

## HIGH-MOBILITY GROUP PROTEIN HMGA2-DERIVED FRAGMENTS STIMULATE THE PROLIFERATION OF CHONDROCYTES AND ADIPOSE TISSUE-DERIVED STEM CELLS

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### Abstract

In previous research, it was shown that recombinant HMGA2 protein enhances the proliferation of porcine chondrocytes grown *in vitro*, opening up promising applications of this embryonic architectural transcription factor for tissue engineering, such as in cartilage repair. In this paper, we describe the development and analyses of two synthetic fragments comprising the functional AT-hook motifs of the HMGA2 protein, as well as the nuclear transport domain. They can be synthesised up to large scales, while eliminating some of the problems of recombinant protein production, including unwanted modification or contamination by the expression hosts, or of gene therapy approaches such as uncontrolled viral integration and transgene expression even after therapy. Application of one of these peptides onto porcine hyaline cartilage chondrocytes, grown in *in vitro* monolayer cell culture, showed a growth-promoting effect similar to that of the wild type HMGA2 protein. Furthermore, it also promoted cell growth of adult adipose tissue derived stem cells. Due to its proliferation inducing function and vast availability, this peptide is thus suitable for further application and investigation in various fields such as tissue engineering and stem cell research.

**Keywords:** High-mobility-group protein A2, cartilage, chondrocytes, adipose tissue derived stem cells, tissue regeneration.

### Introduction

Recombinant High-Mobility-Group A2 (HMGA2) proteins have been shown to significantly increase the proliferative activity of chondrocytes in a dose-dependent manner in an *in vitro* system of porcine origin (Richter *et al.*, 2009). Since articular cartilage defects are a major problem in patients from all ages, this opens up future possibilities to improve cell yield for *in vitro* propagation of these cells.

However, in terms of a perspective therapeutic application of this protein and its authorisation, the recombinant HMGA2 protein production process has some disadvantages, including potential unwanted modifications of the protein by the expression hosts, microbial contamination including foreign DNA and proteins, as well as further contamination e.g. from the growth medium that all need to be taken care of. To avoid these issues, we developed two peptide fragments HMGA2-A (60mer) and HMGA2-B (80mer) that are synthesised by microwave assisted solid phase peptide synthesis thus eliminating any steps involving prokaryotic or eukaryotic protein expression systems and providing the ability of large scale synthesis of these biologically active substances for future fields of application in e.g. tissue engineering. This peptide approach also enables the direct application of the active agent only during the phase of treatment, as opposed to e.g. virus based delivery systems, where uncontrolled viral integration, genomic instability, and transgene expression even after therapy may occur, and also makes possible the direct delivery of the molecules into the cells due to the small molecule nature of the developed peptides. The two peptides or fragments thereof are expected to show comparable effects to the native protein by comprising the eponymous biologically active AT-hooks of HMGA2, which bind to the minor groove of AT-rich DNA and thereby influence DNA conformation and binding of various transcription factors, finally influencing the activity of a large number of genes (Reeves and Beckerbauer, 2001).

As HMGA2 has virtually no secondary structure (Reeves and Beckerbauer, 2003), it allows for the use of synthesised peptides of parts thereof, i.e. in this case the DNA binding AT-hooks, without further modification. In previous research, it had been shown that synthetic peptides comprising only the 3 AT-hooks still possess the DNA-binding capability of the native protein (Geierstanger *et al.*, 1994); however, the focus was not set on any function besides DNA-binding. Later it was shown that truncated HMGA2 lacking the carboxy-terminus led

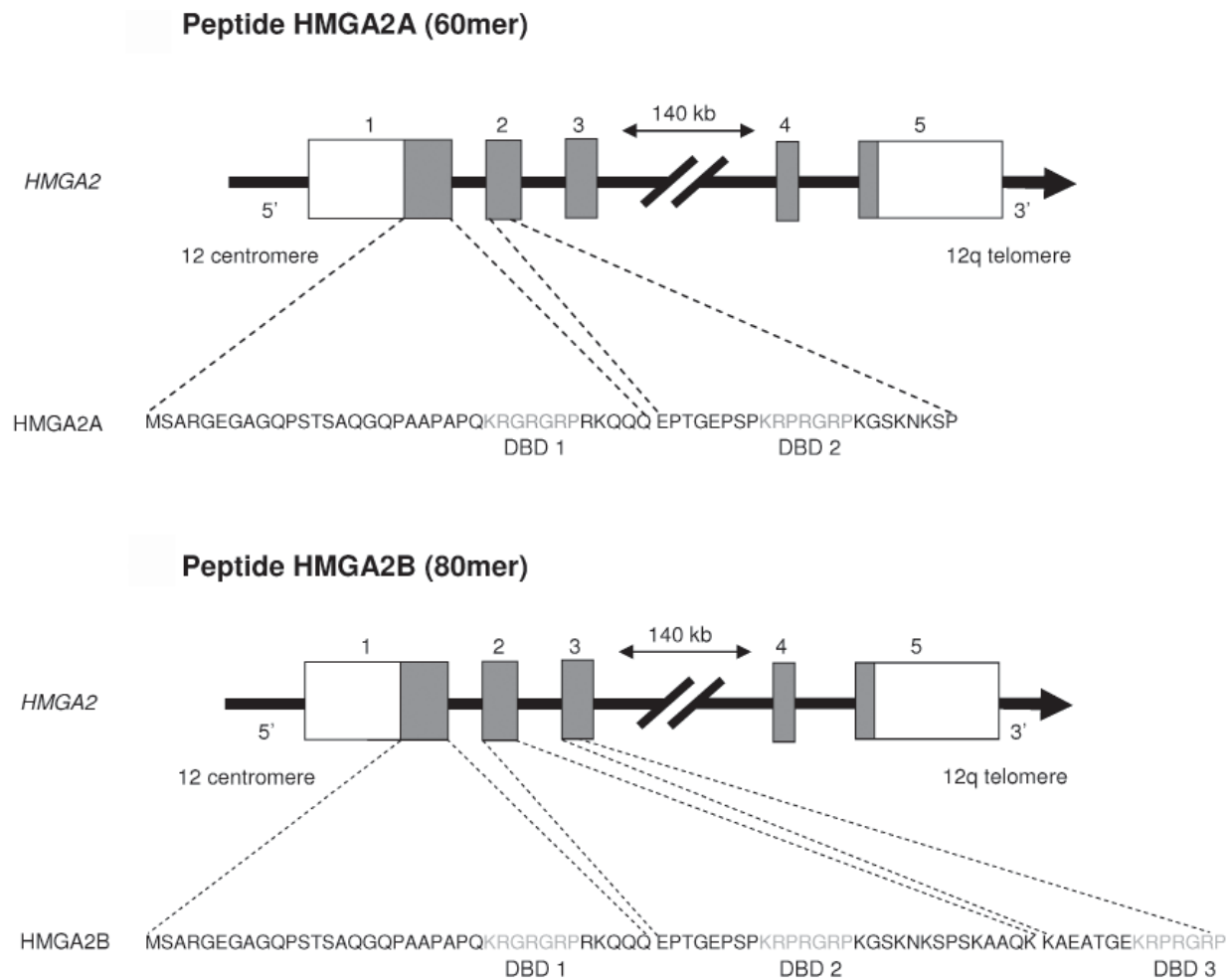
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**Fig. 1.** Amino acid sequence and structure of the two HMGA2 peptides. DBD = DNA binding domain.

to increased growth of NIH3T3 cells as compared to wild type HMGA2 (Fedele *et al.*, 1998) and remains functional in recombinant mice (Battista *et al.*, 1999).

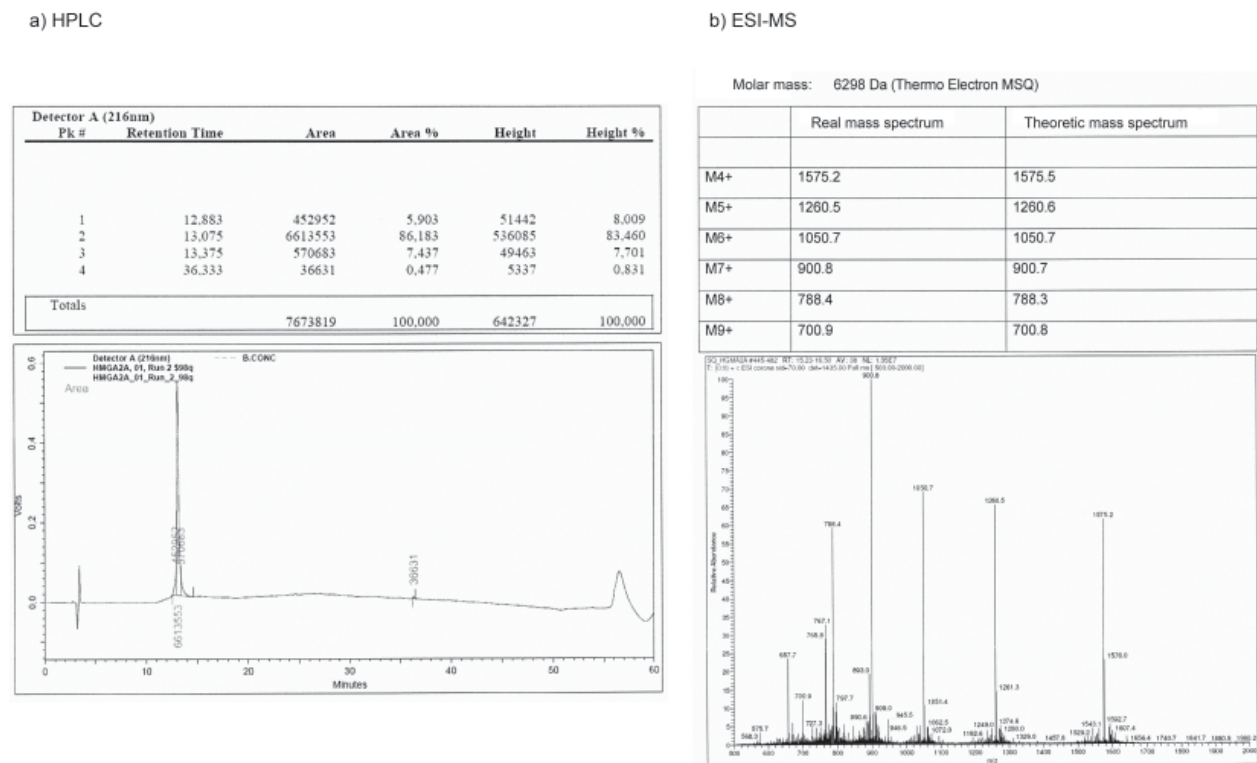
While the function of the AT-hooks is thus well-known and described (Reeves and Nissen, 1990; Geierstanger *et al.*, 1994; Chau *et al.*, 1995; Goodwin, 1998; Reeves, 2000; Cattaruzzi *et al.*, 2007; Cleynen and Van de Ven, 2008), the role of the acidic C-terminus is still not well understood, but it is assumed that it is responsible in parts, amongst others, for the protein binding capacity of HMGA2 (Noro *et al.*, 2003), and sequence specificity of DNA-binding (Yie *et al.*, 1997).

The aim of this study therefore was the evaluation of the two newly synthesised fragments of HMGA2 in respect to cell growth in the same *in vitro* cell culture system as described before (Richter *et al.*, 2009), as well as to the cell penetration / DNA-binding capacity which was determined by fluorescence microscopy of a labelled variant of the HMGA2-B peptide (HMGA2-CC). In addition to porcine chondrocytes, the peptides were also applied to canine adult adipose tissue derived stem cells (ADSCs), as these can be differentiated into chondrogenic cells in the presence of lineage-specific induction factors including insulin and TGF- $\beta$ , leading to the formation of hyaline cartilage (Zuk *et al.*, 2001; Huang *et al.*, 2004).

## Materials and Methods

### HMGA2 fragment synthesis

Two fully synthetic HMGA2 fragments (60 AA and 80 AA, Fig. 1) were synthesised at a scale of 0.25 mmol by microwave assisted solid phase peptide synthesis in an automated Liberty unit (CEM, Kamp-Lintfort, Germany). The growing peptide chain was assembled on PAL ChemMatrix resin (Matrix Innovation, Montreal, Canada). Deprotection was achieved by adding 10 mL Piperidine (5% in dimethylformamide / 0.1M HOBt (Biosolve BV, Valkenswaard, Netherlands)) and irradiation with 65 W for 3 min. Coupling of the next amino acid was achieved using a fivefold excess of reagents (amino acid, Pyclock (Livchem, Frankfurt/Main, Germany), Cl-HOBt (Biosolve, Valkenswaard, Netherlands), Diisopropylethylamine (Iris Biotech, Marktredwitz, Germany)) in 10 mL dimethylformamide and irradiation with 40 W for 5 min. All couplings were done by a double coupling procedure including capping with Z-2-Br-OSu (Iris Biotech, Marktredwitz, Germany). After washing with dichloromethane, the peptide was cleaved by adding 40 mL cleavage cocktail (94% trifluoroacetic acid, 1.0% triisopropylsilane (Sigma, Deisenhofen, Germany), 2.5% H<sub>2</sub>O, 2.5% 3,6-Dioxa-1,8-octandithiol (Sigma,



**Fig. 2.** HPLC and ESI-MS analysis of the HMGA2-A synthesis product.

Deisenhofen, Germany)) and reaction at room temperature for 3 h. The peptide was then precipitated in 160 mL cold ether, solved in acetonitrile/water and purified by Liquid chromatography-mass spectrometry (LC-MS) using a Nebula (Gilson) purification system (Column: C18 repositil 100 10  $\mu$ m 250 x 20mm; flow rate: 20 mL/min; eluent A: water and 0.1% TFA; eluent B: acetonitrile & 0.085% TFA). Analysis of the purified peptides was performed by HPLC (Column: Phenomenex Gemini 5  $\mu$ m 110 A 4.6 mm x 250 mm, eluent A: water & 0.1% TFA; eluent B: acetonitrile & 0.085% TFA; flow rate: 1 mL/min) and electrospray-ionisation mass spectrometry (ESI-MS) (Fig. 2: HMGA2-A and Fig. 3: HMGA2-B).

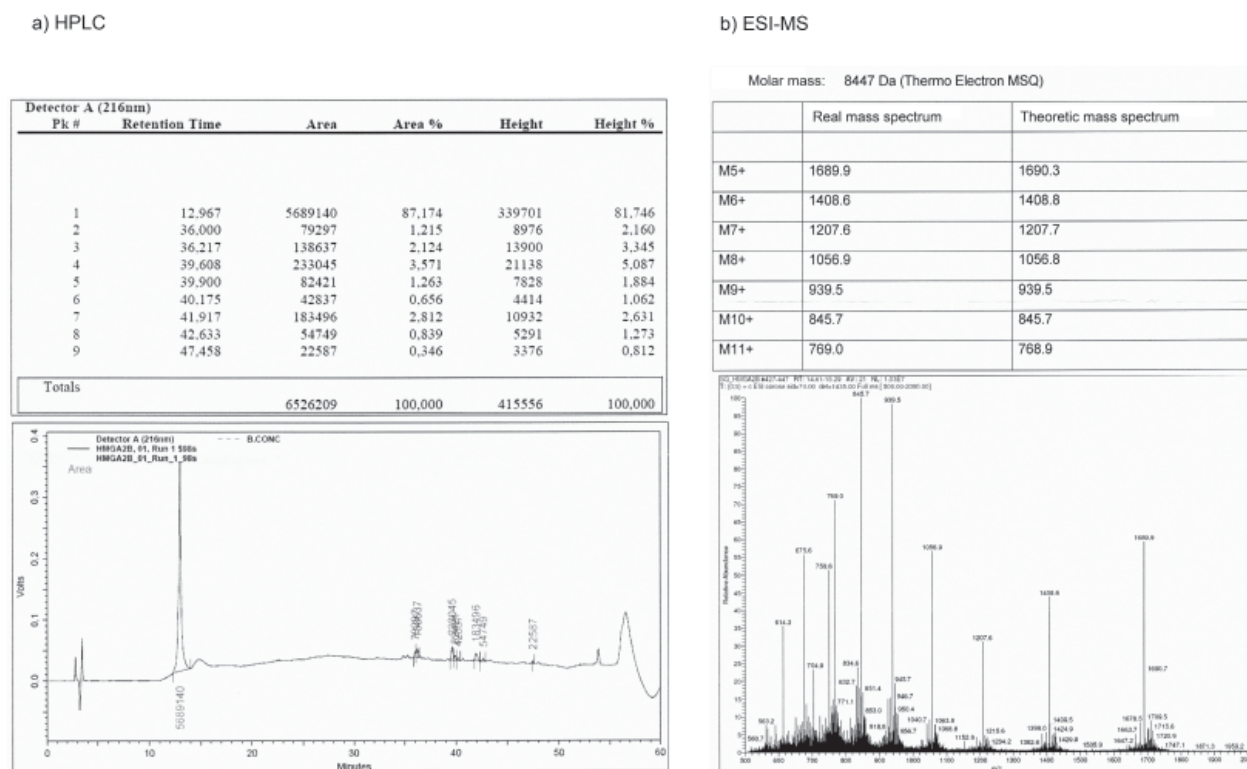
#### Synthesis of the carboxyfluorescein labelled HMGA2-B variant

The carboxyfluorescein labelled variant of the HMGA2-B peptide was synthesised as described above, with additional coupling of 5/6-carboxyfluorescein to the N-terminus at the final step of the solid phase synthesis. In this procedure, the label was coupled to the solid phase, i.e. all reactive groups excluding the N-terminus were still protected leading to selective N-terminal labelling of the peptide. For this cause, no further validation of the N-terminal coupling of the label was performed as it can be assumed from the procedure that the label can only bind to the N-terminus of the peptide. Coupling was performed by the use of N,N'-Diisopropylcarbodiimid (Sigma) / HOBt and coupling for 12 h at room temperature. The labelled fragment was then purified as described above.

#### Expression and purification of recombinant HMGA2 protein

Recombinant HMGA2 produced using the Novagen pET System (Merck Chemicals, Darmstadt, Germany) following a protocol adapted from Schwanbeck (1995) served as a positive control. The complete coding sequences of HMGA2 was inserted into the pET3a vector. Subsequently, transformation of the non-expression host *E. coli DH5 $\alpha$*  with these plasmids was performed for verification by sequencing and long term storage. Protein expression was performed with freshly transformed *E. coli BL21(DE3) Rosetta pLysS* (Merck Chemicals). A preparatory culture was grown to an OD of 0.6 in 10 mL LB broth supplied with 100  $\mu$ g/mL ampicillin, 34  $\mu$ g/mL chloramphenicol and 1% (w/v) glucose. After inoculation, 1L of expression culture (LB broth supplied with 100  $\mu$ g/mL ampicillin, 34  $\mu$ g/mL chloramphenicol and 0.5% (w/v) glucose) was grown to an OD of 0.6, followed by induction of protein expression with 1 mmol/l IPTG. Expression was performed for 120 min at 37  $^{\circ}$ C, the culture was thereafter stored on ice and then pelleted by centrifugation.

HMGA2 and other acid soluble proteins were recovered from the bacteria by performing a combined three cycle freeze (-20  $^{\circ}$ C) and thaw and acidic precipitation procedure with 3x (w/v) 5% perchloric acid, resulting in lysis of bacterial cells and precipitation of bacterial debris and non-acid soluble proteins. Following centrifugation, the HMGA2-containing supernatant was removed and acidified with 37% HCl to a final concentration of 0.35 M HCl. Precipitation of HMGA2 proteins was performed by



**Fig. 3.** HPLC and ESI-MS analysis of the HMGA2-B synthesis product.

adding 6x vol. of  $-20\text{ }^{\circ}\text{C}$  acetone. Protein was pelleted by centrifugation in 80 mL glass tubes, the acetone supernatant carefully removed, the pellet frozen to  $-80\text{ }^{\circ}\text{C}$  and dried under vacuum. Pelleted protein was resolved using 50 mM ammonium hydrogen carbonate at  $4\text{ }^{\circ}\text{C}$ . This crude acid soluble protein mix containing mostly HMGA2 was stored until further processing at  $-80\text{ }^{\circ}\text{C}$ .

Final purification of HMGA2 protein was performed using a 2-step HPLC procedure. In the first step, acid soluble bacterial proteins as well as bacterial DNA which were carried over from acetone precipitation were removed using a cation exchange column (TSKgel SP5 PW 20  $\mu\text{m}$ , Tosoh Bioscience GmbH, Stuttgart, Germany) and a gradient of 25 mM  $\text{H}_3\text{BO}_3$  pH 9.4 and 25 mM  $\text{H}_3\text{BO}_3/1\text{M}$  NaCl pH 9.4. Due to the lack of aromatic amino acid in HMGA2 protein, identification of the HMGA2 fraction was achieved using a 215 nm absorption peak proximal to the absorption maximum of the peptide bond at 205 nm. Following this first purification step, a reverse phase HPLC purification (Grom-Sil 300 ODS-5 ST, Grom, Rottenburg-Hailfingen, Germany) with a gradient of 0.1% TFA and 0.1% TFA/70% ACN of the fraction obtained was performed enabling the removal of degraded or fragmentary HMGA2, leading to a very pure fraction of intact protein. Quality control was performed by PAGE (data not shown). For removal of eluent, gravimetric quantitation, and long term storage, fraction volume was first reduced using Amicon Ultra 10000 MWCO Centrifugation devices (Millipore, Schwalbach, Germany) and then lyophilised using a freeze dryer. Gravimetric quantitation of the resulting HMGA2 pellets was performed on an analytical balance (Sartorius, Goettingen, Germany), and the pellets

frozen at  $-80\text{ }^{\circ}\text{C}$  for long term storage. A stock solution of  $10\text{ }\mu\text{g/mL}$  HMGA2 was then prepared from the lyophilisate for use in the cell proliferation assays.

### Cell culture

Chondrocytes were obtained from the knee joint of an approximately four month old pig for slaughter, which was culled for meat production at a butchery that is subject to the German animal protection law and licensed for meat production by the Federal Office of Consumer Protection and Food Safety, which implies ethical treatment of the animals slaughtered. Cartilage was carefully removed excluding the calcified inner cartilage layer using a punch and a scalpel. The cartilage pieces were further chopped and digested for 6 h under continuous shaking at  $37\text{ }^{\circ}\text{C}$  / 5%  $\text{CO}_2$  in 50% v/v 0.26% collagenase NB8 (Serva Electrophoresis GmbH, Heidelberg, Germany) and tissue culture medium (medium 199 containing Earle's salts and L-glutamine (Invitrogen, Karlsruhe, Germany), 200 IU Penicillin & 200 mg/mL Streptomycin (Biochrom, Berlin, Germany), and 10% foetal bovine serum (Invitrogen, Karlsruhe, Germany)) until the chondrocytes were released from the extracellular cartilage matrix. Following digestion, the released cells were filtered from the undigested cartilage residue, washed and transferred to a  $25\text{ cm}^2$  tissue culture flask (Nunc, Wiesbaden, Germany). They were incubated at  $37\text{ }^{\circ}\text{C}$  / 5%  $\text{CO}_2$  / 5 mL tissue culture medium as described previously until  $\frac{3}{4}$  confluency of the TC-flask was reached. This was followed by one further passage.

Canine subcutaneous adipose tissue of the abdominal region was taken during surgery from dogs admitted to the

Small Animal Clinic, University of Veterinary Medicine (Hanover, Germany). The adipose tissue was transferred into sterile Hank's solution and minced into small pieces followed by treatment with 0.26% collagenase NB8. After 1-2 h, the dissociated cells were transferred into sterile flasks containing 5 mL tissue culture medium 199 / 20% FCS. The flasks were incubated at 37 °C / 5% CO<sub>2</sub> and medium was replaced every 2-3 d. Stem cells were selected for by their adherence to the plastic surface of the cell culture flask (Dominici *et al.*, 2006) and passaged when they reached approximately 80% confluence.

#### Cell penetration assay and fluorescence microscopy

Porcine chondrocytes were seeded onto glass plates (A=1.76 cm<sup>2</sup>) positioned in a sterile plastic 12-well microtiter plate and incubated for 24 h at 37 °C/5% CO<sub>2</sub> (100,000 cells / well in 1 mL of medium 199/10% FCS). Following incubation, the medium was replaced with 0.5 mL of medium 199/10% FCS (non peptide control), 0.5 mL of medium 199/10% FCS + 10 µM HMGA2-CC, and 0.5 mL of medium 199/10% FCS + 50 µM HMGA2-CC, respectively, and the cells incubated a further 4 h at 37 °C/5% CO<sub>2</sub>. Thereupon, the medium was replaced with 1 mL of medium 199/10% FCS + 25 µg/mL DAPI + 1 µg/mL propidium iodide (PI) followed by a further incubation time of 5 min at room temperature. Cells were washed three times with 1 mL medium 199/10% FCS to remove residual stains. The glass plates were removed from the 12-well plate and embedded onto glass slides, using rubber cement, for analysis. Uptake of fluorescently labelled HMGA2-CC peptides was determined by fluorescence microscopy utilising a Carl Zeiss Axioskope and documented electronically by Axiovision 4.5 software using green (GFP, FITC), blue (DAPI), and red (PI) filters (Carl-Zeiss-AG, Oberkochen, Germany).

Canine ADSCs were subjected to a slightly modified procedure, where 50,000 cells/well in M199 + 10% FCS were seeded into a 24-well plate such that the next day a density of about 50% was achieved. The medium was removed and for each condition to be tested a solution of M199/10% FCS with HMGA2-CC (100 µM, 50 µM, 10 µM and 0 µM, respectively), bisbenzimidazole (0.1 µg/mL) and PI (1 µg/mL) was added to the cells. Cells were incubated at 37 °C for 4 h, washed in M199 and put in Hank's buffered saline solution (HBSS) for immediate analysis of fluorescence. Living cells were checked and photographed by fluorescence microscopy using green (GFP, FITC), blue (bisbenzimidazole), and red (PI) filters and the 20x objective. Overlay pictures were produced using Leica application suite software.

#### *In vitro* cell proliferation assay

The proliferative effects of HMGA2-A and HMGA2-B were examined using the BrdU cell proliferation ELISA kit (colorimetric) (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions. Conditions to be tested for porcine chondrocytes were 10 µM and 50 µM of HMGA2-A as well as 10 µM and 50 µM of HMGA2-B, with 10 µM recombinant HMGA2 and 0 µM HMGA2 serving as controls. Chondrocytes were harvested using TrypLE Express (Gibco, Karlsruhe, Germany), washed and

resuspended in fresh tissue culture medium as described previously and adjusted to 150,000 cells/mL. For each parameter or control, 8 consecutive wells of a 96-well cell culture microtiter plate (Nunc, Wiesbaden, Germany) were seeded with 7,500 cells/well in 50 µL of M199/10%FCS. Cells were left to settle for three hours at 37 °C / 5% CO<sub>2</sub>. To each test condition, dilutions of HMGA2-A and HMGA2-B as well as recombinant HMGA2 solution and the non-peptide/protein control were added to the final concentrations of 0 µM, 10 µM or 50 µM, respectively, in a total volume of 100 µL. Cells were left at 37 °C / 5% CO<sub>2</sub> for 17 h followed by the addition of BrdU. After an incubation period of 7 h at 37 °C / 5% CO<sub>2</sub>, BrdU incorporation was detected with the BioTek Synergy HT microtiter plate reader (BioTek Instruments GmbH, Bad Friedrichshall, Germany) and interpreted by spreadsheet analysis. Data was normalised using the non-HMGA2 control as reference value.

The *in vitro* cell proliferation assay of ADSCs was performed with the same BrdU ELISA procedure, with some cell type specific modifications. Conditions to be tested were 5 µM and 50 µM HMGA2-A, as well as 5 and 50 µM HMGA2-B, compared to a non peptide (0 µM) control. Cells were grown in M199/10% FCS and seeded into the wells of a 96 well flat bottom plate at a density of 10<sup>4</sup> cells in 100 µL M199 per well. Following attachment of cells to the bottom of the wells, the culture medium was replaced with M199/1%FCS which was left on the cells for 24 h. Finally, this was replaced with M199/1%FCS containing the peptides at the concentrations mentioned above as well as the non peptide control. After 6 h of incubation, BrdU was added to each well to a final concentration of 10 µM. Another incubation of 18 h followed after which the measurement of cell proliferation was performed as described in the chondrocyte section.

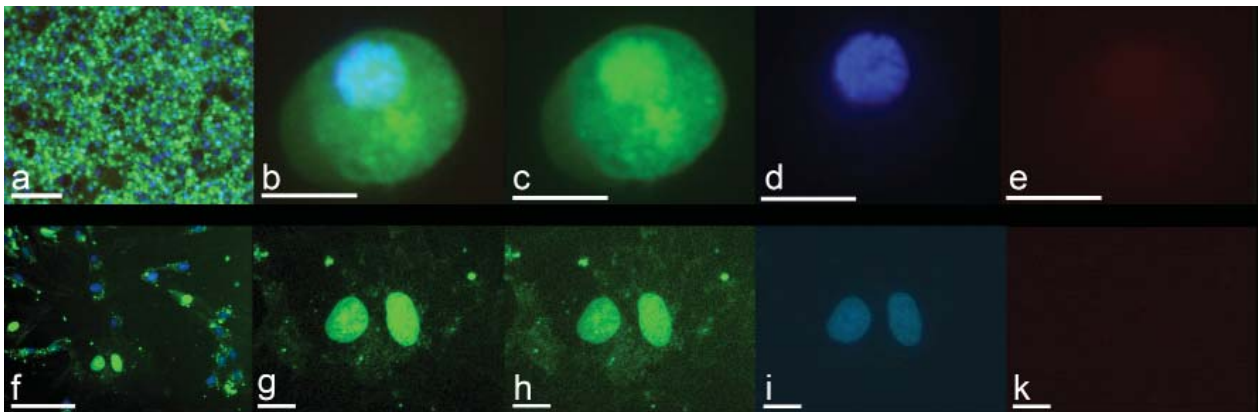
#### Statistics

Statistical analysis was performed with GraphPad Instat and GraphPad Prism 5 software (GraphPad Software, La Jolla, CA, USA) using the one-way ANOVA (*ANALYSIS Of VARIANCE*), with pairs of group means being compared using the Tukey-Kramer Multiple Comparisons Test. The significance level was set at  $P < 0.01$ . Identity of standard deviations was checked using Bartlett's test, and Gaussian distribution of sample data was tested by the Kolmogorov-Smirnov method.

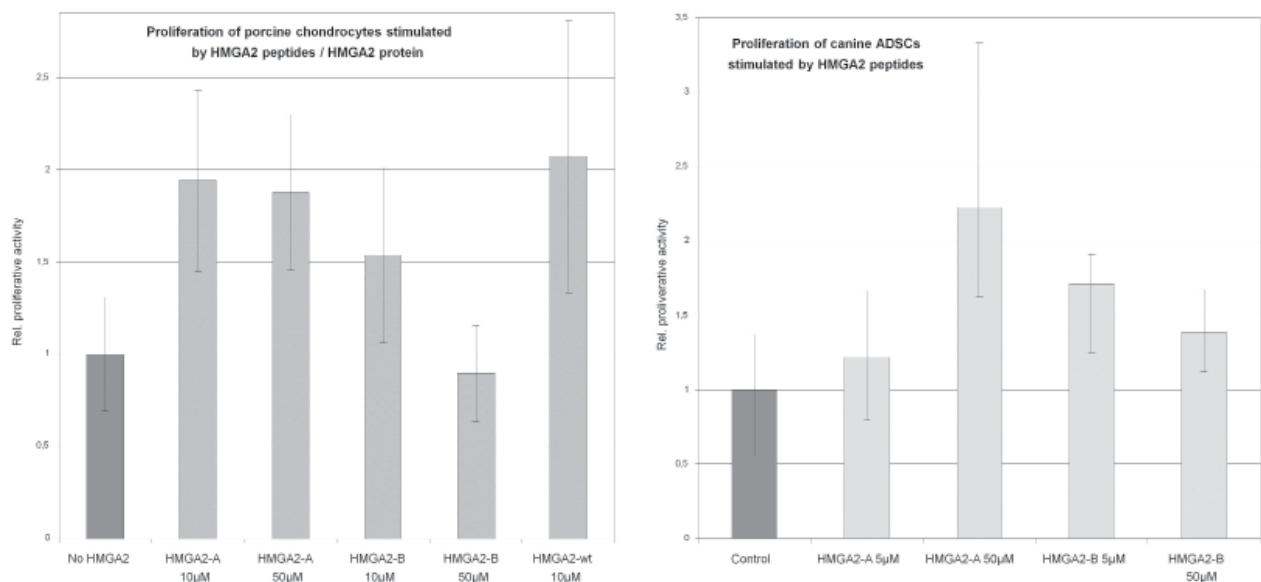
## Results

#### Cell penetration assay

By fluorescence microscopy, a green stain caused by the accumulation of fluorescently labelled HMGA2-CC peptides was detected in the nuclei of 51% of chondrocytes for both cells incubated with 10 µM peptide (not shown) and 50 µM peptide (Fig. 4 a-e), respectively, and in 24% of ADSCs at 100 µM peptide (Fig. 4 f-k, 10 µM and 50 µM peptide not shown). Green fluorescence could also be detected in the cytoplasm of most chondrocytes and ADSCs, even when no intensely stained green nuclei were present. Nevertheless, all cells displayed the typical blue



**Fig. 4.** Transfection of porcine chondrocytes (**a-e**) and canine ADSCs (**f-k**) in *in vitro* monolayer cell culture with the fluorescently labelled HMGA2-CC peptide. (**a**) Overview of porcine chondrocytes showing the green fluorescence of HMGA2-CC in 51% of the cells as well as the blue DAPI stain in the nuclei of all cells. (**b**) Single chondrocyte with combined green HMGA2-CC and blue DAPI stains. (**c**) Single chondrocyte with green HMGA2-CC stain showing the presence of HMGA2-CC in the cytoplasm as well as the nucleus. (**d**) Single chondrocyte with blue DAPI stain in the nucleus. (**e**) Single chondrocyte showing no signs of cell damage or death as no PI stain could be detected. (**f**) Overview of canine ADSCs showing the green fluorescence of HMGA2-CC in 24% of the cells as well as the blue DAPI stain in the nuclei of all cells (**g**) two ADSCs with combined green HMGA2-CC and blue DAPI stains (**h**) green channel only (HMGA2-CC) (**i**) blue channel only (DAPI), (**k**) red channel only (PI). Scale bars: 100  $\mu$ m in **a** and **f**; 10  $\mu$ m in **b-e**; 20  $\mu$ m in **g-k**.



**Fig. 5.** Cell proliferation of porcine hyaline cartilage cells (**a**) and canine ADSCs (**b**) induced by the addition of HMGA2 peptides. Values obtained by the microtiter plate reader were normalised to the absorbance of the non-HMG control which was set as 1. Error bars: standard deviation.

DAPI or bisbenzimidine stain in the nucleus. No dead cells were detected as revealed by lack of simultaneous red fluorescence (PI) of the nuclei.

#### Colorimetric Cell Proliferation ELISA

Proliferation of porcine chondrocytes and canine ADSCs in response to two different peptides of human HMGA2, as well as wild type HMGA2 protein in case of the chondrocytes, was examined in *in vitro* ELISA-based assays. For comparability, the absorbance values obtained from the microtiter plate reader were normalised to the values of the non-HMGA2 control, which was set to a value of 1 (Fig. 5).

To see if the data were suitable for ANOVA, they were subjected to the Bartlett's test which did not show significant differences between the standard deviations of the different groups in both the chondrocyte and ADSC assays. Gaussian distribution of data was tested by the Kolmogorov-Smirnov method with all populations passing the normality test. Deducing from these test results, data were usable for ANOVA. The one way ANOVA resulted in a  $P$  value  $<0.0001$  for both chondrocytes and ADSCs, showing that variation among column means is significantly greater than expected by chance. For determination of group differences, pairs of group means were compared

**Table 1.** Tukey-Kramer Multiple Comparisons on the effect of different HMGA2 peptides and wild type HMGA2 protein on the proliferation of porcine chondrocytes (a), as well as HMGA2 peptides on the proliferation of canine ADSCs (b). Grey cells indicate significant differences with a *P* value < 0.01.

(a)	Control	HMGA2-A 10 $\mu$ M	HMGA2-A 50 $\mu$ M	HMGA2-B 10 $\mu$ M	HMGA2-B 50 $\mu$ M	HMGA2- wt 10 $\mu$ M
Control						
HMGA2-A 10 $\mu$ M	< 0.01					
HMGA2-A 50 $\mu$ M	< 0.01	> 0.05				
HMGA2-B 10 $\mu$ M	> 0.05	> 0.05	> 0.05			
HMGA2-B 50 $\mu$ M	> 0.05	< 0.01	< 0.01	> 0.05		
HMGA2-wt 10 $\mu$ M	< 0.001	> 0.05	> 0.05	> 0.05	< 0.001	

(b)	Control	HMGA2-A 5 $\mu$ M	HMGA2-A 50 $\mu$ M	HMGA2-B 5 $\mu$ M	HMGA2-B 50 $\mu$ M
Control					
HMGA2-A 5 $\mu$ M	> 0.05				
HMGA2-A 50 $\mu$ M	< 0.001	< 0.001			
HMGA2-B 5 $\mu$ M	< 0.01	> 0.05	> 0.05		
HMGA2-B 50 $\mu$ M	> 0.05	> 0.05	< 0.01	> 0.05	

using the Tukey-Kramer Multiple Comparisons Test as shown in Table 1.

Compared to the non-HMGA2 control, whose proliferative activity was set as 1 for comparability, a statistically significant increase in proliferation could be detected in chondrocytes treated with either HMGA2-A peptides at both 10  $\mu$ M (1.94x increase) and 50  $\mu$ M (1.88x increase), or wild-type HMGA2 protein (10  $\mu$ M, 2.1x increase). However, no dosage-dependency could be detected for the HMGA2-A peptide, as there was no statistically significant difference of the measured data for both 10  $\mu$ M and 50  $\mu$ M. There was also no significant difference in the proliferation inducing activities of wild type HMGA2 and 10  $\mu$ M or 50  $\mu$ M HMGA2-A peptide, respectively, indicating a similar proliferation enhancing effect of the HMGA2-A peptide to the wild type protein.

The measured data for the HMGA2-B peptides, on the other hand, showed no statistically significant differences at both 10  $\mu$ M (1.54x increase) and 50  $\mu$ M (0.90x decrease) compared to the non-HMGA2 control, indicating this peptide does not influence the proliferation

of chondrocytes as intended. However, there was also no significant difference between HMGA2-B at 10  $\mu$ M and HMGA2-wt, while HMGA2-B at 50  $\mu$ M significantly differed from the wild type protein, even leading to a slight decrease in proliferative activity. This indicates at least some proliferation enhancing effect of the HMGA2-B peptide on the cells that is comparable to wild-type HMGA2, albeit only at the lower concentration, with the effect being diminished or rather reversed at the higher concentration of 50  $\mu$ M.

Concerning ADSCs, there was a highly significant increase of proliferation compared to the untreated control at the higher concentration of 50  $\mu$ M HMGA2-A, but not at the lower concentration of 10  $\mu$ M. In contrast, only the treatment of ADSCs with 5  $\mu$ M HMGA2-B provided a statistically significant increase of proliferative activity compared to the control, but not the higher concentration of 50  $\mu$ M, which even led to a significantly decreased proliferation of cells compared to the HMGA2-A peptide at 50  $\mu$ M.

## Discussion

Recombinant proteins of the human High-Mobility-Group A (HMGA) family including HMGA2 had been shown to significantly increase the proliferative activity of chondrocytes in a dose-dependent manner in an *in vitro* system utilising cells of porcine origin (Richter *et al.*, 2009). These embryonic proteins are usually absent in fully differentiated adult cells (Rogalla *et al.*, 1996); however, their application to adult chondrocytes led to enhanced growth opening up future possibilities e.g. in hyaline cartilage repair (Richter *et al.*, 2009). To overcome problems caused by e.g. viral delivery of the target gene sequence to cells, which is subject to several factors such as gene dosage, integration into the host genome, and expression of the transgene even after therapy, as well as obstacles of recombinant protein expression in bacterial or eukaryotic expression systems, we developed two synthetic fragments HMGA2-A and HMGA2-B comprising the biologically active parts of HMGA2, the so-called AT-hooks (Reeves and Nissen, 1990; Geierstanger *et al.*, 1994; Chau *et al.*, 1995; Goodwin, 1998; Reeves, 2000; Cattaruzzi *et al.*, 2007; Cleynen and Van de Ven, 2008). These peptides can be delivered directly to the affected areas and are eliminated over the course of time when not re-applied as needed by therapy, solving the problems mentioned. For assessing their function, the peptides were tested on porcine chondrocytes for their cellular localisation as well as their effects on proliferation in comparison to a non-HMGA2 control and recombinant HMGA2 protein in the same *in vitro* cell culture system as described before (Richter *et al.*, 2009). In addition, the new peptides were also evaluated on canine adipose tissue derived stem cells, as ADSCs can be differentiated into chondrogenic cells in the presence of lineage-specific induction factors (Zuk *et al.*, 2001; Huang *et al.*, 2004), and are thus another important subject in the field of cartilage repair. Due to HMGA2 proteins being highly conserved between mammals, especially in regard to the functional motifs of the AT-hooks, the sequence of the human HMGA2 were chosen for both peptide and protein. This allows for the use of only one peptide variant across several species as e.g. pig, dog, and human.

Most likely, the detected internalisation of the fluorescently labelled variant of the HMGA2-B peptide (HMGA2-CC) into the nucleus of chondrocytes as well as ADSCs, although the fragment lacks the carboxyterminal acidic part of the wild type protein, is due to the nuclear localisation signal on the second AT-hook of the HMGA2 peptide. From this it can be assumed that either the complete peptide or at least a fragment thereof comprising the labelled N-terminus up to the second AT-hook (this would correspond somehow to the shorter HMGA2-A peptide which lacks the 20 C-terminal amino acids of HMGA2-B including AT-hook 3) are located into the nucleus, as the presence of the first AT-hook alone is not sufficient for nuclear transport, while the presence of the second AT-hook is necessary (Cattaruzzi *et al.*, 2007). The fluorescence found in the cytoplasm would then be caused by either or both intact peptides not yet transported into the

nucleus as well as peptide fragments lacking the nuclear transport signal of the second AT-hook.

However, concerning cell proliferation, the administration of recombinant HMGA2 to porcine chondrocytes could reproduce the same positive effect on proliferation as described before (Richter *et al.*, 2009), as there was highly significant difference in proliferation to the untreated control. The synthetic HMGA2-A peptide led to a comparable effect on proliferation to the wild type protein as there was no statistically significant difference in cell proliferation at both 10  $\mu$ M and 50  $\mu$ M HMGA2-A compared to HMGA2-wt, leading to the conclusion that it is an adequate alternative to the recombinantly produced protein. On the other hand, HMGA2-B did not show any significant difference to the untreated control group at both 10  $\mu$ M and 50  $\mu$ M, and only had comparable effect to the wild type protein at 10  $\mu$ M, while there was a statistically significant lower proliferation at 50  $\mu$ M, showing that this larger peptide might probably be detrimental to chondrocyte proliferation at higher levels. The fact that no increase (HMGA2-A) or rather a decrease (HMGA2-B), respectively, occurs at 50  $\mu$ M compared to 10  $\mu$ M, might also indicate that there is saturation of cells or their DNA, respectively, at higher concentrations of the peptides, which in case of HMGA2-B might also inhibit cellular processes required for proliferation. As the peptides bind to the minor groove of AT-rich DNA, there are probably only a limited number of binding sites available for the peptide molecules which might be occupied from a certain concentration, leading to either no or detrimental effects on the cells.

Concerning ADSCs, a beneficial effect on the proliferation of these cells could be detected as well, at least at the higher concentration of 50  $\mu$ M HMGA2-A. On the other hand, the larger HMGA2-B peptide caused a significant increase of proliferative activity only at the lower concentration of 5  $\mu$ M, while the higher dose of 50  $\mu$ M led to a reduced proliferation compared to 5  $\mu$ M HMGA2-B, which is similar to the results obtained from chondrocytes, albeit at a lower degree. Differences in response to HMGA2 peptides between chondrocytes and ADSCs – besides comparing distinct cell types – might probably be due to possible different chromatin stages of these cell types. For example, anti-proliferative activities of HMGA2 have been described depending on the cellular context (Narita *et al.*, 2006).

However, the proliferative effects evoked by the HMGA2-A peptide open up further possibilities in e.g. tissue engineering, comparable to those of HMGA2-wt. Due to the presence of two AT-hooks and the nuclear localisation signal, this peptide comprises similar functions to the recombinant protein, but instead can be synthesised up to industrial scale without the need of recombinant expression systems. Thus, this peptide as a proliferation inducing/enhancing agent is of great importance for future applications in e.g. cartilage repair and further tissue engineering applications, as well as in further fields such as e.g., stem cell research.



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**Editor's Note:** Since all questions/comments by the reviewers were answered by text changes, there is no "Discussion with Reviewers" section.