Abstract The bone morphogenetic proteins BMP-2 and BMP-4 and the homeobox gene MSX-2 are required for normal development of many embryonic tissues. To elucidate their possible roles during the remodeling of the tubular heart into a fully septated four-chambered heart, we have localized the mRNA of Bmp-2, Bmp-4, Msx-2 and apoptotic cells in the developing mouse heart from embryonic day (E) 11 to E17. mRNA was localized by in situ hybridization, and apoptotic cells by TUNEL (TdT-mediated dUTP-biotin nick end-labeling) as well as by transmission electron microscopy. By analyzing adjacent serial sections, we demonstrated that the expression of Msx-2 and Bmp-2 strikingly overlapped in the atrioventricular canal myocardium, in the atrioventricular junctional myocardium, and in the maturing myocardium of the atrioventricular valves. Bmp-4 was expressed in the outflow tract myocardium and in the endocardial cushion of the outflow tract ridges from E12 to E14. Msx-2 appeared in the mesenchyme of the atrioventricular endocardial cushion from E11 to E14, while Bmp-2 and Bmp-4 were detected between E11 and E14. Apoptotic cells were also detected in the mesenchyme of the endocardial cushion between E12 and E14. Our results suggest that BMP-2 and MSX-2 are tightly linked to the formation of the atrioventricular junction and valves and that BMP-4 is involved in the development of the outflow tract myocardium and of the endocardial cushion. In addition, BMP-2, BMP-4 and MSX-2 and apoptosis seem to be associated with differentiation of the endocardial cushion.

Keywords Heart development · BMP-2 · BMP-4 · MSX-2 · Apoptosis · Mouse (NMRI)

Introduction Cardiovascular malformations are the most common birth defects (Eisenberg and Markwald 1995) and the majority are due to abnormal septum and valve formation (Clark 1987). The remodeling of the AV cushion into valves is a critical event in the developing heart and its abnormal development leads to congenital heart malformations in man (Goor and Lillehei 1975). The goal of this study was to characterize the expression patterns of the closely related candidates BMP-2, BMP-4 and MSX-2 during critical phases of cardiac patterning and endocardial cushion remodeling of the developing mouse heart.

Bone morphogenetic proteins (BMPs) are members of the transforming growth factor (TGF) β superfamily of growth factors. They are involved in many developmental processes including epithelial-mesenchymal interactions, with key roles in the development of practically all organs analyzed (Vainio et al. 1993; Hogan 1996). In addition, they may exhibit several differences in their expression patterns between the mouse and chick (Hogan 1996). Numerous developing tissues produce mRNA of both Bmp ligands, as well as Bmp receptors at various sites in which epithelial-mesenchymal interactions occur (Dewulf et al. 1995; Jones et al. 1991; Lyons et al. 1990; Mishina et al. 1995; Zhang and Bradley 1996; Nakajima et al. 2000). In the mouse embryo, Bmp-2 has been found to be expressed during formation of the four-chambered heart.
in the myocardium of the regions where the formation of endocardial cushion takes place (Lyons et al. 1990), in the promyocardium and surrounding mesodermal cells at embryonic day (E) 8 and in the atrioventricular canal of the embryonic heart at E9.5 (Zhang and Bradley 1996). In the embryonic chick heart, Bmp-2 transcripts have also been detected in the myocardium adjacent to the endocardial cushion but not in the endocardial cushion itself (Yamagishi et al. 1999a). Similarly, in the rat embryo, Bmp-2 expression has been detected in the atrioventricular region of the heart at E15 and E18 (Ikeda et al. 1996). Information about Bmp-2 expression in the endocardial cushion has remained questionable. On the other hand, Bmp-4 has been detected in the atrium of the rat heart at E13, E15 and E18 (Ikeda et al. 1996). In the mouse heart, Bmp-4 expression has been detected by Jones et al. (1991) in the outer myocardial layer of the developing atrioventricular canal at E9 and in the truncus arteriosus at E10.5; however, the same study reported Bmp-4 expression between E11.5 and E17.5 in other embryonic tissues but not in the heart. In addition, Bmp-6 (Vgr-1), Bmp-3, Bmp-5, and Bmp-7 (OP-1) are all expressed in the developing heart (Jones et al. 1991; Dudley and Robertson 1997).

Previous investigations in the developing mouse heart have reported the expression of transforming growth factors: TGFβ1 in endothelial cells, TGFβ2 in the myocardium adjacent to the endocardial cushion tissue region and TGFβ3 in the condensed mesenchyme (Akhurst et al. 1990; Pelton et al. 1990; Millan et al. 1991; Dickson et al. 1993; Nakajima et al. 2000). In the embryonic chick heart, TGFβ3 mRNA and protein are localized to the myocardium, endocardium, and mesenchyme of the outflow tract and the atrioventricular regions (Nakajima et al. 1994, 1998). Furthermore, development of the endocardial cushion occurs under the potential control of TGFβs (Potts and Runyan 1989; Choy et al. 1990; Ramsdell and Markwald 1997; Qu et al. 1998; Boyer et al. 1999; Yamagishi et al. 1999a, 1999b; Nakajima et al. 2000).

The MSX-2 gene is the mammalian counterpart of the Drosophila msh (muscle segment homebox) gene (Davidson 1995). In the developing chick heart, Msx-2 has been found to be restricted to a distinct subpopulation of myocardial cells (Chan-Thomas et al. 1993). Msx-1 but not Msx-2 has been detected in the embryonic chick endocardial cushion (Chan-Thomas et al. 1993). Interestingly, Msx-2 has been detected at E13.5 in the mouse endocardial cushion (Lakkis and Epstein 1998), and at E9.5 in the developing atrioventricular myocardium of the mouse (Tanaka et al. 1999). Information about other embryonic days is incomplete.

Signs of cell death have been found previously in the embryonic heart (Pexieder 1975; Hurle and Ojeda 1979). Apoptotic DNA fragmentation has been observed in the outflow tract of the chicken (Watanabe et al. 1998; Poellmann et al. 1998), of the rat (Takeda et al. 1996) and of the mouse (Zhao and Rivkees 2000). Apoptosis was found in the mouse atrioventricular endocardial cushion at E12.5 (Lakkis and Epstein 1998) and in the developing mouse cushion forming the atrioventricular valves after E14.5 (Zhao and Rivkees 2000). Apoptosis at other stages critical for valvogenesis and remodeling of the endocardial cushion has not been reported. In addition, detection of apoptosis in the mouse endocardial cushion has been dependent only on TUNEL (DTD-mediated dUTP-biotin nick end-labeling) analysis. Thus, the ultrastructural gold standard demonstration of the apoptosis observed by TUNEL in the endocardial cushion of the mouse heart is essential. It has been shown that BMP-2, BMP-4 and MSX-2 function in common interacting signaling pathways (Maas and Bei 1997; Stock et al. 1997; Thesleff and Pispa 1998; Thesleff and Sharpe 1997; Ferrari et al. 1998). They are generally involved in morphogenesis, cell differentiation and also in the induction of apoptosis (Graham et al. 1994; Zou and Niswander 1996; Coucouvanis and Martin 1999; Rice et al. 1999; Ferrari et al. 1998). Thus, although data on these genes have been published, it is important to detail and clarify their patterns of expression. It is hoped that this will help to characterize the mechanisms that pattern the four-chambered heart. Here we mapped their temporal and spatial relationships and the distribution of apoptotic cells in the mouse heart from E11, when the heart is a primitive loop, until formation of the prenatal heart at E17. Our results suggest that BMP-2 and MSX-2 are tightly linked to the formation of the atrioventricular junction and valves and that BMP-4 is involved in the development of the outflow tract myocardium and of the endocardial cushion. In addition, BMP-2, BMP-4 and MSX-2 and apoptosis seem to be associated with differentiation of the endocardial cushion.

### Materials and methods

#### Preparation of the tissues

Approval for the use of laboratory animals was obtained from the Ethics Committee of the University of Turku. NMRI mice embryos were collected at E11, E12, E13, E14, and E17 of gestation. Mating was confirmed by the presence of a vaginal sperm plug at noon. This was designated day 1 of embryonic development. Whole embryos were rapidly transferred to freshly prepared 4% paraformaldehyde in phosphate-buffered saline (PBS) and fixed overnight at +4°C, dehydrated, and embedded in paraffin. Serial sections, 5–7 μm in thickness, were cut and mounted onto silanized Superfrost slides and stored at +4°C. For histology, sections were stained with hematoxylin and eosin. To confirm possible overlapping of apoptotic cells with gene expression patterns, consecutive sections were cut and placed on different slides. They were then hybridized with mRNA probes for Bmp-2, Bmp-4 and Msx-2, as well as checked for apoptosis by TUNEL reaction.

#### Detection of Bmp-2, Bmp-4 and Msx-2 mRNA

by in situ hybridization

Preparation of the mRNA probes has been described previously (Vainio et al. 1993; Jernvall et al. 1998). In situ hybridization on tissue sections was performed using [35S]-UTP-labeled riboprobes as described previously (Vainio et al. 1991; Kim et al. 1998). Following in situ hybridization, the sections were stained with Delafield’s hematoxylin and mounted with DePeX (BDG). Images were taken using a Cohu 4912–5000 CCD (Cohu, SanDiego, CA) camera and a Scion LG-3 Frame Grabber card (Scion, Frederick, Maryland, USA).