NTNU – Trondheim
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Biotechnology
Submission date: May 2012
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PREFACE

This master thesis was carried out at the Department of Biotechnology (IBT) and the work was performed at the Department of Cancer Research and Molecular Medicine (IKM) at NTNU, St. Olav's University Hospital.

I would very much like to use this opportunity to thank:

My teaching supervisor Toril Holien (IKM), for all the support and guidance throughout my work. Your cheerful mood and broad knowledge has inspired me to learn more and work hard. You have taught me to work independently, and given me a great experience. For this I am very thankful, and am confident that I could not have had a better supervisor.

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All the other employers in the myeloma- and toll group for all the help and understanding they have shown me during my work. They have all been kind and helpful by answering my questions, given me advice and shown me the way around the lab. A special thanks goes to Hanne Hella, Berit Størdal and Lill Anny Grøseth for taking care of and preparing cells for my experiments.

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Oddrun Elise Olsen

ABSTRACT

Multiple myeloma (MM) is a malignancy of antibody producing plasma cells located in the bone marrow. MM is also called bone marrow cancer and is the second most common hematologic cancer. The exact cause for this type of cancer is still unknown and the disease is today generally thought to be incurable. One hallmark of MM is the degradation of bone. Bone morphogenetic proteins (BMP) are a group of signaling molecules that have multiple roles in normal and malignant cells. BMPs regulate growth, differentiation and apoptosis in myeloma cells, but also have a role in the induction of bones. This means that BMPs have the potential to inhibit the growth of cancer cells and reduce the degradation of bones.

In this thesis BMP-9 was studied by looking at how it affects apoptosis and proliferation in different types of human myeloma cell lines (hMCLs). Experiments were also performed to see if any of the common BMP antagonists had an effect on this protein. Efforts were also made to identify by which receptors BMP-9 signals in myeloma cells and which downstream signaling pathways are activated.

It was found that BMP-9 induced apoptosis and/or inhibited proliferation in several hMCLs. The SMAD pathway was activated by BMP-9 in myeloma cells, and BMP-9 most likely uses ALK-2 as Type I receptor and ACVR2A or ACVR2B as Type II receptor in these cells. The antagonists CHL-1 and Tsg, the Type III receptor endoglin and the SMAD inhibitor DMH1 were found to interfere with BMP-9 signaling. Another interesting discovery was that CpG-ODN did not have the same inhibitory effect on BMP-9 as has been shown on other BMPs. Further experiments have to be done to investigate what makes BMP-9 different from other BMPs in this regard.

SAMMENDRAG

Myelomatose, også kjent som beinmargskreft, er en kreftype som rammer plasmaceller lokalisert i beinmargen. Myelomatose er den nest vanligste formen for hematologisk kreft, men den eksakte grunnen for at denne sykdommen oppstår er enda ukjent. Denne typen kreft kan per i dag ikke helbredes. Noe som kjennetegner myelomatose er degradering av ben. Benmorfogene proteiner (BMP) er en gruppe signalmolekyl som har flere roller i normale celler, blant annet regulering av vekst, differensiering, apoptose og induksjon av ben. Dette vil si at BMP potensielt både kan hemme vekst av kreftceller og redusere degraderingen av ben.

I denne oppgaven ble BMP-9 studert ved å se på dens effekt på apoptose og proliferasjon i humane myelomcellelinjer (hMCLs). Effekten av ulike antagonister av BMP ble undersøkt, i tillegg ble det utført forsøk for å identifisere hvilke reseptorer BMP-9 benytter seg av og hvilke signaliseringsveier som er i bruk.

Fra forsøkene ble det funnet at BMP-9 induserer apoptose og/eller hemmer proliferasjon i flere hMCLs. SMAD signaliseringsveien ble aktivert av BMP-9 i myelomceller og BMP-9 benytter mest sannsynlig type I reseptor ALK-2 og type II reseptorene ACVR2A eller ACVR2B for signalering i disse cellene. Antagoistene CHL-1 og Tsg, type III reseptoren endoglin og SMAD-hemmeren DMH1 hadde alle hemmende effekt på BMP-9 signalering. Et annet interessant funn var at CpG-ODN ikke hadde samme hemmende effekt på BMP-9 som andre BMPer. Dette må undersøkes videre for å finne ut hva som skiller BMP-9 fra andre medlemmer av BMP familien i denne sammenhengen.

TABLE OF CONTENTS

ABBREVIATIONS

aa	amino acid
ACVR	activin receptor
ALK	activin receptor-like kinase
ATP	adenosine triphosphate
BMP	bone morphogenetic protein
BMPR	bone morphogenetic protein receptor
BSA	bovine serum albumin
CHL	chordin like
Co-SMAD	common-partner SMAD
CpG-ODN	cytosine-phosphate-guanosine oligodeoxynucleotide
CR domain	cysteine-rich domain
CV-2	crossveinless-2
DAN	differential screening-selected gene aberrative in neuroblastoma
DM	dorsomorphin
DMH1	dorsomorphin analogue 1
EBV	Epstein Barr virus
ERK	extracellular signal-regulated kinase
FITC	fluorescein isothiocyanate, a derivative of fluorescein
FS	follistatin
GAG	glycosaminoglycan
GDF	growth differentiation factor
GS domain	glycine/serine-rich domain
HGF	hepatocyte growth factor
hMCL	human myeloma cell line
HSPG	heparan sulfate proteoglycan
Id	inhibitor of differentiation

I-SMADs	inhibitory SMADs
IL	interleukin
MAPKs	mitogen-associated protein kinases
MM	multiple myeloma
mRNA	messenger RNA
OD	orphan domain
OP	osteogenic protein
PCL	plasma cell leukemia
PBS	phosphate buffered saline
PDZ	combination of first letters of the three proteins: post synaptic density protein (PSD95), Drosophila disc large tumor suppressor (Dlg1) and zonula occludens-1 protein (Zo-1).
PI	propidium iodide
PS	phosphatidylserine
RGD	arginine-glycine-aspartate tripeptide motif
RNAi	RNA interference
R-SMADs	receptor-regulated SMADs
RT-qPCR	reverse transcription-quantitative real time polymerase chain reaction
siRNA	small interfering RNA
SMAD	(small) mothers against decapentaplegic (dpp) homolog
SOST	sclerostin
STAT-3	signal transducer and activator of transcription-3
TBST	0.1 % Tween-20-Tris Buffered Saline, pH 7.4
TGF-β	transforming growth factor-β
Tsg	twisted gastrulation
VGR	vegetal related
ZPD	zona pellucida domain

1 INTRODUCTION

The background for this thesis was the desire to find out how different signaling molecules, in this case bone morphogenetic proteins (BMPs), in multiple myeloma (MM) actually work. MM is a malignancy of antibody producing plasma cells located in the bone marrow.[1] These cells are called myeloma cells and it is important to learn more about them and their interactions with other components in the body, since the cellular mechanism of the cancer is still unknown. Studying different signaling molecules, the intracellular signaling pathways and the mechanisms of apoptosis and growth arrest can provide useful information in finding a cure for MM.[2]

Hence, the aim of this study was to find out how BMP-9 affects human myeloma cell lines (hMCLs) by looking at apoptosis and proliferation. The effect of different antagonists of BMP-9 was also investigated, and efforts were made to identify the receptors used for signaling and downstream signaling pathways.

1.1 MULTIPLE MYELOMA

MM, also called bone marrow cancer, is the second most common hematological cancer, and is most often diagnosed around age 70.[3] This type of cancer arises when abnormal plasma cells located in the bone marrow divide unrestrictedly and gather up.[4,5] Abnormal plasma cells origin from normal plasma cells that has gone through changes both genetically and environmentally.[5] The myeloma cells are dependent on the microenvironment of the bone marrow that has shown to play a crucial role for their ability to survive, continuously divide and migrate. The reason for this is that other cells in the bone marrow produce different cytokines, and that positive signals to the cells are produced after binding to adhesion molecules in the extracellular matrix or on neighboring cells. Two of these factors are interleukin (IL)-6 and -10, which in some cases also can be produced by the myeloma cells.[2] The exact cause for this type of cancer is still unknown. This makes it hard to find a cure, and this disease is today generally thought to be incurable.[1,4]

Myeloma cells contribute to a weaker bone structure and as a result of this patients often have pain in bones and bone fractures.[6] Myeloma cells produce an abnormal antibody called monoclonal (M)-protein, and accumulation of this antibody can lead to kidney failure.[7] To be

diagnosed with MM there are some criteria that has to be met: the serum M-protein has to be present in serum and/or urine; the level of clonal bone marrow plasma cells has to be higher than 10 %; and it is required to have impairment of function in related organs or tissue.[1,6] The diagnosis is mainly based upon blood tests and bone marrow biopsies. Despite treatment with high dosages of chemotherapy, radiation and stem cell transplantation, the relapse rate is high since the patients in time will become resistant to it. The treatment is given with the intention to prolong life and improve the quality of life, but the prognosis for survival is still low with a median of about five to seven years.[1,2,8]

1.1.1 HUMAN MYELOMA CELL LINES

Cell lines are cell cultures that are able to grow and divide continuously. A cell line must have the capacity to proliferate indefinitely when given space and fresh growth medium.[9] Human myeloma cell lines (hMCLs) have been established from bone marrow or peripheral blood from patients with MM, or a more aggressive variant known as plasma cell leukemia (PCL) where the MM has invaded extramedullary sites.[1,9,10] Most hMCLs have been established from samples obtained from patients with a mean age of 60±12 years suffering from relapse.[9]

The first hMCLs were established in 1966 and 1968 and were named RPMI-8226 and U266 respectively.[9] During the last two decades a large number of hMCLs have been derived (assortment given in Table 1). The hMCLs are usually grown in suspension, mainly as single cells but sometimes also in small clusters.[9] One reason for the difficulty of establishing hMCLs is the slow proliferative activity, which can lead to the outgrowth of an Epstein Barr Virus (EBV) positive cell line. This is not desirable since one of the hallmarks for genuine hMCLs is negative EBV status.[10]

Table 1. General description of an assortment of hMCLs.

hMCL	Year	Sex, age	Disease, status*	Specimen site**	EBV status	Cytokine dependency	Comments	Ref
ANBL-6	1992	F, 67	MM (IgGλ), R	PB	EBV-	IL-6	Produces HGF. Tolerates high concentrations of BMP	[9-13]
CAG	---	---	MM	BM	EBV-		Tolerates high concentrations of BMP	[14]
IH-1	1999	M, 76	MM (IgAλ)	PF	EBV-	IL-6	Sensitive to BMP	[11]
INA-6	1988	M, 80	PCL (IgGκ), T	PE	EBV-	IL-6	Sensitive to BMP	[9,15,16]
JJN-3	1987	F, 57	PCL (IgAκ), D	BM	EBV-		Produces HGF. Tolerates high concentrations of BMP	[9,10,17,18]
KJON	2010	F, 66	PCL (Igκ), R/T	PB	EBV-	IL-6	Tolerates high concentrations of BMP	[19]
RPMI-8226	1966	M, 61	MM (IgGλ), D	PB	EBV-		Tolerates high concentrations of BMP	[9,10,20,21]
U266	1968	M, 53	MM (IgEλ), R/T	PB	EBV-	IL-6	IL-6 autocrine loop. Tolerates high concentrations of BMP	[9,10,20,22]

*MM - multiple myeloma; PCL - plasma cell leukemia; D - diagnosis; R - relapse; T - terminal

**BM - bone marrow; PB - peripheral blood; PE - pleural effusion; PF - peripheral fluid

--- not found

1.2 TRANSFORMING GROWTH FACTOR-B SUPERFAMILY

The transforming growth factor (TGF)-β superfamily is a comprehensive family of regulatory proteins that are structurally related.[23] The mature protein is released upon proteolytic cleavage of the large synthesized precursor.[24] In most cells TGF-β controls essential mechanisms such as proliferation, apoptosis and cellular differentiation.[23,25] The TGF-β superfamily can be divided into subgroups (Figure 1), including: TGF-βs; BMPs; growth and differentiation factors (GDFs); activins; inhibins; and nodals.[26,27]

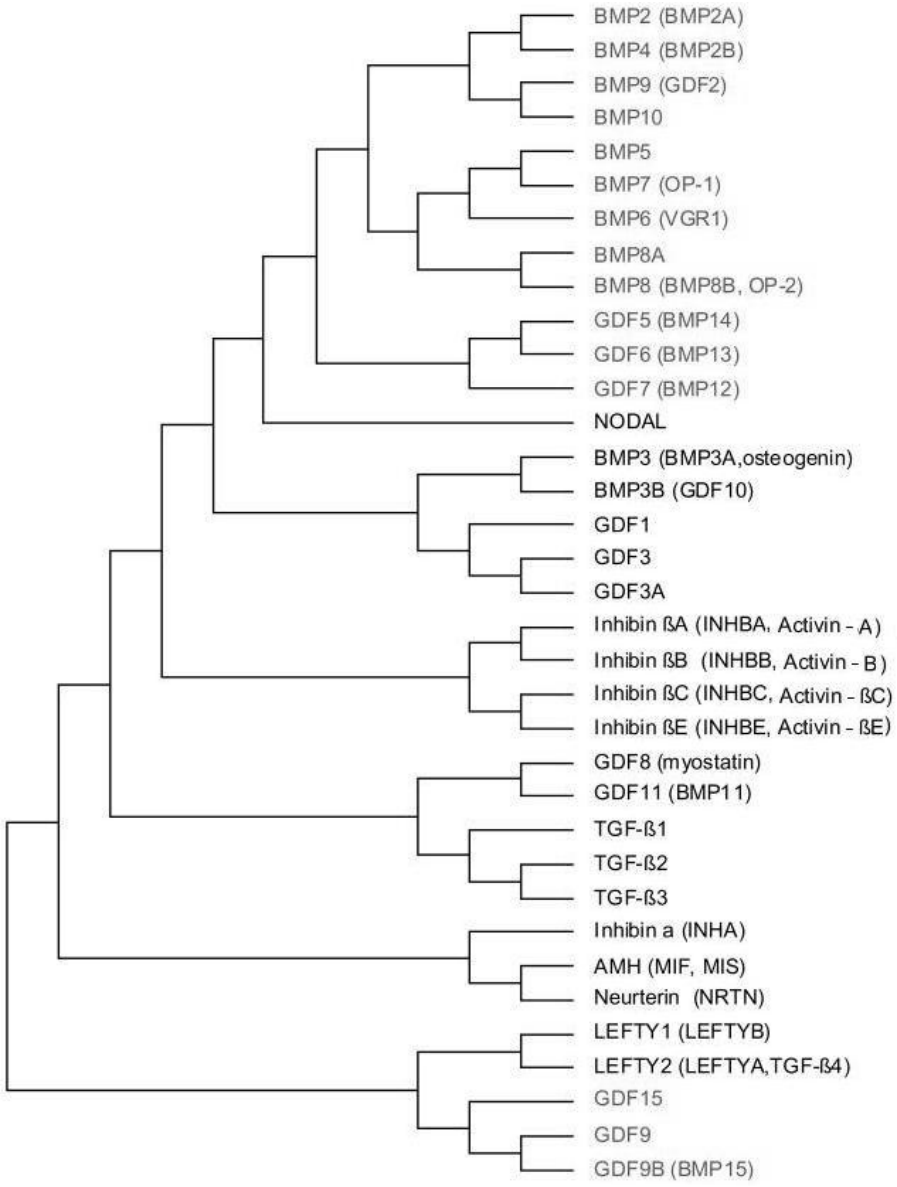


Figure 1. Members of TGF-β superfamily and their evolutionary relation. Synonyms are given in parentheses and members written in grey have been shown to activate BMP signaling by phosphorylating SMADs.[27, Figure 2 adapted]

1.3 ACTIVINS

Activins are dimeric proteins linked by disulphide bonds and can be found as homo- or heterodimers. In humans four β-subunits of activin (βA, βB, βC and βE) is found, where only βA and βB have been shown to be biologically active. These two subunits form the activins known as; activin-A (βAβA), activin-B (βBβB) and activin-AB (βAβB).[28] Activin-A is known to regulate apoptosis, wound healing, cell proliferation, apoptosis and metabolism.[29] Activin-A signals through the Type I receptors activin receptor-like kinase (ALK)-2/ACVR1A and ALK-4/ACVR1B and the Type II receptor ACVR2A. The canonical signaling pathway is further explained in section 1.4.2.[28-30]

1.4 BONE MORPHOGENETIC PROTEINS

BMPs are a group of cytokines (signaling molecules) that constitute the largest part of the TGF-β superfamily.[31] Earlier it has been shown that BMPs play multiple roles in the regulation of growth, differentiation and apoptosis in normal cells as well as various cancer cells. BMP was first identified as a group of proteins capable of inducing formation of cartilage and bone, but are now known as multifunctional cytokines that have involvement in embryogenesis, hematopoiesis and neurogenesis.[30,32,33] The BMP family consists of several genetically related members, and today at least 20 human members are identified, including BMP-1 to BMP-15 (assortment in Table 2).[34]

The activities of BMP can be regulated by certain classes of molecules in different ways, including: BMP-BMP receptor interaction inhibition, presence of dominant negative non-signaling membrane pseudoreceptors, blocking of BMP signaling by inhibitory SMADs (I-SMADs) or intracellular SMAD binding proteins, and ubiquitination or proteasomal degradation of BMP signaling effectors.[35]

The molecules responsible for the inhibition of BMP-BMP receptor interaction are recognized as BMP antagonists.[35,36]

Table 2. Some BMPs, their subfamily, generic name and known function.

BMP- **(Generic name)** ***Subfamily***	**Known function**	**Ref**
BMP-2 (BMP-2A) *BMP 2/4*	Dominant role in development of extra embryonic tissue, heart and limb bud during embryogenesis in addition to neurogenesis. FDA approved (with BMP-7) for clinical applications, and currently used in fractures of long bones, regeneration of invertebral disc and spinal fusion. BMP-2 also is approved for certain dental applications.	[27,31-34,37]
BMP-4 (BMP-2B) *BMP 2/4*	Development of kidney, heart, lung and limb bud formation during embryogenesis. Mesoderm induction and formation, induction of apoptosis of neural precursors.	[24,27,31,32,38,39]
BMP-6 (VGR-1) *OP-1*	Regulates cell growth, differentiation and apoptosis. Also have a role in development of cartilage, neurogenesis during embryonic development.	[27,31]
BMP-7 (OP-1) *OP-1*	Key role in development of kidney, heart and small intestine. By implanting BMP-7 in an adequate matrix, new bone formation will be induced. FDA approved for clinical applications, see section about BMP-2.	[27,31-33,40,41]
BMP-9 (GDF-2) *OP-1*	Production of ectopic bone growth. Involved in induction of osteo- and chondrogenesis in differentiation of cholinergic neurons, differentiation of mesenchymal stem cells into cartilage and proliferation of cultured liver cells.	[27,31,42,43]

In myeloma cells it has been shown that BMP-2, -4, -5, -6 and -7 induces growth arrest and apoptosis. SMAD-1, -5 and -8 are central in the BMP signal transduction.[11,30,44]

1.4.1 SMADs

SMAD is derived from the genes SMA and MAD (mothers against decapentaplegic (dpp)) in *Caenorhabdittis elegans* (*C. elegans*) and *Drosophila* respectively, and is an abbreviation for (small) mothers against dpp.[45] SMADs are proteins that convert signals from TGF-β ligands, as BMPs, to the nucleus and regulate transcription.[45,46] The role of SMADs in BMP signaling is further explained in chapter 1.4.2.

There are three subgroups of SMADs, including 1) receptor-regulated (R-SMADS) which consist of SMAD-1 to -3, -5 and -8; 2) common-partner (Co-SMAD) which is SMAD-4 in humans; and 3) inhibitory (I-SMADs) which consist of SMAD-6 and -7.[46]

1.4.2 BMP SIGNALING AND RECEPTORS

BMP signals by binding to BMP receptors (BMPRs) with serine/threonine kinase activity (assortment of TGF-β superfamily ligands and their receptors is given in Table 3).[31] There are two types of receptors, Type I (also called activin receptor-like kinases (ALK)) including ALK-1 to -3 and Type II including activin receptor (ACVR)2A, ACVR2B and BMPR2. Type II receptors are constitutively active and will phosphorylate Type I receptors upon binding.[27] Type I and Type II receptors all contain a cysteine rich extracellular domain, a transmembrane region and a serine/threonine intracellular domain. The main difference between the two types is the glycine/serine (GS) rich domain which is a short regulatory sequence found in the cytoplasmic domain of Type I receptors, N-terminal to the kinase domain.[47,48] BMPR2 differs from the other members of Type II receptors by containing a long tail in the cytoplasmic domain, C-terminal to the kinase domain (Figure 2).[49] The tail is involved in regulation of p38 and p42/44 mitogen-associated protein kinase (MAPK) pathways, which are alternate, non-SMAD signaling pathways of BMPs. The complete function of this tail has yet to be discovered.[50]

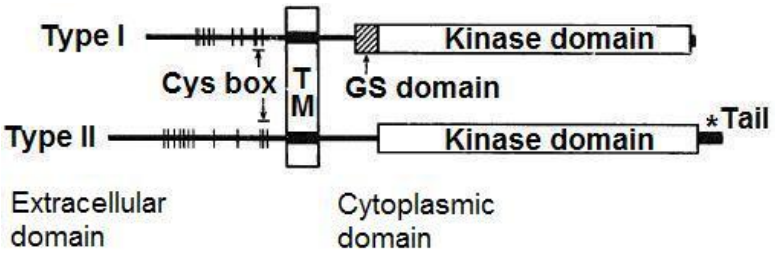


Figure 2. General structure of Type I and Type II BMP receptors. Vertical bars represent cysteins in the extracellular domain and TM denotes the transmembrane region. GS is a glycine- and serine rich domain. The tail (*) on Type II receptor is only found in BMPR2 in humans.[47, Figure 3 adapted]

Table 3. Assortment of TGF-β superfamily ligands and their receptors.

Ligand	Receptor Type I	Receptor Type II	Ref
BMP-2	ALK-2/ACVR1A	ACVR2A	27,31,51-53
	ALK-3/BMPR1A	ACVR2B	
	ALK-6/BMPR1B	BMPR2	
BMP-4	ALK-3	ACVR2A	27,31,52-55
	ALK-6	BMPR2	
BMP-6	ALK-2	ACVR2A	27,31,56
	ALK-3	ACVR2B	
	ALK-6	BMPR2	
BMP-7	ALK-2	ACVR2A	27,31,52,54,57,58
	ALK-3	ACVR2B	
	ALK-6	BMPR2	
BMP-9	ALK-1/ACVRL1	ACVR2A	27,31,42,59,60
	ALK-2	ACVR2B	
		BMPR2	
Activin-A	ALK-2	ACVR2A	28-30
	ALK-4/ACVR1B		

BMPs bind as homo- or heterodimers to receptor Type I and II with different affinities. The binding can occur to a preformed receptor complex, or to a receptor complex which is formed by ligand binding to receptor Type I that thereafter recruits receptor Type II.[61] When BMP-BMP receptor interacts, heterotetrameric receptor complexes will be created. When this complex is formed Type II receptors will phosphorylate Type I receptors, enabling binding and phosphorylation of intracellular proteins called R-SMADs.[33] There are two classes of R-SMADs: 1) BMP-activated: SMAD-1, -5 and -8 which are activated by ALK-1 to -3 and ALK-6; and 2) TGF-β/Activin-activated: SMAD-2 and -3 which are activated by ALK-4, -5 and -7.[46,62] The phosphorylated R-SMADs can then bind to SMAD-4, which is the Co-SMAD in humans, translocate to the nucleus and regulate transcription (Figure 3). This BMP signaling is called the SMAD pathway.[31,33] Activation of SMADs can be detected by western blot, which is a technique used to detect target proteins by the use of gel electrophoresis, membrane blotting and specific antibodies.[63] In this case antibodies against e.g. the phosphorylated forms of SMAD-1/-5/-8 and SMAD-2/-3 can be used.

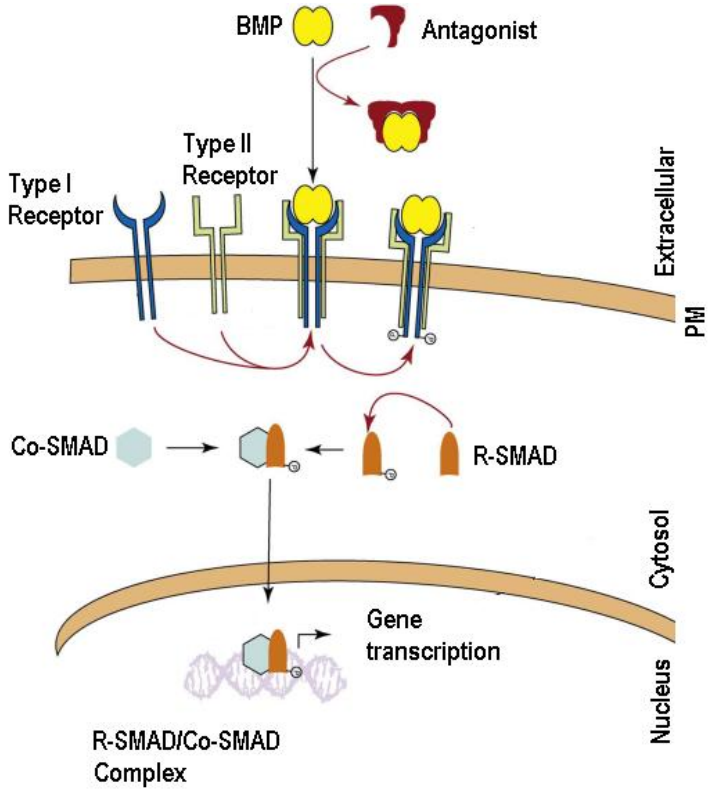


Figure 3. BMP signaling pathway. Binding of BMP enables Type II receptor to bind to and activate Type I receptor, which lead to the phosphorylation of R-SMADs. R-SMAD will form a complex with Co-SMAD, translocate to the nucleus and regulate gene transcription.[33, Figure 1 adapted]

1.4.3 Regulation of BMP signaling

BMP signaling can be regulated on several levels. BMP Type III receptors are membrane-associated and interfere with the BMP-BMP receptor binding by competing for the BMP binding sites on the BMP receptor.[27] Two Type III receptors are identified (Table 4), which respond to different BMPs. Heparan sulfate proteoglycan (HSPG) syndecan-1 is a potential co-receptor since it binds to various factors, including signaling molecules, enzymes and mitogens.[64] In addition syndecan-1 is shown to have both favorable and unfortunate effect for myeloma patients.[65,66]

Table 4. BMPs and their Type III receptor.

Type III receptor	BMP-	Ref
Endoglin	2, 7, 9,10	[27,67]
Betaglycan	2, 4, 7	[27,68]

ENDOGLIN

Endoglin, or CD105, is a transmembrane glycoprotein and one of the Type III receptors for the TGF-β superfamily, predominantly expressed on endothelial cells.[68] The Type III receptors, unlike Type I and II, is involved in the modulation of signal responses from various members of the TGF-β superfamily.[69] Endoglin is expressed in chondrocytes, vascular endothelial cells and in neural stem cells in lower amounts, and plays a role in regulating systematic and pulmonary vascular homeostasis, cancer, angiogenesis, preeclampsia, and neovascularization.[27,69] Endoglin has a large extracellular domain which can undergo proteolytic cleavage, creating a soluble form.[27] The membrane form and the soluble form of this co-receptor are known to present BMPs to their receptors, and to prevent BMP-BMP receptor interaction.[68] An exception is BMP-9 and -10, where endoglin also can bind directly with high affinity and in that way inhibit the SMAD signaling pathway.[67]

The extracellular domain contains an orphan domain (OD) composed of N-linked glycans with no existing similarity to known proteins, and a zona pellucida domain (ZPD) which is involved in the interaction between ligand and Type III receptors. Human endoglin also contains an RGD domain (ArgGlyAsp) which acts as an interaction motif for integrins. The PDZ domain exists in the cytoplasm and is a consensus binding motif of 80-90 amino acid (aa), in this case sequence SerSerMetAla (Figure 4). The abbreviation PDZ comes from combining first letters of the three proteins post synaptic density protein (PSD95), Drosophila disc large tumor suppressor (Dlg1) and zonula occludens-1 protein (Zo-1). [68,69]

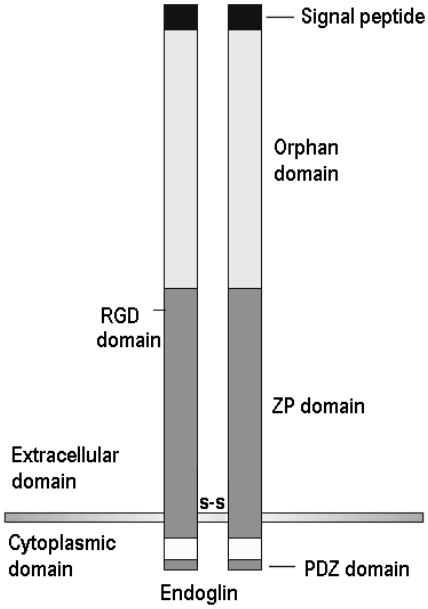


Figure 4. Representation of endoglin, which is a homodimeric Type III receptor linked with a disulphide bridge (s-s). The extracellular domain is large, and contains an orphan domain (OD) and a zona pellucida domain (ZPD). The PDZ domain is phosphorylated at SerSerMetAla residues. The RGD domain (ArgGlyAsp) is only present in humans.[69, Figure 1 adapted]

BETAGLYCAN

Betaglycan, TGF-β RIII, is a transmembrane glycoprotein and is known to have a role in BMP signaling. Betaglycan shares a high degree of aa homology with, and acts like endoglin in enhancing the BMP-BMP receptor binding. This Type III receptor also exists both in membrane bound and soluble form.[68] The soluble form of betaglycan is known to inhibit proliferation, growth and motility by binding to BMPs or to the BMP receptors.[68,70] In many myeloma cells betaglycan is poorly expressed or not present at all, which may contribute to the progression of MM. By restoring expression of betaglycan, the growth and proliferation of myeloma cells was inhibited, independent of ligand presentation.[70]

The structure of betaglycan is very much similar to endoglin. The main differences between these two co-receptors are that betaglycan not contain the RGD domain, the PDZ domain sequence is SerSerThrAla, and the interactions between monomers are non-covalent (Figure 5).[68]

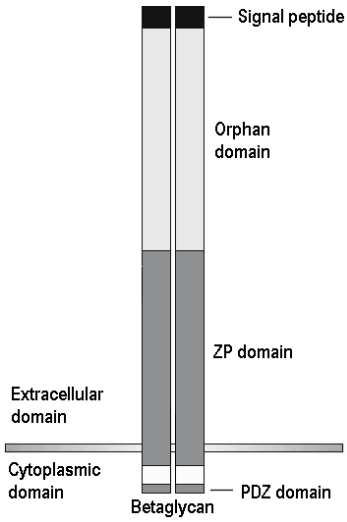


Figure 5. Representation of betaglycan, which is a homodimeric Type III receptor linked by non-covalent interactions. The extracellular domain is large and it contains an orphan domain (OD) and a zona pellucida domain (ZPD). The PDZ domain is phosphorylated at SerSerThrAla residues.[68, Figure 2 adapted]

SYNDECAN-1

HSPG syndecan-1, CD138, is a transmembrane protein and a member of the syndecan family (Figure 6). The syndecan family is Type I integral membrane proteins that contain homologous transmembrane and cytoplasmic domains, and syndecan-1 contains an extracellular region with glycosaminoglycan (GAG) attachment sites.[71] This protein is shed with a high rate by myeloma cells and is expressed on myeloma cell surfaces, epithelial cells and terminally differentiated B cells such as plasma cells.[64,72] In most cases, syndecan-1 increase the binding of growth factors to their respective receptors by leading growth factors closer, but shed syndecan-1 can sequester growth factors and reduce the concentration of them at the cell surface. Syndecan-1 binds to various factors, including signaling molecules, enzymes and mitogens. It is thus likely that the expression of syndecan-1 depends on the concentration of these factors.[64]

Soluble syndecan-1 in MM facilitates growth, angiogenesis and metastasis.[64] Since syndecan-1 has been shown to have both favorable effect for patients by inducing apoptosis and unfortunate effect for prognosis when found in high levels in serum, there is a possibility that the shed syndecan-1 can have a harmful effect *in vivo*.[65,66]

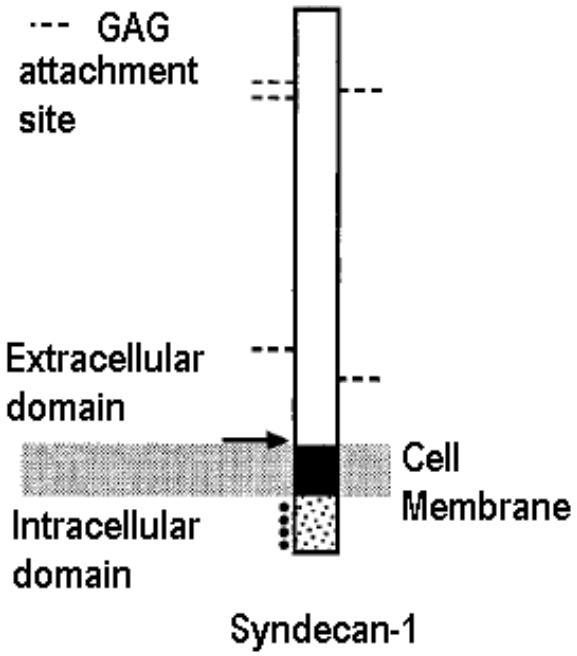


Figure 6. Representation of syndecan-1, which is one of four homologous transmembrane proteins of the syndecan family. Syndecan-1 contains an extracellular region with potential GAG attachment sites (dotted lines) and a transmenbrane domain (black). The potential cleavage site is indicated with an arrow.[73, Figure 1 adapted]

INHIBITORS

There are several small molecule inhibitors of BMPs described in current literature, e.g. dorsomorphin (DM) and different analogues of DM.[74,75]

DM is a small molecule that prevents activation of R-SMADs, and has been shown to inhibit BMP-2, -4, -6 and -7 (Figure 7 A). The mechanism for this inhibition is still unclear, but likely it happens by inhibiting the Type I receptors ALK-2, -3 and -6.[75]

An analogue of this inhibitor is dorsomorphin analogue 1 (DMH1), which is both potent and selective (Figure 7 B). DMH1 has higher specificity to the Type I receptors than DM, and has been shown to inhibit ALK-2 directly.[74]

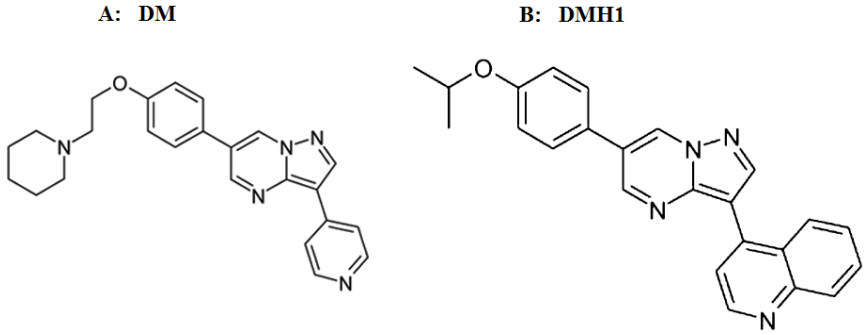


Figure 7. Structure of inhibitors. **A:** dorsomorphin (DM) and **B:** dorsomorphin analogue 1 (DMH1).[74,] Figure 1 and 3 adapted

1.4.4 BMP ANTAGONISTS

The activity of BMPs is in some part modified by extracellular BMP antagonists that function by binding to various BMPs with different affinity, and in that way inhibit BMP-BMP receptor interaction.[36] Several antagonists have been identified and they usually contain between 170-250 aa. Two exceptions are chordin with 948 aa, and crossveinless-2 (CV-2) with 685 aa. The antagonists differ in their binding specificity and affinity to different BMPs.[33] The protein sequence of the extracellular BMP antagonists contains cysteine-rich (CR) domains, giving them a cystine-knot structure.[35] This structure is made of two pairs of intramolecular disulfide bonds, that together form a recognizable ring; and a third bond

penetrating the ring, forming the knot-like structure.[76] Variations in residue numbers between the two bonding cystines give the origin to a ring of odd sizes, and allow the division of the antagonists into three classes. The classes of extracellular BMP antagonists include: 1) noggin, chordin and follistatin (FS) that contain a 10-membered ring; 2) twisted gastrulation (Tsg) that contain a nine-membered ring; and 3) the DAN (differential screening-selected gene aberrative in neuroblastoma) family that contain an eight-membered ring (Figure 8).[36,76]

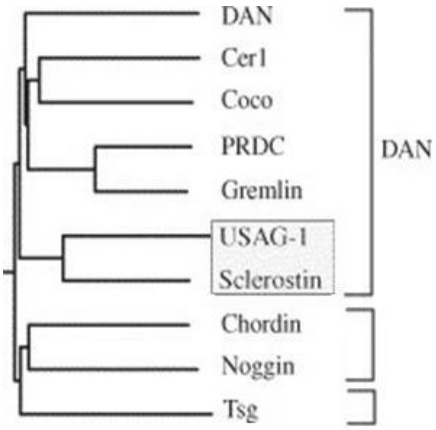


Figure 8. Phylogenetic tree where the subgroups of BMP antagonists are given. The antagonists are divided based on the similarity of the overall aa sequence.[36, Figure 1 adapted]

THE CHORDIN FAMILY, FOLLISTATIN AND BRORIN

The chordin family includes the antagonist chordin, the newly identified antagonist brorin (2007)[77], and chordin like (CHL) antagonists e.g. CHL-1 and CV-2.[33,35] These antagonists consist of a 10-membered cystine ring, and the spacing between the cysteins has a structural homology.[35] The antagonists noggin and FS also fall under this class.[31] Brorin is considered to be a unique member since the only similarity with the other members is the 10-membered cystine ring.[77]

Chordin is secreted as a glycosylated homodimer and is regulated by interaction with other secreted proteins from the extracellular matrix. Chordin binds with high affinity to BMP-2, -4 and -7, preventing the BMP-BMP receptor interaction.[35,45] Chordin does not bind to other members of the TGF-β superfamily.[35] CHL-1 is structurally similar to chordin, and is thought to have similar function in the inhibition of BMP-BMP receptor by binding to BMPs.[78] CHL-1 binds to BMP-2, -4 and -6.[31]

CHL antagonists are in most cases known to antagonize the BMP effect, but some of them, e.g. CV-2, can also promote the BMP effect. CV-2 antagonizes the effect of BMP-2 and -4, but has in addition the ability to bind chordin and in that way promote the BMP effect.[35,79]

Brorin is a recently identified BMP antagonist, and has been shown to inhibit the effect of BMP-2 and -6 by interacting with two von Willebrand factor C (VWFC) domains.[33,77]

FS is an activin binding protein, and prevents the binding of activin-A to receptors.[80] FS has also been shown to bind BMP-2, -4 and -7, with higher affinity to BMP-7.[76,81,82]

TWISTED GASTRULATION

Tsg is a secreted glycoprotein that consists of a nine-membered cystine ring. Tsg can either function as an antagonist or an agonist, but how it works *in vivo* is still to reveal.[35] Tsg functions as an antagonist by binding to BMP-2 and -4.[31] This direct binding inhibits the BMP-BMP-receptor interaction. Tsg has also shown to work as a cofactor for the antagonist chordin by forming a tertiary complex thus enhancing the binding of BMP-4.[83]

Tsg can in addition lead to the cleavage of chordin and in this way work as an agonist. The BMP binding response can also be provoked if Tsg binds to the receptor.[35,83]

THE DAN FAMILY

The DAN family includes the antagonist gremlin, sclerostin (SOST), dante and cerberus.[35] These antagonists are secreted glycoproteins that consist of an eight-membered cystine ring.[76] Antagonists in the DAN family share a common carboxyterminal CR domain, beyond this the homology is vague. In general these proteins are expressed during embryogenesis, but gremlin and SOST have also been shown to regulate the activity of BMP in adult skeleton.[35,45]

Gremlin binds to and antagonizes the effect of BMP-2, -4 and -7 in a similar way as chordin.[45,82,84] The effect of SOST on BMP effect is controversial. According to Kusu[85], SOST binds strongly to BMP-6 and -7 and weakly to BMP-2 and -4. Gazerro[35], on the other hand shows that SOST only bind with low affinity to BMP-2, -4, -5, -6 and -7.

1.5 APOPTOSIS

Apoptosis is a form of cell death and is a mechanism needed to obtain homeostasis in tissue.[86,87] There are different subgroups of cell death that are divided based on measurable biochemical features, including: extrinsic apoptosis, regulated necrosis, autophagic cell death, mitotic catastrophe and cascade-dependent or -independent intrinsic apoptosis.[88]

Caspases are a type of proteins that are activated during early stages of apoptosis, and function by cleaving essential cellular components like DNA repair enzymes and nuclear proteins. The caspases can in addition activate DNases which will cleave nuclear DNA and thereby promote apoptosis.[89,90] The proteases are cystein-dependent aspartate-directed, and are divided into two subgroups based on function. One group is involved pathogen immunity and the maturation of cytokines, and are called the inflammatory caspases (caspase-1, -4 and -5). The other group has a role in regulation of apoptosis. Caspase-2, -8, -9 and -10 are initiators and caspase-3, -6 and -7 are effectors.[90]

Three different pathways for apoptosis have been defined including the death receptor pathway initiated by binding to death receptors leading to recruitment of adaptor proteins; mitochondrial pathway initiated by external and internal stress signals like irradiation; and endoplasmic reticulum (ER)-stress pathway which is especially important in secretory cells such as plasma cells (Figure 9).[87]

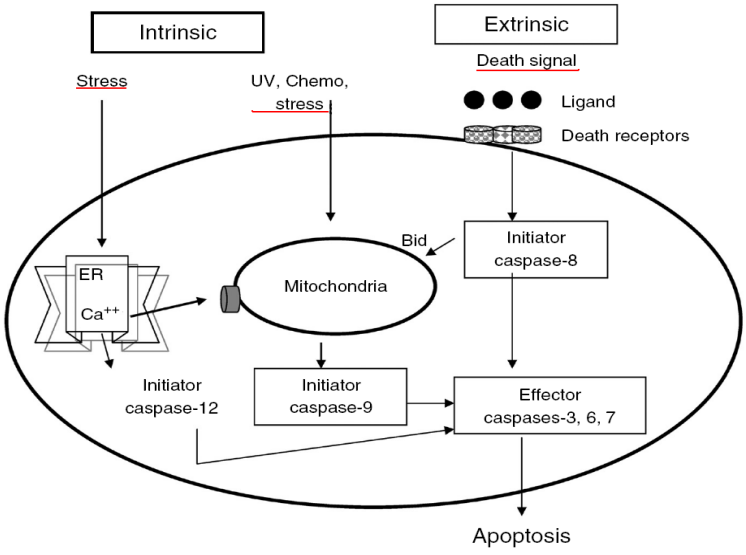


Figure 9. The three different pathways for apoptosis and as the figure shows there is crosstalk between them.[87, Figure 1 adapted]

1.6 PROLIFERATION

Proliferation is a term used to describe the increase in cell number, rapid growth or reproduction of e.g. cells, new tissue or offspring.[91] Proliferation is among other things dependent of the rate of cell division which is the time it takes to complete a cell cycle (Figure 10).[92] The cell cycle consists of several phases. First protein and RNA is synthesized in gap phase-1 (G_1). When the cells are ready to divide they will pass a restriction (R) point before DNA is synthesized in the synthetic (S) phase. The newly synthesized chromosomes will condense in the second gap phase, G_2, and at last the complete replicated chromosomes are segregated to the two daughter cells by mitosis (M). G_0 denotes a resting state where only normal cells can be. The cells have in this phase gone into growth arrest and will not divide. Differentiation is the normal process where a cell matures to possess a more distinct phenotype and function. When normal cells differentiate they generally lose the capacity to continue cell division.[92]

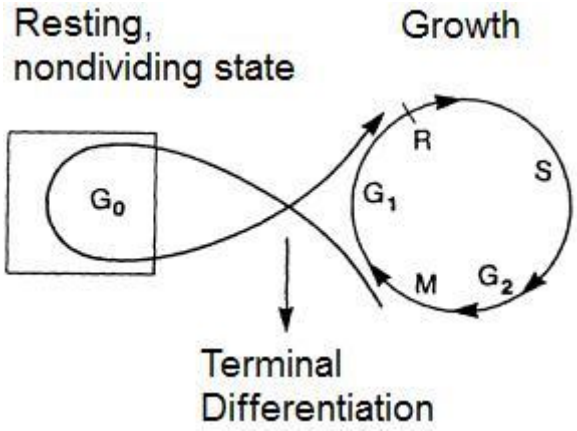


Figure 10. The cell cycle. G_1 (protein and RNA synthesis) and G_2 (condensation of the new duplicated chromosomes) are preparatory gap phases which precedes S and M phase respectively; R (restriction point) is where the cell pass through when they are ready to divide after G_1; S (synthetic period) is where DNA is synthesized; M (mitosis) is the phase where the complete replicated chromosomes are segregated to the two daughter cells by mitosis. G_0 denotes a resting, nondividing state where only normal cells can be.[92, Figure 2.1 adapted]

When cancer cells arise due to the accumulation of genetic changes, they often get a proliferative advantage compared to normal cells. By measuring proliferation of cancer cells the prognosis, management and treatment of patients can be determined.[91,93,94] One of the goals of current treatment is to reduce the number of cancer cells by preventing proliferation, but since the mechanisms for proliferation in both cancer and normal cells are similar this task has been shown to be challenging.[92]

2 METHODS

2.1 CELL LINES AND CULTURE CONDITIONS

The following hMCLs were used: INA-6 (kind gift from Dr. M Gramatzki, Erlangen, Germany), IH-1 (established at St. Olav`s University Hospital, Trondheim, Norway[11]), RPMI-8226 (from America Type Culture Collection (ATCC), Rockville, MD, USA), JJN-3 (kind gift from J. Ball, University of Birmingham, UK), KJON (established at St. Olav`s University Hospital), U266 (from ATCC), ANBL-6 (kind gift from Dr. D Jelinek, Mayo Clinic, Rochester, MN, USA), and CAG (kind gift from Dr. J Epstein, Little Rock, AR, USA). The non-myeloma hematologic cell line U937 was from ATCC and the human hepatoma cell line HepG2 was from The European Collection of Cell Cultures (ECACC, Sigma Aldrich)

For experiments the cells were cultured in different media than the usual growth media (Table 5). All cells were cultured at 37 °C with 5 % CO_2 atmosphere. Washing of cells were performed by resuspension in Hanks` balanced salt solution (HBSS) (Sigma Aldrich, London, UK).

Table 5. Growth- and experiment media for hMCL

hMCL	Growth media	Experiment media
ANBL-6, INA-6	10 % FCS in RPMI, IL-6 (1 ng/ml)	2 % HS in RPMI, IL-6 (1 ng/ml)
CAG, JJN-3	10 % FCS in RPMI	2 % HS in RPMI
IH-1	10 % HS in RPMI, IL-6 (1 ng/ml)	2 % HS in RPMI
KJON	5 % HS in RPMI, IL-6 (1 ng/ml)	2 % HS in RPMI
RPMI-8226	20 % FCS in RPMI	2 % HS in RPMI
U266	15 % FCS in RPMI	2 % HS in RPMI
HepG2*	10 % FCS in EMEM, 1 % NEAA	10 % FCS in RPMI
U937**	10 % FCS in RPMI	2 % HS in RPMI

*HepG2 is a human hepatoma cell line, **U937 is a non-myeloma hematologic cell line
Abbreviation: EMEM - Eagle`s minimal essential medium (Sigma Aldrich) supplemented with L-glutamine (2 mM); FCS - fetal calf serum; HS - heat inactivated human serum (Blood Bank, St. Olav`s University Hospital); NEAA - non-essential amino acid solution 100 X (Sigma Aldrich); RPMI - RPMI (Sigma Aldrich) supplemented with L-glutamine (100 mg/ml) and gentamicin (20 mg/ml); IL-6 (Biosource, CA, USA)

2.2 APOPTOSIS STUDIES

Apoptosis was studied by analyzing annexin-V FITC binding and PI uptake on flow cytometry. In flow cytometry cells in suspension can be detected based on the binding of e.g. dyes or fluorescently labeled antibodies. The scattered light gives a quantitative measurement that reflects the morphology of the cell, and when using fluorescent components the fluorochromes get excited with a higher energy unique to different fluorochromes. The emitted light from the cells is focused before reaching a detector, which converts the light signal to an electric signal proportional to the amount of fluorochrome in the cells.[95]

In early apoptosis the asymmetry of the plasma membrane is lost, which leads to the exposure of phosphatidylserine (PS). Fluorescein isothiocyanate (FITC) labeled annexin-V is often used as a specific probe, and will bind to PS when Ca^{2+} is present. Annexin is not able to bind to normal viable cells, since the phospholipid bilayer is functional (Figure 11).[96]

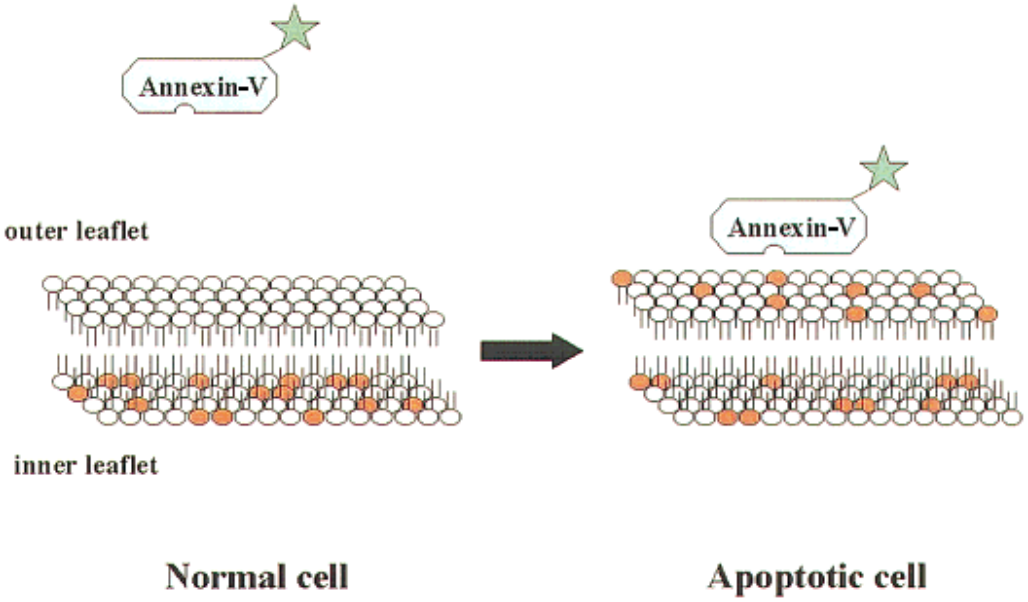


Figure 11. Representation on how annexin-V FITC will bind to PS in an apoptotic cell.[96, Figure 1 adapted]

Propidium iodide (PI) is used in addition to annexin-V to part the apoptotic cells from the dead cells. By using flow cytometry the result is given in a diagram with four separate quadrants named Q1-Q4. Q2 contains dead cells, Q3 contains viable cells and Q4 contains apoptotic cells (Figure 12).[96]

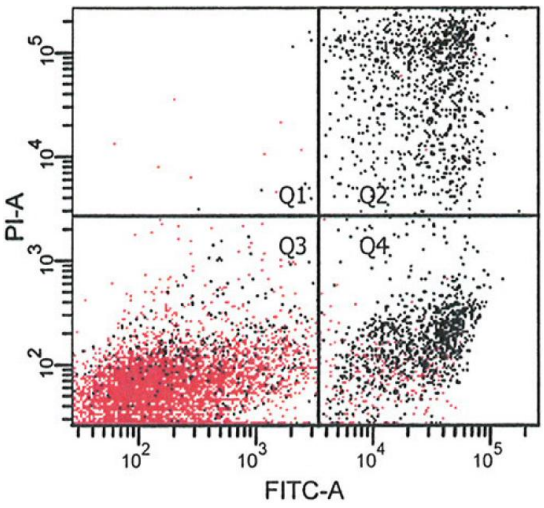


Figure 12. An example on annexin-V FITC binding and PI uptake in hMCL IH-1. The cells are divided in dead-, viable- and apoptotic cells located respectively in quadrant Q2-Q4.[97]

Cells were seeded in 96-well culture plates (Corning Costar, Corning, NY, USA) with different concentrations of cytokines. The cells were incubated for 3 days before they were stained with annexin-V FITC and PI by the following protocol from the manufacturer (APOPTEST-FITC kit, Nexins Research, Hoeven, Netherlands).

In short samples were incubated on ice for 1 hour with annexin-V FITC (0.2 µg/ml) in binding buffer. PI (1.4 µg/ml) was added 5 minutes before the samples were measured and classified as PI- or annexin-V-positive or -negative using BD LSR II flow cytometer (BD Biosciences, USA).

PI positive cells are classified as dead cells, PI-negative and annexin-V positive cells are classified as apoptotic cells, while the remaining cells that are negative for both PI and annexin-V are classified as viable cells.

2.2.1 Effect of BMP-9 on apoptosis

The effect of BMP-9 (R&D systems, Abingdon, UK) on apoptosis in hMCLs INA-6, IH-1, RPMI-8226, U266, JJN-3, KJON, ANBL-6, and CAG was tested by analyzing annexin-V FITC binding and PI uptake on flow cytometry. Cells were seeded with different concentrations of BMP-9 as indicated (Table 6).

Table 6. Concentrations of BMP-9 and amount of cells plated in 96-well plates.

hMCL	Cells/well	Concentration of BMP-9 (ng/ml)
INA-6	30,000	0-5
IH-1	50,000	0-5
RPMI-8226, ANBL-6, CAG, U266, KJON	50,000	0-20
JJN-3	20,000	0-20

2.2.2 Apoptosis studies on regulation of BMP signaling

Endoglin and syndecan-1

Recombinant human variants of the Type III receptors endoglin and betaglycan, and the HSPG syndecan-1 (all from R&D systems), were tested against different BMPs using IH-1 cells. This was done to see if any of them had the ability to counteract the effect of BMPs.

IH-1 cells (50,000 cells/well) were seeded with BMP-2 (200 ng/ml), BMP-4 (20 ng/ml), BMP-6 (300 ng/ml) and BMP-9 (5 ng/ml), and soluble endoglin (0-500 ng/ml) or syndecan-1 (1-10 µg/ml) (all reagents from R&D systems).

The hMCL ANBL-6, CAG, IH-1, INA-6, JJN-3, KJON, RPMI-8226 and U266, the non-myeloma hematologic cell line U937 and stromal cells were in addition checked for contents of surface endoglin by labeling with endoglin antibody (PE Mouse anti- Human CD105, BD Biosciences) before detecting with flow cytometry. Syndecan-1 (CD138, BD Biosciences) was used as a labeling control, and Mouse γ1 PE(x40) (BD Bioscience) was used as isotype control. Samples were incubated 30 minutes with 5 µl antibody before detection.

CpG-ODN

Cytosine-phosphate-guanosine oligodeoxynucleotide (CpG-ODN) (CpG 2006, 5′-TsCsGsTsCsGsTsTsTsTsGsTsCsGsTsTsTsTsGsTsCsGsTsT-3′, TIB MOLBIOL, Berlin, Germany) is a compound that previously has been shown to inhibit SMAD activation.[98] The effect of CpG-ODN on SMAD activation by BMP-9 was tested by performing apoptosis experiments on INA-6 and IH-1 cells (50,000 cells/well) with CpG-ODN (4-8 µM), BMP-4 (5-20 ng/ml), BMP-6 (25-300 ng/ml) or BMP-9 (0.1-4 ng/ml).

DMH1

The small molecule BMP inhibitor DMH1 (Sigma Aldrich) was used as a control of the effect on SMAD-inhibition. DMH1 was tested by performing apoptosis experiments. INA-6 and IH-1 cells (50,000 cells/well) were seeded with BMP-2 (200 ng/ml), BMP-4 (20 ng/ml), BMP-6 (300 ng/ml) and BMP-9 (0.05-5 ng/ml) and DMH1 (0.25-1 µM).

2.3 PROLIFERATION STUDIES

Proliferation can be determined by measuring DNA synthesis or adenosine triphosphate (ATP) content. ATP is the energy unit of the cells and when viable cells are in need of energy ATP will release chemical energy. ATP-bioluminescence assays measures amount of ATP which is directly proportional to the number of viable cells in culture (Figure 13).[99,100] One example of an ATP-bioluminescence assay used to measure proliferation is CellTiter Glo from Promega, and this assay was used in experiments.[100]

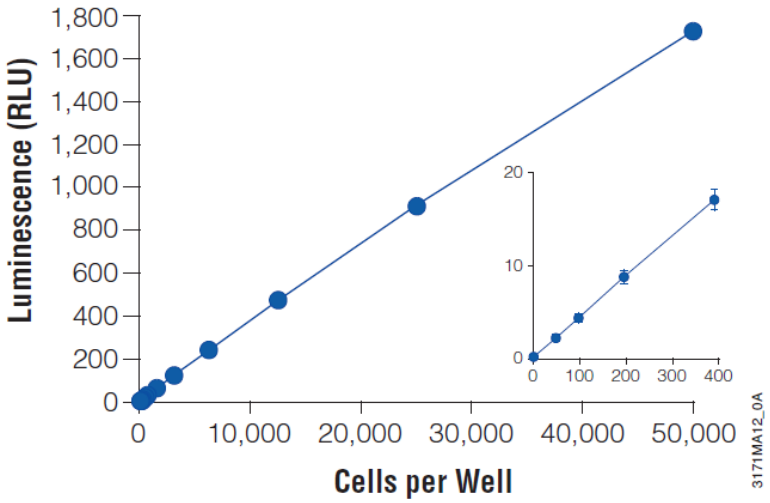


Figure 13. The number of cells in culture is directly proportional to the amount of ATP which is measured as luminescence.[100, Figure 2]

CellTiter Glo is based on a luciferase called Ultra-Glo™ Recombinant Luciferase. The thermostable luciferase will trigger a reaction which produces light that can be detected with a regular plate reader. When the CellTiter Glo reagent is added to the cell culture luciferin will be converted, by a mono-oxygenation, to the inactive oxyluciferin and light will be produced. This reaction is catalyzed by the enzyme Ultra-Glo™ Recombinant Luciferase, and takes place when magnesium (Mg^{2+}), ATP and oxygen (O_2) is present (Figure 14).[100]

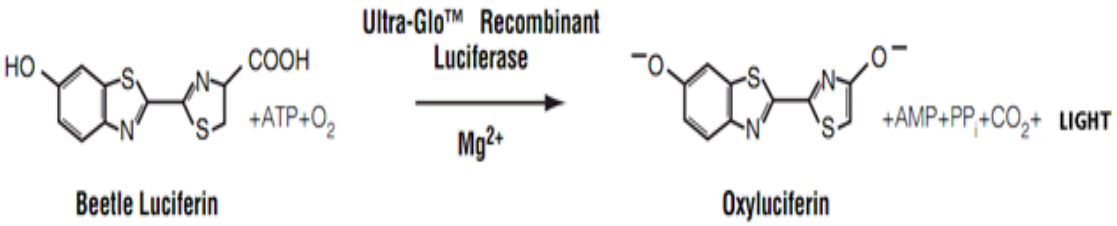


Figure 14. The CellTiter Glo reaction where the enzyme Ultra-Glo™ Recombinant Luciferase will catalyze the mono-oxygenation of luciferin which produces detectable light and inactive oxyluciferin. ATP, oxygen (O_2) and magnesium (Mg^{2+}) have to be present to make this reaction take place.[100, Figure 3]

Cells were seeded in 96-well white culture plates with clear, flat bottom (Corning Costar) with different concentrations of cytokines. The plates were incubated for 3 days before detection with CellTiter Glo® Luminescent Cell Viability Assay (Promega, Madison, WI, USA) following the protocol from the manufacturer. In short the CellTiter Glo reagent was added to the cells in same amounts as total volume (both reagent and cells had room temperature). The plate was placed on a plate shaker for 2 minutes following incubation on bench for 10 minutes before detection of luminescence with Victor Plate reader (PerkinElmer Inc., Waltham, MA).

2.3.1 Effect of BMP-9 on proliferation

The effect of BMP-9 on proliferation in hMCLs INA-6, IH-1, RPMI-8226, U266, JJN-3, KJON, ANBL-6, and CAG was tested by seeding cells for 3 days with different concentrations of BMP-9 (Table 7).

Table 7. Concentrations of BMP-9 and amount of cells plated in 96-well plates.

hMCL	Cells/well	Concentration of BMP-9 (ng/ml)
INA-6, JJN-3	10,000	0-50
ANBL-6, IH-1	20,000	0-50
RPMI-8226, CAG, U266, KJON	50,000	0-50

2.3.2 Effect on proliferation by antagonists

The antagonists chordin-like 1 (CHL-1), gremlin, crossveinless-2 (CV-2), twisted gastrulation (Tsg), follistatin (FS), brorin and sclerostin (SOST) (all from R&D systems) were tested against BMP-9 on INA-6 cells to see if any of them had the ability to antagonize the effect of BMP-9. INA-6 cells (20,000 cells/well) was seeded with BMP-9 (0.25-0.5 ng/ml) and different antagonists (0-10 µg/ml). DM (2 µM) (Calbiochem Merck Chemicals, Nottingham, UK) was used as a positive control.

Control experiments were performed to show that the antagonists were functional. In these experiments IH-1 cells (20,000 cells/well) were seeded with different BMPs or Activin-A, and different antagonists (Table 8).

Table 8. Controls used for testing functionality of the antagonists.

Antagonist (µg/ml)	**Control (ng/ml)**
CHL-1 (5)	BMP-4 (1)
Gremlin (5)	BMP-4 (1)
CV-2 (5)	BMP-4 (1)
Tsg (5)	BMP-4 (1)
FS (5)	Activin-A (100), BMP-2 (100), BMP-4 (1), BMP-7 (100)
Brorin (5)	BMP-2 (100), BMP-4 (1), BMP-6 (75)
SOST (5)	BMP-2 (100), BMP-4 (1), BMP-7 (100)

2.3.3 Proliferation studies on regulation of BMP signaling

Endoglin, betaglycan and syndecan-1

Recombinant human variants of the Type III receptors endoglin and betaglycan, and the HSPG syndecan-1 were tested against different BMPs. INA-6 cells (10,000-20,000 cells/well) were seeded with BMP-9 (0.125-0.25 ng/ml) and soluble endoglin (0-500 ng/ml), betaglycan (0-2 µg/ml) or syndecan-1 (0-500 ng/ml) 3 days before detection.

2.3.4 Soluble receptors

The soluble receptors sALK-1, sALK-2, sALK-3, ALK-6, sACVR2A, sACVR2B and sBMPR2 (R&D systems) was used to find out which receptors BMP-6 and -9 can bind to. INA-6 cells (5,000 cells/well) was seeded with the soluble receptors (5 µg/ml) and either BMP-6 (50 ng/ml) or BMP-9 (0.25 ng/ml) in 3 days before detection with CellTiter Glo.

2.4 Transfections

Nucleofector is a machine used to transfect difficult-to-transfect cell lines, and combines electrical parameters with specific reagents to make pores in the cell membrane which e.g. siRNA can penetrate. This method of transfecting cells is also known as electroporation.[101]

The hMCL INA-6 was transfected by electroporation using Nucleofector™ II from Amaxa biosystems and Amaxa™ Cell Line Nucleofector™ kit R (Lonza, Basel, Switzerland). Cells were transfected with siRNA (1 µM) and cotransfected with selection marker pcDNA-3 CD4 (2.5 µg) (a kind gift from Martin Janz, Berlin). siRNAs used were ON-TARGETplus Non-Targeting pool, ALK-2, -3 and -6, ACVR2A, ACVR2B and BMPR2 (Dharmacon RNAi technologies by Thermo Scientific, Lafayette, CO, USA). Transfected cells were isolated after 18 hours using Dynal® CD4 Positive Isolation kit (Invitrogen by Life Technologies, CA, USA) and the cells were used for further studies when the function of the different siRNA was on top, which was 48 hours after transfection.

2.5 RT-qPCR

Small interfering RNA (siRNA) is double stranded RNA, and is central in RNA interference (RNAi). siRNA may interfere with gene expression by knocking down the messenger RNA (mRNA) of specific genes.[102] Experiments with siRNA can be used to identify e.g. which receptors BMP-9 is using by first transfecting cells with specific siRNAs and then measure gene expression by performing reverse transcription quantitative real time PCR (RT-qPCR).[103]

RT-qPCR can be performed as a two step method where isolated RNA first is reverse transcribed into complementary DNA (cDNA) by traditional PCR, before the cDNA is quantified by real-time PCR.[104] The comparative C_T method is a real-time PCR method based on the difference in the measurement of a fluorescent signal between an experimental and a calibrator sample. The amount of emitted fluorescence is proportional to the amount of PCR product and after each cycle the signal is transformed into a real-time PCR graph which contains four phases (Figure 15): 1) linear ground, where the baseline fluorescence is measured; 2) early exponential, where the fluorescent signal is significantly higher than the baseline, this cycle is known as C_T; 3) log-linear/exponential, where the product doubles each cycle; and 4) plateau, where the reaction stops. The exponential phase gives the most accurate quantification results and is therefore the phase of importance in real-time PCR.[105]

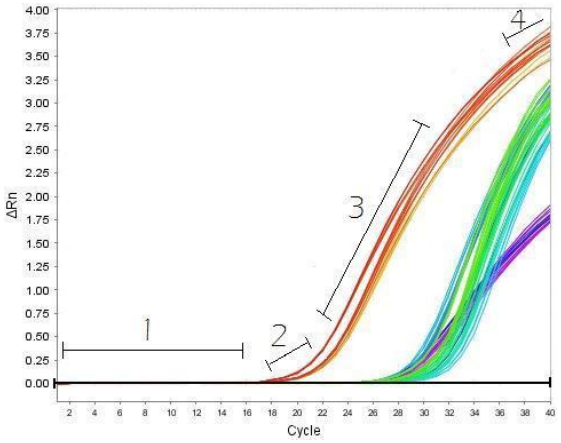


Figure 15. The four different phases in real-time PCR. 1: linear ground phase, where the baseline fluorescence is measured. 2: early exponential phase, where the fluorescent signal is significantly higher than the baseline, this cycle is known as C_T. 3: log-linear/exponential phase, where the product doubles each cycle. 4: plateau phase, where the reaction stops.[97]

To find out which BMP receptors INA-6 cells are expressing RT-qPCR was performed. First total RNA was isolated using RNeasy® Minikit (Qiagen, Crawley, UK) before cDNA synthesis by PCR using High Capacity RNA-to-cDNA kit and Thermal Cycler 2720 (Applied Biosystems). Gene expression was measured on StepOne Plus Real Time PCR System with TaqMan® Universal PCR Master Mix and primers GAPDH (Hs99999905_m1) as housekeeping gene, ALK-1 (Hs00163543_m1), ALK-2 (Hs00153836_m1), ALK-3 (Hs01034909_g1), ALK-6 (Hs00176144_m1), ACVR2A (Hs00155658_m1), ACVR2B (Hs00609603_m1) and BMPR2 (Hs00176148_m1) (Applied Biosystems).

To find out which receptors BMP-9 are using transfected INA-6 cells were seeded in a 24-well culture plate and incubated for 18 hours before stimulation with BMP-9 (0.5 ng/ml) or BMP-6 (150 ng/ml) for 45 minutes. After stimulation total RNA was isolated and gene expression was measured by RT-qPCR as explained previously. Primers used were GAPDH as housekeeping gene, Id1 (Hs00357821_g1), ALK-2, ALK-3, ALK-6, ACVR2A, ACVR2B and BMPR2. Id1 was used to look at gene expression after receptor knockdown, since the expression of this gene is upregulated by BMPs.

2.6 WESTERN BLOT

Western blot is a protein detection technique where gel electrophoresis is used to separate proteins before detection of target protein by specific antibodies.[63] Western blot was used to detect which signaling pathways BMP-9 activates. INA-6 cells were washed and incubated with TGF-β (5 ng/ml) for 10 minutes or BMP-9 (0.5 ng/ml) from 10 minutes to 4 hours before harvesting. Unstimulated cells were used as control.

Cell extracts was prepared by resuspending dry pellets in lysis buffer (20 µl buffer per 500,000 cells) containing Nonidet P40 (NP40) (1 %) (Sigma Aldrich), glycerol (10 %), Tris (50 mM, pH 7.5), NaCl (150 mM), a protease inhibitor cocktail (Complete Mini EDTA-free, Roche, Mannheim, Germany) and 50 mM NaF and 1 mM Na_3VO_4 for phosphatase inhibition.

A NuPAGE® 10 % Bis-Tris Gel (1.0 mm X 10 well, Novex® by Life Technologies, CA, USA) was added 3 µl See BluePlus2 Prestained Standard (Invitrogen) and 15 µl lysate mixed with 5 µl NuPAGE® LDS Sample Buffer (4X) (Invitrogen) added dithiothreitol (DTT) (100 mM). The gels were run with PowerEase 500 (Invitrogen) for 30 minutes with 80- and 150 V

and 1-2 hours with 180 V with NuPAGE® MOPS SDS Running Buffer (20X) (Invitrogen). Gels were then blotted onto a nitrocellulose membrane (using Blot® Gel Transfer Stack Nitrocellulose – Mini and iBlot™, Invitrogen). Membranes were blocked in Odyssey Infrared Imaging System Blocking Buffer (LI-COR Biosciences, NE, USA) for 1 hour at room temperature. Primary antibody (phospho SMAD-1 (Ser463/465)/-5 (Ser463/465)/-8 (Ser426/428), phosoho SMAD-2 (Ser465/467), phospho SMAD-3 (Ser423/425) (C25A9), phospho p38 MAPK (Thr180/Tyr182), phospho p44/42 MAPK (extracellular signal-regulated kinase – ERK-1/-2) (Thr202/Tyr204), phospho signal transducer and activator of transcription (STAT)-3 (Tyr705) (all from Cell Signaling Technology®) and GAPDH (ab8245) (Abcam, Cambridge, UK)) were incubated for 3 days at 4 °C in Tris Buffered Saline pH 7.4 containing 1 % Licor blocking buffer and 0.1 % Tween-20 (BioRad Laboratories, CA, USA) (TBST). Membranes were washed three times with TBST and incubated with suitable secondary antibody (Goat-anti-Rabbit IRDye® 800 CW, LI-COR Biosciences) for 1 hour at room temperature. After three washes with TBST the membrane was then detected on Odyssey (LI-COR Biosciences).

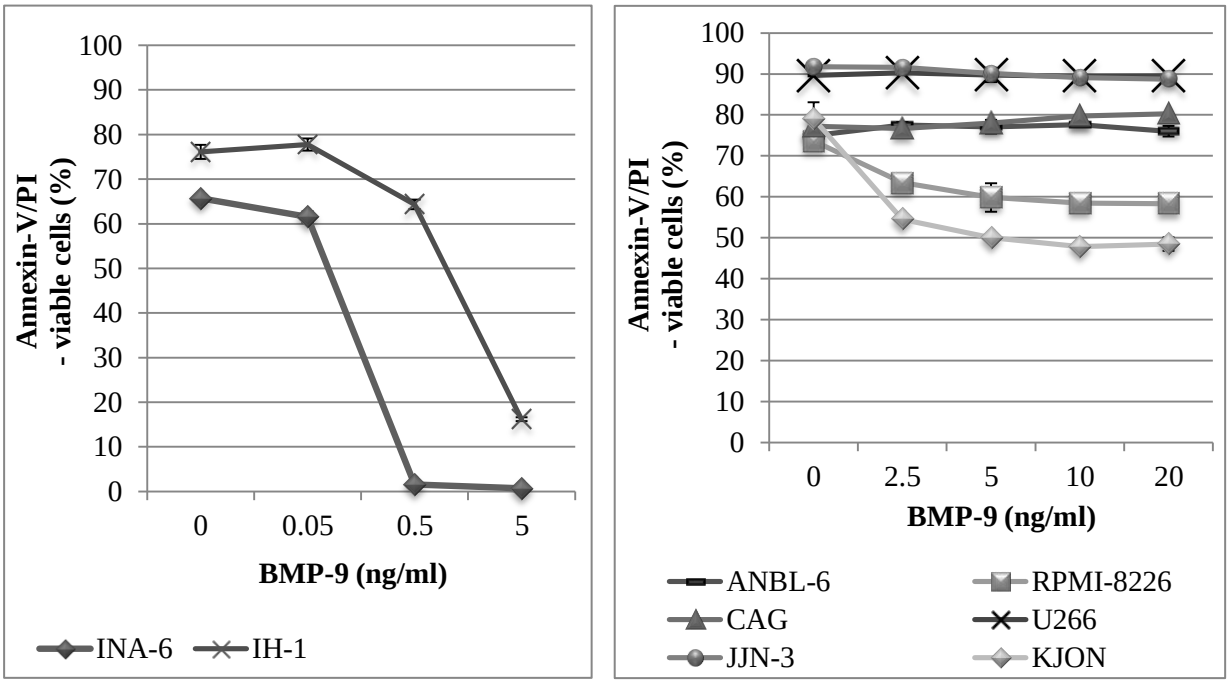
Western blot analysis was also performed on IH-1 and HepG2 cells stimulated for 1 hour with CpG-ODN (1-4 µM), BMP-4 (5 ng/ml), BMP-6 (10 ng/ml), BMP-9 (1-10 ng/ml) or TGF-β (5 ng/ml) (R&D systems) to see if CpG-ODN inhibited BMP-9.

Western blot analysis was also performed to look at effects of receptor knockdown. INA-6 cells were transfected with ON-TARGETplus Non-Targeting pool and ALK-2 and stimulated with BMP-9 (0.5 ng/ml) for 45 minutes before western blot was performed.

3 RESULTS

3.1 APOPTOSIS

The effect of BMP-9 in hMCL INA-6 and IH-1 (Figure 16 A), and RPMI-8226, U266, JJN-3, KJON, ANBL-6, and CAG (Figure 16 B) was tested using annexin-V/PI staining and flow cytometry. The figure shows plots of viable cells which are cells negative for both annexin-V FITC and PI (located in quadrant Q3 Figure 12). In hMCLs IH-1 and INA-6 apoptosis was induced by low concentrations of BMP-9 (0.05-5 ng/ml). High concentrations of BMP-9 (2.5-20 ng/ml) only had a small effect on apoptosis in hMCLs KJON and RPMI-8226, while the remaining hMCLs were unaffected by BMP-9.



A **B**

Figure 16. The effect of BMP-9 (0.05-20 ng/ml) on apoptosis in hMCLs. Cells were stimulated for three days before analysis by flow cytometry. **A:** INA-6 and IH-1; **B:** ANBL-6, RPMI-8226, CAG, U266, JJN-3 and KJON.

3.2 PROLIFERATION

The effect of BMP-9 on proliferation in hMCLs INA-6, IH-1, RPMI-8226, U266, JJN-3, KJON, ANBL-6, and CAG (Figure 17) was tested using CellTiter Glo viability assay that measures ATP-levels. The figure shows measured relative luciferase units (RLU), which reflect relative numbers of viable cells for each condition. Proliferation was inhibited in a high degree in hMCLs IH-1 and INA-6, and in a smaller degree in hMCLs ANBL-6, JJN-3, KJON and RPMI-8226. Proliferation in hMCLs CAG and U266 were unaffected by BMP-9.

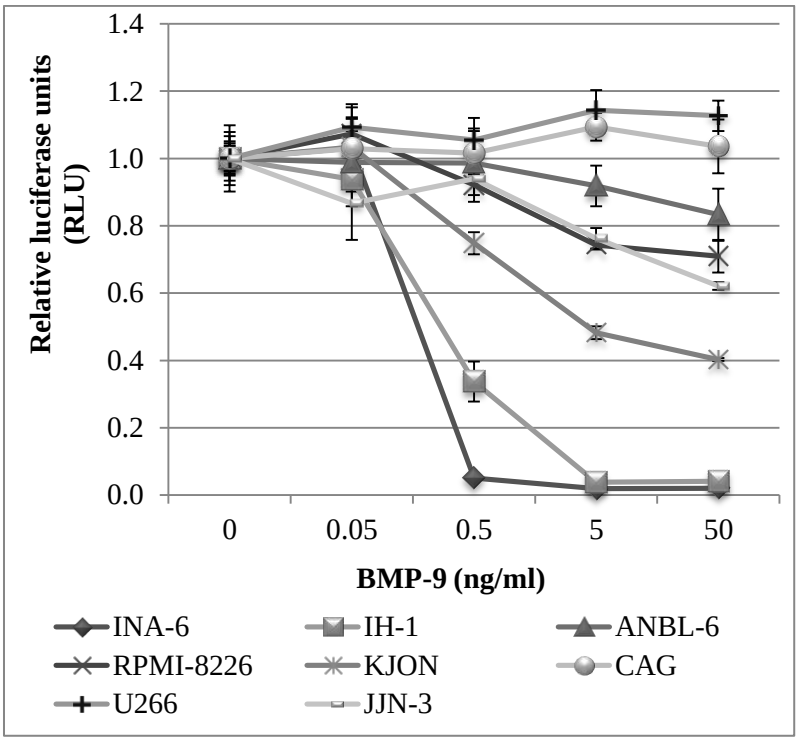


Figure 17. The effect of BMP-9 (0.05-50 ng/ml) on proliferation in hMCLs INA-6, IH-1, ANBL-6, RPMI-8226, CAG, U266, JJN-3 and KJON. Cells were stimulated for three days before detection with CellTiter Glo viability assay.

3.3 ANTAGONISTS

The antagonists gremlin, crossveinless-2 (CV-2), follistatin (FS), brorin and sclerostin (SOST) (Figure 18 A), and chordin-like 1 (CHL-1) and twisted gastrulation (Tsg) (Figure 18 B) were tested against BMP-9 on INA-6 cells. DM was used as a positive control. Control experiments were also performed to show that the antagonists were functional (Figure 19, Table 9). The figures shows measured RLU after treatment with different antagonists and BMP as indicated, which reflect relative number of viable cells for each condition. The antagonists CHL-1 and Tsg antagonized the effect of BMP-9, but only using high concentration of antagonist. CV-2, gremlin, brorin, SOST and FS had no effect on the BMP-9 induced growth suppression. All antagonists, except brorin and SOST, were found to be functional.

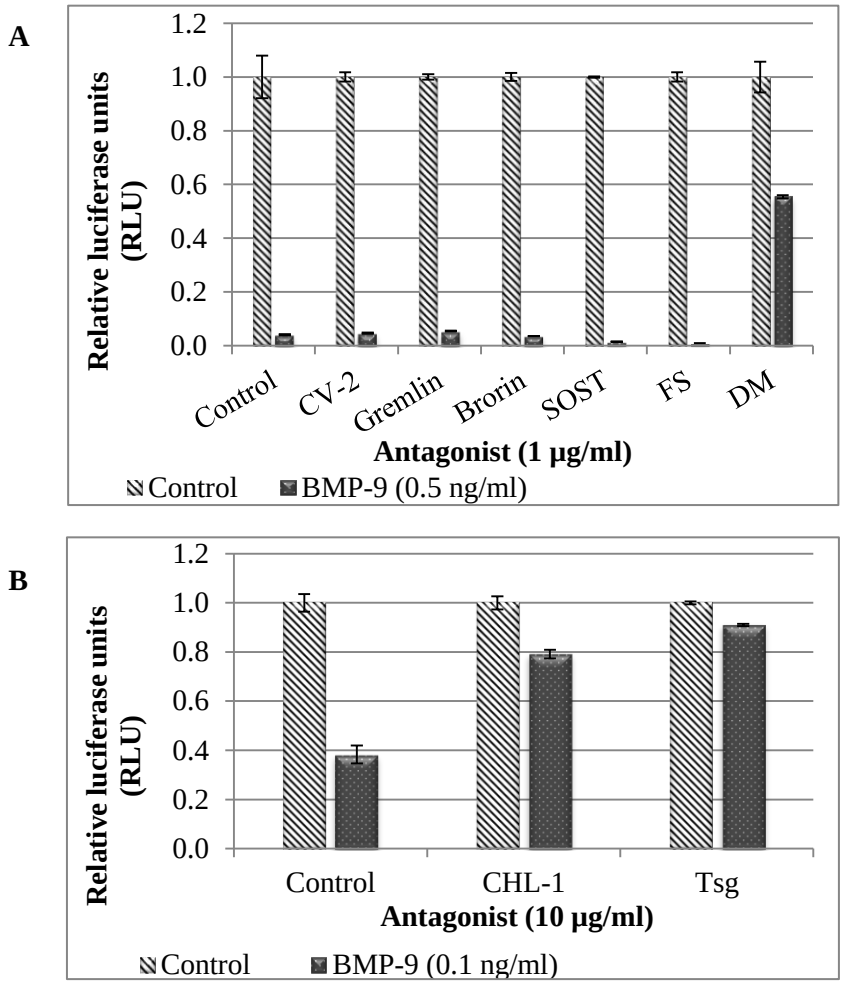


Figure 18. The effect of antagonists on hMCL INA-6. Cells were stimulated for three days before detection with CellTiter Glo viability assay. **A:** CV-2, gremlin, brorin, SOST and FS (1 µg/ml) and BMP-9 (0.5 ng/ml). DM (2 µM) was used as a positive control. **B:** Tsg and CHL-1 (10 µg/ml) and BMP-9 (0.1 ng/ml).

Table 9. Overview over controls used for the different antagonists.

Antagonist (µg/ml)	Control (ng/ml)
CHL-1 (5)	BMP-4 (1)
Gremlin (5)	BMP-4 (1)
CV-2 (5)	BMP-4 (1)
Tsg (5)	BMP-4 (1)
FS (5)	Activin-A (100)
Brorin (5)	---
SOST (5)	---

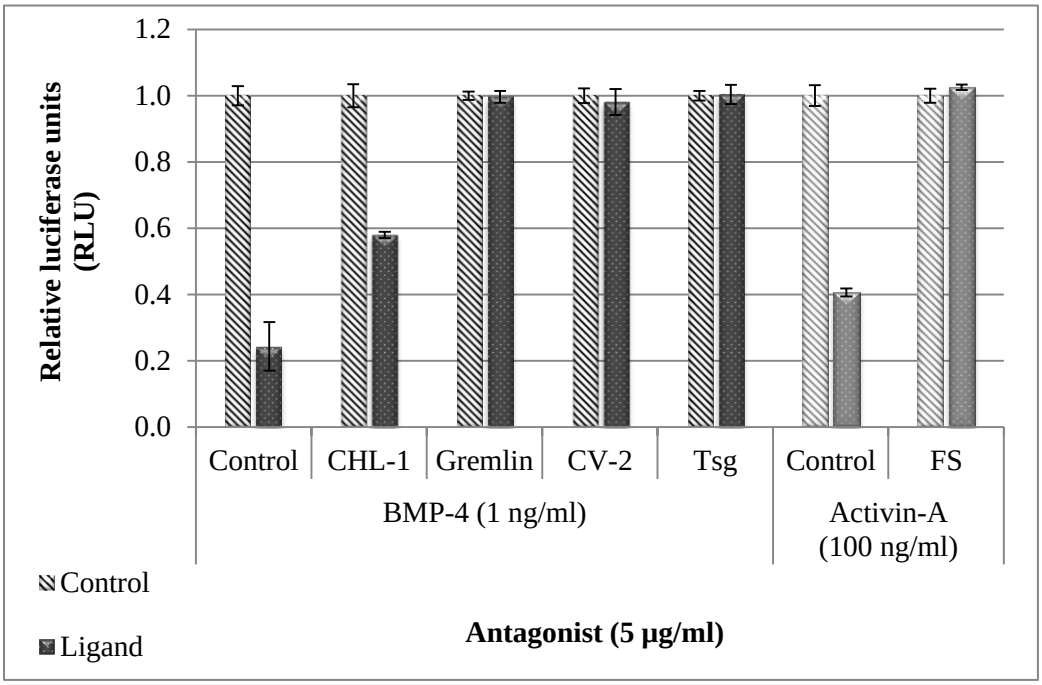


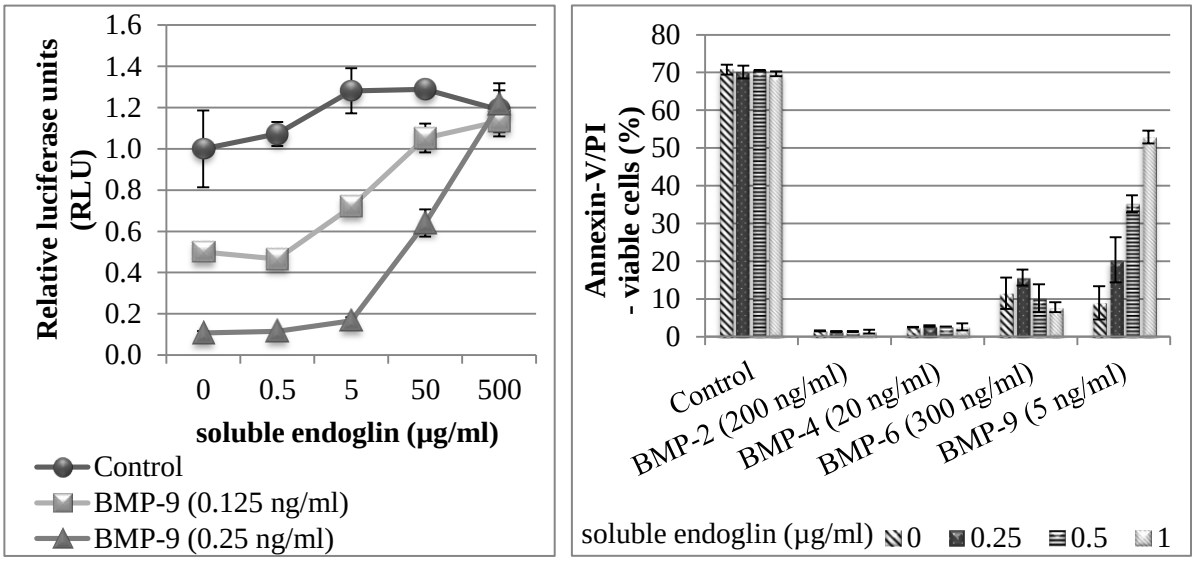
Figure 19. Control experiments on hMCL IH-1 for antagonists (5 µg/ml). Tsg, CHL-1, CV-2 and gremlin were tested against BMP-4 (1 ng/ml), while FS was tested against Activin-A (100 ng/ml). Cells were stimulated for three days before detection with CellTiter Glo viability assay.

3.4 REGULATION OF BMP SIGNALING

The Type III receptors endoglin and betaglycan, the HSPG syndecan-1 and CpG-ODN were tested against different BMPs (BMP-2, -4, -6 and -9) on INA-6 and IH-1 cells. The SMAD inhibitor DMH1 was also tested against different BMPs to see if it could serve as a useful tool.

3.4.1 ENDOGLIN

Soluble endoglin was tested against BMP-9 on INA-6 cells (Figure 20 A). The figure shows measured RLU after treatment with different concentrations of soluble endoglin and BMP as indicated, which reflect relative number of viable cells for each condition. Endoglin was also tested against BMP-2, -4, -6 and -9 on IH-1 cells (Figure 20 B). The figure shows plot of viable cells which are cells negative for both annexin-V FITC and PI (located in quadrant Q3 Figure 12). Soluble endoglin bound and antagonized BMP-9, but did not have any effect on BMP-2,-4 or -6.



A **B**

Figure 20. A: The effect of soluble endoglin (0.5-500 ng/ml) on BMP-9 (0.125-0.25 ng/ml) on hMCL INA-6. Cells were stimulated for three days before detection with CellTiter Glo viability assay. **B:** The effect of soluble endoglin (0.25-1 µg/ml) on BMP-2 (200 ng/ml), BMP-4 (20 ng/ml), BMP-6 (300 ng/ml) and BMP-9 (5 ng/ml) on hMCL IH-1. Cells were stimulated for three days before analysis by flow cytometry.

Expression of membrane bound endoglin (CD105) in stromal cells, the non-myeloma hematologic cell line U937 and the hMCLs CAG, JJN-3, KJON, RPMI-8226, ANBL-6, IH-1, INA-6 and U266 were analyzed in FlowJo by making overlay plots (Figure 21) and looking at mean fluorescence intensity (MFI) (Table 10). Stromal cells and U937 were strongly positive for surface endoglin based on the large shift in MFI in CD105 labeled cells compared to isotype control labeled cells (Figure 21 A-B).The hMCLs CAG, JJN-3, KJON and RPMI-8226 only showed a small shift in MFI and were found to be slightly positive for surface endoglin (Figure 21 C-F). The hMCLs ANBL-6, IH-1, INA-6 and U266 did not have a shift in MFI and thus no expression of CD105 (Figure 21 G-J).

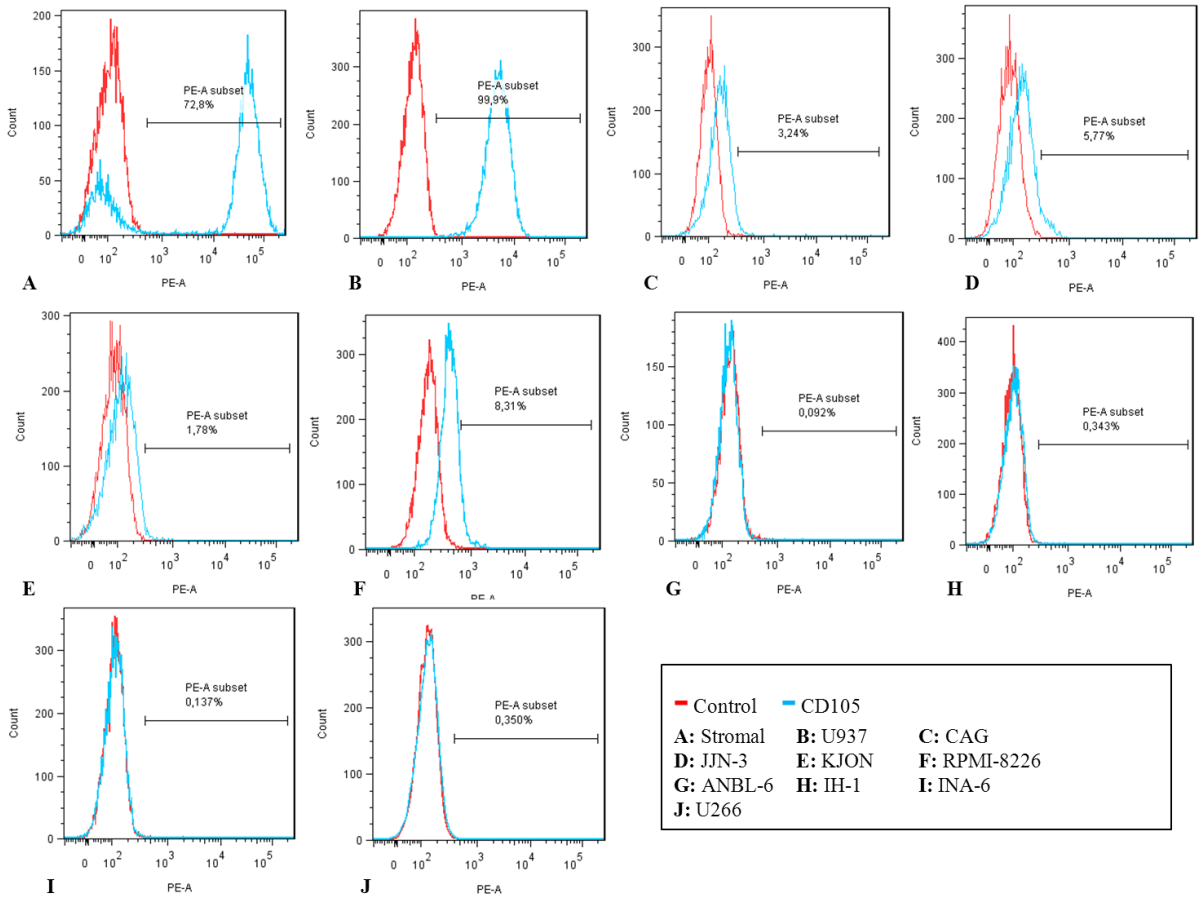


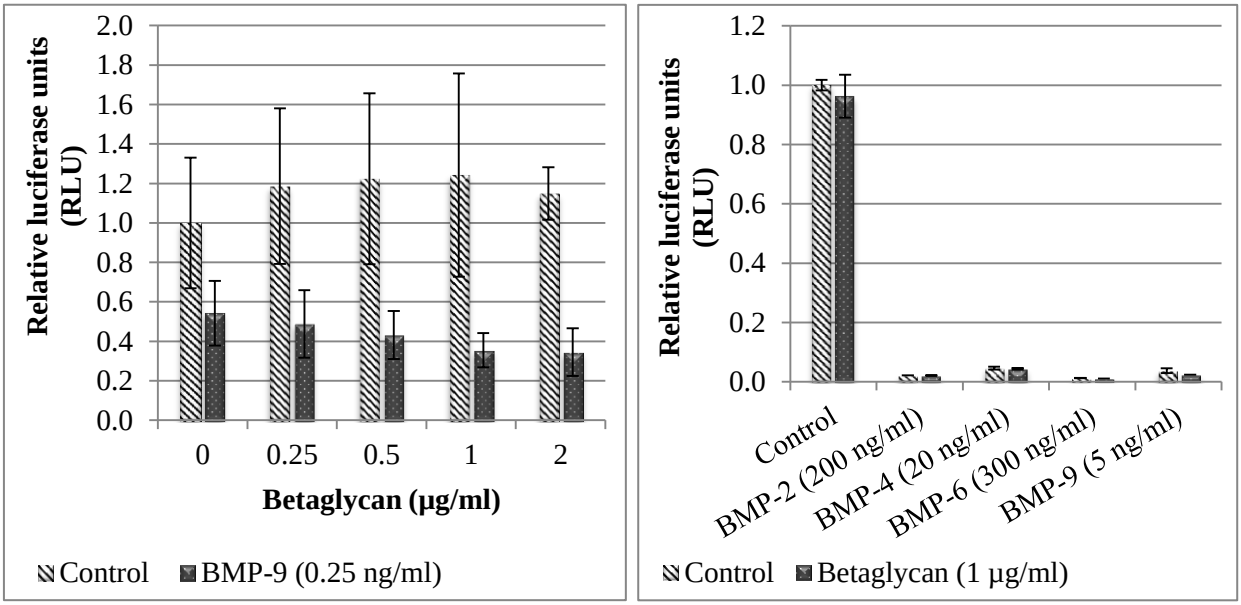
Figure 21. Expression of surface endoglin in cells labeled with CD105. **A:** stromal cells, **B:** the non-myeloma hematologic cell line U937, and hMCLs**: C:** CAG, **D:** JJN-3, **E:** KJON, **F:** RPMI-8226, **G:** ANBL-6, **H:** IH-1, **I:** INA-6 and **J:** U266.

Table 10. Shift in mean fluorescence intensity (MFI) measured by flow cytometry and analyzed by FlowJo. The increase in MFI in cells stained with CD105 compared to control cells demonstrates an increase in expression of endoglin (CD105).

Cells	MFI	
	Control	**CD105**
Stromal	115	38400
U937	134	5084
CAG	97.3	506
JJN-3	78.6	152
KJON	78.4	129
RPMI-8226	183	433
ANBL-6	140	133
IH-1	90.8	103
INA-6	111	113
U266	131	138

3.4.2 BETAGLYCAN

Soluble betaglycan was tested against BMP-9 on INA-6 cells (Figure 22 A), and against BMP-2, -4, -6 and -9 on IH-1 cells (Figure 22 B). The figure shows measured RLU after treatment with different concentrations of betaglycan and BMP as indicated, which reflect relative number of viable cells for each condition. Betaglycan had no effect on BMP-2, -4, -6 or -9 signaling in the performed experiments.

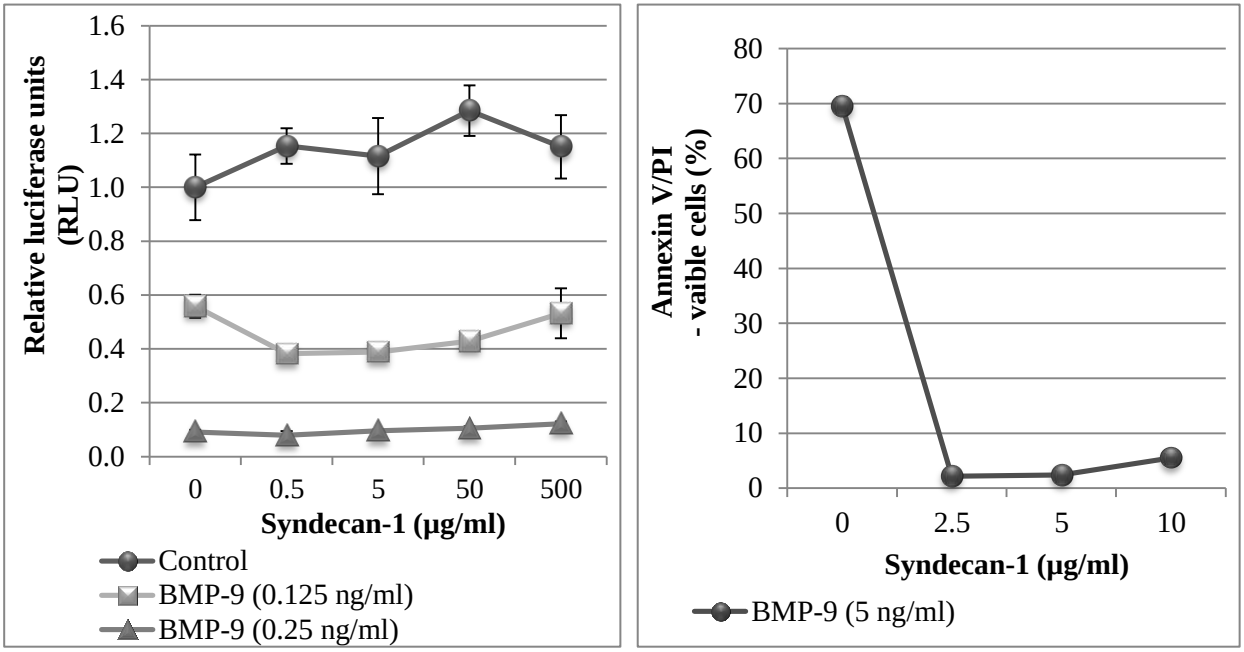


A **B**

Figure 22. Effect of betaglycan after stimulation for three days before detection with CellTiter Glo cell viability assay. **A:** The effect of betaglycan (0.25-2 µg/ml) on BMP-9 (0.25 ng/ml) on hMCL INA-6. **B:** The effect of betaglycan (1 µg/ml) on BMP-2 (200 ng/ml), BMP-4 (20 ng/ml), BMP-6 (300 ng/ml) and BMP-9 (5 ng/ml) on hMCL IH-1.

3.4.3 SYNDECAN-1

Soluble syndecan-1 was tested against BMP-9 on INA-6 cells with CellTiter Glo cell viability assay (Figure 23 A). The figure shows measured RLU after treatment with different concentrations of syndecan-1 and BMP as indicated, which reflect the relative number of viable cells for each condition. Syndecan-1 was also tested against BMP-9 on IH-1 cells with flow cytometry (Figure 23 B). The figure shows plot of viable cells which are cells negative for both annexin-V FITC and PI (located in quadrant Q3 Figure 12). Syndecan-1 did not have an effect on BMP-9 signaling in hMCLs IH-1 and INA-6.



A **B**

Figure 23. A: The effect of syndecan-1 (0.5-500 ng/ml) on BMP-9 (0.125-0.25 ng/ml) on hMCL INA-6. Cells were stimulated for three days before detection with CellTiter Glo cell viability assay. **B:** The effect of syndecan-1 (2.5-10 µg/ml) on BMP-9 (5 ng/ml) on hMCL IH-1. Cells were stimulated for three days before analysis by flow cytometry.

3.4.4 CpG-ODN

The effect of CpG-ODN on SMAD activation by BMP-4, -6 and -9 was checked in hMCL INA-6 (Figure 24 A) and IH-1 (Figure 24 B) by looking at apoptosis. The figures shows plot of viable cells which are cells negative for both annexin-V FITC and PI (located in quadrant Q3 Figure 12). CPG-ODN inhibited both BMP-4 and -6, but did not have any effect on BMP-9.

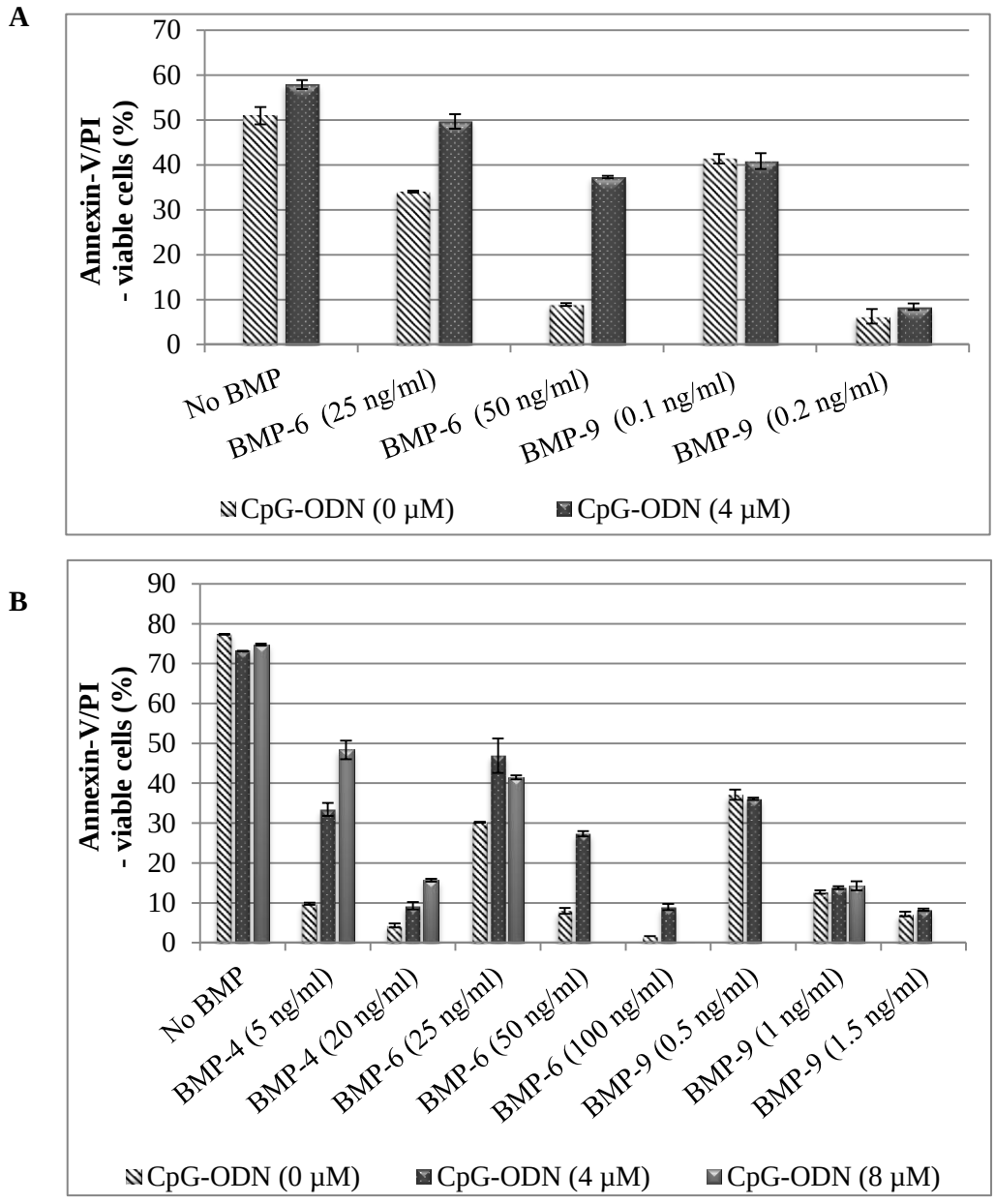


Figure 24: The effect of CpG (4-8 µM) on SMAD activation by BMP-4 (5-20 ng/ml), BMP-6 (25-100 ng/ml) or BMP-9 (0.1-1.5 ng/ml) in hMCLs **A:** INA-6 and **B:** IH-1. Cells were stimulated for three days before analysis by flow cytometry.

Western blot analysis was used to detect SMAD phosphorylation of IH-1 cells (Figure 25 A) and HepG2 cells (Figure 25 B) treated with BMP-4, -6 or -9, TGF-β and CpG-ODN. The figure shows that CpG-ODN counteracts BMP-4 and -6 induced SMAD activation, but this does not apply for BMP-9. GAPDH was used as loading control.

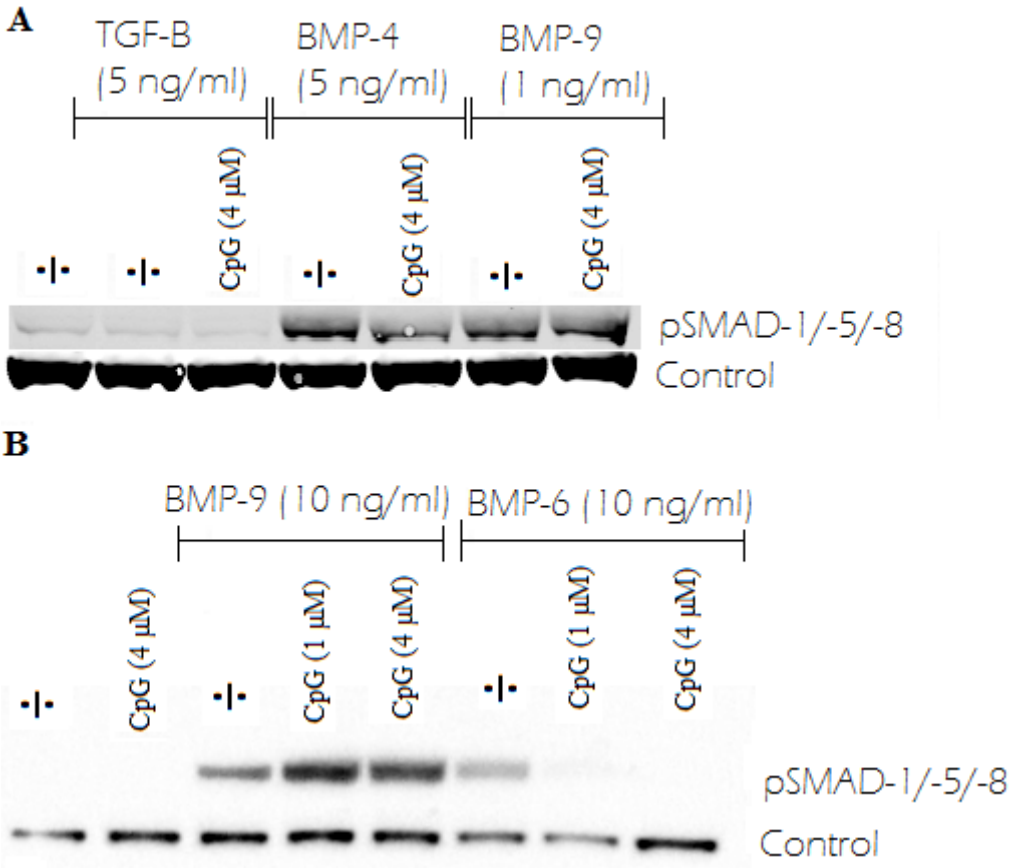


Figure 25. Western blot analysis of **A:** IH-1 cells and **B:** HepG2 cells, stimulated as indicated with TGF-β (5 ng/ml), CpG-ODN (1-4 µM), BMP-4 (5 ng/ml), BMP-6 (10 ng/ml) and BMP-9 (1-10 ng/ml) for 1 hour. The figure shows levels of phosphorylated SMAD-1/-5/-8 in total cell extract. GAPDH was used as loading control.

3.4.5 DMH1

DMH1 were tested against BMP-9 on INA-6 cells (Figure 26 A), and against BMP-4, -6 and -9 on IH-1 cells (Figure 26 B). The figures shows plot of viable cells which are cells negative for both annexin-V FITC and PI (located in quadrant Q3 Figure 12). The inhibitor DMH1 showed to inhibit the effect of BMP-4, -6 and -9, and also a higher dosage of DMH1 is needed to achieve inhibition of BMP-9.

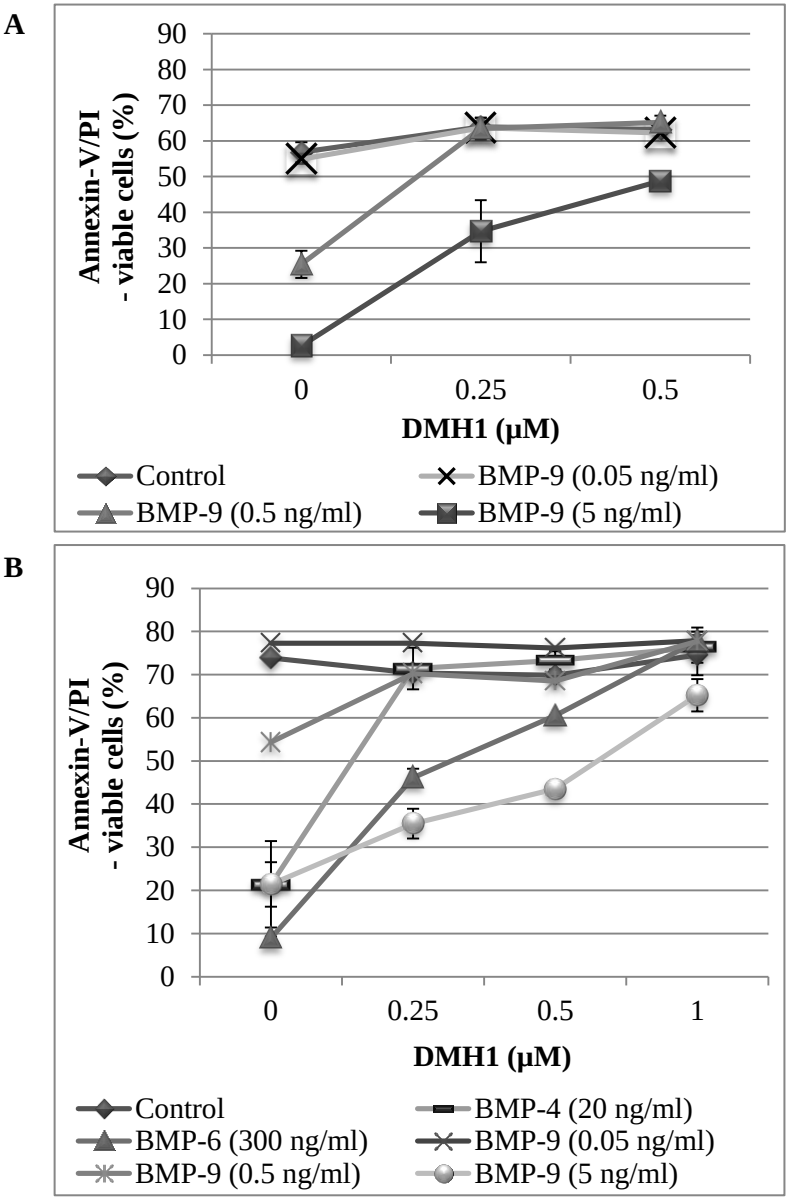


Figure 26. The effect of DMH1 after stimulation for three days before analysis by flow cytometry. **A:** The effect of DMH1 (0.25-0.5 µM) on BMP-9 (0.05-5 ng/ml) on hMCL INA-6. **B:** The effect of DMH1 (0.25-1 µM) on BMP-4 (20 ng/ml), BMP-6 (300 ng/ml) and BMP-9 (0.05-5 ng/ml) on hMCL IH-1.

3.5 RECEPTORS

The soluble receptors sALK-1, sALK-2, sALK-3, ALK-6, sACVR2A, sACVR2B and sBMPR2 were used to find out which receptors BMP-6 and -9 can bind to. This was done by looking at proliferation of INA-6 cells stimulated with soluble receptors and either BMP-6 or -9 (Figure 27). The figure show measured RLU after treatment with BMP-6 or -9 and different soluble receptors as indicated, which reflect relative number of viable cells for each condition. BMP-6 bound to Type I receptors ALK-3 and -6. BMP-9 bound to Type I receptors ALK-1 in a high degree and ALK-3 in a small degree and Type II receptors ACVR2A in a smaller degree, and ACVR2B and BMPR2 in a high degree.

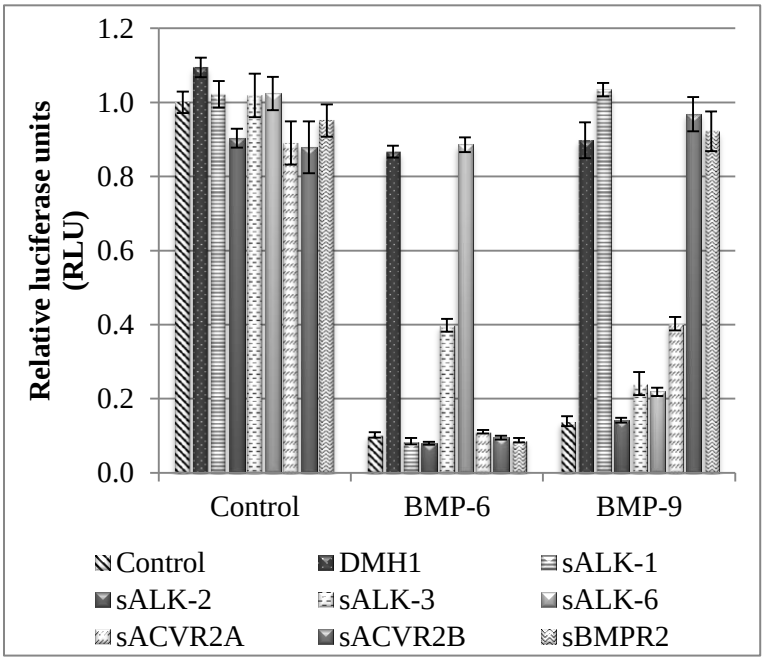


Figure 27. The effect of soluble receptors (5 µg/ml) on BMP-6 (50 ng/ml) and -9 (0.25 ng/ml) in hMCL INA-6. Cells were stimulated for three days before detection with CellTiter Glo cell viability assay.

INA-6 cells were checked for receptor expression by using RT-qPCR (Figure 28). The figure shows relative gene expression of the receptors ALK-1 to -3, ALK-6, ACVR2A, ACVR2B and BMPR2. INA-6 cells expressed the receptors ACVR2B and BMPR2 in a high degree; ALK-2, ALK-3 and ACVR2A to a lesser degree; and ALK-1 and ALK-6 was not expressed at all (Figure 28).

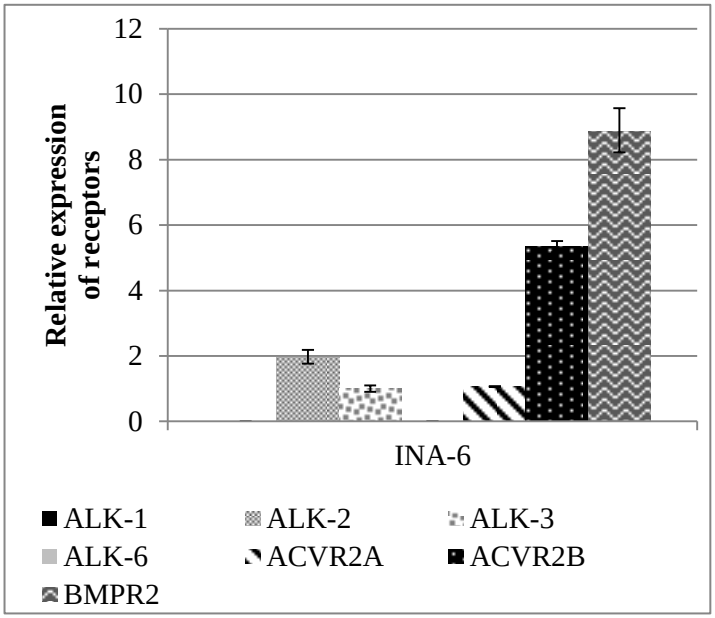


Figure 28. The relative expression of receptors ALK-1 to -3, ALK-6, ACVR2A, ACVR2B and BMPR2 in hMCL INA-6.

To find out which receptors BMP-6 and -9 signals through, INA-6 cells were treated with siRNA (ON-TARGETplus Non-Targeting pool, ALK-2, ACVR2A, ACVR2B and BMPR2), stimulated with BMP-9 and RT-qPCR was performed (Figure 29). Experiments indicate that BMP-9 may be using ALK-2 as Type I receptor and ACVR2A and ACVR2B as Type II receptor (Figure 29 A,C-D), and BMP-6 may be using ALK-2 as Type I receptor (Figure 29 A-B).

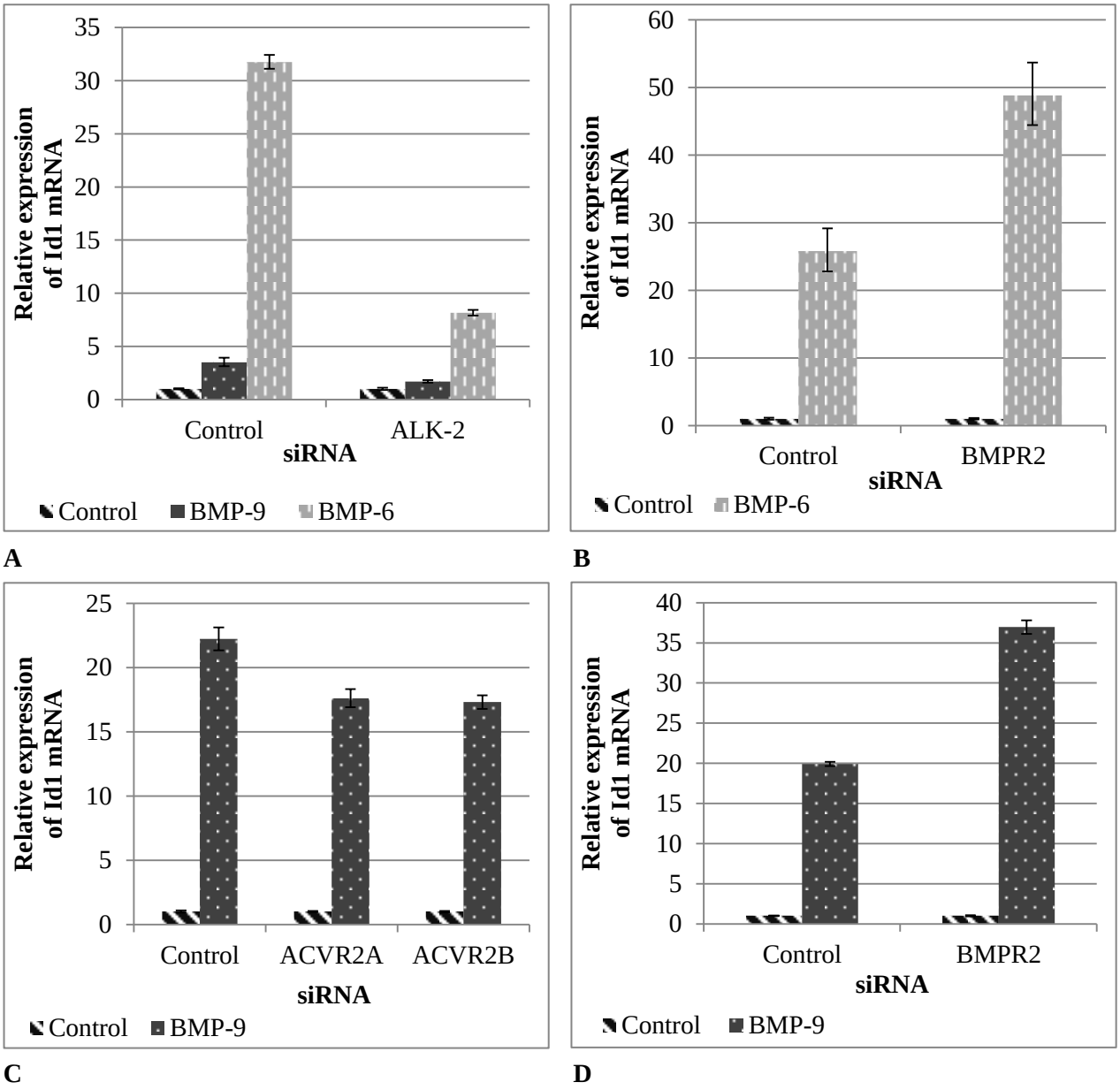


Figure 29. Relative expression of Id1 mRNA to identify which receptors BMP-6 and 9 are using. INA-6 cells transfected with siRNA and stimulated for 45 minutes with BMP-9 (0.5 ng/ml) or BMP-6 (150 ng/ml) before RT-qPCR was performed. **A:** ALK-2 siRNA treated with BMP-6 or -9. **B:** BMPR2 siRNA treated with BMP-6. **C:** ACVR2A and ACVR2B siRNA treated with BMP-9. **D:** BMPR2 siRNA treated with BMP-9. ON-TARGETplus Non-Targeting pool siRNA was used as a control.

Western blot analysis was performed on INA-6 cells to look at effects of receptor knockdown (Figure 30). The figure shows levels of phosphorylated SMAD-1/-5/-8 in total cell extracts from INA-6 cells transfected with ON-TARGETplus Non-Targeting pool or ALK-2 and stimulated with BMP-9. GAPDH was used as loading control. The BMP-9 signal is downregulated in BMP-9 stimulated cells where ALK-2 receptor has been knocked down, compared to BMP-9 stimulated control cells. This further supports the hypothesis that BMP-9 signals through the Type I receptor ALK-2 in myeloma cells.

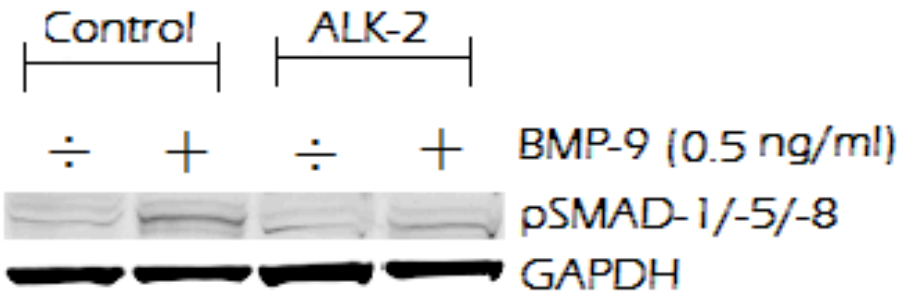


Figure 30. Western blot analysis of INA-6 cells transfected with ON-TARGETplus Non-Targeting pool or ALK-2 (both 1 µM) and stimulated with BMP-9 (0.5 ng/ml) for 45 minutes. The figure shows levels of phosphorylated SMAD-1/-5/-8 in total cell extract from the transfected INA-6 cells. GAPDH was used as loading control.

3.6 SIGNALING PATHWAY

Western blot analysis was used to find out which signaling pathway BMP-9 is using (Figure 31). The figure shows levels of phosphorylated SMAD-1/-5/-8, p38, pERK-1/-2 and STAT-3 in total cell extracts from INA-6 cells treated with TGF-β and BMP-9. GAPDH was used as loading control. Phosphorylated SMAD-2 and -3 were also checked, but no bands were detected. The figure shows that BMP-9 signals through SMAD-1/-5/-8 and that the activity of STAT-3 is inhibited after 2-4 hours.

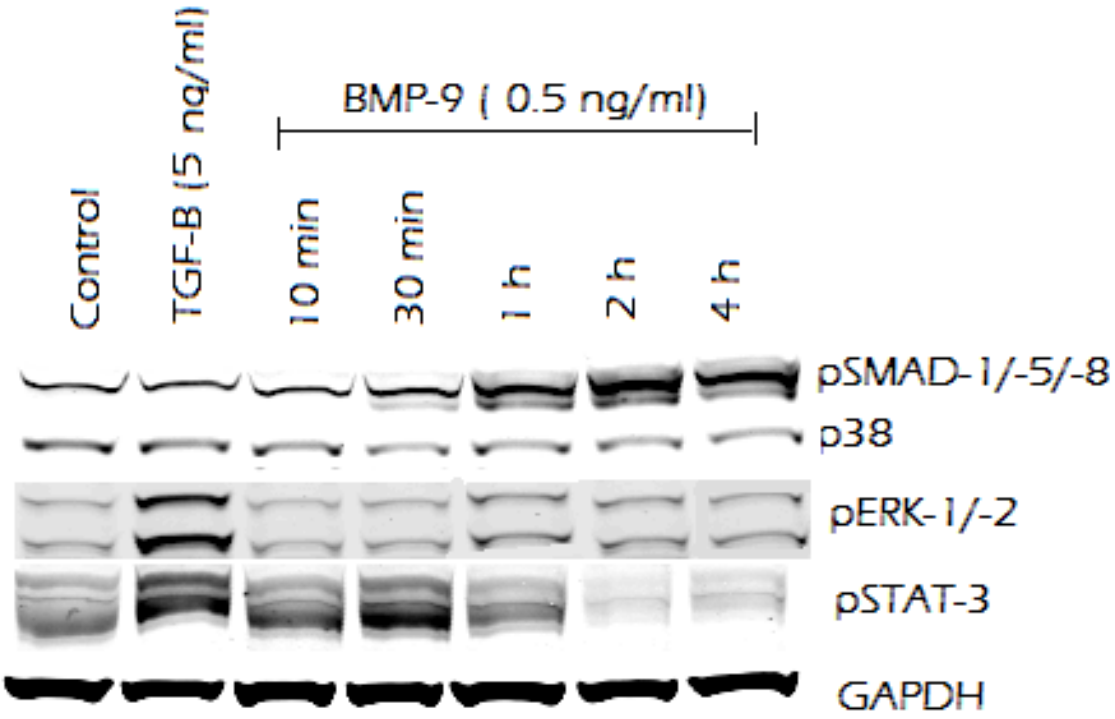


Figure 31. Western blot analysis of INA-6 cells stimulated with TGF-β and BMP-9. The figure shows levels of phosphorylated SMAD-1/-5/-8, p38, ERK-1/-2 and STAT-3 in total cell extracts from INA-6 cells treated with TGF-β (5 ng/ml) for 10 minutes or BMP-9 (0.5 ng/ml) for 10 minutes to 4 hours. GAPDH was used as loading control.

4 DISCUSSION

This thesis was performed with the goal to study and find out how BMP-9 affects different hMCLs. This was done by looking at apoptosis and proliferation. Experiments were also performed to see if any of the common antagonists had an effect on this protein and efforts were made to identify by which receptors BMP-9 signals in myeloma cells and which downstream signaling pathways are activated.

4.1 THE EFFECT OF BMP-9 ON APOPTOSIS AND PROLIFERATION

Apoptosis may be difficult to detect and quantify because of the rapidity of the apoptotic events. One advantage by looking at BMPs is that they induce apoptosis relatively slowly. Apoptosis was in this thesis measured by analyzing annexin-V binding and PI uptake on flow cytometry. This method offers several advantages by giving a rapid and reproducible estimate of apoptosis. Annexin-V binding is considered to be a specific marker of apoptosis based on the high affinity for PS. This method for measuring apoptosis gives a clear result and is generally easy to perform. Some cell types (b-cells and neutrophils) have also been shown to become transiently positive for annexin-V before subsequently reverting to a negative state.[106] This can make interpretation of results difficult. Proliferation is often measured in addition to apoptosis to get an overview of cell growth.

One frequently used method for measuring proliferation is to measure DNA synthesis by incorporation of radioactive thymidine. Recently an adenosine triphosphate (ATP)-bioluminescence assay called CellTiter Glo has been made available. This assay measures ATP in culture which is directly related to viable, healthy and metabolically active cells. The number of ATP molecules is in addition relatively constant from cell to cell. By using an assay like CellTiter Glo the use of radioactive thymidine, which is both a health and environment risk, can be eliminated. Other advantages with this platform are that it is more versatile and sensitive in addition to being less hazardous and laborious. The assay can easily be automated and is less prone to variations since it contains a single reagent, which has a stable luminescent output with a half-life greater than five hours, and no washing steps. Disadvantages with this method for measuring proliferation are that some treatments may have an effect on the amount of ATP and it is more expensive than other methods.

To sum up you can get a clear overview on how specific compounds affect cell cultures by measuring apoptosis and proliferation using annexin-V FITC and CellTiter Glo, respectively. These are both simple methods with many advantages to comparable methods.

In this thesis annexin-V FITC and CellTiter Glo was used to find out how BMP-9 affected the hMCLs INA-6, IH-1, RPMI-8226, U266, JJN-3, KJON, ANBL-6 and CAG. Figure 16 and 17 shows that BMP-9 induced apoptosis and/or inhibited proliferation in several hMCLs. The hMCLs INA-6 and IH-1 were sensitive to BMP-9 stimulation, and this correlates with the effect shown by other BMPs previously.[30] The hMCLs U266, CAG, ANBL-6 and JJN-3 does not undergo apoptosis, however the proliferation results indicate that ANBL-6 and JJN-3 becomes growth arrested. For the remaining hMCLs (KJON and RPMI-8226) where apoptosis is shown in a small degree and proliferation is inhibited, it is difficult to separate apoptosis and growth arrest. In cases where the apoptotic events are strong it is most likely that the decrease in RLU is a result from the strong apoptosis and not growth arrest, but it cannot be ruled out that there is some growth arrest at the same time.

4.1.1 Antagonists

The presence of antagonists in the bone marrow may be an explanation to why myeloma cells survive in an environment with BMPs. CellTiter Glo cell viability assay was used to test the effect of the common BMP antagonists CV-2, gremlin, brorin, SOST, FS, Tsg and CHL-1 against BMP-9 (Figure 18). Only Tsg and CHL-1 were found to antagonize the BMP-9 effect. To get antagonizing effect the concentration of the antagonists had to be 10 µg/ml, which is high compared to amounts of BMP-9 used (0.1 ng/ml). This means that BMP-9 should be able to inhibit proliferation of myeloma cells even in the presence of these antagonists.

All antagonists were in addition tested against TGF-β family members they are known to have an effect on, to test if they were functioning in the correct way (Figure 19). There was not found a positive control for brorin or SOST so the quality of these two antagonists could not be verified. SOST is an antagonist that has been described with a high degree of variation, and it was therefore not expected to find a control.[35,85]

4.1.2 Regulation of BMP signaling

Annexin-V FITC staining and CellTiter Glo cell viability assay was used to test the effect of Type III receptors, potential co-receptors and inhibitors together with BMP-9.

Endoglin

Previously endoglin has been shown to inhibit BMP-2, -7 and 9.[27,67] Soluble endoglin have been shown during experiments to bind and antagonize BMP-9, but not BMP-2,-4 or -6 (Figure 20). A broad spectrum of BMP concentrations was tested. When BMP-9 forms a complex with Type I receptor ALK-2 it resembles binding patterns of BMP-6 and -7, while when it binds to Type I receptor ALK-1 it resembles BMP-2 and -4 more.[60] So why is endoglin only antagonizing BMP-9? Does this indicate that there is an additional regulating point/level of the system? BMP-9 has previously been shown to bind to endoglin in the absence of both Type I and Type II receptors and it has also been shown that endoglin and Type II receptors use the same interface on BMP-9.[67] This may suggest that BMP-9 possess a characteristic property for binding endoglin or an additional level of signaling. Since endoglin is blocking Type II receptors several signaling pathways are inhibited and this may be an advantage for targeting therapy.[67]

It was found that some hMCLs (RPMI-8226, JJN-3, KJON and CAG) possessed some surface endoglin (CD105) (Figure 21, Table 10). This raises the question if cells expressing endoglin are less sensitive to BMP-9 compared to cells without detectable endoglin? No correlation between sensitivity for BMP-9 and the expression of endoglin on the cell surface was found.

Betaglycan

In the literature the soluble form of the Type III receptor betaglycan is described to inhibit BMP-2, -4 and -7[27,68] as well as proliferation in myeloma cells.[68] This trend was not seen in experiments. Soluble betaglycan did not revoke the effect of BMP-9 (Figure 22), and was in addition tested against BMP-2, -4 and -6 without any effect. One reason for this may be that the recombinant form of the Type III receptor used is missing an essential part and thereby cannot function properly.

SYNDECAN-1

Soluble syndecan-1 was tested against BMP-9 since it previously has been shown to induce apoptosis in myeloma cells and since increased levels in serum is associated with poor prognosis for patients with MM.[65,66] Concentrations up to 10 µg/ml of syndecan-1 were tested based on the knowledge that some myeloma patients have very high levels of soluble syndecan-1 in the bone marrow[72], but syndecan-1 was not able to alter BMP-9 signaling (Figure 23).

In experiments a recombinant form of syndecan-1 was used, and this may be a reason for the lack of effect. A possible explanation can be that the recombinant form is missing a property compared to the form found in bone marrow. The structure of glycosaminoglycan (GAG) chains is important for the binding properties of syndecan-1. GAG chains have tissue-specific structure polymorphisms and the recombinant syndecan-1 used here was produced in murine myeloma cells. It may have been a better alternative to isolate syndecan-1 directly from bone marrow from patients with MM to get a human, biologically functioning form.

CPG-ODN

CpG-ODN has previously been shown to inhibit the apoptotic effect of BMP-2 and -6. When testing CpG-ODN against BMP-4, -6 and -9 in this thesis the experiments showed that CpG-ODN had the same effect on BMP-4 and -6 as previously found, but no effect at all on BMP-9 (Figure 24). Western blot was in addition performed on BMP-4 and -9 to confirm these findings (Figure 25). The western blot also showed that the effect of BMP-4 was revoked, while the effect of BMP-9 was similar for cells stimulated with BMP-9 and with or without CpG-ODN. This raises the interesting question of what separates BMP-9 from the other members in the BMP family. Is it something about the BMP-BMP receptor complex that differs, or can this result from binding of an unknown co-receptor? As shown in this thesis, both BMP-6 and -9 seem to use the Type I receptor ALK-2 for signaling in the INA-6 cell line. The finding that CpG-ODN inhibits BMP-6-, but not BMP-9-induced phosphorylation of SMAD-1/-5/-8 may be used as a tool to discover new factors important for BMP signaling.

4.2 BMP-9 RECEPTORS

To find out which receptors the hMCL INA-6 possesses, and which receptors BMP-9 signals through transfection and RT-qPCR was used. Transfection of hMCLs is difficult and the outcome of successfully transfected cells is low. By cotransfecting with a plasmid expressing CD4 (pCD4) the cells can be concentrated. Some of the parameters that influence the outcome are cell condition and cell type (homogeneous cells will increase the probability of a successful transfection), amount of siRNA, time, temperature, and stimulation time. All these parameters have to be optimized to get the best possible result. When using magnetic beads for isolating transfected cells you cannot be entirely sure that all the cells get isolated. There are a lot of washing steps in the protocol where there is a possibility of losing cells. This method also gives a low yield compared to amount of cells used.

RT-qPCR is a technique with a broad dynamic range of quantification in addition to being reproducible and precise it also has both high technical sensitivity and throughput. By using RT-qPCR a single RNA molecule and small changes in gene expression can be detected. This is advantageous, especially when no post-PCR steps have to be performed and so avoiding the possibility of cross-contamination due to PCR products. Limitations with this method are that PCR products increases exponentially which will lead to the increase in variations with cycle number. There can also be an overlap of the emission spectra and an increased risk of false negative results. RT-qPCR generates an abundance of data which has to be reviewed, this often takes some time. The accuracy of the data depends on a number of factors such as sample preparation and choice of genes. RT-qPCR also requires expensive equipment and reagents.

INA-6 cells were tested for the expression of receptors (Figure 28) and it was shown that INA-6 cells expressed different receptors with varying degree. INA-6 showed low expression of ALK-1 and -6, and these receptors were thereby not of interest to investigate further.

The effect of soluble BMP-receptors on the growth inhibitory effect of BMP-6 and -9 on hMCL INA-6 was also performed to identify which receptors BMP-9 can bind. This experiment showed that BMP-9 can bind Type I receptors ALK-1 in a high degree and ALK-3 in a small degree, while BMP-6 bound to Type I receptors ALK-3 and -6 (Figure 27). The lack of receptor expression in cells may be a cause for the difference in response to BMPs and one mechanism for resistance of BMP induced growth inhibition.

To find out which receptors BMP-9 is using the expression of Id1 mRNA was checked with RT-qPCR. Id1 was used for these experiments based on the ability BMPs have to upregulate this gene. Results from experiments (Figure 29) showed that there is a strong possibility that BMP-9 is using ALK-2 as Type I receptor and ACVR2A and ACVR2B as Type II receptor. This was concluded since the relative expression of BMP-9 induced Id1 was lower when receptors were knocked down.

An interesting trend that was found was that the BMP-9 induced Id1 expression was higher in cells where BMPR2 was knocked down (Figure 29 D). This was also seen in cells stimulated with BMP-6 (Figure 29 B). What this means is not clear. One possible reason is that other secreted membrane-associated or intracellular modulators may alter the regulation and thereby the signaling of BMPs. Another reason may be that the specific tail only found on this receptor has an unknown function to regulate the signaling. A possible explanation to why this is seen in both BMP-6 and -9 is that they have similar binding patterns when signaling through Type I receptor ALK-2.

4.3 SIGNALING PATHWAY

Western blot is a technique that is depending on a lot of factors. Among other factors the amount of protein, the accuracy of the loading, and a successful transfer of proteins from gel to membrane is important to obtain the best possible result. The nature of the gel has to be taken into consideration according to the protein of interest, e.g. the use of thinner gels leads to faster transfer. The best option is to run the softest gel that will yield the required resolution. Reagents and equipment also have to be entirely optimized. Quality of western blot also depends of blocking of the membrane, and binding of primary- and secondary antibody. One disadvantage by using nitrocellulose membranes is that the proteins are not bound covalently and the membrane is in addition fragile when dry. The main advantage with western blot is the ability to give information about multiple antigens within the same sample at the same time.

DMH1 and DM are small molecule BMP inhibitors that prevent activation of R-SMADs, and have been shown to inhibit BMP-2, -4, -6 and -7.[74,75] The inhibitor DMH1 showed to inhibit the effect of BMP-4, -6 and -9 (Figure 26), which indicates the importance of the SMAD signaling pathway.

Western blot was performed to identify BMP-9 signaling pathway by labeling with different antibodies. The different antibodies were tested since BMP can signal through the SMAD pathway resulting in activation of SMADs, or other non-SMAD signaling pathways regulating the activation of MAPK p38, ERK-1/-2 or STAT-3. It has previous been shown that STAT-3 signaling is inhibited by BMPs in myeloma cells, and it was therefore of interest to test this against BMP-9.[11] As you can see from the result (Figure 31) it is clear that BMP-9 signals through the SMAD pathway since SMAD-1/-5/-8 gave strong bands where cells have been stimulated with BMP-9. The binding of primary antibody also became stronger in cells stimulated for a longer period of time. Unstimulated cells and cells stimulated with TGF-β were used as control. Staining with the other antibodies phospho p38 and phospho ERK-1/-2 showed no binding. Phospho STAT-3 showed bands in samples stimulated with BMP-9 for a short period, but when the stimulation time exceeded one hour the bands became weaker. This indicated that the activity of STAT-3 was inhibited after 2-4 hours. The activation in TGF-β when using phospho ERK-1/-2 as primary antibody may be a result of HCl since TGF-β is dissolved in a higher concentration of HCl than BMP-9.

5 CONCLUSIONS AND FURTHER STUDIES

In this thesis it was found that BMP-9 induced apoptosis and/or inhibited proliferation in several hMCLs. BMP-9 signals via the SMAD dependent pathway and most likely uses ALK-2 as Type I receptor and ACVR2A and/or ACVR2B as Type II receptor. Several compounds that can have a significance for BMP-9 signaling were also found: 1) the antagonists CHL-1 and Tsg were found to antagonize the effect of BMP-9 when used in high concentration; 2) the Type III receptor endoglin showed to bind and inhibit this protein; 3) CpG-ODN do not have the same effect on BMP-9 as other BMPs, and 4) the small molecule inhibitor DMH1 were found to antagonize BMP-9.

Further experiments have to be done to identify what distinguishes BMP-9 from other members of the BMP family regarding CpG-ODN findings. One idea is to use a different cell line which is easier to work with and show the same effect when treated with BMP and CpG. An example is HepG2 cells which have shown from experiments to have the same effect for BMP-6 and -9.

In the attempt of identify which receptors BMP-9 signals through it was found that the expression of Id1 in cells where BMPR2 was knocked down was higher in BMP-9 stimulated cells versus control cells. This trend was also seen in cells stimulated with BMP-6. What this means is not clear, and has to be investigated further. It may be a suggestion to make a stable transfected cell line, by transfecting with viruses, which expresses specific receptors. This may give a stable knock down and make experiments easier to perform in large scale.

Another interesting experiment is to make a stable transfected construct with e.g. INA-6 cells containing a luciferase construct. The stable transfected cells can then be used to identify the level of BMP-9 in different solutions and to test different potential inhibitors.

As mentioned here there are a number of findings in this thesis that raised interesting questions that should be further investigated.

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