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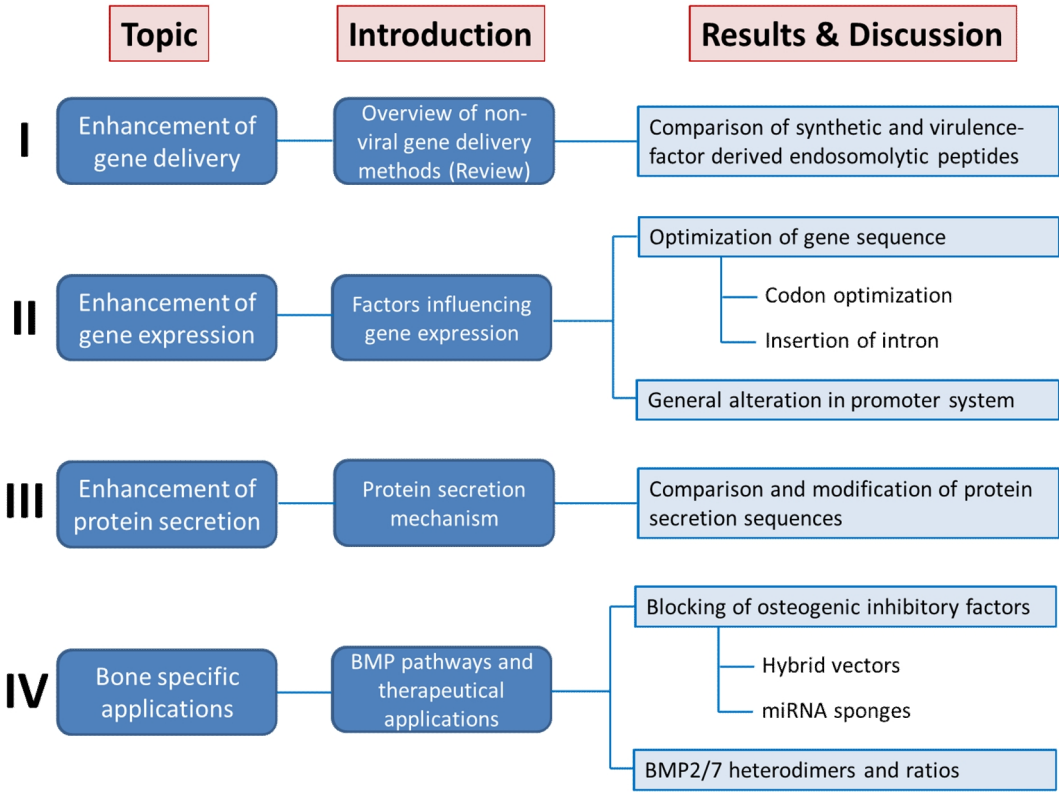
Ազատ կամքով եվ համբերություն
Լավ բաները խառներ եմ
Որ լինի ինչբես հայրի քուքուն
Երեկ չեր, միշտ էսօր եմ

Content - Chapters



A general overview summarizes the barriers of non-viral gene delivery and specifies advantages and disadvantages of different chemical and mechanical non-viral gene delivery methods evolved to date. Subsequently, membrane disrupting activity of artificial and virulence-derived endosomolytic peptides and proteins are examined for the application in gene delivery approchaches. Addition of these lysosomal membrane destructive agents are potential enhancers of gene delivery.

The further two chapters are focused on the enhancement of the general outcome of the delivered exogenous gene by either addition of gene expression enhancers on plasmids or by boosting protein secretion by modification secretion sequences, respectively.

Final chapter is focused on the enhancement of gene therapeutical impact in bone regeneration and bone tissue engineering. Increase of osteogenic differentiation of target cells and tissues by application of different therapeutical plasmid constructs, as well as by knockdown of inhibitors of BMP signaling pathways, are the main targets of the last chapter.

Summary

One of the key components of tissue engineering is the delivery of tissue specific growth factors either as protein level or encoded on therapeutic DNA plasmids (gene therapy).
The application of therapeutic plasmids encoded for growth factors provides a promising tool associated with lower manufacturing costs, higher safety and increased bioactivity of the produced proteins due to host-specific post-translational modifications and correct folding of the locally produced growth factors. In order to introduce exogenous DNA into cells *in vitro* and *in vivo*, several strategies based on viral or non-viral gene transfer approaches can be applied. Unfortunately, non-viral gene transfer methods are limited in their efficacy. Nevertheless, non-viral vectors score concerning their manufacturing cost, easy handling and safety. Therefore, many attempts are ongoing to increase the efficacy of gene delivery using non-viral vectors by specific modifications or additional auxiliary substances, than trying to make viral systems safer. Therefore, in this work, different strategies are introduced to compensate weak gene delivery. Firstly, hemolytic activity of different viruence-factor derived peptides were compared for their potency to enhance in gene transfer systems as mediating disruption of lysosomes.
Compared to other tested endosomolytic peptides, the virulence-factor Listeriolysin O showed unrivalled pH specific hemolytic activity. Secondly, strategies to enhance gene expression itself were introduced in order to compensate weak transfection efficiency. Sequence modifications of the therapeutic gene showed several fold increase in gene expression. Additionally, by constructing a cascade promoter system, bottlenecks of transfection factor depletion was overcomed, leading to further increase in expression. Thirdly, screening of potent secretion signal sequences to mediate enhanced protein secretion by signal sequence exchange was performed. Additionally, by monitoring protein secretion kinetics intersting insights into bottlenecks during protein secretion were observed. Finally, additional strategies to improve specifically osteogenic differentiation. Amongst others, the application of a hybrid vector system for overexpression of a therapeutic gene by simulatenous downregulation of inhibitory factors showed enhanced osteogenic differentiation of target cells compared to conventional vectors.

Zusammenfassung

Eine der Hauptkomponenten der Gewebezüchtung beinhaltet die Anwendung von gewebespezifischen Wachstumsfaktoren entweder auf Proteinebene oder kodiert auf einem theraoeutischen DNA-Plasmid (Gentherapie).

Die Anwendung von therapeutischen Plasmiden kodierend für Wachstumsfaktoren bietet ein vielverpsrechendes Mittel verbunden mit niedrigen Herstellungskosten, höherer Sicherheit und erhöhte Bioaktivität der produzierten Proteine wegen wirtsspezifischer post-translationaler Modifikationen und korrekter Faltung der lokal produzierten Wachstumsfaktoren. Um exogene DNA in Zielzellen (sowohl *in vitro* als auch *in vivo*) einzubringen, können mehrere virale oder nicht-virale Gentransfer-Methoden angewendet werden. Jedoch sind nicht-virale Methoden in ihrer Effektivität beschränkt. Trotzdem punkten nicht-virale Transfersysteme durch niedrige Produktionskosten, leichter Anwendbarkeit und Sicherheit. Deshalb sind viele Versuche laufend, die auf spezifische Modifikationen der Transfersysteme oder durch das Zufügen von Helfersubstanzen (z.B Peptide), um die Wirksamkeit des nicht-viralen Gentransfers zu erhöhen, als viralen Gentransfer sicherer zu machen.

Aus diesen Gründen wurden in dieser Arbeit Methoden vorgestellt um einen schwachen Gentransfer, oder die Effizienz der Genexpression, zu verbessern um die schwache Wirksamkeit zu kompensieren. Erstens wurde die hämolytische Aktivität verschiedener, auf Virulenzfaktoren basierender Peptide miteinander verglichen. sogenannte endosomolytischen Peptide mediieren die Zerstörung der Lysosomenmembran während des Gentransfers und verhindern dadurch den Abbau des Gentransfersystems sowie der therapeutischen DNA.

Weiters wurden Strategien entwickelt, um die Genexpression an sich zu erhöhen, um einen eventuellen schlechten Gentransfer vorzubeugen, unter anderem Modifikationen am Promotersystem der Expressionskasette (Kaskadenpromoter).

Weiters wurde die Stärke von Sekretionssignalen von unterschiedlichen Protein verglichen, um eventuell schwache Signalsequenzen der eingesetzten Wachstumsfaktoren mit potenteren zu ersetzen. Dabei wurden unter anderem auch interessante Engpässe in der Proteinsekretion beobachtet und analysiert.

Letztendlich wurden auch im Fall der Knochenregeneration Strategien vorgestellt, um nicht-virale Anwendungen der Gentherapie speziell in diesem Fall (osteogene Differenzierung) zu verbessern. Unter anderem der Einsatz und Verifizierung von Hybridplasmiden, konzipiert für eine transiente Überexpression eines knochenspezifischen Wachstumsfaktors bei gleichzeitiger erzwungener Unterdrückung (Herunterregulierung) von potentiellen Inhibitoren des osteogenen Signalweges.

1. Introduction

Aim of the work – Enhancement of gene therapeutical applications

A precondition for the success of gene therapy is the development of delivery systems that are suitable for efficient gene transfer in a variety of tissues. However, under *in vivo* conditions, the efficiency of non-viral gene transfer is low at best [1-3]. The reason for this is that many barriers must be overcome for the efficient transfer of endogenous genes into target cells, even before any gene expression can occur. Even then, in many cases gene expression is below the treshold for a significant effect of the overexpressed gene. To compensate potential low gene transfer efficiencies, many concepts are introduced in this work to improve the expression of the endogenous gene, like alteration of promoter system, optimization of codon sequence, or the addition of cis and trans acting sequence elements.

Regarding the latter, it has been demonstrated that the expression and final yield of recombinant proteins does not necessarily correlate with their mRNA levels [4, 5]. Furthermore, for the application of genes encoded for extracellular growth factors, inefficient protein secretion can be a bottleneck. Several studies have demonstrated an enhanced level of protein secretion by optimization of signal sequences. The efficiency of protein secretion is stongly determined by the composition of the signal peptide [5-10].

Therefore, also potencies of different secretion signals were examined in this work as well as secretion kinetics of secretion signals derived from osteogenic factors.

1. Introduction A – Overview of *in vivo* gene delivery methods

Efficient non-viral delivery of exogenous therapeutic genes *in vivo* and *in vitro* is still limited due to different mechanical and chemical barriers. These barriers prevent efficient gene delivery and can be classified into three major obstacles. Firstly, exogenous DNA has to pass the chemical barrier of the cell membrane. Secondly, the entrapment and subsequent degradation inside the lysosomes after endocytic uptake has to be overcome. Finally, introduced DNA has to be imported to the cell nucleus. This review gives an overview of different chemical and mechanical methods specifically developed to counter these specific obstacles. The following sections are subdivided into the three main barriers of successful gene delivery. Furthermore, chemical and mechanical methods based on current knowledge to increase the efficacy of non-viral gene delivery are described. Future aspects and drawbacks are discussed giving an outlook for the upcoming next steps in non-viral gene delivery.

One of the key components of tissue engineering is the delivery of growth factors either as the factors itself or the information of production encoded on therapeutic DNA plasmids (gene therapy).

The introduction of therapeutic plasmids encoded for growth factors provides a promising tool associated with lower manufacturing costs, higher safety and increased bioactivity of the produced proteins due to host-specific post-translational modifications and correct folding of the locally produced growth factors. In order to introduce exogenous DNA into cells *in vitro* and *in vivo*, several strategies based on viral or non-viral approaches can be applied.

Viral gene transfer strategies (retroviruses [11], adenoviruses [12], adeno-associated viruses [13, 14], herpes viruses [15], lentiviruses [16] and Epstein-Barr viruses [17]) show by far the highest transfection efficiencies *in vitro* and *in vivo*, whereas non-viral vectors (polycations, cationic lipids), and mechanical methods (magnetofection, electroporation, sonoporation, gene gun) are limited in their efficacy to deliver genes. The efficiency of gene delivery is especially restricted in the presence of serum and other proteins, which makes non-viral vectors less adequate for *in vivo* approaches. But nevertheless, due to safety aspects, the application of non-viral gene delivery systems *in vivo* gain more popularity due to the lack of disadvantages associated with viral systems like detrimental immune responses. Additionally, the fact that many viruses tend to integrate introduced exogenous DNA into the host genome dramatically increases the probability of tumor cell growth in target cells. Furthermore, non-viral vectors score concerning their manufacturing cost and easy handling during gene therapy approaches.

Therefore, many attempts are ongoing to increase the efficacy of gene delivery using non-viral vectors by specific modifications or additional auxiliary substances, than trying to make viral systems safer.

The use of carrier molecules to enhance DNA delivery into host cells has been the subject of intense examination. The DNA delivery is limited due to 3 major physical and chemical barriers encountered along the delivery pathway (Figure 1).

1. Crossing the cell membrane

2. Overcome lysosomal degradation

3. Import into nucleus through nuclear membrane

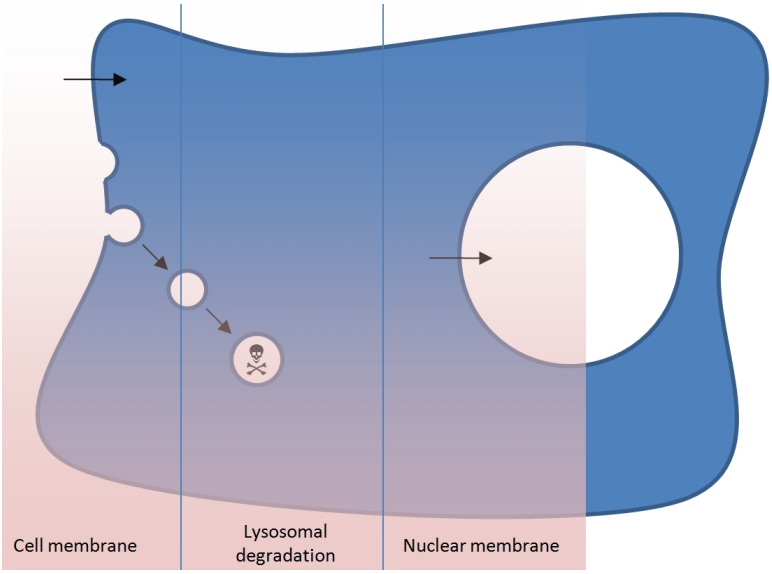


Figure 1: Three main cell barriers hindering efficient gene delivery

The following sections give an overview about physical and chemical barriers hindering the delivery of macromolecules into eukaryotic target cells, and the corresponding experimentally evaluated solutions for overcoming these delivery limiting obstacles (Figure 2). This review helps obtaining a general overview of different approaches to achieve or enhance non-viral delivery of macromolecules, especially of nucleic acids.

Furthermore, the following sections outline the various non-viral methods and formulations that have been explored to overcome these major gene delivery barriers

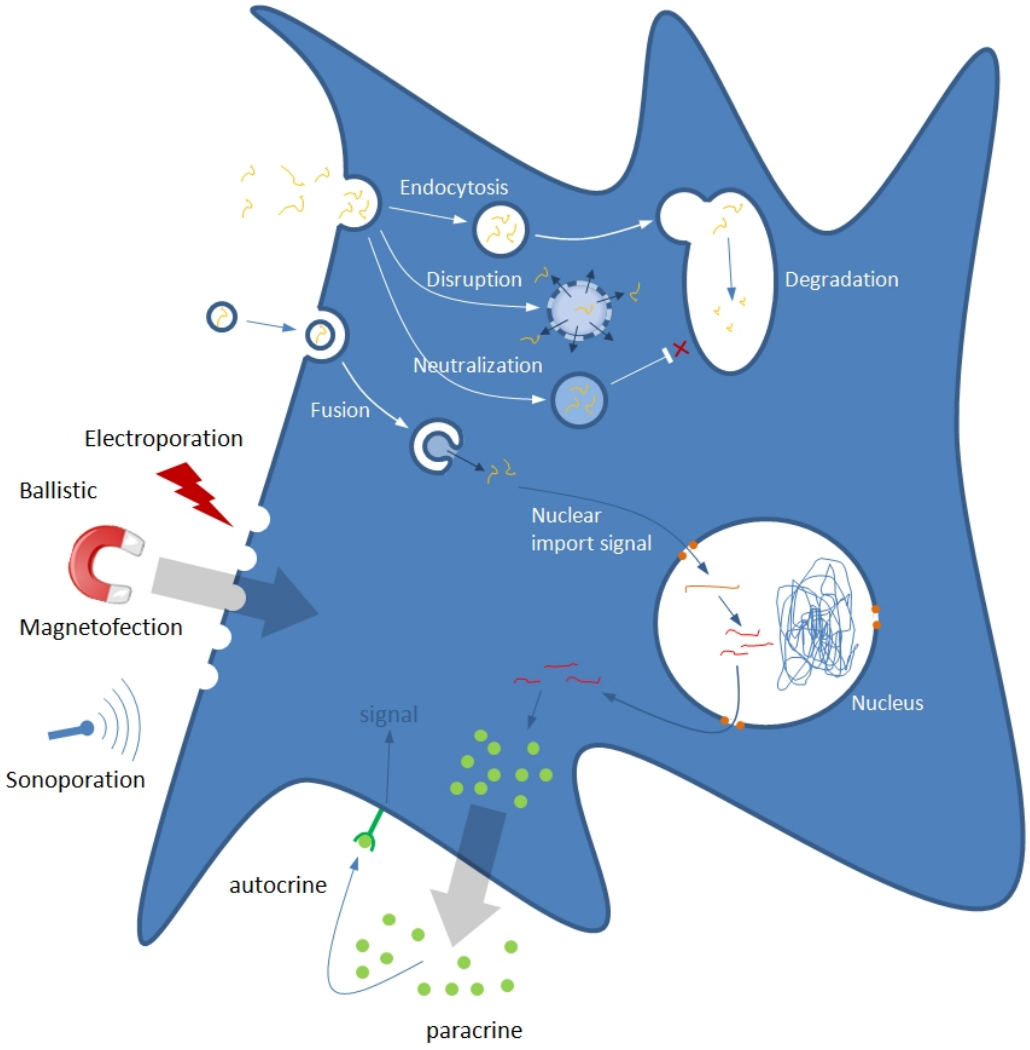


Figure 2: Schematic overview about barriers and solutions for an efficient delivery of DNA

2. Barriers and solutions

2.1. Crossing the cell membrane

Although the transfection efficiency and the level of gene expression is dependent on various factors such as plasmid size and copy number [18], the gene transfer in general using naked DNA (DNA without auxiliary carrier systems), shows weak success, independently of the targeted cell and tissue type *in vitro* and *in vivo,* respectively. Table 1 shows an overview of applications designed to overcome target cell membrane in and enhance or trigger gene delivery.

Application	**Method**	**Description**
Magnetofection	Mechanical/physical	DNA complexed with magnetic particles in magnetic field
Electroporation	Mechanical/physical	Migration of DNA in electric field mediated by two electrodes
Sonoporation	Mechanical/physical	DNA complexed with microbubbles, gene transfer by cavitation induced by ultrasound pulses
Shock wave	Mechanical/physical	Membrane permeabilization
Ballistic gene delivery (gene gun)	Mechanical/physical	DNA complexed with gold particles, acceleration ("shooting") by high gas pressure
Polycations/cationic lipids	Chemical	Complex formation of DNA by electrostatic interactions
Silica nanoparticles	Chemical	Complex formation of DNA by electrostatic interactions
Receptor/ligand interaction	Chemical	Masking and direct cell receptor targeting by coating of DNA complexes with ligands
Cell penetrating peptides	Chemical	Direct translocation of cargo through cemm mebrane by postulated flipping mechanism

Table 1: Overview of applications designed to overcome target cell membrane

2.1.1. Cell penetrating peptides (CPPs)

Cell penetrating peptides (short protein transduction domains), consist of mainly positively charged amino acids which mediate the translocation of proteins through the cell membrane of target cells [19, 20].

Proteins fused with a transduction domain (e.g derived from TAT protein of HIV (GRKKRRQRRRPQ)) show efficient translocation through the cell membrane *in vitro* [21, 22] and *in vivo* [23]. The translocation into the cells occurs rapidly in around 5-20 minutes (rate dependent on the size of the cargo protein attached) [24]. However, the covalent attachment of the transduction domain of the TAT protein to plasmids shows moderate [25] translocation. Table 2 gives an overview of defined potent transduction peptides.

Potent cell penetrating peptides		
Derived from	**Sequence**	**Reference**
Antennapedia Homeobox domain/Penetratin (aa 43-58) (D. *melanogaster*)	RQIKIWFQNRRMKWKK	[26]
Tat protein (aa 48-60) (HIV-1)	GRKKRRQRRRPQ	[27]
Rev protein (aa 34-50) (HIV-1)	TRQARRNRRRRWRERQR	[28, 29]
FHV coat protein (aa 35-49) (FHV)	RRRRNRTRRNRRRVR	[29]
Rex (aa 4-16) (HTLV-II)	TRRQRTRRARRNR	[29, 30]
N coat protein (aa 14-30) (P22)	NAKTRRHERRRKLAIER	[29]
Gag (aa 7-25) (CCMV)	KLTRAQRRAAARKNKRNTR	[29]

Gag (aa 7-25) (BMV)	KMTRAQRRAAARRNRWTAR	[31]
Transportan (galanin + mastoparan)	GWTLNSAGYLLGKINLKALAALAKKIL	[32]

Table 2: Overview of known cell penetrating peptides

2.1.2. Gene activated matrices (for local application)

Biomaterials can be used as a biocompatible and biodegradible carrier/drug release system for the local delivery of therapeutic nucleic acids, especially in wound healing or tissue engineering. Lipoplex- and polyplex-loaded biomaterial sponges (implantable carrier matrices) are superior in mediating sustained and local gene delivery as compared to naked DNA-loaded sponges. Protective copolymers promote the tranfection potential of polyplex-loaded sponges upon local implantation, due to their stabilizing and opsonization-inhibiting properties (Table 3).

Scaffold	**Carrier**	**Growth factor**	**Application**	**Reference**
Fibrin	Lipofectamine 2000	Plasmid-VEGF	Wound healing	[33]
Fibrin	Lipofectin	Plasmid-EGFP, Luciferase + β-Galactosidase	Simultaneous delivery of multiple genes to wound bed	[34]
Fibrin		TNF, Albumin, Plasmid-DNA (Reporter)		[35]
Fibrin		Plasmid-	Ischemic flap	[36]

		VEGF(165)	necrosis	
Collagen	GenePORTER	Plasmid-GDNF	Brain injury	[37]
Chitosan	Chitosan	Platelet derived growth factor (PDGF) gene	Periodontal ligament cells (PDLCs)	[38]
Atelocollagen	calcium-phosphate precipitates (CaP)	Plasmid-BMP2	Critical size bone defect in rat tibiae	[39]
Collagen	Copolymer-protected gene vectors (PEI-DNA-PROCOP)	Plasmid-Luciferase	Subcutaneous implantation, significantly increased expression of luciferase	[40]

Table 3: Application of gene activated matrices in tissue engineering

2.2. Overcoming the lysosomes

Because exogenous DNA is rapidly degraded by lysosomal nucleases, the escape of DNA vectors from the lysosomal compartments is an important step for efficient gene delivery [41-43]. Several approaches to lyse or neutralize lysosomal vesicles are designed, amongst them, photoinduction [44] and endosomolytic (membrane disrupting) agents.

2.2.1. Endosomolytic peptides

Due to their endosome disrupting activity, amphipathic peptides (peptides with both, a hydrophobic domain and polar head group) are adopted for gene delivery in order to overcome the lysosomal barrier [45, 46]. Several tested amphipathic peptides derived from different origins or designed synthetically, give promising results concerning their efficacy in delivering exogenous DNA fragments. The conformational structure of synthetic peptides allows sterical adjacency of glutamic acid and lysine residues, respectively. Under acidic conditions (pH = 5), the protonation of the glutamic acid and lysine residues leads to a mutual repulsion of the cationic chains and hence to a conformational change to an amphipathic alpha-helix. The following exposition of hydrophobic helices promotes the interaction and following rupture of the uncharged lipid bilayer membrane [47-50]. Some of the most common peptides are listed in Table 4.

Peptide	**Sequence**	**Origin/adapted from**	**Membrane interaction**	**Reference**
GALA	WEAALAEALAEALAEHLAEALA EALEALAA	Artificial	Destabilization, pore formation, fusion	[47, 49, 51]
KALA	WEAKLAKALAKALAKHLAKALA KALKACEA	Artificial	Destabilization	[52]
Melittin	GIGAVLKVLTTGLPALISWIKRKR QQ	Bee venom (Melittin)	Destabilization	[47-50]

HA-2	GLFGAIAGFIEGGWTGMIDGW YG	Influenza (HA)	Destabilization, fusion	[53-57]
EB1	LIRLWSHLIHIWFQNRRLKWKKK	Drosophila (Antennapedia homeoprotein)	Destabilization	[58]
HGP	LLGRRGWEVLKYWWNLLQYW SQELC	HIV (gp41)	Destabilization	[59]
INF-7	GLFEAIEGFIENGWEGMIDGWY G	Influenza (HA)	Destabilization, fusion	[56]
E5	GLFGAIAGFIEGGWTGMIDG	Influenza (HA)	Destabilization, fusion	[60]
JTS-1	GLFEALLELLESLWELLLEA	Influenza (HA)	Destabilization	[61]
H5WYG	GLFHAIAHFIHGGWHGLIHGWY G	Influenza (HA)	Destabilization	[62]
ppTG1	GLFKALLKLLKSLWKLLLKA	Influenza (HA)	Destabilization	[63]
HEL	KLLKLLLKLWKKLLKLLK	Artificial	Destabilization	[64]
Peptide 4_6	LARLLARLLARLLRALLRALLRAL	Artificial	Destabilization	[65]

Table 4: Compilation of endosomolytic peptides

2.2.2 Endosomolytic proteins (Listeriolysin O)

Secretion of the hemolytic protein Listeriolysin O (LLO) by Listeria *monocytogenes* causes membrane perforation and releases the bacteria from the lysosomes of eukaryotic host cells [66-68]. The main difference between LLO and other hemolytic proteins such as streptolysin or perfringolysin is the advantage of the pH dependent activity of LLO. In order to prevent the host cell wall from damage, the hemolytic activity of LLO is inhibited in cytosolic non-acidic conditions. For affirmation, activity analysis with purified LLO showed increased hemolytic activity at acidic pH which can be compared with lysosomal conditions [69-71]. Additionally, *in vitro* studies with pH-sensitive liposomes packed with

LLO and a fluorescence dye [72] or toxin [73], respectively, and bacterial cells expressing LLO [74] confirm these observations.

To conclude, by application of LLO in gene transfer approaches, lysosomal degradation can be bypassed and therefore a more efficient delivery of therapeutic DNA vectors, proteins or toxins *in vivo* can be achieved.

2.3. Overcoming the nuclear membrane

Gene delivery into rapidly dividing cells is more efficient due to the dissolved nuclear membrane during the cell division [75, 76]. But in the case of *in vivo* cell transfection, the majority of cells have very slow division rates. Therefore, many types of viruses have to import their DNA as well as other macromolecules into the host cell nucleus to ensure their replication and transcription. In most cases, the host cell´s own import machinery is utilized by targeting host import proteins by nuclear localization signals encoded on viral proteins in order to ensure active import of these macromolecules through the nuclear pore complexes [77, 78]. In contrast to viral vectors, non viral gene delivery methods show low transfection efficiencies *in vitro* and *in vivo*, not at least because of lacking efficient methods to overcome the nuclear membrane of non-dividing cells. Most introduced plasmid DNA remains in the cytosol [44, 79] and is degraded by cytosolic nucleases within 2 hours, whereas the time of the degradation is independent of the cell line or the type of plasmid DNA [80, 81]. Yamaizumi et. al. demonstrate that at least 1000-fold more DNA is required for an efficient expression of injected DNA into the cytosol in contrast to DNA injected directly into the cell nucleus [82]. Moreover, Labat-Moleur et al reported that only 1 of 100 plasmid DNA molecules injected into the cytoplasm of cells were able to reach the nucleus [83]. Furthermore, it has been demonstrated that the efficiency of the DNA import is higher in the case of applying linear DNA fragments smaller than 1.5kb [84]. Beside these observations, the transfection efficiency and level of gene expression is dependent on various factors such as plasmid size [85] and copy number [18]. Therefore, an increased copy number of plasmids inside the nucleus of eukaryotic cells leads to an increase in the gene expression [86].

To find solutions for this rate limiting step, an understanding in the molecular mechanism based on the nuclear transport is essential.

2.3.1. Application of nuclear localization signals for an increased transfection efficiency

Because most cells targeted in gene therapy approaches are non-dividing or slowly-dividing cells, the incorporation of nuclear localization signals (NLS), capable of mediating nuclear import, can significantly increase the expression rate of the introduced exogenous DNA [84, 87-93]. It has been demonstrated that only one nuclear localization sequence conjugated to DNA is sufficient to mediate the nuclear import [94]. As additional examples, increase of the transfection efficiency using conjugated NLS derived from SV40 was obtained with linear DNA molecules using either microinjection approaches [86], cationic lipids [94-96], polycations [97] or electroporation [98], respectively.

But in contrast, other observations showed no increase of NLS-conjugated transfection by using cationic lipids and polycations, respectively [93, 99, 100]. Direct introduction of exogenous DNA by microinjection showed no nuclear detection of the DNA, neither with linear DNA conjugated to NLS bearing peptides alone [100, 101] nor in combination with cationic polymers [93, 102].

2.3.2. mRNA transfection

Avoidance of the nuclear membane barrier can also be achieved by replacing the introduced therapeutic DNA with messenger RNA (mRNA). Instead of circular or linear DNA, the introduction of mRNA has recently appeared as an promising and attractive alternative in gene therapy approaches. Due to the fact that mRNA does not need to enter the cell nucleus in order to be translated into protein, nuclear import issues are eliminated on that way [103]. Other big advantage of mRNA transfection is the impossibility of the introduced ribonucleic acid to integrate into the host genome which otherwise might lead to cancer [104]. On the other side, the challenge of mRNA delivery is the low stability of mRNAs against RNases during the preparation process, leading to more extensive preparation procedures. Furthermore, the generation of mRNA (by *in vitro* transcription reaction) demands an additional subsequent 5´ modification step

(adding a CAP structure) to ensure consistent mRNA stability in target cells. Different CAP analogons are reviewed by Yamamoto et al [104].

Compared to DNA vectors, mRNA shows a different expression kinetic (more rapid appearance of expression product, but short-term expression in contrast to the elongated expression kinetics of plasmid DNA).

Finally, the present immunogenicity of mRNAs (lower immune response compared to DNA due to the lacking of immunogenic CpG motifs [103]) can be further reduced by specific modifications masking the introduced ribonucleic acid as eukaryotic mRNA [105].

3. Conclusion of gene delivery methods

Due to the high clinical benefits and perspectives of gene therapy, a lot of different methods and approaches are tested for the efficient and low toxic delivery of therapeutic genes (Table 5). However, until this date, the field of non-viral gene is still in its infancy. Continuative improvements of the delivery systems, based on a better understanding of cellular pathways and their barriers, is required for the successful clinical application of gene therapy. The big variety of different chemical and mechanical approaches reflects that to date there is no explicit ideal gene delivery system. Nonetheless, it can be expected that ongoing research will bring new delivery strategies specifically optimized and adjusted to particular applications and problems and that the numerous limitations will be solved to smooth the way for routine clinical use of gene therapy.

Method	**Efficiency**		**Toxicity**
	in vitro	***in vivo***	
Needle injection	Low	Low	Safe
Ballistic (gene gun)	Medium	Medium	Tissue damage
Electroporation	High	High	Invasive
Ultrasound	Good	Average	Low
Hydrodynamic delivery	High	High	Low
Polycations	High	Low/Average	High
Cationic lipids	High	Low/Medium	High
Lipid/polymer hybrids	Average/High	Low	Low
Lipid/polymer hybrids	High	Low/Medium	Low

Table 5: Conclusion of advantages and disadvantages of established non viral gene delivery systems

1. Introduction B – Approaches presented in this work to overcome barriers of gene delivery

1.1. Comparison of synthetic and virulence-factor derived endosomolytic peptides

The lysosomal degradation of transferred macromolecules like exogenous DNA for gene therapeutic applications or toxins for cancer treatment is one of the major barriers in gene therapy. The endocytic uptake of DNA (whether pure or complexed with carriers), results in the degradation of the entrapped molecules in the lysosomes of target cells under acidic conditions [41-43, 73]. To overcome this barrier, several endosomolytic peptides and polymers [106-108] are developed by several groups to show functional membrane disrupting activity when exposed to lysosomal conditions. INF7, E5 and K5 are derivates of the Influenza hemagglutinin protein domain HA-2.

The naturally occurring hemolytic protein Listeriolysin O (LLO) is showing the same behavior regarding pH dependent membrane disruption, and is therefore included in this study. It causes membrane perforation and releases the bacteria from the lysosomes [66-68, 109]. Membrane-disrupting proteins from different species similar to LLO like streptolysin or perfringolysin do not show a pH selectivity and would therefore be toxic after subsequent endocytic uptake by target cells [69]. It is therefore one of the aims of this study to perform a direct comparison of several known endosomolytic peptides with LLO regarding pH selectivity, stability and reversibility of bioactivity.

1.2. Enhancement of gene expression

1.2.1 Optimization of gene sequence

1.2.1.1 Codon optimization

Different codons are preferentially used in different organisms, which is dependent on the tRNA concentration insde the cell. Codon usage plays an important role in the

regulation of protein biosynthesis. Therefore, rarely used codons can slow down translation, whereby frequently used codons can accelerate translation [110, 111].
In this work, codon optimization for the adjustment of codon usage frequency of mouse organism was performed in order to achieve higher translation rates for both, BMP2 and BMP7 gene, respectively.

1.2.1.2 Insertion of intron

Additionally, the effect of the insertion of an artificial intron on gene expression rates was examined. Insertion of introns is known to enhance transcription rates and mRNA stability and export into the cytosol, respectively [112, 113]. The expression level of intronless genes is often 10 – 100 times lower than the intron-containing counterpart [114]. An artificially designed intron based on alpha globin gene will be inserted into the coding region of both, BMP2 and BMP7, respectively, and subsequent expression rates will be observed.

1.2.2 General alteration in promoter system (Cascade promoter system)

It is postulated, that genes located under strong viral promoters like CMV promoter, still have the challenge concerning the recruitment of enough transcription factors, which potentially leads to a bottleneck effect. Therefore, a cascade promoter system was introduced for subsequent proof of the mentioned concept. The basic principle is to avoid depletion of transcription factors by introducing own transcription factors, which are encoded on the therapeutic plasmid (Fig. 1). By this system, a theoretical multiplication of mRNA transcript production is postulated.

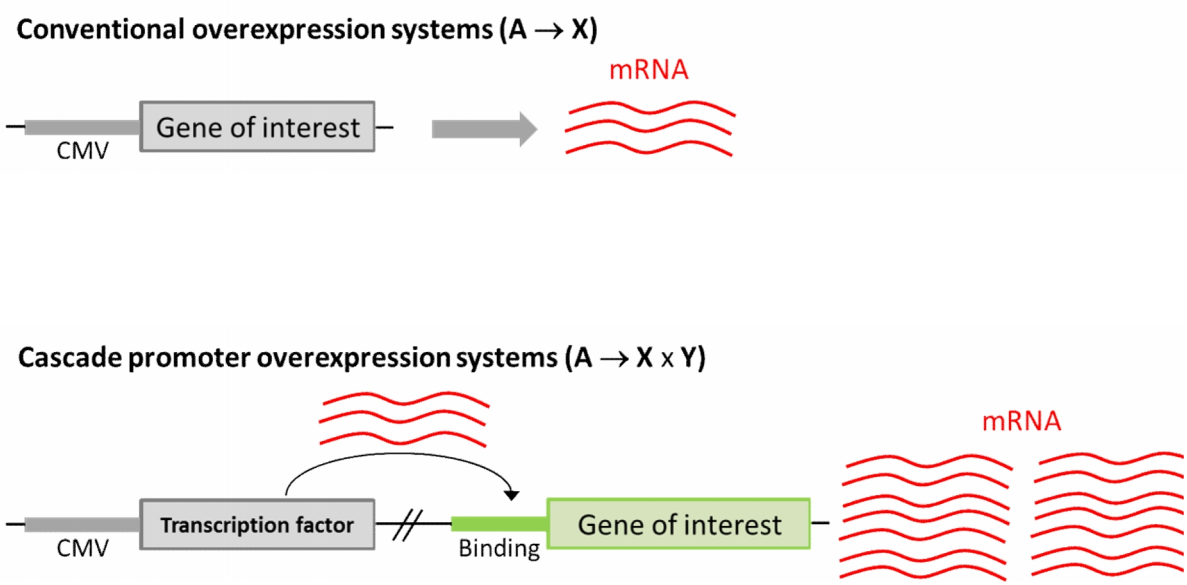


Fig. 1 Scheme of cascade promoter system

1.3. Protein secretion kinetics

Protein secretion sequences are initially expressed as precursors with an N-terminal signal sequence, which is cleaved during the translocation process [115]. Proteins containing an aminoterminal hydrophobic domain are recognized by signal recognition particles during the ribosomal translation process (Fig. 2). Signal recognition particles and signal peptidases recognize structural motifs rather than primary amino acid sequences. They mediate the docking of the translation complex to the rough endoplasmic reticulum (ER) membrane where they are translocated co-translationally into the ER lumen through specific translocator channels [116, 117]. The signal sequence is cleaved by signal peptidases and protein translation continues while the newly synthetized strand translocates through the ER membrane (cotranslational translocation). After subsequent protein modifications in the Golgi apparatus, proteins are sorted into secretory vesicles which are finally secreted by exocytosis.

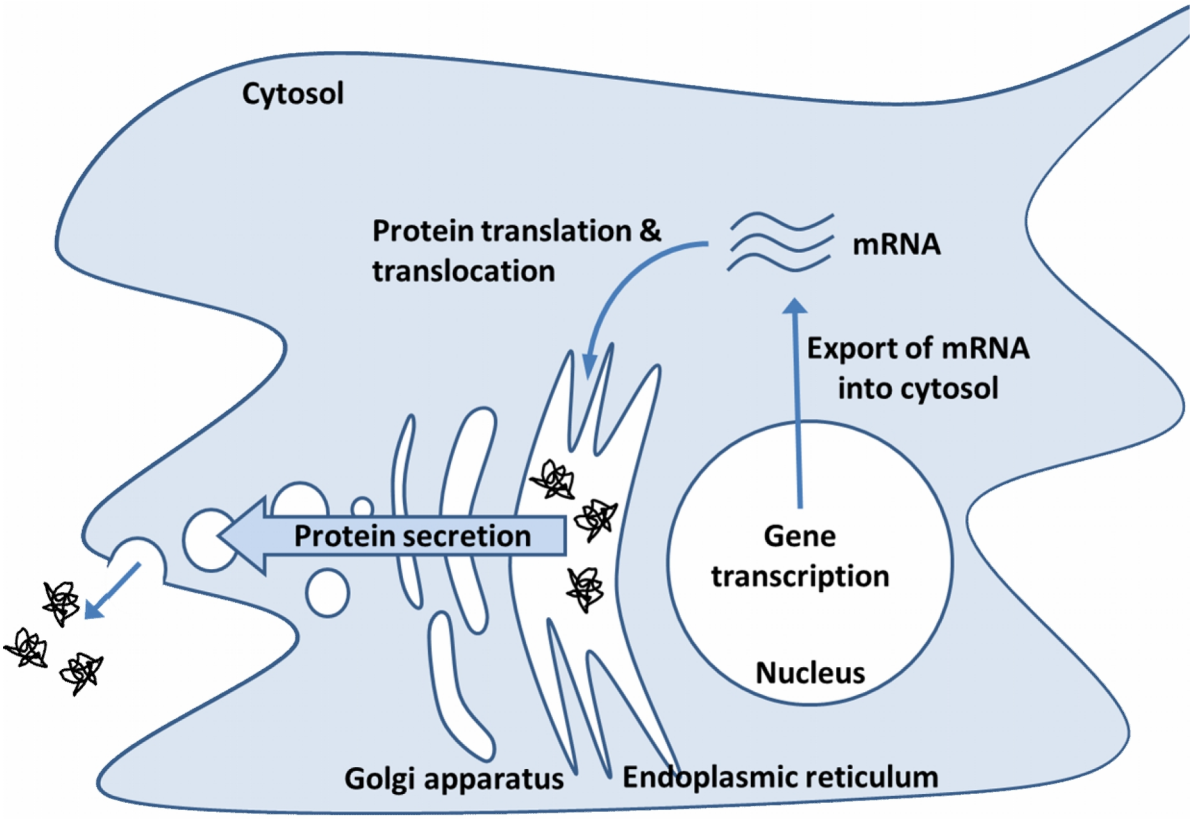


Fig. 2 Schematic depiction of protein secretion pathway

However, secretion signal sequences have different protein secretion potencies and kinetics, which is based on their their amino acid composition [5-10]. Signal recognition particles and signal peptidases recognize structural motifs rather than primary amino acid sequences [118, 119].

Based on this, the third chapter of this study is based on the identification of more potent signal sequences mediating increased protein secretion for gene therapeutical approaches, here particularly for bone tissue regeneration and engineering. Various secretion signals derived either from osteogenic factors or previously verified strong secretion proteins, respectively, were compared among each other. Additionally, artificial altered signals are in focus of this chapter.

1.4. Bone specific applications

Introduction

Pseudoarthrosis: state of the art

Clinical state of the art to treat pseudoarthrosis is accompanied by autologous bone graft derived from the iliac crest. The current procedure is highly invasive, expensive and time consuming representing a high economic burden to health care systems. Around 20% of the patients develop complications at the graft site one year post operation, of which 3% need operative revision [120]. Additionally 55% of all patients still had complaints at the graft site one year after operation.

Growth factor therapy

Recombinant growth factors such as Bone morphogenic protein 2 (BMP2) and Bone morphogenic protein 7 (BMP7) are already approved for use in the treatment of pseudoarthrosis in combination with appropriate carrier materials. Apart from high dosages and high costs involved for these agents, a number of safety issues have been identified with their use e.g unwanted ectopic bone formation. Bone morphogenic proteins (BMPs) are responsible for osteoinduction. Beside this, BMP2 and BMP7 have been identified as a very potent combination to support ossification *in vivo* and *in vitro* [121-123].

Combinatorial gene therapy

Therapeutic DNA is cheap and simple to produce even under GMP conditions required for clinical studies [124, 125]. The reprogramming of target cells with overexpressed growth factor genes results in the *in situ* production of correctly processed proteins with host-specific bioactive post-translational modifications [126, 127].

This ensures higher bioactivity compared to recombinant growth factors, whereby the application of low concentrations is feasible [128, 129].

Furthermore, the application of more than one therapeutic gene can enhance rate of differentiation, since a sufficient stimulus often requires the action of at least two

therapeutic genes simultaneously. For example, heterodimer BMP formation (BMP2/BMP7) has been shown to occur physiologically and to have an increased osteogenic activity compared to BMP homodimers [121, 122, 130] and have therefore been selected as the main osteoinductive therapeutic gene combination to be investigated within this work.

Orthopaedic gene therapy

Viral gene therapy still provides the highest and transfection efficacies [131]. Nevertheless, for *in vivo* approaches the application of viral vector systems is limited by its potential hazards including high host immune responses [132, 133]. Therefore, safe non-viral gene transfer strategies have been developed, but still limited by low transfection efficiencies. To date, osteoinductive gene therapy at orthotopic defect sites, either viral or non-viral, were only used in *ex vivo* approaches with explanted stem cells being transfected *in vitro* prior to implantation [134, 135]. Orthopaedic gene therapy focus on the manipulation of target cells or tissues with exogenous DNA in order to modify gene expression patterns and trigger osteogenic differentiation *in vivo*. Transient overexpression of exogenous DNA in somatic target cells remains active up to several weeks [136-138]. *In vivo* transfection mediates local and transient osteogenic stimulus through delivery of exogenous genes encoded for growth factors that are produced by the transfected cells *in situ*, thereby restricting adverse effects as only limited amounts of highly bioactive growth factors are produced directly at the defect site.

Bone morphogenic proteins in bone regeneration

Bone morphogenic proteins (BMPs) are growth factors mediating osteogenic differentiation of progenitor cells during development in embryos and fracture repair in adults. They serve as clinical agents to enhance bone regeneration due to their high potency to induce or enhance bone and cartilage formation [139-141]. Addition of recombinant human BMP2 protein alone is capable of mediating osteoinduction in various test systems, e.g. in pluripotent mesenchymal precursor C2C12 cells [142]. Furthermore, osteoinduction can be enhanced by the co-introduction of additional osteogenic inducers like BMP7 [121]. Gene therapeutic approaches inducing the

osteogenic differention of target cells by overexpressing bone morphogenic protein 2 (BMP2) alone shows only weak induction when introduced to target cells, compared to the simultanous overexpression of BMP2 and BMP7 [122, 143]. Furthermore, while BMP2 homodimers are efficiently inducing osteogenic differentiation of target cells *in vitro*, clinically effective dosages of homodimers need to be up to milligrams, which is accompanied with high costs and potential side effects [144, 145].

Regarding action of BMPs, after a short induction period of BMPs, several extracellular inhibitors are known to be endogenously produced subsequently and secreted by differentiating cells, generating a feedback loop of inhibition. This negative regulation loop is triggered by extracellular proteins like Noggin [146, 147], Chordin [148] and Gremlin-1 [149, 150]. By binding to BMP2, these extracellular inhibitors prevent further binding of BMP2 to its target receptor on the cell surface, and therefore inhibiting BMP-mediated signal transduction [151-153]. Knock down of for example Noggin by siRNA enhanced osteoinduction of C2C12 *in vitro* and increases bone volume *in vivo* [147] as well as accelerate bone formation *in vivo* [154]. Furthermore, the inhibitory effect of Noggin on osteogenic differentiation was shown in several mouse and rat cell systems [146, 155, 156]. Previously observations revealed inhibition of the osteogenic differentiation of both, mesenchymal precursor cells [151, 155, 157] and pluripotent mesenchymal precursor (C2C12) [146], respectively, by the addition of recombinant Noggin. Furthermore, the expression and secretion of Noggin is temporary induced by BMP-mediated differentiation, followed by a decline of Noggin when cells undergo differentiation [142, 151, 155]. This transient expression pattern indicates that Noggin should be an important factor in the negative feedback response during cell differentiation.

1.4.1 Blocking of osteogenic inhibitory factors

1.4.1.1 miRNA sponges

Neutralization of endogenous anti-osteogenic miRNA was under investigation of this work. Endogenous miRNAs can regulate up to thousands of genes. As an alternative to

chemically modified anti-miRNA oligonucleotides, the introduction of binding sites of target microRNAs downstream of a gene (RNA sponge [158]) can be introduced into plasmids. Here, for proof of concept, RNA polymerase II promoter driven miR-20a-target transcript, a pro-osteogenic miRNA, was expressed, containing only two instead of conventionally used minimal 7 binding sites. When vectors encoding these sponges are transfected into target cells, miRNA sponges bind mir-20a miRNA at least as strongly as artificially derived antisense oligonucleotides and such minimize the reactive endogenous miRNA.

1.4.1.2 Hybrid vectors

The application of eukaryotic vectors for the overexpression of therapeutic genes in target cells is the usual way to manipulate gene expression. Beside the overexpression of exogenous genes, the downregulation of specific genes by artificial siRNA or siRNA/shRNA vectors are additionally in common use [159]. Knock down of an inhibitory gene by siRNA have shown significant enhancement of cell differentiation. Previously described knock down of several either extracellular (Noggin [147], Chordin [148], Gremlin-1 [160]) as well as intracellular (Ubc9 [161, 162], TNF-alpha [163, 164]) inhibitors of the BMP signalling pathway, respectively, confirm osteo-inductive effect during cell differentiation. Furthermore, naturally derived downregulation of inhibitors by microRNAs during osteogenic differentiation are recently reported. For example, the overexpression of the physiological mir20a potentiates the effect of osteoinduction by knock down of several inhibitory factors like Crim1, Bambi and PPARγ [165].

In order to avoid osteogenic dysfunction mediated by BMP inhibitors, we have developed a hybrid expression vector for the simultaneous downregulation of a single or multiple genes during the overexpression of BMP2 under the control of the constitutive active CMV promoter. By the attenuation of inhibitory genes that interfere with the activators of the BMP2 signaling pathway, a stronger osteoinductive potential of the therapeutic vector was aimed.

An intron was inserted into the open reading frame of either BMP2 or fluorescence protein. The artificial intron includes palindromic sequences leading to the formation of a hairpin loop upon transcription into mRNA (Fig. 3). The formed hairpin loop is thereafter recognized and processed by Dicer and RISC complex for the subsequent inactivation of the complementary target mRNAs [166]. The hairpin elements are clustered in the artificial intron sequence ensuring the persistence of mRNA stability upon post-transcriptional microRNA processing. Subsequently, three microRNA candidates were arranged in triplet clusters leading to a multi-cluster structure of nine microRNAs.

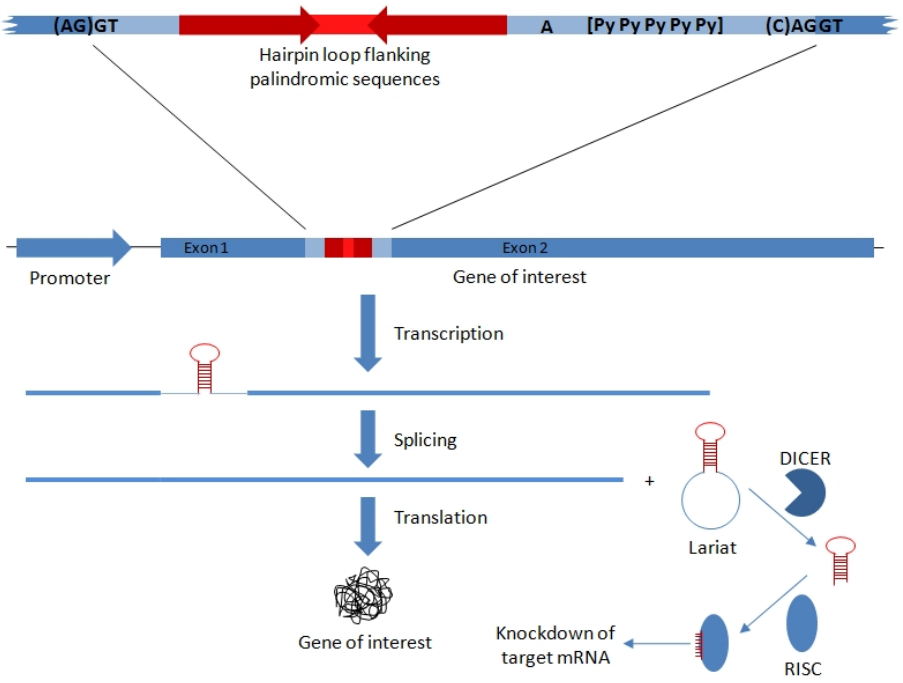


Fig. 3 Pathway of hybrid knock down system

By the introduction of intronic hairpin elements inside the open reading frame of the therapeutic gene, additional shRNA (U6) and siRNA (flanking H1 and U6) promoters are hereby not necessary.

1.4.2 BMP heterodimers (ideal BMP2 to BMP7 ratio)

Several studies showed a dramatic increase in osteogenic induction when BMP2 is used in combination with BMP7 (which also occurs physiologically) and therefore decreasing treshold of effective clinical dosages [130, 143, 167, 168]. The resulting bone induction with BMP2/7 heterodimers is shown to be up to 20 fold higher compared to the application of BMP2 alone [121].

Therefore, the construction of co-expression vectors for simultaneous expression of exogenous BMP2 and BMP7 is of high relevance for gene therapeutical approaches. However, in respect to stoichiometry in co-expression systems, protein secretion kinetics are not considered and could therefore interfere with stoichiometric balance between BMP2 and BMP7, leading to an increased formation of homodimers in the endoplasmic reticulum. Additionally, different osteogenic impacts of BMP2 and BMP7, when co-delivered simultaneously, are of major importance. Mediation of osteogenic differentiation and bone regeneration of BMP2 in the presence of BMP7 (and vice versa) were decribed for future approaches of gene therapy as well as for approaches with recombinant proteins for bone tissue regeneration and engineering. Therefore, this work examined the ratio(s) of BMP2/BMP7, at which the highest osteogenic induction is achieved.

2. Material and Methods

Expression vectors & secretion signals

Mammalian expression vectors used in this study and corresponding designers are summarized in Table 1. Applied secretion signals are listed in Table 2.

Vector	Design	Origin of backbone
pVax1-BMP2	This study	Life Technologies
pVax1-BMP2-Advanced	This study	Life Technologies
pMetLuc2-BMP2-Advanced (BMP2-Norris)	This study	Clontech
pDouble-BMP2-Advanced	This study	Clontech
pVax1-BMP2/7	[169]	Life Technologies
pTeton-BMP2/7	[169]	Clontech

Table 1 Mammalian overexprssion vectors used in this study

Name	Origin	Sequence	Abb
Metridia luciferase	*Metridia* longa	MDIKVVFTLVFSALVQA	Met
Gaussia luciferase	*Gaussia* princeps	MGVKVLFALICIAVAEA	Gau
Bone morphogenic protein 2	Human	MVAGTRCLLALLLPQVLLGGAAG	hB2/hBMP2
Bone morphogenic protein 2	Mouse	MVAGTRCLLVLLLPQVLLG	mB2/BMP2

Bone morphogenic protein 7	Human/mouse	MHVRSLRAAAPHSFVALWAPLFLLRSALA	mB7/BMP7
Bone morphogenic protein 6	Human/mouse	MPGLGRRAQWLCWWWGLLCS	mB6/BMP6
Noggin	Mouse	MERCPSLGVTLYALVVVLGLRAAPAGG	Nog
Chordin	Mouse	MPSLPAPPAPRLLLGLLLLGSRPASG	Cho
Gremlin-1	Mouse	MNRTAYTVGALLLLLGTLLPTAEG	Gre
Azurocidin preprotein	Human	MTRLTVLALLAGLLASSRA	Azu
Serum albumin	Mouse	MKWVTFLLLLFVSGSAFS	Alp
Trypsinogen	Mouse	MNLLLILTFVAAAVA	Try
TGF-beta 1	Mouse	MPPSGLRLLPLLLPLPWLLVLTPGRPAAG	TGF
Osteocalcin	Mouse	MRTLSLLTLLALAALCLSDLTDA	Osc
Osteopontin	Mouse	MRIAVICFCLLGIASA	Osp
VEGF	Human	MNFLLSWVHWSLALLLYLHHAKWSQA	hVEGF
Gaussia luciferase tuned		**MGVKVLFALICIAVAEA**	**Gau**
Gaussia luciferase	*Gaussia* princeps/artificial	MGVKVLLFALICIAVAEA	GAU-L2
		MGVKVLLLLFALICIAVAEA	GAU-L4
		MGVKVLLLLLLFALICIAVAEA	GAU-L6
		MGVKVLLLLLLLLFALICIAVAEA	GAU-L8
		MGVKVLFALLICIAVAEA	GAU-2L
		MGVKVLFALLLLICIAVAEA	GAU-4L
BMP2 tuned		**MVAGTRCLLALLLPQVLLGGAAG**	**hB2/hBMP2**
Bone morphogenic	Human/artificial	MVAGTRCLLALLLLLLPQVLLGGAAG	B2-L6
		MVAGTRCLLALLLLLLLLPQVLLGGAAG	B2-L8

protein 2		MRRMVAGTRCLLALLLPQVLLGGAAG	MRL10
		MRPLLLLLLLLLLGAAG	MRPL10
		MRRMVAGTRCLLALLLPQVLGGAAG	MRRB2

Table 2 Overview of origin and sequence of applied secretion signals and their particular modifictaions

mir20a-target	**Sequence inserted into 3´ untranslated region of luciferase mRNA**
Seed right	TAA**TGCACTTT**AATTGACACATGTAA**TGCACTTT**AAT
Seed reverse	ATT**AAAGTGCA**TTACATGTGTCAATT**AAAGTGCA**TTA
Seed mutated	AA**TGTACTCT**AATTGACACATGTAA**TGTACTCT**AAT

Table 3 MiR-20a sponge seeds. MiR-20a binding sites indicated in bold and underlined

Construction of hybrid vectors

Oligonucleotides coding for particular hairpins were resuspended in annealing buffer (10 mM Tris, pH = 8.0, 50 mM NaCl, 1 mM EDTA) and annealed by step-wise cooling to room temperature. Annealed oligos were ligated into linearized BMP2-Advanced (EcoRI/XhoI).

Target	**Target sequence**	**Reference**
Noggin	CCATCATTTCCGAGTGTAAGTG	[147]
Chordin	CAGGTGCACATTGCCAGTCAG	[148]
Gremlin-1	GCACATCCGAAAGGAGGAA	[170]
Ubc9	CAGGTGGAACTAAGGGACTTT	[162]
mir20a	CTACCTGCACTATAAGCACTTTA	natural

Table 4 Targets used for knock down in hybrid vector system

Vector	Overexpressed gene	Target(s)	Number of shRNAs	Vector backbone
DsRed-Empty	DsRed	-	0	pDsRed-Express-c1 (Clontech)
DsRed-Anti-Noggin		Noggin	3	
DsRed-Anti-Ubc9		Ubc9	3	
DsRed-Triple1		Noggin, Ubc9, TNF-alpha	9	
DsRed-Triple2		Chordin, Gremlin-1, mir20a targets	9	
BMP2-Empty	BMP2	-	0	pVax1 (Life Technologies)
BMP2-Anti-Noggin		Noggin	1	
BMP2-Anti-Chordin		Chordin	1	
BMP2-Pseudo-mir20a		mir20a targets	1	
BMP2-Anti-Noggin*		Noggin (reverse)	1	
BMP2-Anti-Gremlin*		Gremlin-1 (reverse)	1	
BMP2-Anti-Chordin*		Chordin (reverse)	1	
BMP2-mir20a		mir20a targets	1	

Table 5 Hybrid vector conctructs

Construction of secretion signal reporter vectors

Metridia luciferase derived from pMetLuc2-Control (Clontech, USA) was amplified using Phusion polymerase (Biozym, Germany) with overhang primers featuring HindIII and XhoI.

Purified PCR amplicon was digested with above mentioned restriction enzymes and subsequently cloned into the mammalian expression vector pVax1 (Life Technologies, USA). Complementary oligonucleotides coding for different secretion signals were mixed in annealing buffer (10 mM Tris, pH = 8.0, 50 mM NaCl, 1 mM EDTA) and cooled down stepwise in order to mediate annealing reaction, leading to desired overhangs appropriate for the restriction enzymes NheI and HindIII, respectively. Linearized pVax1-MetLuc (NheI/HindIII) lacking secretion signal was ligated with the different pre-annealed oligonucleotides and transformed into E. *coli* for subsequent amplification and plasmid isolation.

Cell culture and transfection

Mouse muscle myoblast precursor cell line C2C12 (#ACC565) was cultured in DMEM high glucose (4.5 g/L glucose), supplemented with 2 mM L-glutamine (Sigma Aldrich) and 5 % fetal calf serum (Lonza).

Cells were seeded into 24-well plates at a cell density of $0.5x10^5$/well 24-h prior transfection. The next day, the cells were transfected with 1 µg of indicated plasmid. All transfections were carried out using Peqfect (Peqlab). The medium was changed 3 h post transfection.

Recombinant human bone morphogenetic protein 2 (rhBMP2) was purchased from Wyeth (InductOS).

ALP assay

ALP assays were applid to quantify the grade of osteogenic differentiation. The cells were lysed with 100 µL lysis buffer (ALP buffer containing 0.5 % Triton X-100) per well and incubated for 1 h at room temperature. Enzymatic activity was quantified in cleared supernatants after centrifugation by adding 20mM p-nitrophenylphosphate in 50 µL ALP buffer as substrate. The reaction was stopped after 20 min after with 50 µL of 0.2 M NaOH. The conversion of p-nitrophenylphosphate to p-nitrophenol by alkaline phosphatase was quantified by its absorbance at 405 nm. Depicted ALP activities are refered to nmol of p-nitrophenol liberated per minute.

Real time quantification

C2C12 cells were harvested and total RNA was extracted using Trifast reagent (Peqlab) according to the manufacturer's instructions. RNA concentrations were determined using a Hitachi U-5100 photometer and the complementary DNA (cDNA) was synthesized from the total RNA (2 µg) using a DyNAmo RT Reagent Kit (Biozym, Germany) in accordance with the manufacturer's instructions. Subsequently, the cDNA was added to real-time PCR mix using a KAPA SYBR Fast Mix (Peqlab). Each real-time PCR reaction consisted of 4 µl of diluted RT product (40ng), 10 µl KAPA SYBR Fast Mix and specific primer pairs (250 nM) in a total volume of 20 µl. The reactions were performed on a C1000/CFX96 (Biorad) cycler using a 3 step protocol (95°C/3min, [95°C/10s, TA_{primer}/30s/read plate, 72°C/10s/read plate)x40, 95°C/10s, TA_{primer}/30s/read plate, Melt curve from Primer Annealing Temperature to 95°C, increment 0.5°C, 0:05 + Read plate/END].

The fold change in the mRNA levels of each gene was calculated using Samples (2^-(dCt)) divided by mean (2^-(dCt)) of control group.The housekeeping gene GAPDH served for normalization. All PCR primers were synthesized by Microsynth (Switzerland). The primer sequences and the amplified lengths used in the study are listed in the table beneath.

Primername	**Sequence s/as**	**T_A (°C)**	**T_M (°C)**
Osteopontin	AAAGCAATTCCAATGAAAGCC	53	81.5
	GGGACGATTGGAGTGAAAGT		
Runx2	CAAGTAGCCAGGTTCAACGA	57.5	83
	CTGAGGCGATCAGAGAACAA		

Table 6 qPCR target primer for hybrid vector verification

Codon optimization

The coding regions were optimized using the GeneOptimizer® expert software, based on a deterministic sliding window algorithm [171].

Luciferase assay

50 µl of supernatant of transfected cells were removed for subsequent quantification using 40 µl coelenterazine substrate (NanoLight Technology, USA). Luciferase quantification time points are indicated in the particular description.

EXPERIMENTAL PROCEDURES

Endosomolytic peptides

Peptides (Table 1) were synthesized at Genscript.

Cloning of Listeriolysin O into bacterial expression vector (pET11a-LLO-His)

The *hlyA* gene from the *Listeria monocytogenes* strain DP-L2723 encoding LLO lacking its secretion signal sequence was amplified from the plasmid pDP3615 (a kind gift of Daniel Portnoy, University of California, Berkeley [74]) using the sense primer LLO-s (5´-GAATTCCATATGAAGGATGCATCTGCATTCAAT-3´) generating an NdeI restriction site at the 5´ end, and the antisense primer LLO-His-as (5´-GGATCCTTAATGATGATGATGATGTTCGATTGGATTATCTACT-3´), generating a BamHI restriction site with a caboxy-terminal 6x-poly-histidinde tag at the 3´end. The *hlyA* gene was directly ligated into the subcloning vector pCR2.1 (Invitrogen) following transfer into the bacterial expression vector pET11a (Novagen) (NdeI, BamHI) for subsequent protein expression after transformation into BL21(DE3).

Expression and purification of LLO

E. *coli* (BL21(DE3)) housing the LLO expression vector pET11a-LLO-His were inoculated in 50ml LB medium with 100 µg/ml ampicillin and bacterial growth was stopped at a density of A_{600} = 0.6. Protein expression was initiated by 0.3 mM Isopropyl β-D-1-thiogalactopyranoside (IPTG). The following expression was performed either at room temperature or 37 °C, respectively, and aliquots were collected for subsequent PAGE gel

analysis at defined time points (1h – 16h). After protein expression, the bacterial suspension was harvested by centrifugation at 4000rpm at 4 °C for 30 min.

The lysis of harvested E. *coli* was carried out using 5ml of Lysis buffer (1mg/ml Lysozyme, 1 tablet complete EDTA-free protease Inhibitor (Roche diagnostics), 50 µg Dnase I in 1X PBS). After incubation at room temperature for 30min under permanent shaking, the cell suspension was sonicated three times for 15 seconds on ice. Inclusion bodies were spun down for 20min at 4000 rpm (4 °C) and washed two times with 20 ml of 100 mM Tris (pH = 8) with 2 % Triton X-100 following sonication and centrifugation at the same conditions as above. Furthermore, three additional washing steps were performed with 20 ml of 100 mM Tris (pH = 8) alone.

For solubilizing the proteins in the inclusion bodies, the pellet was resuspended in 5ml Solubilization buffer (6 M Urea, 100 mM Tris (pH = 8), 500 mM NaCl) following incubation over night at 4 °C for the subsequent purification directly by affinity chromatography at 4 °C. Refolding prior elution was performed with ice-cold washing buffer at either pH = 5 or pH = 7, respectively.

Purification of recombinant LLO by affinity gel chromatography

The solubilized recombinant LLO was directly loaded onto a Ni-Sepharose affinity column (GE Healthcare, Amsterdam, the Netherlands) at 4 °C (to prevent deactivation) and washed with three column volumes of washing buffer (20mM phosphate buffer, 500 mM NaCl, 20 nM Imidazole). LLO was eluted with 1 column volume of elution buffer (20 mM phosphate buffer, 500 mM NaCl, 500 nM Imidazole). Eluted protein was immediately quantified and diluted with different volumes of acidic buffer for pH adjustment (pH = 5). Protein concentration was measured using the Bradford method.

SDS-Page and Western blot

SDS-Page was performed in 10 % polyacrylamide gels (Bio-Rad). Proteins were stained with the non-specific protein dye Coomassie Brilliant Blue G-250.

Western blot was performed by blocking the nitrocellulose membrane with 5 % low-fat milk for 2 hours at 4 °C. Incubation of 1:2000 diluted monoclonal anti-his-antibody conjugated with horseradish peroxidase (Abcam) in 1X PBST was carried out in the

presence of 0.25 % low-fat milk for 1 hour in the dark at room temperature following washing three times for 5 minutes with PBST.

Detection was performed using 1.25mM Luminol with 2 mM 4-iodophenylboronic acid (4IPBA) and 5.3 mM H_2O_2 stock in 100 mM Tris-HCl (pH = 8.8).

Hemolytic assay

To quantify the membrane disrupting activity of LLO and the synthetic peptides, washed red blood cells (RBCs) were applied as ligands for the purified hemolytic proteins. Human blood was isolated and RBCs were washed three times with 1x PBS to remove serum components.

The pellet containing red blood cells was washed with 20ml 1x PBS following centrifugation at 1000 rpm for 20 min at 4 °C. The washing procedure was repeated three times. For positive control, 100% lysis was performed with ddH_2O. Diluted RBCs were incubated at the indicated temperature, pH and time conditions.

After incubation with the respective peptide or protein, the RBCs were centrifuged at maximum speed (13000 rpm) for 5 minutes. The amount of lysed RBCs was photometrically detected by the measurement of released hemoglobin at a wavelength of 541 nm. Additionally, to give a relative benchmark for the activity of hemolytic proteins. As negative control, RBCs were diluted in 1x PBS alone (without hemolytic protein or peptide).

Regain of hemolytic activity after exposure to pH = 7 at 37°C

Prior addition to RBCs, 10µM of purified LLO or endosomolytic peptide was incubated at pH = 7 at 37°C for 30min. Subsequent incubation of peptides with RBCs (either in absence or presence of 2 mM DTT, respectively), following measurement of hemoglobin release, gave insight about the reversibility of the pH dependent hemolytic activity of the tested protein or peptide.

Concentration dependent stability of LLO

The stability of purified LLO at different concentrations (2.5-10 µM) was elucidated by dilution in increasing volumes of acidic buffer after purification with affinity chromatography and subsequent quantification.

Cell viability

Cytotoxicity of the transfection reagents was evaluated by (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium), MTS assay (Promega, Madison, USA).

C2C12 cells (0.5×10^6/ml) in 1ml of DMEM high glucose supplemented with 5% FBS were seeded in 24-well plates and incubated for 24 hours with particular endosomolytic peptides at a final concentration of 10µM. Negative control was performed with incubation with 0.9% Triton X-100. After 24 hours, cells were washed and 5 mg/ml MTT reagent in 1X PBS (50 µl/well) and 100µl fresh medium was added into the plate. After one hour of incubation at 37 °C, the absorbance at 490 nm was determined with a plate reader (POLARstar Omega, BMG LABTECH, Austria) (Data Analysis Software: MARS 2.30 R2).

Statistical analysis

Statistical analysis was performed by One-way ANOVA (Tukey post test) after confirmation of data following a Gaussian distribution using Graphpad. $P < 0.05$ was considered as statistically significant. Results are represented as average±standard deviation.

3. Results

3.1 Enhancement of gene delivery

Name	Sequence	Theoretical pI	MW	Origin/adapted from	Membrane interaction	Ref
INF7	GLFEAIEGFIENGWEGMIDGWYG	3.45	2590	Influenza (HA)	Destabilization, fusion	[56]
E5	GLFEAIAEFIEGGWEGLIEG	3.51	2137	Influenza (HA)	Destabilization, fusion	[60, 172]
K5	GLFKAIAKFIKGGWKGLIKG	10.6	2132	Influenza (HA)	Destabilization, fusion	[172]
GALA	WEAALAEALAEALAEHLAEALAEALEALAA	3.83	3032	Artificial	Destabilization, pore formation, fusion	[47, 173]
H5WYG	GLFHAIAHFIHGGWHGLIHGWYG	7.16	2583	Influenza (HA)	Destabilization	[62]
Listeriolysin O (LLO)		6.71	59,000	L. *monocytogenes*	Pore formation	P13128

Table 1: Overview of used endosomolytic peptides

Expression and purification of LLO

A screening with different expression conditions was performed (Fig. 4A, B). Alternating IPTG concentration and induction times showed optimal expression conditions with 0.3 mM IPTG and incubation times of 3-4 hours.

The expressed and purified LLO was verified by Western blot using antibodies recognizing the C-terminally attached His-tag to LLO (Fig. 4C, D).

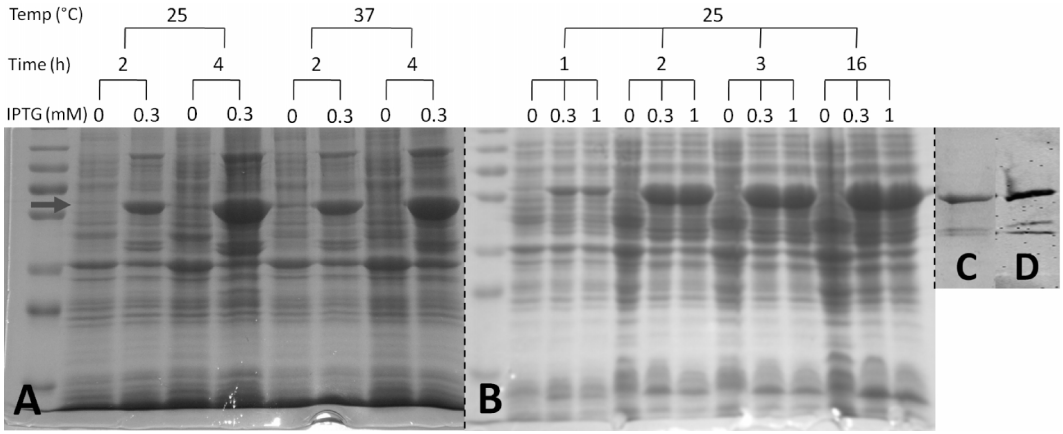


Fig. 4 Listeriolysin O expression screen with altered IPTG concentration and induction times (A, B), respectively. After indicated conditions, *E. coli* BL21(DE3) whole cell proteom was directly loaded onto the PAGE gel. The arrow indicates height of LLO on gel. IPTG = final concentration of applied IPTG (mM). PAGE gel (C) and Western blot (D) after Ni-NTA purification of Listeriolysin O.

Note: Gel was done during diploma thesis of author, but showed here for the sake of completeness of the described project

pH dependent hemolytic activity

Purified LLO showed maximum capability to lyse red blood cells at pH = 5.5 (Fig. 5). The hemolytic activity was dramatically decreased upon increase of the pH level above 6. Furthermore, in comparison to endosomolytic peptides, LLO showed a remarkable higher activity. LLO was able to lyse more than 95 % of red blood cells within 10 minutes, whereas the different peptides succeed to lyse up to 16 % in 45 min. Furthermore, relating to the tested endosomolytic peptides, all candidates except K5 showed pH dependence membrane disrupting potential. However, K5 showed only decreased bioactivity at a pH level of 7.

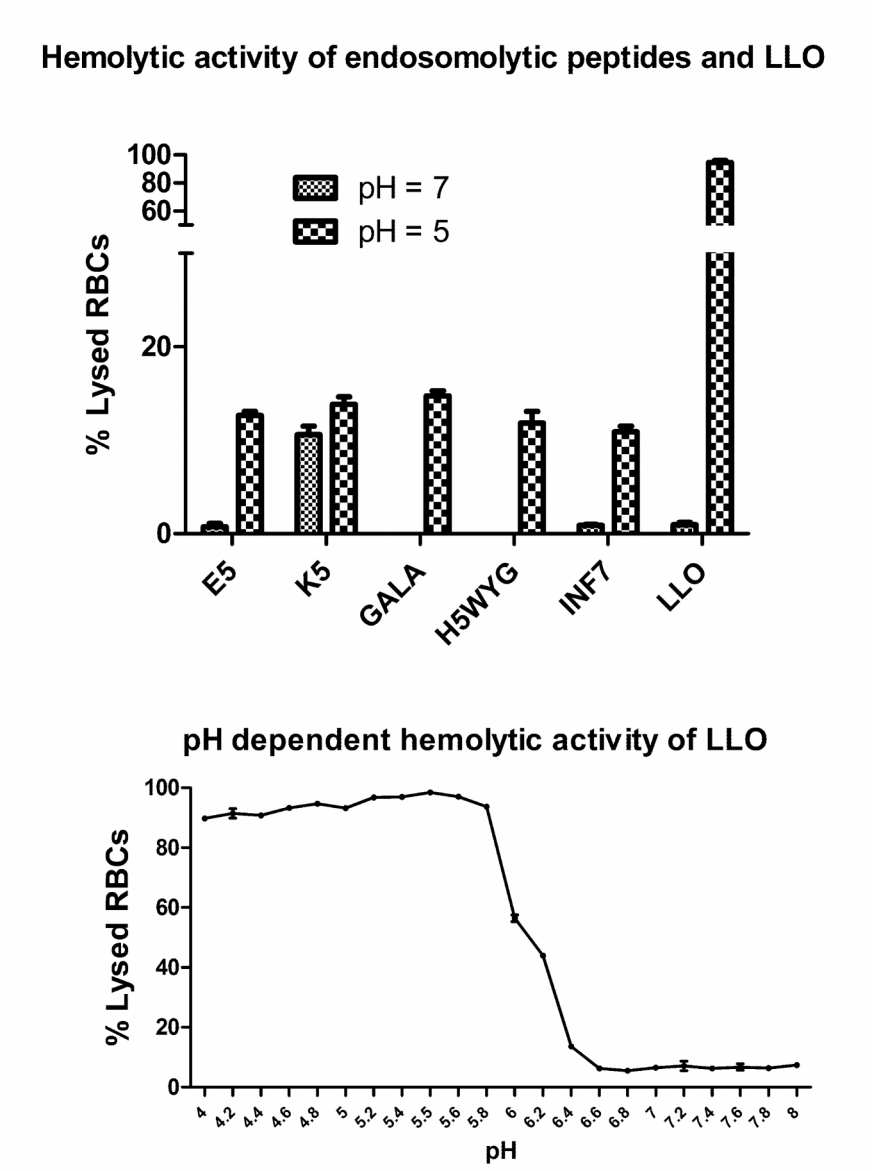


Fig. 5 pH dependent hemolytic activity of hemolytic candidates. Upper panel: Different endosomolytic peptides or Listeriolysin O (LLO) protein (final concentration of 10 µM) were incubated in pH = 5 and pH = 7, respectively, for 45 min at 37 °C. Lower panel: Purified LLO (final concentration of 5 µM) was incubated in increasing pH conditions for 10min at 37 °C. Results are presented as mean ± SD, n = 3.

Regain of hemolytic activity

For stability tests, the candidates were first exposed to physiological conditions (pH = 7, 37 °C). After incubation for 30 min, LLO and peptides were further incubated at pH = 5 in

either presence or absence of 2 mM DTT, respectively. All endosomolytic peptides showed regained biological activity at pH = 5. However, LLO was not capable to convert its active conformation back after the subsequent decrease of pH (Fig. 6). Even after the decrease of the exposition time at pH = 7 to 5 minutes, LLO was irreversibly inactivated and not longer capable to lyse red blood cells at pH = 5 (data not shown).

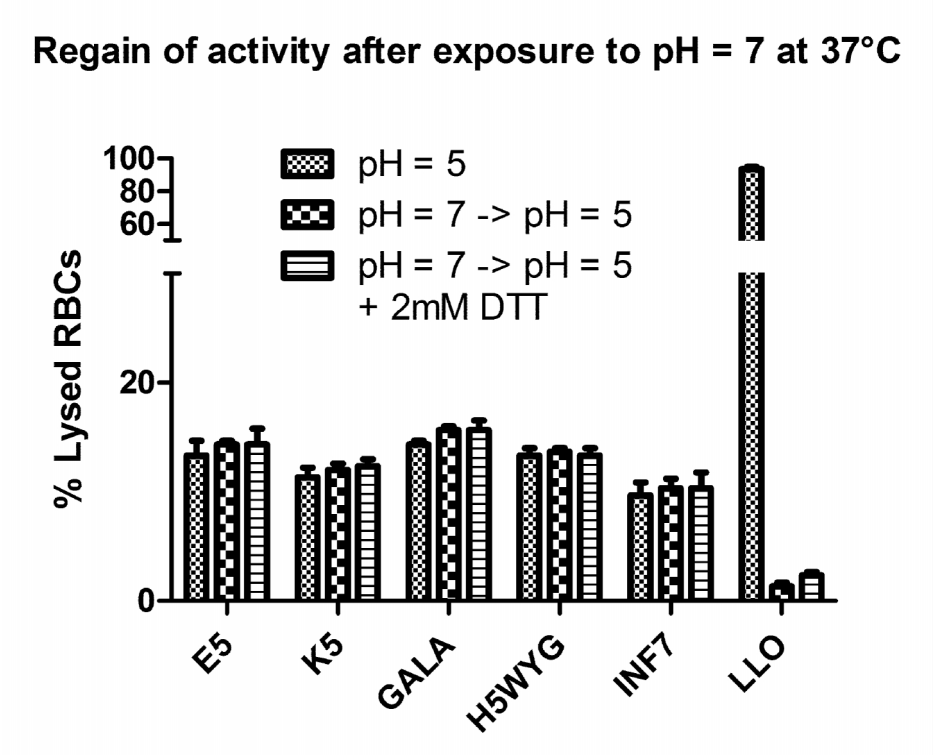


Fig. 6 Test for reversibility of hemolytic activity. Candidates were pre-incubated at pH = 7 at 37 °C for 30 min (final concentration of 10 µM). Hemolytic activity after subsequent decrease of pH and addition of red blood cells, in the presence and absence of 2 mM DTT, was observed. Expect Listeriolysin O, all tested candidates showed recovered hemolytic activity by the decrease of pH. Results are presented as mean ± SD, n = 3.

LLO stability at different protein concentrations

LLO was incubated at 4 °C at pH = 5 and aliquots were taken at different time points for subsequent confirmation of hemolytic activity. Hemolytic assays with same amounts of LLO but altered concentrations (2.5-10 µM) showed increased inactivation by self-aggregation with increasing concentrations (Fig. 7).

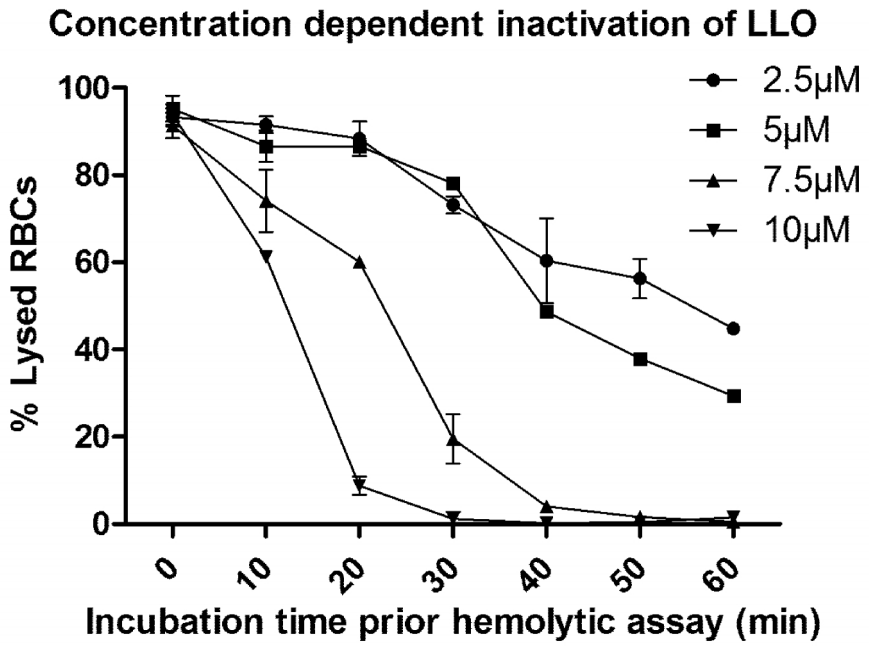


Fig. 7 Concentration dependent aggregation and deactivation of Listeriolysin O. LLO was incubated at pH = 5 and 4 °C at different concentrations (10-2.5 µM). Subsequently, RBC lysis assay was performed after indicated time points for 5min at 37 °C. Results are presented as mean ± SD, n = 3.

Note: Experiment was done during diploma thesis of author, but showed here for the sake of completeness of the described project

Long-term storage

This experiment was designed to determine whether LLO can be frozen in liquid nitrogen, stored at -80 °C, and thawed again without any forfeiture of the protein activity. Aliquots of 500 µM concentration were transferred immediately after the refolding and elution process to liquid nitrogen and stored at -80 °C without addition of any cryoprotectants. The protein samples were thawed on ice at different time points and tested for their hemolytic activity (Fig. 8). Freezing and thawing processes showed no remarkable loss in the protein activity in the time period of one year (and therefore can be readily stored over long periods at -80 °C).

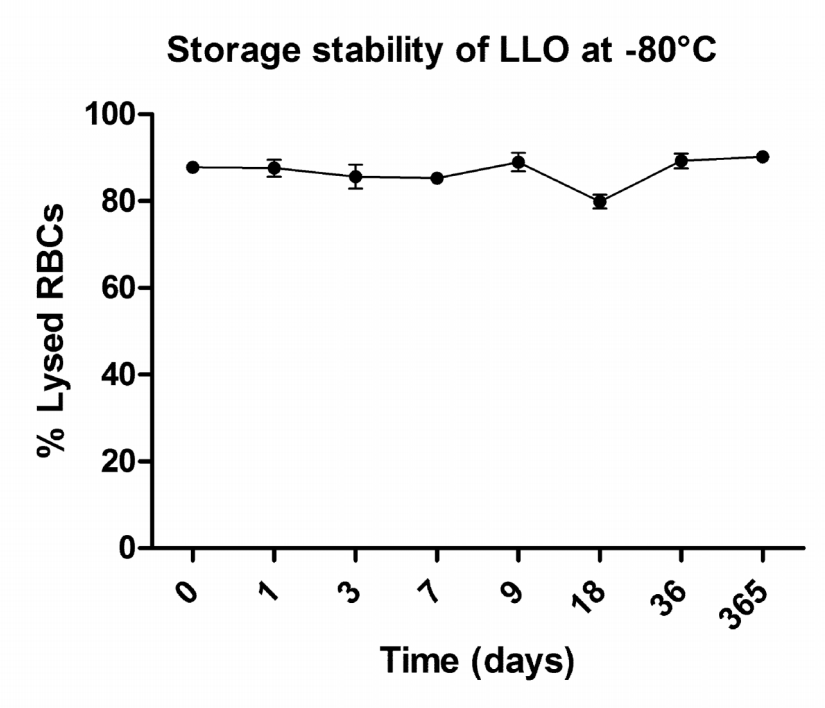


Fig. 8 Storage stability of Listeriolysin O at -80 °C. LLO (500 µM) was frozen in liquid nitrogen and stored at -80 °C were thawed at different time points for the subsequent measurement of maintained hemolytic activity at a final concentration of 5 µM. Results are presented as mean ± SD, n = 3.

Cell viability

Peptide concentration of 1 µM (data not shown) and 10 µM (Fig. 9) were incubated with C2C12 for 24 hours. All tested peptides showed no significant cytotoxicity when exposed to C2C12 cells for 24 hours, as determined by MTS assay.

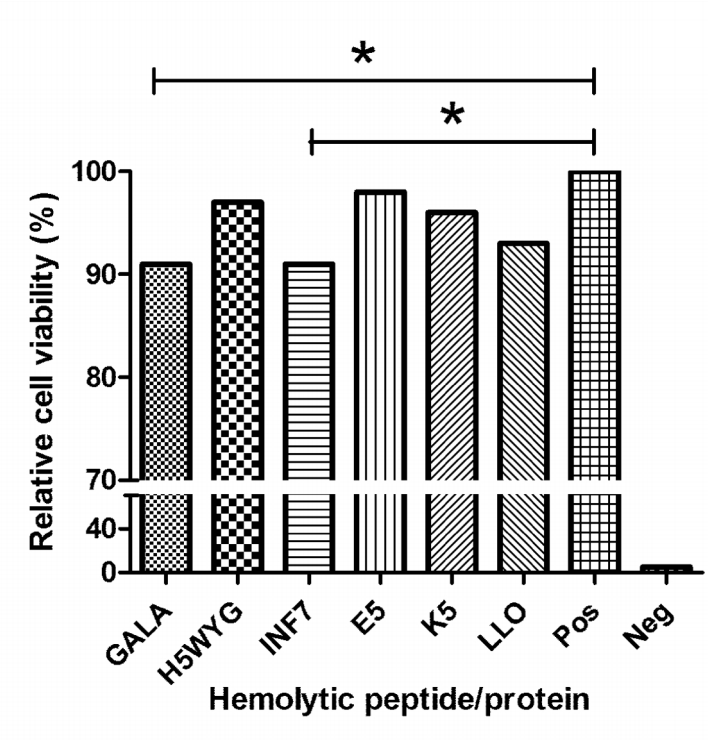


Fig. 9 Cell viabilities of C2C12 treated with particular endosomolytic peptides for 24 hours in fresh culture medium. Relative cell viabilities were expressed in percentage based on the control without peptide treatment. Results are presented as mean ± SD, n = 6.

3.2. Enhancement of gene expression

3.2.1 Optimization of gene sequence

BMP2 gene sequence was modified by codon optimization using the GeneOptimizer® expert software and additionally by the insertion of an artificial intron. Subsequent quantification of bone differentiation by ALP assay showed over five fold increase when C2C12 myoblast cell line was transfected with advanced BMP2 casette (BMP2-Advanced) compared to conventional BMP2 gene after four days (Fig. 10).

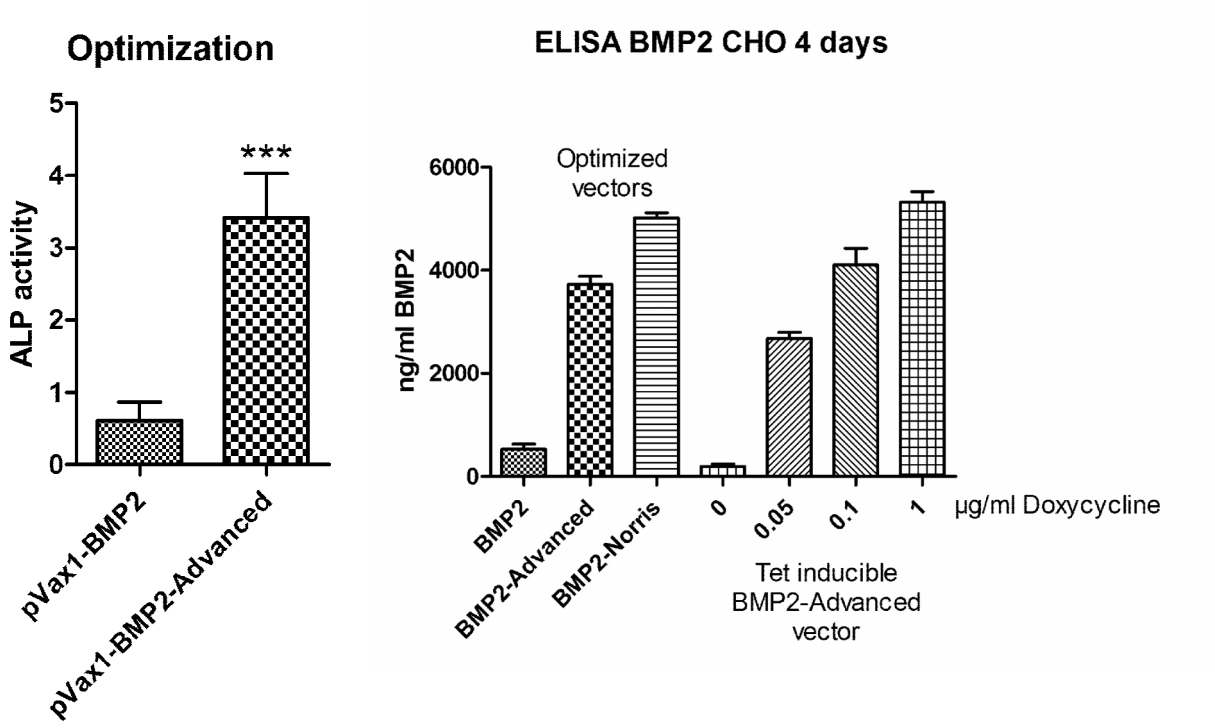


Fig. 10 *Left panel*: C2C12 were transfected with either pVax1-BMP2, pVax1-BMP2-Advanced (1 µg/ml), respectively. Quantification of induced Alkaline phosphatase activity 4 days post transfection showed ober five fold increase in potency to mediate osteogenic differentiation. *Right panel*: BMP2 secretion in CHO cells. Cells were either transfected with 1µg BMP2 plasmid, or as co-transfection in case of tetracycline inducible system (per well: 0,7µg inducible BMP2-Advanced vector with 0,3µg transactivator plasmid (pTeton)).

3.2.2 General alteration in promoter system (cascade promoter system)

For proof of concept, the basic structure of the commercially available Tet-response system (minimal CMV promoter) was used.

Tetracycline-controlled transcriptional activation is a method of inducible gene expression where transcription is reversibly turned on in the presence of the tetracycline or its more stable analogue doxycycline. Therefore, a cascade promoter is triggered. The transcription factor, in this case the tetracycline transactivator (tTA) protein fused to the strong viral activation domain VP16, is overexpressed under the influence of the constitutive minimal viral CMV promoter. This transcription factor on the other hand binds to the response element upstream of the gene of interest and mediates the strong

expression of the therapeutic gene. Therefore, lack of transcription factor due to a surplus of therapeutic vector is supposed to be avoided.

The showed results demonstrate the increase in growth factor expression by the addition of transcription factor encoded on a separate plasmid (pTeton-Advanced) in comparison to conventional bidirectional/bicistronic expression vectors (Fig. 11).

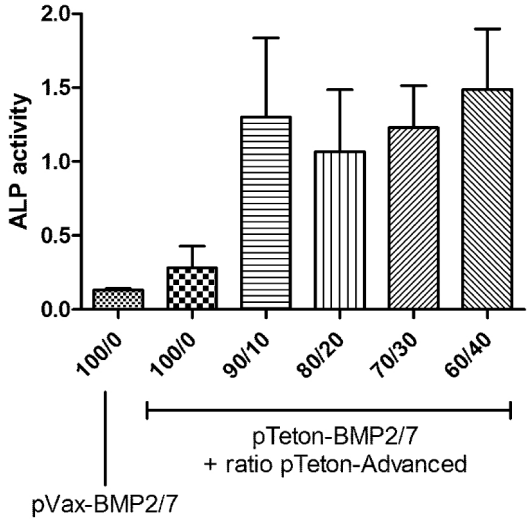


Fig. 11 Cascade promoter system to overcome a potential bottleneck of transcription factor

Cascade system showed increased potenty when compared to CMV promoter alone. By the stepwise addition of transcription factor (Tet repressor), the osteogenic signal was even more increased. This relational increase indicates the influence of the amount of transcription factor on transcription rates.

3.3. Protein secretion kinetics

For the exchange of secretion signal of BMP2, eleven different secretion signals were tested concerning protein secretion potency, among them inhibitory factors as well as activators of osteogenic differentiation (Fig. 12). Secretion sequence of BMP2, as well as the secretion sequences of all inhibitory factors (Noggin, Chordin, Gremlin-1), showed

highest potential regarding the mediation of protein secretion in C2C12 cells. Previously described effectivity of Gaussia luciferase secretion signal showed only moderate secretion potential. Additional modifications of the hydrophobic domain of Gaussia luciferase signal was necessary to increase the secretion potential to levels comparable to BMP2 secretion signal strength. Attempts to upgrade the BMP2 secretion signal by either the insertion of additional hydrophobic amino acids, or the addition of positively charged N-terminal amino acids, unfortunately led to the decreased protein secretion capacity.

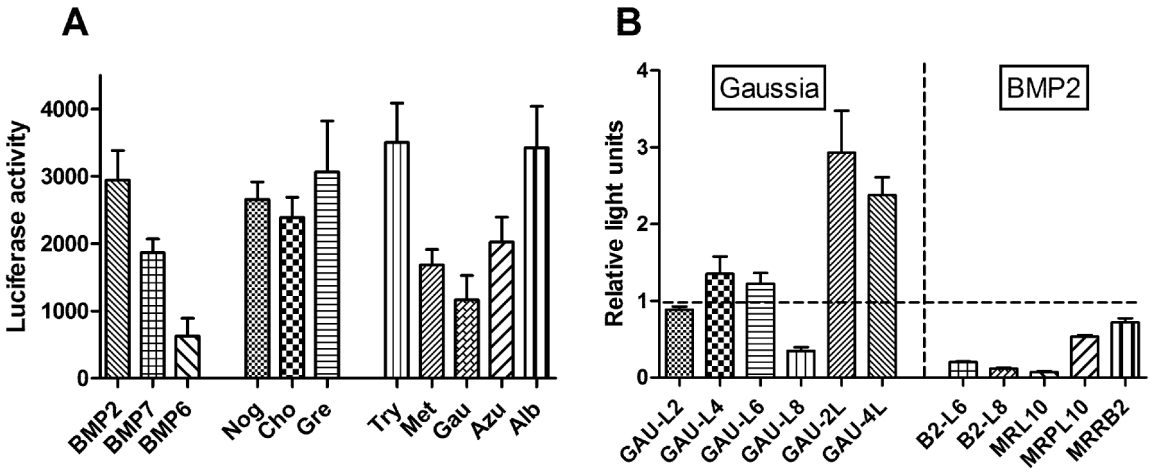


Fig. 12 Testing of native and modified secretion signals

A: 11 secretion signal were tested for their protein secretion potency. The secretion signal of Metridia luciferase was replaced with the particular selected candidates. C2C12 cells were transfected (in presence and absence of 300ng rhBMP2) with the particular vector constructs and secreted luciferase was measured after 3 days. The data is representative of two experiments performed as triplicates.

B: The secretion signal of Gaussia luciferase and BMP2 was modified resulting in 10 different candidates. C2C12 cells were transfected (in presence and absence of 300ng rhBMP2) with the particular vector constructs and secreted luciferase was measured after 3 days. The data is representative of two experiments performed as triplicates.

Impact of presence of secretion signals to secretion of other proteins and osteogenic differentiation

For future application of bicistronic vectors encoded for two secreted growth factors, the secretion behaviour and kinetics of osteogenic cofactors was examined.

Transfection of two plasmids simultaneously revealed secretion or expression priorities and different secretion kinetics when two signals were in competition.

BMP2 gene was co-delivered with same stoichiometric amounts of “competitor”, in this case Metridia luciferase gene fused to either BMP2, Gremlin-1 or BMP6 signal sequence (Fig. 13). Additionally, a plasmid encoded for Metridia luciferase lacking a secretion signal (0-Luc) was applied as fill-in to avoid differences of transfection efficiencies and transcription by keeping overall DNA amount at same level (2 µg). Subsequent potential of BMP2 gene for osteogenic induction of target cells was examined. The results indicate different impact of the various secretion signals (BMP2, Gremlin-1, BMP6) on osteogenic differentiation.

Osteogenic differentiation in the presence of Metridia luciferase fused to Gremlin-1 secretion signal was considerably blocked more efficiently compared to the differentiation in presence of BMP2 secretion signal. Concomitant, luciferase quantification after 3 days showed same levels of Metridia luciferase secretion. The data showed a decreased osteogenic differentiation of target cells in the presence of Gremlin-1 secretion signal compared to BMP2 secretion signal, indicating that Gremlin-1 signal has a negative influence on either osteogenic differentiation or BMP2 release, respectively. BMP6 secretion signal showed weakest impact regarding the inhibition of osteogenic differentiation, but also bit weaker luciferase secretion.

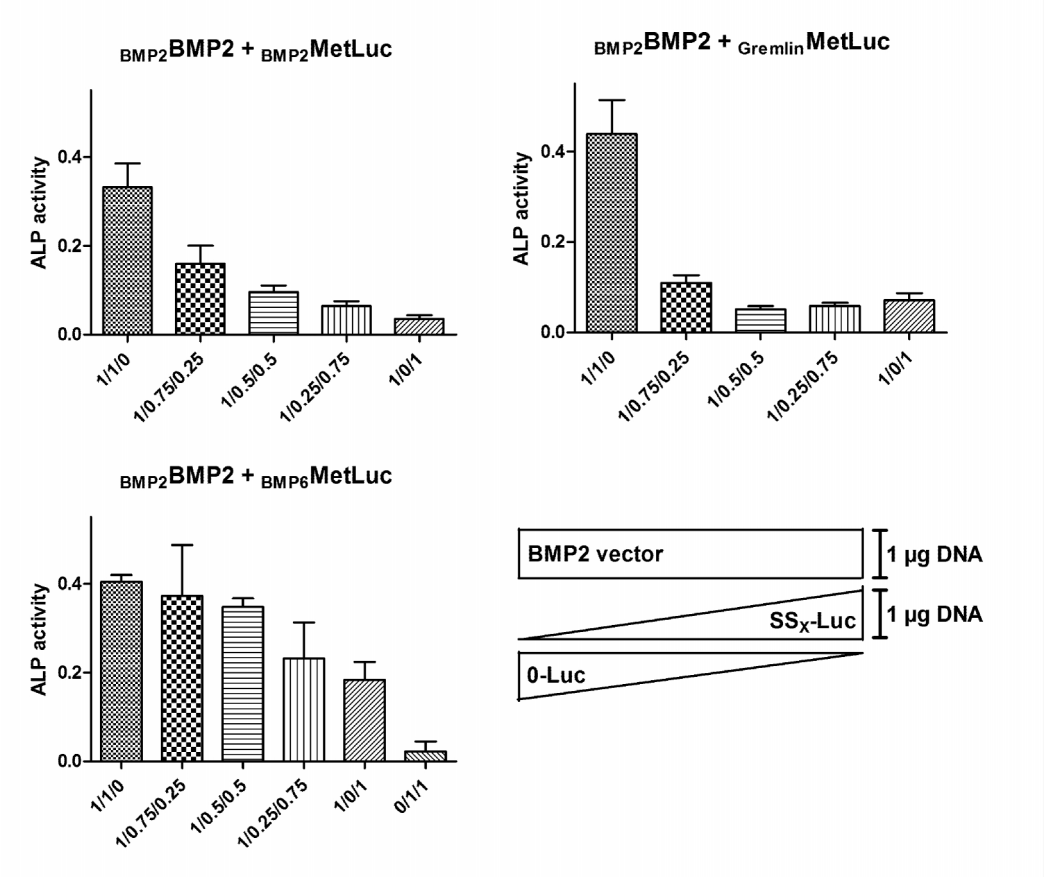


Fig. 13 Competition triple transfections (Luciferase data showed no significant changes in luciferase levels in the supernatant (data not shown)) of BMP2 and Gremlin-1 secretion signals). ALP was quantified 4 days post transfection. Gremlin signal sequence showed stronger negative influence on BMP2 secretion (or osteogenic differentiation) compared to BMP2 or BMP6 signal sequence.

Further screening of inhibition potential of secretion signals to osteogenic differentiation indicated potent luciferase secretion mediated by the secretion signals of Noggin and Chordin, without high negative influence in osteogenic differentiation (Fig. 14 and Fig. 15). Additionally, BMP6 and BMP7 secretion signals showed also low negative influence on osteogenic differentiation, however, their mediation of luciferase secretion showed high volatility, ranging from low to average luciferase secretion.

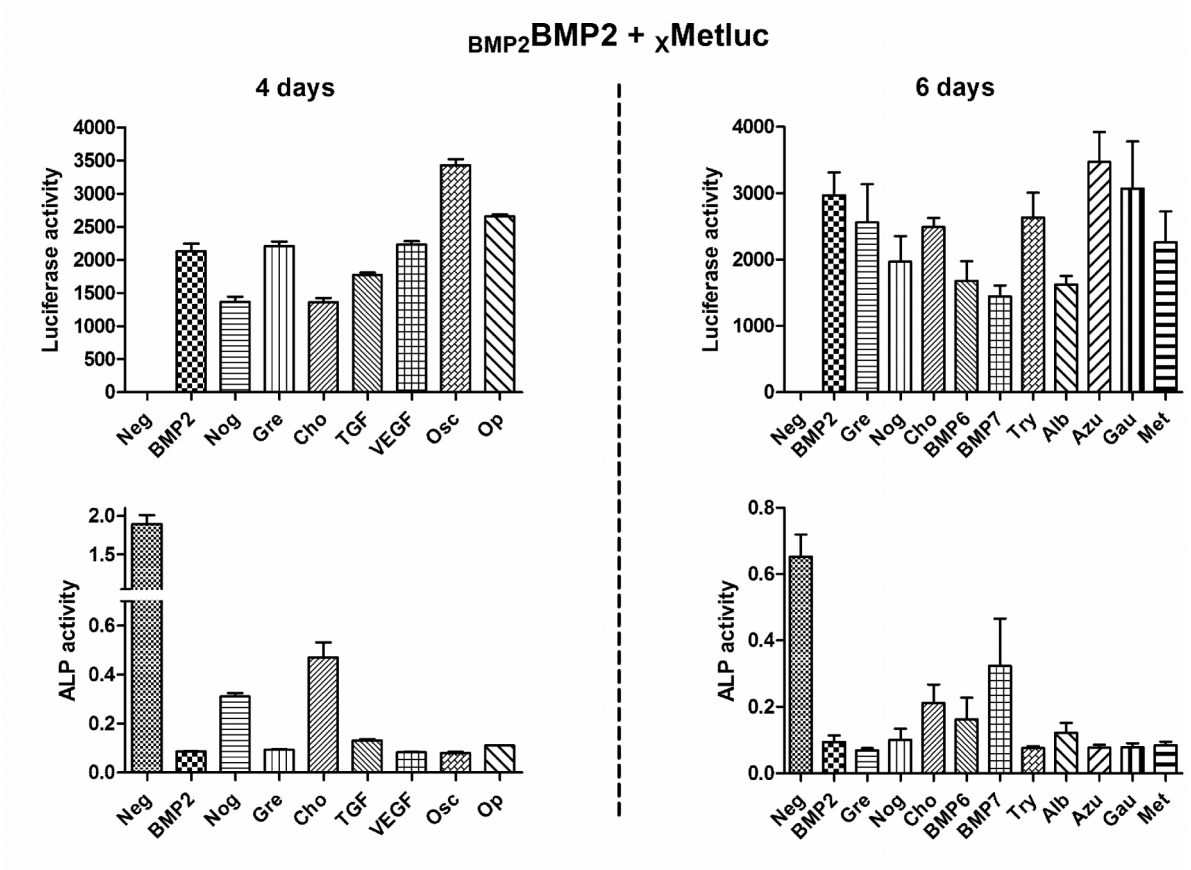


Fig. 14 Influence of presence of competitonal secretion signal on BMP2 release/osteogenic differentiation.

Cotransfection of 0.5 µg BMP2 gene (with natural BMP2 secretion signal) and 0.5 µg competitonal secretion signal (BMP2, Gremlin-1, Noggin, Chordin, BMP6, BMP7, Trypsinogen, Albumin, Azurocidin, Gaussia, Metridia) into C2C12 cells. Quantification of ALP assay either 4 days (n = 5) or 6 days (n = 6) post transfection, respectively.

Neg = Metridia luciferase lacking secretion signal.

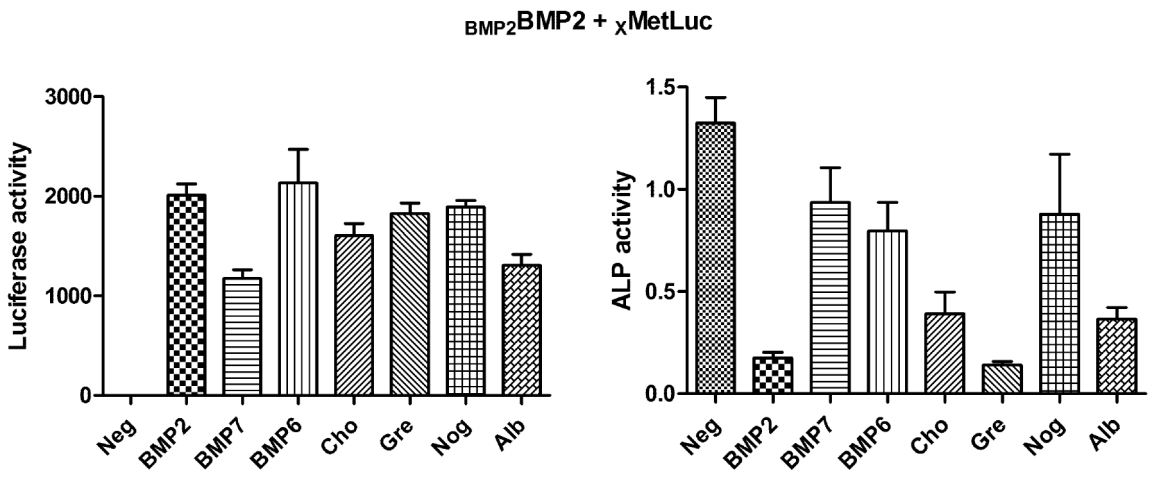


Fig. 15 Influence of presence of competitonal secretion signal derived from factor of osteogenic pathway on BMP2 release/osteogenic differentiation.

Cotransfection of 0.5 µg BMP2 gene (with natural BMP2 secretion signal) and 0.5 µg competitonal secretion signal (BMP2, Gremlin-1, Noggin, Chordin, BMP6, BMP7, Albumin) into C2C12 cells. Quantification of ALP assay 5 days post transfection.

Neg = Metridia luciferase lacking secretion signal.

Examination of behaviour of multiple secretion signals when coexpressed simultaneously

To examine possible mutual influences among protein secretion signals (e. g. mutual disturbance or amplication), double and triple plasmid transfections were performed in presence of 300ng recombinant human BMP2 (Fig. 16). Subsequent cell differentiation was quantified using enzymatic ALP assay. Main focus was the examination of possible outcancelling or compensation effects among secretion signals which was monitored based on the inhibition of osteogenic differentiation of target cells.

Again, the presence of secretion signals of inhibitors of the osteogenic pathway (Noggin, Gremlin-1, Chordin), either individually or in combination with one another, showed lowest inhibition of osteogenic differentiation (luciferase signals were at comparable levels).

Furthermore, except in combination with Gremlin-1 and Gaussia luciferase secretion signals (2GGau), the presence of BMP2 secretion signal disturbed osteogenic differentiation when triggered by 300ng BMP2 protein.

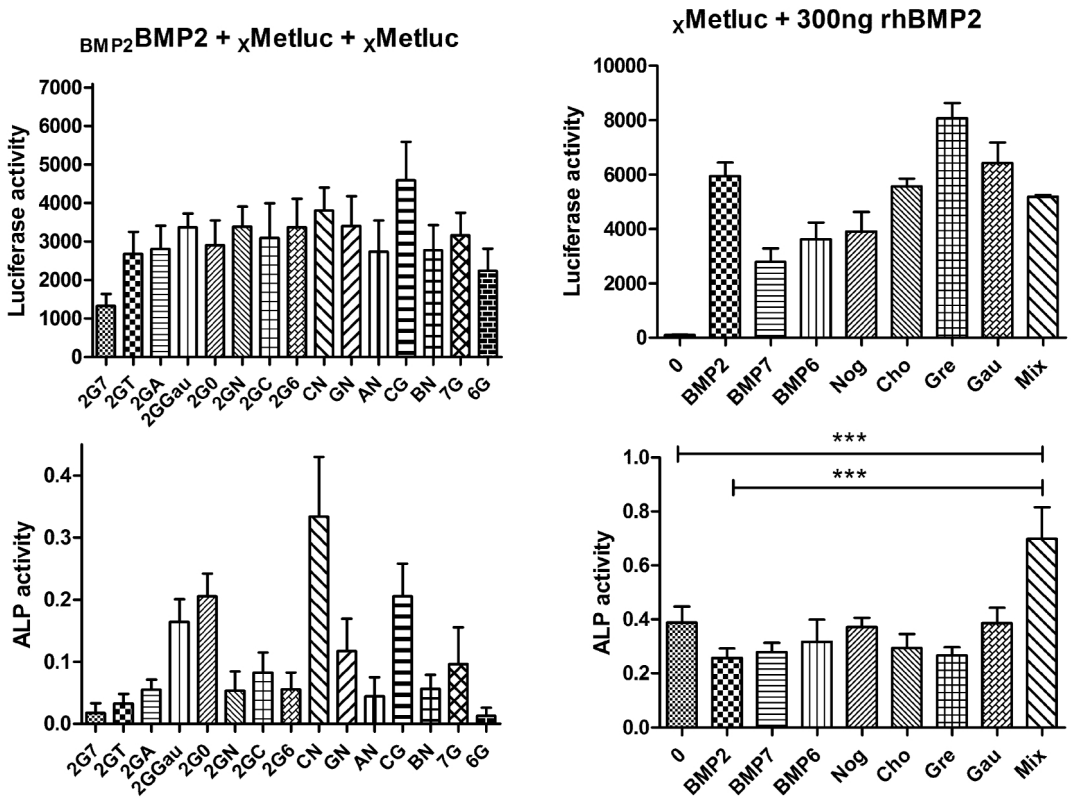


Fig. 16 Influence of presence of either multiple (left panel) or a single (right panel) secretion signal on BMP2 release/osteogenic differentiation induced by recombinant BMP2 protein. Screening of possible potentiating or out canceling effects of secretion signals when expressed simultaneously.

Competitonal secretion signals (overall amount always 1 µg) were transfected into C2C12 cells in the presence of 300ng recombinant BMP2. Quantification of ALP assay was performed 4 days post transfection (n = 6).

0 = Metridia luciferase lacking secretion signal.

Hybrid transfection - better secretion by broad-band effect?

The experiment was based on the hypothesis that several different secretion signals could possibly potentiate protein secretion by occupying several finely different secretion pathways and therefore overcome bottleneck effects during protein secretion.

Deeper examination of luciferase secretion showed evidence of possible broad-band effect by the combination of two types of plasmids, each encoding for same gene of interest but fused to different secretion signals (in this case BMP2 secretion signal combined with Gaussia luciferase secretion signal). However in return, repeating experiments with different signals (Chordin, Gremlin, Noggin secretion signals) revealed no significant evidence for an increased protein secretion (Fig. 17).

Interestingly, by the application of multiple secretion signals simultaneously, a better osteogenic differentiation of target cells was observed (Fig. 16, “Mix”).

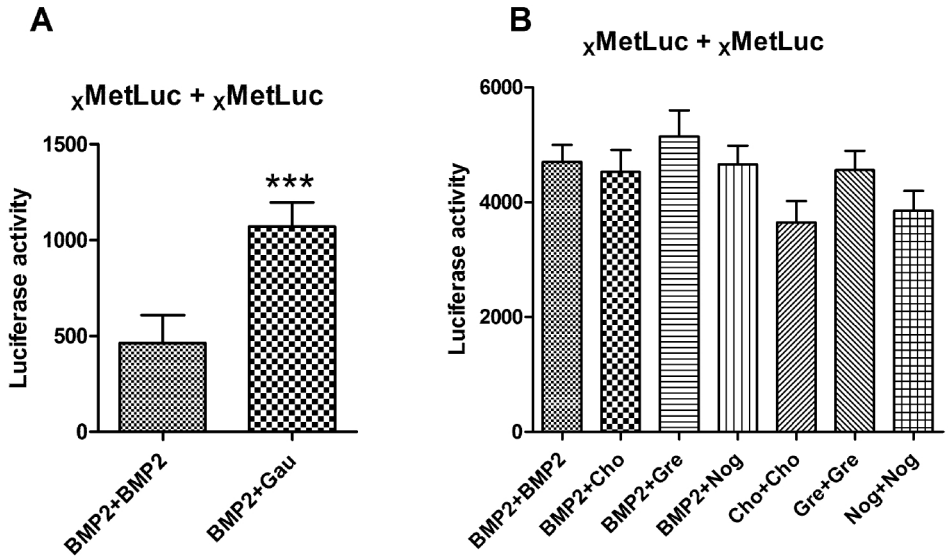


Fig. 17 Hybrid transfection. Combination of different secretion signals for screening of pontential additive effects concerning secretion of luciferase reporter protein. Transfection of either homogenic (one signal) or heterogenic (two signals) protein secretion signal mixtures into C2C12 cells (overall DNA amount of 1 µg) with subsequent quantification of luciferase release 2 days post transfection (n = 6).

Influence of the residual N-terminal amino acid after restriction by signal peptidase

In order to test if the residual N-terminal amino acid after signal peptide cleavage has influence in either secretion, signal peptide cleavage or stability of the mature protein, BMP7 secretion signal was modified resulting in six additional candidates, each consisting of a different N-terminal amino acid at the cleavage site. C2C12 cells were transfected with different constructs and quantification of secreted luciferase was performed two days post transfection. Secretion potencies of modified sites were compared to the naturally derived BMP7 sequence (Asp). Except for valine, all tested amino acids either negatively influenced protein stability or attenuated protein secretion up to 50% (Fig. 18).

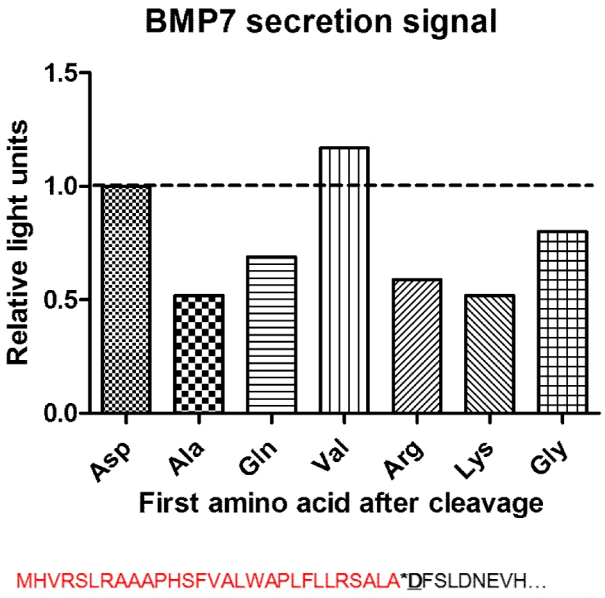


Fig. 18 Impact of N-terminal residual amino acid after secretion peptide cleavage on protein secretion

Residual amino acid after claevage of BMP7 secretion signal showed influence in protein secretion capacity.

C2C12 cells were transfected with Metridia luciferase vector equipped with BMP7 secretion signal harbouring different single amino acid mutations after cleavage site. Luciferase quantification was performed 2 days post transfection. Asterisk indicates signal peptidase cleavage site, bold underlined amino acid shows mutation site (n = 12).

3.4. Bone specific applications

3.4.1 Blocking of osteogenic inhibitory factors

3.4.1.1 miRNA sponges

When vectors encoding RNA sponges are transfected into target cells, the expressed RNA sponges bind endogenous microRNA targets at least as strong as artificially derived antisense oligonucleotides.

For proof of concept, different variants of the mir20a seed sequences (aGCACTTTa) were inserted into the 3´ untranslated region of luciferase gene under the control of the inducible tetracycline response promoter (two copies of each seed sequence, respectively) (Table 3). C2C12 cells were transfected with indicated constructs leading to thinning out of endogenous mir20a miRNAs. Addition of recombinant BMP2 protein induced the increase of endogenous mir20a, which was titrated out by exogenous mRNA harbouring the miRNA seed sequences. Therefore, mir20a was inhibited in his function to promote osteogenic differentiation, which was shown by significantly decreased ALP levels even in the presence of 300ng recombinant BMP2. At the same time, luciferase signals of mir20a seed sequences of right orientation was hardly detectable, indicating efficient degradation upon mir20a binding to the seed sequences (Fig. 19).

After the demonstration of the effectiveness of miRNA sponges with the pro-osteogenic miR-20a, fourteen anti-osteogenic miRNA candidates, were under future investigation for potential miRNA sponge targets in order to - this time - enhance osteogenic differentiation.

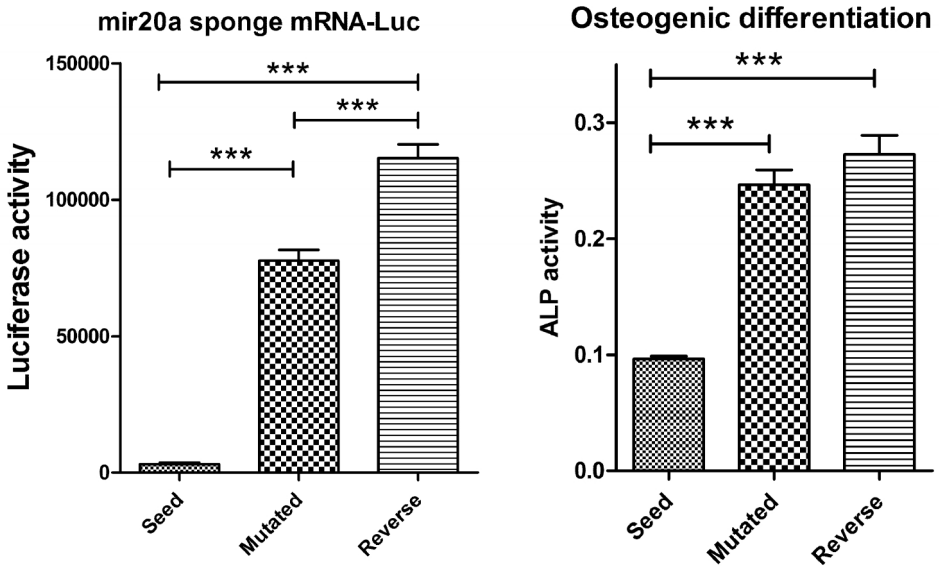


Fig. 19 Effectivity of mir20a sponge: A negative control for proof of concept.

C2C12 cells were transfected with inducible Tet-on vector (two repeats of seed, mutated or reversed target sites of mir-20a on 3´ UTR of luciferase gene). 0.8 µg pTRE-Tight-Luc-mir20a seed/mutated/reverse + 0.2 µg pTeton per well in presence of 300ng rhBMP2.

Left: C2C12 cells were incubated with 1 µg/ml doxycycline for 16 hours. After removal of doxycycline, cells were incubated for additional 24 hours prior quantification of luciferase activity, n = 6.

Right: C2C12 cells were incubated with 1µg/ml doxycycline for 24 hours. ALP activity was measured 6 days post-transfection, n = 6.

3.4.1.2 Hybrid vector system

Hybrid vectors were applied to C2C12 cells for overexpression of BMP2 and simultaneous downregulation of selected targets by short hairpin RNA (shRNA) encoded in intronic sequences of either a red fluorescence protein (DsRed) or BMP2, respectively. The ability to enhance the osteoinduction by the knock down of the individual targets are tested in particular followed by a combination thereof arranged in multi-hairpin structures.

Preliminary results with fluorescence proteins demonstrated successful splicing of the artificial intron confirmed fluorescence microscopy. Furthermore, no remarkable changes in fluorescence levels and cell morphology was observed when the artificial intron was inserted into the open reading frame of the gene of interest (Fig. 20B). Furthermore, preliminary knock down experiments were performed by the insertion of short hairpin RNAs targeted against either Noggin or Ubc9, respectively. Transfection of C2C12 and subsequent enzymatic ALP assay revealed increased osteogenic differentiation potential of both vectors compared to control vector (red fluorescence gene with empty intron element) (Fig. 20A). Additionally, resulting cell morphologies confirmed efficient knock down effect of Ubc9-shRNA, an important key regulator of myotube formation (Fig. 20C), by demonstrating the inability of target cells to form myotubes when Ubc9 is repressed. Finally, the combination of shRNAs into a single intron revealed an up to six fold better osteogenic response when compared to same vector construct without shRNAs (Fig. 21).

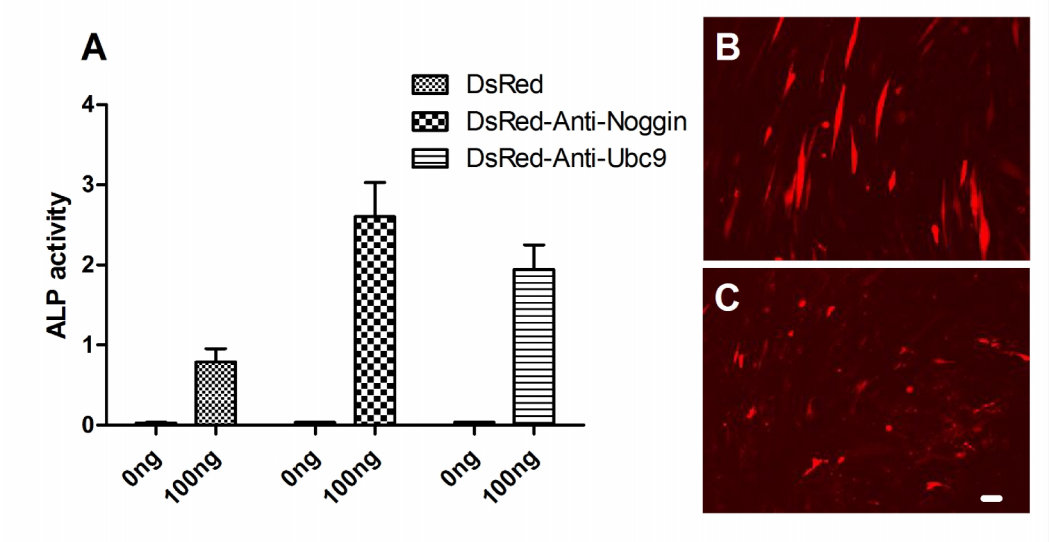


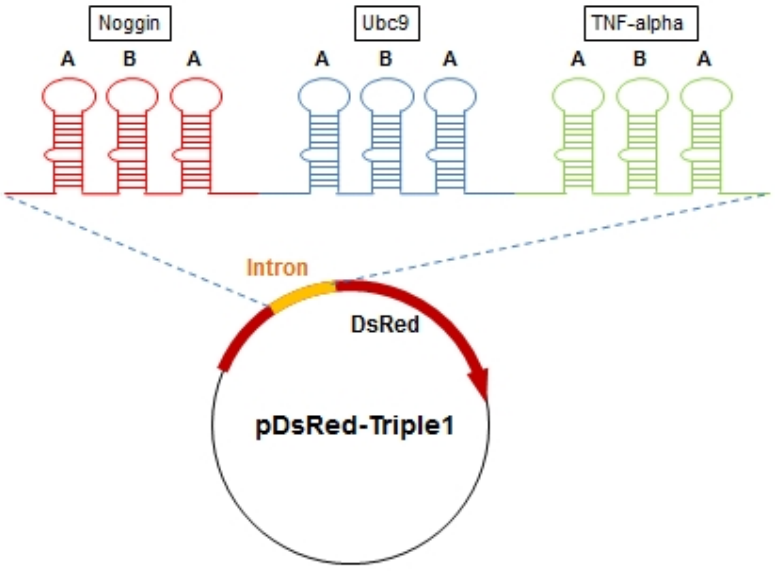
Fig. 20 4d, C2C12, 100ng rhBMP2. Scale bars represent 50µM.

A: DsRed-Empty vs DsRed-Anti-Noggin vs DsRed-Anti-Ubc9

B: Fluorescence: DsRed-Empty

C: Fluorescence: DsRed-Anti-Ubc9

The knockdown of Ubc9, an essential protein for myotube formation, leads to a fusion-defective phenotype in differentiating C2C12 myoblasts



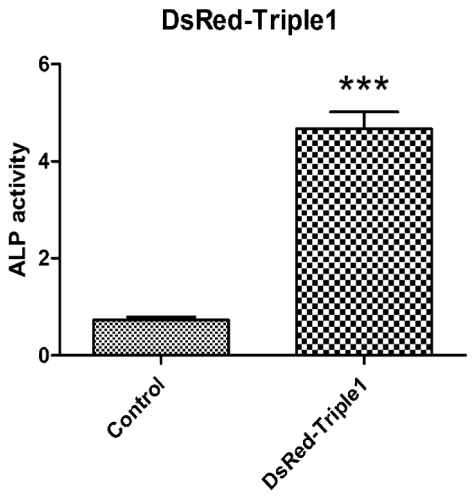


Fig. 21 C2C12 cells were transfected with 1 µg DsRed-Triple1 in presence of 100 ng recombinant human BMP2. Quantification of ALP activity showed over six fold increase in osteogenic response 4 days post transfection.

Introns with hairpins for different targets were inserted into the BMP2 gene to construct an all-in-one vector system. The amount of BMP2 expression and secretion of different vector constructs was quantified by ELISA (Fig. 22). No significant influence of different intronic insertions on BMP2 levels was observed.

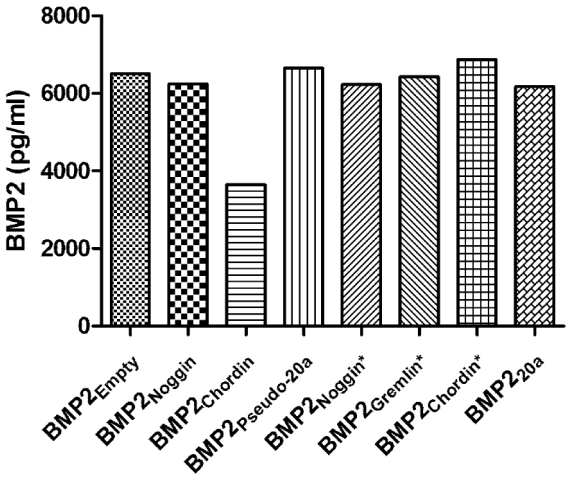


Fig. 22 BMP2 secretion of transfected C2C12 cells. BMP2 levels in supernatant were quantified 5 days post transfection using ELISA (triplicates)

Furthermore, vector constructs with hairpins showed increased mRNA levels of the osteogenic factors Osteopontin and Runx2 five days after transfection. Amongst them, knock down of Gremlin-1 showed highest improvement in osteogenic differentiation (Fig. 23). Additionally, ALP activity demonstrated increased osteoinductivity with BMP2 constructs harbouring shRNA against specified targets (Fig. 23).

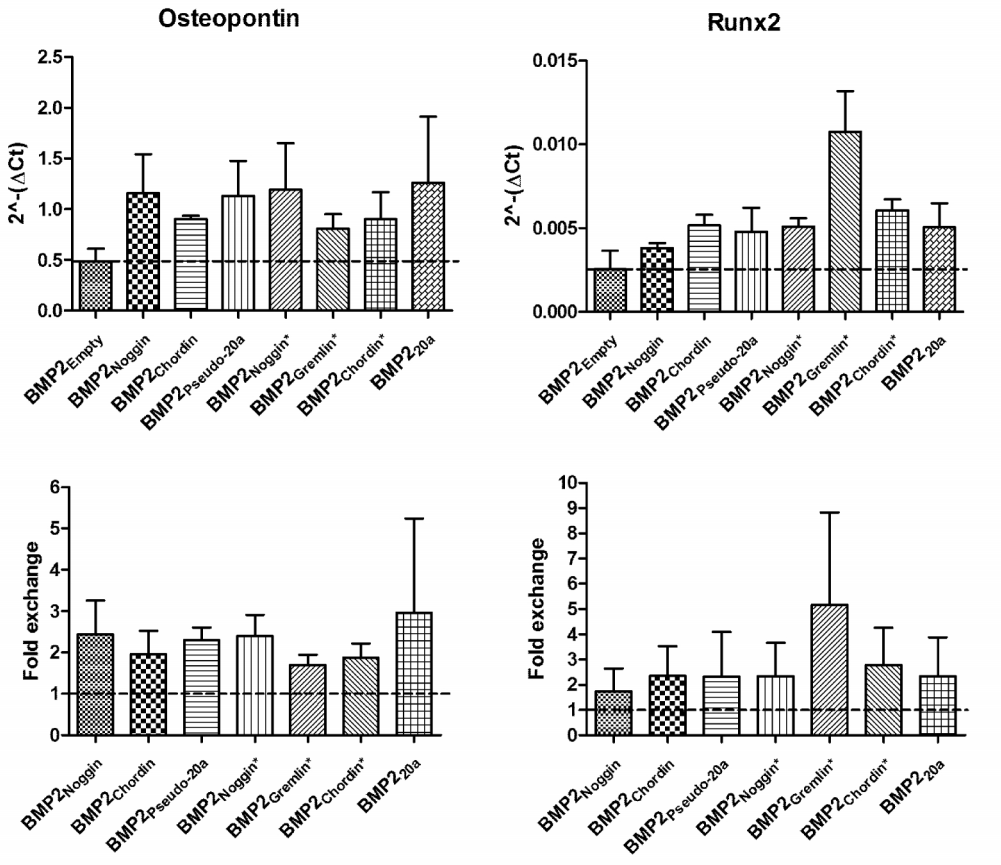


Fig. 23 Real time PCR displayed mRNA levels of Osteopontin and Runx2 5 days post transfection. All tested vector construct showed higher quantities of the osteogenic factors Osteopontin and Runx2. Amongst them, knock down of Gremlin-1 showed highest improvement in osteogenic differentiation.

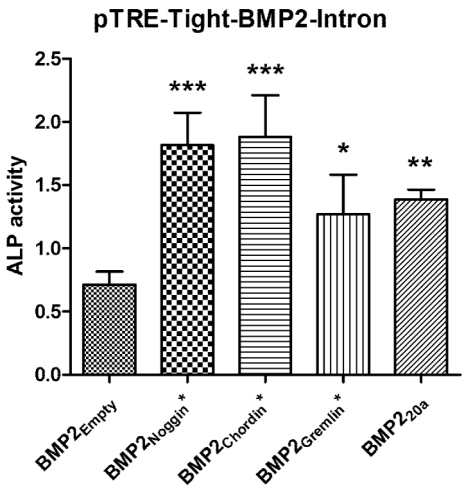


Fig. 24 Osteogenic differentiation of C2C12 mediated by BMP2 gene with particular hairpin elements under the control of the inducible tetracycline response system. 0.8 µg DNA vector constructs were co-transfected with 0.2 µg pTeton-Advanced and C2C12 cells were incubated for 4 days in presence of 1 µg/ml doxycycline.

3.4.2 BMP heterodimers (ideal BMP2 and BMP7 ratio)

Several studies demonstrate an up to 20 fold increase in osteogenic induction when BMP2 is co-applied with BMP7. Especially for the design of BMP2/BMP7 coexpression vectors, one interesting fact will be the ideal ratio of BMP2/BMP7, at which best osteogenic response is achieved. To get better insight into the ideal stoichiometric ranges of BMP2 to BMP7, different ratios of BMP2 and BMP7 plasmid was transfected into C2C12 cells. Interestingly, the results showed a very high tolerance concerning the ratio (and treshold) of the two growth factors being potent osteoinducers (Fig. 25). Therefore, variations in gene expression as well as secretion (BMP7 secretion significantly lower than BMP2 secretion) should not carry too much weight concerning the formation of efficient heterogenic dimerization and therefore increased osteogenic differentiation of target cells. Additionally, different ratios of the advanced BMP vectors (BMP2-Advanced/BMP7-Advanced), which have several fold higher expression, showed comparable less need for heterodimer formation to mediate high levels of osteogenic differentiation.

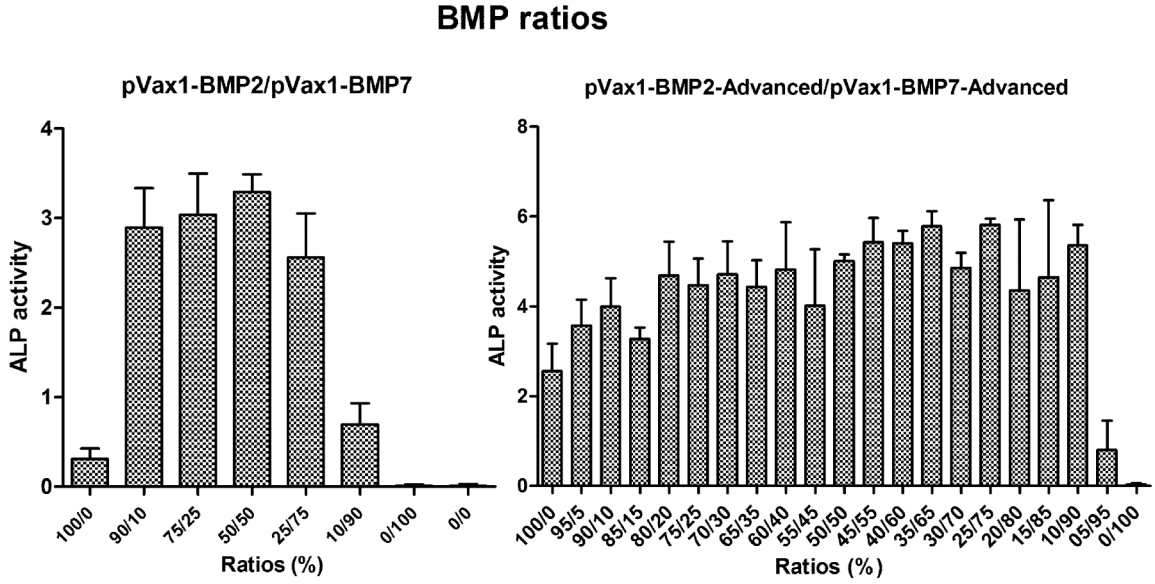
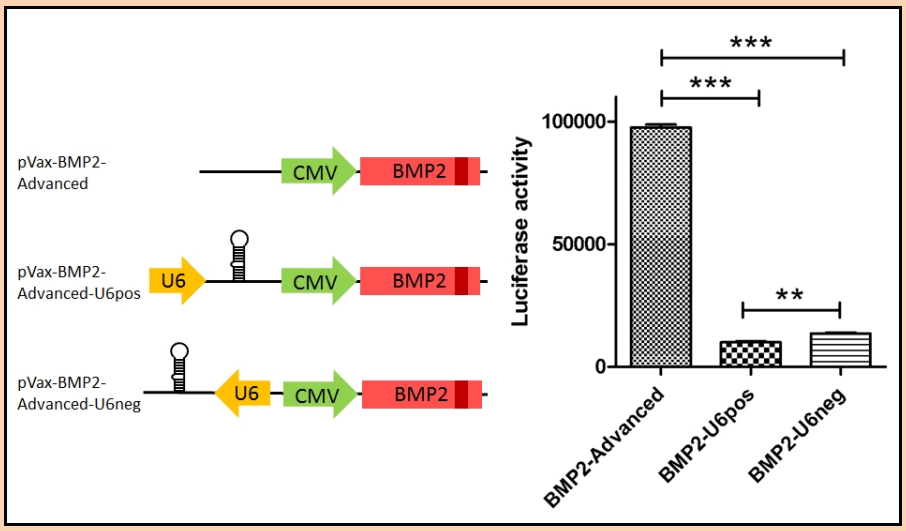


Fig. 25 The application of different ratios of BMP2 and BMP7 showed high tolerance concerning particular aberrations. C2C12 cells were transfected with different ratios of BMP2 and BMP7 plasmids, respectively. Overall DNA amount was set to 1 µg. ALP assay was performed 4 days post transfection (100 = 100% = 1 µg DNA)

Left panel: Ratio of pVax1-BMP2/pVax1-BMP7 (n = 6)

Right panel: Ratio of pVax1-BMP2-Advanced/pVax1-BMP7-Advanced (n = 3)



Additional information: *Despite above mentioned knockdown phenotype, ongoing experiments with shRNA under the control of the polymerase III promoter (U6) demonstrate a tremendously higher knock down effect of mRNA targets (here: Luciferase) compared to polymerase II promoter driven shRNA expression (CMV promoter)* (Applications of polymerase III hybrid systems for bone regeneration (replacement of luciferase gene with BMP2 gene) are in progress). Discussion on page 89.

4. Discussion

4.1. Transfection

4.1.1 Non-viral gene delivery methods

Due to the high clinical benefits and perspectives of gene therapy, a lot of different methods and approaches are tested for the efficient and low toxic delivery of therapeutic genes (Table 1). However, until this date, the field of non-viral gene is still in its infancy. Continuative improvements of the delivery systems, based on a better understanding of cellular pathways and their barriers, is required for the successful clinical application of gene therapy. The big variety of different chemical and mechanical approaches reflects that to date there is no explicit ideal gene delivery system. Nonetheless, it can be expected that ongoing research will bring new delivery strategies specifically optimized and adjusted to particular applications and problems and that the numerous limitations will be solved to smooth the way for routine clinical use of gene therapy.

Method	**Efficiency**		**Toxicity**
	in vitro	***in vivo***	
Needle injection	Low	Low	Safe
Ballistic (gene gun)	Medium	Medium	Tissue damage
Electroporation	High	High	Invasive
Ultrasound	Good	Average	Low
Hydrodynamic delivery	High	High	Low
Polycations	High	Low/Average	High
Cationic lipids	High	Low/Medium	High
Lipid/polymer	Average/High	Low	Low

hybrids			
Lipid/polymer hybrids	High	Low/Medium	Low

Table 1: Conclusion of advantages and disadvantages of established non viral gene delivery systems

4.1.2 Comparison of synthetic and virulence-factor derived endosomolytic peptides

In contrast to other hemolytic proteins like streptolysin O or perfringolysin O, Listeriolysin O has the advantage of its pH dependent hemolytic activity which is displayed only inside the lysosomes and therefore its low cytotoxicity [174, 175]. Furthermore, LLO is degraded when located inside the cytosol, therefore featuring an additional protection of the cell membrane of the host cell [176], which makes the protein an interesting candidate to increase specifically increase lysosomal escape of the gene therapeutic payload and therefore subsequent gene expression. Unfortunately in spite of this advantage, we have shown that the natural pH and temperature dependent inactivation of LLO is an irreversible process hampering an easy-to-use add-on for enhanced delivery of macromolecules. Once the protein is inactivated, there is no known way of reactivation . Therefore, it is difficult to apply LLO together with a DNA transfer systems because target cells *in vitro* and *in vivo* are always exposed to physiological conditions concerning pH and temperature (pH under 5.5, temperature over 33°C). Initially, naturally derived LLO is not exposed to such deactivating conditions, since the protein expression and secretion is triggered exclusively when Listeria *monocytogenes* is trapped inside the lysosomes. Deactivation is only taking place after the lysis of the lysosomes after subsequent exposition of LLO to cytosolic conditions.

Bavdek et al have recently shown stabilization of LLO by dimerization through disulfide bridges at acidic conditions [177]. To test, whether oxidation states can mediate reactivation, LLO was re-incubated in acidic buffer in the presence of 2mM DTT. To conclude, mimicking lysosomal oxidation states showed no effect on reversing the inactivation of hemolytic ability of LLO in our experiments.

The major problem is the irreversible inactivation of LLO by self-aggregation induced by conformational changes when exposed to physiological pH conditions [69, 70]. To underline this assumption, we showed that higher concentrations of LLO led to faster deactivation by aggregation even when stored at pH levels around 5. Our attempts to decrease the concentration of LLO showed therefore an increased stability of LLO. This

natural property of LLO has to be taken into account during the optimization of ideal storage conditions giving rise to prolonged protein stability. Additionally, recent findings showing a higher stability of LLO at acidic conditions by dimerization have to be considered regarding short and long term storage [177].

Finally, in contrast to the observations described by Schuerch et al [178], no significant differences in stability in the presence and absence of 500mM NaCl was observed (data not shown).

Despite to these observations, some confirm an enhanced gene delivery by the addition of LLO. For example packed liposomes with special conditions [72, 73, 179] or conjugated directly to a protamine based DNA binding domain [180].

Alternative endosomolytic peptides

Beside LLO, we have tested several common alternative endosomolytic peptides. Firstly, we have confirmed the pH dependent hemolytic activity of the selected endosomolytic peptides and compared their activity in direct relation among each other. Interestingly, all peptides showed similar levels of activity regarding the lysis of washed red blood cells at pH = 5. Furthermore, all tested peptides except K5 showed little or no activity at pH levels above 7, which is one of the most important requirements for ideal candidates for additives in gene transfer systems. Possibly, pH sensitive properties of the DNA binding endosomolytic peptide K5 could be adjusted by alteration of the N/P ratio of bound DNA.

Despite the fact that their hemolytic activity was dramatically lower when compared with LLO, all peptides showed reactivation of their membrane disrupting potential when exposed to lower pH after inactivation at pH 7 at 37°C. This advantage is important concerning the ease of use and storage of these additives.

Additionally, toxicity tests revealed the harmlessness of all candidates even at high doses and prolonged exposition.

Conclusion

Most of the tested endosomolytic peptides are possible additives for improving gene delivery systems. In spite of their decreased activity when compared to LLO, the peptides still outperform due to their stability at different conditions. Because of this reversibility,

the hemolytic activity, after exposition to physiological conditions *in vitro* and *in vivo* prior to the endocytic uptake by target cells, is maintained until the peptides reach their destination point. One possible approach using the strong hemolytic protein LLO could be the encapsulation of low concentrations of active LLO in closed compartments like liposomes containing an acidic content to ensure the maintenance of the hemolytic activity of its cargo.

4.2. Enhancement of gene expression

This chapter was focused on the construction of a strong gene therapeutical system, in order to compensate low transfection efficiencies and cross the treshold concerning quantities of expressed endogenous gene to increase the gene therapeutical impact. Nevertheless, there is no overall restriction concerning the application of the described modifications exclusively for non-viral gene systems.

4.2.1 Optimization of gene sequence

Codons of BMP2 gene were optimized according to Raab et al [171] using the GeneOptimizer® expert software, which is based on a deterministic sliding window algorithm, adjusted for mouse organism. Additionally, an artificial intron was inserted in the coding region of the gene. Insertion of introns is known to enhance transcription rates and mRNA stability and export into the cytosol, respectively [112, 113]. The expression level of intronless genes is often 10 – 100 times lower than intron-containing counterpart [114]. Here, an over five fold increase in gene expression was achieved. Due to the fact that the codon optimization and the insertion of the intron were done simultaneously, the particular influence of the single modifications (even potential negative influence) to the enhancement of gene expression will remain unclear. Additionally, positive effect of codon optimization to gene expression levels is not exclusively explainable by codon usage and particular tRNA levels of the respective organsims. Supplementary, by alteration of nucleotide sequence during codon optimization process, possible binding sites of regulatory and transcription factors, which would hinder efficient transcription, could possibly be eradicated. Positive outcome of codon optimization is not guaranteed and codon alterations should not be applied blindly. It is rather dependent on the selected gene as well as the particular test system and resulting alterations in gene expression should be under supervision.

4.2.2 General alteration in promoter system (cascade promoter system)

The cascade promoter system was constructed according to the hypothesis, that insufficient endogenous transcription factors act as bottleneck in gene transcription causing a deceleration of gene expression. As a competing system to the strong constitutive viral CMV promoter, a tetracycline response system was used for demonstration purposes. For the conventional vector, BMP2 and BMP7 were inserted under a bidirectional CMV promoter. The constitution of the cascade promoter was comparable to the conventional CMV vector, except having the transcription factor (tet repressor) controlled by the CMV promoter. BMP2 and BMP7 were cloned into the bidirectional Tet promoter as control. The conventional CMV vector could not compete with the cascade promoter vector. Furthermore, the cumulative increase of transcription factor (encoded on pTeton-Advanced) increased the output remarkably on a concentration dependent manner. This increase indicates the meaningful influence and therefore the importance of the amount of transcription factor on transcription rates, and therefore for the gene expression outcome.

To conclude, introducing own transcription factors (preferably under the influence of a moderate inconspicuous promoter rather than a strong viral promoter) to cells is a method to bypass possible transcriptional bottlenecks triggered by depletion of transcription factors. Additionally, by introducing exogenous or non-conventional, but strong transcription factors (like the strong Gal4-VP16 fusion protein transcriptional activator), endogenous transcription regulation remains undisturbed.

After successful proof of the potency of the cascade promoter vector, further tetracycline independent systems (e.g. Gal4-UAS system) are under construction for the construction of efficient second generation mammalian gene expression systems.

4.3. Protein secretion kinetics

The application of BMP2 gene induce osteogenic differentiation of target cells *in vitro*, but clinically effective dosages of homodimers *in* vivo need to be up to milligrams, which is

accompanied with high costs and potential side effects. Additionally, it is known that BMP2 in combination with BMP7 increase osteogenic differentiation up to 20 fold [130, 143, 167, 168]. One of the main reason is the previously shown higher affinity of BMP2/7 hetero dimers to heterologous BMP receptors. Therefore, it was obvious, that the facilitation of BMP2/BMP7 hetero dimer formation (stoichiometrically 1:1) would also increase osteogenic potency compared to BMP2 homo dimers.

The expression of exogenous BMPs is a promising potential therapy for healing bone defects. For this, co-expression vectors are constructed in order to simulatenously express BMP2 and BMP7 genes under strong promoters. However, when these genes are under the control of the same promoter and consequently their transcription rates are comparable, a stoichiometrical disbalance can arise when the export of the produced proteins varies. This study demonstrates for the first time that BMP2 exhibits a twice higher secretion rate compared to BMP7, hence leading to an increased probability for accumulation of BMP2 homodimers in the endoplasmic reticulum prior secretion.

The main idea was that a bicistronic BMP2/BMP7 vector, when controlled by the same promoter, will indeed have stoichiometrically comparable amounts of transcription and therefore mRNA (apart from eventually different expression rates due to structural differences), but still would accumulate at different concentration in the endoplasmic reticulum (ER). This would happen due to the diverging potency of their different secretion signals regarding the mediation of protein translocation into the ER, prior to protein secretion through the Golgi apparatus.
Indeed, comparison of native BMP2 and BMP7 secretion signals revealed that the BMP7 signal is approximately half as potent as the BMP2 signal. Therefore, it is assumable, that approximately twice as much BMP2 would accumulate in the ER than BMP7, probably facilitating BMP2 homo dimer formation due to sterical issues. Interestingly, experiments with different ratios of plasmids encoded for BMP2 and BMP7 gene, respectively, showed a high tolerance concerning aberrations of the theoretically ideal BMP2/BMP7 ratio of 1:1. Already as low as 10% amount of BMP7 plasmid content in respect to the amount of BMP2 was sufficient to achieve nearly same grade of osteoinduction like the theoretically

ideal 1:1 ratio. One possible explanation could be that already small amounts of BMP2/BMP7 hetero dimers act as a kind of starters and mediate a positive feedback trigger for amplified osteogenic differentiation. To conclude for these set-up, a fine-tuning of BMP2/BMP7 secretion by aligning their secretion signals is assumed not to play a big role regarding better osteogenic impact, which was hypothesized at the beginning of the experiments. For other growth factors, balance of protein secretion still might be an important need to improve outcomes.

Furthermore, attempts to improve the secretion signal of BMP2 in order to enhance protein secretion were not successful. Interestingly, among many others, the BMP2 secretion signal was one of the top most potent secretion signals. Therefore, the initial plan to replace the secretion signal with a more potent one turned out to be pointless in this particular case. Zhang et al. (2005) have shown that the secretion of tested proteins could be increased several fold by increasing the basicity and the hydrophobicity of the IL-2 signal sequence [7]. Adapting these alterations to the BMP2 secretion signal merely led to decrease of protein secretion potential. Additionally, increasing the hydrophobic domain of Gaussia luciferase secretion signal only had low efficacy of improving protein secretion.

Secretion of other proteins and osteogenic differentiation in the presence of competitive secretion signals

To date, no evidence exists of potential interplays of secretion signals, which can be of inportance to achieve better outcomes in gene therapeutical approaches. Different combinations of signals indicate possible priorities and mutual influences regarding protein secretion.

The data presented in this work (Fig. 13 - Fig. 16) encourages one of the two following hypothesis:

I. **A priority order consisting protein secretion exists, which is based on the signal sequence composition**

It is possible that in the example of expression of BMP2 gene in presence of simultaneously expressed luciferase gene with competing secretion signal, BMP2 secretion is hindered. The grade of inhibition could occur according to the strength of the competing secretion signal. The results show a better osteogenic differentiation (and assumably therefore better release of BMP2 protein) in presence of BMP2 or BMP6 secretion signal competitor than compared with BMP2 release in presence of the secretion signal of Gremlin-1. Comparison of these data suggests that Gremlin-1 secretion signal has a higher priority than BMP2 or BMP6. This could make sense, given that Gremlin-1 expression is chronologically delayed given that its expression is induced by BMP2 as a response and plays a negative feedback role during osteogenic differentiation.

II. Cleaved secretion signals interfere with osteogenic differentiation procedures in either a positive or negative manner

Second explanation could be that the cleaved secretion signal itself play a role in regulating cellular processes during, in this case, osteogenic differentiation.

This again could make sense for the negative feedback role of Gremlin-1, probably consigning its secretion signal for additional negative osteogenic feedback regulation.

Although less is known about the general fate of secretion signal after protein translocation into the ER, some special post-translocational effect have been observed in mammalian cells [181-184].

Unfortunately, there was no option to track *de novo* created and cleaved secretion signals, to get a better insight into possible post-translocational effects.

Nevertheless, the results reveal that the secretion signal of Chordin (and Noggin) act as potential candidate for the application in therapeutical vectors to induce osteogenic

differentiaion. I has comparably less influence in disturbing osteogenic differentiation mediated by BMP2 overexpression. Therefore, co-expression vectors with BMP2 and Chordin (or Noggin) secretion signals should possibly be prefered.

One potential evidence of the influence of secretion signals (whether in cleaved or non-cleaved form) to osteogenic differentiation of target cells was given by the observation of osteogenic differentiation potential mediated by the application of recombinant BMP2 protein during simultaneous overexpression of luciferase linked to selected particular secretion signals.
Here, luciferase signal strength slightly negatively correlated with the grade of osteogenic differentiation, indicating the higher protein secretion the lower cell differentiation occur. Therefore, generally no significant evidence was given to show any influence of overexpressed secretion signal protein in osteogenic differentiation (even when it has to be mentioned, that the induction of osteogenic differentiation was based on the application of recombinant BMP2 protein, whichever had no need to undergo all the translocation steps required for efficient protein secretion at all).

Additional notes: Bottleneck - Does overexpression influence protein secretion of endogenous genes?

For future gene therapeutical studies, the influences of overexpression of secretion proteins on the overall balance of the secretome should be examined. This should be performed by the overexpression of an exogenous gene which has no influence on cellular processes, ensuring positive or negative influence of secretome exclusively based on secretion procedures alone.

Hybrid transfection - better secretion by broad-band effect?

One additional presumption is a possible broad-band effect when multiple secretion signals are applied simultenously, possibly based on the utilization of different binding proteins and sub-routes of known protein secretion machinery. Quantification of secreted luciferase by combination of two types of plasmids, each encoding for same gene of

interest but fused to different secretion signals showed indications of the theory. However in return, additional experiments with different signals (Chordin, Gremlin, Noggin secretion signals) revealed no significant evidence.

Interestingly, by the application of multiple secretion signals simultaneously, a better osteogenic differentiation of target cells was observed (Fig. 16) compared to single signal expression.

To explore possible underlying mechanisms for this phenotype, additional experiments have to be performed, but the indication of possible work-sharing effect of during protein secretion is assumable.

Influence of the residual N-terminal amino acid after restriction by signal peptidase

Furthermore, residual amino acid after claevage of BMP7 secretion signal showed influence in protein secretion capacity. Additionaly experiments are required to get a better insight about the background procedures leading to this phenotype. One possible explanation could be a decreased stability of the residual protein located in the ER. Varshavsky et al had described influence of N-terminal amino acid residue on stability (N-end rule) of proteins located in the cytosol [185], but indications exist for other compartments too [186]. Therefore, possible analogies of secretion behaviour and stability of mature proteins consisting of different N-terminal amino acid residues cannot be exluded.

4.4. Bone specific applications

4.4.1 Blocking of osteogenic inhibitory factors

4.4.1.1 miRNA sponges

Additional potential space for vector improvement is provided by the application of miRNA sponges. It has been described previously, that exogenous mRNA transcripts can bind endogenous miRNAs by serving seed sequences complementary to the miRNAs. By this, endogenous miRNAs are redirected and neutralized.

For proof of concept, mir20a was chosen as target miRNA. mir20a is described as supporting osteogenic differentiation, and therefore osteogenic differentiation should be hindered or at least attenuated by introduction of mir20a sponges. Actually, by inhibiting mir20a by the tetracycline-inducible luciferase vector, osteogenic differentiation was successfully blocked.

To focus on the increase of osteogenic differentiation of cells, seed sequences complementary to probably anti-osteogenic miRNAs are under further investigation. These candidates will be chosen to design anti-mir analogues, which can be co-delivered with the therapeutic vector, like it is in use with artificial siRNAs. Amongst them, miRNAs 125, 135, 138, 141, 200, 181a, 206, 23a, 27a, 24-4, 26a and 204/211) will be examined in more detail for subsequent sponge studies.

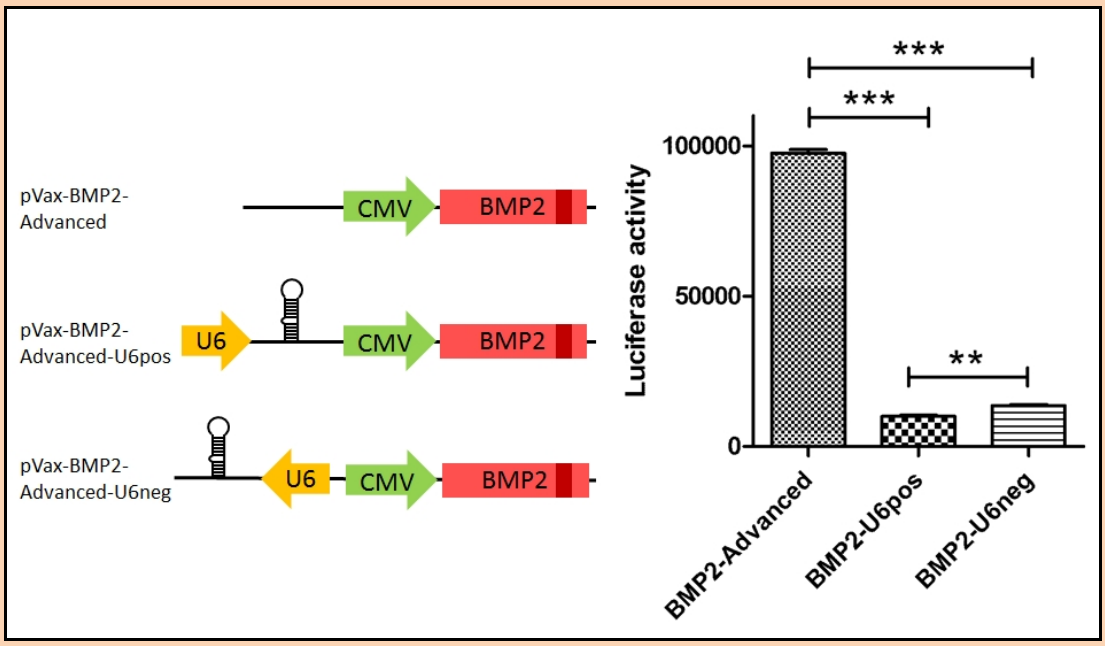
4.4.1.2 Hybrid vector system

In respect to the applicability of the hybrid vector system, we demonstrate its ability to constantly knock down target mRNAs during the time of overexpression of the therapeutic gene (BMP2 as selected gene). The designed hybrid vector, a combination of the advantages of both, an overexpression and an siRNA vector, respectively, shows a broad range of possible applications. This hybrid system offers a good alternative to former approaches, in which siRNAs had to be introduced continously by repeated addition of new siRNA to maintain a constant knock down level of target genes. By combining, a sustained release of shRNA and therefore a constant knock down of target genes can be reached.

The repression of a broad range of specific inhibitory proteins (either secreted extracellular factors preventing BMP binding to receptors, or cytosolic factors inhibiting for example Smad downstream activation), can easily be induced to rearrange specific gene expression patterns in order to enhance the efficacy of the overexpressed therapeutic gene.

Here, it was observed that the inserted hairpins impaired efficent expression of the gene of interest (probably by impairment of splicing of the intron) when more than 4 to 5 shRNAs were inserted in tandem repeats into a single intron (data not shown). The

addition of a second intron could possibly overcome this limitation, and has to be further investigated.



<u>Additional information:</u> Despite above mentioned knockdown phenotype, ongoing experiments with shRNA under the control of the polymerase III promoter (U6) demonstrate a tremendously higher knock down effect of mRNA targets (here: Luciferase) compared to polymerase II promoter driven shRNA expression (CMV promoter). Despite few reports about putative toxicity of polymerase III promoters, polymerase III driven hairpins are the method of choice due to their compact promoter, small hairpin size and the short termination signal (5 nucleotides).

4.4.2 BMP heterodimers (ideal BMP2 and BMP7 ratio)

Several studies demonstrate an up to 20 fold increase in osteogenic differentiation of target cells when BMP2 is co-applied with BMP7.

To get better insight into the ideal stoichiometric ranges of BMP2 to BMP7, different ratios of BMP2 and BMP7 plasmid were transfected into C2C12 cells. Interestingly, the results showed a very high tolerance regarding the ratio (and treshold) of the two growth factors concerning the mediation of osteoinduction (Fig. 25). Therefore, ratio variations in gene expression as well as secretion of BMP2 and BMP7 (BMP7 secretion was significantly

lower than BMP2 secretion) should not be a big issue concerning the mediation of osteogenic differentiation of target cells.

Interestingly, different ratios of enhanced BMP genes (BMP2-Advanced/BMP7-Advanced) showed comparable less need for heterodimer formation to mediate high levels of osteogenic differentiation. Despite still higher impact on osteogenic differentiation, the data indicates a slight compensation of the impact of BMP2/BMP7 heterodimers when BMP expression levels are high enough. This could be possibly explained by triggering enhanced osteogenic differentiation when gene expression exceed possible tresholds concerning BMP2, and especially BMP7 levels, one important fact when transfection levels are only moderate in non-viral *in vivo* approaches.

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6. Curriculum vitae

	2001 to date	Studies in Molecular Biology at the University of Vienna, Campus Vienna BioCenter
	2006	First Degree (equal to Bachelor) in Molecular Biology at the University of Vienna
	2009	Master degree
Military service	**2000-2001**	in Wiener Neustadt
Work experience	**From 2012**	Group leader and head of the department of molecular biology at Ludwig Boltzmann Institute for Experimental and Clinical Traumatology
	June 2009 – to date	Doctoral thesis at the Ludwig Boltzmann Institute for Experimental and Clinical Traumatology (Non-viral gene therapy)
	June 2009	Master thesis at Ludwig Boltzmann Institute for Experimental and Clinical Traumatology (Enhancement of non-viral cell transfection)
	May-June 2007	Campus Vienna Biocenter (Max F.Perutz Laboratories) (RNases in microorganisms)
	February 2006 - April 2007	Department of Pediatrics, Medical University of Vienna and Department of Neurology at Vienna General Hospital

(Mass spectrometrical and computational analyses of alterations in the protein expression pattern after spinal cord injury in rats)

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