1. Introduction

1.1 Bone Morphogenetic Proteins

1.1.1 From historic perspective

Already in the early 1960s fundamental research yielded indices anticipating the contribution of heterogeneous and diffusible factors to the formation of bony tissue [Goldhaber, 1961; Vainio et al., 1962]. However, it was Marshall Urist who paved the way for a profound understanding of the processes involved in bone formation and regeneration in 1965 by an observation which shaped up as an unique key discovery. The experiment that mapped the way to BMP included the implantation of decalcified diaphyseal bone which was partially pretreated with calcium chloride to enable intramuscular nucleation in rodents. In contrast to the preliminary hypothesis, the non pretreated control samples led to a pronounced formation of new and well functionalized bony tissue within a few weeks [Urist, 1965]. Urist attributed the observed “autoinduced” process, which followed the typical route of endochondral ossification, to the presence of a substance in bone matrix. A few years later Urist was able to identify this substance to be a complex mixture of glycoproteins, which he named bone morphogenetic protein [Urist et al., 1971].

This breakthrough was followed by numerous studies focused on the development of procedures to purify the mixture of bone inducing proteins. First advances were achieved by applying dissociative extractants [Sampath et al., 1981] and mixtures of aqueous and non aqueous solvents [Urist et al., 1979]. However, these methods faced the problem of extremely low yields which solely added up to 1 mg/kg of wet weight of fresh bone [Urist et al., 1983]. A few years later isolations of various single components of the heterogeneous BMP mixture were successfully conducted by utilizing different chromatographic principles [Urist et al., 1984; Hauschka et al., 1986; Sampath et al., 1987]. Subsequently, Wang and coworkers were able to identify the amino acid sequences of three of these proteins [Wang et al., 1988]. These molecules (BMP-1, BMP-2 and BMP-3) stated the first members of the BMP nomenclature. By means of these amino acid sequences respective oligonucleotide probes could be synthesized and enabled the screening of the human genes for the encoding DNA sequences [Wang et al., 1987]. Subsequent progresses in gene technology

led to the recombinant production of human BMPs [Wang et al., 1989]. The method developed by Wang was performed in Chinese hamster ovary (*CHO*) cells by using mammalian expression vectors. A few years later Kubler et al. [1998] successfully conducted the production of rhBMP-2 and rhBMP-4 in an *E. coli* based expression system and overcame the problem of extremely low yields associated with *CHO* based expression systems especially found for BMP-4 without any loss in biological activity. Indeed, the physicochemical behavior of rhBMPs derived from bacterial expression systems is altered due to absent glycosylation [Uludag et al., 2000].

1.1.2 Biochemistry

The BMP family represents a subfamily of the transforming growth factor (TGF)–β super-family. BMPs are intracellularly synthesized as precursor forms, whereby a subsequent proteolytic cleavage in the C-terminal region leads to the mature protein composed of approximately 130 amino acids [Rueger, 2002]. The systematic nomenclature of BMPs is depicted in *Table 1.1-1.* It has to be mentioned that BMP-1 is not ranked among actual bone morphogenetic proteins. Instead, BMP-1 has been revealed to contribute to the formation of extracellular matrix by acting as procollagen-C-proteinase and has therefore been categorized to the group of metalloproteinases [Hofbauer and Heufelder, 1996].

Table 1.1-1: Overview of bone morphogenetic proteins, their nomenclature and potential functions [modified according to Reddi, 2000 and Saito et al., 2008]; CDMP = cartilage derived morphogenetic protein; GDF = growth and differentiation factor; OP = osteogenic protein.

Morphogene	Generic names	Potential function
BMP-2	BMP-2A	cartilage and bone morphogenesis/heart
BMP-4	BMP-2B	cartilage and bone morphogenesis
BMP-3	-	osteogenesis bone formation/brain
BMP-3B	GDF-10	craniofacial bones
BMP-5	-	bone morphogene
BMP-6	-	hypertrophy of cartilage/skin
BMP-7	OP-1	bone differentiation, eye and kidney development
BMP-9	GDF-2	n.a.
BMP-8	OP-2	bone formation

Morphogene	Generic names	Potential function
BMP-10	-	n.a.
BMP-11	GDF-11	n.a.
BMP-12	GDF-7, CDMP-3	ligament and tendon development
BMP-13	GDF-6, CDMP-2	cartilage development and hypertrophy
BMP-14	GDF-5, CDMP-1	mesenchymal condensation, chondrogenesis
BMP-15	GDF-9B	ovarian physiology
BMP-16	-	n.a.
BMP-17	-	n.a.
BMP-18	-	n.a.

Interestingly, BMP-like proteins could not only be isolated from mammals but also from invertebrates including *Drosophila* and *Caenorhabditis elegans*. Thereby it was shown that some of these isolated forms like the *Drosophila decapentaplegic* gene product (DPP) exhibited a high structural similarity to some mammalian BMPs like BMP-2 and BMP-4. For DPP it was even revealed that a subcutaneous implantation in mammals yielded a pronounced induction of bone formation [Sampath et al., 1993].

Already in the early 1990s Sampath et al. exemplarily investigated the basic structure of mature BMPs by means of an osteogenic protein derived from bovine bone [Sampath et al., 1990]. They revealed that the osteogenic protein was composed of the two disulfide-linked polypeptides OP-1 and BMP-2A. Similarly, they elucidated that this dimeric structure was essential for the biological activity. Furthermore, it was shown that the mature domain of nearly all BMPs is structurally marked by a highly conserved pattern of seven cysteine residues [Nickel et al., 2002; Rueger, 2002; Wozney, 1998]. Thereby six cysteine residues form intramolecular disulfide linkages, whereas the seventh cysteine residue participates in the intermolecular disulfide linkage [Nickel et al., 2002]. In contrast to preliminary assumptions, Sieber et al. [2006] could show that the interchain disulfide bond is not essential for the biological activity of rhGDF-5 *in vitro* as it was supposed before. The group of BMPs and related proteins can be subdivided according to structural and functional features. One subgroup is formed by BMP-2, BMP-4 and DPP, a second subgroup comprises BMP-5, BMP-6, BMP-7, BMP-8 and the *Drosophila gbb-60A* gene product, and finally, BMP-12, BMP-13 and BMP-14 are summarized in a third subgroup.

1.1.3 BMP receptor signaling

As members of the TGF-β superfamily, BMPs act as ligands for serine/threonine kinase receptors. The group of receptors targeted by BMPs can be subdivided as displayed in *Fig. 1.1-1*. In this context, it has to be highlighted that only type II receptors are constitutively active.

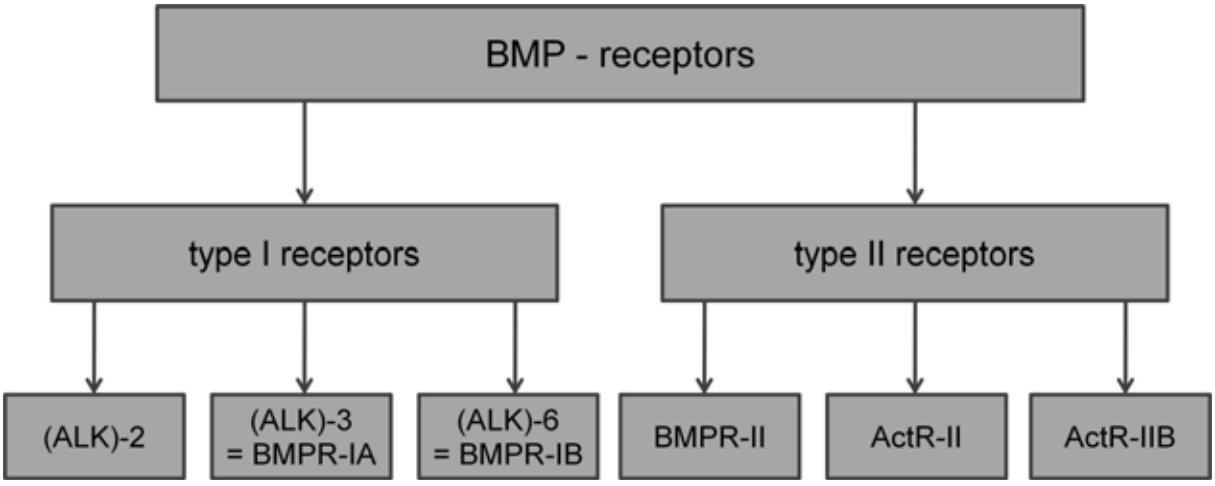

Fig. 1.1-1: Subgroups of BMP – receptors according to Chen et al. [2004]; ALK = activin receptor-like kinase, BMPR-I = BMP type I receptor, BMPR-II = BMP type II receptor, ActR-II = activin type II receptor.

As far as known, the signal cascade is initiated by ligand stimulation and activation of BMP receptors, forming a heterotetrameric receptor complex resulting in the phosphorylation of receptor-regulated Smads (R-Smads). This group of downstream molecules includes Smad 1,5 and 8 which are activated by BMP type I receptors, Smad 2 and 3 activated by (ALK)-2 and TGF-β type I receptors [Miyazono et al., 2005]. Subsequently, activated R-Smads build up a complex with Smad 4, the only identified Co-Smad in mammals. The phosphorylated complex is then translocated in the nucleus, where Smads pilot the transcription of target genes through direct binding to specific DNA sequences. Within this process Smads orchestrate with additional DNA binding proteins like transcriptional factors [Ito and Miyazono, 2003], transcriptional co-activators or co-repressors [Miyazono et al., 2000]. The described activating pathway is negatively regulated both by inhibitory and competing processes. Thereby, I-Smads prevent type I receptors from activating R-Smads [Imamura et al. 1997], whereas Smurfs mediate the degradation of R-Smads [Zhu et al., 1999] and specific transcription factors (e.g. Runx2) [Zhao et al., 2003]. Aforementioned processes are summarized by *Fig. 1.1-2*. A further BMP antagonist, which is named Noggin was described

by Brunet et al. [1998]. It is supposed to capture BMP-2,4 and 7 and therefore inhibits the initiation of BMP signaling by these osteogenic factors [Chen et al., 2004]. Recently, Zhang et al. [2007], were able to elucidate the mechanism by which von Willebrand factor type C domains (VWC) containing proteins like chordin, chordin like-2 and crossveinless 2 interfere with the BMP signaling cascade. According to the authors, the binding competent domains of these proteins utilize specific subsets of BMP-2 binding determinants, which overlap with the binding sites of type I and type II receptor respectively.

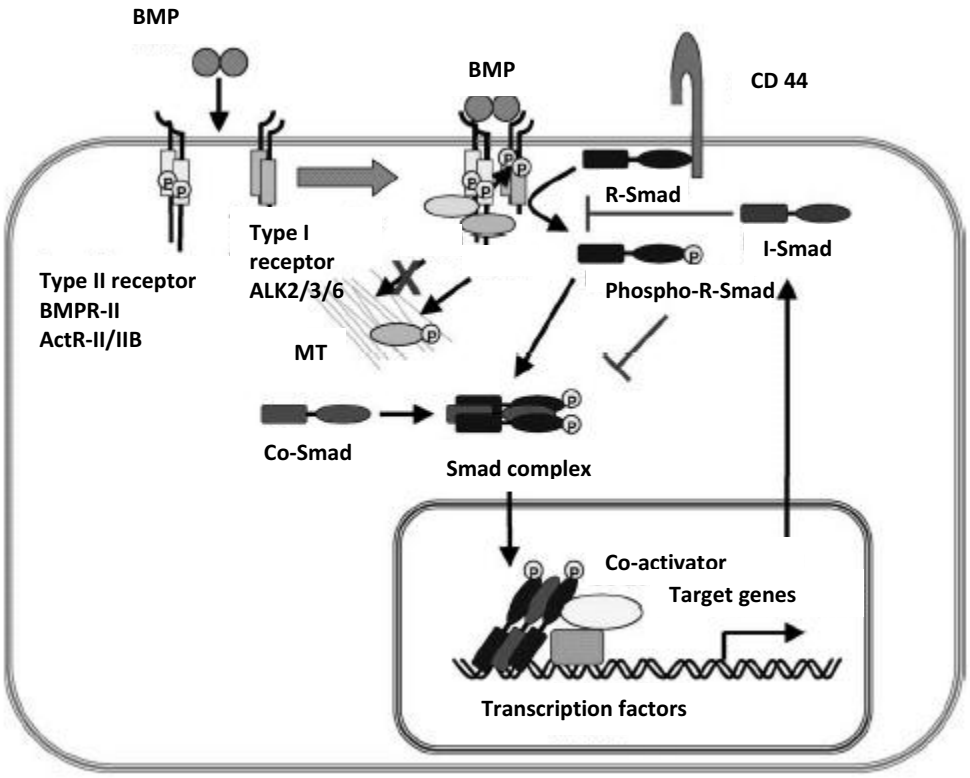

Fig. 1.1-2: BMP signaling according to Miyazono et al. [2005]

1.1.4 Biologic activity and osteoinduction

It is frequently described in literature that BMPs exhibit qualitatively and quantitatively varying activities. For instance, BMP-2 [Wang et al., 1988], BMP-4 [Hammonds et al.,1991], BMP-5 [D'Alessandro et al., 1991] and BMP-7 [Sampath et al., 1992] proved their osteogenic and chondrogenic activity, whereas GDF-5, 6 and 7 showed their potential to induce the formation of tendon and ligament-like structures. Interestingly, postnatal bone regeneration mirrors the pathways of embryonic bone development [Carrrington and Reddi, 1991]. The pioneering work of Wozney [1992] and Reddi [1992] contributed to an enriched knowledge

about the role of BMPs within *de novo* bone formation, which are now considered to act as soluble signals of tissue morphogenesis. According to literature, bone healing can proceed along two different routes. Within the scope of endochondral ossification a cartilage intermediate is formed, whereas this stage is circumvented by intramembranous ossification [Zhao et al., 2002a]. The process of endochondral ossification involves the migration of mesenchymal stem cells, which subsequently condensate and differentiate into chondrocytes. These cells further proliferate and finally produce extracellular matrix which constitutes the primordial cartilage. Afterwards proliferating chondrocytes in the core of the new formed cartilage differentiate into hypertrophic chondrocytes. These cells initiate the production of new extracellular matrix which deviates from the proliferating cartilage with respect to its composition. Meanwhile, the hypertrophic cartilage is vascularized. Thus, the invasion of osteoblasts, osteoclasts and hematopoietic cells is promoted. Thereby, primary ossification centers are established, wherein hypertrophic cartilage is replaced by bony tissue. With respect to endochondral ossification, the role of BMP signaling is not fully understood up to now due to the great variety of inducing factors and antagonists which interplay over predominantly unknown crosstalk pathways. However, it is generally accepted that BMPs affect the hypotrophy of chondrocytes [De Luca et al., 2001; Minina et al., 2001; Brunet et al., 1998]. Additionally, it was shown that BMPs also affect bone mineral densities and bone formation rates [Devlin et al., 2003; wu et al., 2003; Zhao et al., 2002b].

1.2 State of the art in bone growth factor delivery

1.2.1 Demands on potential bone substitutes

Today a fast-growing demand for bone substitutes suitable for orthopedic indications emerges. This can be explained by an increase in life expectancy, a higher number of traumatic injuries associated with new “lifestyle” sports and the aim to improve quality of life for patients affected by endemic diseases like osteoporoses. Moreover, there are noticeable tendencies in surgery and orthopedics focusing on a regenerative treatment of bony defects. Materials that are supposed to be applied within this scope have to fulfill several basic requirements summarized in *Table 1.2-1*.

Although, it is desirable to match all mentioned demands by formulation development, the achievement of a prolonged retention of the active agent at the treatment site has to be emphasized since it is of paramount importance for successful bone regeneration. This is