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Research Article

# Comparison of cell-based versus cell-free mammalian systems for the production of a recombinant human bone morphogenic growth factor

The human bone morphogenetic protein-2 (hBMP2) is a glycoprotein, which induces *de novo* bone formation. Here, recombinant production in stably transfected Chinese Hamster Ovary (CHO) cells is compared to transient expression in Human Embryo Kidney (HEK) cells and cell-free synthesis in CHO cell lysates containing microsomal structures as sites of post-translational processing. In case of the stably transfected cells, growth rates and viabilities were similar to those of the parent cells, while entry into the death phase of the culture was delayed. The maximum achievable rhBMP2 concentration in these cultures was 153 pg/mL. Up to 280 ng/mL could be produced in the transient expression system. In both cases the rhBMP-2 was found to interact with the producer cells, which presumably contributed to the low yields. In the cell-free system, hBMP2 yields could be increased to almost 40 $\mu$g/mL, reached within three hours. The cell-free system thus approached productivities for the active (renatured) protein previously only recorded for bacterial hosts, while assuring comprehensive post-translational processing.

**Keywords:** Cell-free expression / CHO cell lysate / Glycosylation / hBMP2 / Mammalian cell culture

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## 1 Introduction

Human bone morphogenetic proteins (hBMPs) are members of the TGF-$\beta$ family. They are primary cellular differentiation factors, which induce the differentiation of mesenchymal stem cells into bone and cartilage cells [1]. Recombinant hBMP2 has therapeutic uses in aiding bone healing [2, 3], but also for improving implant ingrowth and stability in dentistry [4]. Their complex nature predestines the BMPs for production in mammalian hosts, such as Chinese Hamster Ovary (CHO) cells [5], in particular since material produced in *E. coli* is known to be of limited therapeutic benefit [6, 7]. However, product titers reported in the literature for recombinant growth factors produced in CHO cells tend to be low, e.g., 2.4 ng/mL for hBMP2 [8], 80 pg/mL for hFGF2 [9], or 358 ng/mL for hVEGFA [10]. This makes the hBMPs interesting candidates for a fundamental investigation of possible biosynthetic bottlenecks in mammalian cells and alternative production strategies.

Even though registered biopharmaceuticals are currently produced in permanently transfected cell lines, alternatives have been suggested. Transient gene expression allows the production of milligram to gram amounts of recombinant protein within a short period of time (5–10 days) [11]. High-density cultures of suspension-adapted Human Embryo Kidney (HEK293) cells have shown a superior performance in this context [12–14]. Finally, cell-free protein synthesis in cell lysates presents an alternative, which supposedly circumvents some limitations of the cell-based systems [15]. For production, the crude lysates need to be supplemented with additional "energy components" (ATP/creatine phosphate/creatine kinase), free amino acids and the target gene DNA or mRNA [16]. Both prokaryotic and eukaryotic lysates are in use. Prokaryotic lysates tend to produce higher yields, but are limited where the synthesis of complex, post-translationally modified proteins is concerned [17].

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**Abbreviation: hBMP2**, morphogenetic protein-2

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Established eukaryotic systems include yeast [18], wheat germ [19,20], insect [21,22], tobacco [23] and mammalian [24,25] cell lysates. The wheat germ system is highly popular due to its high yields, but limited in regard to the correct post-translational modification of human proteins. CHO cell lysates are more promising, since they are closely related to the most commonly used mammalian host in industry and may even constitute an innovative prescreening platform. CHO lysates have been shown to contain endogenous microsomal structures derived from the endoplasmatic reticulum [21, 26], which contain many of the enzymes required for post-translational modification. Proteins that translocate into these vesicles undergo comprehensive processing including disulfide bond formation, phosphorylation, lipidation, and most importantly glycosylation [27]. In a recent comparison of various eukaryotic cell-free systems for the production of recombinant proteins [24], the CHO cell lysates gave the highest yields.

In this contribution, cell-based (stable, transient transfection) and cell-free production strategies for recombinant hBMP2 are compared. Neither stable nor transient expression gave satisfactory yields, while the cell-free system resulted in product titers approaching 40 mg $L^{-1}$.

## 2 Materials and methods

### 2.1 Materials

Plastic materials and chemicals were from established suppliers and used as received. Linear poly(ethyleneimine) (l-PEI) was from Polysciences Europe (Eppelheim, Germany). High quality water was produced by a Millipore unit. Cell culture media were from Lonza (Verviers, Belgium) and Sigma Aldrich (Taufkirchen, Germany). L-Glutamine, antibiotics, G418, and fetal calf serum were from Biochrom (Berlin, Germany). Protease inhibitors were from Carl Roth (Karlsruhe, Germany). The recombinant human BMP-2 standard material produced in *E. coli* (ErhBMP2) or CHO cells (CrhBMP2) was from PeproTech GmbH (Hamburg, Germany). Oligonucleotides were synthesized at Operon (Ebersberg, Germany and IBA GmbH, Göttingen, Germany), primer sequences are given in Table 1.

### 2.2 DNA constructs

phBMP2-EGFP (5.9 kb, Fig. 1A) was cloned in house according to standard laboratory practice and used for hBMP2 expression as pre-pro-protein in CHO and HEK293 cells. Cells transfected with this plasmid co-express the enhanced green fluorescent protein (EGFP). The translation of hBMP2 is initiated by a 5′-end cap region and that of the EGFP by an IRES sequence. Plasmids $pH_2B$-EGFP (5.1 kb, cloned in house according to [28]), encoding only EGFP and pCDNA3.1-ScFv49M (6.3 kb, cloned in house), encoding a secreted single chain antibody, were used in control transfections. pMK-CRPV-Mel-hBMP2 (3.9 kb, Fig. 1B) encoding the hBMP2 gene with a melittin signal peptide, an internal ribosome entry site (IRES) from the intergenic region (IRG) of the cricket paralysis virus (CRPV), and regulatory sequences (T7 promoter) was from GeneArt (ThermoFisher Scientific, Dreieich, Germany) and used in the cell-free expression

**Table 1.** Oligonucleotide primers

| Target gene/primer name | Primer | Sequence (5′ – 3′) | Tm (°C) | Amplicon size (bp) |
|---|---|---|---|---|
| $\beta$-actin | Sense | AACAAGATGAGATTGGCATGGC | 58.4 | 108 |
| | Antisense | TCACCTTCACCGTTCCAGTTTT | 58.4 | |
| hBMP2 | Sense | ACTACCAGAAACGAGTGGGAA | 57.9 | 113 |
| | Antisense | GCATCTGTTCTCGGAAAACCT | 57.9 | |
| EGFP | Sense | ATCATGGCCGACAAGCAGAAGAAC | 62.7 | 258 |
| | Antisense | GTACAGCTCGTCCATGCCGAGAGT | 66.1 | |
| BMP2-CFS[a)] | Sense | TACATTTCTTACATCTATGCGGACGCGGCTGGCCTCGTTCCG | 84.6 | 1174 |
| | Antisense | *CTTGGTTAGTTAGTTATTA*CTAGCGACACCCACAA | 67.7 | |
| Mel-SP | Fusion Oligonucleotide | TAAGAAGGAGATAAACAAAAGCAAAAATGTGATCTTGCTTGT AAATACAATTTTGAGAGGTTAATAAATTACAAGTAGTGCTATT TTTGTATTTAGGTTAGCTATTTAGCTTTACGTTCCAGGATG CCTAGTGGCAGCCCCACAATATCCAGGAAGCCCTCTCTGCG GTTTTTCAGATTAGGTAGTCGAAAAACCTAAGAAATTTACCT GCTAAATTCTTAGTCAACGTTGCCCTTGTTTTTATGGTCGTA TACATTTCTTACATCTATGCGGAC | | |
| N-ter | Sense | ATGATATCTCGAGCGGCCGCTAGCTAATACGACTCACTATAG GGAGACCACAACGGTTTCCCTCTAGAAATAATTTTGTTTAA CTTTAAGAAGGAGATAAACA | 85.6 | 1538 |
| C-ter | Antisense | *TAATAACTAACTAACCAAG*ATCTGTACCCCTTGGGGCCTCTA AACGGGTCTTGAGGGGTTTTTTGGATCCGAATTCACCGGT GATATCAT | 88.4 | |

a) Oligonucleotides for the first PCR step for generation of templates for the cell-free expression system. Underlined sequence encodes for the melittin signal peptide, italic sequence corresponds to the regulatory 3' sequence, bold sequence corresponds to the CRPV IGR IRES. Accession N°: NM_001101.3 (ß-actin), NM_001200.2 (hBMP2) and U55762.1 (EGFP).

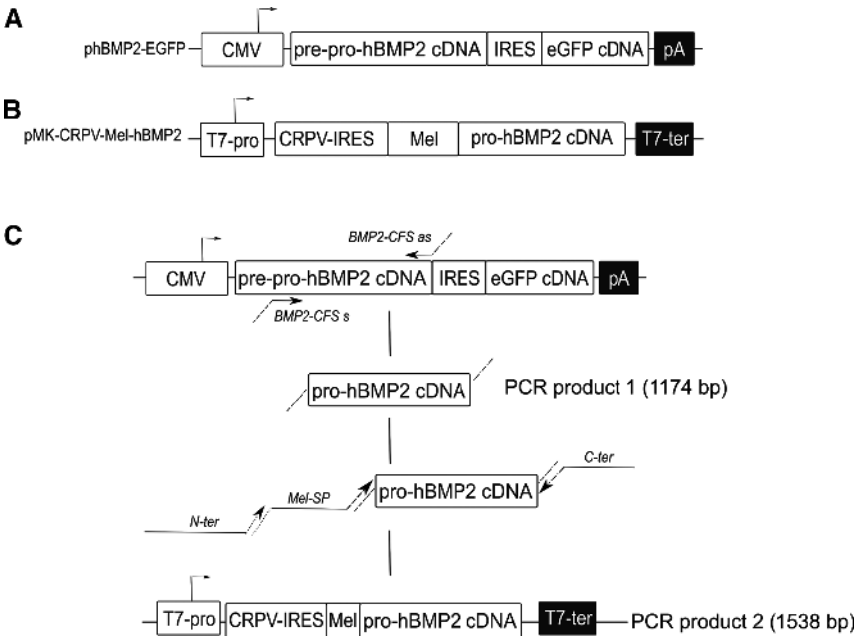


**Figure 1. DNA constructs.** (A) Expression cassette within plasmid phBMP2-EGFP (B) Expression cassette within plasmid pMK-CRPV-Mel-hBMP2 (C) Schematic presentation of the DNA amplification procedure used to produce for the linear DNA sequence for cell-free protein synthesis including the introduction of the necessary regulatory sequences.

system. The CRPV IGR IRES sequence, a 5′ UTR translation initiation sequence, which does not require any translation initiation factor, was integrated, since this sequence has been described previously to result in a significant increase in protein production in CHO-lysate based cell-free systems [24]. Plasmids were amplified in *E. coli* DH5$\alpha$ in LB medium using standard molecular biology techniques, followed by harvesting and purification with Qiagen's Maxi- or Giga-Prep kits (Qiagen, Hilden, Germany). Plasmid concentration and quality (> 80 % supercoiled topology) were determined by $A_{260/280}$ ratio (> 1.8) and agarose gel electrophoresis. In addition, a linear template for cell-free expression was generated by a two-step PCR procedure, as previously described [29]. A schematic overview of the procedure, including the introduction of the necessary regulatory sequences for cell-free protein synthesis in CHO cell lysates, is given in Fig. 1C.

## 2.3 Cell culture and transfection

Cells were maintained in a standard cell culture incubator (5.0% $CO_2$, 37°C, 95% humidity). Chinese Hamster Ovary cells (CHO-K1, CCL-61, ATCC) were cultivated in R10 (RPMI 1640 medium supplemented with 2 mM L-glutamine, 0.1 mg $L^{-1}$ penicillin/streptomycin, 10 % fetal calf serum). Suspension adapted Human Embryo Kidney cells (HEK293sus, CRL-1573.3, ATCC) were cultivated in serum-free ExCell 293 medium supplemented with 6 mM L-glutamine and 0.1 mg $L^{-1}$ penicillin/streptomycin. The recombinant cell line 4D6 was derived from the CHO-K1 parent cell line by transfection with phBMP2-EGFP, using PEI as transfection agent, followed by G418-selection and EGFP-based screening/adaptation to suspension culture as previously described [30]. 4D6 cells and CHOsus (CHO-K1 similarly adapted to suspension cultivation) were maintained in ProCHO5 medium supplemented with 4 mM L-glutamine and 0.1 mg $L^{-1}$ penicillin/streptomycin. HEK293sus cells were transfected (HEK293trans) using the high-density transient transfection protocol proposed in [12]. Suspension cells were cultivated in spinner flasks (IIBS, Chur, Switzerland, 100 mL medium, 50 rpm, inoculation cell density $1 \times 10^5$ cells/mL).

## 2.4 Cell-free protein synthesis

CHO cell lysate preparation and cell-free protein synthesis were performed as previously described [26,29]. Briefly, cells were suspended in lysis buffer (40 mM HEPES, pH 7.5, 100 mM NaOAc, 4 mM DTT) at approximately $5 \times 10^8$ cells/mL and syringed through a 20-gauge needle. After removal of cell debris by centrifugation (10 000 × *g*, 10 min, 4°C), the coupled transcription-translation reactions were performed in Eppendorf tubes (total volume 25 $\mu$L) using 3 U $\mu$L of T7 RNA polymerase. In addition, 0.3 mM of UTP and CTP and 0.1 mM of the cap analogue m7G(ppp)G were provided. For protein synthesis, translationally active lysates were supplemented with HEPES-KOH (pH 7.5, 30 mM), sodium acetate (100 mM), $Mg(OAc)_2$ (3.9 mM), KOAc (150 mM), amino acids (100 $\mu$M), spermidin (0.25 mM), DTT (2.5 mM), and energy generation components (20 mM creatine phosphate, 1.75 mM ATP, 0.3 mM GTP). Approximately 20 ng/$\mu$L of the linear PCR product or the indicated amount of pMK-CRPV-Mel-hBMP2 was added. Finally, lysates were supplied with 50 $\mu$M $^{14}$C-leucine (specific radioactivity 66.67 dpm/pmol) (Perkin Elmer, Rodgau, Germany) to facilitate product detection, and the mixture was placed for 3 h at 30°C and 600 rpm in a thermomixer. To confirm the glycosylation of the de novo synthesized hBMP2, lysates were supplied with 1.72 $\mu$M $^{14}$C-mannose (American Radiolabeled Chemicals) instead of $^{14}$C-leucine and the reaction was performed as described above.

### 2.5 Analytics

Cell number and viability were determined automatically after Trypan blue staining using a Vi-Cell XR (Beckman Coulter, Krefeld, Germany). Lactate and glucose concentrations were quantified with commercial kits (UV-method) from R-Biopharm AG (Darmstadt, Germany). EGFP expression was measured as previously described by flow cytometry [31] or spectrophotometrically [30]. In HEK293trans cells, EGFP was also quantified in cell lysates. For this, cells were suspended at a concentration of $1 \times 10^6$ cells per mL in lysis buffer (1% Triton X-100 in PBS, pH 7.4) and incubated for 2 h at 37°C. After removal of the cell debris by centrifugation (16 000 × *g*, 10 min, 4°C) aliquots were transferred into black, non-binding 96 microtiter plates (200 $\mu$L per well). The fluorescence (Ex 485 (20) /Em 535 (25)) was measured using a GeniosPro a plate reader (Tecan, Männedorf, Switzerland). Lysates of non-transfected cells were used as blanks.

hBMP2 concentrations in solution were determined by ELISA (kit: PeproTech, Hamburg, Germany); standard curve range: 0.016 – 2 ng/mL, only data points, which fell into this range, were used. In the cell-free system, hBMP2 yields were determined in 5 $\mu$L aliquots, which were subjected to hot trichloroacetic acid (TCA) precipitation, followed by liquid scintillation counting as previously described [22, 24]. Molecular masses were estimated by SDS-PAGE (10% gels, non-reducing conditions) followed by autoradiography. For verification of the glycosylation, glycan residues were enzymatically removed (PNGase F and Endo H, New England Biolabs, Frankfurt am Main, Germany), according to the supplier's recommendations, followed by differential SDS-PAGE analysis in comparison to sample aliquots processed under similar experimental conditions, but in the absence of the glyco-specific enzymes.

For mRNA quantification by RT-qPCR, total RNA was extracted by standard phenol – chloroform protocol followed by ethanol precipitation and resuspension in sterile Milli-Q water pre-treated with diethylpyrocarbonate. Remaining genomic DNA was digested with DNase I and the poly-dT primed total RNA reverse-transcribed into cDNA at 65°C with Maxima H minus reverse transcriptase. All enzymes were from ThermoFisher Scientific (Dreieich, Germany) and used according to the supplier's protocols. No-template-controls (NTC, sample without cDNA) and reverse transcriptase-minus-controls (sample without reverse transcriptase, $RT^-$) were always included. qPCR was performed using 20 to 250 ng of cDNA, 200 nM of each primer (Table 1) and 2 × KAPA SYBR-FAST universal master mix (PeqLab, Erlangen, Germany) in a final volume of 20 $\mu$L. Cycling parameters were 95°C for 10 min followed by 40 cycles of 95°C for 60 s, 63°C for 60 s, and 72°C for 30 s. The $2^{-\Delta\Delta}$Cq method [32] was used to quantify target cDNAs, with $2^{-\Delta\Delta}$Cq giving the quantity of target gene normalized to $t = 0$. $\beta$-actin served as reference gene. Data were not taken into consideration when Cq-NTC ≤ 40.

The RNA secondary structure predictions, including the Gibbs free energy ($\Delta G$) calculation for folding were done with the prediction server GeneBee-Net: http://www.genebee.msu.su/services/rna2_reduced.html [33]. Codon usage was evaluated using the tool available at the Kazusa DNA Res. Inst. website: http://www.kazusa.or.jp [34]. The isoelectric point of the mature hBMP2 protein was calculated using the tool provided at the Expasy website: http://web.expasy.org/compute_pi/ [35].

## 3 Results and discussion

### 3.1 Production of human BMP2 in stably transfected CHO cells

CHO-K1 cells were transfected with phBMP2-EGFP and stable clone 4D6 was selected for further study. In phBMP2-EGFP, the hBMP2 and EGFP genes are linked by an internal ribosome entry site (IRES). In consequence, both proteins arise from a common mRNA. The translation of the hBMP2 sequence is initiated by a 5'end cap region, that of the EGFP by the IRES. While the EGFP is produced as intracellular protein, the rhBMP2 is excreted.

Figure 2A summarizes the viable cell densities and viabilities from batch spinner cultivations of clone 4D6 in comparison to that of the suspension adapted parent cell line (CHOsus). For the first 120 h, the growth rate of the 4D6 cells ($\mu_{max}$: 0.013 $h^{-1}$) is similar to that of the CHOsus cells ($\mu_{max}$: 0.015 $h^{-1}$). Afterward, the viability of the CHOsus cultures declines, while that of the 4D6 cultures remains at nearly 80 % for another 72 h. Several groups have reported an anti-apoptotic effect of BMP proteins on mammalian cells [36–38]. A similar effect may be operative here. The metabolic markers glucose and lactate showed no significant differences between the two cultures, data not shown.

The product titers for EGFP and hBMP2 are shown in Fig. 2B, while Fig. 2C summarizes the results from the RT-qPCR quantification of the corresponding mRNA sequences. The hBMP2 protein concentrations were approximately three orders of magnitude lower (pg/mL-range) than the EGFP concentrations (ng/mL-range). It is possible that this is due to differences in the translation initiation efficiencies of the 5'-end cap structure (hBMP2) and the IRES (EGFP). The fact that such differences can be of significant influence has recently been highlighted [39] and may have contributed here as well. Another difference may be more important, however. In these experiments, only EGFP was produced at steady rates throughout the cultivation. The hBMP2 concentration reached a maximum (153 pg/mL) after 120 h and dropped afterwards below the detection limit of the ELISA within 48 h. The RT-qPCR gave significantly lower relative values for the relative abundances of hBMP2 compared to the EGFP mRNA, in spite of the fact that both sequences should be part of a common transcript. A simulation of the two RNA secondary structures, showed a much higher degree of structuring in the hBMP2 than in the EGFP mRNA sequence. Secondary structures in the mRNA may affect reverse transcription [40], but also interfere with translation, e.g. by slowing or blocking initiation or the movement of the ribosomes along the strand [41]. Secondary structures may therefore be responsible for both the weaker signal of the hBMP2 mRNA in the RT-qPCR and for the lower protein titers during the first 120 h of cultivation.

However, although low, the relative abundance of the hBMP2 mRNA increased throughout the cultivation. The rapid decrease in the hBMP2 protein titer after 120 h can therefore not be attributed to a disappearance of the BMP2 mRNA. Proteolytic degradation was also ruled out, since a repetition of the

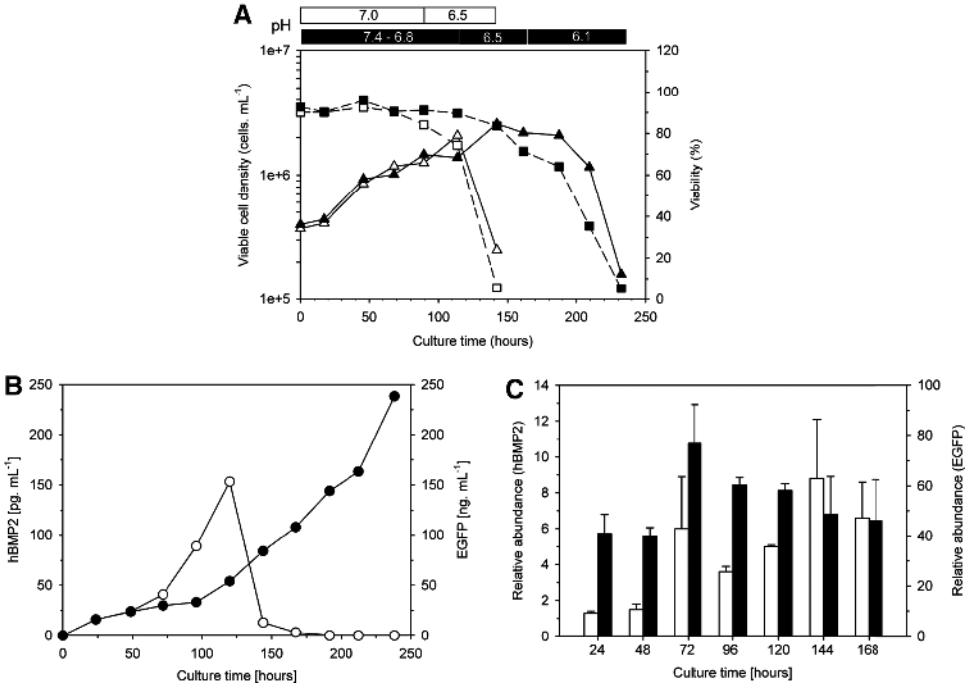


**Figure 2. Production of hBMP2 in recombinant CHO cells (clone 4D6).** (A) Viable cell densities (triangles) and viabilities (squares) of 4D6 (■,▲) and CHOsus (□,△) cells. (B) Protein titers in the 4D6 cell culture (hBMP2: ○; EGFP (expression estimated in the cell lysates): ●). (C) Relative mRNA abundance ($2^{-\Delta Cq}$, hBMP2: white bars; EGFP: black bars), with $C_q$: quantity of target gene normalized to reference gene $\beta$-actin ($C_q$,target - $C_q$,$\beta$-actin), Mean ± SD ($n = 3$).

cultivation in the presence of a protease inhibitor mix (aprotinin, trypsin inhibitor and phenylmethylsulfonyl fluoride (PMSF) 10 $\mu$g/mL each) did not increase titers or prevent the disappearance of hBMP2 after 120 h. The pH of the culture supernatant was another concern, as this value became as low as 6.1 towards the end of the cultivations and the first value below 7 coincided with the start of product loss. However, an evaluation of the pH sensitivity of hBMP2 using commercially produced material (ErhBMP2) gave no indication of a pronounced instability in the pH-range of interest (6.0–7.0).

Expression of a BMP receptor (BMP receptor II) has been shown in postnatal (d15) hamster ovarian cells [42] and linked to the already mentioned anti-apoptotic affect [36–38]. To investigate a possible interaction of hBMP2 with the producing cells, CHOsus cells (1 and 3 × $10^6$ cells/mL) were incubated at 37°C in ProCHO5 medium supplemented with 5 ng/mL ErhBMP2. Within 10 min the hBMP2 concentration in the supernatant had decreased by more than 50 %, whereas no decrease was observed in the absence of the cells. Moreover, we were subsequently able to delay the entry into the death phase of CHOsus cultures by almost two days via the addition of 10 ng/mL CrhBMP2 to the culture medium, i.e. by a similar time span as observed for the hBMP2 producing 4D6 cells, data not shown. Israel et al. showed that mature hBMP2 binds to the cellular membrane of CHO-DUKX cells [43]. These authors already hypothesized that beyond a mere "stripping", such bound hBMP2 is internalized by the cells, possibly initiating a physiological control mechanism down-regulating protein synthesis in a manner analogous to autocrine regulation. In our case, this would explain the observed drop in product titer after a certain cultivation time even though the mRNA was still produced.

### 3.2 Transient expression of recombinant hBMP2 in HEK cells

Analysis of the cDNA sequences of hBMP2 and EGFP showed that for an expression in CHO cells the hBMP2 gene contains 73 rare and 24 very rare codons (5 of them forming a cluster), whereas the EGFP gene contains only 9 rare and 1 very rare codon. Codon optimization is a viable option in such cases, however, it would not have resolved the main problem of an interaction of the produced hBMP2 with the CHO cells. To investigate at least the possibility of improving the product titers by switching to a human system, where incidentally the hBMP2 gene still contains 43 rare and 13 very rare codons (7 rare and 1 very rare for EGFP), high-density cultures of human cells (HEK293sus) were transiently transfected with phBMP2-EGFP. In parallel, plasmids encoding either EGFP alone ($pH_2B$-EGFP) or a secreted single chain antibody (pCDNA3.1-ScFv49M) were transfected into these cells.

The development of the viable cell density in the transfected cultures was similar to that of the parent cell culture in all cases, indicating little influence of the expressed transgenes on the cells (Fig. 3A). The EGFP concentration developed as expected for transient expression in HEK293trans cells, i.e. expression was highest on day 2 and then slowly declined, due to the dilution of the episomal pDNA during cell division (i.e., diminution of the number of cells expressing EGFP). By comparison, the maximum rhBMP2 titers were reached on day 5 (20 ± 0.8 ng/mL) and day 6 (18.8 ± 3.6 ng/mL), while the hBMP2 mRNA levels peaked on day 3 (Fig. 3A). Even when compared on a per cell basis, hBMP2 productivities were thus considerably higher in the HEK293trans cells (4.5 ng per $10^6$ viable cells), than in the recombinant CHO cells (7.4 pg per $10^6$ viable cells).

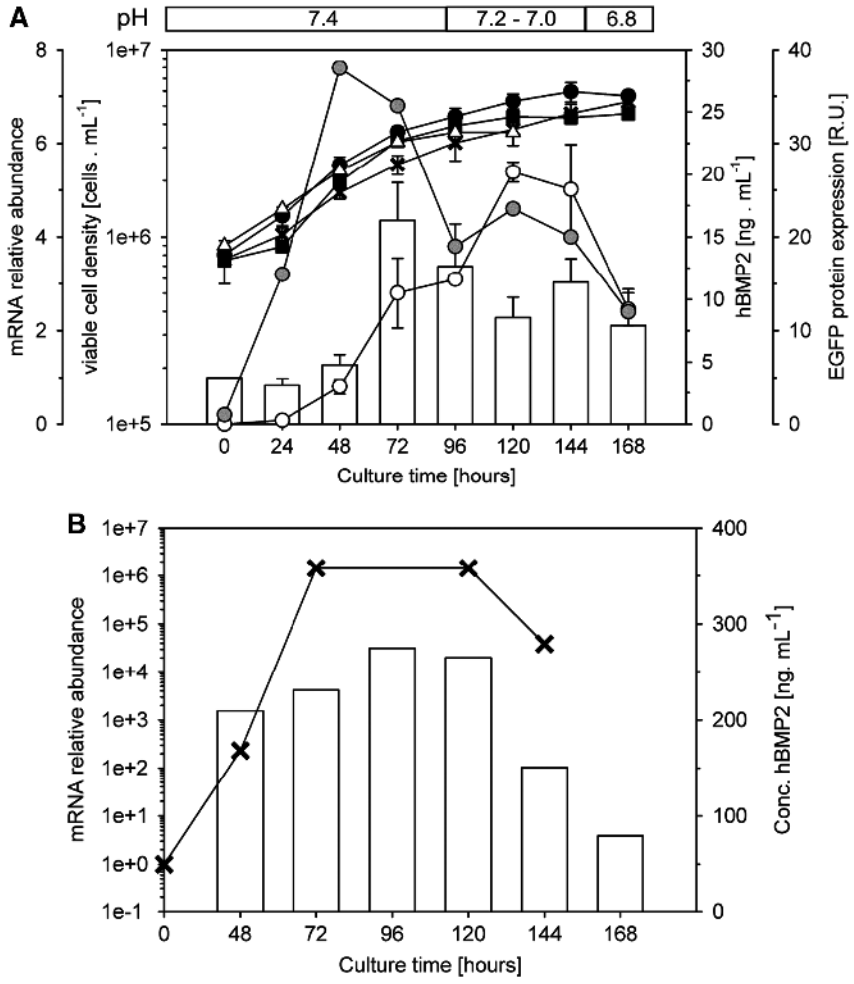


**Figure 3. Transient expression of hBMP2 in suspension adapted HEK293 cells.** (A) Cultivation/production in standard ExCell medium. **x**: growth kinetics of HEK293sus (parental cells) compared to HEK293trans transiently transfected with●: phBMP2-EGFP,■: $pH_2B$-EGFP,△: pCDNA3.1-ScFV-49M-His.○: hBMP2 titers (measured by ELISA),●: EGFP expression estimated in the cell lysates, white bars: relative abundance of the hBMP2 mRNA measured by qRT-PCR. (B) Cultivation / production in ExCell medium supplemented with 25 mM HEPES. white bars: hBMP2 titers, **x**: relative abundance of the hBMP2 mRNA measured by qRT-PCR.

This can probably be attributed to a more efficient translation (codon usage), but also to the higher DNA copy number typical for transient compared to stable transfection/expression [11, 44]. However, the fact that the maximum protein titers were reached later for hBMP2 than for EGFP, argues that even for the human cells hBMP2 is more difficult to produce than EGFP.

A pH ≥ 7.0 could be maintained in the HEK293 cultures at least up to 144 hours. Beyond that the bicarbonate buffer system of the medium was no longer able to maintain the pH above 7, which coincided again with a decrease in product titer. When additional experiments were conducted in the presence of 25 mM HEPES, it was possible to maintain a pH ≥ 7 throughout the cultivation. HEPES was chosen as it is known to stimulate ATP production and to modulate energy dependent efflux and uptake processes [45]. In the presence of HEPES, the hBMP2 titer increased to 274.9 ng/mL (Fig. 3B). The maximum hBMP2 mRNA levels were again reached on day 3, but could be maintained at that level for another 48 h. However, in spite of the stable pH, the hBMP2 concentration detected in the supernatant declined after 120 h.

### 3.3 Stability of hBMP2 in the cell culture supernatants

Product loss toward the end of batch cultivations is not unusual and typically ascribed to product instabilities. To further investigate and quantitate the effect, stability tests were done using rhBMP2 commercially produced in *E. coli* or CHO cells. The material (5 ng/mL) was incubated in ProCHO5 or ExCell medium (as such, containing 25 mM HEPES, or end-of-culture) at 4°C or 37°C. In the ProCHO5 medium almost 90 % (87.8 ± 6.9 %) of the added ErhBMP2 was still detectable after 120 h incubation at 4°C. At 37°C, on the other hand, less than 30 % (26.3 ± 3.8 %) of the material could be recovered at this point. This instability at 37°C undoubtedly also contributed to the extremely low hBMP2 titers obtained with the 4D6 cells.

For the investigated ExCell media (Table 2), no statistically significant temperature effect was observed. Recoveries after 120 h from the medium itself, however, were below 60%. Since the temperature had no effect, we excluded a (bio-)chemical degradative reaction. Instead we hypothesized that this decrease in the ErhBMP2 concentration was due to unspecific

**Table 2.** Recovery of recombinant hBMP2 after incubation in end-of-culture ExCell media

| | | Recovery (%) | | |
|---|---|---|---|---|
| | | Standard medium | HEPES-buffered (25 mM) medium | Heat-inactivated[a] medium |
| **Condition 1** | Incubation (h) | | | |
| | 120 | 10.5 ± 0.5/*14.8 ± 0.4*[b] | 83.2 ± 1.3/*85.1 ± 3.1* | n.d. |
| **Condition 2** | | | | |
| | 48 | 62.6 ± 4.8/**83.2 ± 2.2**[b] | 96.3 ± 3.4/**90.9 ± 1.9**[b] | 82.4 ± 6.2/**102.0 ± 1.7**[b] |
| | 120 | 70.2 ± 1.0/**82.6 ± 3.5**[b] | 96.9 ± 14.9/**86.6 ± 0.02** | 89.7 ± 5.6/**87.4 ± 2.4** |
| | 168 | 54.7 ± 8.4/**61.1 ± 0.7** | 75.6 ± 5.4/**65.4 ± 0.1**[b] | 65.3 ± 6.2/**67.4 ± 1.7** |

**Condition 1**: ErhBMP2 incubated in plastic tissue dishes at 37°C (regular print) and 4°C (italic print) – For comparison, in fresh ExCell medium, the recovery was 51.3 ± 7.0% at 37°C and 56.4 ± 6.3% at 4°C. **Condition 2**: ErhBMP2 (regular print), CrhBMP2 (bold print) incubated in glass dishes at 37°C. In all case, initial concentration 5 ng/mL, pH of the media ≥ 7.0. n.d.: not determined.
[a] 15 min, 95°C.
[b] Both values are statistically different (Student's *t*-test, $p<0.001$).

adsorption of the protein to the plastic tissue culture dish, as observed before for other proteins [43]. Plastic surfaces are attractive to proteins in particular at their isoelectric points (theoretical p*I* of mature hBMP2: 7.5). Addition of HEPES to the ExCell medium improved the recovery to >80%, indicating that this buffer indeed stabilizes hBMP2 in solution, perhaps due to its surfactant activity [46]. In the end-of-culture ExCell medium, recovery of ErhBMP2 was dramatically reduced (<15%). Moreover, the loss was significantly more pronounced at 37°C than at 4°C, arguing for some (bio-)chemical degradation (Table 2).

The *E. coli* derived ErhBMP2 lacks glycosylation and may therefore be more prone to degradation. Experiments with ExCell medium were thus repeated in glass dishes to reduce adsorption including also commercially available glycosylated CrhBMP2 commercially produced in CHO cells (Table 2). Recoveries were significantly improved, in particular when a HEPES supplemented or a heat inactivated (15 min, 95°C) end-of-culture medium was used. In particular in the untreated end-of-culture ExCell medium the glycosylated, CrhBMP2 was significantly more stable than the *E coli* derived material. However, neither adsorption nor active degradation can fully explain the development of the hBMP2 titers observed in the (glass) spinner batch cultivations of transiently transfected HEK293sus cells, when a HEPES-buffered ExCell medium was used.

When nontransfected HEK293sus cells ($4 \times 10^6$ cells/mL) were added (37°C) to the ExCell medium supplemented with 5 ng/mL rhBMP2, rapid stripping was observed, in particular for the glycosylated CrhBMP2 (<20% recovery after 10 min versus 50% for ErhBMP2). Expression of a putative BMP receptor has been reported for a HEK293 cell derivative (293FT cells) [47]. Just as discussed for the recombinant CHO cells above, interaction with the producer cells may thus be responsible for the loss in product titer toward the end of the HEK293sus cultivation, even though no corresponding anti-apoptotic effect is seen. In consequence, the efficient expression of hBMP2 may not be possible in mammalian cells unless extensive additional genetic engineering is done.

### 3.4 Production of hBMP2 in a cell-free mammalian expression system

Cell-free protein synthesis has been proposed as alternative for "difficult-to-express" proteins. Since native hBMP2 is glycosylated, a cell-free system based on eukaryotic cell lysates is required for its production. CHO cell lysates were chosen, since they had previously been established as the most productive eukaryotic system in our hands [24], while concomitantly enabling a direct comparison to the production of hBMP2 in cell line 4D6. A particular advantage of the cell-free approach is the possibility to directly use the PCR product for a facile, cloning-free approach to protein production [48]. When added to the cell-free reaction mixture, the PCR product (for details see Fig. 1) triggered the *de novo* synthesis of approximately 6 $\mu$g/mL of hBMP2 within 3 h, yielding a well-defined band at the expected molecular weight (49 kDa) in the autoradiogram. This corresponds to a 20-fold increase in protein yield compared to the best results from the cell-based expression systems.

Previous observations have shown that adding specific *circular* DNA templates (plasmids) to CHO cell-free protein synthesis systems instead of linear DNA, may further increase protein yields [49]. To exclude any background translational activity of the lysates, a blank control, lacking the supplemented DNA, was prepared. Whereas the Coomassie stained SDS-gel clearly indicates the presence of proteins, no corresponding signal can be seen in the autoradiogram (Fig. 4A). Besides demonstrating the lack of any endogenous translation activity, this result underlines the specificity of the CHO-based cell-free expression system. When pMK-CRPV-Mel-hBMP2 was added to the CHO cell lysates in the range of 60 $\mu$g/mL to 300 $\mu$g/mL, protein titers ranging from 1 $\mu$g/mL to 30 $\mu$g/mL were obtained (Fig. 4B). A higher plasmid DNA concentration in the cell lysates increases the template concentration for the T7 RNA polymerase. Presumably this leads to a higher abundance of the mRNA and in consequence more protein. Finally, cell-free protein *de novo* synthesis is known to be temperature dependent [21]. For hBMP2, a range of 27–30°C was optimal in terms of product titer (37 $\mu$g/mL) (Fig. 4C). At higher temperatures, hBMP2 titers decreased, most

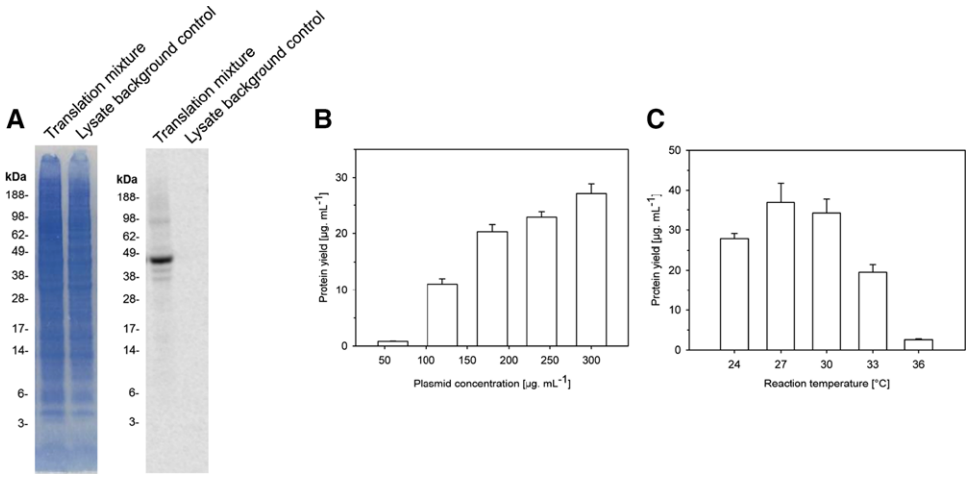


**Figure 4. Cell-free production of hBMP2 using a plasmid DNA template.** (A) SDS-PAGE (left) and autoradiography (right) of the end-of-reaction mix after incubation with (Translation mixture) and without (Lysate background control) DNA template. The migration of molecular weight standards (kDa) is indicated. (B) Yields of de novo synthesized, radio-labeled hBMP2 as a function of the plasmid concentration added to the lysates (30°C) (protein quantification by scintillation measurement of the $^{14}$C-leucine labeled hBMP2). Mean ± SD ($n$ = 3). (C) Yields of de novo synthesized, radio-labeled hBMP2 as a function of the reaction temperature (300 $\mu$g/mL pDNA) (protein quantification by scintillation measurement of the $^{14}$C-leucine labeled hBMP2). Mean ± SD ($n$ = 3).

likely reflecting a less efficient coupling of transcription and translation, as previously observed for other proteins [50].

The ability to perform post-translational modifications is a major advantage of eukaryotic systems. In this context the fact is of particular relevance that CHO lysates prepared by the protocol used here have previously been shown to contain significant amounts of endogenous microsomes derived from the endoplasmatic reticulum during lysis [26]. To enforce translocation of the target protein into the microsomal structures, a melittin signal peptide was fused to the hBMP2 cDNA. When the microsomal fraction was separated from the lysates by centrifugation, approximately 35% of the total detected hBMP2 was found there. Co-localization of larger protein aggregates into this fraction cannot be excluded. However, the accompanying

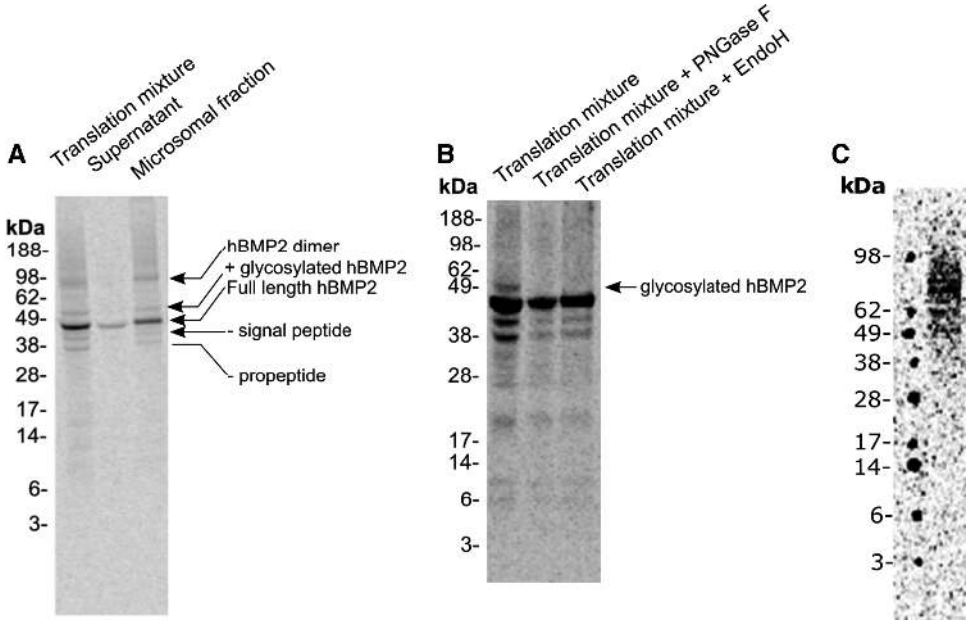


**Figure 5. Protein maturation and glycosylation of the *de novo* synthesized hBMP2.** (A) Analysis by SDS-PAGE and autoradiography of the cell-free end-of-reaction mix as well as the microsomal and non-microsomal fractions ("supernatant") thereof (separated by centrifugation). The expected positions of bands corresponding to the various hBMP2 processing stages are indicated. (B) Verification of hBMP2 glycosylation by enzymatic digestion: differential SDS-PAGE analysis and autoradiography of the original translation mixture and samples treated with deglycosylating enzymes PNGaseF and EndoH. (C) Qualitative analysis of $^{14}$C-mannose-labeled, *de novo* synthesized hBMP2 by SDS–PAGE and autoradiography. All reactions were performed at 30°C using 300 $\mu$g/mL pDNA as template.

**Table 3.** Comparative analysis of the three production systems in term of yield and specific productivity

| Production system | Total amount produced [$\mu$g] | Time to peak hBMP2 titer [h] | Peak hBMP2 titer [ng/mL] | Specific productivity [ng/(mL h] |
|---|---|---|---|---|
| Recombinant cell line (CHO) | 0.015 | 120 | 0.15 | 0.0013 |
| Transient production (HEK293) | 25 | 120 | 274.9 | 2.3 |
| Cell-free system (CHO) | 0.75 | 3 | 37000 | 12333.3 |

SDS-PAGE and autoradiogram (Fig. 5A) give no indication of pronounced amounts of BMP2 aggregates. Instead the bands can be assigned to various hBMP2 species, corresponding to the expected processing stages including, pro-peptide cleavage, signal peptide cleavage, glycosylation, and dimerization.

To verify glycosylation, the produced hBMP2 was treated with two glyco-residue removing enzymes, namely PNGaseF and EndoH (Fig. 5B). The autoradiogram shows the disappearance of a band from the high molecular weight region after enzyme treatment. To confirm the glycosylation of the protein, a sample aliquot was run in the presence of $^{14}$C-mannose instead of $^{14}$C-leucine. In that case, the $^{14}$C-mannose is incorporated into different macromolecules as judged from the gel patterns, revealing a broad fuzzy smear in which discrete bands are absent (Fig. 5C). It is therefore highly probable that the band observed in Fig. 5B corresponds to the glycosylated hBMP2 and not, e.g. to protein aggregates or other high molecular weight hBMP2 variants.

Cell-free protein synthesis increased hBMP2 titers by two orders of magnitude compared to CHO and HEK293 cell based production. The difference becomes even more dramatic, when productivities are considered (Table 3), i.e. the fact that maximum product titers are reached within 3 h in the cell-free system compared to 120 h in the cell-based ones. The performance of the cell-free system thus approaches overall productivities of bacterial hosts, where the active protein has to be renatured from inclusion bodies [51], but with full post-translational processing. However, at present cell-free production in CHO lysates is restricted in volume to the microliter scale, while 8.1 mg of DNA is required per mg of product protein. Whereas first alternatives to the small-scale batch production process exist, e.g. the continuous exchange cell-free system (CECF) [52–54], which in principle could be adapted to CHO lysates and would allow a scale up of the production, the high DNA consumption remains an unsolved problem.

## 4 Concluding remarks

Human growth factors are complex molecules, which make their production in mammalian cells desirable. However, low product titers caused by a variety of both cell and process related effects may hinder the development of highly productive processes. In such cases, cell-free protein production using CHO cell lysates containing endogenous microsomes for post-translational processing, may eventually present an attractive alternative. In particular since these lysates can be used under tightly controlled conditions assuring a higher degree of reproducibility, than, e.g. transient transfection systems. Cell-free systems are known to circumvent typical bottlenecks of cell-based ones, such as metabolic regulation and cell maintenance mechanisms. In consequence, the production of a recombinant protein is neither inhibited by its accumulation nor by any interaction with the cells, e.g. through the activation of inhibitory signaling pathways. However, the broader use of cell-free protein synthesis for production purposes would require that the challenges of scale up and of providing the high amount of DNA template are resolved.

### Practical application

Mammalian cells, in particular CHO cells, have become standard solutions for the recombinant production of complex human proteins. Yet not all proteins are easily produced in these cells, in particular when the cells interact with the product. Here production of a human growth factor (hBMP2) is investigated in a pertinent case study. The efficient production in a stably transfected CHO cell line was not possible. In consequence options und limits of alternatives such as transient expression in HEK cells and cell-free expression in CHO cell lysates were investigated. Synthesis in the CHO cell lysates was not hindered by the various phenomena impeding the cell-based systems, while comprehensive posttranslational modification (in particular glycosylation) was assured. In addition, a benchmark is supplied for CHO cell based production. Provided issues of scale up and plasmid use can be resolved, cell-free production could become a powerful alternative for other "difficult-to-express" proteins.

*Sören Blum, Florian Gruber, and Jennifer Nack produced some of the data as part of their Master theses. Nicole Andersen and Franziska Ehlicke produced some of the data as part of their Diploma thesis. Andreas Kuck provided excellent help in the lab as an intern.*

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## Permission statements

The manuscript does not contain experiments using animals or human studies

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