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Review

Role of bone morphogenetic proteins in cardiac differentiation

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Abstract

Bone morphogenetic proteins (BMP) are involved in the regulation of a plethora of processes underlying cardiovascular development. This review summarizes the effects of BMP and the signaling pathways that regulate the differentiation of cardiomyocytes from mesoderm in the heart-forming region and at the distal borders of the heart tube from the second heart field. Subsequently, the role of BMPs in the formation of the ventricular chambers and septovalvulogenesis in the atrioventricular canal and outflow tract is described. Finally, the effects of BMPs in stem cell biology and cardiac regeneration are discussed.

Keywords: BMP signaling; Cardiovascular development; Septation; Chamber formation

1. Overview of cardiac development

Heart development starts when the first mesodermal cells migrate anterolaterally and form the bilateral heart-forming regions during gastrulation. Due to folding of the embryo, the heart-forming regions meet at the anterior side, forming the cardiac crescent. At the anterior side of this crescent, a trough and subsequently a linear heart tube is formed, comprising a myocardial outer layer and an endocardial inner layer. The primary heart tube does not yet comprise all cardiac compartments but will largely give rise to the left ventricle (Fig. 1). Subsequently the heart tube elongates due to recruitment of cardiogenic mesoderm into the cardiac lineage. The elongating heart tube bends to the right. At the outer curvatures of this S-shaped heart the atrial and ventricular chambers form by local differentiation and proliferation, whereas the inflow, atrioventricular canal (AVC), outflow tract (OFT), and inner curvatures retain the initial phenotype. During septation, the heart tube is separated into a right, pulmonary, and a left, systemic, halve. The atrial chamber is septated by the formation of the atrial septum which grows as a crescent shape from its roof, the ventricle by the formation of the ventricular septum, which elongates due to apposition of cardiomyocytes. What remains to be septated are the remnants of the primary heart tube. Within these parts extensive extracellular matrix deposits are laid down in between the myocardial and endocardial layers, which are molded into two cushions in both the AVC and OFT. The cushions fuse and separate the AVC, the ventricular part of the primary heart tube, and the OFT into a left and a right component. Although these cushions are initially a-cellular, they become filled with mesenchyme. Considering the extent of the cushions, one would anticipate that large mesenchymal septa would be present in the adult heart, which is, however, not the case. During development the mesenchyme is replaced by myocardium. This process is referred to as myocardialization (for reviews see [1–3]).

2. BMP signalling

Bone morphogenetic proteins (BMP) are multi-potential proteins that regulate a plethora of cellular functions during development and adult life. Because BMP signal transduction is reviewed in several excellent accounts [4–6], we will focus on BMP signaling during heart formation. BMPs belong to the

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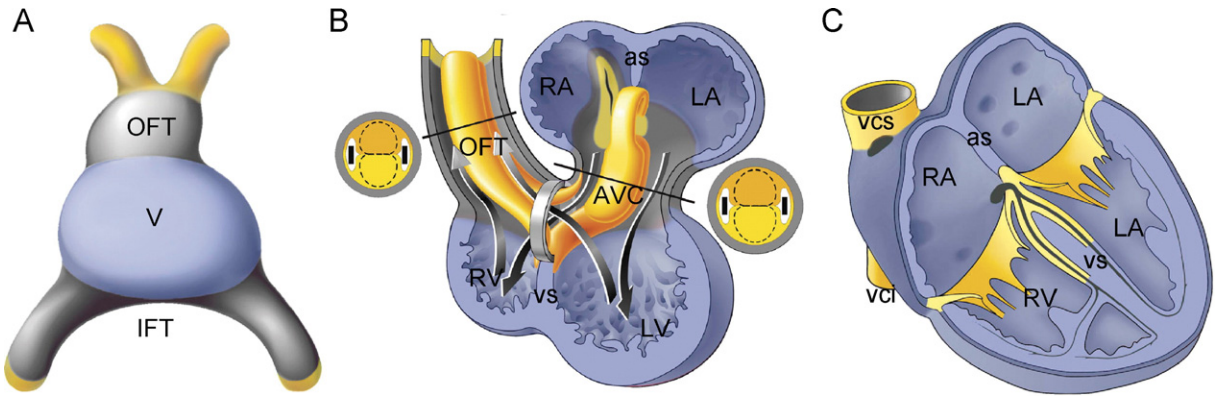

Fig. 1. Formation of the four-chambered heart. A: early in development the heart comprises a straight primary heart tube (grey) in which ventrally the ventricle (V, blue) develops; IFT: inflow tract; OFT: outflow tract; connecting vessels (yellow). B: prototypical embryonic four-chambered heart; in blue the differentiating and expanding atria (LA and RA) and ventricles (LV and RV). In grey the primary heart tube in which the cushions that separate the right and left flow of blood (arrows) are indicated in yellow. The black lines indicate the level of the adjacently shown cross-sections of the OFT and AVC. C: the adult four-chambered heart. Abbreviations: AVC: atrioventricular canal; as: atrium septum; vs: ventricle septum; vcs: vena cava superior; vci: vena cava inferior.

transforming growth factor β superfamily and comprise a subfamily of more than 20 members. BMPs are produced as biological inactive precursor proteins that are activated by endoproteolytic cleavage. BMPs are glycosylated and secreted as homo- and heterodimers. Secreted BMPs form a complex with two type I and two type II receptors (Fig. 2). Thus far, three out of seven type I and three out of five type II receptors have been identified to transduce the BMP signal into the cell. Unlike the other TGFβ-family members, BMPs have a higher affinity for the type I than the type II receptors. The type I receptors are ACVR1 (ALK2, ActRI), BMPRIA (ALK3, BRK-1), and BMPR1B (ALK6, BRK-2), and the type II are BMPR2 (BMPRII, BRK-3), ACVR2A (ActRIIA) and ACVR2B (ActRIIIB). Subsequent to the formation of the heteromeric complex, the type II receptors phosphorylate the Type I receptors in their intracellular kinase domain upon which they phosphorylate a SMAD1, SMAD5, or SMAD8 that is recruited into the receptor complex to convey the signal intracellularly. The activated SMAD binds to common SMAD4 upon which this SMAD-complex accumulates in the nucleus and effects gene expression directly and indirectly. In recent years it has become evident that the simple presence of a BMP is not sufficient to regulate a developmental process, but timing, duration, and local concentration are of

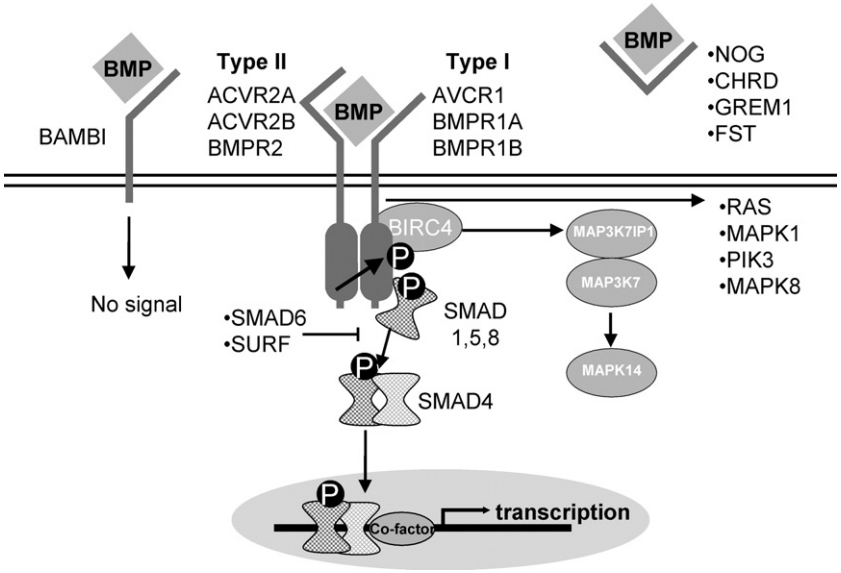

Fig. 2. BMP signaling pathways. BMPs form a heteromeric complex with type I and type II BMP receptors. Subsequent to this complex formation the type II receptor phosphorylates the type I receptor, upon which the SMAD1, SMAD5 or SMAD8 are phosphorylated. Phosphorylated SMAD forms a complex with the common SMAD4 and is transported into the nucleus. Besides signaling via SMADs, the BMP signal can also be transduced via MAP3K7(TAK1)/MAP3K7IP1 (TAB1), RAS, MAPK1 (ERK) or PIK3. Extracellularly, BMPs can be inhibited by secreted inhibitors, like NOG, CHRD (chordin), GREM1 (Gremlin) and FST (follistatin), or by the decoy receptor BAMBI, which lacks the intracellular domain for signal propagation. BMP signal transduction is intracellularly inhibited by SMAD6 or SMURF.

importance, as well. To achieve this subtle regulation, BMP signaling is modulated extracellularly, at the membrane level, and intracellularly. Extracellular proteins, e.g. NOG (Noggin), directly bind BMPs, inhibiting their interaction with the receptors. At the membrane level co-receptors either inhibit signal transduction by interfering with receptor complex formation, e.g. BAMBI [7], or enhance signaling by presenting the BMPs to the receptors, e.g. RGMB (DRAGON) [8]. Intracellularly BMP signal transduction is influenced by modulating SMAD phosphorylation and transport into the nucleus, e.g. SMURF [9] or SMAD6 [10]. Also cross-talk with other signaling pathways has been observed to affect the phosphorylation status of the SMADs and nuclear accumulation [11]. Although SMAD-mediated signaling is the most extensively studied, BMP signaling can be mediated by MAP3K7/MAP3K7IP1 (Tak1/Tab1) leading to the activation of MAPK14 (p38 MAPK), as well as of PIK3 (PI3 kinase), RAS, MAPK1 (ERK), and MAPK8 (JNK) [12–15].

3. Formation of cardiomyocytes in heart-forming regions

Cardiomyocyte formation is extensively studied during chicken development, because of the easy accessibility of the embryo and the extended developmental period during which the initial phase of development occurs. The cardiac progenitor cells are among the first mesodermal cells to form during gastrulation at stage 3 in chicken or at Carnegie stage (CS) 6 in human [16–18]. During gastrulation the progenitors are in a WNT1, WNT3A, and WNT8A expression domain which inhibit the cardiac gene expression program via the canonical CTNNB1 (β-catenin) signaling pathway. With ongoing development the progenitors migrate anterolaterally and form the bilateral heart-forming regions in the visceral mesodermal layer (Fig. 3). With the anterior movement of the progenitors, WNT signaling is inhibited by DKK1 (Dickkopf) and FRZB2 (Crescent), relieving the block on the initiation of the cardiac gene program. Upon arrival of the progenitors at their lateral position, the cardiac gene program is induced by BMPs expressed in the overlaying endoderm and ectoderm. The ectoderm expresses BMP4 and BMP7, and the endoderm expresses BMP2 and BMP5 [19,20]. In mouse, however, BMP2, BMP4, BMP5 and BMP7 are expressed in the anterior mesoderm, including the heart-forming regions [21–23]. The first cardiac-enriched genes expressed are the transcription factors NKX2-5, GATA5 and GATA4 at stage 4 in chicken (cf. human CS7) [19,24] and NKX2-5 and GATA4 at E7.5 in mouse [25,26]. With subsequent development expression of MEF2, SRF, TBX2, TBX5, and TBX20 is initiated at chicken stage 5–6 (cf. human CS8). [27–29] and from stage 7 onward, sarcomeric proteins become expressed [30]. In chicken at stage 10, in human at CS10 and in mouse at embryonic day (*E*) 8.5 the heart starts to contract spontaneously [31].

Application of the BMP inhibitor NOG to stage 4 precardiac mesendoderm explants of chicken, prevents the initiation of the cardiac gene program and, as a consequence,

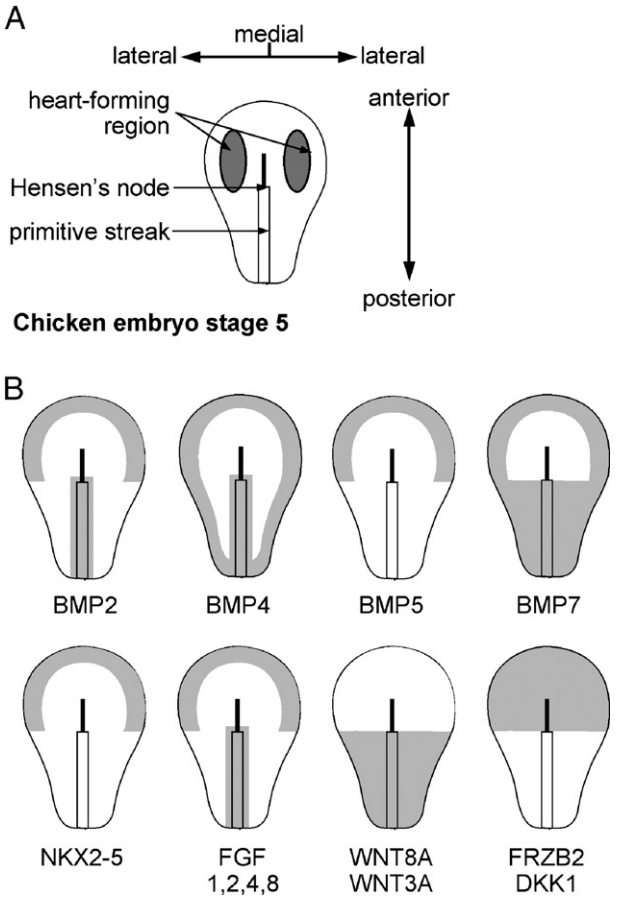

Fig. 3. Patterns of gene expression in the heart-forming regions. Schematic representation of a stage 5 chicken embryo viewed from the amniotic cavity showing the patterns of expression of genes involved in the differentiation of mesodermal cells into the cardiac lineage. (Modified from [133].)

formation of spontaneous contracting cardiomyocytes [19,27]. When NOG is applied to stage 5 explants, the cardiac gene program is initiated but the myocytes do not contract spontaneously, whereas at stage 6 they fully differentiate [32]. In line with these findings (1) BMP inhibits cardiomyogenesis in chicken epiblast explants [33], (2) NOG mRNA was found to be expressed in the heart-forming regions at *E*7.5 in mice [34] whereas in chicken NOG mRNA was not detected in the heart-forming regions at stage 5–6 [35], and (3) NOG stimulated mouse embryonic stem cells to differentiate into cardiomyocytes [34]. These findings point to a role of NOG just after mesoderm formation and before cardiomyocyte differentiation. Ectopic delivery of BMP2 medial to the heart-forming regions results in an expansion of the expression domain of NKX2-5, TBX2, GATA4, GATA5, GATA6 and SMAD6, but not of TBX5, SMAD1, SMAD5, HAS2 and TNNT2 (cardiac Troponin T), whereas lateral delivery does not affect the expression domains of these genes [19,27,28,36–38]. In both the promoter of NKX2-5 and SMAD6 a binding site for SMAD was identified [39,40]. Mutation of the SMAD-binding site in the NKX2-5 promoter abolishes NKX2-5 expression in the cardiac crescent [40]. The finding that

SMAD6 is a direct target of BMP signaling suggests that a negative feedback loop is operational in the regulation of cardiomyocyte formation.

The positioning of the medial border of the heart-forming regions is most likely the result of NOG and CHRD, which are both expressed in the embryonic midline, and of WNT3A and WNT1 expressed in the neural tube [41]. Lateral expansion of the NKX2-5 expression domain is achieved by ectopic delivery of FGF8 [42], suggesting that FGF8 is downstream of BMP signaling. However, ablation of the anterior endoderm, which is the source of FGF8 and BMP2, the cardiac gene program can be rescued by FGF8, but not by BMP2 [42]. On the other hand, culturing posterior mesendoderm, which in vitro and in vivo does not differentiate into the cardiac lineage, can be induced to do so by a brief exposure to FGF2 and contiguous presence of BMP2 [43], showing that BMP and FGF cooperate in cardiomyocyte formation and that timing and exposure duration are important parameters. Not only direct, but also indirect effects of SMADs on the cardiac gene program are reported. MYOCD (Myocardin), which is a cardiac and smooth muscle-specific transcriptional cofactor for SRF and induced by BMP2 stimulation, was found to directly interact with activated SMAD1 and synergistically activate cardiac reporter genes in a SMAD-binding site independent manner [44].

Recently, WNT11 was found to activate MAPK8 (JNK) and induce expression of cardiac marker genes in vitro [45–48]. However, its role in vivo is still a matter of debate and discussed in depth in two recent reviews [49,50]. In mice whole mount staining suggests that WNT11 mRNA is expressed in the cardiac crescent [51], whereas in chicken reports are conflicting [45,52]. Although in *Xenopus* and zebrafish, the ortholog of mammalian and chicken WNT11, WNT11-R is only expressed in the heart after myocardial differentiation, WNT11-R is also able to induce cardiomyocyte formation in non-precardiac mesoderm [53]. Surprisingly, cardiomyocyte formation is not inhibited upon introduction of WNT11 or WNT11-R morpholinos into a *Xenopus* or zebrafish embryos [47,53,54]. These findings suggest that MAPK8-mediated WNT11 signaling does not directly induce cardiac marker genes expression. The high level over-expression of WNT11 down-regulates CTNNB1 signaling via MAPK8, which has previously been found to be essential for activation of the cardiac gene program by BMPs [55]. Nevertheless, a direct effect of MAPK8-mediated WNT11 signaling on cardiomyocyte formation can as yet not be excluded. Interestingly, in other developmental process MAPK8-mediated BMP signaling has been suggested [4,56], which would allow cross-talk between BMP and WNT signaling. Further analysis is needed to support this idea.

The BMPs expressed in and adjacent to the precardiac mesoderm are conveyed by type I and II receptors. The patterns of expression of the type I receptors are comparable between mouse [57,58] and chicken (unpublished observations). ACVR1 is expressed in Hensen's node and the primitive streak, BMPR1B in the forming somites, and BMPR1A being broadly expressed during early development. The patterns of expression of the type II receptors show considerable differences between mouse and chicken. In chicken the BMPR2 and ACVR2A are potential candidates to mediate BMP signal during early cardiac differentiation as BMPR2 is ubiquitously expressed at these stages and ACVR2A is expressed in the mesoderm and becomes confined to the heart-forming regions [59,60]. ACVR2B is expressed in Hensen's node and the primitive streak and becomes subsequently confined to the neural tube [59]. In mouse both ACVR2B and BMPR2 are ubiquitously expressed during cardiomyocyte formation, whereas ACVR2A becomes only expressed after cardiomyocyte formation at *E*9.5 [61,62].

4. Knockout mice and early cardiac development

Functional disruption of the different BMPs did not unveil a role in early cardiac differentiation; BMP2 knockout mice die after formation of the heart tube [21], BMP4 Knockout mice die during gastrulation [63], and BMP5 [64] or BMP7 [22] knockout mice are viable and do not show any gross cardiac abnormalities. Since BMP5 and 7 are closely related, functional redundancy might underlie the observed phenotypes. A double knock-out, indeed, showed embryonic lethality at *E*10.5, in these mice the chambers were formed but the cushions were severely affected [23]. Although BMP6 is expressed during early embryogenesis, it is not expressed in or close to the heart-forming regions [65]. Because BMP6 is the third member of the subgroup including BMP5 and 7, it cannot be excluded that BMP6 might complement the loss of BMP5 and/or BMP7. BMP6 knockout mice are viable and have no cardiac defect [66], whereas BMP6 and BMP7 double knockout mice show defective cushion formation, being a late cardiac defect [65]. A triple knockout is not yet available.

BMPR1B is not expressed during early heart development and functional disruption results in viable mice [67]. Knockout of ACVR1 [58,68], or BMPR2 [62] results in embryonic lethality during gastrulation, obscuring a possible role in early heart formation. Disruption of ACVR2A [69] does not affect heart development, whereas impairment of ACVR2B results in postnatal death due to cardiac septation and alignment defects [70]. The double knockout of both ACVR2A and ACVR2B, on the other hand, results in embryonic death at gastrulation, pointing to redundancy [71]. Using conditional knockout mice their role in early heart development needs to be established, as yet.

At the time of cardiomyocyte formation three extracellular inhibitors of BMP signaling are expressed in the mesoderm and/or Hensen's node; NOG [72], CHRD (chordin) [73] and FST (follistatin) [74]. Functional disruption of either of these does not result in an early

cardiac phenotype, though a late cardiac phenotype that resembles DiGeorge Syndrome is observed in the CHRD knockout [73]. The double knockout of CHRD and NOG does not unveil an early cardiac phenotype, either [75]. Because functional redundancy is likely between these proteins, a triple knockout might uncover a role in early cardiac development.

Immunohistochemistry showed enrichment of the SMADs activated by the BMP-receptors in the heart-forming regions of chickens [35]. In mouse SMAD1 and SMAD5, but not SMAD8, mRNA is expressed in the embryonic mesoderm at the time of cardiomyocyte formation [76]. Functional disruption of either SMAD1 or SMAD5 did not uncover a role in cardiomyocyte formation. Nevertheless, mice in which SMAD5 was disrupted showed cardiac looping abnormalities which are most probably secondary to a loss of BMP signaling which results in aberrant left–right signaling [77]. Due to the fact that SMAD1 and SMAD5 are co-expressed in the region of cardiomyocyte formation and their comparable function, a double knockout might be needed to unveil their role in heart formation. Embryos, in which SMAD4 is deleted, do not gastrulate, obscuring a possible role in heart development [78]. Surprisingly, conditional removal of SMAD4 from epiblast cells allows the formation of a heart tube and expression of NKX2-5 [79], suggesting that BMP-mediated SMAD signaling is dispensable for initial heart development or that during early development SMAD4 is not needed for nuclear accumulation of SMADs.

Functional disruption of SMAD6 does not impair early heart development, but results in hyperplastic valves and abnormal septation of the OFT [10]. This phenotype is rather unexpected based on its spatiotemporal expression pattern in chicken [36] but is in line with its expression in the endocardium and cushion mesenchyme of the AVC and OFT [10].

5. Elongation of the linear heart tube

The initially formed heart tube only comprises the left ventricle and possibly the atrioventricular canal and part of the atria [80–82]. With subsequent development, the heart tube elongates by recruitment of cardiomyocytes from flanking mesoderm at both the anterior and posterior side [3].

The source of mesoderm at the anterior side is referred to as the anterior or secondary heart field [83–85]. In chicken the mesodermal cells of the secondary heart field express the cardiac-enriched transcription factors and GATA4. The differentiation of these cells into cardiomyocytes is, like in the heart-forming regions induced by BMP2 that is expressed in the OFT myocardium and by FGF8 that is expressed in the flanking endoderm and ectoderm [84]. In mouse, however, BMP4 is expressed in the OFT myocardium rather than BMP2. Deletion of BMP4 from the myocardium of the developing heart (NKX2-5-Cre) resulted in aberrant septation of the proximal OFT. When the expression level of BMP4 was severely reduced in a BMP7 negative background not only the cushions were hypoplastic, but also the OFT was shorter [86]. Because (1) the BMP7 knockout does not show any cardiac abnormalities [87], (2) BMP7 is expressed throughout the myocardium [87], and (3) the BMP4/7 heterodimer is more potent than the homodimers [88], recruitment of cardiomyocytes at the anterior border might be regulated by the heterodimer BMP4/7.

Analysis of BMP-induced cardiomyocyte-formation in embryonic stem cells has shown that BMP signals are mediated by MAP3K7 [89]. In kidney, the signal transduction pathway of MAP3K7 via MAP2K6 (MEK6) and MAPK14 was found to negatively regulate CCND1 (cyclin D1) and cell cycle progression [90]. It has recently been shown that (1) there is a negative relation between cardiomyocyte proliferation and expression of active MAPK14 [91], (2) the OFT myocardium shows very low proliferation rate [92], and (3) proliferation of the OFT myocardium is increased when BMP4 is deleted from the myocardium (NKX2-5-Cre) [86]. Taken together these findings suggest a role of BMP signaling via MAP3K7 rather than SMAD in regulating the rate of proliferation of the OFT.

In vitro cultures of chicken explants point to a role of BMPs in recruitment of mesodermal cells to the posterior site of the heart, as well [93,94]. These recruited cardiomyocytes are negative for NKX2-5, but express TBX18 [95], suggesting that an alternative gene expression program is activated in these cardiomyocytes than at the posterior site of the heart.

6. Chamber formation

Upon folding of the embryo the bilateral heart-forming regions fuse medioventrally, forming the linear heart tube. With subsequent development the heart tube loops to the right and at the outer curvature chambers are formed. [96,97]. Ventricle formation becomes evident with the formation of trabecules at *E*8 in mice and stage 12 in chicken. Both in mice [98] and chicken [99] BMP10 expression becomes first evident in the heart tube at the site of chamber formation. Ventricle formation is absent in BMP10 knockout mice that die at E9 [98]. In BMP6 and BMP7 double knockouts mice die from *E*10.5 onward showing hypoplastic ventricles with reduced trabeculations [65]. These BMP signals are probably transduced via BMPR1A, because deletion of BMPR1A from the myocardium (MYH6(αMHC)-Cre) results in abnormal trabeculations and compact myocardium formation [100].

The transcriptional repressor TBX2, which is regulated by BMP2 and BMP4 [28] and is expressed in the AVC and OFT, was found to be both sufficient and necessary to prevent differentiation of chamber myocardium [101]. Moreover, cardiac deletion (NKX2-5-Cre) of BMP2, results in down-regulation of TBX2 and, as a consequence,

extension of ventricular formation into the region of the AVC [102]. In TBX20 knockout mice, TBX2 was found to be up-regulated in the cardiac crescent and expressed throughout the entire heart tube, which consequently results in abrogation of ventricular formation [103]. TBX20 is, like TBX2, a transcriptional repressor and was found to regulate TBX2 expression via a direct interaction with its promoter [104]. Although these data document a role of BMPs and TBX transcriptional repressors in the induction and formation of the chambers, their interaction is not clear.

7. Cushion formation

In the looped heart, cushions form as a result of expansion of the extracellular matrix in the AVC and OFT. In these regions the myocardium induces part of the overlaying endocardium to loosen from their epithelial connection and transform into mesenchymal cells. These mesenchymal cells migrate into the cushion and produce extracellular matrix. Besides endocardial-derived mesenchyme [105], the OFT cushions are also populated by cardiac neural crest cells (CNCs), and the AVC cushions by epicardial-derived cells. The cushions fuse and contribute to the developing valves and septa.

The formation of endocardial-derived mesenchyme is induced at stage 13 in chicken and *E*9.5 in mouse in the AVC and slightly later in the OFT [106,107]. In mice, BMP2 and BMP4 are expressed in the myocardium of the AVC and OFT, with BMP2 being more prominent in the AVC and BMP4 in the OFT [108]. BMP6 shows a very dynamic pattern of expression and is expressed in the OFT myocardium and endocardium at the time of endocardial transformation [65]. Although BMP5 is expressed in the entire heart tube, its expression is (completely) down-regulated at the time of endocardial transformation, which suggests that BMP5 is not involved in the induction and regulation of this process [22]. In chicken, BMP4 is expressed in the OFT, and BMP2 and BMP5 in both the OFT and the AVC [20,109]. BMP6 and BMP7 are not likely to be involved in the regulation of endocardial-to-mesenchymal transformation, because BMP6 is not expressed in the heart, and BMP7 is expressed in the entire myocardium at this stage [20].

Cardiac deletion of BMP2 (NKX2-5-Cre) results in a reduction of extracellular matrix and mesenchyme formation in the AV cushions [102,110]. The finding that the OFT cushions develop normally, either suggests that BMP4 complements the lack of BMP2, or that cardiac neural crest-derived mesenchyme substitutes the endocardial-derived mesenchyme. Although cardiac deletion of BMP2 (NKX2-5-Cre) unveiled a role in AVC development, the effect of BMP can either be direct or indirect on the process of endocardial transformation (Fig. 4).

BMPR1A ablation in the myocardium only (MYH6 (αMHC)-Cre), resulted in hypoplastic AV cushions and a ventricle septum defect [100], indicating that the cushion defect is not due to direct BMP signaling to the endocardium. The cushion defect is most probably mediated via TGFB2 (TGFβ2), because TGFB2 expression was found to be decreased in the AVC myocardium of these mutant mice. In line with this finding, BMP2 stimulation of mouse endocardial AVC explants was found to induce mesenchyme

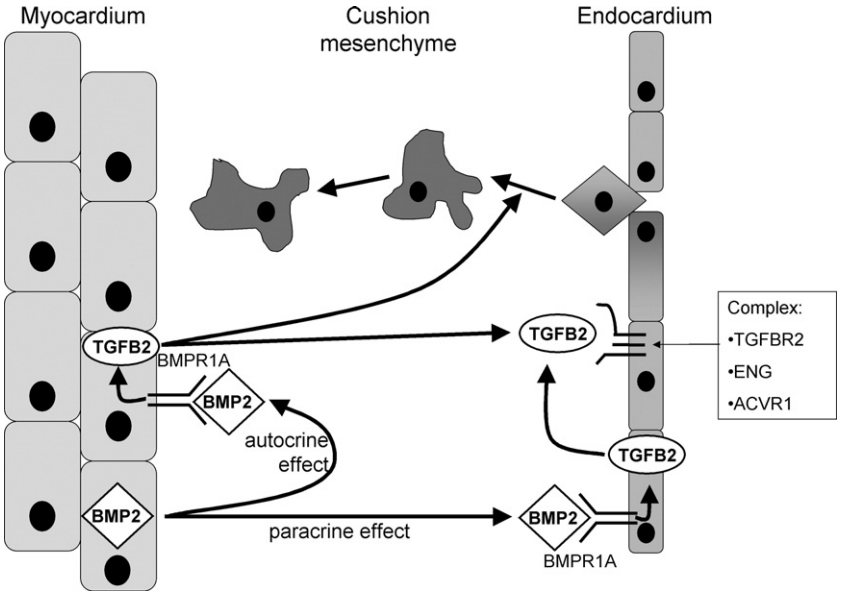

Fig. 4. Formation of cushion mesenchyme in the atrioventricular canal. During cushions formation in the AVC, cardiac jelly is replaced by mesenchyme derived from the endocardium upon BMP signaling by the AV myocardium. BMP2 produced by the AVC myocardium has both an autocrine and a paracrine effect. Both the autocrine and paracrine effects are transduced via the type I receptor BMPR1A and an unknown type II receptor. The autocrine effects lead to up-regulation of TGFB2 production in the AVC myocardium and the paracrine effect in the AVC endocardium. The secreted TGFB2 forms a complex with TGFBR2, ACVR1 and ENG expressed on a subset of the overlying endocardium, inducing it to undergo endocardial–mesenchymal transformation.

formation and TGFB2 expression [111]. In chicken BMP2 stimulation of AVC endocardial explants does not induce mesenchyme formation [112]. Addition of TGFBs was found to be essential to induce mesenchyme formation. In these explants, TGFB2 was found to induce dislodgement from the endocardium monolayer and TGFB3 (TGFβ3) migration of the activate mesenchyme into the matrix [113]. In mice, these two processes are regulated by TGFB2, alone [106]. These differences, rather than being fundamental, are most probably due to differences in isoform effects.

The effect of BMP on the endocardium (paracrine effect) was substantiated by deletion of BMPR1A from the endocardium (TEK(Tie2)-Cre). In these mutant mice, P-SMAD1/5/8 expression was reduced in the endocardium and in two-third of the embryos the cushions were hypoplastic [102]. Interestingly, in the remaining embryos cushions were formed, pointing towards BMP receptor redundancy: ACVR1 being a likely candidate based on its expression in the endocardium [114]. Endocardial deletion (TEK(Tie2)-Cre), but not myocardial deletion (MYH6(αMHC)-Cre), of the ACVR1 receptor did cause failure of mesenchyme formation in the AV cushion [114]. This finding was further substantiated by the observations (1) that genes previously reported to be crucial for endocardial-to-mesenchymal-transformation, MSX1 and SNAI1, were down-regulated in the ACVR1/TEK(Tie-2)-Cre knockout [114], (2) that in vitro ACVR1 negative endocardial AVC cells did not form mesenchyme [114], and (3) that chicken ventricular endocardial cells can be induced to transform into mesenchymal cells by ectopic over-expression of a constitutive active ACVR1 receptor [115].

Comparable to cardiomyocyte formation in the heart-forming regions SMAD6 is up-regulated in AVC endocardium upon ectopic expression of constitutive active ACVR1 [36,115]. In line with this finding hyperplastic valves were observed in SMAD6 knockout mice [10]. In the ACVR1 negative endocardium not only BMP-SMAD1/5/8, but also the TGFβ-SMAD2 was found to be reduced [114], suggesting that besides BMP also TGFβ signals can be transduced via the ACVR1 receptor. It is generally thought that TGFβ signaling is mediated via a heteromeric complex comprising the TGFβ type II receptor and the type I receptors ACVRL1 (ALK1) or TGFBR1 (ALK5). In vitro and in vivo experiments in chicken have shown that ENG (endoglin), a type III co-receptor, is essential for allowing TGFB2 signaling via ACVR1 in the AVC and endocardial transformation [115]. Interestingly, ectopic expression of ENG in ventricular endothelium allows transformation into mesenchymal cells in response to TGFB2 stimulation [116].

Although endocardium contributes mesenchyme to the OFT ridges, in none of the above discussed transgenic animals OFT abnormalities were reported. This finding might not only be due to redundancy by other BMPs but also by cardiac-neural-crest-derived mesenchyme. When ACVR1 or BMPR1A were deleted in neural crest cells (WNT1-Cre), aberrant invasion of CNCs and septation defects in the OFT are observed [117,118]. Cardiac deletion of BMP4 (NKX2-5-Cre) showed failure of proximal OFT septation and reduced proliferation of OFT mesenchyme [86]. Interestingly, in these knockouts the CNCs enter the OFT cushions but do not migrate down into the proximal part of the OFT, suggesting that a BMP concentration gradient regulates the distance of invasion of CNC cells into the OFT cushions. This idea is substantiated by the following observations. (1) In mice, in which the BMP4 concentration is moderately lowered (PRRX1(prx1)-Cre), no abnormalities were detected in the OFT. In these mutants BMP7 was found to be up-regulated, suggesting redundancy between BMP4 and BMP7. If the BMP4 concentration was reduced in a BMP7 negative background, the OFT was shortened and the cushions were hypoplastic [86]. (2) Although mice, in which the BMPR2 is deleted, die at gastrulation [62], defects in septation of the proximal OFT and absence of the semilunar valves are observed in mice in which the affinity of the BMPR2 for BMPs is reduced [119]. In these mutants the AVC valves appeared normal indicating that the AVC can develop normally when BMP signaling is impaired. (3) Chicken embryos in which NOG was ectopically expressed using a retrovirus showed a range of OFT defects, from complete absence of septation and valves to septal defects with normal valves. Also in these embryos the AVC was not affected [109]. Taken together these findings suggest that septation and valve formation is more sensitive to small changes in BMP signaling in the OFT than in the AVC. Alternatively, an earlier induction of mesenchyme formation in the AVC than in the OFT, might underlie this observation [107].

BMPs are also needed for ongoing mesenchyme formation in both the AVC and OFT cushions and the formation and maturation of functional valves. BMP6 and BMP7 double knockouts showed a reduced mesenchyme formation, whereas the induction of endocardial-of-mesenchymal-transformation was unaffected [65]. In the formed valves, cartilage related genes are expressed in the leaflets and tendon related genes in the chordea tendineae. In an in vitro culture system using chicken AVC explants BMP2 was found to induce the expression of cartilage markers, like SOX9 and AGC1 (Aggrecan) [120]. Moreover, in most semilunar valves that are replaced, histological analysis showed the presence of bone elements and expression of BMP2 and/or BMP4, suggesting that re-expression of BMPs induces bone formation [121].

8. The role of BMP in the differentiation of in stem cell into cardiomyocytes

Trauma to the adult myocardium leads to irreversible loss of myocardium due to its limited regenerative capacity. The self-renewal capacity and ability of stem cells to form specialized cell types lead to the idea to cure ischemic damage of the heart by stem cell replacement. A reliable selective differentiation method of stem cells into functional

cardiomyocytes is prerequisite to succeed in the attempts to transplant cells. The knowledge of cardiomyocyte differentiation is used to force stem cells into cardiomyocytes. Stem cells can be obtained from two possible sources; adult tissues (somatic stem cells) and embryos (embryonic stem cells) [122]. The ability of somatic stem cells to substantially contribute to the myocardium during regeneration is controversial [123]. Nevertheless, (1) ISL1 (Islet 1)-positive cells [124], (2) ATXN1 (Sca1)-positive [125], and (3) KIT (c-kit)-positive cells [126], which are relatively undifferentiated and resided in the adult heart, as well as bone marrow derived cells [127] are able to differentiate into cardiomyocytes. The future will learn whether these cells are able to provide a substantial and sufficient contribution in regeneration of the ischemic heart.

Mouse and human embryonic stem cells are used to identify the signaling pathways that are necessary and sufficient for differentiation into cardiomyocytes. BMPs seem to play an important role both in self-renewal of stem cells and their differentiation into cardiomyocytes [4,128]. Cardiomyocyte formation can be divided into four stages (Fig. 5); (1) pluripotent stem cell formation (2) precardioblast stage, (3) cardioblast stage and (4) cardiomyocyte stage [129]. In each of these stages BMPs play an important role. The cells in the pluripotent stem cell stage express POU5F1 (Oct4), SOX2 and NANOG [122] and self-renewal of this population is regulated by BMPs. [130]. BMPs stimulate the expression of inhibitor of differentiation (ID) genes [131], which in turn reduce the levels of MAPK1 and MAPK14, which are involved in maintaining the cells undifferentiated. To allow differentiation into precardioblasts, BMPs needed to be transiently inhibited by NOG [33,34]. After this transient BMP inhibition, BMPs stimulate the up-regulation of cardiac transcription factors, like NKX2-5 and GATA4, hereby promoting the transition of precardioblasts into cardioblasts. Using P19CL6 embryonic carcinoma cells BMP signaling was found to induce NKX2-5 and GATA4 expression via both SMAD and MAP3K7 signaling which is integrated on ATF2 [132]. In the final differentiation step of cardioblasts into cardiomyocytes again BMPs signaling via SMAD and MAP3K71 was found to activate the expression of sarcomeric proteins, like MYH7 (βMHC) [132].

Taken together these findings point to a crucial role of BMP signaling in the regulation of self-renewal of stem cells and differentiation of stem cells into cardiomyocytes. Nevertheless, the precise regulation of these processes is still poorly understood and involves probably additional intrinsic factors and cross-talk to other signaling pathways. Unraveling these interactions is a major challenge, but is probably essential for efficient differentiation of stem cells into cardiomyocytes.

9. Concluding remarks

BMPs play a critical role in cardiac development and are one of the inducers of cardiac differentiation not only in the heart-forming regions but also in the recruitment of cardiomyocytes at the distal borders of the heart. During the formation of the four-chambered heart, BMP are crucial in the regulation of septovalvular development. In these processes BMP signaling is mainly transduced via SMAD, but recently, also signal transduction via MAP3K7, MAPK1 (ERK), MAPK14 (p38) and PIK3 has been described. Moreover, BMPs have distinct effects on cardiac cells depending on temporal and spatial differences and interactions with other extracellular stimuli. These individual alternative signaling pathways have to be explored to assess their cross-talk with one another and with other signaling pathways. Studying normal and abnormal cardiac development might provide insights on the precise

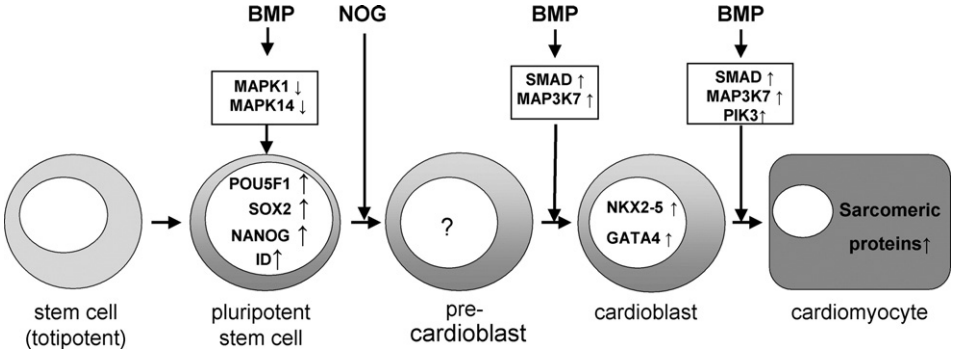

Fig. 5. Role of BMPs in the differentiation of embryonic stem cells into cardiomyocytes. Differentiation of totipotent embryonic stem cells into cardiomyocytes can be divided into four stages. In the first stage, *pluripotent stem cells*, the cells are undifferentiated and have the potential to differentiate into several cell types, including cardiomyocytes. During this stage, BMPs are important in self-renewal. BMP signaling was found to down-regulate MAPK1 (ERK) and MAPK14 (p38) and to stimulate expression of the transcription factors POU5F1 (Oct4), SOX2, NANOG and ID, which are important to retain the rather undifferentiated state of the cells. When these cells are transiently exposed to Noggin they can become committed to develop into cardiomyocytes by BMP. This stage is referred to as the *pre-cardioblast stage*. Subsequent stimulation by BMPs, which is transduced by both SMAD and MAP3K7 signal pathways, induces expression of cardiomyocyte-enriched transcription factors. These cells are referred to as *cardioblasts*. Ongoing stimulation of these cardioblasts by BMPs, which mediated via SMAD, MAP3K7 and PIK3 signal transduction pathways, induces the expression of sarcomeric proteins which consequently leads to the differentiation of cardioblasts into rhythmically contracting *cardiomyocytes*.

molecular mechanisms of BMP signaling. These findings might be helpful in understanding (1) stem cell biology and (2) adult pathology.

In spite of the extensive knowledge of the effects of BMPs on cardiac differentiation during development and in stem cell biology, regenerative medicine using stem cells is still rather disappointing because stem cells cannot be efficiently induced to differentiate into adult cardiomyocytes. Nevertheless, large resources are currently allocated to treat ischemic heart disease using various types of stem cells. An alternative approach for this problem might be to reprogram cardiac fibroblasts into cardiomyocytes. This approach has several advantages: (1) cardiac fibroblasts already populate the infarcted area of the heart, (2) making ex vivo manipulation not necessary. (3) No ethical burdens or (4) rejection problems are involved when reprogramming the patients own cardiac fibroblasts. The challenge for the near future will be to identify the factors that are necessary and sufficient to drive the cardiac fibroblasts into the cardiac lineage. So far, pro-epicardial mesothelial cells, which are among others the progenitors of the interstitial cardiac fibroblasts, were found to differentiate into cardiomyocytes by BMPs [93,94].

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