ra, right atrium. Arrowheads and arrows indicate the ascending and descending aortas, respectively. F: Hybridization with a sense riboprobe as a negative control. G: Higher magnification of the pulmonary artery (PA) and right ventricular outflow tract (rvot). The *Lox* transcript is most predominantly seen at the vessel wall of the main PA (see arrowheads), but it is also expressed in the mesenchymal cells within the endocardial cushion tissue of the right ventricular outflow tract (see arrows). *Lox* is not expressed in the infundibular myocardium (M). H: Higher magnification of the ascending aorta (AsAo) and left ventricular outflow tract (lvot). Similarly, *Lox* transcript is predominantly expressed within the aortic wall and, to a lesser extent, in the mesenchymal cells of left ventricular outflow tract cushion tissue (arrows). I: This is an area highlighted in part E, wherein *Lox* transcript is moderately expressed in the mesenchymal cells within the avc cushion tissue (arrows). The number of mesenchymal cells is significantly increased compared with E11.5 (part C). J: Another low magnification of the embryo in a sagittal section, more dorsal and caudal to part E. *Lox* transcript is also seen at the wall of the descending aorta (DsAo), vertebral bodies (arrowheads), and lung parenchyma (arrows). K: *Lox* mRNA expression at E14.5 embryonic heart. The *Lox* transcript is now predominantly seen in the epicardial cells (arrowheads) and, to a lesser extent, in the ventricular myocardium, probably in the interstitial cells (arrows). ca, coronary artery. The results of mRNA expression pattern by in situ hybridization were reproducible in three different embryos at E11.5 and E13.5, and in two at E14.5. Magnification bars = 100 μm.

1999). The development of the compact myocardium and the coronary vascular supply have to be closely linked. The up-regulation of *Lox* in the epicardium and in the interstitial tissue of the compact myocardium may be related to ECM remodeling during the coronary vascular development. In addition to the cardiovascular system, *Lox* is expressed in the lungs, chondrocytes of the vertebral bodies (see Fig. 2J), and skin (presumptive dermis (not shown)) at E13.5. Because all of these developing tissues are enriched with collagens and elastin, the mRNA localization is consistent with a specific role for *Lox* in stabilizing early ECM assembly by cross-linking collagens and elastin for optimal tissue differentiation.

Our studies show that *Lox* is spatiotemporally regulated during early embryonic heart development, specifically in differentiation of myocardial cells, smooth muscle cells of aortic arch vessels, mesenchymal cells of endocardial cushion tissue, epicardial cells, and (probably) coronary vessels. *Lox* expression is markedly up-regulated at E13 during mouse embryonic development, and is most intense in the aortic arch vessels. Because elastin is the major constituent of the aortic arch vessels and regulates the proliferation of smooth muscle cells (Li et al., 1998), the expression pattern of *Lox* suggests an essential role associated with elastogenesis during cardiovascular development (Hurle et al., 1994; Davis, 1995).

ACKNOWLEDGMENTS

We thank Hui Wang for her technical assistance.

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