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High Mobility Group Proteins in Tissue Regeneration

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1. Introduction

Articular cartilage damages affect a large number of the human population including both young and elderly patients, as well as domestic animals and livestock. Structure and disease thereof were described scientifically as early as in the 18th century by William Hunter (1742), indicating problems in cartilage regeneration that are still present today: “If we consult the standard Chirurgical Writers from Hippocrates down to the present Age, we shall find, that an ulcerated Cartilage is universally allowed to be a very troublesome Disease; that it admits of a Cure with more Difficulty than carious Bone; and that, when destroyed, it is not recovered”.

The lack in cure or recovery is thus a major aspect in the treatment of cartilage damage, as articular cartilage usually does not achieve *“restitutio ad integrum”*, but instead often develops further deterioration during the course of the disease (Hunziker 2002). Cartilage regeneration is hampered by the specific tissue properties of articular cartilage. In adult humans, only about one percent of cartilage volume is made of cells, the other 99% being the avascular and aneural extracellular matrix (Buckwalter et al. 1997). In case of damage, this aggravates the healing process, which in vascularised tissues is usually dependent on processes involving blood supply. In these tissues, response to trauma, e.g. a cut, is almost always divided into three subsequent stages, i.e. necrosis, inflammation, and repair. Necrosis due to cellular damage and the resulting loss of blood supply in the affected tissue triggers inflammation. This in turn leads to increased blood flow, dilatation of vascular channels, increase of vessel wall permeability, as well as onset of further processes that finally fill up the extracellular spaces in the traumatised area, e.g. through the formation of fibrin clots. Finally, repair processes including recruiting of undifferentiated cells and new cell growth lead to repair or regeneration of the injury (Mankin 1982). In articular cartilage, on the other hand, these processes do not occur due to the avascular nature of the tissue. In case of injuries that do not affect the subchondral bone, necrosis happens in the cartilage tissue after injury, but no inflammation arises. Additionally, there are no processes supporting the formation of repair tissue or recruiting of undifferentiated cells to fill up the defect, leaving the repair to the small number of chondrocytes already present in the cartilage tissue (Mankin 1982). While chondrocytes proliferate and increase matrix synthesis near the injury as a response to tissue damage, this is not sufficient to fill up the defective area (Buckwalter 1998). In injuries involving the vascular subchondral plate though, cells, growth factors, and cytokines may migrate into the lesion and initiate processes

that lead to the formation of fibrocartilage to fill up the defect (Frenkel et al. 2004). Nevertheless, the newly built fibrocartilage is less resilient than the original articular cartilage (Coletti et al. 1972) and is thus prone to premature wear leading to further damage of the affected joint.

Cartilage damage can occur due to multiple causes, including trauma e.g. from accidents, high impact sports (Buckwalter 2003), repetitive loading (Clements et al. 2001), defective positions of joints, e.g. patellar malalignment (Vasiliadis et al. 2011), muscle weakness (Herzog et al. 2003; Herzog et al. 2007) and ligament injuries (Hunziker 2002), as well as sex and weight (Ding et al. 2006). Another factor for cartilage damage is age, where age-related modifications due to senescence of the muscosceletal system (Aigner et al. 2004; Loeser 2009) are playing an important role. Finally, disease affecting the underlying bone may lead to cartilage damage, as in case of Osteochondritis dissecans (OD). OD occurs in humans, but is also present in e.g. dogs (Newton et al. 1985), horses (Riley et al. 1998), and pigs (Busch et al. 2011).

Once damaged, cartilage is prone to further degeneration due to the poor healing capability and in addition, initial cartilage damage may also lead to the onset of secondary osteoarthritis (OA) (Buckwalter et al. 1996; Buckwalter et al. 1997; Ding et al. 2006), although the exact mechanisms thereof are not yet clear. However, besides these “mechanic” impacts, OA can also be caused without any preceding injury (primary OA), with risk factors including ethnicity, hormonal status, bone density, nutritional factors, and genetics (Felson et al. 2000).

Several therapeutic strategies were developed for treating cartilage defects, which were initially based on surgery or arthroscopy alone, including cleaning of the affected joints, abrasion, debridement, Pridie drilling, or spongialisation, some of them trying to trigger the natural tissue healing response (Hunziker 2002). Transplantation techniques were developed in addition, e.g. by transplanting cartilage from lesser loaded areas into the defect (autologous osteochondral grafting, autologous osteochondral mosaicplasty) (Hangody et al. 1997; Hangody et al. 2008). However, lack of intact transplantable cartilage tissue and problems deriving from surgery are still a challenge in this kind of cartilage regeneration, as transplantation of cartilage or cartilage and bone plugs do not lead to complete recovery in the regions where the plugs are positioned. The transplanted cartilage does usually not integrate well into the adjacent intact cartilage, and spaces between the plugs do not get filled (Lane et al. 2001; Kock et al. 2004). Furthermore, necrosis may occur at the explantation sites (McGregor et al. 2011).

Another strategy that has thus emerged in cartilage regeneration in the last couple of years is the use of tissue engineering methods, trying to overcome some of the problems mentioned above. In tissue engineering, combined developments in biology, material science, engineering, manufacturing, and medicine are used in biologically based mechanisms (as opposed to mechanical devices) to achieve healing of damaged and diseased tissues (Hardingham et al. 2002). In case of cartilage, autologous chondrocyte transplantation (ACT) is used to amplify chondrocytes *in vitro*, easing the lack of chondrocytes/cartilage to fill up the defect (Brittberg et al. 1994). Chondrocytes are harvested from lesser loaded areas too, but in contrast to autologous chondral grafting, cells are amplified in *in vitro* cell culture first and subsequently implanted into the defective area. Second generation ACT includes cell-seeded bioabsorbable and biodegradable scaffolds (e.g. alginate beads, chitosan, or hybrids thereof) to provide the cells with growth conditions mimicking their natural localisation in the extracellular matrix of articular cartilage (Guo et al. 1989; Iwasaki et al. 2004). Further enhancements are the use of cartilage-specific growth factors (single factors or combinations thereof) and/or specialised growth media, trying to achieve faster growth rates as well as improved regeneration and maintenance of the chondrogenic state of the cultured cells, which usually de-differentiate when grown in culture (Harrison et al. 2000; Blunk et al. 2002; Malpeli et al. 2004), and the advent of stem-cell based procedures for cartilage formation (Sekiya et al. 2002). However, ACT still has room for improvement as there is a number of patients with complications arising from ACT (Harris et al. 2011).

In regard to chondrocyte yield for ACT, besides factors such as IGF1 (Insulin-like growth factor 1 (somatomedin C)), IL4 (Interleukin 4), or PDGF (Platelet-derived growth factor), the use of embryonic proteins usually involved in developmental processes might be a promising strategy for increase of growth rates. This might be especially valid for proteins of the high mobility group (HMG) family, as variants thereof were shown to be involved in processes of chondrogenic differentiation (Kubo et al. 2006).

HMG proteins are small (<30kDa) nuclear proteins that were first discovered in 0.35M NaCl-extractable proteins from calf thymus chromatin (Goodwin et al. 1973). They were named according to their mobility in the electric field in acid polyacrylamide gel electrophoresis (PAGE), and could be separated from the Low Mobility Group proteins of the extracts due to their solubility in 2% trichloroacetic acid (Goodwin et al. 1973). HMGB1 and HMGB2 (formerly named HMG1 and HMG2) were the first proteins to be isolated and characterised from this fraction (Goodwin et al. 1973), which was followed by the isolation of HMGB3 (formerly HMG3) and

HMGN2 (formerly HMG17) (Goodwin et al. 1975), and HMGN1 (formerly HMG14) (Goodwin et al. 1977). Later, HMGA1a (formerly HMGI) and HMGA1b (formerly HMGY) proteins were discovered in HeLa-S3 cells (Lund et al. 1983), and were confirmed to be splicing variants of the *HMGA1* (formerly *HMGI(Y)*) gene (Johnson et al. 1989). Furthermore, HMGA2 (formerly HMGIC) was detected in extracts from virus transformed cells (Giancotti et al. 1985; Goodwin et al. 1985; Giancotti et al. 1987).

HMG proteins can be divided into distinct protein families that are defined by the respective functional domains of the proteins. The functional motif also served as the basis for the new HMG nomenclature that was established when it became obvious that the initial protein names were causing confusion due to e.g. the similarity in their names (Bustin 2001), as for example in the case of HMG1 and HMGI. The root symbols chosen for the HMG protein families were thus HMGA for the AT-hook proteins (e.g. HMGA1, HMGA2), HMGB for proteins containing HMG-boxes (e.g. HMGB1, HMGB2, HMGB3), and HMGN for proteins with a nucleosome binding domain (e.g. HMGN1, HMGN2).

HMGA proteins selectively bind to the minor groove of AT-rich deoxyribonucleic acid (DNA), but with no sequence specificity (Solomon et al. 1986). DNA binding is enabled by the so-called AT-hooks, which are highly conserved regions that are closely related to the consensus amino acid (aa) sequence of T-P-K-R-P-R-G-R-P-K-K found in all HMGA proteins (Reeves et al. 1990). Proteins of the HMGB family bind to DNA facilitated by their highly conserved HMG-boxes. DNA binding is sequence independent, but structure specific, e.g. when binding to four-way junctions (Bianchi et al. 1992). HMGN proteins bind to the nucleosome core particle by use of their nucleosome binding domain, in particular to nucleosomal DNA and histone H2A (Abercrombie et al. 1978; Cook et al. 1989; Crippa et al. 1992).

Concerning their function, although distinguished by their different functional domains, all HMG family proteins were named architectural transcription factors due to their involvement in transcription regulation by influencing DNA and protein structure and DNA-/protein interaction. For example, HMGA1 proteins were shown to play a key architectural role in the assembly and stability of transcription enhancer complexes in both the *IFNB1* (interferon, beta 1, fibroblast) and *SELE* (selectin E) gene promoters (Whitley et al. 1994), while it was found out that HMGA2 enhances NF-kappaB (Nuclear factor NF-kappa-B p50/p65 heterodimer) mediated transcriptional activation through interacting with the PRDII (positive regulatory domain II) element of the *IFNB1* enhancer (Mantovani et al. 1998). HMGB1 was shown to modulate DNA structure by bending, thus facilitating the formation of

higher-order DNA-protein structures (Grosschedl et al. 1994), and HMGN proteins were suggested to be architectural elements which assist in the assembly of an unfolded chromatin fibre thereby decreasing the repressive activity of histones and facilitating transcriptional processes (Bustin et al. 1995).

Thus, while not being involved in direct transcription processes, by binding DNA and proteins and thus influencing their binding and structure, they are involved in the regulation of a large number of genes, for example those that are target genes of the NF-kappaB pathway (Henriksen et al. 2010), which correlates with the findings of Mantovani et al. (1998) mentioned above.

Besides their initially known nuclear function in transcription regulation, further research on HMG proteins revealed additional extracellular functions in case of HMGB1. It was shown that HMGB1 promotes neurite outgrowth (Merenmies et al. 1991), is a late mediator of endotoxin lethality in mice (Wang et al. 1999), and also plays a role in other inflammatory processes (Scaffidi et al. 2002). Later it was revealed that HMGB1 is involved in angiogenesis when it is released from necrotic cells e.g. in hypoxic regions of growing tumours (Schlueter et al. 2005). HMGA2 was recently shown to be highly expressed in human embryonic stem (hES) cells (Li et al. 2006), acting as a regulator of human genes linked to mesenchymal cell differentiation, adipogenesis, and hES cell growth (Li et al. 2007), as well as regulating chromatin structure and maintenance of the undifferentiated cell state (Pfannkuche et al. 2009), thus having influence on the cells' "stemness". However, in mouse embryonic fibroblasts (MEF), it was shown that down-regulation of *Hmga2*, amongst other MEF-specific genes, increased the efficiency of induced pluripotent stem cell generation (Yang et al. 2011).

As HMG proteins are involved in transcriptional regulation, with HMGA proteins being usually absent or only expressed at very low levels in non proliferating adult tissues (Rogalla et al. 1996), deregulation of HMG expression has widely been associated with the formation of both benign and malignant tumours (Hess 1998; Zhou et al. 1998; Tallini et al. 1999; Wisniewski et al. 2000; Reeves et al. 2001; Evans et al. 2004; Sgarra et al. 2004; Fusco et al. 2007; Young et al. 2007; Cleynen et al. 2008; Fedele et al. 2010; Tang et al. 2010). Intron 3 of the *HMGA2* gene was shown to be the most frequent target of chromosomal aberrations in human tumours, leading to truncated HMGA2 or fusion proteins with other ectopic sequences (Kazmierczak et al. 1998).

Concerning HMGA, especially mechanisms of *HMGA2* gene regulation and de-regulation were studied in detail, showing an involvement of the 3' untranslated region (UTR) (Borrmann et al. 2001), which has been linked to interaction with micro-

ribonucleic acids (miRNAs) of the *let-7* family (Lee et al. 2007; Mayr et al. 2007). Binding of *let-7* miRNAs to several regions of the 3'-UTR led to degradation of *HMGA2* messenger ribonucleic acid (mRNA) and thus suppression of HMGA2 protein levels (Lee et al. 2007). Loss of *HMGA2* repression by *let-7*, e.g. through truncation of the 3'-UTR of *HMGA2* or reduced *let-7* miRNA levels can thus lead to *HMGA2* re-expression due to reduced *HMGA2* mRNA degradation and is associated with oncogenic transformation (Lee et al. 2007; Mayr et al. 2007). Involvement of miRNAs in *HMGA1* regulation has been described also (Kaddar et al. 2009; Palmieri et al. 2011; Wei et al. 2011). The re-expression of *HMGA2* and further *let-7* regulated genes such as *IGF2BP1* (insulin-like growth factor 2 mRNA binding protein 1), *LIN28B* (lin-28 homolog B (C. elegans)), as well as *KRAS* (v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog), *NRAS* (neuroblastoma RAS viral (v-ras) oncogene homolog), and *MYC* (v-myc myelocytomatosis viral oncogene homolog (avian)) was shown to lead to a process termed "reverse embryogenesis" (Johnson et al. 2005; Park et al. 2007; Peter 2009), meaning that their presence in adult cells promotes dedifferentiation and increase of "stemness", which, if uncontrolled, might lead to the onset of tumour growth.

However, controlled use of the stemness- and growth-inducing properties of HMGA proteins might also be used as a tool to regulate cell growth in situations where it is a wanted event such as in tissue engineering and -regeneration, i.e. in case of this thesis in the treatment of articular cartilage defects. Re-expression of HMGA proteins was shown to be involved in a large percentage of examined pulmonary chondroid hamartomas (Kazmierczak et al. 1995; Kazmierczak et al. 1996; Kazmierczak et al. 1996; Wanschura et al. 1996; Kazmierczak et al. 1999; Rogalla et al. 2000; Tallini et al. 2000; Lemke et al. 2002). These benign tumours of the lung are characterised, amongst others, by their growth of chondroid, adipose and smooth muscle cells, and other cells of mesenchymal origin (Fletcher et al. 1995). Regulation and improvement of chondrocyte growth by HMGA proteins might thus be an important aspect overcoming some of the problems in cartilage tissue regeneration. On the one hand, increased cell proliferation might reduce the time needed for growing cells for autologous chondrocyte transplantation when cells are exposed to HMGA proteins or transfected with suitable vectors/agents inducing the expression of HMGAs. On the other hand, influence of HMGA proteins on chondrogenesis (Kubo et al. 2006) might prove beneficial for the cells' differentiation stage, in the ideal case overcoming some of the problems of chondrocyte dedifferentiation in *in-vitro* cell culture (Harrison et al. 2000; Veilleux et al. 2004; Chiang et al. 2010).

Concerning scaffolds, in addition to the already established biomaterials such as alginate or chitosan, non-biological matrices such as beta-tricalcium phosphate (β-TCP) might prove beneficial in cartilage regeneration, e.g. in case of defects involving both cartilage and the underlying bone like for example OD. Beta-TCP is an established material in bone repair and restoration, and usually gets degraded and replaced with newly-built tissue over time (Anker et al. 2005; Hirata et al. 2006). Preliminary studies with β-TCP in combined bone and cartilage regeneration were successfully conducted in sheep and pigs (Guo et al. 2004; Gotterbarm et al. 2006; Jiang et al. 2007). No data was available for dogs, although some breeds are prone to develop OD leading to lameness. Seeding or connecting β-TCP with chondrocytes thus might be a useful strategy in treating these kinds of defects, both for the benefit of the affected dog, as well as for humans using the dog as a model.

Taken these aspects into consideration, application of HMGA proteins and derivatives thereof in cartilage tissue engineering seemed to be an auspicious therapeutic strategy. Therefore, the main focus of the work conducted during this thesis was set on this issue. HMGA proteins and peptides were examined in an *in vitro* animal model utilising porcine cartilage. In addition, a preliminary study concerning the applicability of β-TCP structures for use in canine cartilage regeneration was investigated in an *in vitro* study, focusing on biocompatibility of these constructs with canine chondrocytes.

Additional work included aspects of *HMG* and further genes in canine genetics and disease, as the dog has been established as a suitable model for various human diseases, promising benefit in treatment and cure for both dogs and their human owners (Ostrander et al. 1997; Kuska 1999; Ostrander et al. 2000; Ostrander et al. 2000; Starkey et al. 2005; Shearin et al. 2010).

2. Materials and Methods

2.1. Porcine cartilage

Porcine cartilage was obtained from knee or elbow joints of freshly slaughtered pigs that were raised for meat production. The surrounding articular capsule was carefully removed and the cartilage cut from the subchondral bone in small chips by both a 4mm biopsy punch and a scalpel. Cartilage was then washed in phosphate buffered saline (PBS) and transferred to a fresh petri dish containing the respective growth medium used in the adjacent procedures and experiments (see the particular Materials and Methods sections in the enclosed publications for details of medium used).

2.2. Canine cartilage

Canine cartilage was provided by the Small Animal Clinic, University of Veterinary Medicine Hannover, Foundation, Germany, and the Small Animal Clinic, Duisburg Asterlagen, Germany, from dog patients that underwent therapeutic surgery for either femoral head and neck excision or hip joint prosthesis implantation during therapy. Cartilage chips removed from the femoral head were immediately transferred into Hanks solution until further use.

2.3. HMGA1a, HMGA1b, and HMGA2 expression vectors

pET3a expression vectors (Merck Chemicals, Darmstadt, Germany) containing the complete coding sequences of the human *HMGA1a*, *HMGA1b*, and *HMGA2* gene, respectively, were provided by the Centre for Human Genetics of the University of Bremen, Germany. For plasmid preparation, sequence verification, and long term storage, the expression vectors were transformed into the non-expression host *Escherichia coli (E. coli) DH5α* (Merck Chemicals) following the protocol of Inoue et al. (1990). Glycerol stocks were prepared by adding aliquots of 1ml bacterial culture to aliquots of 1ml 60% (v/v) glycerol in a sterile reaction tube for storage at -80°C. Plasmids were isolated using the QIAprep Spin Miniprep Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Sequencing was done by Eurofins MWG GmbH (Ebersberg, Germany).

2.4. Recombinant protein expression

The different HMGA proteins were recombinantly expressed in *E. coli BL21(DE3) Rosetta pLysS* (Merck Chemicals) following a protocol adapted from Schwanbeck (2000). Deviating from Schwanbeck's protocol, the *Rosetta pLysS* strain was chosen and additional glucose was added to the growth medium leading to acceptable transformation efficiency through reduced background expression of the transgene, which proved impedimental to the previously used expression host *E. coli BL21(DE3)*. Recombinant HMGA and other acid soluble proteins were recovered from the pelleted bacteria in a combined three cycle freeze and thaw acidic precipitation procedure in 5% (w/v) perchloric acid to remove non-acid soluble proteins with subsequent precipitation of HMGA by 6x vol. acidified -20°C acetone. The resulting pellet was freeze dried under vacuum and the proteins resolved in 50mM ammonium hydrogen carbonate at 4°C. This crude acid soluble protein mix containing mostly HMGA was stored until further processing at -80°C.

2.5. High-performance liquid chromatography

Final purification of HMGA protein from the crude acid soluble protein mix was performed using a two-step high-performance liquid chromatography (HPLC) procedure. In the first step, acid soluble bacterial proteins as well as bacterial DNA, which were carried over from the acetone precipitation, were removed using a cation exchange column (TSKgel SP5 PW 20µm, Tosoh Bioscience GmbH, Stuttgart, Germany) and a gradient of 25mM H_3BO_3 pH 9.4 and 25mM H_3BO_3/1M NaCl pH 9.4 at a flow rate of 1ml/min. 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% trifluoroacetic acid and 0.1% trifluoroacetic acid / 70% acetonitrile and a flow rate of 0.5ml/min of the fraction obtained from the cation exchanger was performed, enabling the removal of degraded or fragmentary HMGA, leading to a very pure fraction of intact protein.

2.6. Sodium dodecyl sulphate polyacrylamide gel electrophoresis

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was used for quality control of the purified HMGA protein fractions, in which an aliquot of the final HPLC-eluate was run on a discontinuous polyacrylamide gel (4% stacking gel, 12% separating gel), followed by subsequent detection of protein bands by Coomassie staining.

2.7. Protein quantification

For removal of eluent, gravimetric quantification, and long term storage of the complete protein batch, the fraction volume was first reduced using Amicon Ultra 10000 MWCO (molecular weight cut off) centrifugation devices (Millipore, Schwalbach, Germany) and the proteins were subsequently lyophilised using a freeze dryer. Gravimetric quantification of the resulting HMGA protein pellets was performed on an analytical balance (Sartorius, Göttingen, Germany), and the pellets were frozen at -80°C for long term storage. For use in the particular experiments, aliquots of 10µg/µl protein in double distilled water (Invitrogen, Karlsruhe, Germany) were prepared and stored at -80°C.

2.8. HMGA2 fragments

The HMGA2 fragments (HMGA2-A: M-S-A-R-G-E-G-A-G-Q-P-S-T-S-A-Q-G-Q-P-A-A-P-A-P-Q-K-R-G-R-G-R-P-R-K-Q-Q-Q-E-P-T-G-E-P-S-P-K-R-P-R-G-R-P-K-G-S-K-N-K-S-P, 60 aa; HMGA2-B:, M-S-A-R-G-E-G-A-G-Q-P-S-T-S-A-Q-G-Q-P-A-A-P-A-P-Q-K-R-G-R-G-R-P-R-K-Q-Q-Q-E-P-T-G-E-P-S-P-K-R-P-R-G-R-P-K-G-S-K-N-K-S-P-S-K-A-A-Q-K-K-A-E-A-T-G-E-K-R-P-R-G-R-P, 80 aa) were provided by Aplagen GmbH, Baesweiler, Germany. They were synthesized by microwave assisted solid phase peptide synthesis and purified by liquid chromatography-mass spectrometry. The carboxyfluorescein labelled HMGA2-CC variant of the HMGA2-B peptide was synthesised and purified as described above, with additional coupling of 5/6-carboxyfluorescein to the N-terminus at the final step of the solid phase synthesis.

2.9. Primary cell culture

The excised cartilage chips were further chopped and digested in 50% (v/v) collagenase NB8 (Serva Electrophoresis GmbH, Heidelberg, Germany) and tissue culture medium (for details of medium used, see the particular Materials & Methods sections in the enclosed publications) until the chondrocytes were released from the extracellular cartilage matrix. Following digestion, released cells were washed in tissue culture medium and transferred to a 25cm² tissue culture flask (Nunc, Wiesbaden, Germany) and grown at 37°C / 5% CO_2 / 5ml tissue culture medium until 75% confluency of the tissue culture flask. Cells were then detached with TrypLE (Invitrogen) and passaged once. Cells were again grown to 75% confluency of the flask and afterwards employed in the respective experiments.

2.10. Cell proliferation assay

Cell proliferation was measured using the Cell Proliferation ELISA, BrdU (colorimetric) kit (Roche Diagnostics, Mannheim, Germany) following the manufacturer's instructions. Absorption reading was performed using the Anthos 2001 microtitre plate reader (Anthos Mikrosysteme, Krefeld, Germany) or the BioTek Synergy HT microtitre plate reader (BioTek Instruments GmbH, Bad Friedrichshall, Germany). Data editing and visualisation was done with Excel 2003 software (Microsoft, Unterschleissheim, Germany).

2.11. Cell penetration assay

Uptake of the fluorescently labelled HMGA2-CC fragments was examined in a cell penetration assay with porcine chondrocytes in monolayer cell culture. Porcine chondrocytes were seeded onto glass plates in a 12-well microtitre cell culture plate (100,000 cells/well), and incubated with medium 199/10% foetal bovine serum (FBS) containing the fluorescently labelled HMGA2-CC peptide for 4h with peptide concentrations of 0µM (control), 10µM, and 50µM, respectively. Cells were washed to remove any residual peptides. To rule out any false positive signals caused by diffusion of peptides into permeable dead cells, vitality staining was performed prior to fluorescence microscopy.

2.12. Beta-tricalcium phosphate cylinders (Cerasorb®)

Cerasorb® cylinders were provided by Curasan AG, Kleinostheim, Germany. They consist of beta-tricalcium phosphate (β-TCP) with a phase purity of more than 99% and possess intrinsic interconnective porosity as caused by the material's properties. Furthermore, so-called macropores (1mm diameter) are drilled throughout the cylinders, by which the complete inner and outer surfaces of the cylinder could be reached by medium flow-through and thus be colonised with cells. Also, the drill holes could be used to mount cartilage chips to the cylinders.

2.13. Cerasorb® colonisation

Colonisation of Cerasorb® cylinders with isolated canine chondrocytes was done in two steps. In the first step, the cylinders were stored in small volume containers (2 ml Gibco cryotube, Invitrogen) and inoculated with 1.2ml of tissue culture medium 199/20% FBS containing 100,000 cells/ml each, so that the cells could settle on the cylinders and throughout the cylinders' macropores. After the cells had attached (6h incubation), the cylinders were transferred to and fastened in a specially prepared

cell culture flask and covered with 15ml of medium 199/20% FBS. The cells were left to grow at 37°C 5% CO_2 for one week, after which the cylinders were removed for analysis, i.e. vitality staining, visible light and fluorescence microscopy, and scanning electron microscopy. Medium was exchanged every three days. In addition, as no microscopic monitoring of the cells on the constructs was possible during the incubation period due to the specific experimental setup, control cells were seeded into 6-well multidish plates and grown under identical incubation conditions as the cells on the cylinders. These cells were microscopically checked during the incubation period and also subjected to the viability tests.

Canine cartilage chips were cut to size so they would fit the drill holes and mounted to the cylinder using forceps. They were incubated under the same conditions as the isolated cells for one week, and examined accordingly.

2.14. Vitality staining

Viability of porcine chondrocytes following HMGA2-CC peptide application was determined by combined staining with propidium iodide (PI, detector stain, Sigma-Aldrich Chemie GmbH, Munich, Germany) and 4′,6-Diamidin-2-phenylindol (DAPI, background stain, Roche Diagnostics, Mannheim, Germany), allowing the distinction of dead cells by the red PI stain in the nucleus. Medium (medium 199/10% FBS) was removed from the respective wells and replaced with medium 199/10% FBS containing 25µg/ml DAPI + 1µg/ml PI. After incubation for 5 minutes at room temperature, cells were washed with PBS to remove residual stains. In preparation for fluorescence microscopy, the glass plates were removed from the 12-well plate's wells and embedded onto glass slides by rubber cement.

Viability of canine chondrocytes on the Cerasorb® constructs as well as viability of the control cells grown in 6-well multidish plates was determined by trypan blue staining, followed by additional DAPI staining of the cells on the constructs, allowing the employment of fluorescence microscopy for determination of complete cell numbers and growth patterns. The control cells were not DAPI-stained, as these could easily be examined by phase contrast microscopy. The cylinders were removed from the cell culture flasks, washed in PBS, and incubated for 5 min in 0.2% trypan blue solution (Invitrogen). Afterwards, the constructs were washed with PBS to remove any residual trypan blue. The control cells were stained in the multidish plate following the same procedure. The washed cylinders were then incubated in 1µg/ml DAPI/methanol solution (Sigma-Aldrich Chemie GmbH) for 15 min and washed with methanol to remove any residual DAPI stain.

2.15. Visible light and fluorescence microscopy

Microscopic evaluation was performed using an Axioskop 2 microscope (Carl-Zeiss-AG, Oberkochen, Germany). An external light source was used for reflected visible light microscopy, while the built in UV-light source was used for fluorescence microscopy. Electronic documentation was done using Axiovision software releases 4.5 and 4.8 (Carl-Zeiss-AG).

2.16. Scanning electron microscopy

Constructs were prepared for scanning electron microscopy (SEM) by fixation in 2% glutaraldehyde. SEM itself was conducted elsewhere on a LEO 1530 VP FE-SEM (Carl-Zeiss-AG).

2.17. Statistical analysis

Statistical analysis was performed with GraphPad Instat and GraphPad Prism 5 software (GraphPad Software, La Jolla, USA) using the one way analysis of variance (ANOVA), with pairs of group means being compared by the Tukey test. A p value of <0.01 was deemed statistically significant. Before ANOVA, measured data were checked for identity of standard deviations by Bartlett's test. In case of significant differences between the standard deviations of the different groups, logarithmic transformation (log10) of the raw data was performed, leading to identical standard deviations as is necessary for ANOVA. Gaussian distribution of sample data was checked for by the Kolmogorov-Smirnov test.

2.18. *In silico* sequence analysis of the porcine HMGA genes

During the work conducted for this thesis, the porcine genome had not been sequenced completely, and especially in regard to *HMGA1* and *HMGA2*, information in the sequence databases was sparse. Thus, *in silico* sequence analysis of the porcine *HMGA* genes was conducted utilising known human sequence information, e.g. concerning exon / intron boundaries, and the porcine sequences that were available from the Entrez nucleotide and protein databases (National Center for Biotech Information (NCBI), National Library of Medicine, Bethesda, MD, United States of America) at that time. Sequence and structure of the porcine *HMGA1* and *HMGA2* mRNAs were determined using the Basic Local Alignment Search Tool (BLAST) (Altschul et al. 1990) provided by the NCBI.

3. Results

3.1. High mobility group proteins in cartilage regeneration

In tissue engineering and regeneration of damaged articular cartilage, improved and controlled growth of chondrocytes is an important aspect. Usually in damaged areas, fibrocartilage formation occurs leading to less resilience of the affected joint and further damage due to premature abrasion of the recovered areas. Tissue engineering based therapies are hampered by the low number of viable cells available for growing new cartilage, as well as dedifferentiation of chondrocytes usually occurring when grown in culture. These strategies including the transplantation of autologous cells (autologous chondrocyte transplantation, ACT) amplified in cell culture or grown on matrices and in scaffolds thus still have room for improvement.

Aberrations of *HMGA* genes were shown to be involved in the formation of pulmonary chondroid hamartomas (Kazmierczak et al. 1996; Kazmierczak et al. 1999; von Ahsen et al. 2005), benign tumours of the lung that show, amongst others, focal differentiation into areas of articular cartilage (Koss 1990). Interestingly, overexpression of the *HMGA2-LPP* fusion gene was shown to promote expression of the chondrogenic *COL11A2* (collagen, type XI, alpha 2) gene (Kubo et al. 2006).

Therefore, the effect of recombinantly produced HMGA proteins on chondrocytes was evaluated in this study in regard to proliferation of the cells, a strategy that might improve tissue engineering techniques based on chondrocyte amplification.

Due to the low availability of human articular cartilage specimens, porcine cartilage was chosen instead in these experiments. Protein sequence analysis showed complete agreement of the human and porcine HMGA1a and HMGA1b proteins, while the HMGA2 protein only showed one amino acid difference, but not in the functional motif of the AT-hooks. Thus, porcine cartilage was deemed sufficient for use in these trials.

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Application of high-mobility-group-A proteins increases the proliferative activity of chondrocytes *in vitro*

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The recombinant expression of the high mobility group proteins HMGA1a, HMGA1b, and HMGA2 was established in *E. coli BL21(DE3) Rosetta pLysS* (Merck Chemicals). While the procedure was described before (Schwanbeck 2000), it needed some adaptation to provide for successful transformation and growth efficiencies of the expression host as well as appropriate protein yield for the subsequent purification steps. The expression strain *E. coli BL21(DE3)* as used by Schwanbeck proved sensitive to transformation with the expression plasmids, and the small number of transformed clones showed impaired growth and protein production in culture. Therefore, a different strain *E. coli BL21(DE3) Rosetta pLysS* with reduced transgene background expression due to its additional pLysS plasmid, as well as growth medium supplemented with glucose for further reduction of background expression, was chosen for protein expression and led to sufficient amounts of crude recombinant protein.

The basic HPLC purification procedure was also described before (Schwanbeck 2000), and was successfully adapted to the HPLC equipment available.

Application of these recombinant HMGA proteins to isolated porcine chondrocytes in cell culture showed some significant effects on proliferation of these cells in a dose dependent manner. Compared to the untreated control, at the lowest concentration of 1µg/ml protein, a 1.5x increased growth could be detected for the HMGA1b protein, but not for HMGA1a and HMGA2 (there is a slight increase in measured cell proliferation, which however proved not statistically significant). However, at the increased amount of 10µg/ml, significant growth enhancing effects (about 1.5x) could be observed for all three proteins HMGA1a, HMGA1b, and HMGA2, when compared to the untreated control. Finally, at the highest administered dose of 100µg/ml, once again a significant increase in proliferation could be detected for all proteins HMGA1a, HMGA1b, and HMGA2 when compared to the untreated control, albeit with a protein specific effect of 1.7x in case of HMGA1a, and about 2x in case of HMGA1b and HMGA2. Concerning dosage, significant differences in proliferation could be seen between the application of 1µg/ml, 10µg/ml, and 100µg/ml in HMGA1a and HMGA2, but not HMGA1b.

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Application of high-mobility-group-A proteins increases the proliferative activity of chondrocytes *in vitro*

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Own contribution:

Study design

Recombinant expression of high mobility group proteins

High performance liquid chromatography

Cell culture

In vitro cell proliferation assay

Statistics

Writing of the manuscript

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Application of High-Mobility-Group-A Proteins Increases the Proliferative Activity of Chondrocytes *In Vitro*

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The low capability of self-repair in hyaline cartilage tissue and chondrocytes de-differentiating when grown *in vitro* (e.g., for tissue engineering approaches) limits articular cartilage repair. It has been shown that the embryonic architectural transcription factors of the high-mobility-group-A (HMGA) protein family affect the regulation of cell differentiation by influencing the state of cell chromatin and are involved in hyaline cartilage development by affecting the expression of chondrocyte-specific marker genes. Thus, the control of cartilage cell proliferation and differentiation by HMGA proteins promises to be an important aspect in cartilage tissue repair. To elucidate the effects on the proliferative activity of hyaline chondrocytes, HMGA proteins were recombinantly expressed, highly purified, and applied to porcine hyaline cartilage cells growing in *in vitro* monolayer cell culture. Direct application of HMGA1a, HMGA1b, and HMGA2 proteins onto porcine chondrocytes was shown to have a highly significant influence on cell proliferation. Greater proliferation of chondrocytes was achieved than in the untreated control group, indicating a promising approach to enhancing cartilage tissue repair.

Introduction

Articular cartilage defects affect a large number of patients from all age groups. Although normal wear and tear affects a high percentage of the elderly, in younger people, overstressing or injuring articular cartilage through, for example, high-impact sports or defective positions of joints may lead to damage and degradation.

In contrast to many other tissues, because of the absence of a blood supply, damaged articular cartilage has only limited capacity for self-repair, with joint surface defects above a critical size healing poorly and eventually leading to osteoarthritis.[1] Furthermore, the intrinsic repair mechanism of articular cartilage usually leads to the formation of fibrocartilage, which is unable to sustain the pressures it is usually exposed to in the joint, leading to new lesions that can affect the underlying bone as well.[2]

Apart from applied surgical interventions such as lavage and arthroscopy, shaving, debridement, laser abrasion and chondroplasty, abrasion chondroplasty, and Pridie drilling, tissue-engineering techniques are gaining a larger share in the treatment of articular cartilage defects.[3] Tissue engineering uses biologically based mechanisms for repairing and healing of damaged and diseased tissues, employing techniques from various disciplines such as biology, material science, engineering, manufacturing, and medicine.[4] A tissue-engineering approach that has gained attention in recent years in cartilage regeneration is the transplantation of autologous chondrocytes (autologous chondrocyte transplantation, ACT) that have been grown *in vitro*, with recent approaches including the use of matrices or scaffolds for facilitating cell growth. Nevertheless, the low quality of the engineered cartilage hampers regeneration of the damaged regions because chondrocytes usually dedifferentiate over time *in vitro*.[5] Growth of cells in agarose gels,[6] alginate beads,[7] or collagen matrices[8] has a beneficial effect on the differentiation state of cells, and the application of growth factors such as insulin-like growth factor-I, interleukin-4, and transforming growth factor-beta 1 has led to an increase in the rate of cartilage tissue growth and extracellular matrix deposition.[9]

Another approach for directed cartilage cell growth and differentiation is the specific use of embryonic proteins that are involved in the control of cartilage formation in the developing organism (i.e., those of the high-mobility-group-A (HMGA) family). HMGA proteins are chromatin-associated nonhistone proteins strongly conserved in mammals that act as architectural transcription factors, having no direct influence on transcription but acting indirectly by enabling the interaction of various transcription factors (e.g., enhanceosome formation in interferone-beta).[10] By acting as "master switches" influencing the expression of more than 5000 genes, HMGA proteins also influence the expression of genes involved in chondrogenesis, such as *COL11A2*.[11] Furthermore, it has been shown that *HMGA2* is expressed at high

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levels in human embryonic stem cells, regulating key developmental genes that are linked to mesenchymal cell differentiation.[12,13] The *HMGA* family genes being predominantly expressed in embryonic tissue, no expression of *HMGA2* had been identified after birth or in fully differentiated adult tissue[14] until the recent advent of more-sensitive detection methods such as quantitative real-time polymerase chain reaction, which in some apparently normal adult tissues also provided evidence for basal *HMGA2* expression.[15–17] Alterations of *HMGA* genes could be detected in a variety of tumors, many of them showing areas with entopic or ectopic cartilage growth. In soft tissue and skeletal chondromas, nonrandom involvement of the region 12q13–15, which includes the *HMGA2* locus, has been shown with expression of truncated (exons 1–3), fusion (e.g., *HMGA2/LPP*), or full-length transcripts of *HMGA2*, respectively.[18]

In pulmonary chondroid hamartomas (PCHs), re-expression of *HMGA* genes is a highly important aspect in tumor formation. PCHs, which are the most common benign tumors of the lung,[19] consist of focal overgrowths of articular cartilage, smooth muscle, mature adipocytes, and respiratory tract epithelium.[20] Chromosomal rearrangements of *HMGA* genes or parts thereof (the DNA-binding AT-hooks), including one of the most frequent fusion genes found in human tumors, *HMGA2-LPP*, commonly cause re-expression of HMGA proteins in PCHs.[21] In a series of 191 PCHs, cytogenetic and fluorescence *in situ* hybridization analyses showed 6p21.3 (*HMGA1*) aberrations in 25% of cases and 12q14–15 (*HMGA2*) aberrations in 58.7% of cases.[22]

Because *HMGA*-family genes are frequently involved in PCH formation and growth of articular cartilage is one prominent feature of these tumors, the effect of HMGA proteins on articular cartilage cell differentiation and growth is an important aspect in the regeneration of articular cartilage. The control of chondrocyte growth by HMGA proteins might prove useful for the application of cell engineering methods in articular cartilage repair, especially by facilitating and speeding up the production of cells used for seeding structures and by keeping the cells in a differentiated state. Thus, the aim of this study was the evaluation of the influence of HMGA family proteins on chondrocyte growth by examining the proliferational effect of recombinantly produced HMGA1a, HMGA1b, and HMGA2 proteins on porcine chondrocytes grown in *in vitro* monolayer cell culture.

Materials and Methods

Expression and purification of recombinant HMGA proteins

Recombinant HMGA proteins were produced using the Novagen pET System (Merck Chemicals, Darmstadt, Germany) following a protocol adapted from Schwanbeck *et al*.[23] The complete coding sequences of the human *HMGA1a*, *HMGA1b*, and *HMGA2* were inserted into the pET3a vector. Subsequently, transformation of the nonexpression host *Escherichia coli DH5α* with these plasmids was performed for verification using sequencing and long-term storage. Protein expression was performed using freshly transformed *E. coli BL21(DE3) Rosetta pLysS* (Merck Chemicals). A preparatory culture was grown to an optical density (OD) of 0.6 in 10 mL Luria-Bertani (LB) broth supplied with 100 μg/mL ampicillin, 34 μg/mL chloramphenicol, and 1% (w/v) glucose. After inoculation, 1 L of expression culture (LB broth supplemented with 100 μg/mL ampicillin, 34 μg/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 isopropyl-beta-D-thiogalactopyranoside. Expression was performed for 120 min at 37°C, and the culture was thereafter stored on ice and then pelleted using centrifugation.

HMGA and other acid-soluble proteins were recovered from the bacteria by performing a combined three-cycle freeze (−20°C) and thaw and acidic precipitation procedure with 3× (w/v) 5% perchloric acid, resulting in lysis of bacterial cells and precipitation of bacterial debris and non-acid-soluble proteins. After centrifugation, the HMGA-containing supernatant was removed and acidified with 37% hydrochloric acid (HCl) to a final concentration of 0.35 m HCl. Precipitation of HMGA proteins was performed by adding 6× volume of −20°C acetone. Proteins were pelleted by centrifugation in 80-mL glass tubes, and the acetone supernatant was carefully removed and the pellet frozen to −80°C and dried under vacuum. Pelleted proteins were resolved using 50 mM ammonium hydrogencarbonate at 4°C. The crude acid-soluble protein mix containing mostly HMGA was stored until further processing at −80°C. Final purification of HMGA proteins was performed using a two-step high-performance liquid chromatography procedure. In the first step, acid-soluble bacterial proteins and bacterial DNA that were carried over from acetone precipitation were removed using a cation exchange column (TSKgel SP5 PW 20 μm, Tosoh Bioscience GmbH, Stuttgart, Germany) and a gradient of 25 mM boric acid pH 9.4 and 25 mM boric acid/1 M sodium chloride, pH 9.4. Because of the lack of aromatic amino acids in HMGA proteins, identification of the HMGA fraction was achieved using a 215-nm absorption peak proximal to the absorption maximum of the peptide bond at 205 nm. After this first purification step, reverse-phase high-performance liquid chromatography purification (Grom-Sil 300 ODS-5 ST, Grom, Rottenburg-Hailfingen, Germany) with a gradient of 0.1% trifluoroacetic acid and 0.1% trifluoroacetic acid/70% acetonitrile of the fraction obtained was performed, enabling the removal of degraded or fragmentary HMGA, leading to a very pure fraction of intact protein. Quality control was performed using polyacrylamide gel electrophoresis (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 lyophilized using a freeze dryer.

Cell culture

Chondrocytes were obtained from the elbow joint of an approximately 4-month-old pig for slaughter that was culled for meat production at the local abattoir. This company operates under the German animal protection law and is licensed for meat production by the Federal Office of Consumer Protection and Food Safety, which indicates ethical treatment of the animals slaughtered. Cartilage was carefully removed, excluding the calcified inner cartilage layer, using a scalpel. The cartilage pieces were further chopped and digested for at least 6 h at 37°C/5% carbon dioxide (CO_2) in 50% v/v 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% pig serum (Invitrogen, Karlsruhe, Germany)) until the chondrocytes were released from the extracellular cartilage matrix. After digestion, released cells were washed, transferred to a 25-cm^2 tissue culture flask (Nunc, Wiesbaden, Germany), and incubated at 37°C/5% CO_2/5 mL tissue culture medium as described previously until three-quarters confluency of the tissue culture flask was reached. This was followed by one further passage.

In vitro *cell proliferation assay*

Cells were harvested using TrypLE Express (Gibco, Karlsruhe, Germany), resuspended in fresh tissue culture medium as described previously, and counted using a hemocytometer. Concentration was adjusted to 75,000 cells/mL. HMGA proteins HMGA1a, HMGA1b, and HMGA2 were adjusted to concentrations of 1 µg/mL, 10 µg/mL, and 100 µg/mL, respectively, in tissue culture medium. For each protein, assays with concentrations of 0 µg/mL, 0,1 µg/mL, 1 µg/mL, and 10 µg/mL were performed using a bromodeoxyuridine (BrdU) cell proliferation enzyme-linked immunosorbent assay (ELISA) kit (Roche Diagnostics, Mannheim, Germany). For each parameter, cells were seeded in eight wells of a 96-well cell culture microtiter plate (Nunc, Wiesbaden, Germany) with a concentration of 7500 cells per well (100 µL of cell solution). Cells were left at 37°C/5% CO_2 overnight, followed by the addition of the corresponding amounts of protein. After an incubation period of 24 h at 37°C/5% CO_2, BrdU was added followed by incubation at 37°C/5% CO_2 for another 24 h. BrdU incorporation detection was performed according to the manufacturer's instructions using the Anthos 2001 microtiter plate reader (Anthos Mikrosysteme, Krefeld, Germany). Data were normalized using the non-HMGA control as the reference value.

Statistics

Statistical analysis was performed using GraphPad Instat and GraphPad Prism 5 software (GraphPad Software, La Jolla, Ca) using one-way analysis of variance ANOVA, with pairs of group means being compared using the Tukey test. The significance level was set at $p < 0.01$. Homogeneity of variances was checked using Bartlett's test, which showed significant differences between the standard deviations of the different groups. Therefore, logarithmic transformation (log10) of raw data was performed as suggested by the software, leading to identical standard deviations as assumed by ANOVA. Gaussian distribution of sample data was demonstrated using the Kolmogorov-Smirnov test with all populations passing the normality test.

Results

The proliferative response of porcine chondrocytes to human recombinant HMGA proteins *in vitro* was examined in an ELISA-based assay measuring the incorporation of BrdU into the newly synthesized DNA of dividing cells. For comparability, the absorbance values obtained from the microtiter plate reader were normalized to the values of the non-HMGA control, which was set to a value of 1 (Figure 1).

The one-way ANOVA resulted in a p-value < 0.001 indicating extremely significant variation between the different groups, leading to the conclusion that at least two groups were significantly different. For determination of group differences, pairs of group means were compared using the Tukey test, as shown in Table 1.

Deducing from the statistical analysis, proliferative effects of three human recombinant HMGA proteins, including the splicing variants HMGA1a and HMGA1b and the closely related HMGA2, on porcine hyaline cartilage cells were compared with those of the non-HMGA-treated control group.

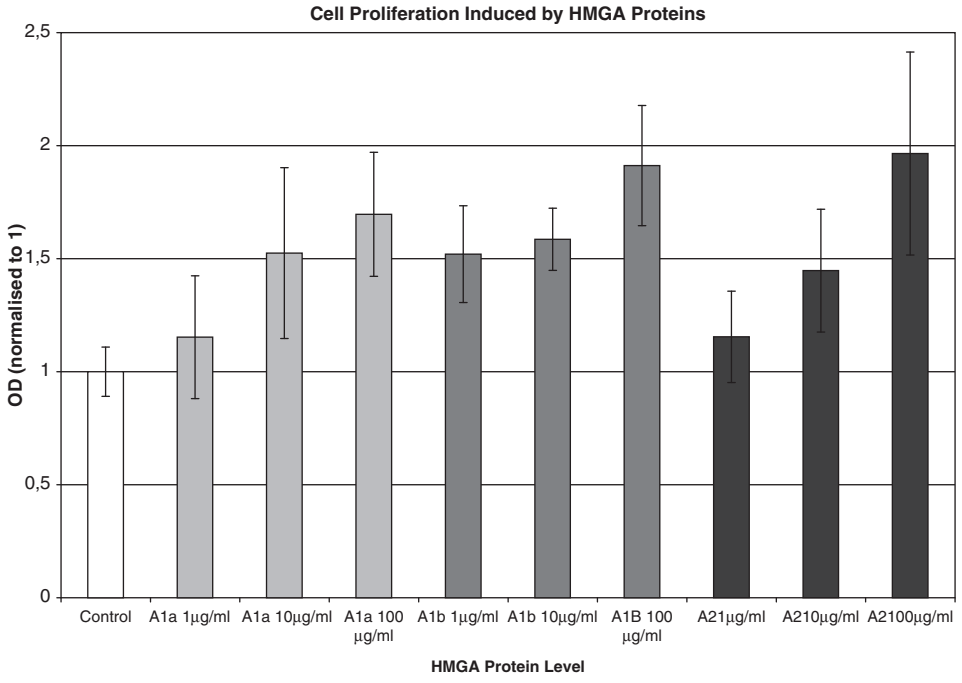

FIG. 1. Cell proliferation of porcine hyaline cartilage cells induced by the addition of high-mobility-group-A (HMGA) proteins at different concentrations. Values obtained using the microtiter plate reader were normalized to the absorbance of the non-high-mobility-group control, which was set as 1. The error bars indicate the standard deviation. OD, optical density.

Although at the lowest concentration of 1 μg/mL protein, no differences from the control group were seen with HMGA1a and HMGA2, significant greater cell proliferation was visible for HMGA1b, leading to 1.5 times greater cell proliferation than in the control group. At concentrations of 10 μg/mL of protein, a significant greater proliferative rate is apparent for each protein, HMGA1a, HMGA1b, and HMGA2, than for the non-HMGA-treated control group. When adding 100 μg/mL of protein, a significant difference in proliferation from the control group can also be seen for all proteins, with HMGA1b and HMGA2 nearly doubling the proliferative rate of cells and HMGA1a leading to 1.7 times greater cell proliferation.

As to the different concentrations, significant differences between the application of 1μ/mL and 100 μg/mL were seen for HMGA1a and HMGA2 but not for HMGA1b.

Discussion

Usually being expressed only in developing embryonic tissue or at very low levels in adults,[15–17] re-expression of members of the HMGA protein family was found to be of significant influence in a variety of benign tumors, some of them displaying growth of differentiated chondrocytes at ectopic (pulmonary chondroid hamartomas) or entopic (chondromas) sites.[18,22] Furthermore, involvement of the complete *HMGA2* and the *HMGA2/LPP* fusion gene consisting of the first three exons of *HMGA2* (encoding the eponymous functional motifs of the DNA-binding AT-hooks) and exons 9–11 of *LPP* in expression regulation of the chondrogenous *COL11A2* has been demonstrated.[11] Based on these findings, the proliferative effect of recombinantly produced HMGA proteins (human HMGA1a, HMGA1b, and HMGA2) on porcine chondrocytes grown *in-vitro* in monolayer cell culture was compared with an untreated control. Because HMGA proteins are highly conserved between mammals, with the porcine HMGA1a and HMGA1b showing only one AA exchange from threonine to alanine at the first AA position of the third AT hook compared with their human counterparts (no sequence data on porcine HMGA2 is available yet in the public databases), the coding sequences of the human HMGA genes were used for the expression vector. In this study, administration of human HMGA1a, HMGA1b, and HMGA2 proteins to porcine chondrocytes showed a highly significant effect on cell proliferation at levels of 10 μg/mL or 100 μg/mL of protein. In addition, application of HMGA1b had a significant effect at 1 μg/mL protein. Although HMGA proteins do not directly regulate transcription, they indirectly influence gene expression by acting as architectural transcription factors (e.g., by interacting with (AT-rich) DNA and several transcription factors). For example, in the beta-interferon enhancer, the activity of the transcription factor nuclear factor-kappaB is enhanced under the influence of HMGA2 bound to its AT-rich target DNA sequence.[24] In human embryonic stem cells, HMGA2 plays an important role in chromatin structure, which in turn is a key factor determining stem cell identity. By influencing the state of embryonic stem cell chromatin, HMGA2 might be an important factor in differentiation initiation.[14] Concerning PCHs, re-expression of HMGA proteins might lead to differentiation of mesenchymal stem cells found in the tumor toward the chondrocyte phenotype. Furthermore, initial de-differentiation of fully differentiated chondrocytes caused by HMGA proteins in conjunction with the chondrogenic activity of HMGA might initiate growth of these cells while keeping their chondrocyte phenotype. As to the mechanisms by which extracellular HMGA proteins can support the growth of chondrocytes, two explanations can be advanced. First, an uptake of the proteins may lead to higher intracellular protein concentrations, the latter directly influencing the chromatin structure of the target cells and enhancing their proliferation. Second, extracellular functions of the proteins that are unknown may account for the findings. HMGB1 is a

Table 1. Tukey Test Comparing the Effect of Different High-Mobility-Group-A (HMGA) Proteins and Protein Concentrations on the Proliferation of Porcine Chondrocytes

	Control	*HMGA1a 1μg/ml*	*HMGA1a 10μg/ml*	*HMGA1a 100μg/ml*	*HMGA1b 1μg/ml*	*HMGA1b 10μg/ml*	*HMGA1b 100μg/ml*	*HMGA2 1μg/ml*	*HMGA2 10μg/ml*	*HMGA2 100μg/ml*
Control										
HMGA1a 1μg/ml	>0.01									
HMGA1a 10μg/ml	<0.01	>0.01								
HMGA1a 100μg/ml	<0.01	<0.01	>0.01							
HMGA1b 1μg/ml	<0.01	>0.01	>0.01	>0.01						
HMGA1b 10μg/ml	<0.01	>0.01	>0.01	>0.01	>0.01					
HMGA1b 100μg/ml	<0.01	<0.01	>0.01	>0.01	>0.01	>0.01				
HMGA2 1μg/ml	>0.01	>0.01	>0.01	<0.01	>0.01	>0.01	<0.01			
HMGA2 10μg/ml	<0.01	>0.01	>0.01	>0.01	>0.01	>0.01	>0.01	>0.01		
HMGA2 100μg/ml	<0.01	<0.01	>0.01	>0.01	>0.01	>0.01	>0.01	<0.01	>0.01	

Grey cells indicate significant differences with a p-value < 0.01.

protein of yet another subfamily of the high-mobility group proteins. Initially, it has been described as a nuclear protein as well, but it has been discovered that HMGB1 has extracellular functions as well when, for example, being released by necrotic cells or actively secreted by activated macrophages. Generally, it cannot be ruled out that HMGA2 also has a yet-unknown extracellular function. The application of HMGA proteins in chondrocyte growth provides a promising way to enhance the methods of cartilage tissue repair.

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References

1. Marlovits, S., Zeller, P., Singer, P., Resinger, C., and Vecsei, V. Cartilage repair: generations of autologous chondrocyte transplantation. Eur J Radiol **57,** 24, 2006.
2. Frenkel, S.R., and Di Cesare, P.E. Degradation and repair of articular cartilage. Front Biosci **4,** D671, 1999.
3. Hunziker, E.B. Articular cartilage repair: basic science and clinical progress. A review of the current status and prospects. Osteoarthritis Cartilage **10,** 432, 2002.
4. Hardingham, T., Tew, S., and Murdoch, A. Tissue engineering: chondrocytes and cartilage. Arthritis Res **4 Suppl 3,** S63, 2002.
5. Marlovits, S., Hombauer, M., Tamandl, D., Vecsei, V., and Schlegel, W. Quantitative analysis of gene expression in human articular chondrocytes in monolayer culture. Int J Mol Med **13,** 281, 2004.
6. Benya, P.D., and Shaffer, J.D. Dedifferentiated chondrocytes reexpress the differentiated collagen phenotype when cultured in agarose gels. Cell **30,** 215, 1982.
7. Guo, J.F., Jourdian, G.W., and MacCallum, D.K. Culture and growth characteristics of chondrocytes encapsulated in alginate beads. Connect Tissue Res **19,** 277, 1989.
8. Veilleux, N.H., Yannas, I.V., and Spector, M. Effect of passage number and collagen type on the proliferative, biosynthetic, and contractile activity of adult canine articular chondrocytes in type I and II collagen-glycosaminoglycan matrices *in vitro.* Tissue Eng **10,** 119, 2004.
9. Blunk, T., Sieminski, A.L., Gooch, K.J., Courter, D.L., Hollander, A.P., Nahir, A.M., Langer, R., Vunjak-Novakovic, G., and Freed, L.E. Differential effects of growth factors on tissue-engineered cartilage. Tissue Eng **8,** 73, 2002.
10. Reeves, R., and Beckerbauer, L. HMGI/Y proteins: flexible regulators of transcription and chromatin structure. Biochim Biophys Acta **1519,** 13, 2001.
11. Kubo, T., Matsui, Y., Goto, T., Yukata, K., and Yasui, N. Overexpression of HMGA2-LPP fusion transcripts promotes expression of the alpha 2 type XI collagen gene. Biochem Biophys Res Commun **340,** 476, 2006.
12. Li, O., Li, J., and Droge, P. DNA architectural factor and proto-oncogene HMGA2 regulates key developmental genes in pluripotent human embryonic stem cells. FEBS Lett **581,** 3533, 2007.
13. Li, O., Vasudevan, D., Davey, C.A., and Droge, P. High-level expression of DNA architectural factor HMGA2 and its association with nucleosomes in human embryonic stem cells. Genesis **44,** 523, 2006.
14. Rogalla, P., Drechsler, K., Frey, G., Hennig, Y., Helmke, B., Bonk, U., and Bullerdiek, J. Am J Pathol **149,** 775, 1996.
15. Gross, K.L., Neskey, D.M., Manchanda, N., Weremowicz, S., Kleinman, M.S., Nowak, R.A., Ligon, A.H., Rogalla, P., Drechsler, K., Bullerdiek, J., and Morton, C.C. HMGA2 expression in uterine leiomyomata and myometrium: quantitative analysis and tissue culture studies. Genes Chromosomes Cancer **38,** 68, 2003.
16. Sarhadi, V.K., Wikman, H., Salmenkivi, K., Kuosma, E., Sioris, T., Salo, J., Karjalainen, A., Knuutila, S., and Anttila, S. Increased expression of high mobility group A proteins in lung cancer. J Pathol **209,** 206, 2006.
17. Meyer, B., Loeschke, S., Schultze, A., Weigel, T., Sandkamp, M., Goldmann, T., Vollmer, E., and Bullerdiek, J. HMGA2 overexpression in non-small cell lung cancer. Mol Carcinog **46,** 503, 2007.
18. Dahlen, A., Mertens, F., Rydholm, A., Brosjo, O., Wejde, J., Mandahl, N., and Panagopoulos, I. Fusion, disruption, and expression of HMGA2 in bone and soft tissue chondromas. Mod Pathol **16,** 1132, 2003.
19. Kazmierczak, B., Rosigkeit, J., Wanschura, S., Meyer-Bolte, K., Van de Ven, W.J., Kayser, K., Krieghoff, B., Kastendiek, H., Bartnitzke, S., and Bullerdiek, J. HMGI-C rearrangements as the molecular basis for the majority of pulmonary chondroid hamartomas: a survey of 30 tumors. Oncogene **12,** 515, 1996.
20. Koss, M. Surgical Pathology of Lung Neoplasms. New York: Dekker, 1990.
21. von Ahsen, I., Rogalla, P., and Bullerdiek, J. Expression patterns of the LPP-HMGA2 fusion transcript in pulmonary chondroid hamartomas with t(3;12)(q27 approximately 28;q14 approximately 15). Cancer Genet Cytogenet **163,** 68, 2005.
22. Kazmierczak, B., Meyer-Bolte, K., Tran, K.H., Wockel, W., Breightman, I., Rosigkeit, J., Bartnitzke, S., and Bullerdiek, J. A high frequency of tumors with rearrangements of genes of the HMGI(Y) family in a series of 191 pulmonary chondroid hamartomas. Genes Chromosomes Cancer **26,** 125, 1999.
23. Schwanbeck, R. Die Phosphorylierung der architektonischen HMGI(Y)-Chromatinproteine: Modulation der Struktur und DNA-Bindung. Göttingen: Cuvillier, 1995.
24. Mantovani, F., Covaceuszach, S., Rustighi, A., Sgarra, R., Heath, C., Goodwin, G.H., and Manfioletti, G. NF-kappaB mediated transcriptional activation is enhanced by the architectural factor HMGI-C. Nucleic Acids Res **26,** 1433, 1998.

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3.2. Development of HMGA2 fragments for use in tissue engineering

As was shown in the previous paper, the application of recombinant HMGA proteins could improve the growth of porcine chondrocytes in an *in vitro* proliferation assay (Richter et al. 2009). However, for application in a perspective therapeutic context, the use of recombinant agents from biological systems also has some disadvantages, such as potential unwanted modifications of the protein by the expression host or contaminations that still might remain in the final product in trace amounts albeit purification procedures.

Thus, a variant of the active agent produced in a non-biological system might be of advantage diminishing any potential biological contamination provided that it shows comparable biological activity to the recombinantly produced protein.

Due to their lack of secondary structure (Reeves et al. 2001), HMGA proteins can be synthesised synthetically e.g. by solid phase protein synthesis (SPPS). This method is constrained by the length of the synthesised fragment as the efficiency of every single coupling step adds up to the final yield of the product, leading to a maximum of 70 amino acids that can be synthesised satisfactorily in SPPS. Longer fragments can be gained by chemically coupling two peptide strains. However, as it had been shown before that truncated HMGA2 remains active albeit the loss of the C-terminal part of the protein (Battista et al. 1999) as long as its functional motifs the AT-hooks are present (Geierstanger et al. 1994), two shorter variants of 60 aa and 80 aa, respectively, containing either two (HMGA2-A) or three (HMGA2-B) AT-hooks were synthesised, overcoming the limitations that would arise when trying to synthesise the complete HMGA2 protein (109 aa).

- II -

High-mobility group protein HMGA2-derived fragments stimulate the proliferation of chondrocytes and adipose tissue-derived stem cells

Richter et al., European Cells and Materials, 2011

Porcine chondrocytes were subjected to the same proliferation assay procedure as described before (Richter et al. 2009), where they were trialled for growth inducing effects of HMGA1a, HMGA1b, and HMGA2. For better comparability due to the differing sizes of the HMGA2 protein and the HMGA2 fragments, molarities instead of weight were used for determining the amount of active agent used in the experiments. In addition, only fragments of HMGA2 and the HMGA2 protein were used in this experimental setup. Supplementary to chondrocytes, canine adipose tissue derived stem cells (ADSCs) were trialled as well, as they can be differentiated into the chondrogenic lineage when exposed to specific factors (Zuk et al. 2001; Zuk et al. 2002). Supplementing the cell proliferation ELISA, fluorescence microscopy was performed for determination of the cellular uptake and localisation of a fluorescently labelled variant of one of the fragments as well for detection of cell viability allowing the exclusion of any false positive results in peptide take-up due to the permeability of dead cells.

Fluorescence microscopy revealed an accumulation of the labelled HMGA2-CC variant in the nuclei of about 50% of the chondrocytes at both concentrations of 10µM and 50µM peptide, while in ADSCs, a percentage of 24 could be detected at the concentration of 100µM. Nearly 100% of the cells showed green fluorescence in the cytoplasm, indicating accumulation of the peptide instead of nuclear transport. Only a minimum amount of cells showed red fluorescence indicating cell death.

In chondrocytes, the cell proliferation ELISA revealed a significant doubling of proliferation caused by the 60 aa HMGA2-A peptide at both concentrations of 10µM and 50µM, as well as for the 109 aa recombinantly produced HMGA2 protein at 10µM (there was no 50µM control for the HMGA2 protein), when compared to the untreated control. Between HMGA2-A and HMGA2, no significant difference could be detected in the proliferation inducing effect, indicating that the smaller 60 aa peptide is a suitable replacement for the complete HMGA2 protein. The HMGA2-B peptide on the other hand only showed a 1.5x increase at the lower concentration of 10µM, however, this was not statistically significant when compared to the untreated control. At 50µM, no significant difference could be seen when compared to the control.

In canine ADSCs, there was a slightly different picture as the shorter HMGA2-A peptide could induce significant proliferation only at the higher concentration of

50μM, while the longer HMGA2-B peptide induced significant proliferation at the lower concentration of 5μM. No positive control utilising recombinant HMGA2 was performed.

- II -

Richter A, Lübbing M, Frank HG, Nolte I, Bullerdiek JC, von Ahsen I

High-mobility group protein HMGA2-derived fragments stimulate the proliferation of chondrocytes and adipose tissue-derived stem cells

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Own contribution:

Study design of the chondrocyte part of the paper

Recombinant expression of HMGA2

High performance liquid chromatography

Chondrocyte cell culture

Chondrocyte *in vitro* cell proliferation assay & vitality testing

Examination and interpretation of results in the chondrocyte part of the paper

Statistics

Graphics design (figures 2 – 5 and table 1)

Writing of the chondrocyte part of the manuscript and adaptation of the adipose tissue derived stem cell part into the final manuscript

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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.

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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

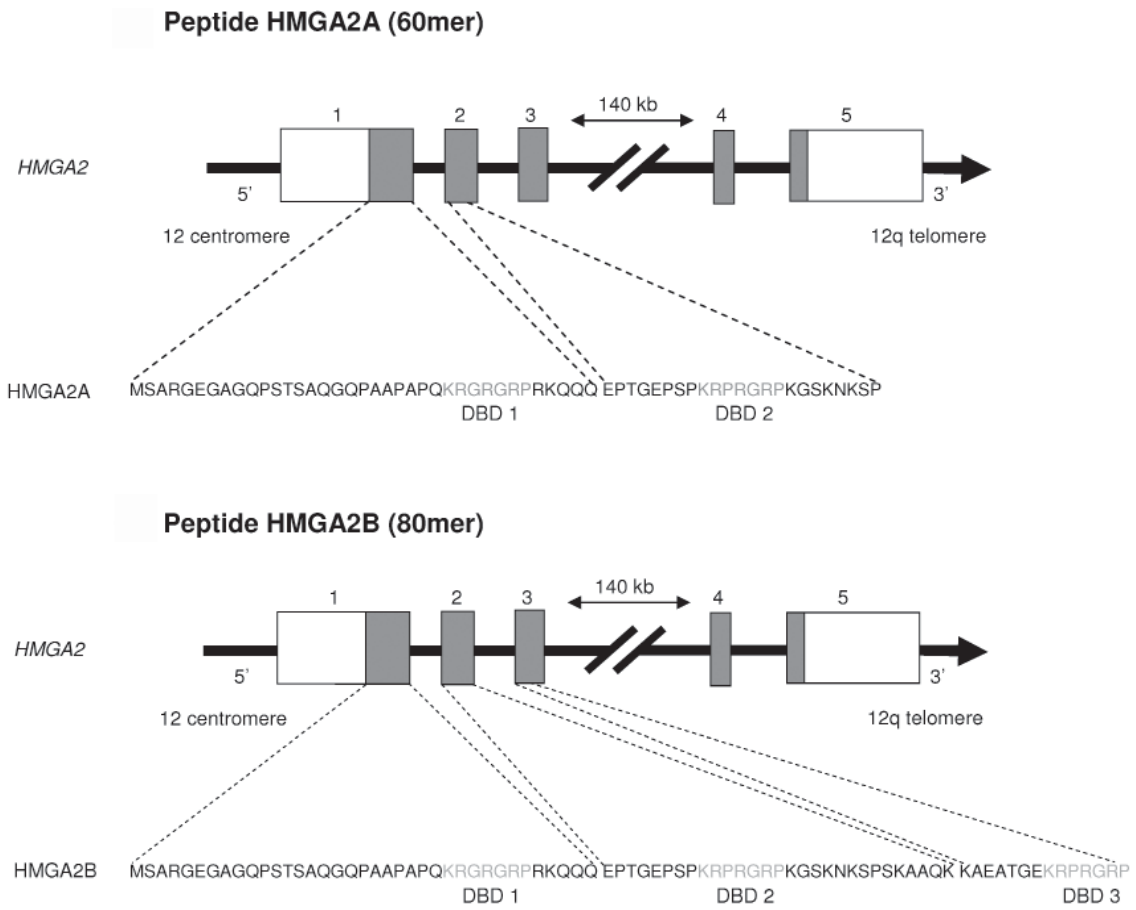


Fig. 1. Amino acid sequence and structure of the two HMGA2 peptides. DBD = DNA binding domain.

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-β, leading to the formation of hyaline cartilage (Zuk *et al.*, 2001; Huang *et al.*, 2004).

Materials and Methods

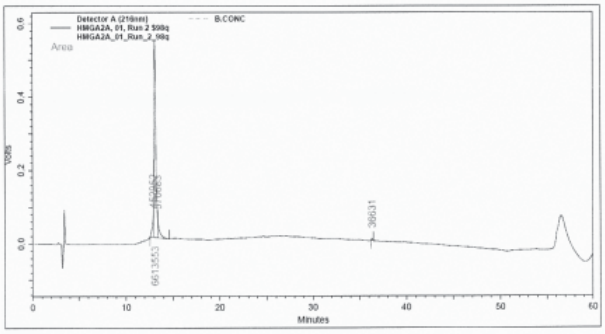
HMGA2 fragment synthesis

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 irridation 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_2O, 2.5% 3,6-Dioxa-1,8-octandithiol (Sigma,

a) HPLC

Detector A (216nm)

Pk #	Retention Time	Area	Area %	Height	Height %
1	12.883	452952	5.903	51442	8.009
2	13.075	6613553	86.183	536085	83.460
3	13.375	570683	7.437	49463	7.701
4	36.333	36631	0.477	5337	0.831
Totals		7673819	100.000	642327	100.000



b) ESI-MS

Molar mass: 6298 Da (Thermo Electron MSQ)

	Real mass spectrum	Theoretic mass spectrum
M4+	1575.2	1575.5
M5+	1260.5	1260.6
M6+	1050.7	1050.7
M7+	900.8	900.7
M8+	788.4	788.3
M9+	700.9	700.8

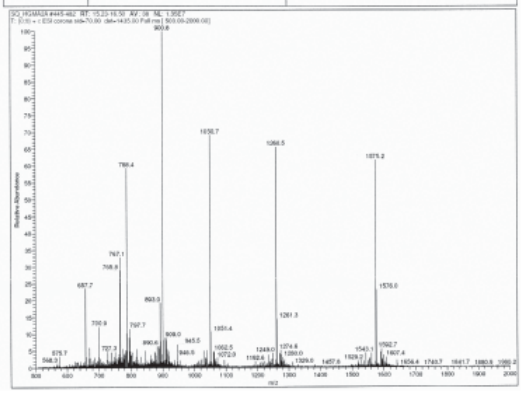


Fig. 2. HPLC and ESI-MS analysis of the HMGA2-A synthesis product.

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 reprosil 100 10 µm 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 µm 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

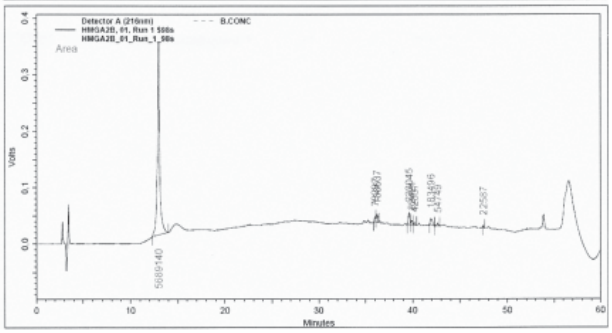
Recombinant HMGA2 produced using the Novagen pET System (Merck Chemicals, Darmstadt, Germany) following a protocol adapted from Schwanbeck (1995) served as a positive control. The complete coding sequences of *HMGA2* was inserted into the pET3a vector. Subsequently, transformation of the non-expression host *E. coli DH5α* 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 µg/mL ampicillin, 34 µg/mL chloramphenicol and 1% (w/v) glucose. After inoculation, 1L of expression culture (LB broth supplied with 100 µg/mL ampicillin, 34 µg/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 °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 °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

a) HPLC

Detector A (216nm)

Pk #	Retention Time	Area	Area %	Height	Height %
1	12.967	5689140	87.174	339701	81.746
2	36.000	79297	1.215	8976	2.160
3	36.217	138637	2.124	13900	3.345
4	39.608	233045	3.571	21138	5.087
5	39.900	82421	1.263	7828	1.884
6	40.175	42837	0.656	4414	1.062
7	41.917	183496	2.812	10932	2.631
8	42.633	54749	0.839	5291	1.273
9	47.458	22587	0.346	3376	0.812
Totals		6526209	100.000	415556	100.000



b) ESI-MS

Molar mass: 8447 Da (Thermo Electron MSQ)

	Real mass spectrum	Theoretic mass spectrum
M5+	1689.9	1690.3
M6+	1408.6	1408.8
M7+	1207.6	1207.7
M8+	1056.9	1056.8
M9+	939.5	939.5
M10+	845.7	845.7
M11+	769.0	768.9

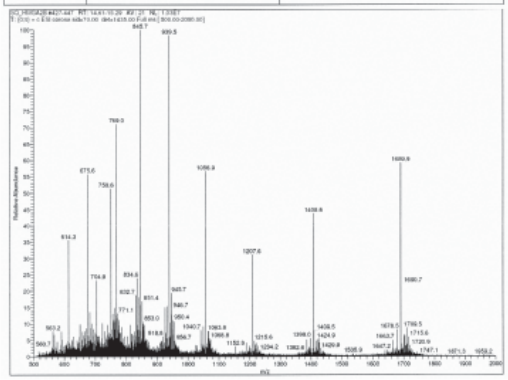


Fig. 3. HPLC and ESI-MS analysis of the HMGA2-B synthesis product.

adding 6x vol. of -20 °C acetone. Protein was pelleted by centrifugation in 80 mL glass tubes, the acetone supernatant carefully removed, the pellet frozen to -80 °C and dried under vacuum. Pelleted protein was resolved using 50 mM ammonium hydrogen carbonate at 4 °C. This crude acid soluble protein mix containing mostly HMGA2 was stored until further processing at -80 °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 µm, Tosoh Bioscience GmbH, Stuttgart, Germany) and a gradient of 25 mM H_3BO_3 pH 9.4 and 25 mM H_3BO_3/1M 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 °C for long term storage. A stock solution of 10 µg/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 °C / 5 % 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 cm² tissue culture flask (Nunc, Wiesbaden, Germany). They were incubated at 37 °C / 5% CO_2 / 5 mL tissue culture medium as described previously until ¾ 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_2 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 cm²) positioned in a sterile plastic 12-well microtiter plate and incubated for 24 h at 37 °C/5% CO_2 (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_2. Thereupon, the medium was replaced with 1 mL of medium199/10% 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), bisbenzimide (0.1 µg/mL) and PI (1 µg/mL) was added to the cells. Cells were incubated at 37 ºC 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 (bisbenzimide), 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_2. 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_2 for 17 h followed by the addition of BrdU. After an incubation period of 7 h at 37 °C / 5% CO_2, 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 10^4 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

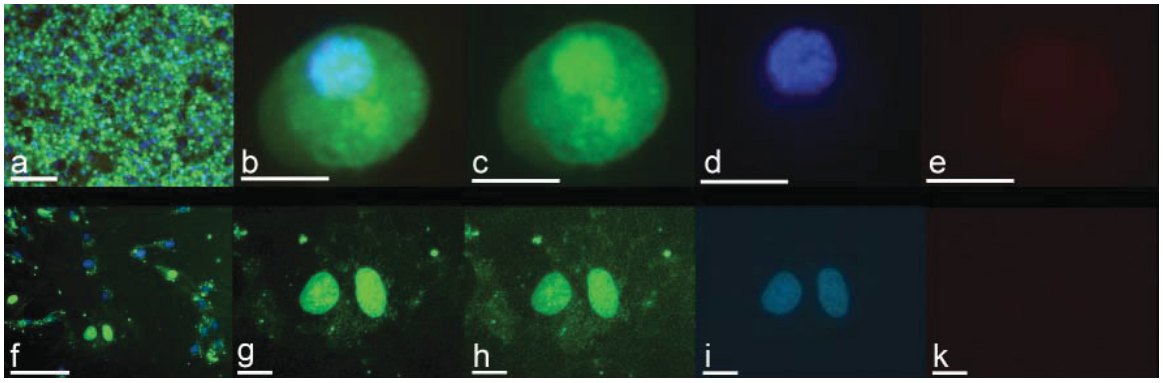


Fig. 4. Transfection of porcine chondrocytes **(a-e)** and canine ADSCs **(f-k)** in *in vitro* monolayer cell culture with the fluorescently labelled HMGA2-CC peptide. **(a)** Overview of porcine chondrocytes showing the green fluorescence of HMGA2-CC in 51% of the cells as well as the blue DAPI stain 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 µm in **a** and **f**; 10 µm in **b-e**; 20 µm in **g-k**.

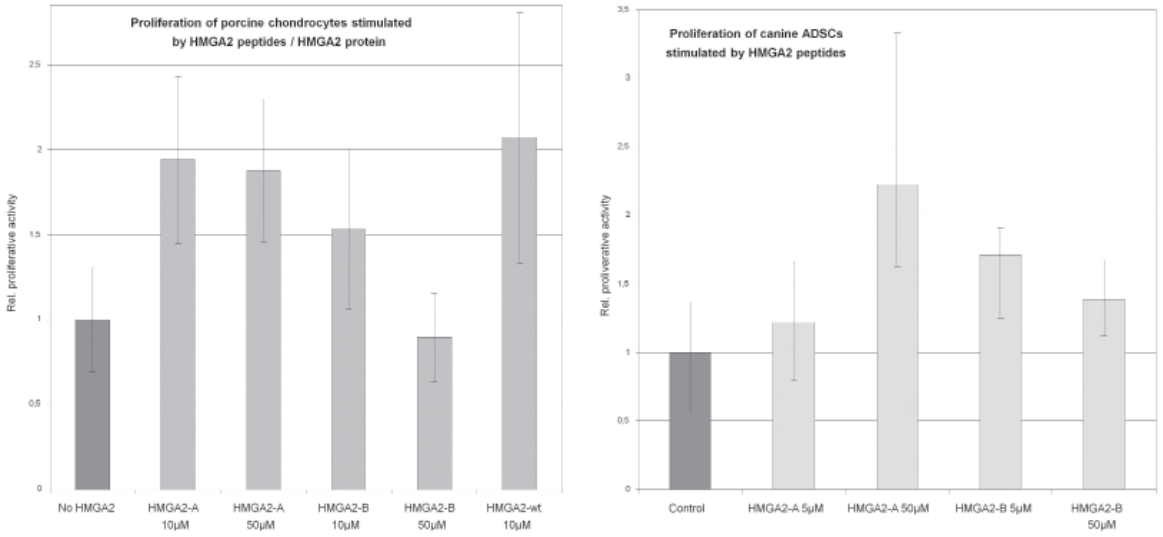


Fig. 5. Cell proliferation of porcine hyaline cartilage cells **(a)** and canine ADSCs **(b)** induced by the addition of HMGA2 peptides. Values obtained by the microtiter plate reader were normalised to the absorbance of the non-HMG control which was set as 1. Error bars: standard deviation.

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 µM	HMGA2-A 50 µM	HMGA2-B 10 µM	HMGA2-B 50 µM	HMGA2-wt 10 µM
Control						
HMGA2-A 10 µM	< 0.01					
HMGA2-A 50 µM	< 0.01	> 0.05				
HMGA2-B 10 µM	> 0.05	> 0.05	> 0.05			
HMGA2-B 50 µM	> 0.05	< 0.01	< 0.01	> 0.05		
HMGA2-wt 10 µM	< 0.001	> 0.05	> 0.05	> 0.05	< 0.001	

(b)	Control	HMGA2-A 5 µM	HMGA2-A 50 µM	HMGA2-B 5 µM	HMGA2-B 50 µM
Control					
HMGA2–A 5 µM	> 0.05				
HMGA2–A 50 µM	< 0.001	< 0.001			
HMGA2–B 5 µM	< 0.01	> 0.05	> 0.05		
HMGA2–B 50 µM	> 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 µM (1.94x increase) and 50 µM (1.88x increase), or wild-type HMGA2 protein (10 µM, 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 µM and 50 µM. There was also no significant difference in the proliferation inducing activities of wild type HMGA2 and 10 µM or 50 µM 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 µM (1.54x increase) and 50 µM (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 µM and HMGA2-wt, while HMGA2-B at 50 µM 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 µM.

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

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 μM and 50 μM 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 μM and 50 μM, and only had comparable effect to the wild type protein at 10 μM, while there was a statistically significant lower proliferation at 50 μM, 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 μM compared to 10 μM, 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 μM 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 μM, while the higher dose of 50 μM led to a reduced proliferation compared to 5 μM 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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References

Battista S, Fidanza V, Fedele M, Klein-Szanto AJ, Outwater E, Brunner H, Santoro M, Croce CM, Fusco A (1999) The expression of a truncated HMGI-C gene induces gigantism associated with lipomatosis. Cancer Res **59**: 4793-4797.

Cattaruzzi G, Altamura S, Tessari MA, Rustighi A, Giancotti V, Pucillo C, Manfioletti G (2007) The second at-hook of the achitectural transcription factor HMGA2 is determinant for nuclear localization and function. Nucleic Acids Res **35**: 1751-1760.

Chau KY, Patel UA, Lee KL, Lam HY, Crane-Robinson C (1995) The gene for the human architectural transcription factor HMGI-C consists of five exons each coding for a distinct functional element. Nucleic Acids Res **23**: 4262-4266.

Cleynen I, Van de Ven WJ (2008) The HMGA proteins: A myriad of functions (Review). Int J Oncol **32**: 289-305.

Dominici M, Le Blanc K, Mueller I, Slaper-Cortenbach I, Marini F, Krause D, Deans R, Keating A, Prockop D, Horwitz E (2006) Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy Position Statement. Cytotherapy **8**: 315-317.

Fedele M, Berlingieri MT, Scala S, Chiariotti L, Viglietto G, Rippel V, Bullerdiek J, Santoro M, Fusco A (1998) Truncated and chimeric HMGI-C genes induce neoplastic transformation of NIH3T3 murine fibroblasts. Oncogene **17**: 413-418.

Geierstanger BH, Volkman BF, Kremer W, Wemmer DE (1994) Short peptide fragments derived from HMG-I/Y proteins bind specifically to the minor groove of DNA. Biochemistry **33**: 5347-5355.

Goodwin G (1998) The high mobility group protein, HMGI-C. Int J Biochem Cell Biol **30**: 761-766.

Huang JI, Zuk PA, Jones NF, Zhu M, Lorenz HP, Hedrick MH, Benhaim P (2004) Chondrogenic potential of multipotential cells from human adipose tissue. Plast Reconstr Surg **113**: 585-594.

Narita M, Krizhanovsky V, Nunez S, Chicas A, Hearn SA, Myers MP, Lowe SW (2006) A novel role for high-mobility group a proteins in cellular senescence and heterochromatin formation. Cell **126**: 503-514.

Noro B, Licheri B, Sgarra R, Rustighi A, Tessari MA, Chau KY, Ono SJ, Giancotti V, Manfioletti G (2003) Molecular dissection of the architectural transcription factor HMGA2. Biochemistry **42**: 4569-4577.

Reeves R (2000) Structure and function of the HMGI(Y) family of architecturaltranscription factors. Environ Health Perspect **108 Suppl 5**: 803-809.

Reeves R, Beckerbauer L (2001) HMGI/Y proteins: Flexible regulators of transcription and chromatin structure. Biochim Biophys Acta **1519**: 13-29.

Reeves R, Beckerbauer LM (2003) Hmga Proteins as Therapeutic Drug Targets. Prog Cell Cycle Res **5**: 279-286.

Reeves R, Nissen MS (1990) The A.T-DNA-binding domain of mammalian high mobility group I chromosomal proteins. A novel peptide motif for recognizing DNA structure. J Biol Chem **265**: 8573-8582.

Richter A, Hauschild G, Murua Escobar H, Nolte I, Bullerdiek J (2009) Application of high-mobility-group-A proteins increases the proliferative activity of chondrocytes *in vitro*. Tissue Eng Part A **15**: 473-477.

Rogalla P, Drechsler K, Frey G, Hennig Y, Helmke B, Bonk U, Bullerdiek J (1996) HMGI-C Expression patterns in human tissues. Implications for the genesis of frequent mesenchymal tumors. Am J Pathol **149**: 775-779.

Schwanbeck R (1995) Die Phosphorylierung der architektonischen HMGI(Y)-Chromatinproteine: Modulation der Struktur und DNA-Bindung. Cuvillier Verlag, Goettingen.

Yie J, Liang S, Merika M, Thanos D (1997) Intra- and intermolecular cooperative binding of high-mobility-group protein I(Y) to the beta-interferon promoter. Mol Cell Biol **17**: 3649-3662.

Zuk PA, Zhu M, Mizuno H, Huang J, Futrell JW, Katz AJ, Benhaim P, Lorenz HP, Hedrick MH (2001) Multilineage cells from human adipose tissue: Implications for cell-based therapies. Tissue Eng **7**: 211-228.

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

3.3. Cartilage replacement in dogs

As in humans, the dog is suffering from articular cartilage damage, which may be age or injury related, as well as disease induced e.g. in case of Osteochondritis dissecans (Newton et al. 1985). Problems occurring in human cartilage regeneration such as fibrocartilage formation and the associated onwards degradation of the affected areas (Frenkel et al. 1999; Marlovits et al. 2006) also occur. Thus, the establishment of a replacement therapy for damaged cartilage in dogs is useful from a double perspective, i.e. for both the canine patient as part of its therapy, as well as for humans where the dog could act as a model organism for transfer of methods and experience gained to human therapy. There are some therapeutical approaches in the treatment of damaged cartilage such as lavage, shaving, debridement, (laser) abrasion chondroplasty, and Pridie drilling, where damaged tissue is removed and/or underlying bone marrow stimulated to enhance the formation of new cartilage to fill up the damage. However, they mostly still lead to formation of less resilient areas mainly consisting of fibrocartilage (Hunziker 2002). To overcome some of these problems, the use of scaffolds seeded with cells amplified *in vitro* or combined with transplanted intact tissue from less heavily loaded areas might be a promising approach to replace cartilage in the damaged areas. For this study, a biosynthetic structure consisting of beta-tricalcium phosphate (β-TCP) was chosen, as this compound was already proven as highly suitable in the treatment of bone defects in humans and animal models (Anker et al. 2005; Hirata et al. 2006; Suba et al. 2006; Frota et al. 2011), but no data was available at that time for the colonisation with canine chondrocytes.

- III -

Cartilage replacement in dogs - A preliminary investigation of colonization of ceramic matrices

Hauschild et al., Veterinary and Comparative Orthopaedics and Traumatology, 2009

In this study, β-TCP cylinders were colonised with isolated canine chondrocytes or cartilage chips, respectively. As a control, isolated chondrocytes from the same donor were kept in cell culture and subjected to the same vitality test as the cells for colonisation. The cylinders that were inoculated with a solution of isolated chondrocytes showed an even distribution of cells across the cylinder surface and within the cylinders' drilled macropores, as far as could be detected by visible light and fluorescence microscopy. Around the macropores' edges, less cell density, but still even distribution of cells could be detected. Vitality staining revealed viability of most cells, and the cells showed partial cell-to-cell contact. Scanning electron microscopy allowed the detection of cells' filopodia that anchored onto the construct surface and entered the substrate's micropores, as well as it confirmed the direct cell to cell contact. The cell culture control also showed even distribution of mostly viable cells and only sporadic cell death in the vitality test.

In contrast, the cylinders that were studded with cartilage chips showed only little colonisation with small groups of cells or individual cells growing around or within the drill holes where the cartilage chips were fastened. Nevertheless, vitality staining proved the viability of these cells.

- III -

Hauschild G, Muschter N, Richter A, Ahrens H, Gosheger G, Fehr M, Bullerdiek J

Cartilage replacement in dogs -- A preliminary investigation of colonization of ceramic matrices

Vet Comp Orthop Traumatol. 2009. 22:216-21.

Own contribution:

Study design together with G. Hauschild and J. Bullerdiek

Design and execution of experiments excluding scanning raster electron microscopy

Examination and interpretation of experiment results together with G. Hauschild and J. Bullerdiek

Keyword compilation for the methods section of the manuscript

Proofreading of the manuscript

Cartilage replacement in dogs

A preliminary investigation of colonization of ceramic matrices

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Keywords

Cartilage replacement, bioartificial graft, matrix

Summary

The objective of this study was to examine the behaviour of canine chondrocytes following colonisation of a β-tricalcium phosphate (β-TCP, Cerasorb®, Curasan) matrix. In total, five of these cylinders were inoculated with 1.5 ml of cell suspension and subsequently incubated for about one week. In the second part of the experiment, another five Cerasorb® cylinders were each studded with two cartilage chips of variable size and then incubated for about one week. The series of experiments were analyzed using cell staining and imaging techniques that included scanning electron microscopy. Cell migration onto the matrix was proven for both colonisation methods. It was observed that colonising the cylinders by pipetting cell suspension on them produced far better results, with respect to both growth rate and spreading of the cells, than did colonisation by studding with cartilage chips. A homogenous, surface-covering colonisation with predominantly living cells was demonstrated by scanning electron microscopy in the chondrocyte morphology. In comparison to cell-culture controls, there was a clearly better colonisation, with cells attached to both the material's primary grains and its micropores. The ceramic studied is well accepted by canine chondrocytes, and appears to be fundamentally well-suited as a matrix for bio-artificial bone-cartilage replacement. Additional qualitative analyses and a series of experiments aiming to accelerate cell proliferation are planned for subsequent studies.

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Introduction

Damage to articular cartilage in canine or human patients is a serious orthopaedic problem and a therapeutic challenge. The tendency of cartilage to self-heal is severely limited due to its avascularity, lack of innervation and lymphatic circulation, and relatively low cellularity (1). Intrinsic repair mechanisms of hyaline cartilage do not bring about healing that leads to reconstitution or regeneration. Instead, usually only a reduction of the lesion is achieved, or the deficient area is filled with biomechanically weaker fibrocartilage, which possesses sufficient tensile strength yet cannot adequately absorb the compression loads acting on joints (1–3). This inadequate tendency to regeneration is further limited by the separation of the chondrocytes from the deficient area by the extracellular matrix (4). Conventional therapeutic methods, such as lavage and debridement, bone-cartilage Pridie drilling, Steadman microfracturation, and Johnson abrasion arthoplasty, all attempt to promote or improve the self-heal potential of the cartilage by stimulating mesenchymal stem cells, growth factors, and progenitor cells from the bone marrow. The result of these various treatments nonetheless remains at best the formation of fibrocartilage, for which a quarter of all patients leads to no improvement of symptoms, even in the long-term, or to renewed deterioration (5–9).

The use of biological materials and bio-artificial grafts represents an alternative to these techniques. The latter are, as a rule, composite materials from a natural or synthetic carrier and autologous or heterologous cells. The use of natural carriers is limited though by their usually low mechanical stability, uncontrollable degradation, and sterilisation difficulties associated with possible pathogen transfer. In contrast, synthetic matrices have form and surface textures that can be controlled much better and exhibit excellent mechanical and physiochemical properties (10). For osteochondral grafts, especially as they are implemented in the context of a modified Autologous Transfer System (OATS), the benefits of using a well-established bone replacement material as a matrix are obvious. The therapeutic concept of OATS has been based so far on grafting autologous bone-cartilage cylinders, taken from areas of low biomechanical stress of the affected joint, onto the prepared deficient area. Significant disadvantages of the procedure include not only graft mount complications, such as central necrosis, resorption, non-orthograde positioning and marrow oedema in the subchondral bone area (11, 12), but also above all, the limited availability and the morbidity at the donor site. In contrast, there is an unlimited availability of synthetic bone

replacement materials, including the β-TCP tested in the present study, which has been successfully used clinically in orthopaedics and dental orthopaedics (13–17). In the case of proven osteoconductivity and successful use as a bone replacement material, the suitability of that bioceramic as a matrix for canine chondrocytes would currently remain unconfirmed to our knowledge. Colonisation of the matrix with autologous chondrocytes or their progenitor cells *in vitro* and *in vivo* is imperative for the imitation of autologous bone-cartilage cylinders used in OATS with a biosynthetic material. Guo et al. (18) were able to achieve promising results with respect to regeneration by implanting pulverised β-TCP colonised with mesenchymal stem cells into osteochondral defects in sheep. The goal of the present study is to investigate the behaviour of canine chondrocytes following colonisation of a pure-phase cylindrical β-TCP ceramic.

Methods

Cylinders

Five Cerasorb® cylinders were colonised by chondrocytes replicated in a cell culture, and five other cylinders were studded with cartilage chips and then incubated. In each case, two of the constructs were stained with 4',6-diamidino-2-phenylindole (DAPI) to evaluate cell growth. Four additional cylinders (two for each study condition above) were stained with Trypan blue to assess cell viability and were then treated with DAPI. Two of these cylinders (one per study condition) were subjected to scanning electron microscopy in order to determine the cell morphology.

Matrix

β-TCP cylinders[a] measuring 8.5 x 20 mm were used as a matrix. The phase purity of the material is over 99%. In addition to the material's intrinsic interconnective porosity, the cylinders had vertical and horizontal drill holes with a diameter of 1 mm (macropores), whereby the horizontal holes ran the entire length of the construct, but the vertical holes did not penetrate the bottom surface of the cylinder (▶Fig. 1)

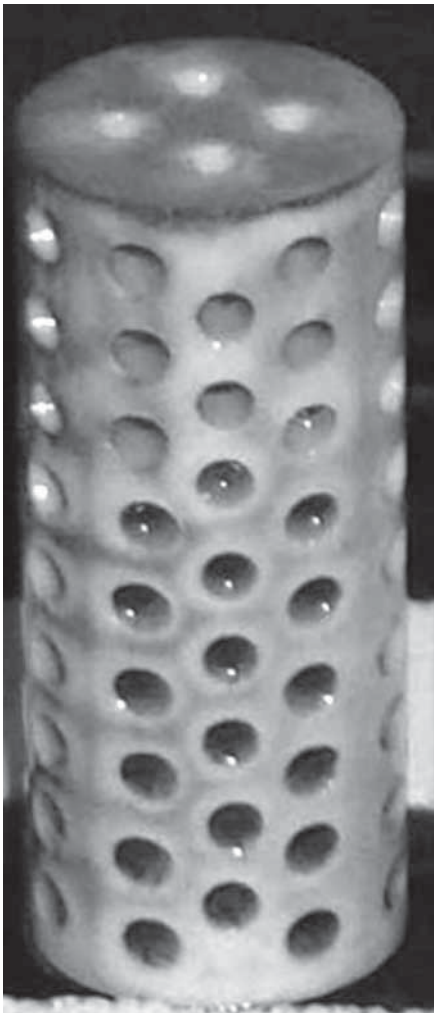

Fig. 1 Cerasorb® cylinder.

Isolation of cartilage cells

The canine cartilage used in the study came in the form of small cartilage chips from animals treated at the Veterinary Clinic of the Hannover Veterinary College and the Asterlagen Veterinary Hospital in Duisburg-Rheinhausen. The chips were obtained from excised femoral heads that were available as a result of a therapeutically conducted hip joint prosthesis implantation or a femoral head and neck excision. They were stored in Hanks' medium[b] until further use. To separate the cells, the cartilage was briefly centrifuged, and the medium was siphoned off using a vacuum pump[c]. The chips were then washed in 10 ml phosphate buffered saline (PBS)[b] and transferred to a 25 cm² cell culture flask[d]. After adding 4 ml of equal parts collagenase NB8[e] and Medium 199[f], the chips were incubated for at least six hours at 37°C in 5% CO_2 to free the cells[g]. During this time, the cell-containing supernatant underwent repeated resuspension by carefully shaking the cell culture flask. Following incubation, the cells were washed with 10 ml of Medium 199 to remove the collagenase and finally resuspended in 5 ml of Medium 199.

Cell cultivation

The resuspended cells were transferred to a 25 cm² cell culture flask, where cell expansion took place between three and seven days. The culture medium was exchanged twice weekly, until approximately three fourths of the flask surface was covered with cells (incubation at 37°C in 5% CO_2). To collect the cells, the medium was first siphoned off. The cells were then washed in 5 ml PBS; 1 ml TrypLE (trypsin replacement)[f] was added to detach chondrocytes from the flask surface, and then the cells were washed in 10 ml Medium 199. The cell count was determined using a hemacytometer[h] after resuspension in 2 ml Medium 199. The final cell count was 1 x 10^5 cells per ml medium.

Matrix colonisation with cultured cells

Each of the five β-TCP cylinders had a liquid intake volume of 1.2 ml. In order to ensure a dense cell colonisation and to minimise colonisation of the culture flask instead of the matrix, the construct inoculation took place in a small CryoTube[i] with a 2 ml capacity. The cylinders were transferred into the tube with

[a] Cersorb®, Curasan, Kleinostheim, Germany

[b] Biochrom KG, Berlin, Germany

[c] Jürgens Omnilab, Bremen, Germany

[d] Nunc, Wiesbaden, Germany

[e] Serva Electrophoresis GmbH, Heidelberg, Germany

[f] Invitrogen GmbH, Karlsruhe, Germany

[g] Thermo Electron Corporation, Waltham, MA, USA

[h] Menzel-Gläser, Braunschweig, Germany

[i] Nalge Nunc International, Rochester, NY, USA

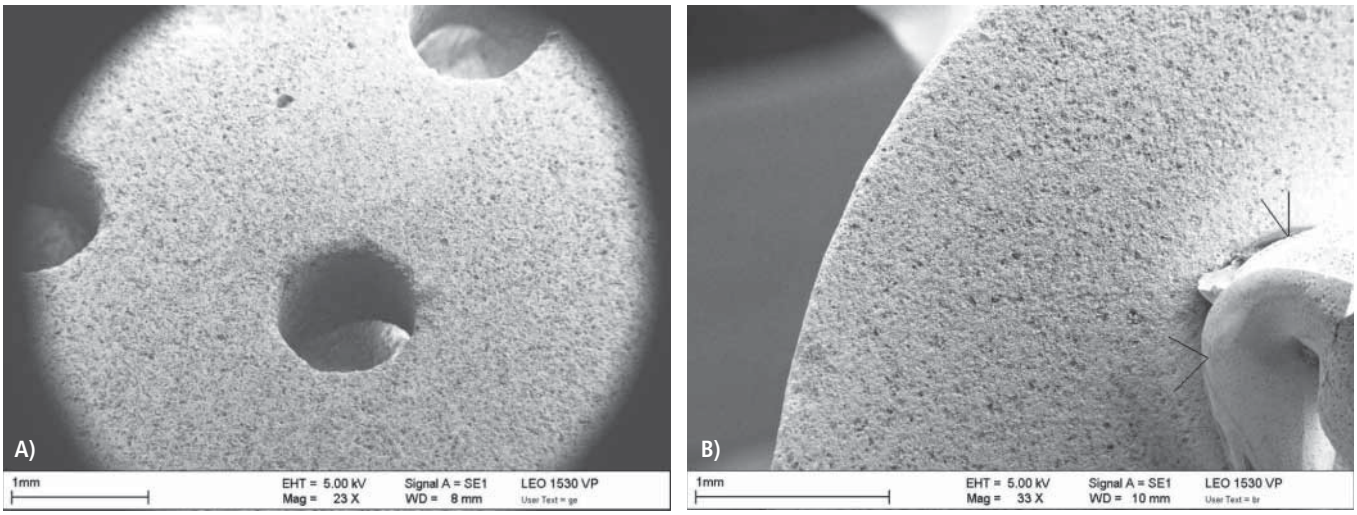

Fig. 2 Scanning electron microcope Images of a Cerasorb® cylinder showing three vertical macropores **(A)** and one macropore **(B)** studded with cartilage chips (arrow heads).

the closed base surface facing down. Then, 2 x 600 µl of cell suspension was pipetted into the top macropores (vertical drill holes) of the cylinder, so that the matrix was filled and covered with cell suspension. To allow gas exchange, CryoTube lids were loosely placed. The cylinders were then incubated for at least six hours at 37°C in 5% CO_2 in order to allow the cells to anchor themselves to the matrix. Next, each construct was transferred to a cell culture flask with a special mounting, covered with 15 ml Medium 199 and incubated for one week at 37°C in 5% CO_2. During this period, the medium was renewed twice, each time after three days.

Matrix colonisation with canine cartilage chips

In a second group, five β-TCP cylinders were each studded with two canine cartilage chips of variable size by placing the chips into the vertical drill holes of the construct (▶Fig. 2). This group was also covered with 15 ml Medium 199 and incubated for one week at 37°C in 5% CO_2. The medium was renewed as previously described.

Analytical process

The cell viability in culture and after colonisation was tested using Trypan blue staining[f] as previously described by Freshney (19), and microscopic analysis and documentation was performed using a Zeiss Axioskop 2 Plus and an Axiocam HR[j]. To assess cell growth in culture and on the surface and inside the cylinders, and to register living cells that were not detected by Trypan blue staining, the constructs were stained with DAPI[k] (4',6-diamidino-2-phenylindole) (20), positioned on a microscope slide and subsequently analyzed using fluorescent microscopy with a DAPI filter. Documentation was carried out as described before. Visualization and measurements were performed using a software-programme(Axiovision)[j]. For a more in-depth examination of cell behaviour on the cell matrix, the constructs were also examined using scanning electron microscopy (REM 1530VP)[j]. To do so, the colonised cylinders were fixed for six hours using 2% glutaraldehyde in phosphate buffer[l], dehydrated in a rising alcohol series, completely evacuated inside a Sputter Coater[m], and finally coated with an approximately 6 nm thick silver layer.

Results

Cell culture controls

Cell culture controls in 6-well plates showed an evenly distributed colonisation of the surface with viable, round or spindle-shaped cells, ranging from 10 to 30 µm in size, with filopodia extending up to 100 µm (▶Fig. 3). Blue staining of cells indicating cell death occurred only sporadic.

Colonisation with expanded cells

On all five cylinders that were inoculated with cell suspension, Trypan blue staining and subsequent DAPI staining showed evenly distributed, mainly viable, round cells with a circumference measuring between 5 and 30 µm. Taken together, both visualization methods showed a surface-covering colonisation of the matrix and, to the extent of their visibility, the vertical drill holes. The constructs stained only with DAPI likewise showed a homogenous surface colonisation with migration of cells into the macropores of the matrix. The cells were positioned on the surface next to one another, with partial cell-to-cell contact. Around the macropores, the cells were grouped less densely but were still evenly distributed (▶Fig. 4). The scanning electron microscopy revealed evenly distributed cells, each displaying several filopodia that anchored onto the primary matrix structure and entered the interconnecting micropores of the ceramic. These projections also demonstrated a predominantly direct cell-to-cell contact (▶Fig. 5A-C).

j Zeiss, Jena, Germany
k Roche Diagnostics GmbH, Mannheim, Germany
l Merck Eurolab GmbH, Darmstadt, Germany
m Polaron Equipment, Watford, England

Studding the matrix

Studding the vertical drill holes of the matrix with cartilage chips produced a different picture.

In both the Trypan blue staining and in the DAPI staining, mainly viable, round cells with a circumference of 5 and 30 µm were observed only in the areas of some of the macropores studded with cartilage chips; sometimes individually or in small groups, sometimes homogenously distributed. With increasing distance from the drill hole, both the cell count and the homogeneity of the colonisation decreased drastically. Colonisation of the matrix could also be observed inside these macropores. In the remaining studded vertical drill-holes, no cell migration with subsequent colonisation of the matrix surface was detected.

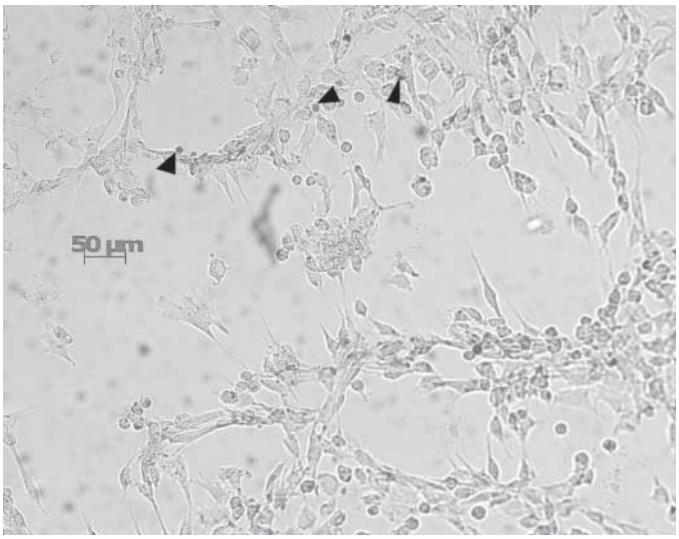

Fig. 3 Cell culture control (6-well plate); Trypan blue stain (40x magnification, exposure 229.64 ms) showing viable and non-viable (arrow heads) cells.

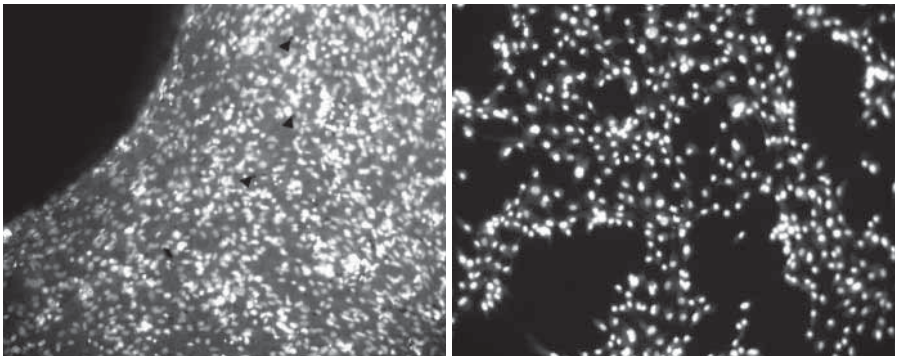

Fig. 4 **Left:** Cerasorb® cylinder colonised with chondrocytes with pore (Trypan blue staining followed by DAPI staining, fluorescent microscopy [10x magnification, exposure 99.5 ms]). Notice sporadically occurring non-viable cells (arrow heads). **Right:** Cell culture control (6-well plate) (Trypan blue staining followed by DAPI staining, fluorescent microscopy [10x magnification, exposure 39.8 ms]).

Discussion

Presently, cartilage defects are treated with the goal of achieving the most complete functional recovery of the joint surface possible. Among the treatments available, mosaicplasty, or OATS, is considered to be highly promising in humans as well as in canine patients (5, 21–27). The extraction of bone-cartilage cylinders is associated though with the risk of damage to the chondrocytes and morbidity at the donor site. Additionally, the procedure is restricted by the limited availability of the material. Synthetic matrices, such as the ceramic β-TCP matrix used in this study, are not restricted by this limitation. In order to minimise the disadvantages of mosaicplasty, a synthetic matrix must first be colonised *in vitro* with autologous chondrocytes. This bioartificial composite material will then be grafted onto the defective area according to OATS technical procedure. The present study investigated the feasibility of colonizing β-TCP cylinders with canine chondrocytes. Different methods of colonisation were compared in respect to their effect on growth rate and cell spreading.

The Cerasorb® ceramic matrix acts as a bone replacement material and thus forms the osteal base for the chondral component of the future graft. In addition to the material's osteoconductive properties, its biodegradation concurrent with osteogenesis, its biocompatibility, its interconnecting porosity, and its phase purity have been confirmed in several studies (15–17, 28, 29). Stimulation of osteal structures to grow directly into the pores of the ceramic usually results in a boundary-free integration into the recipient's natural bone without encapsulating the connective tissue (30). During the assimilation of the matrix into the deficient area, it is expected that a vital connection between the graft and the recipient organism can be attained, due to the described graft behaviour. This is an important prerequisite for the survival of the chondral component of the graft. A further requirement for the graft's functionality is the adequate connection between the subchondral matrix and the cartilage surface. Guo et al. (18) demonstrated that colonisation of the ceramic matrix with ovine chondrocytes is possible but did not report on the morphology of the cell-matrix interface.

The present study used β-TCB cylinders that were colonised with canine chondrocytes or studded with cartilage chips. Compared to cell culture controls, constructs treated with cell suspension exhibited clearly higher cell counts with higher viability. The generally good cell growth on the matrix points to the ceramic's positive influence on cell adhesion and proliferation. The scanning electron microscopy showed that the evenly-distributed cells each had multiple filopodia, which both anchored onto the primary grain structure of the matrix and also entered into the micropores of the ceramic. These projec-

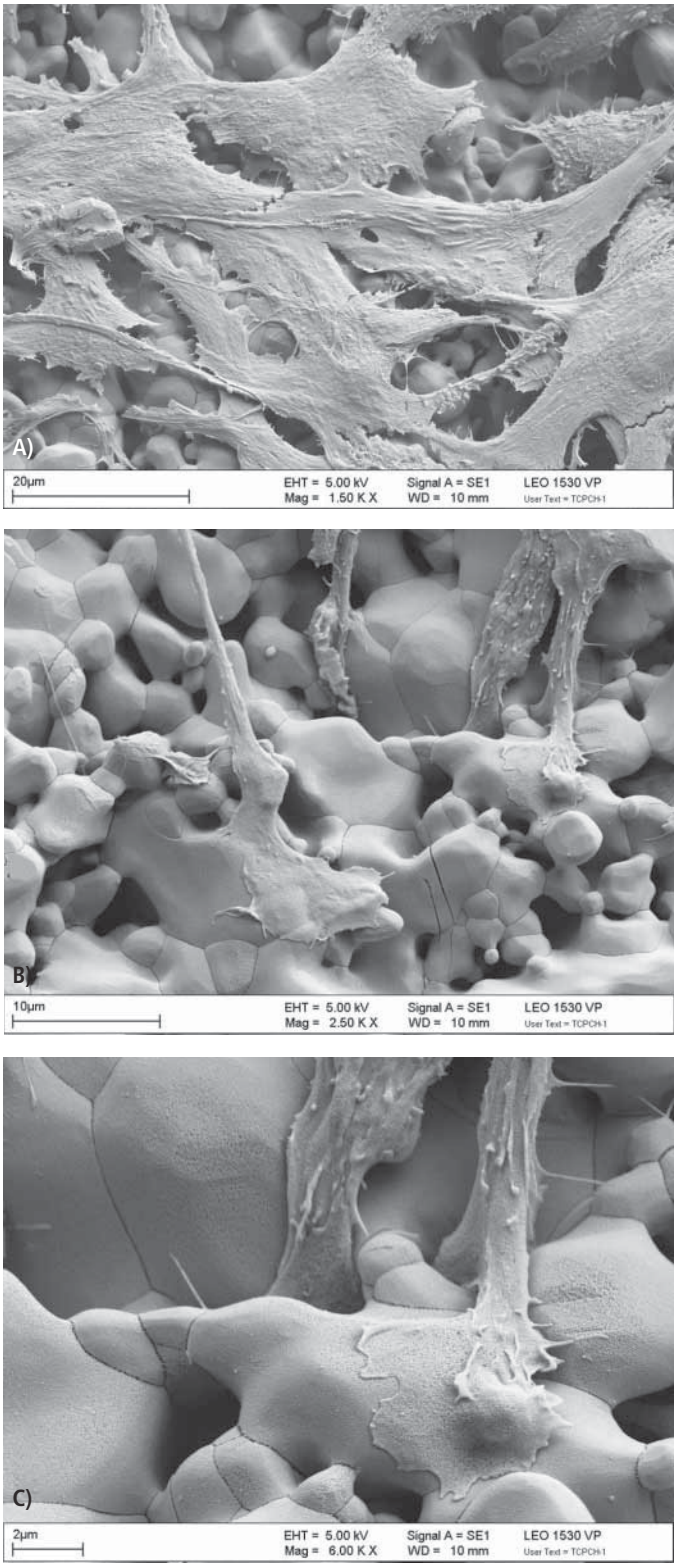

Fig. 5
Scanning electron microscope Images of a Cerasorb® cylinder colonised with a cell suspension. **A)** Interconnecting cells overlapping and covering the TCP-surface. ***B)*** Notice the filopodia anchoring the TCP-surface as well as entering the micropores. **C)** Cell-surface-interface in detail.

tions also demonstrated direct cell-to-cell contact. The direct interlocking between cells and matrix points to the resilience of the bond between the graft components, which is important for the graft's long-term stability and its vital integration into the recipient organism. The absence of this strong connection creates a condition comparable to osteochondrosis dissecans (31, 32), which increases the probability of the *in vitro* generated cartilage surface separating from the ceramic, and later the bone base, leading to graft failure.

Cell migration onto the construct was demonstrated for both colonisation methods. Nonetheless, it was apparent that the technique of studding the construct with cartilage chips was clearly inferior to direct colonisation with cell suspension in respect to cell growth rate and the spreading of cells. Whereas pipetting cell suspension led to cells spreading over the surface of the entire cylinder including the macropores as far as visible, studding the constructs limited cell growth to the area immediately surrounding the cartilage chips. With increasing distance from the drill hole, the cell count and homogeneity became drastically reduced. At a certain distance from the chip, cell growth could not be detected. The expected cell behaviour can probably be attributed to the individual chondrocytes having to first free themselves from the cartilage cell structure. The time loss arising from this process and the larger travel distances in comparison to planar inoculation with cell suspension can explain the significantly lower surface coverage achieved by studding. The observation that single cells do in fact leave the chip and engage the ceramic matrix is an additional indicator for the suitability of β-TCP as a chondral matrix, especially given that equal proportions of living and dead cells appeared in both groups.

It was not possible with the methods of this initial feasibility study *(determination of cell viability by Trypan blue staining, detection of cell growth with DAPI staining, and scanning electron microscopy examination)* to provide indisputable evidence that the cells populating the matrix are hyaline cartilage-producing chondrocytes. Nevertheless, important clues are provided by cell morphology, observed especially with the aid of scanning electron microscopy. Despite the system's inherent disadvantages, that make it impossible to rule out a small-scale change in cell morphology or deterioration of the imaging quality for cell probes (33, 34), the examined cells can be classified with a high degree of confidence as chondrocytes based on their morphology. RT-PCR will be used in upcoming experiments to provide qualitative identification, and it will be able to differenti-

ate between cartilage-specific collagens and aggrecan and versican proteoglycans, and consequently, between hyaline cartilage and fibrocartilage. The same method can also be used to rule out or confirm chondrocyte dedifferentiation on the matrix.

To reach the objective of developing a bone-cartilage replacement for clinical application it is still necessary to determine that the colonisation of a β-TCP ceramic ad modum Cerasorb® by canine chondrocytes is generally possible and that the matrix is well accepted by the cells. Furthermore, the colonisation process must be made faster for use in clinical practice. It is necessary to reduce the required amount of extracted material, in order to eliminate the risk of morbidity at the donor site, which is reduced but not excluded in the modified OATS procedure. Further research will focus on examination of induction of cell replication using the proliferation-promoting High mobililty group AT-hook-2 protein (HMGA2).

References

1. Metz J. Makroskopie, Histologie und Zellbiologie des Gelenkknorpels. In: Gelenkknorpeldefekte. Erggelet C, Steinwachs M (eds). Darmstadt: Steinkopff 2001; 3–14.
2. Liebich HG. Knorpelgewebe (Textus cartilagineus). In: Funktionelle Histologie der Haussäugetiere. Lehrbuch und Farbatlas für Studium und Praxis, vol 3. Liebich HG (ed). Stuttgart, New York: Schattauer 1999; 69–72.
3. Rohen W, Lütjen-Drecoll E. Funktionelle Histologie. Stuttgart: Schattauer 2001.
4. Hardingham TE, Fosang AJ, Dudhia J. Aggrecan, the chondroitin/keratan sulfate proteoglykan from cartilage. In: Articular Cartilage and Osteoarthritis. Kuettner KE (ed). New York: Raven Press 1992; 5–20.
5. Gaissmaier C, Fritz J, Mollenhauer J et al. Verlauf klinisch symptomatischer Knorpelschäden des Kniegelenks. Ergebnisse ohne und mit biologischer Rekonstruktion. Dtsch Ärztebl 2003; 100: 2448–2453.
6. Johnson LL. Arthroscopic abrasion arthroplasty: Historical and pathological perspective: Present status. J Arthroscopy 1986; 2: 54–69.
7. Marlovits S, Vécsei V. Möglichkeiten zur chirurgischen Therapie von Knorpeldefekten – Teil 1: Grundlagen der Knorpelbiologie und der Heilung von Knorpeldefekten. Acta Chir Austriaca 2000; 32: 124–129.
8. Pässler HH. Die Mikrofrakturierung zur Behandlung von Knorpeldefekten. Zentralbl Chir 2000; 125: 500–504.
9. Schmidt H, Hasse E. Arthroskopische operative Behandlung von umschriebenen Knorpelschäden mittels Spongialisation oder Pridie-Bohrung. Beitr Orthop Traumatol 1989; 36: 35–37.
10. Wang Y, Kim UJ, Blasioli DJ et al. In vitro cartilage tissue engineering with 3D porous aqueous-derived silk scaffolds and mesenchymal stem cells. Biomaterials 2005; 26: 7082–7094.
11. Sanders TG, Mentzer KD, Miller MD et al. Autogenous osteochondral "plug" transfer for the treatment of focal chondral defects: postoperative MR appearance with clinical correlation. Skeletal Radiol 2001; 30: 570–578.
12. Imhoff AB, Öttl GM, Burkart A et al. Osteochondrale autologe Transplantation an verschiedenen Gelenken. Orthopäde 1999; 28: 33–44.
13. Foitzik C, Staus H. Phasenreines β-Tricalciumphosphat zum Knochenersatz bei parodontaler Indikation. Quintessenz 1999; 10: 1049–1058.
14. Gruber AJ. Erfahrungen mit Cerasorb® in der Praxis des niedergelassenen Chirurgen. Der niedergelassene Chirurg 1999; 14: 1–3.
15. Hauschild G, Merten HA, Bader A et al. Bioartificial bone grafting: Tarsal joint fusion in a dog using a bioartificial composite bone graft consisting of ß-tricalciumphosphate and platelet rich plasma – A case report. Vet Comp Orthop Traumatol 2005; 1: 52–54.
16. Hauschild G, Bader A, Uhr G et al. Klinischer Einsatz von ß-Tricalciumphosphat – Erfahrungen mit einem matrixorientierten Ansatz zur Osteoregeneration. Tierärztl Prax (K) 2007; 35: 5–13.
17. Szabo G, Suba Z, Hrabak K et al. Autogeneous bone versus beta-tricalcium phosphate graft alone for bilateral sinus elevations (2– and 3-dimensional computed tomographic, histologic, and histomorphometric evaluations): preliminary results. Int J Oral Maxillofac Implants 2001; 5: 681–692.
18. Guo X, Wang C, Duan C et al. Repair of osteochondral defects with autologous chondrocytes seeded onto bioceramic scaffold in sheep. Tissue Engineering 2004; 10 (11/12): 1830–1840.
19. Freshney R. Culture of Animal Cells: A Manual of Basic Technique. Alan R. Liss, Inc., New York 1987; 117.
20. Lydon MJ, Keeler KD, Thomas DB. Vital DNA staining in cell sorting by flow microfluorometry. J Cell Phys 1980; 102: 175–181.
21. Frank M. Einsatz der osteochondralen Transplantation (Mosaicplasty®) in der Therapie der Osteochondrosis dissecans (OCD) des Kniegelenkes beim Hund. Tierärztl Prax (K) 2003; 31: 346–355.
22. Huntley JS, Bush PG, MC Birnie JM et al. Chondrocyte death associated with human femoral osteochondral harvest as performed for mosaicplasty. J Bone Joint Surg Am 2005; 87: 351–360.
23. Marlovits S, Vécsei V. Möglichkeiten zur chirurgischen Therapie von Knorpeldefekten – Teil 2: Chirurgische Behandlungsoptionen zur biologischen Knorpelreparatur. Acta Chir. Austriaca 2000; 32 (4): 185–195.
24. Wagner H. Operative Behandlung der Osteochondrosis dissecans des Kniegelenkes. Z Orthopädie 1964: 62–64.
25. Matsusue Y, Yamamuro T, Hma H. Case report: Arthroscopic multiple osteochondral transplantation to the chondral defect in the knee associated with cruciate ligament disruption. Arthroscopy 1993; 9: 318–321.
26. Bobic V. Arthroscopic osteochondral autograft transplantation in anterior cruciate ligament reconstruction: a preliminary clinical study. Knee Surg Sports Traumatol Arthosc 1996; 3: 262–264.
27. Hangody L, Karpati Z, Szerb I et al. Autologous osteochondral mosaic-like graft technique for replacing weight bearing cartilage defects. Abstract, 7th Congress of the ESSKA 1996, Budapest, Hungary ((**author: please complete**)).
28. Foitzik C, Stamm M. Einsatz von phasenreinem ß-Tricalciumphosphat zur Auffüllung von ossären Defekten – Biologische Materialvorteile und klinische Erfahrungen. Quintessenz 1997; 48: 1365–1377.
29. Heide H, Karbe E, Kling HG et al. Entwicklung und tierexperimentelle Untersuchungen von implantierbaren, porösen keramischen Werkstoffen. Zwei Teilberichte für das Bundesministerium für Forschung und Technologie, Bonn, Referat III B 3–7/12 (1973).
30. Soost F. (2000): Validierung des Knochenumbaus von Knochenersatzmaterialien in der Mund-, Kiefer- und Gesichtschirurgie. Berlin 2000; Humboldt-Universität, Habilitationsschrift
31. Dämmrich K, Loppnow H. Knorpelgewebe. In: Allgemeine Pathologie für Tierärzte und Studierende der Tiermedizin, vol 8. Stünzi H, Weiss E (eds). Berlin, Hamburg: Verlag Paul Parey 1990; 134–136.
32. Fox SM, Walker AM. The etiopathogenesis of osteochondrosis. Vet Med 1993; 88: 116–118.
33. Goodhew PJ, Humphreys FJ. Elektronenmikroskopie: Grundlagen und Anwendung, vol 1. London: McGraw-Hill Verlag 1991.
34. Schmidt PC, Weyhing K. Pulverinhalte aus der Nähe betrachtet. Dtsch Apoth Ztg 2005; 145, Nr. 20: 68–75.

3.4. Canine genetics

As was mentioned in the previously described work and in many recent papers (Ostrander et al. 1997; Kuska 1999; Ostrander et al. 2000; Ostrander et al. 2000; Starkey et al. 2005), the dog is a suitable model organism for the research and treatment of human disease including cancer, while at the same time it benefits from the knowledge gained for its own therapy (Shearin et al. 2010). Therefore, knowledge about canine genetics is a prerequisite for research on dogs and the transfer of the respective results to human medicine. As the canine genome had not been sequenced completely at the beginning of conducted research, and even after completion certain genes were not sequenced successfully in total, own sequencing and analyses of canine genes or parts thereof was performed for genes involved in both canine and human diseases, i.e. the canine *HMG* and *RAS* genes. In addition, expression plasmids for canine HMG proteins were constructed, allowing for research into gene therapeutic approaches of diseases involving this protein family as well as tissue engineering utilising these genes. Supplementing the previously presented work, the following papers resulted from research into the canine genome.

- IV -

Petersen S, Soller JT, Wagner S, Richter A, Bullerdiek J, Nolte I, Barcikowski S, Murua Escobar H.

Co-transfection of plasmid DNA and laser-generated gold nanoparticles does not disturb the bioactivity of GFP-HMGB1 fusion protein

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Own contribution:
Generation of GFP-HMGB1 expression plasmids

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Co-transfection of plasmid DNA and laser-generated gold nanoparticles does not disturb the bioactivity of GFP-HMGB1 fusion protein

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Abstract

Ultrashort pulsed laser ablation in liquids represents a powerful tool for the generation of pure gold nanoparticles (AuNPs) avoiding chemical precursors and thereby making them especially interesting for biomedical applications. However, because of their electron accepting properties, laser-generated AuNPs might affect biochemical properties of biomolecules, which often adsorb onto the nanoparticles. We investigated possible effects of such laser-generated AuNPs on biological functionality of DNA molecules. We tested four differently sized and positively charged AuNPs by incubating them with recombinant eGFP-C1-HMGB1 DNA expression plasmids that code for eGFP fusion proteins and contain the canine architectural transcription factor HMGB1. We were able to show that successfully transfected mammalian cells are still able to synthesize and process the fusion proteins. Our observations revealed that incubation of AuNP with the plasmid DNA encoding the recombinant canine HMGB1 neither prevented the mediated uptake of the vector through the plasma membrane in presence of a transfection reagent nor had any effect on the transport of the synthesized fusion proteins to the nuclei. Biological activity of the recombinant GFP-HMGB1 fusion protein appears to have not been affected either, as a strong characteristic protein accumulation in the nucleus could be observed. We also discovered that transfection efficiencies depend on the size of AuNP. In conclusion, our data indicate that laser-generated AuNPs present a good alternative to chemically synthesized nanoparticles for use in biomedical applications.

Findings

Gold nanoparticles (AuNPs) are used widely for various biomedical applications including cell imaging [1], diagnostics [2], targeted drug delivery [3], and sensing [4]. Various methods have been established for AuNP generation. Many of these rely on several chemical reactions or gas

pyrolysis, showing the risk of impurities or agglomeration [5]. Laser ablation in liquids showed to be a powerful tool with many advantages, having almost no restriction in the choice of source material and the ability of yielding highly pure colloidal particles[6-11]. These pure AuNPs are characterised by their unique surface chemistry free of surfactants, a feature unattainable by other methods [12-14]. X-ray photoelectron spectroscopy of such AuNPs revealed the presence of the oxidation states Au^{+} and Au^{3+} at the AuNP surface [15]. In previous studies we demonstrated that unmodified DNA oligonucleotides adsorb easily onto these positively charged nanoparticles [16,17], probably via amino- and keto-groups, which interact with the electron accepting surface of the generated AuNPs. However, these findings raised the possibility that more complex biomolecules could also be attracted and bound to such nanoparticles' surfaces, if incubated intentionally or unintentionally with colloidal laser-generated gold nanoparticles, even if no additional conjugation is envisaged. Such binding could have a strong effect on the properties of biomolecules and should be characterised with a view of their potential toxicity [18].

We therefore decided to analyse the possible effects of laser-generated AuNPs on DNA functionality. For this reason we incubated the charged particles with recombinant eGFP-C1-HMGB1 expression plasmids and subsequently transfected them into mammalian cells. As the HMGB1 protein is normally highly abundant in the cell nuclei, we were able to show that the treated expression plasmids are still functional and suitable for use as transcription matrix, because the transfected cells were still able to synthesize the fusion proteins, to process them and to transport them to their biofunctional destination. The effect of four differently sized nanoparticles on the activity of the eGFP-C1-HMGB1 plasmid was investigated by fluorescence microscopy. We additionally performed a binding assay to investigate structural effects on the plasmid due to AuNP co-incubation.

Nanoparticle generation

AuNPs were generated by laser ablation in water, as recently reported in detail [17]. Briefly, the beam of a femtosecond laser system (Spitfire Pro, Spectra-Physics), delivering 120 fs laser pulses at a wavelength of 800 nm, was focused with a 40 mm lens on a 99.99% pure gold target placed at the bottom of a Petri dish filled with 2 mL of bidistilled water. A pulse energy of 200 μJ at 5 kHz repetition rate was employed for 12 min. According to observations of Kabashin et al. [9] the focal position was lowered from one generation experiment to the other (0 mm, -2 mm, -4 mm relative to the focus in air) in order to obtain colloidal suspensions containing AuNPs with mean hydrodynamic diameters of d_h = 89 nm, d_h = 59 nm and d_h = 24 nm. The remaining small particles were removed by centrifugation at 15000 rpm for 10 min. To generate 14 nm AuNPs, laser ablation was carried out at a focal position of -4 mm, followed by a second irradiation for 5 min at 1 mJ with an Nd-YLF laser system (pulse length: 27 ns, 1047 nm, 5 kHz), as was described recently [19,20]. Characterization of nanoparticle suspensions was performed by dynamic light scattering using a Malvern Zetasizer and by UV-Vis spectroscopy using a Shimadzu 1650. The hydrodynamic number distributions and the average zeta potential of the colloids are shown in Figure 1. The zeta potential seems to be independent of the nanoparticle size, which might be explained by a similar surface charge density.

The particle mass concentration in the suspensions was determined by weighing the sediment after water evaporation.

Au-NP and eGFP-C1-HMGB1 vector in vitro *transfection assay*

The synthesised Au-NP suspensions were sterilized by filtration through a 0.2 μm filter device (Millex-GV Sterilizing Filter Unit, Millipore, Billerica, USA). Subsequently, 250 ng of each differently sized Au-NPs were incubated for 24 h at room temperature with 1 μg of recombinant plasmid eGFP-C1-HMGB1 in a total volume of 47 μl of ddH_2O. The time of co-incubation was intentionally kept that long as we aimed to investigate possible effects on the vector due to nanoparticle interferences. This was only possible as the circular double-stranded plasmid is not susceptible to rapid degenerative processes.

The recombinant plasmid encodes an eGFP-HMGB1 fusion protein. The HMGB1 coding sequence was derived from canine cDNA using PCR amplification (primer pair EcoR1_B15'CGGAATTCACCATGGGCAAAGGAGA3'/ KpnI_B1 (5'GCGGTACCTTATTCATCATCATC-3'). The obtained PCR products were separated on a 1.5% agarose gel, recovered with QIAquick Gel Extraction Kit (QIAGEN, Hilden, Germany), cloned into the pEGFP-C1 vector plasmid (BD Bioscience Clontech) and sequenced. Twelve hours prior to transfection, 3×10^5 cells from canine mammary cell line MTH53a were seeded into 12 multi well plates. The cells were grown at 37°C and 5% CO_2 in medium 199 (Invitrogen, Karlsruhe, Germany) supplemented with 20% FCS, penicillin, and streptomycin. For transfection, 3 μl aliquots of Fugene HD (FHD) reagent (Roche, Mannheim, Germany) were added to 47 μl of different Au-NP/eGFP-C1-HMGB1 plasmid suspensions in a total volume of 50 μl and incubated for 15 min. The three control sample sets were: (i) 1 μg of eGFP-C1-HMGB1 DNA without nanoparticles, (ii) 250 ng of Au-NPs without any plasmid DNA, and (iii) a set of Au-NPs with DNA, but without the FHD.

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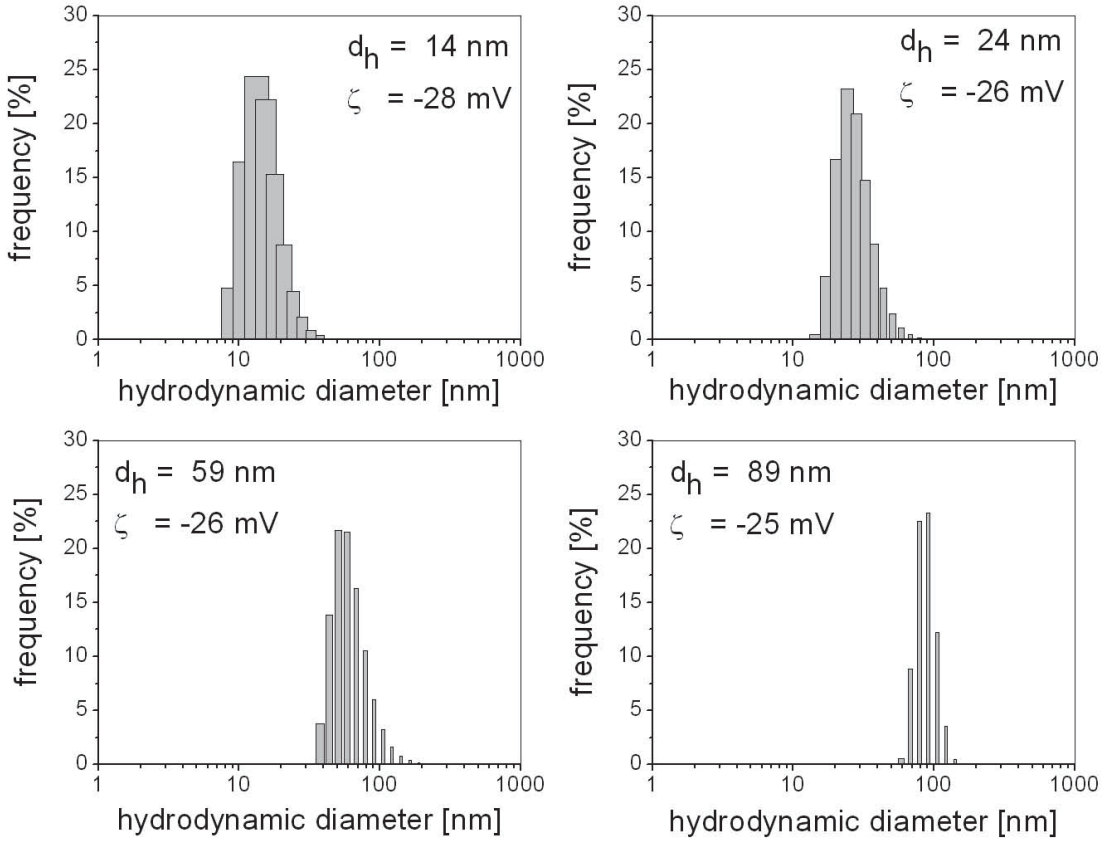

Figure 1

Size distribution and surface charge of laser generated gold nanoparticles. Gold nanoparticles (Au-NP) were generated by laser ablation in water using a femtosecond laser system (Spitfire Pro, Spectra-Physics) delivering 120 fs laser pulses at a wavelength of 800 nm (working pulse energy: 200 μJ per pulse, beam diameter: 4 mm). In order to generate four suspensions containing differently sized nanoparticles, the focal position was lowered from one generation experiment to the other (0 mm, -2 mm, -4 mm relative to the focus in air) resulting in the colloidal suspensions containing nanoparticles with mean hydrodynamic diameters of d_h = 89 nm, d_h = 59 nm and d_h = 24 nm. For the generation of 14 nm sized nanoparticles, laser ablation was carried out at a focal position of -4 mm and then reirradiated for 5 min at 1 mJ with an Nd-YLF laser system (pulse length: 27 ns, 1047 nm, 5 kHz). The hydrodynamic size distribution was analysed by Dynamic Light Scattering.

Following 15 min incubation at 23°C, the respective 50 μl transfection mixtures were added to cell cultures. The cells were incubated for 48 hours in medium 199 (20% FCS) at 37°C and 5% CO_2. The uptake of plasmid DNA and expression of the eGFP-C1-HMGB1 fusion protein were verified by fluorescence microscopy. All experiments were performed in quadruples.

Fluorescence microscopy

Transfected cells were washed with PBS, fixed in a 4% paraformaldehyde/PBS solution (pH 7.5) for 30 min at room temperature and washed again with PBS. Afterwards, the cells were incubated with 10 μl of mounting medium containing DAPI (4',6-diamidino-2-phenylindole) for fluorescent visualization of nucleic DNA (Vecta Laboratories, Burlingame, USA). Fluorescence microscopy was performed using the Carl Zeiss Axioskop 2 and images were recorded with the Axiovision Software. eGFP fluorescence was measured employing wavelength filter set 10 (Carl Zeiss MicroImaging, Göttingen, Germany), while DAPI fluorescence was measured employing wavelength filter set 2 (Figure 2A to 2M). Both fluorescence images were taken with a Zeiss 2-channel Axiocam MRm camera. Both images were then merged in a single image. Full colour images were taken with a Zeiss Axiocam HRc (Figure 2M and 2N). The uptake of plasmid DNA (efficiency of transfection) was estimated taking into account the quantity of cells within an ocular's visual field. Thus the estimation was done comparing the number of cells showing green fluorescence protein expression (green

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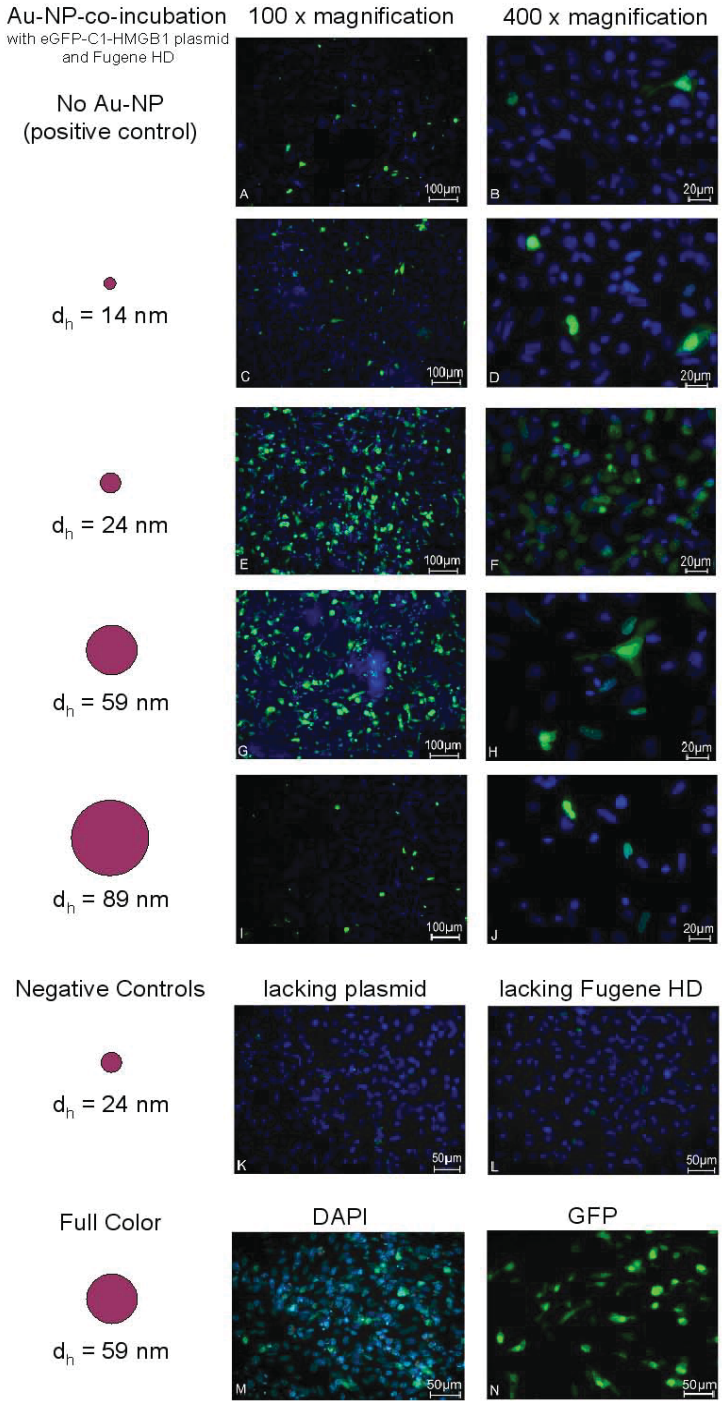

Figure 2

The effect of co-transfecting plasmid DNA and laser generated gold nanoparticles on the bioactivity of GFP-HMGB1 fusion protein. Images A to I (vertical) show a 100 fold magnification and B to J (vertical) a 400 fold magnification. Images A and B represent the positive control I: a transient transfection of MTH53a cells by Fugene HD reagent with the eGFP-C1-HMGB1 plasmid without Au-NP incubation. Cells in images C to J are treated like control I but include incubation of the plasmid with 14 nm sized Au-NP (C and D), 24 nm sized Au-NP (E and F), 59 nm sized nanoparticles (G and H) and 89 nm sized Au-NP (I and J), respectively. Image K and L represent the negative controls II and III. M and N are full color images of DAPI and GFP fluorescence.

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Table 1: Summary of estimated transfection efficiencies

Size Au-NP (d_h)	Estimated Transfection Efficiency (%)	Figure
Positive controls	10 ± 2	A and B
14 nm	15 ± 5	C and D
24 nm	50 ± 5	E and F
59 nm	50 ± 10	G and H
89 nm	8 ± 3	I and J
Negative controls	-	K and L

Differently sized Au-NPs were incubated with plasmid DNA and transfected into the canine MTH53a cellline

staining) and cells showing blue DAPI fluorescence dye staining.

Co-transfection of plasmid DNA and laser-generated gold nanoparticles

As the HMGB1 protein is a transcription factor, it binds strongly to nuclear DNA. We therefore may assume that cell nuclei containing strong eGFP fluorescence represent successful functional transfection events. All cells transfected with AuNP-incubated plasmid DNA showed strong colocalised eGFP and DAPI staining (Figure 2), whilst the negative controls, cells treated with Au-NP and FHD (AuNP of d_h = 24 nm), showed no eGFP fluorescence (Figure 2K). We therefore conclude that co-incubation of AuNP with the plasmid DNA encoding the recombinant canine HMGB1 neither prevents the mediated uptake of the vector in presence of a transfection reagent nor has any visible effect on the transport and biological functionality of the synthesised fusion proteins.

By comparing fluorescence images of the cells co-incubated with the AuNPs of different sizes and to cells incubated without AuNPs, we were able to compare transfection efficiencies in each case. We estimate that the achieved efficiency of DNA transfection for the sample containing 14 nm AuNPs was approx. 15 ± 5% (Figure 2C and 2D).

The highest observed transfection efficiencies were achieved using 24 nm and 59 nm Au-NPs (50 ± 5% and 50 ± 10% respectively, see Figure 2E to 2H). The Au-NPs showed size dependent effects concerning the observed transfection efficiencies (see Table 1). Exemplarily, Au-NPs of a medium size (d_h: 24 and 59 nm) showed the highest effects. Thus, the observed GFP fluorescence of the respective fusion proteins was so intense that it even leaked into the DAPI channel (Fig 2M and 2N respectively for AuNP of d_h = 59 nm). Further negative control samples containing DNA- co-incubated AuNPs missing FHD, showed no recombinant protein expression, proving that our AuNPs did not act as transfection reagent themselves. (Figure 2L). The cell population seems to go along with transfection efficiency, as the observed seeding density was in all wells similar prior to transfection.

Shift assay

We performed binding experiments with plasmid DNA (eGFP-C1-HMGB1) and respective Au-NPs of different sizes and with various concentrations. We digested the co-incubated batches with a *NcoI* restriction enzyme (Fermentas, St Leon Rot, Germany) and separated the resulting DNA fragments in a 1.5% agarose gel. No significant shift alterations could be observed in the DNA mobility pattern. To ensure that this phenomenon is also valid in presence of proteins we added purified HMGB1 protein (Centre for Human Genetics, Bremen, Germany) to the

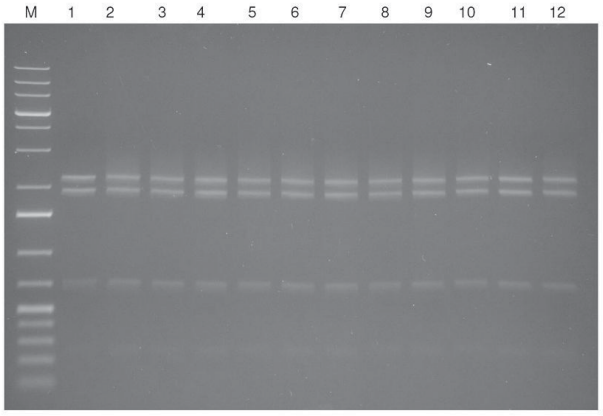

Figure 3
Au-NPs/DNA and HMGB1 protein mobility shift assay. **M**: GeneRuler 1 kb Plus (Fermentas), **lane 1**: 170 ng plasmid (NcoI digested); **lane 2**: 170 ng plasmid (NcoI digested) and 1.5 μg HMGB1; **lanes 3-6**: 170 ng plasmid 170 (NcoI digested) and 1.5 μg HMGB1 in 0.1 nM, 0.5 nM, 1.0 nM and 2.5 nM AuNPs suspensions, size d_h 24 nm; **lane 7**: 170 ng plasmid (NcoI) digested and 1.5 μg HMGB1 and 90 ng pure Au suspension, size d_h 24 nm; **lanes 8-11**: 170 ng plasmid (NcoI digested) and 1.5 μg HMGB1 in 0.1 nM, 0.5 nM, 1.0 nM and 2.5 nM AuNPs suspensions, size d_h 59 nm; **lane 12**: 170 ng plasmid (NcoI) digested and 1.5 μg HMGB1 and 50 ng pure Au suspension, size d_h 59 nm.

batches. Akin to the DNA mobility pattern of digested Plasmid DNA and HMGB1 without Au-NPs (lane 2, Figure 3) we could not detect any significant change in the shift pattern (see lanes 3 to 12, Figure 3). Consequently the DNA/Au-NPs complexes serve as substrates for the DNA-bindig protein HMGB1.

Conclusion

In conclusion, incubation of uncoated, positively charged AuNPs with a DNA plasmid that encodes recombinant eGFP-C1-HMGB1 fusion protein for 24 hours before cellular transfection does not seem to alter the protein expression and the protein functionality (DNA binding), while the presence of AuNPs seems to have a significantly positive effect on the transfection efficiencies. The observed effect was size-dependent: medium sized AuNPs enhanced transfection efficiency nearly 6 fold. These results support the hypothesis that laser-generated AuNPs present a good alternative to chemically synthesized nanoparticles and are especially suitable for biomedical applications.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

SP carried out the nanoparticle generation and partial drafting of the manuskript, JTS carried out the transfections, fluorescence microscopy analysis and partial drafting of the manuscript, SW performed cell culture and DNA preparation, AR generated the recombinant eGFP-C1-HMB1 plasmid, SB principal study design, manuscript drafting and supervision of nanoparticle work, HME principal design, partial manuscript drafting and supervision of molecular and cell biologic work. IN and JB participated in the conception design of the study. All authors read and approved the final manuscript.

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References

1. Chen J, Saeki F, Wiley BJ, Chang H, Cobb MJ, Li ZY, Au L, Zhang H, Kimmey MB, Li X, Xia Y: *Nano Lett* 2005, **5:**473-477.
2. Chen J, Wiley B, Campbell D, Saeki F, Cahng L, Au L, Lee J, Li X, Xia Y: *Adv Mater* 2005, **17:**2255.
3. Yang PH, Sun X, Chiu JF, Sun H, Qing-Yu H: *Bioconjugate Chem* 2005, **16:**494-496.
4. Liu GL, Yin Y, Kunchakarra S, Mukherjee B, Gerion D, Jett SD, Bear DG, Gray JW, Alivisatos AP, Lee LP, Chen FF: *Nat Nanotechnol* 2006, **1:**47-52.
5. Dahl JA, Maddux BLS, Hutchison JE: *Chem Rev* 2007, **107:**2228-2269.
6. Mafuné F, Kohno J, Takeda Y, Kondow T, Sawabe H: *J Phys Chem B* 2000, **104:**9111-9117.
7. Mafuné F, Kohno J, Takeda Y, Kondow T: *J Phys Chem* 2001, **105:**9050-9056.
8. Dolgaev SI, Simakin AV, Voronov VV, Shafeev GA, Bozon-Verduraz F: *Appl Surf Sci* 2002, **186:**546-551.
9. Kabashin AV, Meunier M: *J Appl Phys* 2003, **94:**7941-7943.
10. Barcikowski S, Hahn A, Kabashin AV, Chichkov BN: *J Appl Phys A* 2007, **87:**47-55.
11. Barcikowski S, Menéndez-Manjón A, Chichkov B, Brikas M, Raèiukaitis G: *Appl Phys Lett* 2007, **91:**083113-1.
12. Sylvestre JP, Kabashin AV, Sacher E, Meunier M, Luong JHT: *J Am Chem Soc* 2004, **126:**7176-7177.
13. Sylvestre JP, Poulin S, Kabashin AV, Sacher E, Meunier M, Luong JHT: *J Phys Chem* 2004, **108:**16864-16869.
14. Kabashin AV, Meunier M, Kingston C, Luong JHT: *J Phys Chem B* 2003, **107:**4527-4531.
15. Sylvestre JP, Poulin S, Kabashin AV, Sacher E, Meunier M, Luong JHT: *Phys Chem B* 2004, **108:**16864-16869.
16. Petersen S, Jakobi J, Barcikowski S: *Appl Surf Sci* 2009, **255:**5435-5438.
17. Petersen S, Barcikowski S: *Adv. Funct. Mater.* 2009, **19:**1167-1172.
18. de Jong W, Borm PJA: *Journal of Nanomedicine* 2008, **3:**133-149.
19. Mafuné F, Kohno J, Takeda Y, Kondow TJ: *Phys Chem B* 2001, **105:**9050-9056.
20. Amendola V, Meneghetti M: *Phys Chem Chem Phys* 2009, **11:**3805-3821.

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Own contribution:

Generation of GFP-HMGA1 expression plasmids in collaboration with M. Muth

Establishment and analysis of experiments regarding the localisation of the HMGA1 proteins in canine cells in collaboration with M. Muth

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Genomic characterisation, chromosomal assignment and *in vivo* localisation of the canine High Mobility Group A1 (HMGA1) gene

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Abstract

Background: The high mobility group A1 proteins (HMGA1a/HMGA1b) are highly conserved between mammalian species and widely described as participating in various cellular processes. By inducing DNA conformation changes the HMGA1 proteins indirectly influence the binding of various transcription factors and therefore effect the transcription regulation. In humans chromosomal aberrations affecting the *HMGA1* gene locus on HSA 6p21 were described to be the cause for various benign mesenchymal tumours while high titres of HMGA1 proteins were shown to be associated with the neoplastic potential of various types of cancer. Interestingly, the absence of HMGA1 proteins was shown to cause insulin resistance and diabetes in humans and mice.

Due to the various similarities in biology and presentation of human and canine cancers the dog has joined the common rodent animal model for therapeutic and preclinical studies. Accordingly, the canine genome was sequenced completely twice but unfortunately this could not solve the structure of canine *HMGA1* gene.

Results: Herein we report the characterisation of the genomic structure of the canine *HMGA1* gene consisting of 7 exons and 6 introns spanning in total 9524 bp, the *in vivo* localisation of the HMGA1 protein to the nucleus, and a chromosomal assignment of the gene by FISH to CFA12q11. Additionally, we evaluated a described canine *HMGA1* exon 6 SNP in 55 Dachshunds.

Conclusion: The performed characterisations will make comparative analyses of aberrations affecting the human and canine gene and proteins possible, thereby providing a basis for revealing mechanisms involved in HMGA1 related pathogenesis in both species.

Background

The high mobility group A (HMGA) proteins are small chromatin associated non-histone proteins named according to their characteristic motility in acid-urea polyacrylamide gel electrophoresis. The protein family consists of the three proteins HMGA1a, HMGA1b and HMGA2 which are encoded for by two different genes (HMGA1 and HMGA2). The functional motifs of these proteins, named AT-hooks, bind to the minor groove of DNA causing conformational changes of the DNA molecule. On genomic level these structural changes influence the binding of various transcription factors and thus indirectly influence the transcription regulation, which classifies the HMGA proteins as so called architectural transcription factors (for detail see [1]).

In previous studies we characterised the canine HMGA1 cDNAs and proteins and in comparative analyses of these molecules showed that they are highly conserved between different mammalian species. The observed number of amino acid changes seen across mammalian species (cattle, dog, hamster, horse, mouse, pig, and rat) vary between 0 to 3 when compared to the human molecules [2-10]. Interestingly, only the canine HMGA1 proteins are 100% identical to their respective human counterparts [11].

The HMGA1 proteins are well known to play a significant role in the pathogenesis of various diseases including cancer. In humans, chromosomal aberrations affecting the HMGA1 gene locus on HSA 6p21 were described for various benign mesenchymal tumours, e.g. endometrial polyps, lipomas, pulmonary chondroid hamartomas, and uterine leiomyomas [12-14]. The observed aberrations are supposed to lead to an up-regulation of the *HMGA1* gene in the affected tumours, as opposed to adult healthy tissues where *HMGA* gene expression is low or hardly measurable [9,15,16]. In malignant neoplasias *HMGA1* expression is reported to be associated with an aggressive behaviour of tumours. Accordingly, *HMGA1* overexpression was detected in various malignancies including thyroid, lung, prostatic, pancreatic, uterine cervical, and colorectal carcinoma [17-22]. Thus *HMGA* expression is supposed to present a powerful diagnostic and prognostic molecular marker due to the described correlation between *HMGA* expression and tumour aggressiveness.

Whilst overexpression of *HMGA1* is clearly associated with cancerogenesis the disruption of the *HMGA1* gene and thus induced loss of *HMGA1* expression shows significant pathogenic effects. Heterozygous and homozygous Hmga1 knock-out mice develop cardiac hypertrophy combined with hematologic malignancies e.g. B cell lymphoma and myeloid granuloerythroblastic leukemia [23]. Additional research with Hmga1 knock-out mice targeting diabetes presented by Foti et al. (2005) showed that loss of Hmga1 expression is clearly associated with significantly decreased insulin receptor expression and thus causes a characteristic diabetes type 2 phenotype in mice [24].

The various similarities in presentation and biology of numerous canine and human diseases including cancer suggest similar mechanisms to be involved in the respective pathogenic events. Accordingly, at least a dozen distinct canine cancers are hypothesized to be appropriate models for their human counterparts, among those osteosarcoma, breast carcinoma, oral melanomas, lung carcinomas and malignant non-Hodgkin's lymphomas [25].

The characterization of disease related genes and their protein biology will allow for comparative studies to reveal the molecular mechanisms involved therein and serve as a basis for future clinical studies.

Results and discussion

The *HMGA1* gene and its proteins HMGA1a and HMGA1b are described as regulating multiple cellular processes and are widely reported to be associated with various diseases including diabetes and cancer. In previous studies we characterised the canine *HMGA1* cDNAs and proteins completely and did comparative analyses of these molecules to the respective counterparts of different species and showed high evolutionary conservation. The fact that several canine and human cancer types show striking similarities in presentation and biological behaviour, e.g. spontaneous occurrence and metastasis patterns, strongly suggests similar mechanisms to be involved in the respective pathogenic events of both species. Thus, various canine tumours are currently used as models for several human cancer types. Accordingly, comprehension of the canine gene and its gene products is precondition for comparative analyses, allowing the revelation of molecular effects involved in these pathogenic presentations. Understanding and comparison of the respective genes will thus benefit both species. The exact mechanism for the emergence of the pathogenic effects caused by chromosomal aberrations affecting the human *HMGA1* gene in benign mesenchymal tumours, e.g. endometrial polyps, lipomas, pulmonary chondroid hamartomas, and uterine leiomyomas [12-14] are not completely understood. However, it is currently supposed that the aberration causes up-regulation of the *HMGA1* gene in the affected neoplasias. The principal aim of the study was to characterize the genomic structure of the canine *HMGA1* gene allowing the comparison of its genomic structure to the counterparts of other mammals and thus allowing a further evaluation of evolutionary conservation of the gene and a comparative analysis of chromosomal aberrations in both species. Additional aims were the *in vivo* localization of the canine HMGA1 protein and the evalu-

ation of a previously described point mutation which causes a disrupted protein.

Genomic structure, BAC Screening and FISH

A canine *HMGA1* genomic PCR reaction was established and used for screening of a canine BAC for identification of the canine HMGA1 gene locus by FISH. The verified BAC 572 P20 K12 RC was used for FISH experiments. Ten well spread metaphases were analysed and showed signals on both chromatides of both chromosomes CFA 12q11 (Figure 1). The chromosomal localisation was done following the nomenclature established by Reimann et al. [26]. Existing painting probe based synteny studies and RH analyses [27] indicated that the canine CFA 12 shares homology with the human chromosome 6 on which the *HMGA1* gene is located at HSA 6p21. Chromosomal aberrations affecting CFA 12 are not or barely reported to be significantly associated with canine neoplasias [28,29]. While previous studies reported the localization of a HMGA1 gene positive BAC to CFA 23 [30], the performed *in silico* analyses and the recently published canine genome assembly [31] support the herein described assignment of the canine *HMGA1* gene to CFA 12q11 by FISH described in this study. Comparative chromosomal *in silico* analyses using the "Evolutionary Highway" http://evolutionhighway.ncsa.uiuc.edu/results.html showed similar results.

The genomic structure of the canine *HMGA1* gene consists in total of the 7 exons and 6 introns. Overall the canine *HMGA1* gene spans 9524 bp. The exon/intron structure, size and the homologies to their human counterparts were analysed and defined (Figure 2, Table 1). The total identity to the corresponding human region is 62.8%. In detail, the identities of the exons vary between 74.6% and 97.8% to their human counterpart, while the introns show identities between 58.9% and 92.4% (for details see Table 1). The newly characterized sequences combined with the analyses performed *in silico* revealed that the exon 4, which exists in humans, is missing on genomic level in the canine genome. This exon 4 deletion also exists in the mouse genome and affects the respective mRNAs of both species in their 5' UTR. As the genomic characterization of the canine HMGA1 gene was not available when the exons were named previously, the numbering at that time was based on the respective human exon numbers as defined by Friedmann et al. [32]. Consequently, as it is now known that the canine genomic sequence is lacking an equivalent to human exon 4, the previously used canine exon numbering should be revised with the then named canine exon 5 now being canine exon 4 and so on (Figure 2, Table 1). However, a part of intron 2 remains unsequenced due to an extensive CG repeat which also exists in the human counterpart (90%CG), and only the number of nucleotides (311 bp) could be identified. The genomic sequences were submit-

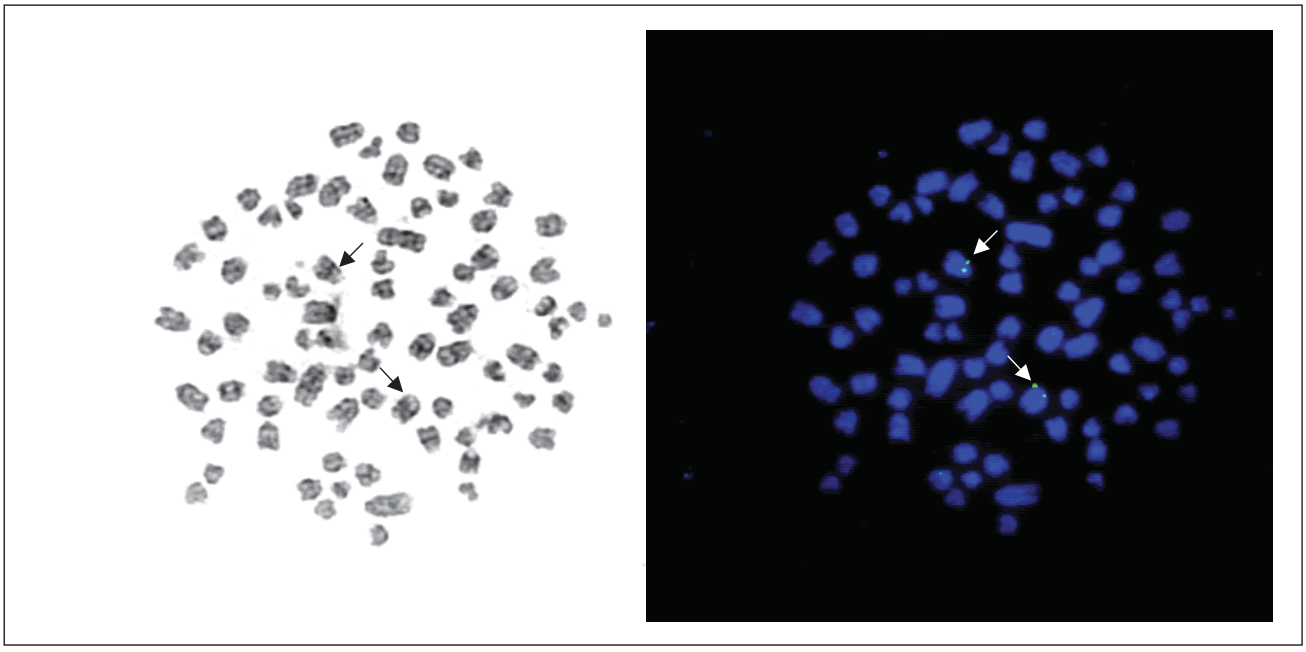

Figure 1
FISH-Mapping of the canine *HMGA1*. Canine metaphase spread after GTG-banding (left) and the same metaphase after fluorescence *in situ* hybridisation with BAC MGA 572 P20 K12 RC showing signals on both chromosomes 12 (right).

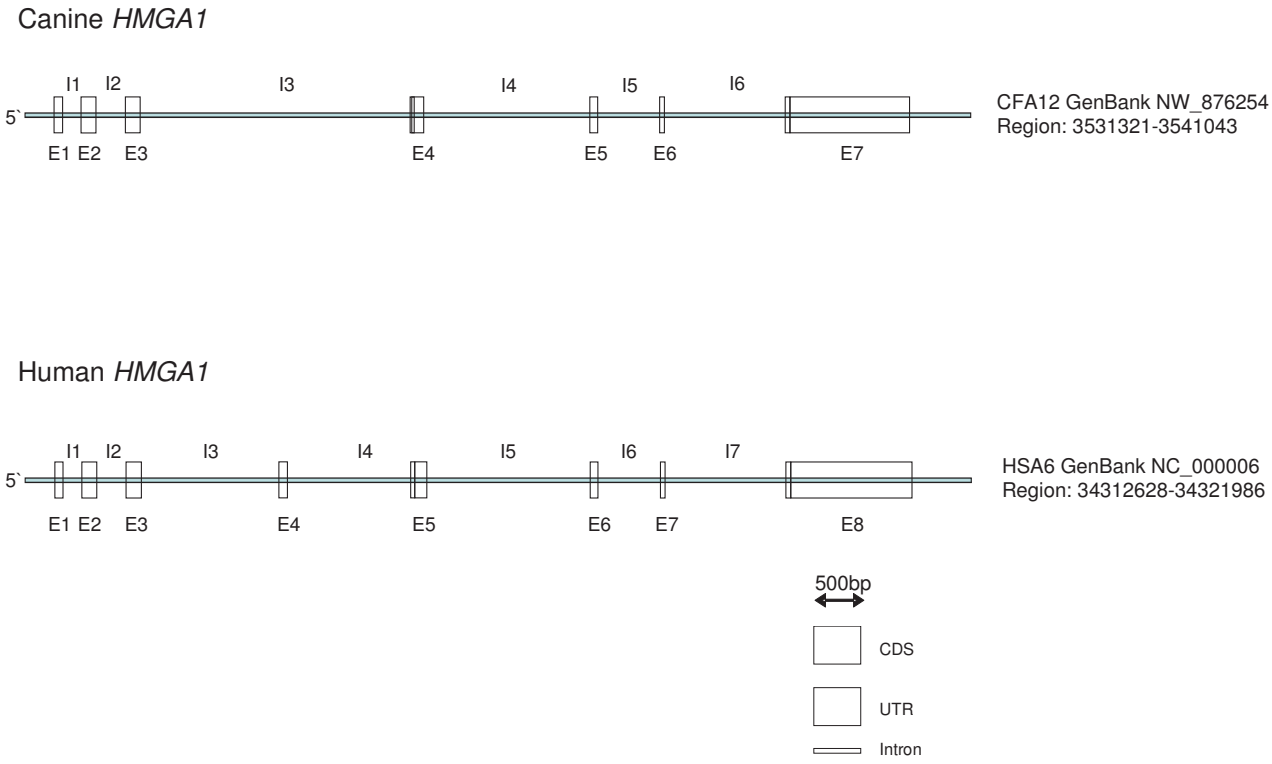

Figure 2
Genomic structure of the canine *HMGA1* gene. Detailed structure of the genomic organisation of the canine *HMGA1* gene.

ted to the NCBI database (bankit1078465, bankit1078536, bankit1078968).

Exon 6 SNP evaluation

While characterising the canine *HMGA1* gene we screened twelve different canine breeds for point mutations affect-

Table 1: Detailed analysis of the canine *HMGA1* gene genomic elements

Element of canine *HMGA1* gene	Size in bp	Identity to human counterpart in % (GenBank NC_000006))
Total gene	9524	62.8
Detail exons/introns (revised numbering)*		
Exon 1	94	97.8
Intron 1	196	92.4
Exon 2	164	95.8
Intron 2	311	-
Exon 3	162	74.6
Intron 3	3096	58.9
Exon 4 (5)	179	93.9
Intron 4 (5)	1761	51.1
Exon 5 (6)	84	96.4
Intron 5 (6)	584	57.5
Exon 6 (7)	51	94.1
Intron 6 (7)	1459	58.1
Exon 7 (8)	1386	75.4

Identity comparison of the genomic elements of the canine *HMGA1* gene with its respective human counterparts.

ing the protein coding region. A Dachshund sample showed a transition from A to G in exon 6 (according to revised exon numeration) leading to an amino acid exchange from threonine to alanine causing a mutated HMGA1 protein [9]. To elucidate if the observed exchange is frequently existent in the Dachshund population we screened 55 Dachshunds for the respective mutation (Figure 3). The results obtained by sequencing and restriction fragment analysis clearly showed that the previously found mutation is a rare event, as none of the screened 55 Dachshunds showed the mutation. Thus our findings suggest that the previously found aberrant *HMGA1* allele leading to a mutated protein form is unlikely to play a major role in HMGA1 pathogenesis in Dachshunds.

In general, different species show significant differences considering the number and probability of described SNPs. This fact surely is directly dependent on total numbers of studies and sequencing reactions performed for the different species. While in 2001 Sachidanandam et al. [33] detected 1.42 million SNPs in the human genome with one SNP per 1.9 kb the currently estimated total number reported SNPs in the public databases is approx. 9 million for the human genome [34]. For the dog Lindblad-Toh et al. reported 2.5 million SNPs, whereas the probability differs depending on the breed between one SNP per 1500 bp and 900 bp [31]. Comparable to the human genome the total numbers of reported SNPs in the other different species is expected to increase significantly according to the performed research efforts, leading to increased knowledge of effects caused by SNPs in general.

HMGA1 in vivo *localization*

The *in vivo* localization of the canine HMGA1 proteins via expression of a canine HMGA1a-GFP fusion protein showed that equivalently to its human counterpart the protein is located in the nucleus (Figure 4). Proteins of the HMGA family are described to be architectural transcription factors, and thus a localisation in the nucleus seems obvious. However, further localisation and function of these proteins seem to be very likely, due to the fact that application of recombinant HMGA1 proteins to porcine cartilage cells *in vitro* showed significant increase of cell proliferation (Richter et al. accepted for publication). For a further member of the HMG proteins called HMGB1 the existence of an extracelluar function was recognised only a long time after its initial characterisation as an architectural transcription factor, revealing a direct influence of

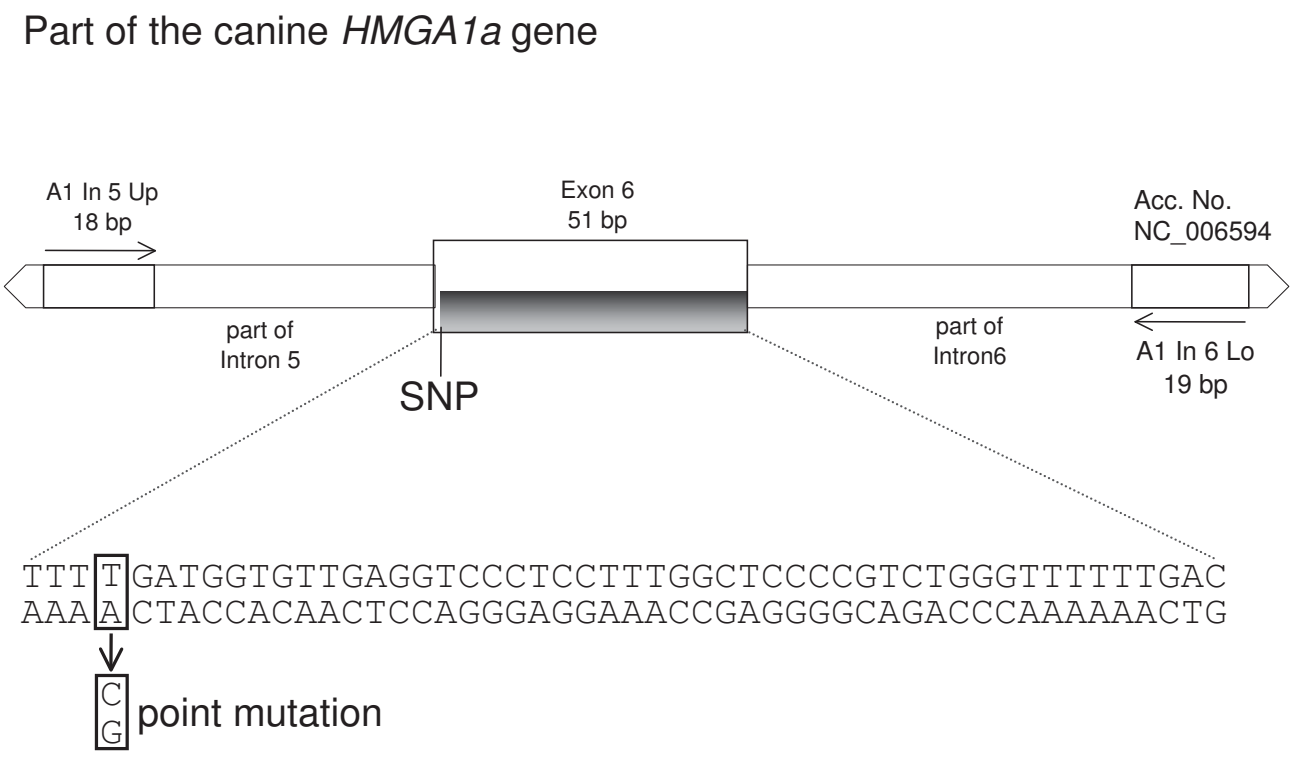

Figure 3
Position of the evaluated Dachshund point mutation. Strategic position of the evaluated point mutation screened in 55 Dachshunds.

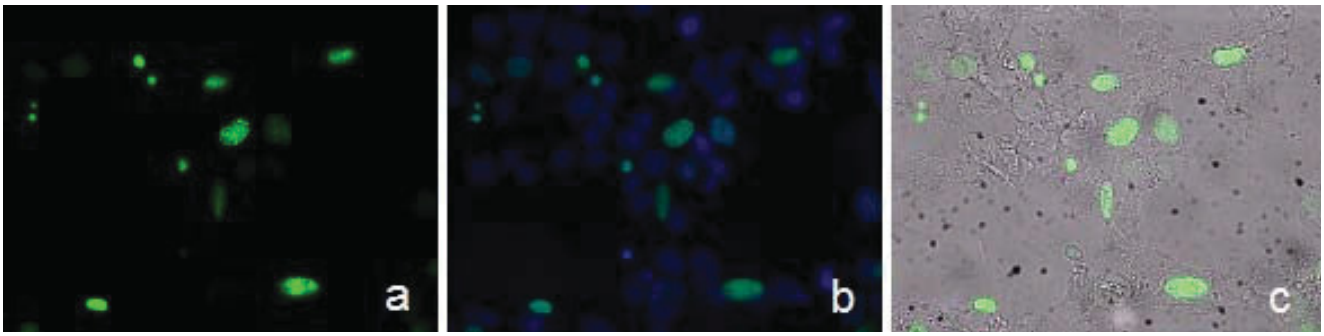

Figure 4
***In vivo* localisation of the canine HMGA1 protein**. *In vivo* localization of a canine HMGA1a-GFP fusion protein in culture canine MTH53A cells, 24 h posttranslational. a) GFP expression in canine mammary cell line MTH53A, b) DAPI fluorescent staining of cell nuclei, merged GFP and DAPI image, c) merged GFP and transmitted light image (magnification ×400).

extracellular HMGB1 on metastatic events [35-37]. Thus, we suppose that a similar mechanism could also exist for HMGA proteins and are currently working towards its identification.

Conclusion

Knowledge about the structure of genes and proteins is precondition to use them as potential therapeutic targets, markers or for revealing mechanisms involved in relevant pathogenic events. The canine and human HMGA genes and proteins have widely been shown to be involved in various diseases especially in cancer. Due to the numerous reasons for using the dog as a model system for human cancer research the characterisation of canine genes and proteins is of special interest. The performed characterisations of the canine HMGA1 gene and proteins will allow performing comparative analyses of aberrations affecting the human and canine genes and proteins as basis for revealing mechanisms involved in HMGA1 related pathogenesis in both species.

Methods

BAC library screening

A PCR reaction for the use in PCR-based screening of the *Canis familiaris* DogBAC library (Schelling et al., 2002) (Institute of Animal Genetics, Nutrition and Housing, University of Berne, Berne, Switzerland) for a BAC clone containing *HMGA1* was established using canine genomic DNA derived from blood. The primers A1In5up (5' GGCATCCGGTGAGCAGTG 3') and A1In6lo (5' CAGGCAGAGCACGCAGGAC 3') were designed using GeneBank sequences AY366395 &NW_876254. PCR parameters were: 95°C for 5 min, followed by 30 cycles of 95°C 30 sec, 59.3°C 30 sec, 72°C 30 sec, and a final elongation of 72°C for 10 min. The corresponding 201 bp PCR product was cloned into the pGEM-T Easy vector system (Promega, Mannheim, Germany) and verified by sequencing. The DNA contigs and alignments were done with Lasergene software (DNAstar, Madison, USA) and various sequences from the NCBI database (AY366395, NW_876254). The verified BAC clone MGA 572 P20 K12 RC was used as probe for the following FISH experiments.

Slide Preparation

1 ml of canine whole blood was incubated for 72 h in Chromosome Medium B (Biochrom, Berlin, Germany). Subsequently, colcemide (0.1 μg/ml) (Biochrom, Berlin, Germany) was added for 2 hours. The cells were centrifuged at 135 × g for 10 min and incubated for 20 min in 0.05 M KCl. Finally the cells were fixed with methanol/glacial acetic acid. This suspension was dropped on ice-cold slides and dried for at least 7 days at 37°C. The chromosomes were stained by GTG banding for karyotype description. Prior to use in FISH investigations, the slides were destained with 70% ethanol.

Fluorescence in situ *Hybridization*

MGA 572 P20 K12 RC BAC-DNA was digoxigenin labelled (Dig-Nick-Translation-Kit, Roche, Mannheim, Germany). The hybridization mixture contained 200 ng probe, 40 ng ssDNA, 600 ng sonicated dog DNA, 2 × SSC, 2 × SSPE, 50% formamide and 10% dextran sulfate. 50 μl of this mixture were applied to each slide and the cover slips were sealed with rubber cement. Probe and chromosomes were denatured at 75°C on an Eppendorf Thermocycler gradient, using the *in situ* adapter. Afterwards, the slides were incubated in a moist chamber at 37°C over night. Cover slips were carefully removed and the slides were incubated in 0.1 × SSC at 61°C and 1 × PBS at RT. Slides were then covered with 100 μl non fat dry milk (NFDM) for 20 min. at 37°C in a moist chamber. For signal detection 100 μl NFDM containing 3 μg of Anti-Digoxigenin-Rhodamine, Fab fragments (Roche, Mannheim, Germany), were added to each slide and again incubated for 20 min at 37°C in a moist chamber, followed by washes with 1 × PBS, 3 × 3 min. at RT. Slides were air dried

before chromosomes staining was performed with 25 μl of Vectashield Mounting Medium with DAPI (Vector Laboratories, Burlingame, CA, USA)

Ten well spread metaphases were examined indicating a signal on CFA 12q11 on both chromatids of both chromosomes CFA 12q11 (Fig. 1). The determination of chromosomes follows the nomenclature of the canine karyotype as described previously [26].

Genomic characterisation

For genomic characterisation of the canine *HMGA1* gene the missing parts were amplified by PCR on the screened BAC clone MGA 572 P20 K12 RC. For the missing part 1 a 858 bp fragment (bankit 1078968) was generated with primer pair A1_6640-6997_upa (5'-GGCGCGGCTCCAAGAA-3'), A1_6_lo_2 (5'-CCAACAGAGCCCTGCAAA-3'), a 1879 bp fragment (bankit 1078465 for the missing part 2 was generated by the primer pair A1_8864-10549_upa (5'-GTCTCACCGTCTGGAGAAT-3'), A1_8864-10549_loa (5'-TCACCGGAGGCTGCTT-3') and for the third missing part a 979 bp fragment (bankit 1078536) was generated with primer pair A1_11223-11834_upa (5'-CTGAGCCCATGCCAGATAA-3'), A1_11223-11834_loa (5'-AGAGATCCCTGCCGTAGT-3'). The obtained PCR products were separated on a 1.5% agarose gel, recovered with QIAquick Gel Extraction Kit (QIAGEN, Hilden, Germany), cloned in pGEM-T Easy vector system (Promega, Mannheim, Germany) and sequenced for verification. The final genomic canine *HMGA1* contig and the identity alignments were created with Lasergene software (DNASTar, Madison, USA) with the generated sequences from the cloned cDNAs described previously and various sequences from the NCBI database derived from the canine genome sequencing (AY366394, AY366395, AY366396, NM_001003387, NW_876254).

SNP screening

Genomic DNA was isolated from the collected 55 Dachshunds samples using the QiaAmp kit (QIAGEN, Hilden, Germany). A specific genomic PCR using the primer pair A1In5up (5' GGCATCCGGTGAGCAGTG 3') and A1In6lo (5' CAGGCAGAGCACGCAGGAC 3') was established allowing the amplification of the complete exon 6 and flanking regions of intron 5 and 6, respectively (Figure 3). In detail the PCRs were performed in a 25 μl volume containing 0.5 μM of both primers (MWG Biotech, Martinsried, Germany), 0.1 mM of each dNTP (Invitrogen, Karlsruhe, Germany) 0.6 units Taq-DNA polymerase (Promega, Mannheim, Germany), 1.5 mM $MgCl_2$ (Promega, Mannheim, Germany), PCR buffer (Promega, Mannheim, Germany) and 2.5 μl template DNA, containing averaged 26.5 ng/μl.

After an initial denaturation step of 5 min at 95°C, the amplification followed in 30 cycles (30 sec. at 95°C, 30 sec at 59.3°C and 30 sec at 72°C). To complete, a final elongation step for 10 min. at 72°C completed the process. The obtained PCR products were purified using the QIAquick PCR Purification Kit (Qiagen, Hilden, Germany), directly sequenced by MWG Biotech (Martinsried, Germany), and additionally digested enzymatically with AluI (Fermentas, St. Leon-Rot, Germany). The occurrence of the described SNP creates a new restriction site for the enzyme AluI (5' AG▼CT 3'). Thus, a digestion with AluI cuts the 201 bp PCR product in two fragments of 69 bp and 132 bp, respectively allowing a verification of the sequencing results.

HMGA1 in vivo *localisation*

For the HMGA1 *in vivo* localisation the protein coding sequence of the canine HMGA1a was amplified by PCR using primer pair EcoR1_IY-upATG (5'-CGGAATTCCACCATGAGCGAGTCGAGCTCGA-3'), BamH1_IY-loSTOP (5'-CGGGATCCTCACTGCTCCTCTTCGGAGGACT-3'). The obtained PCR products were separated on a 1.5% agarose gel, recovered with QIAquick Gel Extraction Kit (QIAGEN, Hilden, Germany), ligated into the pEGFP-C1 vector plasmid (BD Bioscience Clontech) and sequenced for verification.

Cells from canine mammary tumour cell line MTH53a were cultivated using medium 199 (Invitrogen, Karlsruhe, Germany) supplemented with 20% FCS, penicillin, and streptomycin. The transfection was performed according to the manufacturer's instructions using 3 μl FugeneHD reagent (Roche, Mannheim, Germany) in 100 μl PBS (without Mg^{2+}) containing 2 μg of recombinant pEGFP-C1-HMGA1a. After treatment, the cells were incubated for 48 hours in the culture media. The uptake and expression of DNA was verified by fluorescence microscopy.

Authors' contributions

CB: collected the Dachshund samples and performed the point mutation screening, JB: head of the centre for human genetics, took part in the conception design of the study, GD: constructed the screened BAC library, JTS: *in silico* analyses and construction of the *HMGA1* gene structure, MM: construction of expression vectors for the *in vivo* localisation, HME: principal study design, IN: head of the small animal clinic, took part in the conception design of the study, NR-B: karyotyping, AR: transfection of cells for in vivo localisation, CS: screening of the canine BAC library, SiW: molecular cloning of the newly characterised HMGA1 fragments, SaW: supervision point mutation screening, SuW: performed the FISH experiments.

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References

1. Bustin M, Reeves R: **High-mobility-group chromosomal proteins: architectural components that facilitate chromatin function.** *Prog Nucleic Acid Res Mol Biol* 1996, **54:**35-100.
2. Fujisaki S HH Eguchi T, Watanabe Y, Honma D, Saitou T, and Yasue H: **Construction of a full-length library of swine olfactory bulb and its preliminary characterization.** *Unpublished manuscript* 2003.
3. Johnson KR, Lehn DA, Elton TS, Barr PJ, Reeves R: **Complete murine cDNA sequence, genomic structure, and tissue expression of the high mobility group protein HMG-I(Y).** *The Journal of biological chemistry* 1988, **263(34):**18338-18342.
4. Aldrich TL RR Lee CC, Thomas JN, and Morris AE: **HMG-I(Y) proteins implicated in amplification of CHO cell DNA.** *Unpublished manuscript* 1999.
5. Strausberg RL, Feingold EA, Grouse LH, Derge JG, Klausner RD, Collins FS, Wagner L, Shenmen CM, Schuler GD, Altschul SF, Zeeberg B, Buetow KH, Schaefer CF, Bhat NK, Hopkins RF, Jordan H, Moore T, Max SI, Wang J, Hsieh F, Diatchenko L, Marusina K, Farmer AA, Rubin GM, Hong L, Stapleton M, Soares MB, Bonaldo MF, Casavant TL, Scheetz TE, Brownstein MJ, Usdin TB, Toshiyuki S, Carninci P, Prange C, Raha SS, Loquellano NA, Peters GJ, Abramson RD, Mullahy SJ, Bosak SA, McEwan PJ, McKernan KJ, Malek JA, Gunaratne PH, Richards S, Worley KC, Hale S, Garcia AM, Gay LJ, Hulyk SW, Villalon DK, Muzny DM, Sodergren EJ, Lu X, Gibbs RA, Fahey J, Helton E, Ketteman M, Madan A, Rodrigues S, Sanchez A, Whiting M, Madan A, Young AC, Shevchenko Y, Bouffard GG, Blakesley RW, Touchman JW, Green ED, Dickson MC, Rodriguez AC, Grimwood J, Schmutz J, Myers RM, Butterfield YS, Krzywinski MI, Skalska U, Smailus DE, Schnerch A, Schein JE, Jones SJ, Marra MA: **Generation and initial analysis of more than 15,000 full-length human and mouse cDNA sequences.** *Proceedings of the National Academy of Sciences of the United States of America* 2002, **99(26):**16899-16903.
6. Sgarra R, Diana F, Bellarosa C, Dekleva V, Rustighi A, Toller M, Manfioletti G, Giancotti V: **During apoptosis of tumor cells HMGA1a protein undergoes methylation: identification of the modification site by mass spectrometry.** *Biochemistry* 2003, **42(12):**3575-3585.
7. Sgarra R DF Bellarosa C, Rustighi A, Toller M, Manfioletti G, and Giancotti V: **Increase of HMGA1a protein methylation is a distinctive characteristic of tumor cells induced to apoptosis.** *Unpublished manuscript* 2002.
8. Vandenplas M CPMM Suzuki Y, Sugano S, Moore JN, Liang C, Sun F, Sullivan R, Shah M, and Pratt LH: **An EST database from equine (Equus caballus) unstimulated peripheral blood leukocytes.** *Unpublished manuscript* 2003.
9. Murua Escobar H, Soller JT, Richter A, Meyer B, Winkler S, Flohr AM, Nolte I, Bullerdiek J: **The canine HMGA1.** *Gene* 2004, **330:**93-99.
10. Sonstegard TS VTCP Matukumalli LK, Harhay GP, Bosak S, Rubenfield M, and Gasbarre LC: **Production of EST from cDNA libraries derived from immunologically activated bovine gut.** *Unpublished manuscript* 2004.
11. Murua Escobar H, Soller JT, Richter A, Meyer B, Winkler S, Bullerdiek J, Nolte I: **"Best friends" sharing the HMGA1 gene: comparison of the human and canine HMGA1 to orthologous other species.** *The Journal of heredity* 2005, **96(7):**777-781.
12. Kazmierczak B, Dal Cin P, Wanschura S, Borrmann L, Fusco A, Van den Berghe H, Bullerdiek J: **HMGIY is the target of 6p21.3 rearrangements in various benign mesenchymal tumors.** *Genes Chromosomes Cancer* 1998, **23(4):**279-285.
13. Tallini G, Vanni R, Manfioletti G, Kazmierczak B, Faa G, Pauwels P, Bullerdiek J, Giancotti V, Van Den Berghe H, Dal Cin P: **HMGI-C and HMGI(Y) immunoreactivity correlates with cytogenetic abnormalities in lipomas, pulmonary chondroid hamartomas, endometrial polyps, and uterine leiomyomas and is compatible with rearrangement of the HMGI-C and HMGI(Y) genes.** *Lab Invest* 2000, **80(3):**359-369.
14. Williams AJ, Powell WL, Collins T, Morton CC: **HMGI(Y) expression in human uterine leiomyomata. Involvement of another high-mobility group architectural factor in a benign neoplasm.** *Am J Pathol* 1997, **150(3):**911-918.
15. Chiappetta G, Avantaggiato V, Visconti R, Fedele M, Battista S, Trapasso F, Merciai BM, Fidanza V, Giancotti V, Santoro M, Simeone A, Fusco A: **High level expression of the HMGI (Y) gene during embryonic development.** *Oncogene* 1996, **13(11):**2439-2446.
16. Rogalla P, Drechsler K, Frey G, Hennig Y, Helmke B, Bonk U, Bullerdiek J: **HMGI-C expression patterns in human tissues. Implications for the genesis of frequent mesenchymal tumors.** *The American journal of pathology* 1996, **149(3):**775-779.
17. Fedele M, Bandiera A, Chiappetta G, Battista S, Viglietto G, Manfioletti G, Casamassimi A, Santoro M, Giancotti V, Fusco A: **Human colorectal carcinomas express high levels of high mobility group HMGI(Y) proteins.** *Cancer research* 1996, **56(8):**1896-1901.
18. Bandiera A, Bonifacio D, Manfioletti G, Mantovani F, Rustighi A, Zanconati F, Fusco A, Di Bonito L, Giancotti V: **Expression of HMGI(Y) proteins in squamous intraepithelial and invasive lesions of the uterine cervix.** *Cancer research* 1998, **58(3):**426-431.
19. Abe N, Watanabe T, Masaki T, Mori T, Sugiyama M, Uchimura H, Fujioka Y, Chiappetta G, Fusco A, Atomi Y: **Pancreatic duct cell carcinomas express high levels of high mobility group I(Y) proteins.** *Cancer research* 2000, **60(12):**3117-3122.
20. Diana F, Di Bernardo J, Sgarra R, Tessari MA, Rustighi A, Fusco A, Giancotti V, Manfioletti G: **Differential HMGA expression and post-translational modifications in prostatic tumor cells.** *International journal of oncology* 2005, **26(2):**515-520.
21. Frasca F, Rustighi A, Malaguarnera R, Altamura S, Vigneri P, Del Sal G, Giancotti V, Pezzino V, Vigneri R, Manfioletti G: **HMGA1 inhibits the function of p53 family members in thyroid cancer cells.** *Cancer research* 2006, **66(6):**2980-2989.
22. Sarhadi VK, Wikman H, Salmenkivi K, Kuosma E, Sioris T, Salo J, Karjalainen A, Knuutila S, Anttila S: **Increased expression of high mobility group A proteins in lung cancer.** *The Journal of pathology* 2006, **209(2):**206-212.
23. Fedele M, Fidanza V, Battista S, Pentimalli F, Klein-Szanto AJ, Visone R, De Martino I, Curcio A, Morisco C, Del Vecchio L, Baldassarre G, Arra C, Viglietto G, Indolfi C, Croce CM, Fusco A: **Haploinsufficiency of the Hmga1 gene causes cardiac hypertrophy and myelo-lymphoproliferative disorders in mice.** *Cancer research* 2006, **66(5):**2536-2543.
24. Foti D, Chiefari E, Fedele M, Iuliano R, Brunetti L, Paonessa F, Manfioletti G, Barbetti F, Brunetti A, Croce CM, Fusco A, Brunetti A: **Lack of the architectural factor HMGA1 causes insulin resistance and diabetes in humans and mice.** *Nature medicine* 2005, **11(7):**765-773.
25. MacEwen EG: **Spontaneous tumors in dogs and cats: models for the study of cancer biology and treatment.** *Cancer metastasis reviews* 1990, **9(2):**125-136.
26. Reimann N, Bartnitzke S, Bullerdiek J, Schmitz U, Rogalla P, Nolte I, Ronne M: **An extended nomenclature of the canine karyotype.** *Cytogenetics and cell genetics* 1996, **73(1-2):**140-144.
27. Breen M, Bullerdiek J, Langford CF: **The DAPI banded karyotype of the domestic dog (Canis familiaris) generated using chromosome-specific paint probes.** *Chromosome Res* 1999, **7(5):**401-406.
28. Reimann N, Bartnitzke S, Nolte I, Bullerdiek J: **Working with canine chromosomes: current recommendations for karyotype description.** *The Journal of heredity* 1999, **90(1):**31-34.
29. Reimann N, Nolte I, Bartnitzke S, Bullerdiek J: **Re: Sit, DNA, sit: cancer genetics going to the dogs.** *Journal of the National Cancer Institute* 1999, **91(19):**1688-1689.
30. Becker K, Murua Escobar H, Richter A, Meyer B, Nolte I, Bullerdiek J: **The canine HMGA1 gene maps to CFA 23.** *Animal genetics* 2003, **34(1):**68-69.
31. Lindblad-Toh K, Wade CM, Mikkelsen TS, Karlsson EK, Jaffe DB, Kamal M, Clamp M, Chang JL, Kulbokas EJ 3rd, Zody MC, Mauceli E, Xie X, Breen M, Wayne RK, Ostrander EA, Ponting CP, Galibert F, Smith DR, DeJong PJ, Kirkness E, Alvarez P, Biagi T, Brockman W, Butler J, Chin CW, Cook A, Cuff J, Daly MJ, DeCaprio D, Gnerre S, Grabherr M, Kellis M, Kleber M, Bardeleben C, Goodstadt L, Heger A, Hitte C, Kim L, Koepfli KP, Parker HG, Pollinger JP, Searle SM, Sutter NB, Thomas R, Webber C, Baldwin J, Abebe A, Abouelleil A, Aftuck L, Ait-Zahra M, Aldredge T, Allen N, An P, Anderson S, Antoine C, Arachchi H, Aslam A, Ayotte L, Bachantsang P, Barry A, Bayul T, Benamara M, Berlin A, Bessette D, Blitshteyn B, Bloom T, Blye J,

Boguslavskiy L, Bonnet C, Boukhgalter B, Brown A, Cahill P, Calixte N, Camarata J, Cheshatsang Y, Chu J, Citroen M, Collymore A, Cooke P, Dawoe T, Daza R, Decktor K, DeGray S, Dhargay N, Dooley K, Dooley K, Dorje P, Dorjee K, Dorris L, Duffey N, Dupes A, Egbiremolen O, Elong R, Falk J, Farina A, Faro S, Ferguson D, Ferreira P, Fisher S, FitzGerald M, Foley K, Foley C, Franke A, Friedrich D, Gage D, Garber M, Gearin G, Giannoukos G, Goode T, Goyette A, Graham J, Grandbois E, Gyaltsen K, Hafez N, Hagopian D, Hagos B, Hall J, Healy C, Hegarty R, Honan T, Horn A, Houde N, Hughes L, Hunnicutt L, Husby M, Jester B, Jones C, Kamat A, Kanga B, Kells C, Khazanovich D, Kieu AC, Kisner P, Kumar M, Lance K, Landers T, Lara M, Lee W, Leger JP, Lennon N, Leuper L, LeVine S, Liu J, Liu X, Lokyitsang Y, Lokyitsang T, Lui A, Macdonald J, Major J, Marabella R, Maru K, Matthews C, McDonough S, Mehta T, Meldrim J, Melnikov A, Meneus L, Mihalev A, Mihova T, Miller K, Mittelman R, Mlenga V, Mulrain L, Munson G, Navidi A, Naylor J, Nguyen T, Nguyen N, Nguyen C, Nguyen T, Nicol R, Norbu N, Norbu C, Novod N, Nyima T, Olandt P, O'Neill B, O'Neill K, Osman S, Oyono L, Patti C, Perrin D, Phunkhang P, Pierre F, Priest M, Rachupka A, Raghuraman S, Rameau R, Ray V, Raymond C, Rege F, Rise C, Rogers J, Rogov P, Sahalie J, Settipalli S, Sharpe T, Shea T, Sheehan M, Sherpa N, Shi J, Shih D, Sloan J, Smith C, Sparrow T, Stalker J, Stange-Thomann N, Stavropoulos S, Stone C, Stone S, Sykes S, Tchuinga P, Tenzing P, Tesfaye S, Thoulutsang D, Thoulutsang Y, Topham K, Topping I, Tsamla T, Vassiliev H, Venkataraman V, Vo A, Wangchuk T, Wangdi T, Weiand M, Wilkinson J, Wilson A, Yadav S, Yang S, Yang X, Young G, Yu Q, Zainoun J, Zembek L, Zimmer A, Lander ES: **Genome sequence, comparative analysis and haplotype structure of the domestic dog.** *Nature* 2005, **438(7069):**803-819.

32. Friedmann M, Holth LT, Zoghbi HY, Reeves R: **Organization, inducible-expression and chromosome localization of the human HMG-I(Y) nonhistone protein gene.** *Nucleic acids research* 1993, **21(18):**4259-4267.
33. Sachidanandam R, Weissman D, Schmidt SC, Kakol JM, Stein LD, Marth G, Sherry S, Mullikin JC, Mortimore BJ, Willey DL, Hunt SE, Cole CG, Coggill PC, Rice CM, Ning Z, Rogers J, Bentley DR, Kwok PY, Mardis ER, Yeh RT, Schultz B, Cook L, Davenport R, Dante M, Fulton L, Hillier L, Waterston RH, McPherson JD, Gilman B, Schaffner S, Van Etten WJ, Reich D, Higgins J, Daly MJ, Blumenstiel B, Baldwin J, Stange-Thomann N, Zody MC, Linton L, Lander ES, Altshuler D: **A map of human genome sequence variation containing 1.42 million single nucleotide polymorphisms.** *Nature* 2001, **409(6822):**928-933.
34. Kim S, Misra A: **SNP genotyping: technologies and biomedical applications.** *Annual review of biomedical engineering* 2007, **9:**289-320.
35. Muller S, Scaffidi P, Degryse B, Bonaldi T, Ronfani L, Agresti A, Beltrame M, Bianchi ME: **New EMBO members' review: the double life of HMGB1 chromatin protein: architectural factor and extracellular signal.** *The EMBO journal* 2001, **20(16):**4337-4340.
36. Huttunen HJ, Fages C, Kuja-Panula J, Ridley AJ, Rauvala H: **Receptor for advanced glycation end products-binding COOH-terminal motif of amphoterin inhibits invasive migration and metastasis.** *Cancer research* 2002, **62(16):**4805-4811.
37. Taguchi A, Blood DC, del Toro G, Canet A, Lee DC, Qu W, Tanji N, Lu Y, Lalla E, Fu C, Hofmann MA, Kislinger T, Ingram M, Lu A, Tanaka H, Hori O, Ogawa S, Stern DM, Schmidt AM: **Blockade of RAGE-amphoterin signalling suppresses tumour growth and metastases.** *Nature* 2000, **405(6784):**354-360.

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***RAS* gene hot-spot mutations in canine neoplasias**

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RAS Gene Hot-Spot Mutations in Canine Neoplasias

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Abstract

Point mutations in the cellular homologues *HRAS*, *KRAS2*, and *NRAS* of the viral Harvey and Kirsten rat sarcoma virus oncogenes are commonly involved in the onset of malignancies in humans and other species such as dog, mouse, and rat. Most often, three particular hot-spot codons are affected, with one amino acid exchange being sufficient for the induction of tumor growth. While *RAS* genes have been shown to play an important role in canine tumors such as non-small lung cell carcinomas, data about *RAS* mutations in canine fibrosarcomas as well as *KRAS2* mutations in canine melanomas is sparse. To increase the number of tumors examined, we recently screened 13 canine fibrosarcomas and 11 canine melanomas for point mutations, particularly within the mutational hot spots. The results were compared to the already existing data from other studies about these tumors in dogs.

A family of genes often involved in human tumors are the well-characterized *RAS* genes, which comprise *HRAS*, *KRAS2*, and *NRAS*, coding for closely related, small, 189 amino acid, 21 kDa, membrane-bound, intracellular proteins. The human cellular *HRAS* and *KRAS2* genes were identified to be homologues of the Harvey and Kirsten rat sarcoma virus oncogenes *v-Ha-ras* and *v-Ki-ras2*, respectively (Der et al. 1982; Parada et al. 1982), with *NRAS* being only weakly homologous to both *v-Ha-ras* and *v-Ki-ras2* (a *v-N-ras* gene has not been described) (Shimizu et al. 1983). *Ras* genes have been found in a variety of mammals, showing high sequence similarity across species, with sequence variation most often not affecting the amino acid sequence of the encoded proteins (Watzinger et al. 1998).

The RAS proteins function in relaying mitogenic growth signals into the cytoplasm and nucleus, influencing proliferation, differentiation, transformation, and apoptosis of cells (Watzinger and Lion 1999). Regulation of RAS protein activity occurs through intrinsic GTPase activity in the wild-type RAS, which switches the protein from an active (guanosine triphosphate [GTP]-bound) to an inactive (guanosine diphosphate [GDP]-bound) state. Point mutations in a number of particular hot-spot codons in exon 1 (mostly codons 12 and 13) and exon 2 (mostly codon 61) lead to diminished GTPase activity, bringing about constant signal transduction and facilitating uncontrolled cell division and tumor growth (Park 1995).

Alterations in *RAS* genes are among the most important incidents in the onset of malignancies in humans (Arber 1999; Hahn et al. 1994), and have been described in dog, mouse, and rat, among others. Studies indicate that in man, up to 13% of brain tumors, 30% of lung tumors, 30% of liver tumors, 30% of acute myelogenous leukemia, 53% of follicular and 60% of undifferentiated papillary thyroid tumors, 50% of tumors of the gastrointestinal tract, and 90% of pancreatic tumors are affected by a mutation in the hot-spot codons of one of the three known *RAS* genes (Bos 1989; Knapp and Waters 1997; Spandidos et al. 2002; Tang et al. 2002).

Studies about the involvement of *RAS* genes in canine tumors have been performed by a number of groups investigating several types of tumors. Gumerlock et al. (1989) described the formation of activated *NRAS* through the substitution of glycine by aspartartic acid at position 12 of the protein in a case of a gamma radiation-induced canine acute nonlymphocytic leukemia.

KRAS2 activation was observed in non-small cell lung cancer of the dog (Kraegel et al. 1992). Out of 21 tumors, which included adenocarcinomas, adenosquamous carcinomas, and one large cell carcinoma, 5 were shown to be affected by mutations mostly of codon 12 of the *KRAS2* gene, being similar to the overall frequency of *KRAS2* involvement in non-small cell lung cancer in man (25%). This was confirmed by a later study investigating a wide range of

canine lung tumors where 19 out of 117 tumors (16%) showed *KRAS2* alterations in the hot-spot codons (Griffey et al. 1998). On the other hand, *NRAS* was shown to be infrequently activated in canine malignant lymphomas, with only 1 from 28 examined cases showing an amino acid substitution from glycine to aspartate at position 13 (Edwards et al. 1993).

Similar to malignant lymphomas, *RAS* gene mutations at the hot-spot loci were shown to be rarely or not involved in canine mammary tumors (Castagnaro 1995; Mayr et al. 1998). Furthermore, Watzinger et al. (1998) have shown in a variety of canine tumors that *RAS* genes are, compared to humans, rather infrequently involved in the onset of malignancies. In that study, only three fibrosarcomas were included, none of which showed *RAS* gene alterations. Since Guerrero et al. (2002) showed that fibrosarcomas can be induced in nude mice by subcutaneously injecting transfected fibroblasts with *KRAS2* point mutations in codon 12, we recently screened a larger number of 13 canine fibrosarcomas for *KRAS2* and *NRAS* mutations in the particular hot-spot codons. In addition, we also recently screened 11 canine melanomas for *KRAS2* and *NRAS* mutations (Murua Escobar et al. 2004). However, none of the screened tumors showed the characteristic *RAS* alterations in the hot-spot codons. A low rate of *NRAS* involvement in canine melanomas has been shown before, with 2 of 16 tumors showing *NRAS* mutations (Mayr et al. 2003).

In summary, the data from the available studies on canine fibrosarcomas and melanomas (Mayr et al. 2003; Murua Escobar et al. 2004; Watzinger et al. 2001) strongly indicate that *KRAS2* and *NRAS* mutations at the hot-spot loci are essentially very rare in the investigated canine tumor entities. To the best of our knowledge, from the total number of 32 screened canine fibrosarcomas and 17 screened canine melanomas, only 2 melanoma samples have been found to have exon 61 of the *NRAS* gene affected. For *KRAS2*, no mutations in the hot-spot codons have been found. However, to allow for a comparison of these canine tumors with research results from, for example, man and mouse, with vast amounts of data being available, a larger number of canine tumors will have to be screened in the future, as it is still too early to draw conclusions from the relatively small number of canine tumors examined.

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References

Arber N, 1999. Janus faces of ras: anti or pro-apoptotic? Apoptosis 4:383–388.

Bos JL, 1989. Ras oncogenes in human cancer: a review. Cancer Res 49:4682–4689.

Castagnaro M, 1995. [Ras gene analysis in mammary tumors of dogs by means of PCR-SSCP and direct genomic analysis]. Ann 1st Super Sanita 31:337–341.

Der CJ, Krontiris TG, and Cooper GM, 1982. Transforming genes of human bladder and lung carcinoma cell lines are homologous to the ras genes of Harvey and Kirsten sarcoma viruses. Proc Natl Acad Sci USA 79:3637–3640.

Edwards MD, Pazzi KA, Gumerlock PH, and Madewell BR, 1993. c-N-ras is activated infrequently in canine malignant lymphoma. Toxicol Pathol 21:288–291.

Griffey SM, Kraegel SA, and Madewell BR, 1998. Rapid detection of K-ras gene mutations in canine lung cancer using single-strand conformational polymorphism analysis. Carcinogenesis 19:959–963.

Guerrero S, Figueras A, Casanova I, Farre L, Lloveras B, Capella G, Trias M, and Mangues R, 2002. Codon 12 and codon 13 mutations at the K-ras gene induce different soft tissue sarcoma types in nude mice. FASEB J 16: 1642–1644.

Gumerlock PH, Meyers FJ, Foster BA, Kawakami TG, and deVere White RW, 1989. Activated c-N-ras in radiation-induced acute nonlymphocytic leukemia: twelfth codon aspartic acid. Radiat Res 117:198–206.

Hahn KA, Bravo L, Adams WH, and Frazier DL, 1994. Naturally occurring tumors in dogs as comparative models for cancer therapy research. In Vivo 8:133–143.

Knapp DW and Waters DJ, 1997. Naturally occurring cancer in pet dogs: important models for developing improved cancer therapy for humans. Mol Med Today 3:8–11.

Kraegel SA, Gumerlock PH, Dungworth DL, Oreffo VI, and Madewell BR, 1992. K-ras activation in non-small cell lung cancer in the dog. Cancer Res 52:4724–4727.

Mayr B, Dressler A, Reifinger M, and Feil C, 1998. Cytogenetic alterations in eight mammary tumors and tumor-suppressor gene p53 mutation in one mammary tumor from dogs. Am J Vet Res 59:69–78.

Mayr B, Schaffner G, Reifinger M, Zwetkoff S, and Prodinger B, 2003. N-ras mutations in canine malignant melanomas. Vet J 165:169–171.

Murua Escobar H, Gunther K, Richter A, Soller JT, Winkler S, Nolte I, and Bullerdiek J, 2004. Absence of ras-gene hot-spot mutations in canine fibrosarcomas and melanomas. Anticancer Res 24:3027–3028.

Parada LF, Tabin CJ, Shih C, and Weinberg RA, 1982. Human EJ bladder carcinoma oncogene is homologue of Harvey sarcoma virus ras gene. Nature 297:474–478.

Park M, 1995. Genetic abnormalities of cell growth. In: The metabolic and molecular basis of inherited disease, 7th ed (Scriver CR, Beaudet AL, Sly WS, and Valle D, eds). New York: McGraw-Hill.

Shimizu K, Goldfarb M, Suard Y, Perucho M, Li Y, Kamata T, Feramisco J, Stavnezer E, Fogh J, and Wigler MH, 1983. Three human transforming genes are related to the viral ras oncogenes. Proc Natl Acad Sci USA 80: 2112–2116.

Spandidos DA, Sourvinos G, Tsatsanis C, and Zafiropoulos A, 2002. Normal ras genes: their onco-suppressor and pro-apoptotic functions (review). Int J Oncol 21:237–241.

Tang MS, Pfeifer GP, Denissenko MF, Feng Z, Hu W, Pao A, Zheng Y, Zheng JB, Li H, and Chen JX, 2002. Mapping polycyclic aromatic hydrocarbon and aromatic amine-induced DNA damage in cancer-related genes at the sequence level. Int J Hyg Environ Health 205:103–113.

Watzinger F and Lion T, 1999. RAS family. Atlas of Genetics and Cytogenetics in Oncology and Haematology (last modified March 1999) http://www.infobiogen.fr/services/chromcancer/Deep/ras.html.

Watzinger F, Mayr B, Gamerith R, Vetter C, and Lion T, 2001. Comparative analysis of ras proto-oncogene mutations in selected mammalian tumors. Mol Carcinog 30:190–198.

Watzinger F, Mayr B, Haring E, and Lion T, 1998. High sequence similarity within ras exons 1 and 2 in different mammalian species and phylogenetic divergence of the ras gene family. Mamm Genome 9:214–219.

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Murua Escobar H, Soller JT, Richter A, Meyer B, Winkler S, Bullerdiek J, Nolte I

"Best friends" sharing the *HMGA1* gene: comparison of the human and canine *HMGA1* to orthologous other species

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"Best Friends" Sharing the *HMGA1* Gene: Comparison of the Human and Canine HMGA1 to Orthologous Other Species

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Abstract

HMGA1 nonhistone proteins are reported to participate in various cellular processes including regulation of inducible gene transcription, integration of retroviruses into chromosomes, and the induction of neoplastic transformation and promotion of metastatic progression of cancer cells. Overexpression of *HMGA1* was shown to be characteristic for various malignant tumors, suggesting a relation between the neoplastic phenotype and a high titer of the protein. Also chromosomal aberrations affecting the human *HMGA1* gene at 6p21 were described in several tumors, e.g., uterine leiomyomas, pulmonary chondroid hamartomas, and follicular thyroid adenomas. We characterize the molecular structure of the canine *HMGA1* cDNA, its splice variants, and predicted proteins HMGA1a and HMGA1b. Furthermore, we compared the CDS of both splice variants for 12 different breeds, screened them for SNPs, characterised a basic expression pattern, and mapped the gene via FISH. Additionally, we compared the known human, canine, murine, rat, hamster, bovine, pig, *Xenopus*, and chicken *HMGA1* transcripts.

High mobility group proteins named according to their characteristic mobility in gel electrophoresis are small chromatin-associated nonhistone proteins, which can be subdivided into three families because of their functional sequence motives: the HMGA (functional motive "AT-hook"), HMGB (functional motive "HMG-box"), and HMGN (functional motive "nucleosomal binding domain") protein families (for review see Bustin 2001). By binding DNA with their functional motives, the HMG proteins induce DNA conformation changes influencing the binding of various transcription factors and thus taking indirect influence on transcription regulation as so-called architectural transcription factors (for detail see Bustin and Reeves 1996).

The proteins HMGA1a, HMGA1b, and HMGA2 of the human *HMGA* genes are associated with various human diseases, including cancer. Members of the human HMGA1 protein family presently known are HMGA1a and HMGA1b, which by modifying chromatin structure take influence on transcription and up- and down-regulation of a number of target genes, for example, *ATF2*, *IFN*-β, *NF-κB*, *Interleukin-2 receptor*, *E-Selektin, Interleukin-4*, *Interfeone-A*, *ERCC1*, and *Cyclin A* (Chuvpilo et al. 1993; Du and Maniatis 1994; Thanos and Maniatis 1992; Lewis et al. 1994; John et al. 1995, 1996; Klein-Hessling et al. 1996; Yie et al. 1997; Borrmann et al. 2003).

The expression pattern of the *HMGA* genes in human adult tissues shows only very low levels or even absent expression, whereas it is abundantly expressed in embryonic cells (Rogalla et al. 1996; Chiappetta et al. 1996). In humans the *HMGA1* gene is located on HSA 6p21, a region often affected by aberrations leading to an up-regulation of this gene in various benign mesenchymal tumors, for example, endometrial polyps, lipomas, pulmonary chondroid hamartomas, and uterine leiomyomas (Williams et al. 1997; Kazmierczak et al. 1998; Tallini et al. 2000). This suggests that transcriptional activation due to these chromosomal alterations is probably an early and often even primary event of cancer development. Recently, the canine *HMGA1* gene has been mapped to CFA 23. This cytogenetic assignment

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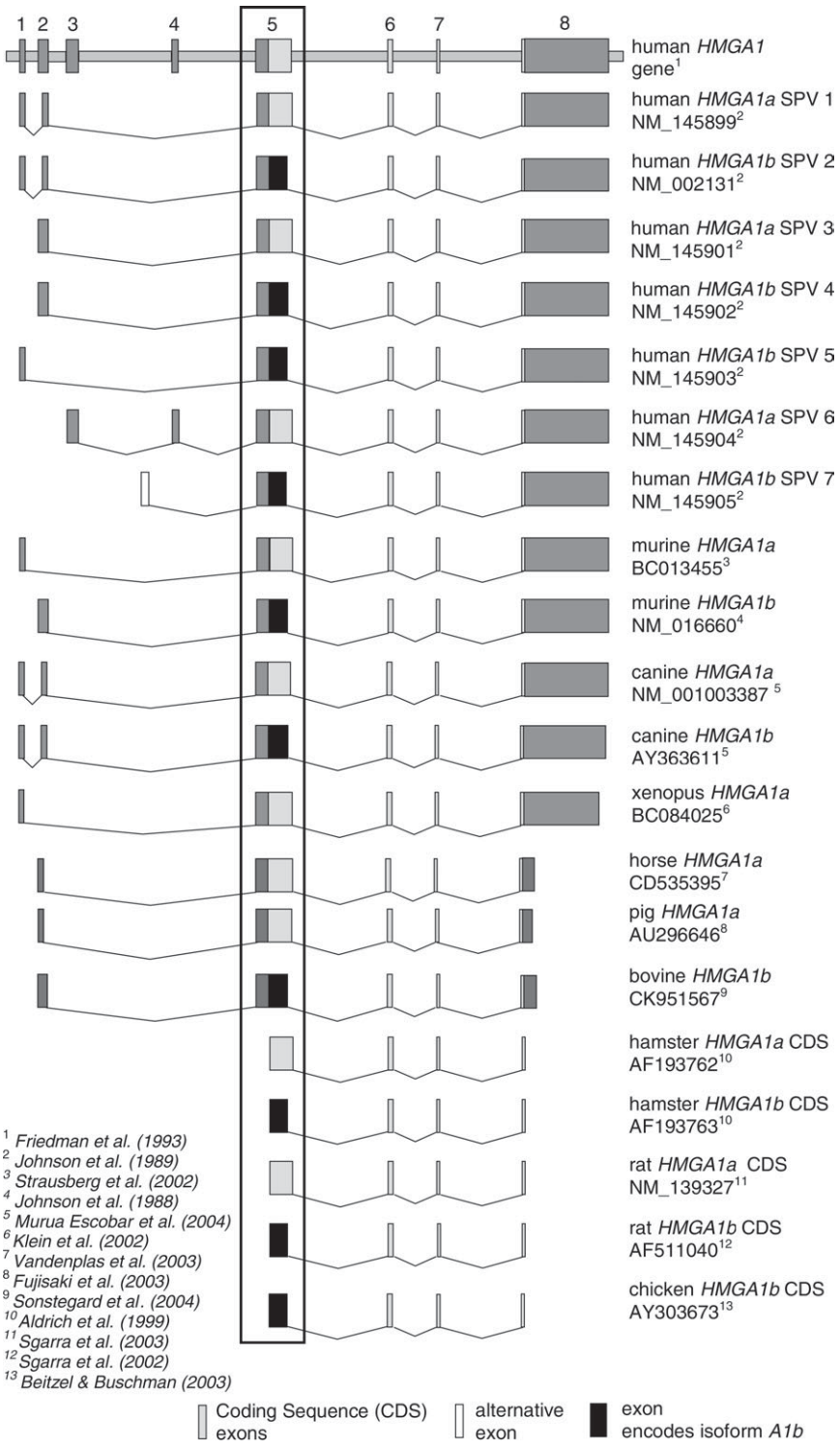

Figure 1. Species comparison of *HMGA1a* and *HMGA1b* transcripts. Exon 5 is enlarged by factor fife for better visualization.

indicates that the canine *HMGA1* gene does not map to a hotspot of chromosomal breakpoints seen in canine tumours (Becker et al. 2003).

HMGA1 expression in human malignant epithelial tumors is reported to be associated with an aggressive behavior of the tumors. Overexpression of *HMGA1* was

detected in a number of malignancies, including thyroid, prostatic, pancreatic, uterine cervical, and colorectal cancer (Tamimi et al. 1993; Chiappetta et al. 1995; Fedele et al. 1996; Bandiera et al. 1998; Abe et al. 1999, 2000; Czyz et al. 2004; Takaha et al. 2004). The correlation between *HMGA* expression and tumor aggressiveness in some of these malignancies has led to the conclusion that *HMGA* expression may present a powerful diagnostic and prognostic molecular marker.

Due to the similarities of various human and canine cancer entities, the characterization of the canine *HMGA* genes could open new fields for experimental and therapeutic approaches. We recently characterized the canine *HMGA1a* and *HMGA1b* transcripts, deduced their proteins, evaluated them as targets for therapeutic approaches, and characterized a basic expression pattern in healthy tissues (Murua Escobar et al. 2004). Sequence comparison showed a 100% identity between the human and canine protein molecules. Although both species showed the identical two proteins, the number of found cDNA transcripts varies. For the human *HMGA1* seven different cDNA transcripts (Figure 1: SPV1–SPV7) were described (Johnson et al. 1988) of which SPV1 and SPV2 are the commonly found variants. The characterized dog variants showed the same composition structure as the mentioned human variants SPV1 and SPV2. Canine counterparts of the human transcript variants SPV3–SPV7 could not be detected using polymerase chain reaction (PCR) amplification approaches. Comparison of the human cDNAs to the known transcripts of other species shows that the dog is the only species showing similar transcripts to those commonly found in humans referring to exon structure and distribution. In detail, human and dog are the only known species showing the presence of exon one and two in both *HMGA1a* or *HMGA1b* transcripts, respectively (Figure 1). Both isoforms (*HMGA1a* and *HMGA1b)* were found in mouse (BC013455, NM_016660), hamster (AF1893762, AF193763), and rat (NM_139327, AF511040), of which for the last two species the described transcripts are limited to the protein coding sequences and the mouse transcripts show either exon one (*HMGA1a*) or exon two (*HMGA1b*) in the respective transcripts (Figure 1).

For the *HMGA1* transcripts of horse (CD535395), pig (AU296646), chicken (AY303673), bovine (CK951567), and *Xenopus* (BC084025), either the *HMGA1a* or the *HMGA1b* isoform are currently (2004) present at the NCBI database. For CDS (coding sequence) and protein identity analysis, we used the described sequences and deduced, if necessary, the corresponding parts for analyses. The in silico analyses were done using Lasergene software programs (DNASTAR, Madison). The coding sequence identities of the canine *HMGA1* transcripts to the sequences from other species vary between 72.0% (chicken AY303673) and 95.7% (pig AU296646, horse CD535395) (Table 1). Identity comparison of the deduced proteins revealed similarities between 69.7% (chicken AY303673) and 100.0% (human: P17096, X14957, horse CD535395) (Table 1). The proteins of all species showed strong conservation in the functional AT-Hook DNA binding domains. Common for all species analysed is that the protein coding sequences are composed of four exons (Figure 1). The described proteins of the different species are composed of 107 amino acids and 96 amino acids, respectively, for HMGA1a and HMGA1b. Also common for those species where both protein isoforms were described is that the difference between the splicing variants is the "typical" 33-bp deletion in the *HMGA1b* transcripts resulting in the lack of 11 amino acids.

Table 1. *HMGA1* identity comparison (CDS and protein) of various species to the canine transcripts and proteins

		Identity (%) to *C. familiaris*	
Species	Isoform	CDS	Protein
Human (*H. sapiens*)	*HMGA1a*	95.1	100.0
Human (*H. sapiens*)	*HMGA1b*	95.1	100.0
Mouse (*M. musculus*)	*HMGA1a*	90.1	96.3
Mouse (*M. musculus*)	*HMGA1b*	90.1	96.9
Rat (*R. norvegicus*)	*HMGA1a*	90.4	96.3
Rat (*R. norvegicus*)	*HMGA1b*	90.4	95.8
Hamster (*C. griseus*)	*HMGA1a*	92.6	98.1
Hamster (*C. griseus*)	*HMGA1b*	92.6	97.9
Pig (*S. scrofa*)	*HMGA1a*	95.7	99.1
Horse (*E. caballus*)	*HMGA1b*	95.7	100.0
Cattle (*B. taurus*)	*HMGA1b*	94.4	99.0
Chicken (*G. gallus*)	*HMGA1b*	72.0	69.7
African clawed frog (*X. laevis*)	*HMGA1a*	90.4	97.2

Previous results describing the comparison of the protein coding sequences in 12 canine breeds revealed that the mentioned deletion is also conserved in the analyzed breeds. SNP screening in these breeds resulted in detection of one-amino-acid change in a single breed. A Teckel showed a nucleotide transition from A to G at the first base of codon 64 in its *HMGA1b* transcript leading to an amino acid replacement from threonine to alanine (Murua Escobar et al. 2004). As far as we know, no other canine *HMGA1* polymorphisms have been described. Summarizing the *HMGA1* transcript and protein comparison data emphasizes the relevance of the canine species as a model organism for the research and development of therapeutic approaches for human disorders.

Due to the mentioned properties of the *HMGA1* gene, its proteins HMGA1a/HMGA1b, and its reported role in development of cancer, future studies targeting HMGA1 proteins could be of significant value.

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References

Abe N, Watanabe T, Masaki T, Mori T, Sugiyama M, Uchimura H, Fujioka Y, Chiappetta G, Fusco A, and Atomi Y, 2000. Pancreatic duct cell carcinomas express high levels of high mobility group I(Y) proteins. Cancer Res 60: 3117–3122.

Abe N, Watanabe T, Sugiyama M, Uchimura H, Chiappetta G, Fusco A, and Atomi Y, 1999. Determination of high mobility group I(Y) expression level in colorectal neoplasias: a potential diagnostic marker. Cancer Res 59:1169–1174.

Aldrich TL, Reeves R, Lee CC, Thomas JN, and Morris AE, 1999. HMG-I(Y) proteins implicated in amplification of CHO cell DNA. Sequence at NCBI AF193763. Unpublished manuscript.

Bandiera A, Bonifacio D, Manfioletti G, Mantovani F, Rustighi A, Zanconati F, Fusco A, Di Bonito L, and Giancotti V, 1998. Expression of HMGI(Y) proteins in squamous intraepithelial and invasive lesions of the uterine cervix. Cancer Res 58:426–431.

Becker K, Murua Escobar H, Richter A, Meyer B, Nolte I, and Bullerdiek J, 2003. The canine HMGA1 gene maps to CFA 23. Anim Genet 34(1): 68–69.

Beitzel B and Bushman F, 2003. Construction and analysis of cells lacking the HMGA gene family. Nucleic Acids Res 31(17):5025–5032.

Borrmann L, Schwanbeck R, Heyduk T, Seebeck B, Rogalla P, Bullerdiek J, and Wisniewski JR, 2003. High mobility group A2 protein and its derivatives bind a specific region of the promoter of DNA repair gene ERCC1 and modulate its activity. Nucleic Acids Res 31:6841–6851.

Bustin M, 2001. Revised nomenclature for high mobility group (HMG) chromosomal proteins. Trends Biochem Sci 26(3):152–153.

Bustin M and Reeves R, 1996. High-mobility-group chromosomal proteins: architectural components that facilitate chromatin function. Prog Nucleic Acid Res Mol Biol 54:35–100.

Chiappetta G, Bandiera A, Berlingieri MT, Visconti R, Manfioletti G, Battista S, Martinez-Tello FJ, Santoro M, Giancotti V, and Fusco A, 1995. The expression of the high mobility group HMGI (Y) proteins correlates with the malignant phenotype of human thyroid neoplasias. Oncogene 10:1307–1314.

Chiappetta G, Avantaggiato V, Visconti R, Fedele M, Battista S, Trapasso F, Merciai BM, Fidanza V, Giancotti V, Santoro M, Simeone A, and Fusco A, 1996. High level expression of the HMGI (Y) gene during embryonic development. Oncogene 13:2439–2446.

Chuvpilo S, Schomberg C, Gerwig R, Heinfling A, Reeves R, Grummt F, and Serfling E, 1993. Multiple closely-linked NFAT/octamer and HMG I(Y) binding sites are part of the interleukin-4 promoter. Nucleic Acids Res 21(24):5694–5704.

Czyz W, Balcerczak E, Jakubiak M, Pasieka Z, Kuzdak K, and Mirowski M, 2004. HMGI(Y) gene expression as a potential marker of thyroid follicular carcinoma. Langenbecks Arch Surg 389(3):193–197.

Du W and Maniatis T, 1994. The high mobility group protein HMG I(Y) can stimulate or inhibit DNA binding of distinct transcription factor ATF-2 isoforms. Proc Natl Acad Sci USA 91:11318–11322.

Fedele M, Bandiera A, Chiappetta G, Battista S, Viglietto G, Manfioletti G, Casamassimi A, Santoro M, Giancotti V, and Fusco A, 1996. Human colorectal carcinomas express high levels of high mobility group HMGI(Y) proteins. Cancer Res 56:1896–1901.

Friedmann M, Holth LT, Zoghbi HY, and Reeves R, 1993. Organization, inducible-expression and chromosome localization of the human HMG-I(Y) nonhistone protein gene. Nucleic Acids Res 21(18):4259–4267.

Fujisaki S, Hiraiwa H, Eguchi T, Watanabe Y, Honma D, Saitou T, and Yasue H, 2003. Construction of a full-length library of swine olfactory bulb and its preliminary characterization. Sequence at NCBI AU296646. Unpublished manuscript.

John S, Reeves RB, Lin JX, Child R, Leiden JM, Thompson CB, and Leonard WJ, 1995. Regulation of cell-type-specific interleukin-2 receptor alpha-chain gene expression: potential role of physical interactions between Elf-1, HMG-I(Y), and NF-kappa B family proteins. Mol Cell Biol 15: 1786–1796.

John S, Robbins CM, and Leonard WJ, 1996. An IL-2 response element in the human IL-2 receptor alpha chain promoter is a composite element that binds Stat5, Elf-1, HMG-I(Y) and a GATA family protein. EMBO J 15:5627–5635.

Johnson KR, Lehn DA, Elton TS, Barr PJ, and Reeves R, 1988. Complete murine cDNA sequence, genomic structure, and tissue expression of the high mobility group protein HMG-I(Y). J Biol Chem 263(34): 18338–18342.

Johnson KR, Lehn DA, and Reeves R, 1989. Alternative processing of mRNAs encoding mammalian chromosomal high-mobility-group proteins HMG-I and HMG-Y. Mol Cell Biol 9(5):2114–2123.

Kazmierczak B, Dal Cin P, Wanschura S, Borrmann L, Fusco A, Van den Berghe H, and Bullerdiek J, 1998. HMGIY is the target of 6p21.3 rearrangements in various benign mesenchymal tumors. Genes Chromosomes Cancer 23:279–285.

Klein SL, Strausberg RL, Wagner L, Pontius J, Clifton SW, and Richardson P, 2002. Genetic and genomic tools for *Xenopus* research: the NIH *Xenopus* initiative. Dev Dyn 225(4):384–391.

Klein-Hessling S, Schneider G, Heinfling A, Chuvpilo S, and Serfling E, 1996. HMG I(Y) interferes with the DNA binding of NF-AT factors and the induction of the interleukin 4 promoter in T cells. Proc Natl Acad Sci USA 93:15311–15316.

Lewis H, Kaszubska W, DeLamarter JF, and Whelan J, 1994. Cooperativity between two NF-kappa B complexes, mediated by high-mobility-group protein I(Y), is essential for cytokine-induced expression of the E-selectin promoter. Mol Cell Biol 14:5701–5709.

Murua Escobar H, Soller JT, Richter A, Meyer B, Winkler S, Flohr AM, Nolte I, and Bullerdiek J, 2004. The canine HMGA1. Gene 14(330): 93–99.

Reeves R, Edberg DD, and Li Y, 2001. Architectural transcription factor HMGI(Y) promotes tumor progression and mesenchymal transition of human epithelial cells. Mol Cell Biol 21:575–594.

Rogalla P, Drechsler K, Frey G, Hennig Y, Helmke B, Bonk U, and Bullerdiek J, 1996. HMGI-C expression patterns in human tissues. Implications for the genesis of frequent mesenchymal tumors. Am J Pathol 149(3):775–779.

Sgarra R, Diana F, Bellarosa C, Rustighi A, Toller M, Manfioletti G, and Giancotti V, 2002. Increase of HMGA1a protein methylation is a distinctive characteristic of tumor cells induced to apoptosis. Sequence at NCBI AF511040. Unpublished manuscript.

Sgarra R, Diana F, Bellarosa C, Dekleva V, Rustighi A, Toller M, Manfioletti G, and Giancotti V, 2003. During apoptosis of tumor cells HMGA1a protein undergoes methylation: identification of the modification site by mass spectrometry. Biochemistry 42(12):3575–3585.

Sonstegard TS, Van Tassell CP, Matukumalli LK, Harhay GP, Bosak S, Rubenfield M, and Gasbarre LC, 2004. Production of EST from cDNA libraries derived from immunologically activated bovine gut. Sequence at NCBI CK951567. Unpublished manuscript.

Strausberg RL, Feingold EA, Grouse LH, Derge JG, Klausner RD, Collins FS, Wagner L, Shenmen CM, Schuler GD, and others, 2002. Generation and initial analysis of more than 15,000 full-length human and mouse cDNA sequences. Proc Natl Acad Sci USA 99(26): 16899–16903.

Takaha N, Resar LM, Vindivich D, and Coffey DS, 2004. High mobility group protein HMGI(Y) enhances tumor cell growth, invasion, and matrix metalloproteinase-2 expression in prostate cancer cells. Prostate 60(2): 160–167.

Tallini G, Vanni R, Manfioletti G, Kazmierczak B, Faa G, Pauwels P, Bullerdiek J, Giancotti V, Van Den Berghe H, and Dal Cin P, 2000. HMGI-C and HMGI(Y) immunoreactivity correlates with cytogenetic abnormalities in lipomas, pulmonary chondroid hamartomas, endometrial polyps, and uterine leiomyomas and is compatible with

rearrangement of the HMGI-C and HMGI(Y) genes. Lab Invest 80: 359–369.

Tamimi Y, van der Poel HG, Denyn MM, Umbas R, Karthaus HF, Debruyne FM, and Schalken JA, 1993. Increased expression of high mobility group protein I(Y) in high grade prostatic cancer determined by in situ hybridization. Cancer Res 53:5512–5516.

Thanos D and Maniatis T, 1992. The high mobility group protein HMG I(Y) is required for NF-kappa B-dependent virus induction of the human IFN-beta gene. Cell 71:777–789.

Vandenplas M, Cordonnier-Pratt MM, Suzuki Y, Sugano S, Moore JN, Liang C, Sun F, Sullivan R, Shah M, and Pratt LH, 2003. An EST database from equine (*Equus caballus*) unstimulated peripheral blood leukocytes. Sequence at NCBI CD535395. Unpublished manuscript.

Williams AJ, Powell WL, Collins T, and Morton CC, 1997. HMGI(Y) expression in human uterine leiomyomata. Involvement of another high-mobility group architectural factor in a benign neoplasm. Am J Pathol 150:911–918.

Yie J, Liang S, Merika M, and Thanos D, 1997. Intra- and intermolecular cooperative binding of high-mobility-group protein I(Y) to the beta-interferon promoter. Mol Cell Biol 17:3649–3662.

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Murua Escobar H, Günther K, Richter A, Soller JT, Winkler S, Nolte I, Bullerdiek J.

Absence of ras-gene hot-spot mutations in canine fibrosarcomas and melanomas

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Absence of Ras-gene Hot-spot Mutations in Canine Fibrosarcomas and Melanomas

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Abstract. *Point mutations within ras proto-oncogenes, particularly within the mutational hot-spot codons 12, 13 and 61, are frequently detected in human malignancies and in different types of experimentally-induced tumours in animals. So far little is known about ras mutations in naturally occurring canine fibrosarcomas or K-ras mutations in canine melanomas. To elucidate whether ras mutations exist in these naturally occurring tumours in dogs, in the present study we screened 13 canine fibrosarcomas, 2 feline fibrosarcomas and 11 canine melanomas for point mutations, particularly within the mutational hot-spots, making this the first study to investigate a large number of canine fibrosarcomas. None of the samples showed a K- or N-ras hot spot mutation. Thus, our data strongly suggest that ras mutations at the hot-spot loci are very rare and do not play a major role in the pathogenesis of the spontaneously occurring canine tumours investigated.*

Dogs and humans often share the same genetic pathways in the development of cancer, as has been described in the literature. Point mutations affecting genes of the *ras*- family are assumed to be among the most important alterations in human tumourigenesis (1). Ras proteins play an important role as signal transmitters. The binding of growth factors activates the ras protein and thus initiates cell division. Mutations in *ras* genes are assumed to lead to enduring activation of pathways that stimulate cell growth, which results in uncontrolled cell division (2). Especially mutations in *K-ras* have been described in human pancreatic cancers and tumours of the gastro-intestinal tract, as well as in tumours of the skin (3-5). *K-ras* screening for hot-spot point mutations in dogs has been described in different types of lung cancer, pancreatic cancer and breast cancer (6-12), showing that the canine gene is also affected by the typical ras mutations observed in humans but at a much lower ratio.

Guerrero *et al.* (13) were able to induce fibrosarcomas in nude mice by subcutaneously injecting transfected fibroblasts with *K-ras* point mutations affecting codon 12. So far little is known about ras mutations in canine fibrosarcomas. Just one report of a ras mutation screening including three canine fibrosarcoma samples has been described (11). There is also a lack of studies about K-ras mutations in canine melanomas. In canine melanomas virtually no hot-spot *N-ras* mutations were described with one exception: Mayr *et al.* (14) found 2 out of 16 melanomas to be affected by mutations in codon 61.

In the present study, we screened 13 canine fibrosarcomas, 2 feline fibrosarcomas and 11 canine melanomas for point mutations, particularly within the mutational hot-spot codons of the *K-ras* and *N-ras* genes, to analyze whether these changes could be detected in these naturally occurring tumours.

Materials and Methods

The tissues used in this study were provided by the Small Animal Clinic, School of Veterinary Medicine, Hanover, Germany. Thirteen canine fibrosarcoma, 2 feline fibrosarcoma and 11 canine melanoma samples were taken and used for analyses. The breeds represented were German Shorthaired Pointer, Irish Terrier, Fox Terrier, Schnauzer, Kuvasz, Berger de Brie, German Shepherd, Standard Poodle, Irish Red Setter, Rottweiler, Cairn Terrier, Beagle and canine and feline crossbreed.

The DNA of the twenty-six canine and feline fibrosarcoma and melanoma samples (10 - 25 mg each) was isolated using QIAamp DNA Kit (QIAGEN, Hilden, Germany) following the manufacturer's tissue protocol. The two feline samples served as internal controls, since they show specific point mutations compared to dogs (15). The PCRs for the screening of the hot-spot exons were performed using the following primer pairs. K-ras: primer pair KEx1up / KEx1lo (5' cgatataaggcctgctgaaa 3' / 5' tgtaggatcatattcatcca 3') and primer pair KEx2up / KEx2lo (5' caggattcctacaggaaaca 3' / 5' aacccacctataatggtgaa 3'). N-ras: primer pair NEx1up / NEx1lo (5' gactgagtacaaactggtgg 3' / 5' gggcctcacctctatggtg 3') and primer pair NEx2up / NEx2lo (5' tcttaccgaaaacaggtggttatag 3' / 5' gtcctcatgtattggtctctcatggcac3'). The PCR products were directly sequenced in the forward and reverse direction and additionally cloned in pGEM-T Easy Vector System

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Key Words: Canis familiaris, fibrosarcoma, hot-spot mutations, melanoma, ras genes.

Table I. *Detected gene base substitutions in N-ras exon 1 and K-ras exons 1 and 2.*

Gene /Exon	Sample	Codon	Substitution	Amino Acid Exchange
K-ras Exon 1	3	23	CTA→TTA	No AA exchange (Leu)
K-ras Exon 2	3	53	TTG→TAG	Leu→Stop
	13	70	CAG→CTG	Gln→Leu
	14	48	GGA→GAA	Gly→Glu
N-ras Exon 1	3	10	GGA→GAA	Gly→Glu
	24	22	CAG→CTG	Gln→Leu

(Promega, Madison, USA) and sequenced once more. The DNA sequences and the homology alignments were created with various sequences from the NCBI database (accession numbers CFU62093, X02751, U62094, S42999, M54968, S64261). In case of single nucleotide exchanges being present, the procedures were repeated for verification.

Results

Four of the twenty-six analysed samples showed nucleotide exchanges in the screened canine exons. None of the exchanges found affected the ras hot-spot codons 12, 13 and 61. One fibrosarcoma sample (Berger de Brie) showed three changes affecting *K-ras* exon 1 codon 23 (CTA→TTA, no amino acid exchange), exon 2 codon 53 (TTG→TAG, Leu→stop codon) and *N-ras* exon 1 codon 10 (GGA→GAA, Gly→Glu). Two other fibrosarcomas (Kuvasz and Poodle) each showed one nucleotide exchange in *K-ras* exon 2 affecting codon 48 (GGA→GAA, Gly→Glu) and codon 70 (CAG→CTG, Gln→Leu), respectively. *N-ras* exon 1 codon 22 (CAG→CTG, Gln→Leu) was affected in a melanoma sample (crossbreed) (Table I). The screening of *N-ras* exon 2 revealed no nucleotide exchanges among the canine sequences. The described nucleotide differences between the canine and feline sequences (15) in *N-ras* exon 2 and *K-ras* exon 2 were detected.

Discussion

Our data strongly suggest that *K-* and *N-ras* mutations at the hot-spot loci are very rare and do not play a major role in the pathogenesis of the spontaneously occurring canine tumours investigated. These results are in accordance with the sparse data available for canine melanomas (twenty-four samples) and fibrosarcomas (three samples) (11, 14). In both studies a total of three mutations at the hot-spot codons could be detected. Compared to the data obtained from different studies in humans that show up to 30% of lung tumours, 90% of pancreatic tumours and 50% of tumours of the gastrointestinal tract to be affected by specific point mutations in the *ras* gene hot-spot codons (16, 17), the data seen in dogs apparently indicate that *ras* mutations do not play a major role in the pathogenesis of these spontaneously occurring canine tumours.

References

1 Arber N: Janus faces of ras: anti or pro-apoptotic? Apoptosis *4(5)*: 383-388, 1999.

2 Park M: Genetic abnormalities of cell growth, *In*: Scriver *et al*: The Metabolic and Molecular Basis of Inherited Disease, 7 edn. New York: McGraw-Hill; 1995.

3 Almoguera C, Shibata D, Forrester K, Martin J, Arnheim N and Perucho M: Most human carcinomas of the exocrine pancreas contain mutant c-K-ras genes. Cell *53(4)*: 549-554, 1988.

4 Belly RT, Rosenblatt JD, Steinmann M, Toner J, Sun J, Shehadi J, Peacock JL, Raubertas RF, Jani N and Ryan CK: Detection of mutated K12-ras in histologically negative lymph nodes as an indicator of poor prognosis in stage II colorectal cancer. Clin Colorectal Cancer *1(2)*: 110-116, 2001.

5 Shukla VK, Hughes DC, Hughes LE, McCormick F and Padua RA: Ras mutations in human melanotic lesions: K-ras activation is a frequent and early event in melanoma development. Oncogene Res *5(2)*: 121-127, 1989.

6 Kraegel SA, Gumerlock PH, Dungworth DL, Oreffo VI and Madewell BR: K-ras activation in non-small cell lung cancer in the dog. Cancer Res *52(17)*: 4724-4727, 1992.

7 Castagnaro M: [Ras gene analysis in mammary tumors of dogs by means of PCR-SSCP and direct genomic analysis]. Ann Ist Super Sanita *31(3)*: 337-341, 1995.

8 Tierney LA, Hahn FF and Lechner JF: p53, erbB-2 and K-ras gene alterations are rare in spontaneous and plutonium-239-induced canine lung neoplasia. Radiat Res *145(2)*: 181-187, 1996.

9 Griffey SM, Kraegel SA and Madewell BR: Rapid detection of K-ras gene mutations in canine lung cancer using single-strand conformational polymorphism analysis. Carcinogenesis *19(6)*: 959-963, 1998.

10 Griffey SM, Kraegel SA, Weller RE, Watson CR and Madewell BR: K-ras mutations in 239PuO2 canine lung neoplasms. Cancer Lett *132(1-2)*: 1-5, 1998.

11 Watzinger F, Mayr B, Gamerith R, Vetter C and Lion T: Comparative analysis of ras proto-oncogene mutations in selected mammalian tumors. Mol Carcinog *30(4)*:190-198, 2001.

12 Mayr B, Schaffner G, Reifinger M: K-ras mutations in canine pancreatic cancers. Vet Rec *153(3)*: 87-89, 2003.

13 Guerrero S, Figueras A, Casanova I, Farre L, Lloveras B, Capella G, Trias M and Mangues R: Codon 12 and codon 13 mutations at the K-ras gene induce different soft tissue sarcoma types in nude mice. Faseb J *16(12)*: 1642-1644, 2002.

14 Mayr B, Schaffner G and Reifinger M, Zwetkoff S and Prodinger B: N-ras mutations in canine malignant melanomas. Vet J *165(2)*: 169-171, 2003.

15 Watzinger F, Mayr B, Haring E and Lion T: High sequence similarity within ras exons 1 and 2 in different mammalian species and phylogenetic divergence of the ras gene family. Mamm Genome *9(3)*: 214-219, 1998.

16 Knapp DW and Waters DJ: Naturally occurring cancer in pet dogs: important models for developing improved cancer therapy for humans. Mol Med Today *3(1)*: 8-11, 1997.

17 Bos JL: ras oncogenes in human cancer: a review. Cancer Res *49(17)*: 4682-4689, 1989.

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- IX -

Richter A, Murua Escobar H, Günther K, Meyer B, Winkler S, Dolf G, Schelling C, Nolte I, Bullerdiek J

The canine *NRAS* gene maps to CFA 17

Anim Genet. 2004. 35:355-6.

Own contribution:

Collaboration in the identification of bacterial artificial chromosome (BAC) clones positive for canine *NRAS*

Collaboration in preparation of DNA from the identified BAC clones for use in fluorescence *in situ* hybridisation (FISH) mapping of the canine *NRAS* gene

Writing of the manuscript

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The canine *NRAS* gene maps to CFA 17

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Introduction: The dog is an emerging model organism for the investigation of mechanisms involved in human disease, including cancer. Several parallels in human and canine tumours have been described, with comparable environmental living conditions and age of tumour onset in both human and canine patients as well as similarities in development and histology of tumours in both species.[1] NRAS is a member of the *ras* proto-oncogene family of proteins that act in growth-related signal transduction and are frequently involved in the development of human tumours, with *ras* point mutations being one of the most important alterations in the onset of malignancies.[2] *Ras* genes show high sequence similarity across different mammalian species such as human, cat, dog, cattle and rodents, with most nucleotide differences representing synonymous changes not affecting the amino acid sequence.[3] In malignancies, most amino acid exchanges in *ras* genes are caused by alterations of the so-called hot spot codons 12, 13, and 61 in exons 1 and 2, respectively, leading to constitutively active ras proteins that bring about constant signal transduction, facilitating uncontrolled cell division. These hot-spot codons have been described to be affected in other mammalian species as well. In dogs, *NRAS* mutations were found in lymphomas[4] and malignant melanomas.[5]

The canine *NRAS* gene had not been mapped so far, therefore, in this study we localized the chromosomal location of the canine *NRAS* gene by fluorescence *in situ* hybridization (FISH).

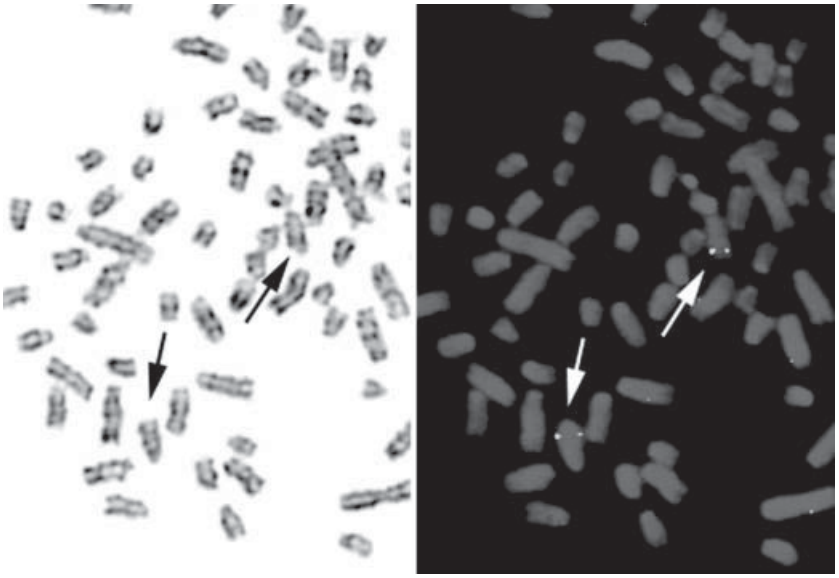

Figure 1 Metaphase spread after fluorescence *in situ* hybridization showing signals on both chromosomes 17 (right) and the same metaphase after GTG-banding (left).

BAC library screening: In order to isolate a FISH probe, the DogBAC canine BAC library[6] (http://www.dogmap.ch/) was polymerase chain reaction (PCR)-screened. Primers were designed using canine mRNA sequence GenBank accession no. U62093 (primer UP: GACTGAGTACAAACTGGTGG and primer LO: GGGCCTCACCTCTATGGTG). The PCR conditions were established on canine blood genomic DNA, the corresponding PCR product cloned and verified by sequencing. The positive BAC clone (DogBAC library ID S050P24H09) was verified by PCR and sequencing.

Gene mapping: For mapping the chromosomal location of the canine *NRAS* gene, metaphase preparations and FISH were performed as described previously.[7] Ten well spread metaphases exhibited a signal on CFA 17 on both chromatids of both chromosomes (Fig. 1), following the nomenclature of the canine karyotype as established by Reimann *et al.*[8]

Comments: NRAS mutations in humans have been found in 30% of liver tumours, 40% of myelodysplastic syndrome, 30% of acute myelogenous leukaemia, 13% of brain tumours and in 53% of follicular and 60% of undifferentiated papillary thyroid tumours.[9] In dogs, depending on tumour type, comparable occurrences exist in malignant melanomas,[5] while fibrosarcomas showed no amino acid alteration of the NRAS protein (H. Murua Escobar, K. Günther, A. Richter, J. T. Soller, S. Winkler, I. Nolte & J. Bullerdiek 2004, personal communication). Overall, data available on involvement of *ras* proto-oncogenes in tumours of dogs are still insufficient. Knowledge of the cytogenetic properties of *NRAS* will further the understanding of this important gene. The mapping results obtained in this study are in accordance with the known homology between canine chromosome 17 and the centromer-proximal regions 11.1–13.3 of the p-arm of human chromosome 1.[10]

References

1 Hahn K A. *et al.* (1994) *In Vivo* **8,** 133–43.
2 Arber N. (1999) *Apoptosis* **4,** 383–8.
3 Watzinger F. *et al.* (1998) *Mamm Genome* **9,** 214–9.
4 Mayr B. *et al.* (2003) *Acta Vet Hung* **51,** 91–4.
5 Mayr B. *et al.* (2003) *Vet J* **165,** 169–71.
6 Schelling C. *et al.* (2002) *J Anim Breeding Genet* **119,** 400–1.
7 Becker K. *et al.* (2003) *Anim Genet* **34,** 68–9.
8 Reimann N. *et al.* (1996) *Cytogenet Cell Genet* **73,** 140–4.
9 Spandidos D. A. *et al.* (2002) *Int J Oncol* **21,** 237–41.
10 Yang F. *et al.* (1999) *Genomics* **62,** 189–202.

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- X -

Winkler S, Murua Escobar H, Günther K, Richter A, Dolf G, Schelling C, Bullerdiek J, Nolte I

The canine *KRAS2* gene maps to chromosome 22

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Own contribution:

Collaboration in the identification of BAC clones positive for canine *KRAS*

Collaboration in preparation of DNA from the identified BAC clones for use in FISH mapping of the canine *KRAS* gene

BRIEF NOTES

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The canine *KRAS2* gene maps to chromosome 22

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Introduction: Dogs and human beings often share the same genetic pathways in development of cancer. Point mutations affecting genes of the *ras* family are assumed to be among the most important alterations in human tumourigenesis.[1] Ras proteins play an important role as signal transmitters. The binding of growth factors activate the ras protein and thus initiates cell division. Mutations in *ras* genes are assumed to remove the time limit of the cell stimulating signals which results in uncontrolled cell division.[2] Mutations in *KRAS2* have been described in human pancreatic cancers and tumours of the gastrointestinal tract as well as in tumours of the skin.[3–5] Hot spot point mutations in *KRAS2* described in different types of human lung tumours and breast cancers are also present in the corresponding canine gene.[6] For further characterization of the gene, we have mapped the canine *KRAS2* gene.

BAC clone and probe: A *KRAS2* DNA probe was generated by polymerase chain reaction (PCR) spanning part of the exon 2 (primer up: 5′-caggattcctacaggaaaca-3′/lo: 5′-aacccacctataatggtgaa-3′ based on NCBI sequence M54968) using genomic canine DNA. The resulting amplicon was cloned and sequenced for verification. These PCR conditions were also used to screen a canine BAC library[7] (URL: http://www.dogmap.ch). To rule out false-positive BAC screening results, a PCR using the initial primer pair was performed, and the resulting amplicon cloned and sequenced for verification. BAC S069P22D02 was positive for *KRAS2* and was used for fluorescence *in situ* hybridization (FISH) analysis.

FISH: Metaphase preparations and FISH were performed as described previously.[8] Ten well spread metaphases were examined and all showed a signal on both chromatids of chromosome 22s (CFA 22) (Fig. 1).

Comments: Different investigations show that 30% of human lung tumours, 90% of human pancreatic tumours and 50% of tumours of the gastrointestinal tract depend on specific point mutations in genes of the *ras* gene family.[9–11] Molecular investigations of the *ras* family are rare in dogs, but existing publications point to the fact that there are the same point mutations affecting hotspot codons 12, 13 and 61, as they are in human malignancies.[6] Up to 24% of cases investigated in dogs showed point mutations in those codons. The canine chromosome to which *KRAS2* was mapped has been involved in a centric fusion of CFA 8/22.[12] According to Yang *et al.*[13] the canine chromosome 22 shares homology with HSA13, whereas the human *KRAS2* gene is located on HSA12. In our FISH studies, there were no metaphase signals on the corresponding canine chromosomes, 29 and 10.

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References

1 Arber N. (1999) *Apoptosis* **4,** 383–8.
2 Scriver *et al.* eds (1995) *The Metabolic and Molecular Bases of Inherited Disease*, McGraw-Hill, New York, 7th edn, pp. 589–611.
3 Almoguera C. *et al.* (1988) *Cell* **53,** 549–54.
4 Belly R. T. *et al.* (2001) *Clin Colorectal Cancer* **1,** 110–6.
5 Shukla V. K. *et al.* (1989) *Oncogene Res* **5,** 121–7.
6 Watzinger F. *et al.* (2001) *Mol Carcinog* **30,** 190–8.
7 Schelling C. *et al.* (2002) *J Anim Breeding Genet* **119,** 400–1.
8 Murua Escobar H. *et al.* (2001) *Cytogenet Cell Genet* **94,** M194–5.
9 Bos J. L. *et al.* (1989) *Cancer Res* **50,** 1352.
10 Tang E. M. *et al.* (2002) *J Natl Cancer Inst* **94,** 1527–36.

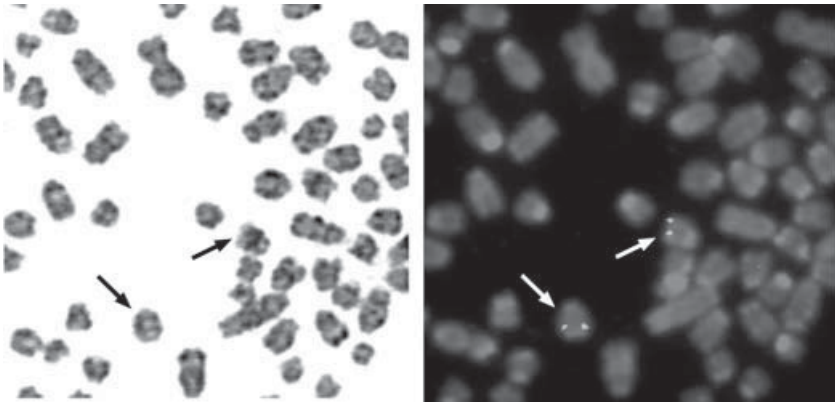

Figure 1 Metaphase spread after fluorescence *in situ* hybridization with signals on both chromosomes 22 (right) and the same metaphase after GTG banding (left).

11 Knapp D. W. & Waters D. J. (1997) *Mol Med Today* **3,** 8–11.
12 Mayr B. *et al.* (1991) *Br Vet J* **6,** 545–8.
13 Yang F. *et al.* (1999) *Genomics* **62,** 189–202.

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- XI -

Murua Escobar H, Soller JT, Richter A, Meyer B, Winkler S, Flohr AM, Nolte I, Bullerdiek J

The canine HMGA1

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Own contribution:

Collaboration in cloning and sequence analysis of fragments of the canine *HMGA1* gene

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The canine *HMGA1*

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Abstract

Due to the emerging advantages of numerous canine diseases as a genetic model for their human orthologs, the dog could join the mouse as the species of choice to unravel genetic mechanisms, e.g. of cancer predisposition, development and progression. However, precondition for such studies is the characterisation of the corresponding canine genes.

Human and murine HMGA1 non-histone proteins participate in a wide variety of cellular processes including regulation of inducible gene transcription, integration of retroviruses into chromosomes, and the induction of neoplastic transformation and promotion of metastatic progression of cancer cells.

Chromosomal aberrations affecting the human *HMGA1* gene at 6p21 were described in several tumours like pulmonary chondroid hamartomas, uterine leiomyomas, follicular thyroid adenomas and others. Over-expression of the proteins of *HMGA1* is characteristic for various malignant tumours suggesting a relation between high titer of the protein and the neoplastic phenotype.

In this study, we characterised the molecular structure of the canine *HMGA1* cDNA, its splice variants and predicted proteins HMGA1a and HMGA1b. Furthermore, we compared the coding sequence(s) (CDS) of both splice variants for 12 different breeds, screened them for single nucleotide polymorphisms (SNPs) and characterised a basic expression pattern.

Keywords: High mobility group proteins; *HMGA1*; *HMGA1a*; *HMGA1b*; Comparative genomics

1. Introduction

As witnessed by a number of recent articles (Kuska, 1996; Kingman, 2000; Ostrander et al., 2000; Vail and MacEwen, 2000), a growing number of scientists predict that human genetics will focus on the dog in this century (Kuska, 1996). Due to the emerging advantages of numerous canine diseases as a genetic model for their human counterparts, the dog could join the mouse as the species of choice to unravel genetic mechanisms, e.g. of cancer predisposition, development and progression.

The proteins of the human *HMGA1* gene HMGA1a and HMGA1b are associated with various human diseases including cancer. Due to the similarities of various human and canine cancer entities, the characterisation of the canine *HMGA1* gene could open new fields for experimental and therapeutic approaches.

Four human members of the HMGA protein family are presently known: the HMGA1a, HMGA1b, HMGA1c and HMGA2 proteins, which can modify chromatin structure by bending DNA, thus influencing the transcription of a number of target genes. The human *HMGA1* gene on 6p21 encodes the well characterised

Abbreviations: A, adenosine; aa, amino acid(s); BAC, bacterial artificial chromosome; bp, base pair(s); cDNA, DNA complementary to RNA; CDS, coding sequence(s); CFA, Canis familiaris; Ci, Curie; D, Dalton; dCTP, deoxycytidine 5′-triphosphate; DNA, deoxy-ribonucleic acid; DNase, deoxyribonuclease; G, guanosine; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HMG, high mobility group; HMGA1, high mobility group protein A1; HMGA2, high mobility group protein A2; HSA, *Homo sapiens*; M-MLV, Moloney murine leukemia virus; mRNA, messenger ribonucleic acid; NCBI, National Center for Biotechnology Information; ORF, open reading frame; PCR, polymerase chain reaction; RACE, rapid amplification of cDNA ends; RNA, ribonucleic acid; SDS, sodium dodecyl sulfate; SNP, single nucleotide polymorphism; UTR, untranslated region.

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HMGA1a and HMGA1b proteins (formerly known as HMGI and HMGY) derived by alternative splicing and the barely characterised HMGA1c variant, while the HMGA2 protein is encoded by a separate gene on chromosome 12 (12q14–15) (for review, Reeves and Beckerbauer, 2001).

Expression of HMGA1 is detectable only at very low levels or is even absent in adult tissues, whereas it is abundantly expressed in embryonic cells (Chiappetta et al., 1996). In humans, 6p21 is often affected by aberrations leading to an up-regulation of *HMGA1* in benign mesenchymal tumours, e.g. lipomas, uterine leiomyomas, pulmonary chondroid hamartomas and endometrial polyps (Williams et al., 1997; Kazmierczak et al., 1998; Tallini et al., 2000). Transcriptional activation due to a chromosomal alteration of *HMGA1* is probably an early and often even primary event of cancer development. In contrast, *HMGA1* expression in malignant epithelial tumours seems to be a rather late event associated with an aggressive behaviour of the tumours. Thus, an overexpression of *HMGA1* was reported for a number of malignancies including thyroid, prostatic, pancreatic, cervical and colorectal cancer (Tamimi et al., 1993; Chiappetta et al., 1995, 1998; Fedele et al., 1996; Bandiera et al., 1998; Abe et al., 1999, 2000). The correlation between *HMGA* expression and tumour aggressiveness in these malignancies has led to the conclusion that *HMGA* expression may present a powerful prognostic molecular marker. The causal role of *HMGA1* expression in the progression of carcinomas has been elucidated by a set of *in vitro* experiments involving *HMGA1* sense and antisense transfection assays (Reeves et al., 2001). An experimental approach aimed at the down-regulation of HMGA protein in tumours has been presented by Scala et al. (2000) who were able to show that an *HMGA1* antisense strategy using an adenoviral vector treatment of tumours induced in athymic mice caused a drastic reduction in tumour size.

Recently, the canine *HMGA1* gene has been mapped to CFA 23. This cytogenetic assignment indicates that the canine *HMGA1* gene does not map to a hotspot of chromosomal breakpoints seen in canine tumours (Becker et al., 2003). However, despite the emerging role of *HMGA1* gene expression in malignancies, the molecular characterisation of the canine *HMGA1* gene had not been carried out before. The characterisation of the molecular structure could permit new therapeutic approaches using the dog as model organism.

In this study, we characterised the molecular structure of the canine *HMGA1* gene on cDNA level, its splice variants and proteins HMGA1a and HMGA1b, and a basic expression pattern. Furthermore, for 12 different canine breeds the coding sequence(s) (CDS) of both splice variants were characterised and screened for SNPs to find out if changes at protein level exist between the different breeds.

2. Materials and methods

2.1. Tissues

The tissues used in this study were provided by the Small Animal Clinic, Veterinary School, Hanover, Germany. The breeds represented were Alsatian, Bull Terrier, Collie, Dachshund, Doberman Pinscher, German Shorthaired Pointer, Golden Retriever, Jack Russell Terrier, Kangal, Munsterland, West Highland Terrier and Yorkshire Terrier. From each breed up to three samples of testis tissue were taken and used for analyses.

2.2. cDNA characterisation

Total RNA was isolated from 150 mg canine testis tissue using TRIZOL LS (Invitrogen, Karlsruhe, Germany) following the manufacturer's protocol. To avoid genomic DNA contamination a DNase digest of each sample was performed using DNA-*free* (Ambion, Huntingdon, Cambridgeshire, UK). cDNA was synthesised using 3′-RACE adaptor primer AP2 (AAGGATCCGTCGACATC(17)T), 5 μg total RNA and M-MLV (Invitrogen) reverse transcriptase according to the manufacturer's instructions. The polymerase chain reactions (PCRs) for the molecular cloning of the cDNA were done using the primer pairs Ex1up and Ex8lo (5′ GCTCTTTTTAAGCTCCCCTGA 3′/5′ CTGTCCAGTCCCAGAAGGAA 3′) and primer pair Ex8up and 3′UTRlo (5′ AGGGCATCTCGCAGGAGTC 3′/5′ ATTCAAGTAACTGCAAATAGGA 3′) which were derived from human cDNA sequences (accession no. X14957). The PCR products were separated on a 1.5% agarose gel, recovered with QIAEX II (QIAGEN, Hilden, Germany), cloned in pGEM-T Easy vector system (Promega, Madison, USA) and sequenced. The cDNA contig and the homology alignments were created with Lasergene software (DNAStar, Madison, USA) and various sequences from the NCBI database (GenBank accession nos. X14957, X14958, NM_002131, NM_145899, NM_145900, NM_145901, NM_145902, NM_145903, NM_145904, NM_145905).

2.3. Characterisation of splice variants

The splice variants *HMGA1a* and *HMGA1b* were detected by amplifying a fragment spanning the CDS with primer pair Up (5′ CATCCCAGCCATCACTC 3′) and Lo (5′ GCGGCTGGTGTGCTGTGTAGTGTG 3′) using the canine testis cDNA samples as template. The primer pair was designed using the cDNA cloned as described in Section 2.2. The obtained PCR products were separated on a 4.0% agarose gel, recovered with QIAEX II (QIAGEN), cloned in pGEM-T Easy vector system (Promega) and sequenced. The contigs and the homology alignments were created with two sequences from the NCBI database (GenBank accession nos. X14957, X14958).

2.4. CDS comparison between breeds

The CDS of both splice variants were characterised for all breeds as described previously in Section 2.3. The contigs and the homology alignments were created using two sequences from the NCBI database (GenBank accession nos. X14957, X14958). In case of single nucleotide exchanges, the samples were sequenced again in both forward and reverse direction. Exchanges causing no amino acid (aa) substitution were not taken into account for further analyses. For all samples with aa substitutions the initial PCR was repeated and the exchange verified by sequencing the product in both forward and reverse direction. If possible, a restriction enzyme digestion was performed additionally.

2.5. Protein sequences

The canine HMGA1a and HMGA1b protein sequences were derived from the open reading frames (ORFs) of the characterised cDNA sequences described previously in Section 2.2. The protein homology alignments were created with two sequences from the NCBI database (GenBank accession nos. X14957, X14958).

2.6. Northern blot

Total RNAs were isolated from canine heart, lung, muscle, kidney and spleen tissue using RNeasy system (QIAGEN). An additional sample of total RNA was isolated from canine heart tissue by TRIZOL LS acid guanidine isothiocyanate–chloroform method (Invitrogen) in order to figure out whether this isolation method would lead to any difference in hybridisation. Further on poly A RNA was purified from canine spleen total RNA with OLIGOTEX (QIAGEN) and total RNA was prepared from human cultured fibroblasts by RNeasy system (QIAGEN). Spleen poly A RNA was placed on the blot in case that *HMGA1* was not detectable in the total RNA samples.

For Northern Blot hybridisation, 20 μg of total RNA from each sample with the exception of 10 μg of muscle and 3.6 μg of spleen poly A RNA were separated on a 1.2% denaturing agarose gel containing 0.65% formaldehyde. RNAs were transferred onto Hybond-N+ positive nylon membrane (Amersham Pharmacia Biotech, Freiburg, Germany) by capillary blot.

A 489-bp cDNA fragment derived from the canine HMGA1a sequence (exon 5/exon 8) served as a molecular probe for hybridisation. The probe was generated by PCR with the primer set Up and Lo (5′ CATCCCAGCCATCACTC 3′/5′ GCGGCTGGTGTGCTGTGTAGTGTG 3′) using the cloned cDNA described in Section 2.2. Probe labelling was performed by random primed labelling (Amersham Pharmacia Biotech) as described in the manufacturer's protocol with 50 μCi(α^{32}P)dCTP (Amersham Pharmacia Biotech). Purification of the labelled probe was performed using Sephadex G-50 Nick Columns (Amersham Pharmacia Biotech) and the probe was stored at −20 °C before use.

Using the PERFECTHYB PLUS hybridisation solution (Sigma-Aldrich, Saint Louis, MO, USA) prehybridisation was carried out for 30 min and hybridisation for 2.5 h at 68 °C. The membrane was washed for 5 min at room temperature in 2×SSC/0.1% SDS, and twice for 20 min at 68 °C in 0.5×SSC/0.1% SDS. Signals were visualised using a STORM phosphorimager (Molecular Dynamics, Sunnyvale, USA).

3. Results and discussion

3.1. The canine HMGA1 cDNA transcripts

For the human *HMGA1* gene various transcripts were described for both splicing variants (*HMGA1a* and *HMGA1b*) that differ in their 5′-UTR. The characterisation of the canine *HMGA1* cDNAs revealed that the complete canine *HMGA1* cDNA spans six exons and codes for two splicing variants *HMGA1a* with 1836 bp and *HMGA1b* with 1803 bp which are similar to the human transcripts (*HMGA1a* GenBank accession no. AY366390 and *HMGA1b* GenBank accession no. AY366392). The exon structure, the UTRs and the ORFs of both splice variants were defined and their homologies to their human counterparts analysed (Fig. 1, Table 1). The splicing variants showed the "typical" 33 bp gap which is conserved across various species such as human, mouse, hamster and rat (GenBank accession nos. BC013455, NM_016660, A7193763, NM_139327, A7511040). The homology of the canine cDNAs to their human counterparts is 80.6% for both splice variants. The 5′-UTR, CDS and the 3′-UTR showed homologies of 95.6%, 95.1% and 74,7%, respectively (Table 1). Homologies of the canine CDS with the CDS from mouse, hamster and rat on nucleotide level vary from 90.4% to 93.1%. The cDNA sequences were submitted to the NCBI database: *HMGA1a*, GenBank accession no. AY366390 and *HMGA1b,* GenBank accession no. AY366392.

3.2. The canine HMGA1a and HMGA1b proteins

The canine HMGA1a and HMGA1b protein sequences were deduced from the respective cDNA sequences. The canine HMGA1a protein is a 107-amino acid molecule with a calculated weight of 11,674.97 D and HMGA1b a 96-amino acid molecule with a calculated weight of 10,677.85 D (Fig. 2). Homology comparison to the human counterparts (GenBank accession nos. P17096, X14957) showed 100% homology of the molecules including the three "AT-hooks" and the acidic carboxy-terminal domain.

Comparison of the canine and human HMGA1a and HMGA1b proteins with the described mouse, rat and hamster molecules showed aa changes in positions 5, 34,

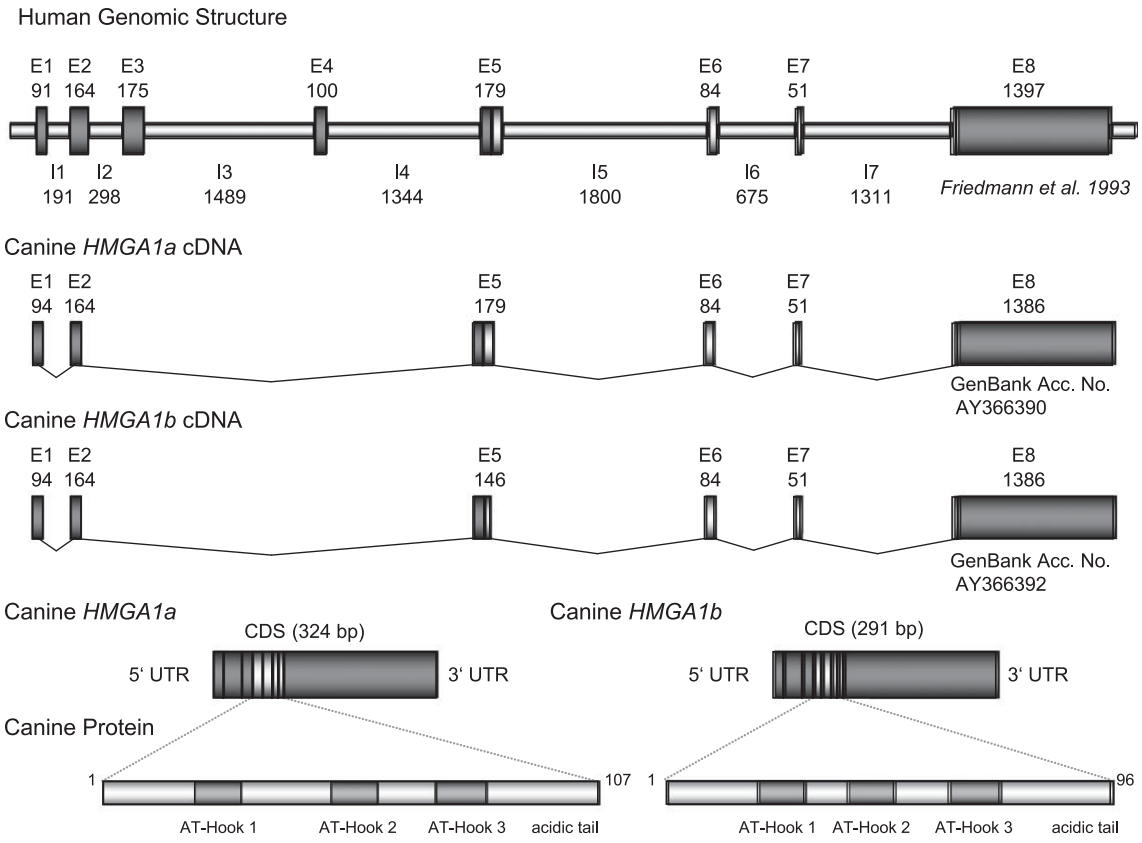

Fig. 1. Structure of the canine *HMGA1a* and *HMGA1b* transcripts and partial genomic structure.

69, 75 and 78 of HMGA1a and positions 5, 34, 58, 64 and 67 of HMGA1b, respectively (Fig. 2) (Johnson et al., 1988, 1989; Friedmann et al., 1993; Aldrich et al., 1999; Sgarra et al., 2000; Strausberg et al., 2002; Sgarra et al., 2003).

According to the definition of the AT-hooks (HMGA1a: I aa 21–31, II aa 53–63, III aa 78–89; HMGA1b: I aa 21–31, II aa 42–52, III aa 67–78) by Reeves and Nissen (1990) and Reeves (2000), none but the aa exchange at position 78 (HMGA1a) or 67 (HMGA1b), respectively, do affect the AT-hooks in either species. The exchange at position 78 leads to a difference in the third AT-hook of mouse and hamster when compared to the other species. According to the definition of the AT-hooks (HMGA1a: I aa 23–31, II aa 55–70, III aa 81–89; HMGA1b: I aa 23–31, II aa 44–59, III aa 70–78) by Huth et al. (1997), this aa exchange does not affect the third AT-hook. Following this definition, the second AT-hook is affected by the aa exchange at position 69 (HMGA1a) or 58 (HMGA1b), respectively.

Table 1
Detailed analysis of the canine *HMGA1a* and *HMGA1b* cDNA

Element of canine *HMGA1* cDNAs	Size in bp	Homology to human counterpart in %
Total cDNA *HMGA1a*	1836	80.6
Total cDNA *HMGA1b*	1803	80.6
5′-UTR	231	95.6
CDS *HMGA1a*	324	95.1
CDS *HMGA1b*	291	95.1
3′-UTR	1332	74.7
Exon 1	94	97.8
Exon 2	114	96.5
Exon 5 *HMGA1a*	179	93.9
Exon 5 *HMGA1b*	146	93.9
Exon 6	84	96.4
Exon 7	51	94.1
Exon 8	1386	75.4

Homology comparison of the cDNA elements of the canine *HMGA1* to its human counterpart (characterisation of the UTRs, the ORF and the exon sizes).

The canine protein sequences were submitted to the NCBI database with GenBank accession nos. HMGA1a AY366390 and HMGA1b AY366392.

Due to the identical structure of the canine HMGA proteins to the respective human molecule, therapeutic approaches applied in dogs could be more suitable in terms of transferability for the development of human therapies than to approaches tested in other organisms.

3.3. HMGA1a and HMGA1b CDS comparison between canine breeds

For twelve different canine breeds the splicing variants *HMGA1a* and *HMGA1b* were detected by amplification of a fragment spanning the CDS using the canine testis cDNA samples as template. The comparison of the characterised protein coding sequences for these twelve canine breeds

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                                  ---------+---------+---------+---------+---------+----------

C. familiaris HMGA1a (AY366390)   1  MSESSSKSSQPLASKQEKDGTEKRGRGRPRKQPPVSPGTALVGSQKEPSEVPTPKRPRGR  60
C. familiaris HMGA1b (AY366392)   1  MSESSSKSSQPLASKQEKDGTEKRGRGRPRKQPP-----------KEPSEVPTPKRPRGR  49
H. sapiens HMGA1a (AAH08832)      1  MSESSSKSSQPLASKQEKDGTEKRGRGRPRKQPPVSPGTALVGSQKEPSEVPTPKRPRGR  60
H. sapiens HMGA1b (AAH04924)      1  MSESSSKSSQPLASKQEKDGTEKRGRGRPRKQPP-----------KEPSEVPTPKRPRGR  49
M. musculus HMGA1a (AAK66159)     1  MSESGSKSSQPLASKQEKDGTEKRGRGRPRKQPPVSPGTALVGSQKEPSEVPTPKRPRGR  60
M. musculus HMGA1b (AAK66158)     1  MSESGSKSSQPLASKQEKDGTEKRGRGRPRKQPP-----------KEPSEVPTPKRPRGR  49
R. norvegicus HMGA1a (NP_647543)  1  MSESGSKSSQPLASKQEKDGTEKRGRGRPRKQPSVSPGTALVGSQKEPSEVPTPKRPRGR  60
R. norvegicus HMGA1b (AAM74157)   1  MSESVSKSSQPLASKQEKDGTEKRGRGRPRKQPS-----------KEPSEVPTPKRPRGR  49
C. griseus HMGA1a (AAF06666)      1  MSESSSKSSQPLASKQEKDGTEKRGRGRPRKQPPVSPGTALVGSQKEPSEVPTPKRPRGR  60
C. griseus HMGA1b (AAF06667)      1  MSESSSKSSQPLASKQEKDGTEKRGRGRPRKQPP-----------KEPSEVPTPKRPRGR  49

                                  ---------+---------+---------+---------+-------

C. familiaris HMGA1a (AY366390)  61 PKGSKNKGAAKTRKTTTTPGRKPRGRPKKLEKEEEEGISQESSEEEQ 107
C. familiaris HMGA1b (AY366392)  50 PKGSKNKGAAKTRKTTTTPGRKPRGRPKKLEKEEEEGISQESSEEEQ  96
H. sapiens HMGA1a (AAH08832)     61 PKGSKNKGAAKTRKTTTTPGRKPRGRPKKLEKEEEEGISQESSEEEQ 107
H. sapiens HMGA1b (AAH04924)     50 PKGSKNKGAAKTRKTTTTPGRKPRGRPKKLEKEEEEGISQESSEEEQ  96
M. musculus HMGA1a (AAK66159)    61 PKGSKNKGAAKTRKVTTAPGRKPRGRPKKLEKEEEEGISQESSEEGQ 107
M. musculus HMGA1b (AAK66158)    50 PKGSKNKGAAKTRKVTTAPGRKPRGRPKKLEKEEEEGISQESSEEGQ  96
R. norvegicus HMGA1a (NP_647543) 61 PKGSKNKGTAKTRKVTTTPGRKPRGRPKKLEKEEEEGISQESSEEEQ 107
R. norvegicus HMGA1b (AAM74157)  50 PKGSKNKGTAKTRKVTTTPGRKPRGRPKKLEKEEEEGISQESSEEEQ  96
C. griseus HMGA1a (AAF06666)     61 PKGSKNKGAAKTRKATTAPGRKPRGRPKKLEKEEEEGISQESSEEEQ 107
C. griseus HMGA1b (AAF06667)     50 PKGSKNKGAAKTRKATTAPGRKPRGRPKKLEKEEEEGISQESSEEEQ  96
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Fig. 2. Comparison of the canine, human, mouse, rat and hamster HMGA1a and HMGA1b proteins.

revealed one amino acid change in a single breed. Nucleotide exchanges causing no amino acid substitution were not taken into account in further analyses. Sample 2 (Teckel) showed in its *HMGA1b* transcript a nucleotide transition from A to G at the first base of codon 64 leading to an aa replacement from threonine to alanine and a new restriction recognition site for *Alu*I causing four (58, 100, 158 and 176 bp) instead of three fragments (58, 100 and 334 bp) to appear in an *Alu*I digest. (data not shown). The substitution was missing in the corresponding *HMGA1a* transcript of the dog suggesting a heterozygous genotype. A possible PCR artifact seems rather unlikely since the nucleotide transition was verified as described in Section 2.4. The CDS cDNA sequences of the twelve breeds were submitted to the NCBI database with GenBank accession nos. AY363606, AY363605, AY363607, AY363604, AY363608, AY363610, AY363609, AY363600, AY363603, AY363599, AY363601, AY363602, AY363994, AY363995, AY363611, AY363999, AY364000, AY364002, AY364001, AY363998, AY363996, AY363997, AY364003.

3.4. Canine HMGA1 expression analysis

Expression of human *HMGA1* is detectable at very low levels or is even absent in adult tissues whereas it is abundantly expressed in embryonic cells (Chiappetta et al., 1996). To elucidate a basic *HMGA1* gene expression pattern in dogs, a canine Northern blot was generated containing total RNA from canine spleen, heart, lung, muscle and kidney tissue samples. In order to detect a possible low level expression of *HMGA1* as reported in adult human tissues, a poly A RNA sample from canine spleen was additionally added to the blot. Hybridisation was performed with a α^{32}P-labelled canine *HMGA1a* cDNA probe. Except for the kidney total RNA and one of two heart samples (Trizol method) all total RNA samples showed a weak signal of approximately 1.8 kb (Fig. 3, Trizol sample not shown), while the poly A RNA spleen sample revealed a distinct signal. After stripping, rehybridisation with a canine *GAPDH* probe showed signals corresponding to approximately 1.3 kb in all but the Trizol method (data not shown) samples, indicating a degradation of the Trizol-prepared RNA.

In humans, *HMGA1* expression in malignant epithelial tumours seems to be associated with an aggressive behaviour of the tumours. Over-expression of *HMGA1* was reported for a number of malignancies including thyroid, prostatic, pancreatic, uterus cervical and colorectal cancer (Tamimi et al., 1993; Chiappetta et al., 1995, 1998; Fedele et al., 1996; Bandiera et al., 1998; Abe et al., 1999, 2000). The correlation between *HMGA* expression and tumour

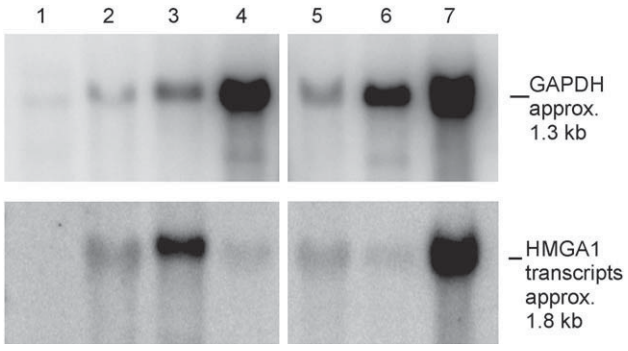

Fig. 3. Northern blot showing 1.8-kb *HMGA1* and 1.3-kb *GAPDH* transcripts. Lanes: (1) canine kidney total RNA, (2) canine spleen total RNA, (3) canine spleen poly A RNA, (4) canine heart total RNA, (5) canine lung total RNA, (6) canine muscle total RNA and (7) human fibroblasts total RNA.

aggressiveness in some of these malignancies has led to the conclusion that *HMGA* expression may present a powerful prognostic molecular marker.

So far no studies analysing the *HMGA1* expression pattern in canine tumours have been carried out. Since these tumours occur spontaneously in dogs as well as in humans a canine *in vivo* analysing system could have significant value for research and drug development.

The causal role of *HMGA1* expression in the progression of carcinomas has been elucidated by a set of *in vitro* experiments involving *HMGA1* sense and antisense transfection assays (Wood et al., 2000a,b; Reeves et al., 2001). A proof of concept for a therapy aimed at the down-regulation of HMGA protein in tumours has been presented by Scala et al. (2000) who were able to show that an *HMGA1* antisense strategy using an adenoviral vector treatment of tumours induced in athymic mice caused a drastic reduction in tumour size.

Due to the similarities of human and canine tumours, the transfer of such experimental approaches could benefit cancer research in either species.

The comprehension of the canine *HMGA1* gene and its gene products could be the precondition for future new experimental approaches and for evaluating the canine gene product as potential target for therapeutic strategies using the dog as model system.

References

Abe, N., Watanabe, T., Sugiyama, M., Uchimura, H., Chiappetta, G., Fusco, A., Atomi, Y., 1999. Determination of high mobility group I(Y) expression level in colorectal neoplasias: a potential diagnostic marker. Cancer Res. 59, 1169–1174.

Abe, N., Watanabe, T., Masaki, T., Mori, T., Sugiyama, M., Uchimura, H., Fujioka, Y., Chiappetta, G., Fusco, A., Atomi, Y., 2000. Pancreatic duct cell carcinomas express high levels of high mobility group I(Y) proteins. Cancer Res. 60, 3117–3122.

Aldrich, T.L., Reeves, R., Lee, C.C., Thomas, J.N., Morris, A.E., 1999. HMG-I (Y) proteins implicated in amplification of CHO cell DNA. Unpublished, GenBank Accession No. AF193763.

Bandiera, A., Bonifacio, D., Manfioletti, G., Mantovani, F., Rustighi, A., Zanconati, F., Fusco, A., Di Bonito, L., Giancotti, V., 1998. Expression of HMGI (Y) proteins in squamous intraepithelial and invasive lesions of the uterine cervix. Cancer Res. 58, 426–431.

Becker, K., Escobar, H.M., Richter, A., Meyer, B., Nolte, I., Bullerdiek, J., 2003. The canine *HMGA1* gene maps to CFA 23. Anim. Genet. 34, 68–69.

Chiappetta, G., Bandiera, A., Berlingieri, M.T., Visconti, R., Manfioletti, G., Battista, S., Martinez-Tello, F.J., Santoro, M., Giancotti, V., Fusco, A., 1995. The expression of the high mobility group HMGI (Y) proteins correlates with the malignant phenotype of human thyroid neoplasias. Oncogene 10, 1307–1314.

Chiappetta, G., Avantaggiato, V., Visconti, R., Fedele, M., Battista, S., Trapasso, F., Merciai, B.M., Fidanza, V., Giancotti, V., Santoro, M., Simeone, A., Fusco, A., 1996. High level expression of the HMGI (Y) gene during embryonic development. Oncogene 13, 2439–2446.

Chiappetta, G., Tallini, G., De Biasio, M.C., Manfioletti, G., Martinez-Tello, F.J., Pentimalli, F., de Nigris, F., Mastro, A., Botti, G., Fedele, M., Berger, N., Santoro, M., Giancotti, V., Fusco, A., 1998. Detection of high mobility group I HMGI (Y) protein in the diagnosis of thyroid tumors: HMGI (Y) expression represents a potential diagnostic indicator of carcinoma. Cancer Res. 58, 4193–4198.

Fedele, M., Bandiera, A., Chiappetta, G., Battista, S., Viglietto, G., Manfioletti, G., Casamassimi, A., Santoro, M., Giancotti, V., Fusco, A., 1996. Human colorectal carcinomas express high levels of high mobility group HMGI (Y) proteins. Cancer Res. 56, 1896–1901.

Friedmann, M., Holth, L.T., Zoghbi, H.Y., Reeves, R., 1993. Organization, inducible-expression and chromosome localization of the human HMG-I (Y) nonhistone protein gene. Nucleic Acids Res. 21, 4259–4267.

Huth, J.R., Bewley, C.A., Nissen, M.S., Evans, J.N., Reeves, R., Gronenborn, A.M., Clore, G.M., 1997. The solution structure of an HMG-I (Y)-DNA complex defines a new architectural minor groove binding motif. Nat. Struct. Biol. 4, 657–665.

Johnson, K.R., Lehn, D.A., Elton, T.S., Barr, P.J., Reeves, R., 1988. Complete murine cDNA sequence, genomic structure, and tissue expression of the high mobility group protein HMG-I (Y). J. Biol. Chem. 263, 18338–18342.

Johnson, K.R., Lehn, D.A., Reeves, R., 1989. Alternative processing of mRNAs encoding mammalian chromosomal high-mobility-group proteins HMG-I and HMG-Y. Mol. Cell. Biol. 9, 2114–2123.

Kazmierczak, B., Dal Cin, P., Wanschura, S., Borrmann, L., Fusco, A., Van den Berghe, H., Bullerdiek, J., 1998. HMGIY is the target of 6p21.3 rearrangements in various benign mesenchymal tumors. Genes Chromosomes Cancer 23, 279–285.

Kingman, S., 2000. Painting a brighter future for dogs and humans. Drug Discov. Today 5, 127–128.

Kuska, B., 1996. Sit, DNA, sit: cancer genetics going to the dogs. J. Natl. Cancer Inst. 91, 204–206.

Ostrander, E.A., Galibert, F., Patterson, D.F., 2000. Canine genetics comes of age. Trends Genet. 16, 117–124.

Reeves, R., 2000. Structure and function of the HMGI (Y) family of architectural transcription factors. Environ. Health Perspect. 108 (Suppl. 5), 803–809.

Reeves, R., Beckerbauer, L., 2001. HMGI (Y) proteins: flexible regulators of transcription and chromatin structure. Biochim. Biophys. Acta 1519, 13–29.

Reeves, R., Nissen, M.S., 1990. The A.T-DNA-binding domain of mammalian high mobility group I chromosomal proteins. A novel peptide motif for recognizing DNA structure. J. Biol. Chem. 265, 8573–8582.

Reeves, R., Edberg, D.D., Li, Y., 2001. Architectural transcription factor HMGI (Y) promotes tumor progression and mesenchymal transition of human epithelial cells. Mol. Cell. Biol. 21, 575–594.

Scala, S., Portella, G., Fedele, M., Chiappetta, G., Fusco, A., 2000. Adenovirus-mediated suppression of HMGI (Y) protein synthesis as potential therapy of human malignant neoplasias. Proc. Natl. Acad. Sci. U. S. A. 97, 4256–4261.

Sgarra, R., Diana, F., Bellarosa, C., Rustighi, A., Toller, M., Manfioletti, G., Giancotti, V., 2000: Increase of HMGA1a protein methylation is a distinctive characteristic of tumor cells induced to apoptosis. Unpublished, GenBank Accession No. AF511040.

Sgarra, R., Diana, F., Bellarosa, C., Dekleva, V., Rustighi, A., Toller, M., Manfioletti, G., Giancotti, V., 2003. During apoptosis of tumor cells HMGA1a protein undergoes methylation: identification of the modification site by mass spectrometry. Biochemistry 42, 3575–3585.

Strausberg, R.L., et al., 2002. Generation and initial analysis of more than 15,000 full-length human and mouse cDNA sequences. Proc. Natl. Acad. Sci. U. S. A. 99, 16899–16903.

Tallini, G., Vanni, R., Manfioletti, G., Kazmierczak, B., Faa, G., Pauwels, P., Bullerdiek, J., Giancotti, V., Van Den Berghe, H., Dal Cin, P., 2000. HMGI-C and HMGI (Y) immunoreactivity correlates with cytogenetic abnormalities in lipomas, pulmonary chondroid hamartomas, endometrial polyps, and uterine leiomyomas and is compatible with rearrangement of the HMGI-C and HMGI (Y) genes. Lab. Invest. 80, 359–369.

Tamimi, Y., van der Poel, H.G., Denyn, M.M., Umbas, R., Karthaus, H.F., Debruyne, F.M., Schalken, J.A., 1993. Increased expression of high

mobility group protein I(Y) in high grade prostatic cancer determined by in situ hybridization. Cancer Res. 53, 5512–5516.

Vail, D.M., MacEwen, E.G., 2000. Spontaneously occurring tumors of companion animals as models for human cancer. Cancer Invest. 18, 781–792.

Williams, A.J., Powell, W.L., Collins, T., Morton, C.C., 1997. HMGI (Y) expression in human uterine leiomyomata. Involvement of another high-mobility group architectural factor in a benign neoplasm. Am. J. Pathol. 150, 911–918.

Wood, L.J., Maher, J.F., Bunton, T.E., Resar, L.M., 2000a. The oncogenic properties of the HMG-I gene family. Cancer Res. 60, 4256–4261.

Wood, L.J., Mukherjee, M., Dolde, C.E., Xu, Y., Maher, J.F., Bunton, T.E., Williams, J.B., Resar, L.M., 2000b. HMG-I/Y, a new c-Myc target gene and potential oncogene. Mol. Cell. Biol. 20, 5490–5502.

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Meyer B, Murua Escobar H, Hauke S, Richter A, Winkler S, Rogalla P, Flohr AM, Bullerdiek J, Nolte I

Expression pattern of the *HMGB1* gene in sarcomas of the dog

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Own contribution:

Collaboration in cloning and sequence analysis of fragments of the canine *HMGB1* gene

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Expression Pattern of the *HMGB1* Gene in Sarcomas of the Dog

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Abstract. *Background: The human high mobility group protein B1 (HMGB1) has attracted considerable interest among oncologists because it sensitises cancer cells to the anticancer drug cisplatin by shielding cisplatin-DNA adducts from nucleotide excision repair. Materials and Methods: Since cisplatin is the cornerstone of adjuvant systemic therapy for osteosarcomas, in both humans and dogs, the expression pattern of the HMGB1 gene in seven canine sarcomas was investigated by Northern blot analysis and semi-quantitative RT-PCR. Results: A strong intertumoural variation of HMGB1 expression was detected by Northern blot analysis and confirmed by the semi-quantitative RT-PCR established herein. Conclusion: The observed variations of HMGB1 expression in canine sarcomas emphasises the role of HMGB1 as a potential marker of clinical interest as its expression level may predict the clinical outcome of therapies based on cisplatin. The semi-quantitative RT-PCR established allows a quick and convenient determination of the HMGB1 expression level as necessary for clinical applications.*

The related platinum compounds cisplatin and carboplatin are widely used antitumour drugs for the treatment of a number of malignancies. The main cytotoxic effect of cisplatin/carboplatin is the formation of cisplatin/carboplatin-DNA adducts characterised by intrastrand cross-links and significantly bended and distorted DNA.

Gel mobility shift assays revealed a selective affinity of high mobility group (HMG) proteins for cisplatin-DNA adducts (1). The recognition of cisplatin damage by HMG is assumed to mediate cisplatin cytotoxicity. HMG proteins are chromatin-associated non-histone proteins characterised by low molecular weight, acid-solubility and a high content of charged amino acids. According to their molecular size, sequence and DNA binding capacity, three families have been distinguished: HMGB (formerly HMG1/2), HMGN (formerly HMG14/17) and HMGA (formerly HMGI(Y)) (2,3). The HMGB family, comprising HMGB1, HMGB2 and HMGB3, is characterised by its two DNA-binding domains called the "HMG-Box" (4,5).

One of the best analysed members of the group of HMG-Box proteins is HMGB1 (synonymously known as HMG1 or amphoterin). Both DNA-binding domains selectively bind with a very high affinity to major cisplatin-DNA adducts (6-8) and interaction between HMGB1 and cisplatin-damaged DNA contributes to its biological activity, as it sensitises cancer cells to cisplatin by shielding its major DNA adducts from nucleotide excision repair (9,10).

Interestingly, *HMGB1* gene expression can be induced by oestrogens in breast cancer cells probably due to an up-regulation of the gene, so that *HMGB1* itself can be considered an oestrogen-responsive gene (11). Recently, we were able to explain this observation by the identification of two oestrogen responsive elements within the first intron of *HMGB1* (12). He *et al.* (10) have shown that, in oestrogen receptor-positive human breast cancer cells, oestrogen can significantly increase the effect of cisplatin by causing an overexpression of *HMGB1*. This finding has led to the conclusion that oestrogen treatment prior to cisplatin therapy may sensitise the cancer cells against that drug. Accordingly, a clinical trial for the treatment of gynaecological tumours with cisplatin has already been approved by the Food and Drug Administration (FDA) (10). On the other hand, the former experiment clearly shows that the quantitation of the intratumoural *HMGB1* expression level may be of high impact for a cisplatin/carboplatin therapy for two reasons. Firstly, it may predict the clinical outcome of the therapy; secondly, it may influence the therapy protocol as, for example, tumours

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Key Words: Osteosarcoma, cisplatin, *HMGB1* expression, semi-quantitative RT-PCR.

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Table I. *Sarcoma samples analysed in this study.*

Sarcoma sample	Tumour	Breed	Sex	Age
OS1	Osteosarcoma	Rottweiler	f	1 yr
OS2	Osteosarcoma	Crossbreed	f	4 yrs
OS3	Osteosarcoma	German Shepherd	m	6 yrs
OS4	Osteosarcoma	Crossbreed	m	9 yrs
OS5	Osteosarcoma	German Shepherd	m	n.r.[1]
FS	Fibrosarcoma	Bobtail	m	5 yrs
LMS	Leiomyosarcoma	Crossbreed	f	10 yrs

[1]n.r. = not reported

showing a high *HMGB1* expression level may be treated with a lower amount of this antitumour drug.

Due to the close similarities of numerous canine diseases to their human counterparts, the role of the dog as a model organism for therapeutic approaches is justified. Furthermore, genes and proteins known to be of high diagnostic and therapeutic impact in man can also be considered to play an important role in the dog.

Osteosarcomas and several types of carcinomas belong to the group of canine malignancies often treated with cisplatin or carboplatin. So far no data are available analysing the expression pattern of the *HMGB1* gene in canine sarcomas. Thus, in this study we analysed the *HMGB1* expression level in five canine osteosarcomas, one fibrosarcoma and one leiomyosarcoma by Northern blot experiments. Based on the observed strong intertumoural variation of *HMGB1* expression, we further established a quick RT-PCR-based diagnostic system for future studies.

Materials and Methods

Tissue samples. All canine tumour samples used in this study (Table I) were provided by the Clinic for Small Animals, Hanover, Germany. Samples were taken during surgery, immediately frozen in liquid nitrogen and stored at -80˚C.

RNA isolation. Total RNA extraction of the canine sarcoma samples was performed according to the RNeasy midi protocol for isolation of total RNA from heart, muscle and skin tissue (Qiagen, Hilden, Germany) including a Proteinase K digest. Enrichment of poly A^+ mRNA was carried out using the Oligotex mRNA kit (Qiagen).

Northern blot hybridisation. For Northern blot analysis, 5 µg of mRNA from each sample were separated on a 1.2% denaturing agarose gel containing 0.65% formaldehyde. RNAs were transferred onto a Hybond-XL charged nylon membrane (Amersham Biosciences, Buckinghamshire, England) by capillary blot overnight. As a probe for hybridisation, a 603 bp cDNA fragment derived from the ORF (exon 2-5) of the canine *HMGB1* gene was generated by PCR using the primer pair ToastUP (5' GGGCAAAGGAGATCCTAAGAAG 3') (13) and Ex5lo (5' TCTTCCTCCTCCTCCTCATCC 3'). A 445 bp cDNA probe detecting the 1.3 kb transcript of the canine *GAPDH* gene was amplified by PCR with the primer set GAPDH2up (5' GTGAAGGTCGGAGTCAAC 3') and GAPDHdog5do (5' AGGAGGCATTGCTGACAAT 3'). Probes were labelled with 50 µCi(α-^{32}P)dCTP (Amersham Biosciences) using the Megaprime Labelling Kit (Amersham Biosciences) for random-primed labelling (14). Hybridisation was performed for 3 h at 68˚C in 10 ml of PerfectHyb Plus Hybridisation Buffer (Sigma-Aldrich, Saint Louis, USA). The membranes were washed for 5 min with low stringency at RT in 2x SSC, 0.1% SDS and twice for 20 min with high stringency at 68˚C in 0.5x SSC, 0.1% SDS. Signals were visualised using a Storm PhosphorImager (Molecular Dynamics, Sunnyvale, USA). Quantitation of the transcripts of *HMGB1* and *GAPDH* was performed using the software program ImageQuant (Molecular Dynamics).

Semi-quantitative RT-PCR. cDNA synthesis was performed using primer AP2 (5' AAGGATCCGTCGACATCT$_{(17)}$ 3') with 500 ng of mRNA with SuperScript Reverse Transcriptase (Invitrogen, Karlsruhe, Germany) according to the manufacturer's instructions. In order to determine the expression of *HMGB1* in relation to that of the housekeeping gene *GAPDH*, a duplex PCR was established using the primer sets ToastUP/Ex5lo and GAPDH2up/GAPDHdog5do (see above). PCR reactions were set up according to the "basic PCR protocol" of Taq DNA Polymerase (Invitrogen) using the following PCR program: initial denaturation for 5 min at 94˚C, 28 cycles of denaturation for 30 sec at 94˚C, primer annealing for 30 sec at 55˚C and extension for 45 sec at 72˚C, followed by a final extension for 10 min at 72˚C. The appropriate number of cycles was previously determined so that for both PCR-products amplification was in the exponential range (data not shown). PCR-products were separated on a 1.2% agarose gel stained with VistraGreen (Amersham) and visualised using a Storm PhosphorImager (Molecular Dynamics). Quantitation of the PCR-fragments of *HMGB1* and *GAPDH* was performed using the software program ImageQuant (Molecular Dynamics) measuring pixel intensities.

Results

Northern blot hybridisation on a series of 5 osteosarcomas, one fibrosarcoma and one leiomyosarcoma sample of the dog (Table I), using a cDNA probe derived from the ORF (Exon 2-5) of the canine *HMGB1* gene, resulted in the detection of two *HMGB1* mRNA transcripts of approximately 1.4 and 2.4 kb (Figure 1), which are similar to that observed in human tissues (15-17) and various canine tissues (18). In order to quantify the expression of *HMGB1*, the blot was rehybridised with a canine *GAPDH*-specific cDNA probe (Figure 1). Summing up the intensities of the 1.4 and 2.4 kb *HMGB1* signals, the *HMGB1*-RNA / *GAPDH*-RNA ratios were calculated. As shown in Figure 1, the analysed canine sarcoma samples revealed a strong intertumoural variation in the relative expression of *HMGB1*. Values obtained by Northern blot analysis for the osteosarcoma samples varied between 0.52 and 1.31, while the fibrosarcoma and the leiomyosarcoma showed ratios of 0.73 and 0.24, respectively (Table II).

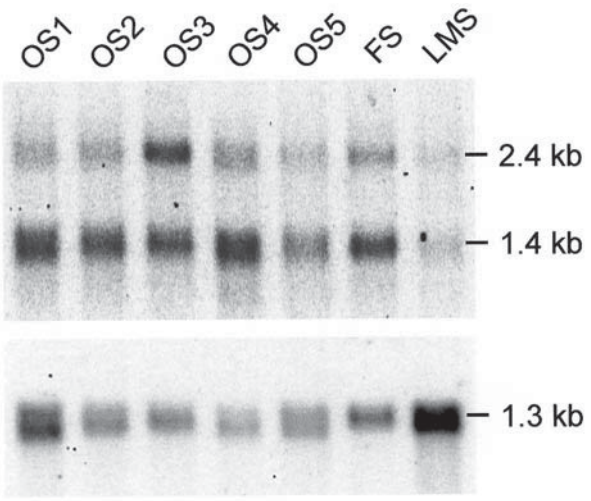

Figure 1. *Northern blot analysis of five osteosarcomas (OS1-5), one fibrosarcoma (FS) and one leiomyosarcoma (LMS) of the dog hybridised with a HMGB1-specific cDNA probe detecting the two canine HMGB1 transcripts of approximately 1.4 and 2.4 kb (upper part). Co-hybridisation of the same membrane with a GAPDH-specific cDNA probe detecting a 1.3 kb transcript (lower part).*

Table II. *Absolute and relative HMGB1-mRNA / GAPDH-mRNA ratios.*

Sarcoma sample	Absolute *HMGB1* / *GAPDH*-RNA ratios		Relative *HMGB1* / *GAPDH*-RNA ratios[1]	
	RT-PCR	Northern blot	RT-PCR	Northern blot
OS1	0.95	0.52	1.09	0.7
OS2	0.99	0.79	1.13	1.06
OS3	1.02	1.05	1.17	1.41
OS4	1.28	1.31	1.47	1.75
OS5	0.72	0.60	0.83	0.79
FS	0.73	0.73	0.84	0.97
LMS	0.42	0.24	0.48	0.32
Mean value	0.87	0.75	1.0	1.0

[1] Calculated with the mean values of the absolute *HMGB1* / *GAPDH*-RNA ratios set to one.

In order to confirm the results and to develop a less time- and material-consuming technique, we established a semi-quantitative duplex RT-PCR suitable for detecting intertumoural variation of *HMGB1* expression in relation to expression of the house-keeping gene *GAPDH* (Figure 2). After quantitation of the signals obtained by RT-PCR, the *HMGB1*-RNA / *GAPDH*-RNA ratios were calculated. The values for the osteosarcoma samples varied between 0.72 and 1.28, while the ratios for the fibrosarcoma and the leiomyosarcoma were 0.73 and 0.42, respectively (Table II). In order to determine the comparability of the results

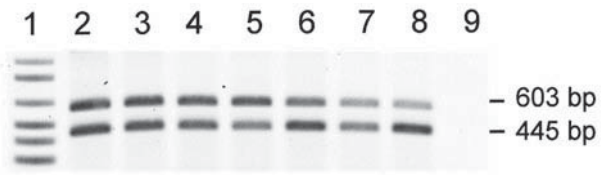

Figure 2. *Semi-quantitative duplex RT-PCR products of HMGB1 (603 bp) and GAPDH (445 bp) using canine cDNAs of five osteosarcomas, one fibrosarcoma and one leiomyosarcoma after electrophoresis and VistraGreen staining (Amersham Biosciences). Lane 1: DNA molecular weight standard 1 Kb Plus DNA Ladder (Invitrogen). Lanes 2-6: osteosarcoma samples 1-5 (OS1-5). Lane 7: fibrosarcoma sample (FS). Lane 8: leiomyosarcoma sample (LMS). Lane 9: H_2O, negative control.*

obtained by the Northern blot hybridisation and RT-PCR analyses, mean values for each test series were calculated, set to one, and relative expression levels were determined (Table II, Figure 3). Statistical analysis using the Pearson's Correlation Test revealed a significant correlation between the relative *HMGB1* expression level obtained by Northern blot hybridisation and the level obtained by the established RT-PCR ($r=0.8919$, $p=0.0071$).

Discussion

Cisplatin and carboplatin are widely used anticancer drugs, manifesting their cytotoxicity to tumour cells by damaging DNA, generating a distorted DNA duplex. HMGB1 proteins selectively bind with high affinity to cisplatin or carboplatin-DNA adducts and several investigations revealed that this interaction contributes to tumour death by blocking excision repair of the major cisplatin-DNA adducts (9,10).

No features have been identified yet allowing clinicians to predict the response to cisplatin or carboplatin therapies in dogs with osteosarcomas at the time of diagnosis or during treatment (19). Hence, it was the aim of this study to analyse the expression level of *HMGB1* in canine sarcomas.

Based on Northern blot and RT-PCR analyses, we were able to show an intertumoural variation of *HMGB1* expression levels among canine sarcomas. Very recently, comparable results were obtained for human breast cancer samples (17,20) and a clinical trial designed to increase *HMGB1* expression by oestrogen treatment has been approved by the FDA (10). The observed intertumoural variances of *HMGB1* expression in seven sarcomas analysed in this study may be of importance for therapeutic approaches based on cisplatin/carboplatin treatment as, for example, tumours showing a high *HMGB1* expression level may be treated with a lower amount of this antitumour drug. However, future clinical studies including a greater number of tumours have to be performed to correlate the

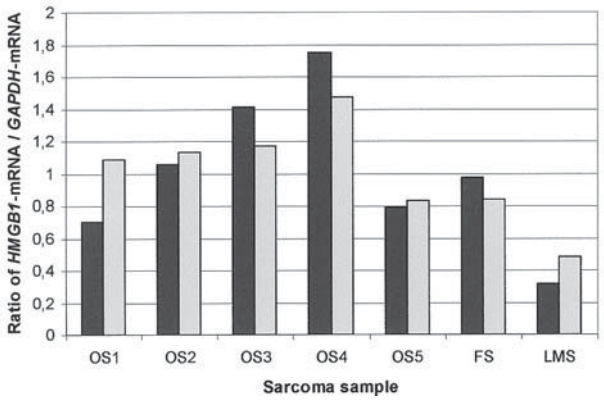

Figure 3. *Variation of relative HMGB1 expression in five osteosarcomas (OS1-5), one fibrosarcoma (FS) and one leiomyosarcoma (LMS) of the dog as revealed by Northern blot analysis (dark grey bars) and semi-quantitative RT-PCR (light grey bars). In order to compare the results obtained by the two methods, mean values for each test series were calculated, set to one and relative expression levels were determined.*

HMGB1 expression level with clinical outcome of cisplatin/carboplatin chemotherapy. The statistically significant correlation of the relative *HMGB1* expression levels obtained by Northern blot analyses as well as duplex RT-PCR makes the established PCR approach a quick and convenient method to determine the intratumoural *HMGB1* expression.

References

1 Kartalou M and Essigmann JM: Recognition of cisplatin adducts by cellular proteins. Mutat Res *478*: 1-21, 2001.

2 Bustin M, Lehn DA and Landsman D: Structural features of the HMG chromosomal proteins and their genes. Biochim Biophys Acta *1049*: 231-243, 1990.

3 Bustin M and Reeves R: High-mobility-group chromosomal proteins: architectural components that facilitate chromatin function. Prog Nucleic Acid Res Mol Biol *54*: 35-100, 1996.

4 Jantzen HM, Admon A, Bell SP and Tjian R: Nucleolar transcription factor hUBF contains a DNA-binding motif with homology to HMG proteins. Nature *344*: 830-836, 1990.

5 Landsman D and Bustin M: A signature for the HMG-1 box DNA-binding proteins. Bioessays *15*: 539-546, 1993.

6 Pil PM and Lippard SJ: Specific binding of chromosomal protein HMG1 to DNA damaged by the anticancer drug cisplatin. Science *256*: 234-237, 1992.

7 Jung Y and Lippard SJ: Nature of full-length HMGB1 binding to cisplatin-modified DNA. Biochemistry *42*: 2664-2671, 2003.

8 Kasparkova J, Delalande O, Stros M, Elizondo-Riojas M-A, Vojtiskova M, Kozelka J and Brabec V: Recognition of DNA interstrand cross-link of antitumor cisplatin by HMGB1 protein. Biochemistry *42*: 1234-1244, 2003.

9 Zamble DB, Mu D, Reardon JT, Sancar A and Lippard SJ: Repair of cisplatin-DNA adducts by the mammalian excision nuclease. Biochemistry *35*: 10004-10013, 1996.

10 He Q, Liang CH and Lippard SJ: Steroid hormones induce HMG1 overexpression and sensitize breast cancer cells to cisplatin and carboplatin. Proc Natl Acad Sci USA *97*: 5768-5772, 2000.

11 Chau KY, Lam HY and Lee KL: Estrogen treatment induces elevated expression of HMG1 in MCF-7 cells. Exp Cell Res *241*: 269-272, 1998.

12 Borrmann L, Kim I, Schultheiss D, Rogalla P and Bullerdiek J: Regulation of the expression of HMG1, a co-activator of the oestrogen receptor. Anticancer Res *21*: 301-305, 2001.

13 Jiang Z, Priat C and Galibert F: Traced orthologous amplified sequence tags (TOASTs) and mammalian comparative maps. Mamm Genome *9*: 577-787, 1998.

14 Feinberg AP and Vogelstein B: A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. Anal Biochem *132*: 6-13, 1983.

15 Wen L, Huang JK, Johnson BH and Reeck GR: A human placental cDNA clone that encodes nonhistone chromosomal protein HMG-1. Nucleic Acid Res *17*: 1197-1214, 1989.

16 Rogalla P, Kazmierczak B, Flohr AM, Hauke S and Bullerdiek J: Back to the roots of a new exon--the molecular archaeology of a SP100 splice variant. Genomics *63*: 117-122, 2000.

17 Flohr AM, Rogalla P, Meiboom M, Borrmann L, Krohn M, Thode-Halle B and Bullerdiek J: Variation of HMGB1 expression in breast cancer. Anticancer Res *21*: 3881-3885, 2001.

18 Murua Escobar H, Meyer B, Richter A, Becker K, Flohr AM, Bullerdiek J and Nolte I: Molecular characterization of the canine HMGB1. Cytogenet Genome Res *101*: 33-38, 2003.

19 Hahn KA, Legendre AM and Talbott JR: The frequency of micronuclei in lymphocytes of dogs with osteosarcomas: a predictive variable for tumor response during cisplatin chemotherapy. Cancer Epidemiol Biomakers Prev *5*: 653-656, 1996.

20 Brezniceanu M-L, Völp K, Bösse S, Solbach C, Lichter P, Joos S and Zörnig M: HMGB1 inhibits cell death in yeast and mammalian cells and is abundantly expressed in human breast cancer carcinoma. FASEB J *17*: 1295-1297, 2003.

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- XIII -

Becker K, Murua Escobar H, Richter A, Meyer B, Nolte I, Bullerdiek J

The canine *HMGA1* gene maps to CFA 23

Anim Genet. 2003. 34:68-9.

Own contribution:

Collaboration in the identification of BAC clones positive for canine *HMGA1*

Collaboration in preparation of DNA from the identified BAC clones for use in FISH mapping of the canine *HMGA1* gene

The canine *HMGA1* gene maps to CFA 23

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Accepted 26 October 2002

Introduction: Parallels between human and canine tumours have often been described. Human chromosomal rearrangements on 6p21 involving the *HMGA1* gene have been described in various benign mesenchymal tumours like pulmonary chondroid hamartomas, uterine leiomyomas, endometrial polyps and lipomas[1,2]. So far, it is not clear if comparable translocations occur in the corresponding canine tumour as well. To further elucidate that question, we have mapped the canine *HMGA1* gene.

BAC clone and probe: A *HMGA1* DNA probe was generated by PCR spanning part of the exon 6, the complete intron 6 and part of exon 7 (primer up: AGC GAA GTG CCA ACA CCT AAG AGA/Lo: CCT TGG TTT CCT TCC TGG AGT TG) on DNA derived from the canine cell line MTH52 (Center for Human Genetics, Bremen, Germany), cloned, sequenced and used for screening of a canine BAC library. Filters were obtained from the BACPAC RESOURCES/Childrens Hospital, Oakland, CA, USA (http://www.chori.org/bacpac). BAC-screening was performed following manufacturers' instructions. To rule out false-positive results, a PCR using the initial primer pair used for the screening probe was preformed, cloned and sequenced.

Fluorescence in situ *hybridization:* Metaphase preparations and fluorescence *in situ* hybridization (FISH) were performed as described previously[3]. Ten well-spread metaphases were examined all showing a signal on CFA 23 on both chromatids of both chromosomes CFA 23 (Fig. 1).

Comments: Chromosomal rearrangements of HSA 6p21 involving *HMGA1* represent the second most frequent specific translocations in human tumours. The assignment of the canine *HMGA1* gene to CFA 23 clearly shows that the chromosomal region to which the canine *HMGA1* gene has been

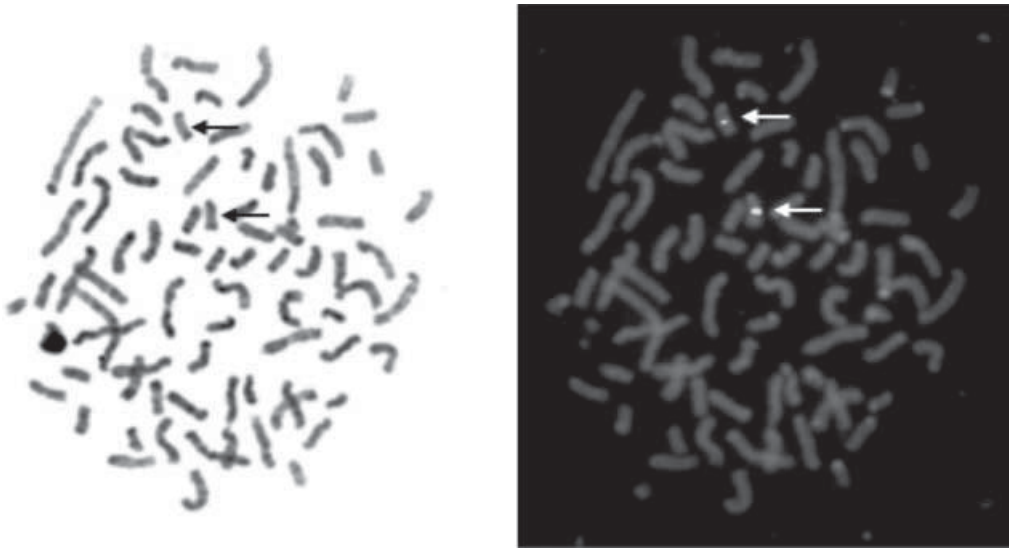

Figure 1 An example of a metaphase spread after fluorescence *in situ* hybridization with signals on both chromosomes 23 (right) and the same metaphase after GTG-banding (left).

mapped, is not a hotspot of chromosomal breakpoints seen in canine tumours. The hotspots that have been found in the dog genome so far, include chromosomes 1, 19 and 25 which are preferentially involved in chromosomal fusions[4]. The X-chromosome of the dog, in contrast, is frequently affected by structural aberrations. Therefore, in contrast to humans, the activation of *HMGA1* as a result of chromosomal translocations does not seem to play a considerable role in canine tumours. This may be due to the fact that the corresponding changes are not able to induce benign tumours in the dog or to stimulate their growth. Alternatively, there may be factors favouring the occurrence of the structural changes in humans which are lacking in dogs.

No homology has been found between human chromosome 6, to which the *HMGA1* is mapped, and canine chromosome 23, rather human chromosome 6 shares homology with canine chromosome 22 and 8[5]. In our FISH studies no metaphase shows signals on these dog chromosomes.

References

1 Kazmierczak B. *et al.* (1996) *J Natl Cancer Inst* **88,** 1234–6.
2 Dal Cin P. *et al.* (1999) *Genes Chromosomes Cancer* **24,** 286–9.
3 Murua Escobar H. *et al.* (2001) *Cytogenet Cell Genet* **94,** 194–5.
4 Reimann N. *et al.* (1999) *J Natl Cancer Inst* **91,** 1688–9.
5 Yang F. *et al.* (1999) *Genomics* **62,** 189–202.

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- XIV -

Murua Escobar H, Meyer B, Richter A, Becker K, Flohr AM, Bullerdiek J, Nolte I

Molecular characterization of the canine HMGB1

Cytogenet Genome Res. 2003.101:33-8.

Own contribution:

Collaboration in cloning and sequence analysis of fragments of the canine *HMGB1* gene

Animal Cytogenetics and Comparative Mapping

Cytogenet Genome Res 101:33–38 (2003)
DOI: 10.1159/000073415

Cytogenetic and Genome Research

Molecular characterization of the canine HMGB1

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Abstract. Due to the close similarities of numerous canine diseases to their human counterparts, the dog could join the mouse as the species of choice to unravel the genetic background of complex diseases as e.g. cancer and metabolic diseases. Accordingly, the role of the dog as a model for therapeutic approaches is strongly increasing. However, prerequisite for such studies is the characterization of the corresponding canine genes. Recently, the human high mobility group protein B1 (HMGB1) has attracted considerable interest of oncologists because of what is called its "double life". Besides its function as an architectural transcription factor HMGB1 can also be secreted by certain cells and then acts as a ligand for the receptor for advanced glycation end products (RAGE). The binding of HMGB1 to RAGE can activate key cell signaling pathways, such as $p38^{MAPK}$, JNK, and $p42/p44^{MAPK}$ emphasizing the important role of HMGB1 in inflammation and tumor metastasis. These results make HMGB1 a very interesting target for therapeutic studies done in model organisms like the dog. In this study we characterized the molecular structure of the canine HMGB1 gene on genomic and cDNA levels, its predicted protein, the gene locus and a basic expression pattern.

As witnessed by a number of recent articles (Kuska, 1999; Kingman, 2000; Ostrander et al., 2000; Vail and MacEwen, 2000) a growing number of scientists predict that human genetics will be "going to the dogs" in this century (Kuska, 1999). Due to the emerging advantages of numerous canine diseases as a genetic model for human orthologs, the dog could join the mouse as the species of choice to unravel genetic mechanisms e.g. of cancer predisposition, development, and progression.

A very interesting group of genes in terms of oncology are the high mobility group (HMG) protein genes. High mobility group proteins named according to their electrophoretic mobility in the electric field are small chromatin-associated nonhistone proteins which can be subdivided into three families because of their functional sequence motifs: the HMGA, HMGB, and HMGN protein families. Three human members of the HMGB protein family are presently known: the HMGB1, HMGB2, and HMGB3 proteins. The best analyzed member of this group, HMGB1 (synonymously known as HMG1 or amphoterin), can modify chromatin structure by bending DNA thus influencing the transcription of a number of target genes (for review see Muller et al., 2001). Recently, HMGB1 has attracted additional interest of oncologists because of what was called its "double life". Besides its function as an architectural transcription factor, HMGB1 can also be secreted by certain cells, e.g. macrophages (Wang et al., 1999). As an extracellular protein HMGB1 is a ligand for the receptor for advanced glycation end products (RAGE) (Parkkinen and Rauvala 1991; Parkkinen et al., 1993; Hori et al., 1995) thus activating key cell signaling pathways, such as $p38^{MAPK}$, JNK, and $p42/p44^{MAPK}$ and playing an important role in inflammation and tumor metastasis (Liotta and Clair, 2000; Taguchi et al., 2000).

The high mobility group protein HMGB1 has a number of features particularly related to the development and progres-

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sion of gynaecological cancers. As an example HMGB1 has been shown to interact with and modify the binding affinity of several transcription factors, e.g. TATA-binding protein (TBP), Hox D9 protein, and steroid hormone receptors (Ge and Roeder, 1994; Zappavigna et al., 1996; Verrier et al., 1997; Boonyaratanakornkit et al., 1998; Romine et al., 1998; Das and Scovell, 2001). Interestingly, HMGB1 gene expression can be induced by estrogens in breast cancer MCF-7 cells probably due to an upregulation of the gene so that HMGB1 itself can be considered an estrogen-responsive gene (Chau et al., 1998). Additionally, it has been shown that HMGB1 is able to bind to cisplatin-DNA adducts (Pil and Lippard, 1992) and sensitizes cancer cells to cisplatin by shielding its major DNA adducts from nucleotide excision repair (He et al., 2000). He et al. (2000) have shown that in estrogen receptor-positive human breast cancer cells estrogen can significantly increase the effect of cisplatin by causing an overexpression of HMGB1. This finding has led to the conclusion that estrogen treatment prior to cisplatin therapy may sensitize the cancer cells against that drug. Accordingly, a clinical trial for the treatment of gynaecological tumors with cisplatin has already been approved by the Food and Drug Administration (He et al., 2000).

However, the canine HMGB1 gene had not yet been characterized molecularly. The enlightenment of the molecular structure could permit new therapeutic approaches using the dog as model organism.

Materials and methods

cDNA characterization

Total RNA was isolated from 50 mg canine testis tissue using TRIZOL LS (Invitrogen) following the manufacturer's protocol. cDNA synthesis was performed using AP2 primer (AAGGATCCGTCGACATC(17)T), 5 µg total RNA and M-MLV (Invitrogen) reverse transcriptase according to the manufacturer's instructions. The PCRs for the molecular cloning of the cDNA were done using the following primer pairs: 5′-AGCTCCATAGAGACAGCACC-3′/5′-TCTTCCTCCTCCTCCTCATCC-3′ and primer pair 5′-AGGCCTCTTGGGTGCATT-3′/5′-AGTATCATCCAGGACTCAGAT-3′.

PCR products were separated on a 1.5% agarose gel, recovered with QIAEX II (QIAGEN), cloned in pGEM T-easy vector system (Promega) and sequenced. The cDNA contig and the homology alignments were created with two sequences from the NCBI database (acc. nos. AF281043, U51677).

Protein sequence

The canine HMGB1 protein sequence was derived from the ORF of the characterized cDNA sequence described previously. The protein homology alignments were created with sequences from the NCBI database (acc. nos. AF281043, U51677).

Northern blot

Total RNAs were isolated from canine heart, lung, muscle, kidney, and spleen tissue using RNeasy system (QIAGEN). An additional sample of total RNA was isolated from canine heart tissue by TRIZOL LS acid guanidine isothiocyanate-chloroform method (Invitrogen) in order to figure out whether this isolation method leads to any difference in hybridization. Furthermore, mRNA was purified from canine spleen total RNA with OLIGOTEX (QIAGEN) and total RNA was prepared from human cultured fibroblasts by RNeasy system (QIAGEN). Spleen mRNA was placed on the blot in case that HMGB1 was not detectable in the total RNA samples.

For Northern blot hybridization 20 µg of total RNA of each sample (except muscle 10 µg and spleen mRNA 3.6 µg) were separated on a 1.2% denaturing agarose gel containing 0.65% formaldehyde. RNAs were transferred onto Hybond-N+ positive nylon membrane (Amersham Pharmacia Biotech) by capillary blot.

A 292-bp cDNA fragment derived from the coding sequence (exon 2/exon 3) of the canine HMGB1 served as a molecular probe for hybridization. The probe was generated by PCR with the primer set 5′-AGGCCTCTTGGGTGCATT-3′/5′-GGGCAAAGGAGATCCTAAGAAG-3′ (Jiang et al., 1998) on the cloned cDNA described previously. Labeling was performed by random primed labeling (Roche Diagnostics) as described in the manufacturer's protocol with 50 µCi [α^{32}P]dCTP (Amersham Pharmacia Biotech). Purification of the labeled probe was done using Sephadex G-50 Nick Columns (Amersham Pharmacia Biotech) and the probe was stored at –20° C before use.

Using the PERFECTHYB PLUS hybridization solution (Sigma-Aldrich) prehybridization was carried out for 30 min and hybridization for 2.5 h at 68° C. The membrane was washed for 5 min at room temperature in 2× SSC, 0.1% SDS, and twice for 20 min at 68° C in 0.5× SSC, 0.1% SDS. Signals were visualized by using a STORM imager (Molecular Dynamics, Sunnyvale, USA).

BAC screening and FISH

An HMGB1 canine genomic DNA probe was used for hybridization of canine RPCI 81 BAC/PAC filter (BACPAC RESOURCES, Children's Hospital Oakland Research Institute, Oakland, USA). The 531-bp probe was generated by PCR (Primer 5′-AGGCCTCTTGGGTGCATT-3′/5′-AGAGGCCTCCGTGAGTATCTTG-3′) on genomic DNA of the canine pleomorphic adenoma cell line ZMTH3 (Center for Human Genetics, Bremen, Germany). The obtained PCR product was separated on a 1.5% agarose gel, recovered with QIAEX II (QIAGEN), cloned in pGEM T-easy vector system (Promega) and sequenced for verification. The probe labeling was performed by random primed labeling (Roche Diagnostics) as described in the manufacturer's protocol with 50 µCi [α^{32}P]dCTP (Amersham Pharmacia Biotech). Purification of the labeled probe was done using Sephadex G-50 Nick Columns (Amersham Pharmacia Biotech) and the probe was stored at –20° C before use.

The filters were placed in a minimum volume of Church Buffer (0.15 mM BSA, 1 mM EDTA, 0.5 M $NaHPO_4$, 7% SDS) and transferred into hybridization bottles. The filters were prehybridized at 65° C for 1 h in 25 ml Church Buffer. Hybridization was performed at 65° C overnight (16–18 h) in the same solution. All further steps were performed according to manufacturer's protocol. Signals were visualized using a STORM imager (Molecular Dynamics, Sunnyvale, USA).

Metaphase preparations were obtained from blood samples of two different dogs. The samples were stimulated with PHA and cultured for 96 h at 37° C. After 1.5 h colcemide (0.1 µg/ml) incubation, the lymphocytes were harvested according to routine procedures. Prior to FISH, chromosomes were stained using the GTG-banding method. After taking photographs of the metaphases, the slides were destained in 70% ethanol for 15 min and air dried.

FISH was performed using the protocol of Reimann et al. (1996) with some modifications. BAC DNA was digoxigenin labeled (Dig-Nick-Translation-Kit, Roche Diagnostics). The hybridization mixture contained 125–175 ng probe, 43.2 µg salmon sperm DNA, 1,000–1,200 ng sonicated dog DNA, 2× SSC, 2× SSPE, 50% formamide and 10% dextran sulfate. The chromosomes were stained with propidium iodide and G-bands were identified according to Reimann et al. (1996).

Genomic characterization

For genomic characterization of the canine HMGB1 gene the introns were amplified by PCR on the screened BAC 24-A16 (BACPAC RESOURCES, Children's Hospital Oakland Research Institute, Oakland, USA) and on genomic DNA of the canine pleomorphic adenoma cell line ZMTH3 (Center for Human Genetics, Bremen, Germany). For intron 2 a 517-bp fragment was generated with primer pair 5′-AGGCCTCTTGGGTGCATT-3′/5′-GGGCAAAGGAGATCCTAAGAAG-3′, a 579-bp intron 3 fragment was generated by the primer pair 5′-GATCCCAATGCACCCAAGAG-3′/5′-GGGGATACTCAGAACAAAACA-3′ and the fourth exon 1,224-bp fragment was generated with pair 5′-GAAGGCTGCTAAGCTGAAGGA-3′/5′-TCTTCCTCCTCCTCCTCATCC-3′. The obtained PCR products were separated on a 1.5% agarose gel, recovered with QIAEX II (QIAGEN), cloned in pGEM T-Easy vector system (Promega) and sequenced for verification. The final genomic canine HMGB1 contig and the homology alignments were

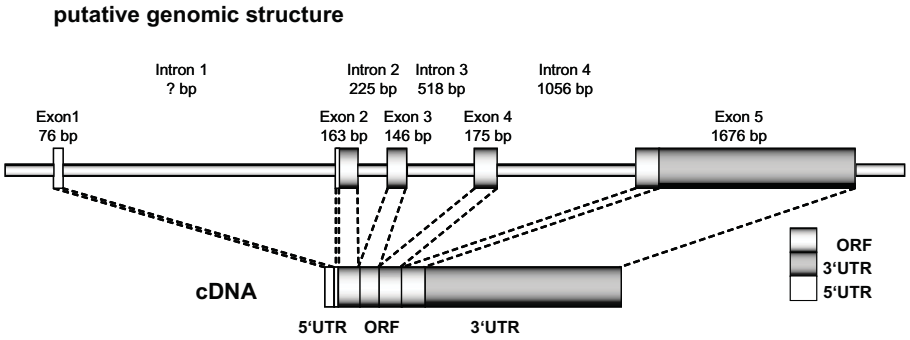

Fig. 1. Structure of the genomic elements and the cDNA of the canine HMGB1.

created with Lasergene software (DNAStar, Madison, USA) with the generated sequences from the cloned cDNA described previously and various sequences from the NCBI database (acc. nos. AF 281043, U 51677, NM_002128).

Results

Herein, we describe the genomic and the cDNA structure, the predicted protein sequence, a basic expression pattern, and the chromosomal locus of the canine HMGB1 gene.

cDNA sequence

The canine cDNA sequence consists of 2,236 bp spanning five exons. The exon structure, the UTRs, and the ORF were defined and their homologies to their human counterpart analyzed (Fig. 1, Table 1). The cDNA sequence was submitted to the NCBI database acc. no. AY135519.

Protein sequence

The canine HMGB1 protein sequence was deduced from the composite cDNA sequence. The protein is a 215-amino-acid (AA) molecule with a calculated weight of 24,892.67 Daltons (Fig. 3, Table 2). The sequence was submitted to the NCBI database acc. no. AY135519. Homology comparison to the human counterpart showed 100% homology of the molecules including the two "HMG boxes" and the acidic carboxy-terminal domain, while mouse and bovine molecules showed differences in the acidic tail.

Genomic structure

The genomic structure of the canine HMGB1 gene consists of the previously described five exons and four introns. Due to the fact that intron 1 could not be cloned a contig spanning exon 2 to exon 5 consisting of 3,959 bp was created. The exon/intron structure, size and the homologies to their human counterparts were analyzed and defined (Fig. 1, Table 1). The genomic sequences were submitted to the NCBI database (acc. nos. AY135520, AY135521).

Northern blot

To elucidate a basic expression pattern, a canine Northern blot was performed using RNA and mRNA from canine spleen, heart, lung, muscle, and kidney tissue samples and hybridized with a ^{32}P-labeled canine HMGB1 cDNA probe. Except for the kidney tissue that showed no detectable signal, all samples revealed two transcripts similar to the signals obtained in human fibroblasts (Fig. 2) of about 1.4 and 2.4 kb. One of two

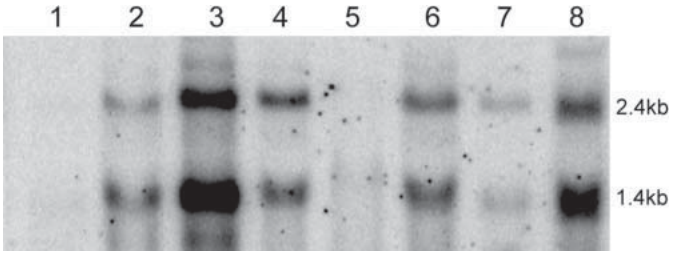

Fig. 2. Northern blot showing 1.4-kb and 2.4-kb HMGB1 transcripts. Lanes: canine: (1) kidney, (2, 3) spleen, (4, 5) heart, (6) lung, (7) muscle; human: (8) fibroblasts.

Table 1. Detailed analysis of the canine HMGB1 cDNA and genomic elements: Homology comparison of the genomic and cDNA elements of the canine HMGB1 to its human counterpart (Characterisation of the UTRs, the ORF and the exon/intron sizes)

Element of canine HMGB1	Size in bp	Homology to human counterpart in %
cDNA elements		
Total cDNA	2236	90.8
5' UTR	89	95.6
cds	648	95.4
3' UTR	1499	88.6
Genomic elements		
Total Exon 2–5	3959	73.3
Exon 1	76	98.7
Intron 1	-	-
Exon 2	163	95.2
Intron 2	225	20.3
Exon 3	146	95.2
Intron 3	518	59.0
Exon 4	175	93.7
Intron 4	1056	61.8
Exon 5	1676	89.4

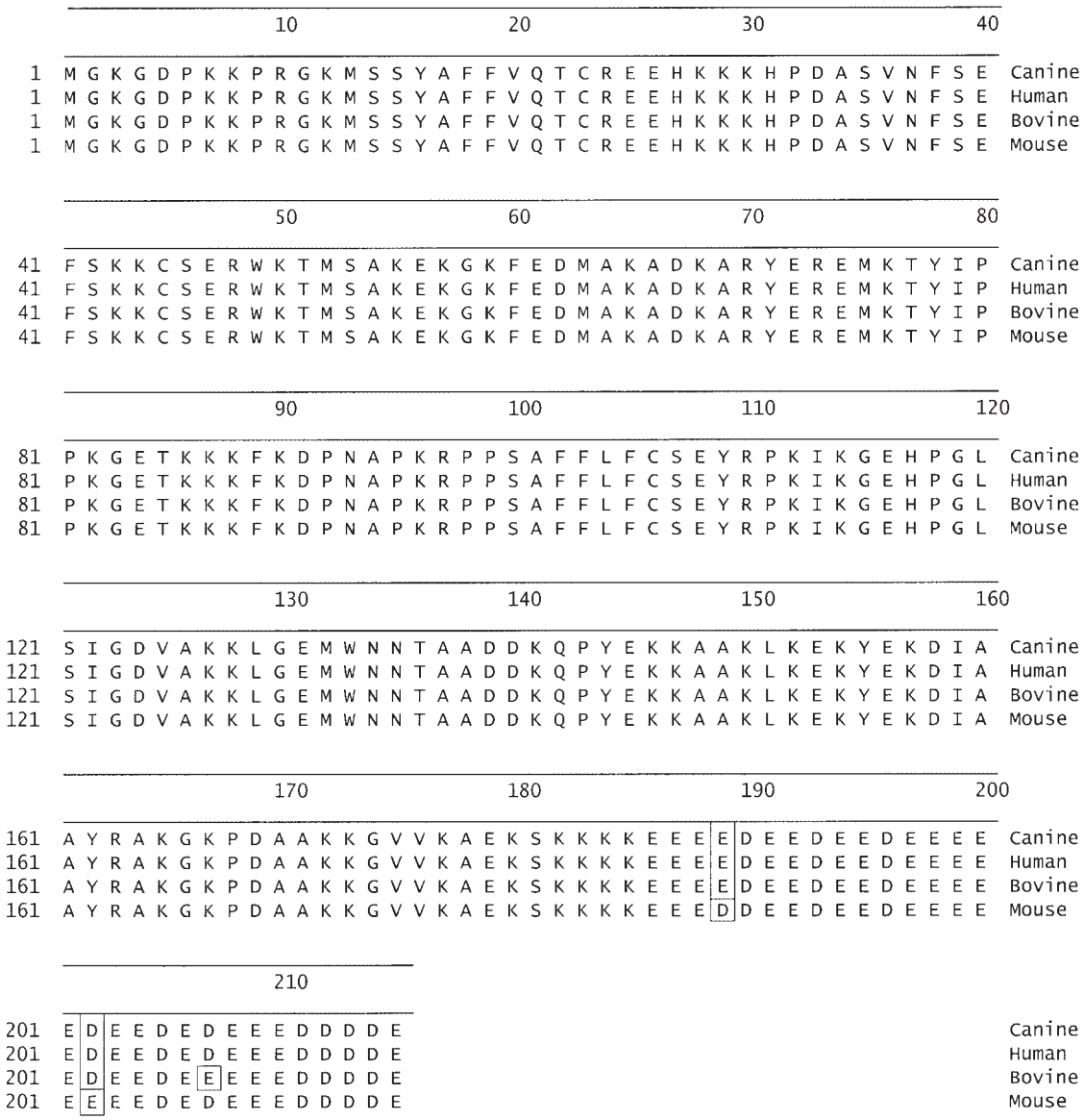

Fig. 3. Comparison of canine, human, mouse, and bovine HMGB1 proteins.

Table 2. Detailed in silico analysis of the canine HMGB1 protein

Amino Acids (AA)	Molecular Weight in Da	Isoelectric Point	Charge at pH 7.0
215	24892.67	5.591	-4.629
Strongly Basic (+) AA	**Strongly Acidic (-) AA**	**Hydrophobic AA**	**Polar AA**
51	56	43	32

canine heart samples showed weak signals probably due to the Trizol sample quantification difficulties. Human HMGB1 transcripts of this size had been detected in multiple normal tissues and several breast cancer samples.

BAC screening and FISH

A canine HMGB1 genomic DNA probe was generated and used for screening a canine BAC for localization of the canine HMGB1 gene locus by FISH.

The screened BAC 24-A16 was first verified according to the manufacturer's protocol which requires two hybridization signals to appear in a specific orientation on the filter spotting panel. Second verification was done by PCR using primer pair UP: 5′-GAAGGCTGCTAAGCTGAAGGA-3′/LO: 5′-TCTTCCTCCTCCTCCTCATCC-3′ spanning intron 4. For final verification the obtained PCR product was separated on agarose gel, recovered, cloned and sequenced as described previously.

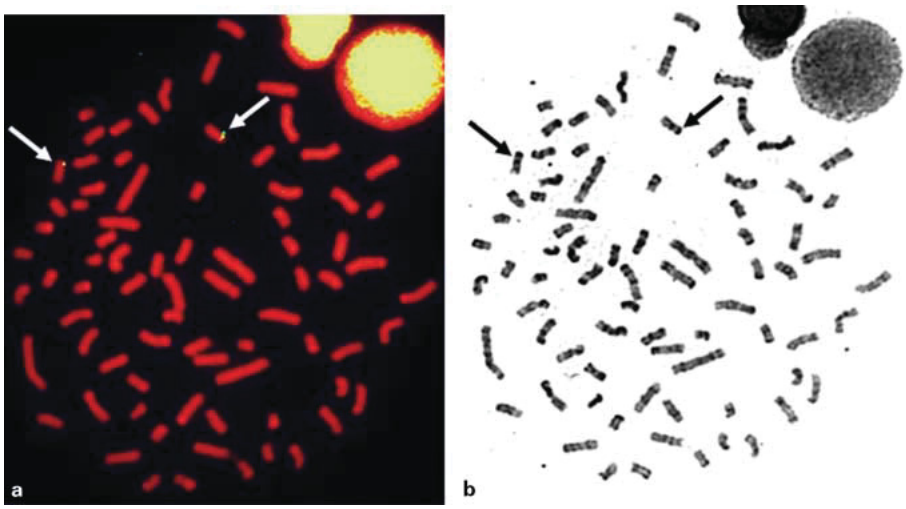

Fig. 4. An example of a metaphase spread after FISH with signals on both chromosomes 25 (**a**) and the same metaphase after GTG-banding (**b**).

The verified BAC was used for FISH experiments. Sixteen well spread metaphases were examined for analysis. Signals were detected on both chromatides of both chromosomes. The obtained signals mapped the canine gene to CFA25 (Fig. 4).

Discussion

The human HMGB1 is considered to have a "double life" with impact on several diseases including tumorigenesis (Taguchi et al., 2000; Flohr et al., 2001). First, by acting as an architectural transcription factor, HMGB1 influences the formation of transcription factor complexes of several target genes. Second, HMGB1 can be released from some cells allowing its binding to the cell surface receptor RAGE. Blockade of RAGE-HMGB1 interaction in rat C6 glioma cells significantly inhibits their growth, motility, and local invasion as well as metastasis (Liotta and Clair, 2000; Taguchi et al., 2000).

The aim of the present study was to characterize the cDNA, protein, genomic structure, gene locus, and a basic expression pattern of the canine HMGB1 gene. Knowledge of its structure would be the precondition to evaluate the canine gene product as a potential target for therapeutic approaches using the dog as model system.

The complete canine HMGB1 cDNA consists of 2,236 bp encoded by five exons similar to the human transcript (acc. no. AY135519). The homology of the complete molecule to its human counterpart is 90.8% whereas the ORF is 95.4%. The derived canine protein consists of 215 AA with a molecular weight of 24,892.67 Da. Comparison to the human protein showed 100% homology of the canine counterpart. Differences between human HMGB1 and the mouse protein were described as two or three AA changes in the acidic carboxy-terminal domain (Paonessa et al., 1987; Ferrari et al., 1994). The bovine molecule (acc. no. P10103) shows one AA change compared to its human and canine counterpart. Due to the previously described properties of the canine HMGB1 and the identical structure to the human molecule, therapeutic approaches in dogs likely can be performed with identical compounds.

Northern blot analysis was performed to define a basic expression pattern in canine heart, lung, muscle, kidney, and spleen tissue. Except for the kidney tissue that showed no detectable signal, all samples revealed two transcripts similar to the signals obtained in human fibroblasts of about 1.4 and 2.4 kb. One of two canine heart samples showed weak signals probably due to the Trizol sample quantification difficulties. Human HMGB1 transcripts of this size had been detected in multiple normal tissues (Rogalla et al., 1998) and several breast cancer samples (Flohr et al., 2001).

At the genomic level the canine HMGB1 gene exon/intron structure is similar to the human ortholog consisting of five exons and four introns. While the homologies of the exons, ranging between 89.4 and 98.7%, are extremely high, the homologies of the amplified introns 2–4 vary between 20.3 and 59.0%. Approaches to amplify the canine intron 1 resulted in unspecific PCR products which were probably due to the putative existence of various CpG islands found in human intron 1 (Borrmann et al., 2001).

Mapping of the canine HMGB1 gene resulted in assignment to CFA25. The G-bands were identified according to Reimann et al. (1996).

Yang et al. (1999) found no conservation of synteny between the human chromosome 13 where the human HMGB1 is located and the canine chromosome 25 where we mapped canine HMGB1.

Nevertheless the molecular characterization of the canine HMGB1 gene and protein showed clearly that man and dog share an identical HMGB1 protein. Considering the similarities of genesis and development of diseases in both species molecular targeting of HMGB1 in dogs can be of significant importance for therapeutic approaches in humans as well.

References

Boonyaratanakornkit V, Melvin V, Prendergast P, Altmann M, Ronfani L, Bianchi ME, Taraseviciene L, Nordeen SK, Allegretto EA, Edwards DP: High-mobility group chromatin proteins 1 and 2 functionally interact with steroid hormone receptors to enhance their DNA binding in vitro and transcriptional activity in mammalian cells. Mol cell Biol 18:4471–4487 (1998).

Borrmann L, Kim I, Schultheiss D, Rogalla P, Bullerdiek J: Regulation of the expression of HMG1, a co-activator of the estrogen receptor. Anticancer Res 21:301–305 (2001).

Chau KY, Lam HY, Lee KL: Estrogen treatment induces elevated expression of HMG1 in MCF-7 cells. Exp Cell Res 241:269–272 (1998).

Das D, Scovell WM: The binding interaction of HMG-1 with the TATA-binding protein/TATA complex. J biol Chem 276:32597–32605 (2001).

Ferrari S, Ronfani L, Calogero S, Bianchi ME: The mouse gene coding for high mobility group 1 protein (HMG1). J biol Chem 269:28803–28808 (1994).

Flohr AM, Rogalla P, Meiboom M, Borrmann L, Krohn M, Thode-Halle B, Bullerdiek J: Variation of HMGB1 expression in breast cancer. Anticancer Res 21:3881–3885 (2001).

Ge H, Roeder RG: The high mobility group protein HMG1 can reversibly inhibit class II gene transcription by interaction with the TATA-binding protein. J biol Chem 269:17136–17140 (1994).

He Q, Liang CH, Lippard SJ: Steroid hormones induce HMG1 overexpression and sensitize breast cancer cells to cisplatin and carboplatin. Proc natl Acad Sci, USA 97:5768–5772 (2000).

Hori O, Brett J, Slattery T, Cao R, Zhang J, Chen JX, Nagashima M, Lundh ER, Vijay S, Nitecki D, Morser J, Stern D, Schmidt AM: The receptor for advanced glycation end products (RAGE) is a cellular binding site for amphoterin. Mediation of neurite outgrowth and co-expression of rage and amphoterin in the developing nervous system. J biol Chem 270:25752–25761 (1995).

Jiang Z, Priat C, Galibert F: Traced orthologous amplified sequence tags (TOASTs) and mammalian comparative maps. Mammal Genome 9:577–787 (1998).

Kingman S: Painting a brighter future for dogs and humans. Drug Discovery Today 5:127–128 (2000).

Kuska B: Sit, DNA, sit: cancer genetics going to the dogs. J natn Cancer Inst 91:204–209 (1999).

Liotta LA, Clair T: Cancer. Checkpoint for invasion. Nature 405:287–288 (2000).

Muller S, Scaffidi P, Degryse B, Bonaldi T, Ronfani L, Agresti A, Beltrame M, Bianchi ME. New EMBO members' review: the double life of HMGB1 chromatin protein: architectural factor and extracellular signal. EMBO J 20:4337–4340 (2001).

Ostrander EA, Galibert F, Patterson DF: Canine genetics comes of age. Trends Genet 16:117–124 (2000).

Paonessa G, Frank R, Cortese R: Nucleotide sequence of rat liver HMG1 cDNA. Nucl Acids Res 15:9077 (1987).

Parkkinen J, Rauvala H: Interactions of plasminogen and tissue plasminogen activator (t-PA) with amphoterin. Enhancement of t-PA-catalyzed plasminogen activation by amphoterin. J biol Chem 266:16730–16735 (1991).

Parkkinen J, Raulo E, Merenmies J, Nolo R, Kajander EO, Baumann M, Rauvala H: Amphoterin, the 30-kDa protein in a family of HMG1-type polypeptides. Enhanced expression in transformed cells, leading edge localization, and interactions with plasminogen activation. J biol Chem 268:19726–19738 (1993).

Pil PM, Lippard SJ: Specific binding of chromosomal protein HMG1 to DNA damaged by the anticancer drug cisplatin. Science 256:234–237 (1992).

Reimann N, Bartnizke S, Bullerdiek J, Schmitz U, Rogalla P, Nolte I, Ronne M: An extended nomenclature of the canine karyotype. Cytogenet Cell Genet 73:140–144 (1996).

Rogalla P, Borda Z, Meyer-Bolte K, Tran KH, Hauke S, Nimzyk R, Bullerdiek J: Mapping and molecular characterization of five HMG1-related DNA sequences. Cytogenet Cell Genet 83:124–129 (1998).

Rogalla P, Kazmierczak B, Flohr AM, Hauke S, Bullerdiek J: Back to the roots of a new exon – the molecular archaeology of a SP100 splice variant. Genomics 63:117–122 (2000).

Romine LE, Wood JR, Lamia LA, Prendergast P, Edwards DP, Nardulli AM: The high mobility group protein 1 enhances binding of the estrogen receptor DNA binding domain to the estrogen response element. Mol Endocr 12:664–674 (1998).

Taguchi A, Blood DC, del Toro G, Canet A, Lee DC, Qu W, Tanji N, Lu Y, Lalla E, Fu C, Hofmann MA, Kislinger T, Ingram M, Lu A, Tanaka H, Hori O, Ogawa S, Stern DM, Schmidt AM: Blockade of RAGE-amphoterin signalling suppresses tumor growth and metastases. Nature 405:354–360 (2000).

Vail DM, MacEwen EG: Spontaneously occurring tumors of companion animals as models for human cancer. Cancer Invest 18:781–792 (2000).

Verrier CS, Roodi N, Yee CJ, Bailey LR, Jensen RA, Bustin M, Parl FF: High-mobility group (HMG) protein HMG-1 and TATA-binding protein-associated factor TAF(II)30 affect estrogen receptor-mediated transcriptional activation. Mol Endocr 11:1009–1019 (1997).

Wang H, Bloom O, Zhang M, Vishnubhakat JM, Ombrellino M, Che J, Frazier A, Yang H, Ivanova S, Borovikova L, Manogue KR, Faist E, Abraham E, Andersson J, Andersson U, Molina PE, Abumrad NN, Sama A, Tracey KJ: HMG-1 as a late mediator of endotoxin lethality in mice. Science 285:248–251 (1999).

Yang F, O'Brien PCM, Milne S, Graphodatsky AS, Solanky N, Trifonov V, Rens W, Sargan D, Ferguson-Smith MA: A Complete Comparative Chromosome Map for the Dog, Red Fox, and Human and its Integration with Canine Genetic Maps. Genomics 62:189–202 (1999).

Zappavigna V, Falciola L, Helmer-Citterich M, Mavilio F, Bianchi ME: HMG1 interacts with HOX proteins and enhances their DNA binding and transcriptional activation. EMBO J 15:4981–4991 (1996).

4. Discussion

High-mobility-group AT-hook (HMGA) proteins are non-histone components of chromatin that function as architectural transcription factors, influencing the activation and transcription of genes in the developing embryo. Initially a target of tumour biology, as re-expressed HMGA proteins are involved in a large number of benign and malignant tumours, recent research led to knowledge on HMGA2 in chromatin structure and pluripotency of stem cells (Pfannkuche et al. 2009). Thus, as HMGA proteins are involved in processes including embryonic cell growth and maintenance of cell stemness (Li et al. 2007), their growth-inducing properties are thought to have the potential of being utilised in applications where enhanced cell growth is an issue, including tissue engineering of cartilage. The connection between HMGA2 and cartilage had been proven before, as the complete HMGA2 and the HMGA2/LPP (LIM domain containing preferred translocation partner in lipoma) fusion protein were demonstrated to positively influence expression of the chondrogenous *COL11A2* gene (Kubo et al. 2006). In addition, Hmga1 (+/-) and (-/-) murine knock-out embryonic stem cells showed decreased expression of the *COL1A2* (Collagen, type I, alpha 2) mRNA coding for the collagen alpha-2(I) chain (Martinez Hoyos et al. 2004), indicating function of HMGA1a and HMGA1b proteins in chondrogenesis. Even earlier, HMGA proteins were shown to be involved in the focal differentiation of areas of articular cartilage in benign tumours of the lung (Kazmierczak et al. 1995; Kazmierczak et al. 1996; Kazmierczak et al. 1996; Wanschura et al. 1996; Kazmierczak et al. 1999; Rogalla et al. 2000; Tallini et al. 2000; Lemke et al. 2002). The connection between HMGA presence and chondrocyte growth was thus the main aspect of the work conducted during this thesis.

Cartilage damage is a common disease present at almost all ages and not limited to humans, as it is also diagnosed in pet as well as production animals. Tissue engineering of cartilage e.g. in the form of autologous chondrocyte transplantation has been the focus of treating cartilage damages in recent years. It is based on amplifying chondrocytes taken from a small biopsy sample and reimplanting the multiplied cells into the defect, either with or without the aid of scaffolds that may be based on biomaterials such as chitosan or synthetic materials as for example polylactic acid. One crucial aspect is thus the growth rate of the explanted chondrocytes, aiming at keeping the time span between explantation and reimplantation as short as possible. While this might be achieved using cartilage-specific growth factors as well as specific growth media, the work conducted in this

thesis was aimed at the investigation of the potential growth inducing effects of the HMGA proteins on chondrocytes. Improved cell growth due to application of HMGA proteins to chondrocytes grown *in vitro* for autologous chondrocyte transplantation, or a combination of HMGA supplemented chondrocytes and scaffolds could thus be a possible enhancement for tissue engineering. For assessing the growth inducing potential of HMGA protein on chondrocytes, it was chosen to supply the cells with recombinantly produced HMGA proteins, as their dosage can be adjusted and proteins will be degraded over time being thus completely removed from the cell, if not re-applied. This is in opposition to gene therapeutic methods by either viral or non-viral gene delivery with limited influence on gene and thus protein dosage and even unwanted side effects such as constitutive transgene expression as well as transactivation of host genes. Thus with viruses, depending on the type chosen, genotoxic effects due to integration into the host genome leading to possible interference with host genes as well as enhancer and promoter sequences have to be taken into consideration. For example, in a patient who underwent gene therapy of human β-thalassaemia, amongst others, the β-globin vector incidentally integrated into the third intron of *HMGA2* introducing a cryptic splice site that resulted in constitutive expression of a truncated *HMGA2* variant insensitive to degradation by *let-7* miRNAs due to missing miRNA binding sites (Cavazzana-Calvo et al. 2010). Integration of viruses into regulative or promoter elements of murine retrovirus-induced interleukin-3 dependent myeloid leukaemia cells caused the activation of *Mecom* (MDS1 and EVI1 complex locus; synonymous with *Evi-1*) due to either the insertion of viral promoter sequences into the 5' non-coding region of the *Mecom* gene (Morishita et al. 1988), or into the genetically linked *Cb-1/fim-3* locus that is located 90 kb 5' of *Mecom*, activating the normal promoter (Bartholomew et al. 1991). Finally, in gene-therapy of human X-linked severe combined immunodeficiency (X-SCID), initially normal high-affinity interleukin-2 receptor expression and function could be achieved (Hacein-Bey-Abina et al. 2002) in a total of 9 from 10 patients treated. However, in two of these patients, uncontrolled exponential clonal mature T-cell proliferation occurred due to retrovirus vector integration in proximity to the *LMO2* (LIM domain only 2 (rhombotin-like 1)) proto-oncogene promoter, leading to aberrant transcription and expression of *LMO2* probably due to retrovirus enhancer activity (Hacein-Bey-Abina et al. 2003).

As undamaged human hyaline cartilage is difficult to obtain, porcine cartilage was chosen as a model for the experiments conducted. The growth inducing properties of HMGA proteins were examined in an *in vitro* system utilising isolated porcine chondrocytes grown in monolayer cell culture that were removed from cartilage of the

elbow joint of a pig for slaughter. Sequence similarity allowed the use of human HMGA proteins on porcine cells, and recombinantly produced human HMGA1a, HMGA1b, and HMGA2 proteins were chosen as potential growth inducing agents. While the actual protocol for recombinant HMGA expression and purification was described in a previous publication (Schwanbeck 2000), it needed some adaptation to provide for efficient production of higher amounts of protein, as the protein yield and purity was not sufficient. Background expression of the HMGA transgenes during expression culture preparation proved to be inhibitory to bacterial growth, probably due to abundant HMGA binding to the bacterial DNA disturbing vital processes within the cells. Furthermore, codon usage of the HMGA proteins proved to be unfavourable in the initially described bacterial host, so that an overall adaptation to a more suitable expression system was necessary. Therefore, a more robust and efficient bacterial strain was chosen that allowed for more stringent background expression control and expressed additional rare transfer ribonucleic acids (tRNAs) not present in the previously used strain. Protein purification also needed some adaptation, as the method had to be adjusted to the HPLC equipment present at that time. In the end, a highly pure fraction of recombinant HMGA1a, HMGA1b, and HMGA2 could be produced for the experiments whose results were published in the Tissue Engineering A journal (Richter et al. 2009).

Administration of HMGA1a and HMGA1b as well as HMGA2 showed a highly significant effect on cell proliferation at levels of 10µg/ml or 100µg/ml protein, respectively, compared to the non-HMGA treated control group, nearly doubling the proliferation rate in case of 100µg/ml HMGA2. HMGA1b was the only variant causing a significant impact on proliferation already at the lower concentration of 1µg/ml protein, but no difference to HMGA1a or HMGA2 could be observed at the higher amounts of protein. Concerning dosage, a statistically significant difference was deduced between 1µg/ml and 100µg/ml for both HMGA1a and HMGA2, but not HMGA1b.

The stimulation of chondrocyte proliferation induced by HMGA proteins is probably related to their proper function in the developing embryo, albeit with different starting conditions in cells derived from fully differentiated adult tissue. HMGA2 was shown to have direct influence on chromatin state in human embryonic stem cell lines (Li et al. 2006), regulating key developmental genes and thus having influence on stemness and proliferation (Li et al. 2007). HMGA1 function in stem cells has been studied to a lesser degree than HMGA2, but, amongst others, involvement in embryonic stem cell lympho-haematopoietic differentiation (Battista et al. 2003) and adipocyte differentiation (Esposito et al. 2009) was demonstrated. Application of HMGA to

chondrocytes derived from adult tissue might thus influence the cells in these regards. Uptake of extracellular HMGA resulting in higher intracellular concentration and subsequent nuclear transport might shift the cells' chromatin into a more embryonic, de-differentiated or stem cell like state, facilitating cell growth usually not found in differentiated cells. A similar mechanism was observed in tumours with re-expression of HMGA2 and further oncofoetal genes, leading to de-differentiation and cell growth in a process subsequently termed reverse embryogenesis (Johnson et al. 2005; Park et al. 2007; Peter 2009). On this account, the tumourigenic potential of HMGA proteins has to be taken into account in a putative therapeutic use. In stem cells, HMGA2 presence has been associated with stemness, i.e. growth- and differentiation potential, which is precondition for volitional steering of cell growth and regeneration of tissues. In tumours however, reversed HMGA2 expression has been associated with genomic instability and unwanted tumour growth. One factor accounting for the tumourigenic potential of HMGA2 is the suppression of non-homologous end joining repair (NHEJ) of DNA double strand breaks (DSBs). HMGA2 overexpression led to accumulation of DNA damage and dysregulation of NHEJ by stimulating phosphorylation of the catalytic subunit of DNA-PKc (DNA-dependent protein kinase) and reducing end binding of the DNA targeting Ku80 subunit, leading to delayed release of DNA-PKcs at DSB sites which is associated with impaired repair (Li et al. 2009). Double strand breaks may result from exogenous sources such as ionising radiation or endogenously generated reactive oxygen species and mechanical stress on the chromosomes, as well as lesions that are encountered by DNA replication forks (Khanna et al. 2001). Furthermore, intended DSBs occur in recombination of homologous chromosomes during meiosis and further cellular processes (Khanna et al. 2001). Increased HMGA2 presence in cells undergoing these processes may thus lead to hampered DNA repair resulting in chromosomal damage that may result in further degradation of cellular integrity. This is proven by results from Li et al. (2009), where ectopic expression of HMGA2 in normal human lung fibroblast WI-38 cells led to spontaneous chromosome aberrations including tetraploidy, nonclonal chromosome breaks and gaps and a translocation. Similar to HMGA2, genome instability has also been associated with ectopic presence of HMGA1 proteins. In human prostate cancer cell lines, induced overexpression of full-length HMGA1a led to enhanced presence and heterogeneity of unbalanced chromosomal rearrangements, which are common in solid human tumours (Takaha et al. 2002). In human transfected MCF-7 (Michigan Cancer Foundation - 7) cells overexpressing HMGA1a, presence of the protein led to increased UV sensitivity by inhibiting global genomic nucleotide excision repair of UV-induced cyclobutane

pyrimidine dimer (CPD) lesions (Adair et al. 2005). This was later attributed to negative regulation of *XPA* (Xeroderma pigmentosum complementation group A) through HMGA1 interacting with the *XPA* promoter (Adair et al. 2007). Further involvement of HMGA proteins in cellular repair processes was shown in the interaction of HMGA1 and ATM (Ataxia-telangiectasia mutated) kinase. In cellular response to DNA damage such as DSBs, ATM induces cell cycle arrest and DNA repair by phosphorylating several substrates intended to reduce chromosomal breakage and enhancing cell survival, including HMGA1b (Pentimalli et al. 2008). Later it was reported that HMGA2 as well interacts with and gets phosphorylated by ATM in irradiated HEK (human embryonic kidney) 293 cells. Both HMGA1 and HMGA2 proteins were demonstrated to positively regulate ATM expression by binding to the ATM promoter (Palmieri et al. 2011).

However, in treating cells with HMGA proteins to induce cell growth, as opposed to tumours with constant HMGA presence e.g. due to loss of *let-7* regulation, the application of recombinant HMGA for growth enhancement of normal cells is a temporary process exposing the cells to HMGA only for a short period of time. This might just provide an initial growth enhancing “start signal” bringing forward enhanced cell growth without the unwanted consequences of constitutive HMGA expression.

Additionally, as opposed to facilitated growth of differentiated cells, the observed cell proliferation might also be caused by stem or progenitor cells present in adult hyaline cartilage. While hyaline cartilage was initially thought to be a post-mitotic tissue without considerable cellular turn-over (Hulth et al. 1972; Aigner et al. 2001), it was later shown that it contains mesenchymal progenitor cells (MPCs) (Alsalameh et al. 2004) that are differentially distributed across the superficial, middle, and deep zones of articular cartilage (Dowthwaite et al. 2004; Grogan et al. 2009), with the highest number present in the superficial zone. Exclusive to the superficial zone are side population cells (SPCs) (Goodell et al. 1996) exhibiting stem cell properties (Hattori et al. 2007). The distribution of both MPCs and SPCs is thus consistent with the postulated appositional mechanism of cartilage growth subsequent to the formation of the secondary centre of ossification (Archer et al. 1994; Hayes et al. 2001). HMGA presence might thus enhance proliferation of these cells leading to increased chondrocyte growth through their influence on stemness of stem or progenitor cells as was proposed above for differentiated chondrocytes, involving processes that are related to stem cell aging or rather stem cell self renewal and subsequent chondrocyte formation. While younger stem cells are generally able to self-renew, stem cells are susceptible to aging as was shown in murine haematopoietic stem

cells (Morrison et al. 1996). In murine neural stem cells, this process could be linked to regulation of $p16^{Ink4a}$ and $p19^{Arf}$ by Hmga2 (Nishino et al. 2008). Hmga2 promoted neural stem cell self renewal by reducing *p16*^{Ink4a} and *p19*^{Arf} expression in young mice. Decreased Hmga2 levels in old mice, i.e. due to increase of *let-7* miRNAs, thus led to an increase of *p16*^{Ink4a} and *p19*^{Arf}, which in turn inhibited self-renewing cell divisions (Nishino et al. 2008). However, recent research conducted on uterine fibroids overexpressing *HMGA2* (Markowski et al. 2010) as well as on human and canine adipose tissue derived stem cells (ADSCs) expressing *HMGA2* via induction with FGF1 (fibroblast growth factor 1 (acidic)) (Markowski et al. 2011) revealed opposite results showing increased expression of *p14*^{Arf}, the human orthologue of murine *p19*^{Arf}, in the presence of HMGA2. Upregulation of *p14*^{Arf} was postulated an endogenous protection mechanism in activated stem cells linking self-renewal with protection of their genome (Markowski et al. 2011), as $p14^{Arf}$ has positive influence on TP53 (tumour protein p53) by interacting with MDM2 (Mdm2 p53 binding protein homolog (mouse)) (Pomerantz et al. 1998; Zhang et al. 1998).

HMGA2 application to cartilage might thus improve stem cell self renewal leading to improved cell growth and regeneration, while at the same time a potential increase of *p14*^{Arf}-expression might prove beneficial for the genomic stability of the proliferating cells. In addition to these processes, the chondrogenic properties of HMGA2 as shown by Kubo et al. (2006), or HMGA1 as indicated by Martinez Hoyos et al. (2004), might also direct the growing cells to remain in or regain the chondrocyte phenotype at the same time. However, further research is required for assessing this possibility. Furthermore, instead of these nuclear processes explaining the growth enhancing effect of HMGA on chondrocytes, also other, yet unknown extracellular functions might play a role. For example, HMGB1, which is a member of the HMGB family of proteins, was initially known to be a nuclear protein but was later shown to have additional extracellular functions. Although there is no evidence yet, similar functions of HMGA proteins cannot be ruled out completely.

During the subsequent research conducted on HMGA2 peptide variants, focus was hence also set onto the cellular localisation of the administered peptides. These peptides were developed to allow for the production of a biologically active agent without the need for biological expression systems, ruling out possible side effects such as unwanted modifications or contaminations that might hamper its putative future therapeutic use, as well as to facilitate the necessary approval for use as a therapeutic agent in the long term. Precondition for a synthetic HMGA-based therapeutic agent is thus a similar or even better growth enhancing property compared to the recombinant protein. Due to constraints of peptide synthesis where

the length of a synthesised peptide is limited by the coupling efficiency of each elongation step, two shorter variants of the HMGA2 protein were synthesised, comprising the first 60 aa (HMGA2-A) or the first 80 aa (HMGA2-B) of HMGA2, respectively. Both peptides thus contained the first two AT-hook domains including the nuclear localisation signal (NLS) of the second AT-hook, but not the carboxyterminal domain, and in case of HMGA2-A, the third AT-hook domain of the complete protein. Truncated HMGA2 comprising the AT-hooks was demonstrated to retain its DNA binding capability (Geierstanger et al. 1994) and remained functional in mice, albeit leading to gigantism and lipomatosis (Battista et al. 1999). However, this unwanted effect in mice is caused by the constitutive presence of the truncated but functional Hmga2 protein due to loss of miRNA binding sites in the 3'-UTR for Hmga2 mRNA regulation, and not to the truncation itself, as both mice over-expressing the wild type Hmga2 or a truncated variant thereof lacking the carboxyterminal part develop a similar phenotype and are susceptible to e.g. pituitary adenomas (Fedele et al. 2002). As the HMGA2 peptides are only administered for a short period of time for enhancing cell growth e.g. during amplification for autologous transplantation, and will be degraded and removed over the course of time if not reapplied, these side effects will most likely be of no importance in a future therapeutic use of the HMGA2 peptides.

To evaluate the growth inducing potential of the peptides, they were administered in the same experimental setup utilising isolated porcine chondrocytes as before, with full length HMGA2 and untreated cells as control. For comparability, molarities were chosen instead of mass (10µM instead of 100µg/ml), and for the two peptides, an additional incubation with 50µM peptide was chosen to check for any higher dosage dependent effect. Supplementing the proliferation ELISA, the cellular localisation of the 80 aa peptide was determined by fluorescence microscopy of cells supplied with a carboxyfluorescein-labelled variant thereof. Concurrently, cell integrity and thus viability was determined. The cell proliferation ELISA revealed that application of the full length HMGA2 protein led to a statistically significant increase in cell proliferation compared to the untreated control as described in the previous paper (Richter et al. 2009). The shorter 60 aa HMGA2-A fragment also indicated this statistically significant growth inducing effect at both concentrations of 10µM and 50µM compared to the untreated control, but there was no difference between 10µM and 50µM HMGA2-A. As there was no statistically significant difference of induced cell growth between the HMGA2-A peptide and the recombinant HMGA2, one can conclude that the HMGA2-A fragment is an adequate alternative to the recombinantly produced full length protein. Conversely, the longer HMGA2-B fragment could not

evoke any statistically significant effects compared to the untreated control group at both concentrations of 10µM and 50µM, although it showed comparable effect to the full length protein at 10µM. At 50µM however, there even was a statistically significant decrease in cell proliferation compared to the full length protein at 10µM, indicating a possible detrimental effect on cell proliferation at higher levels. This and the fact that there is no increase in case of HMGA2-A at 50µM compared to 10µM might indicate that saturation of cells or their DNA occurs at higher concentrations of the peptides, negatively influencing cell proliferation. In human embryonic stem (hES) cells, Li et al. (2006) determined the number of HMGA2 molecules per hES cell to be in the range of 10^5 molecules, considering the interaction of one HMGA2 molecule with one nucleosome core particle (NCP) under normal physiological conditions. Nevertheless, saturation of reconstituted NCPs with HMGA2 was only achieved at a molar ratio of 12:1, indicating that a huge amount of HMGA2 might still bind to DNA respectively nucleosomes, but might lead to detrimental effects on cellular processes. Concerning cellular localisation, internalisation of the fluorescently labelled variant of HMGA2-B (HMGA2-CC) could be detected, with green fluorescence both in the cytoplasm and the nucleus. Concurrent propidium iodide staining indicated intact cell membranes, leading to the conclusion that the HMGA2-CC peptide was taken up by living cells. While the mechanism for cellular uptake is unknown, the nuclear internalisation is probably due to the NLS of the HMGA2 peptide. Cattaruzzi et al. (2007) narrowed down the NLS to amino acid residues 45-53 of HMGA2, which is located within the second AT-hook and present in both the 60 aa and 80 aa peptide variants, as well as the full length recombinant HMGA2 protein. While there were no labelled variants of the 60 aa peptide or the full length protein, nuclear transport can also be assumed for these molecules due to presence of the NLS in both molecules. Concerning peptide integrity, it can be deduced from the green stain in the nucleus that HMGA2-CC comprising at least the fluorescently labelled N-terminus up to the second AT-hook was located into the nucleus, if not the complete peptide. For HMGA2-A or HMGA2, no conclusion can be made concerning molecule integrity, however as both showed proliferation stimulating effects on the cells, a functional molecule entering the nucleus can be assumed. Fluorescence in the cytoplasm might thus be caused by either intact molecules that are not yet transported into the nucleus, or degraded peptides lacking the NLS.

Concluding, the stimulation of cell proliferation caused by the HMGA2-A peptide allows for further research of this fragment's potential use in tissue regeneration of articular cartilage, as it induces comparable proliferation enhancement as the full

length HMGA2. As the peptide can be synthesized up to industrial scales without any biological expression systems it might be a promising agent for enhancing cell proliferation not only in tissue engineering of cartilage, but also in further fields where improved cell growth is an issue.

One potential future application of this peptide in tissue engineering might be the colonisation of scaffolds in treating cartilage defects. These defects are not limited to humans, but also occur in animals such as the dog. Cartilage damage in dogs may be age or injury related, but also happens as the consequence of disease such as Osteochondritis dissecans (Newton et al. 1985), which primarily develops in the larger breeds and is thought to have hereditary nature (Morgan et al. 2000). Development of a valuable replacement therapy in dogs might then be transferred to humans with the dog acting as model organism for human therapy.

Beta-tricalcium phosphate (β-TCP) is an established biodegradable synthetic material for the treatment of bone defects especially in oral surgery, but is also used in other fields where bone rebuilding is an issue. This led to the approach of utilising β-TCP in the related field of cartilage regeneration in the construction of a bone-cartilage replacement. While there was previous research on the suitability of this material for cartilage regeneration in sheep and pig experimental models (Guo et al. 2004; Gotterbarm et al. 2006; Jiang et al. 2007), no data were yet available for dogs. Experiments concerning the colonisation capability of β-TCP cylinders with canine chondrocytes were conducted using both cartilage chips and isolated cells grown in cell culture, albeit, due to the preliminary nature of these trials, no protein or peptide application was done yet. Following colonisation and the subsequent incubation period, the cylinders that were inoculated with a solution of isolated chondrocytes showed an evenly distributed cell growth on the cylinders and within the cylinders' macropores. Viability staining revealed only sporadic cell death, indicating compatibility of the material and the cells vital processes. Further detail revealed by scanning electron microscopy showed the cells' filopodia anchoring onto the matrix surface and entering the construct's micropores. Moreover, cell to cell contact could be detected in visible light as well as scanning electron microscopy. Cells studded with cartilage chips, on the other hand, exhibited much lower colonisation which only occurred within the region of the drill holes with fastened chips. This is comparable to the only marginal growth rate of chondrocytes in damaged areas of the injured joint that is not sufficient to fill up the defective area (Buckwalter 1998). Nevertheless, vitality staining proved the viability of these cells. Concluding, seeding of β-TCP constructs with chondrocytes might be a useful approach in dogs when using isolated chondrocytes that were amplified in cell culture. However, further research is needed

in terms of long term colonisation of these constructs *in vitro* and *in vivo*. Successful trials performed in pig and sheep (Guo et al. 2004; Gotterbarm et al. 2006; Jiang et al. 2007) indicate this being a useful strategy for cartilage regeneration in dogs or in the long term, in humans.

As is evident from the previous experiments and discussion, the dog can serve as a model organism, in this case for the research and treatment of cartilage damage. However, research is not only limited to this field, and as was mentioned before in this thesis and in many research papers, the dog is also an important model for other human diseases, in particular due to comparable disease development because of similar anatomy, physiology and living conditions (Ostrander et al. 1997; Kuska 1999; Ostrander et al. 2000; Ostrander et al. 2000; Starkey et al. 2005; Shearin et al. 2010). The canine was proven as a suitable model for human aging, as elderly dogs also develop similar aspects of neuropathology, show age-related cognitive dysfunction and are deficient in neuropsychological tests of cognitive function (Cummings et al. 1996). Beta-amyloid accumulation in the aged canine brain makes the dog a model of early plaque formation in Alzheimer's disease, with the advantage of better tissue availability and faster disease development due to the shorter lifespan as opposed to some primates (Cummings et al. 1993). Equally in cancer, the dog is an appropriate model for the human counterparts, including lymphomas, mammary tumours, soft tissue sarcomas, and osteosarcomas. Canine lymphoma resembles human non-Hodgkin's lymphoma in clinical presentation, pathology, and treatment response. Mammary tumours are the most common tumour in the female dog, and are also markedly hormonal-dependent. Likewise, soft tissue sarcomas and osteosarcomas closely resemble human tumours (Hahn et al. 1994). Additionally, the dog is the only known nonhuman species to spontaneously develop prostate cancer (Rivenson et al. 1979) sharing similarities in age-dependence of tumour onset, tumour morphology and metastasis (Waters et al. 1998; Cornell et al. 2000). The connection between human prostate cancer and HMG proteins was shown before, as HMGB1 (Amphoterin) was linked to metastatic prostate cancer (Kuniyasu et al. 2003; Ishiguro et al. 2005), and increased expression of HMGA1 was determined in high grade prostatic cancer (Tamimi et al. 1993; Tamimi et al. 1996). Furthermore, HMGA2 expression could be shown in the Dunning rat prostatic cancer model (Diana et al. 2005), as well as in a cell line derived from a canine prostatic cancer (Winkler et al. 2005), and in canine prostate tumours (Winkler et al. 2007). These facts make clear the importance of knowledge on structure and function of canine genes, which resulted in the complete sequencing of the canine genome (Lindblad-Toh et al. 2005). However, during the course of this thesis, the sequencing of the canine

genome was not yet completed, or provided only partial information concerning some genes of interest including the canine orthologues of the HMG proteins. Therefore, work conducted in collaboration included the characterisation of the canine *HMGA1* and *HMGB1* genes as well as the identification and development of tools (e.g. sequences, BAC clones and expression vectors) for investigating their localisation and function. While initially being mapped to CFA 23 (Becker et al. 2003), probably due to cross-hybridisation of the BAC clone chosen for FISH or caused by presence of a pseudo-gene, the true chromosomal localisation of the canine *HMGA1* was later located on CFA 12 (Beuing et al. 2008). This is consistent with synteny of HSA 6p21 and parts of CFA 12 (Yang et al. 1999), as well as with the canine genome assembly (Lindblad-Toh et al. 2005). *HMGB1* was located on CFA 25, but no synteny of HSA 13 and CFA 25 was indicated by Yang et al. (1999). Nevertheless, the later released canine genome assembly confirmed the results obtained from BAC-mapping. Sequencing of parts of the canine *HMGA1* and *HMGB1* genes revealed large identity in the coding sequences (95% for both HMGA1 and HMGB1, respectively), and the resulting proteins (100% for HMGA1a, HMGA1b, and HMGB1), compared to their human orthologues (Murua Escobar et al. 2003; Murua Escobar et al. 2005), facilitating knowledge transfer of the extensive research already conducted in the field of human HMGs to canine genetics and medicine for the benefit of the dog. The other way round, newly evaluated therapeutic approaches in the dog might be easily transferable to human medicine. In addition to the work conducted on canine *HMG* genes, minor focus in this thesis was also set on canine *RAS* genes, including mapping and SNP analyses thereof. The chromosomal location of the canine *NRAS* could be mapped to CFA 17 (Richter et al. 2004), which is consistent with the known synteny of canine chromosome 17 and the centromer-proximal regions 11.1–13.3 of the p-arm of human chromosome 1 (Yang et al. 1999). *RAS* gene hot spot mutations frequent found in human tumours, however, could not be detected for *NRAS* in the canine fibrosarcomas or *KRAS* in the canine melanomas investigated (Richter et al. 2005).

Summarising the main aspects of this thesis, proteins of the HMGA family or peptides derived thereof may prove beneficial for volitional and controlled cell growth e.g. in tissue regeneration. As opposed to uncontrolled re-expression of these embryonic proteins in adult cells that usually promotes tumour development and progression, temporary application of the proteins or peptides might stimulate cell proliferation without the unwanted side-effects of tumourigenesis. While there is a lot of further research necessary for reaching the goal of a clinical application of these proteins or derivated peptides, results gained during the course of this thesis indicate

a possible future role of these proteins in overcoming some of the problems that are still hampering cartilage regeneration up till today. The combination of HMGA enhanced cell growth for the colonisation of scaffolds is a promising aspect in tissue engineering, which might be evaluated in model organisms for human disease such as the dog. Furthermore, as HMGA proteins are involved in the regulation of a large number of genes in the developing embryo not limited to cartilage but also including other tissues such as smooth muscle, liver, skin, etc. (Gattas et al. 1999) the knowledge gained on cartilage in this thesis might be transferrable to other fields as well, opening up further possibilities for improving tissue engineering approaches for regeneration. Thus, HMGA use in tissue engineering might reverse the usually unwanted properties of these proteins in tumours to a volitional, yet time-limited presence for the better of the affected patients.

5. Summary

Hyaline cartilage damage is widespread amongst the population, with injuries usually not healing to a satisfactory degree due to the specific nature of this avascular and aneural tissue that hinders cell renewal and proliferation. Tissue engineering strategies have been developed to overcome these problems by e.g. *in vitro* amplification of chondrocytes from undamaged joint regions for autologous transplantation into the defective area. However, there is room for improvement by e.g. enhancing cell growth for minimising the waiting time between explantation and re-implantation.

One new strategy that was evaluated during the course of this thesis was the suitability of employing proteins of the embryonic high mobility group AT-hook (HMGA) family for influencing cell growth. Usually being involved in processes regulating cell growth during embryonic development, they are also active in processes controlling stemness and aging of stem cells, indicating a possible role in steering cell growth by means of HMGA application. In a study employing recombinantly produced HMGA1a, HMGA1b, and HMGA2 proteins, it could be shown that there is indeed improved cell proliferation with a nearly two-fold increase of chondrocyte proliferation in cells exposed to HMGA1b or HMGA2, respectively, and a slightly lower proliferative effect of cells exposed to HMGA1a (1.7 fold).

Regarding a future potential medical use, shorter peptide variants of the HMGA2 protein comprising two or three of the DNA-binding AT-hooks including the nuclear localisation signal were evaluated, overcoming some of the obstacles of recombinantly produced biological agents including unwanted modifications or contaminations from the expression system. Nuclear transport could be proven and a comparable proliferation enhancing effect comparable to that of the complete recombinant HMGA2 protein was evoked by the shorter 60 amino acid variant. This opens up possibilities of employing this peptide in e.g. tissue engineering of hyaline cartilage, by enhancing cellular growth of chondrocytes.

One future application of HMGA-induced cell growth is the improved seeding of scaffolds with chondrocytes for cartilage regeneration. In a preliminary study for canine cartilage regeneration, yet without the administration of HMGA, beta-tricalcium phosphate cylinders were seeded with isolated cells amplified *in vitro*, or studded with cartilage chips, respectively, to check for acceptance of these constructs by the cells, as there were no data available for the dog. While the construct was not suitable for seeding based on complete cartilage chips, which is

probably due to the low proliferation potential of chondrocytes embedded in their usual extracellular matrix, it was well accepted and colonised by isolated chondrocytes grown in cell culture. In a future combination with the growth enhancing properties of HMGA proteins or the 60 aa HMGA2 peptide, seeding of these structures with amplified chondrocytes might thus be a promising aspect for new strategies of treating cartilage damage.

As the dog is an established model organism for human disease including but not limited to cancer, additional work was done to elucidate structure and function of canine genes whose human orthologues are known to be involved in the onset and development of disease. This included the canine HMGA1 and HMGB1 genes, and, to a lesser degree, *RAS* genes. By contributing to ongoing research on these canine genes, structure and function of the canine *HMGA1* and *HMGB1* genes could be determined, while no involvement of *RAS* hot-spot mutations common in humans could be detected in a subset of canine fibrosarcomas and melanomas. The high structural and functional similarity of the *HMG* genes and the resulting proteins nevertheless promise transferability of data obtained between both humans and dogs for the benefit of both species. Focusing on cartilage regeneration, methods of HMGA-assisted cartilage regeneration initially developed in dogs might thus be readily transferable to humans.

6. Zusammenfassung

Schäden des hyalinen Gelenkknorpels sind in der Bevölkerung weit verbreitet, wobei die Verletzungen in der Regel nicht auf ein zufriedenstellendes Niveau verheilen. Dies liegt in der spezifischen Struktur des Knorpels begründet, der als avaskuläres und aneurales Gewebe die Zellerneuerung und -proliferation erschwert. *Tissue-engineering*-Strategien wurden entwickelt, um einige dieser Probleme zu überwinden, z. B. durch autologe Chondrozytentransplantation von Zellen, die aus nicht betroffenen Gelenkarealen explantiert und zur Reimplantation in den Defekt *in vitro* vermehrt wurden. Dennoch gibt es Bedarf, diese Methoden weiter zu verbessern, z. B. in Hinblick auf verbessertes Zellwachstum zur Minimierung der Wartezeit zwischen der Explantation und Reimplantation.

Eine neue Strategie, die im Rahmen dieser Arbeit evaluiert wurde, hatte die Anwendung der High-Mobility-Group-AT-Hook (HMGA) Proteine zur positiven Beeinflussung des Zellwachstums als Ziel. HMGA-Proteine sind üblicherweise in der Steuerung des Zellwachstums während der Embryonalentwicklung involviert. Sie beeinflussen ebenfalls Stammzelleigenschaften („stemness“) und Stammzellalterung, so dass davon ausgegangen werden kann, dass eine Steuerung des Zellwachstums mittels HMGA-Proteinen möglich ist. Eine im Rahmen dieser Dissertation durchgeführte Studie ergab, dass rekombinant hergestellte HMGA1a-, HMGA1b- und HMGA2-Proteine tatsächlich Einfluss auf das Zellwachstum nehmen konnten und im Falle von HMGA1b und HMGA2 zu einer fast verdoppelten Wachstumsrate der Chondrozyten führten, während es bei HMGA1a eine etwas geringere, 1,7-fach erhöhte Proliferationsrate im Vergleich zur unbehandelten Kontrollgruppe gab.

Im Hinblick auf eine zukünftige medizinische Anwendung wurden zusätzlich kürzere Peptidvarianten des HMGA2-Proteins, die zwei oder drei der DNA-bindenden AT-hooks sowie das Kernlokalisierungssignal umfassten, hergestellt und ebenfalls im Hinblick auf die proliferationsfördernde Wirkung untersucht. Mit der Peptidsynthese sollten eventuelle Probleme bei der Herstellung und Zulassung rekombinanter biologisch aktiver Wirkstoffe umgangen werden, wie z. B. Modifizierung der Proteine oder Kontaminationen aus dem Expressionssystem. Kerntransport konnte für beide Peptide nachgewiesen werden und ein dem rekombinanten Wildtyp-HMGA2 vergleichbarer proliferationsfördernder Effekt zeigte sich bei der kürzeren, 60 Aminosäuren umfassenden HMGA2-Peptidvariante.

Dies ermöglicht weitergehende Versuche in der Geweberegeneration durch *Tissue Engineering*, indem das Zellwachstum des hyalinen Knorpels verbessert wird.

Eine zukünftige Anwendung des HMGA-induzierten Zellwachstums könnte z.B. die verbesserte Besiedlung von Strukturen (*scaffolds*) für die Knorpelregeneration mit Chondrozyten darstellen. In einer Vorstudie zur Knorpelregeneration beim Hund wurde - noch ohne zusätzliche HMGA-Applikation - untersucht, inwieweit sich Beta-Tricalciumphosphatzylinder zur Besiedlung mit caninen Chondrozyten eignen, da hier noch keine Daten für den Hund vorlagen. Dazu wurden vereinzelte und in Zellkultur vermehrte Zellen oder komplette Knorpelchips auf die Zylinder aufgebracht bzw. daran befestigt und die Konstrukte im Hinblick auf die Besiedlung mit Zellen untersucht. Während sich die Besiedlung mit Knorpelchips als Ausgangsbasis als nicht geeignet erwies, höchstwahrscheinlich weil Chondrozyten in hyalinem Knorpel ein geringes Wachstumspotential aufzeigen, wurden die Konstrukte sehr gut von den vereinzelten in Zellkultur vermehrten Chondrozyten angenommen und komplett besiedelt. In Kombination mit dem 60 Aminosäuren umfassenden HMGA2-Peptid zur Verbesserung des Zellwachstums könnte sich dieser Ansatz daher als vielversprechend für die Behandlung von Knorpelschäden des Hundes erweisen.

Da der Hund ein etablierter Modellorganismus für Krebs und andere Krankheiten des Menschen ist, wurden zur Ergänzung dieser Arbeit Studien auf dem Gebiet der Hundegenetik durchgeführt, um Struktur und Funktion von Genen, die beim Menschen mit Krankheitsentstehung assoziiert sind, zu bestimmen. Dabei handelte es sich um Gene der High-Mobility-Group Proteine sowie als Nebenaspekt um *RAS*-Gene. Durch Mitarbeit in laufenden Projekten konnten Struktur und Funktion der *HMGA1*- und *HMGB1*-Gene des Hundes entschlüsselt werden. Hingegen zeigte sich bei den *RAS*-Genen, dass beim Menschen häufig vorkommende Mutationen an bestimmten Hot-Spots zumindest in den untersuchten Hundetumoren, bestehend aus Fibrosarkomen und Melanomen, nicht auftraten.

Jedoch verspricht die hohe strukturelle und funktionale Ähnlichkeit der HMG-Gene und der davon kodierten Proteine bei Mensch und Hund die Möglichkeit, gewonnenes Wissen zwischen den Spezies transferieren zu können. Im Hinblick auf die HMGA-unterstützte Knorpelregeneration besteht daher die Möglichkeit, eine anfänglich beim Hund entwickelte Therapie an den Menschen anzupassen.

7. Complete list of publications

7.1. Peer-reviewed papers

1: Richter A, Lübbing M, Frank HG, Nolte I, Bullerdiek JC, von Ahsen I (2011). High-mobility group protein HMGA2-derived fragments stimulate the proliferation of chondrocytes and adipose tissue-derived stem cells. Eur Cell Mater 21: 355-363.

2: Winter N, Meyer A, Richter A, Krisponeit D, Bullerdiek J. Elevated levels of HMGB1 in cancerous and inflammatory effusions (2009). Anticancer Res 29: 5013-5017.

3: Petersen S, Soller JT, Wagner S, Richter A, Bullerdiek J, Nolte I, Barcikowski S, Murua Escobar H. Co-transfection of plasmid DNA and laser-generated gold nanoparticles does not disturb the bioactivity of GFP-HMGB1 fusion protein (2009). J Nanobiotechnology 7: 6.

4: Richter A, Hauschild G, Murua Escobar H, Nolte I, Bullerdiek J (2009). Application of high-mobility-group-A proteins increases the proliferative activity of chondrocytes in vitro. Tissue Eng Part A 15: 473-477.

5: Hauschild G, Muschter N, Richter A, Ahrens H, Gosheger G, Fehr M, Bullerdiek J (2009). Cartilage replacement in dogs - A preliminary investigation of colonization of ceramic matrices. Vet Comp Orthop Traumatol 22: 216-221.

6: Beuing C, Soller JT, Muth M, Wagner S, Dolf G, Schelling C, Richter A, Willenbrock S, Reimann-Berg N, Winkler S, Nolte I, Bullerdiek J, Murua Escobar H (2008). Genomic characterisation, chromosomal assignment and in vivo localisation of the canine high mobility group A1 (HMGA1) gene. BMC Genet 9: 49.

7: Richter A, Murua Escobar H, Günther K, Soller JT, Winkler S, Nolte I, Bullerdiek J (2005). RAS gene hot-spot mutations in canine neoplasias. J Hered 96: 764-765.

8: Murua Escobar H, Soller JT, Richter A, Meyer B, Winkler S, Bullerdiek J, Nolte I (2005). "Best friends" sharing the HMGA1 gene: comparison of the human and canine HMGA1 to orthologous other species. J Hered 96: 777-781.

9: Murua Escobar H, Günther K, Richter A, Soller JT, Winkler S, Nolte I, Bullerdiek J (2004). Absence of ras-gene hot-spot mutations in canine fibrosarcomas and melanomas. Anticancer Res 24: 3027-3028.

10: Richter A, Murua Escobar H, Günther K, Meyer B, Winkler S, Dolf G, Schelling C, Nolte I, Bullerdiek J (2004). The canine NRAS gene maps to CFA 17. Anim Genet 35: 355-356.

11: Winkler S, Murua Escobar H, Günther K, Richter A, Dolf G, Schelling C, Bullerdiek J, Nolte I (2004). The canine KRAS2 gene maps to chromosome 22. Anim Genet 35: 350-351.

12: Murua Escobar H, Soller JT, Richter A, Meyer B, Winkler S, Flohr AM, Nolte I, Bullerdiek J (2004). The canine HMGA1. Gene 330: 93-99.

13: Meyer B, Murua Escobar H, Hauke S, Richter A, Winkler S, Rogalla P, Flohr AM, Bullerdiek J, Nolte I (2004). Expression pattern of the HMGB1 gene in sarcomas of the dog. Anticancer Res 24: 707-710.

14: Becker K, Murua Escobar H, Richter A, Meyer B, Nolte I, Bullerdiek J (2003). The canine HMGA1 gene maps to CFA 23. Anim Genet 34: 68-69.

15: Murua Escobar H, Meyer B, Richter A, Becker K, Flohr AM, Bullerdiek J, Nolte I (2003). Molecular characterization of the canine HMGB1. Cytogenet Genome Res 101: 33-38.

7.2. Oral presentations

“High-Mobility-Group-Proteins in Canine Disease”
27th Annual VCS Conference, Fort Lauderdale, FL, USA, 1-4 November 2007

7.3. Poster presentations

“Absence of ras-gene hot-spot mutations in canine fibrosarcomas and melanomas”
2nd International Conference on Advances in Canine and Feline Genomics. Utrecht, The Netherlands. 14-16 October 2004.

8. Abbreviations

aa	Amino acid
ACT	Autologous chondrocyte transplantation
ADSC	Adipose tissue derived stem cell
ANOVA	Analysis of variance
ATM	Ataxia-telangiectasia mutated
BAC	Bacterial artificial chromosome
β-TCP	Beta-tricalcium phosphate
BLAST	Basic local alignment search tool
BrdU	Bromodeoxyuridine
CFA	Canis lupus familiaris / Canis familiaris
COL1A2	Collagen, type I, alpha 2
COL11A2	Collagen, type XI, alpha 2
CPD	Cyclobutane pyrimidine dimer
DAPI	4′,6-Diamidin-2-phenylindol
DNA	Deoxyribonucleic acid
DSB	Double strand break
E. coli	Escherichia coli
ELISA	Enzyme-linked immunosorbent assay
FBS	Foetal bovine serum
FGF1	Fibroblast growth factor 1 (acidic)
Fim3	Friend-murine leukemia virus integration site *3* homolog
FISH	Fluorescence *in situ* hybridisation
h	Hour
H2A	Histone H2A
H_3BO_3	Boric acid
HEK 293	Human embryonic kidney 293
hES	Human embryonic stem
HMG	High mobility group (canonical)
HMG1	High mobility group protein 1 (HMGB1)
HMG2	High mobility group protein 2 (HMGB2)
HMG3	High mobility group protein 3 (HMGB3)
HMG14	High mobility group protein 14 (HMGN1)
HMG17	High mobility group protein 17 (HMGN2)
HMGA	High mobility group AT-hook

HMGA1	High mobility group AT-hook 1
Hmga1	Murine high mobility group AT-hook 1
HMGA1a	High mobility group AT-hook 1 isoform a
HMGA1b	High mobility group AT-hook 1 isoform b
HMGA2	High mobility group AT-hook 2
Hmga2	Murine high mobility group AT-hook 2
HMGB	High mobility group box
HMGB1	High mobility group box 1
HMGB2	High mobility group box 2
HMGB3	High mobility group box 3
HMGI	High-mobility group protein isoform I (HMGA1a)
HMGI(Y)	High-mobility group protein isoforms I and Y (HMGA1)
HMGIC	High-mobility group protein isoform I-C (HMGA2)
HMGN	High mobility group nucleosome binding domain
HMGN1	High mobility group nucleosome binding domain 1
HMGN2	High mobility group nucleosome binding domain 2
HMGY	High-mobility group protein isoform Y (HMGA1b)
HPLC	High-performance liquid chromatography
HSA	Homo sapiens
IFNB1	Interferon, beta 1, fibroblast
IGF1	Insulin-like growth factor 1 (somatomedin C)
IGF2BP1	Insulin-like growth factor 2 mRNA binding protein 1
IL4	Interleukin 4
kDa	Kilodalton
KRAS	V-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog
LMO2	LIM domain only 2 (rhombotin-like 1)
LIN28B	Lin-28 homolog B (C. elegans)
LPP	LIM domain containing preferred translocation partner in lipoma
M	Molar
MCF-7	Michigan Cancer Foundation - 7
MDM2	Mdm2 p53 binding protein homolog (mouse)
Mecom	Murine MDS1 and EVI1 complex locus gene
MEF	Mouse embryonic fibroblasts
µM	Micromolar
min	Minute
miRNA	Micro ribonucleic acid

ml	Millilitre
mm	Millimetre
mM	Millimolar
MPC	Mesenchymal progenitor cell
mRNA	Messenger ribonucleic acid
MWCO	Molecular weight cut off
MYC	V-myc myelocytomatosis viral oncogene homolog (avian)
NaCl	Sodium chloride
NCBI	National Center for Biotech Information
NCP	Nucleosome core particle
NF-kappaB	Nuclear factor NF-kappa-B p50/p65 heterodimer
NHEJ	Nonhomologous end joining repair
NLS	nuclear localisation signal
NRAS	Neuroblastoma RAS viral (v-ras) oncogene homolog
OA	Osteoarthritis
OD	Osteochondritis dissecans
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PDGF	Platelet-derived growth factor
PI	Propidium iodide
PRDII	Positive regulatory domain II
SDS	Sodium dodecyl sulphate
SELE	Selectin E
SEM	Scanning electron microscopy
SPC	Side population cell
SPPS	Solid phase protein synthesis
TP53	Tumour protein 53
tRNA	Transfer ribonucleic acid
UTR	Untranslated region
UV	Ultraviolet
Vol	Volume
v/v	Volume per volume
w/v	Weight per volume
X-SCID	X-linked severe combined immunodeficiency
XPA	Xeroderma pigmentosum complimentation group A

9. References

Abercrombie, B. D., G. G. Kneale, C. Crane-Robinson, E. M. Bradbury, G. H. Goodwin, J. M. Walker and E. W. Johns (1978). Studies on the conformational properties of the high-mobility-group chromosomal protein HMG 17 and its interaction with DNA. Eur J Biochem 84: 173-177.

Adair, J. E., Y. Kwon, G. A. Dement, M. J. Smerdon and R. Reeves (2005). Inhibition of nucleotide excision repair by high mobility group protein HMGA1. J Biol Chem.

Adair, J. E., S. C. Maloney, G. A. Dement, K. J. Wertzler, M. J. Smerdon and R. Reeves (2007). High-mobility group A1 proteins inhibit expression of nucleotide excision repair factor xeroderma pigmentosum group A. Cancer Res 67: 6044-6052.

Aigner, T., M. Hemmel, D. Neureiter, P. M. Gebhard, G. Zeiler, T. Kirchner and L. McKenna (2001). Apoptotic cell death is not a widespread phenomenon in normal aging and osteoarthritis human articular knee cartilage: a study of proliferation, programmed cell death (apoptosis), and viability of chondrocytes in normal and osteoarthritic human knee cartilage. Arthritis Rheum 44: 1304-1312.

Aigner, T., J. Rose, J. Martin and J. Buckwalter (2004). Aging theories of primary osteoarthritis: from epidemiology to molecular biology. Rejuvenation Res 7: 134-145.

Alsalameh, S., R. Amin, T. Gemba and M. Lotz (2004). Identification of mesenchymal progenitor cells in normal and osteoarthritic human articular cartilage. Arthritis Rheum 50: 1522-1532.

Altschul, S. F., W. Gish, W. Miller, E. W. Myers and D. J. Lipman (1990). Basic local alignment search tool. J Mol Biol 215: 403-410.

Anker, C. J., S. P. Holdridge, B. Baird, H. Cohen and T. A. Damron (2005). Ultraporous beta-tricalcium phosphate is well incorporated in small cavitary defects. Clin Orthop Relat Res: 251-257.

Archer, C. W., H. Morrison and A. A. Pitsillides (1994). Cellular aspects of the development of diarthrodial joints and articular cartilage. J Anat 184 (Pt 3): 447-456.

Bartholomew, C. and J. N. Ihle (1991). Retroviral insertions 90 kilobases proximal to the Evi-1 myeloid transforming gene activate transcription from the normal promoter. Mol Cell Biol 11: 1820-1828.

Battista, S., V. Fidanza, M. Fedele, A. J. Klein-Szanto, E. Outwater, H. Brunner, M. Santoro, C. M. Croce and A. Fusco (1999). The expression of a truncated HMGI-C gene induces gigantism associated with lipomatosis. Cancer Res 59: 4793-4797.

Battista, S., F. Pentimalli, G. Baldassarre, M. Fedele, V. Fidanza, C. M. Croce and A. Fusco (2003). Loss of Hmga1 gene function affects embryonic stem cell lympho-hematopoietic differentiation. Faseb J 17: 1496-1498.

Becker, K., H. Murua Escobar, A. Richter, B. Meyer, I. Nolte and J. Bullerdiek (2003). The canine HMGA1 gene maps to CFA 23. Anim Genet 34: 68-69.

Beuing, C., J. T. Soller, M. Muth, S. Wagner, G. Dolf, C. Schelling, A. Richter, S. Willenbrock, N. Reimann-Berg, S. Winkler, I. Nolte, J. Bullerdiek and H. Murua Escobar (2008). Genomic characterisation, chromosomal assignment and in vivo localisation of the canine high mobility group A1 (HMGA1) gene. BMC Genet 9: 49.

Bianchi, M. E., L. Falciola, S. Ferrari and D. M. Lilley (1992). The DNA binding site of HMG1 protein is composed of two similar segments (HMG boxes), both of which have counterparts in other eukaryotic regulatory proteins. Embo J 11: 1055-1063.

Blunk, T., A. L. Sieminski, K. J. Gooch, D. L. Courter, A. P. Hollander, A. M. Nahir, R. Langer, G. Vunjak-Novakovic and L. E. Freed (2002). Differential effects of growth factors on tissue-engineered cartilage. Tissue Eng 8: 73-84.

Borrmann, L., S. Wilkening and J. Bullerdiek (2001). The expression of HMGA genes is regulated by their 3'UTR. Oncogene 20: 4537-4541.

Brittberg, M., A. Lindahl, A. Nilsson, C. Ohlsson, O. Isaksson and L. Peterson (1994). Treatment of deep cartilage defects in the knee with autologous chondrocyte transplantation. N Engl J Med 331: 889-895.

Buckwalter, J. A. (1998). Articular cartilage: injuries and potential for healing. J Orthop Sports Phys Ther 28: 192-202.

Buckwalter, J. A. (2003). Sports, joint injury, and posttraumatic osteoarthritis. J Orthop Sports Phys Ther 33: 578-588.

Buckwalter, J. A. and N. E. Lane (1996). Aging, Sports, and Osteoarthritis. Sports Med Arthrosc 4: 276-287.

Buckwalter, J. A. and H. J. Mankin (1997). Articular Cartilage. Part I: Tissue Design and Chondrocyte-Matrix Interactions. J Bone Joint Surg Am 79: 600-611.

Buckwalter, J. A. and H. J. Mankin (1997). Articular Cartilage. Part II: Degeneration and Osteoarthrosis, Repair, Regeneration, and Transplantation. J Bone Joint Surg Am 79: 612-632

Busch, M. E. and H. Wachmann (2011). Osteochondrosis of the elbow joint in finishing pigs from three herds: associations among different types of joint changes and between osteochondrosis and growth rate. Vet J 188: 197-203.

Bustin, M. (2001). Revised nomenclature for high mobility group (HMG) chromosomal proteins. Trends Biochem Sci 26: 152-153.

Bustin, M., L. Trieschmann and Y. V. Postnikov (1995). The HMG-14/-17 chromosomal protein family: architectural elements that enhance transcription from chromatin templates. Semin Cell Biol 6: 247-255.

Cattaruzzi, G., S. Altamura, M. A. Tessari, A. Rustighi, V. Giancotti, C. Pucillo and G. Manfioletti (2007). The second AT-hook of the architectural transcription factor HMGA2 is determinant for nuclear localization and function. Nucleic Acids Res 35: 1751-1760.

Cavazzana-Calvo, M., E. Payen, O. Negre, G. Wang, K. Hehir, F. Fusil, J. Down, M. Denaro, T. Brady, K. Westerman, R. Cavallesco, B. Gillet-Legrand, L. Caccavelli, R. Sgarra, L. Maouche-Chretien, F. Bernaudin, R. Girot, R. Dorazio, G. J. Mulder, A. Polack, A. Bank, J. Soulier, J. Larghero, N. Kabbara, B. Dalle, B. Gourmel, G. Socie, S. Chretien, N. Cartier, P. Aubourg, A. Fischer, K. Cornetta, F. Galacteros, Y. Beuzard, E. Gluckman, F. Bushman, S. Hacein-Bey-Abina and P. Leboulch (2010). Transfusion independence and HMGA2 activation after gene therapy of human beta-thalassaemia. Nature 467: 318-322.

Chiang, H., C. J. Liao, Y. H. Wang, H. Y. Huang, C. N. Chen, C. H. Hsieh, Y. Y. Huang and C. C. Jiang (2010). Comparison of articular cartilage repair by autologous chondrocytes with and without in vitro cultivation. Tissue Eng Part C Methods 16: 291-300.

Clements, K. M., Z. C. Bee, G. V. Crossingham, M. A. Adams and M. Sharif (2001). How severe must repetitive loading be to kill chondrocytes in articular cartilage? Osteoarthritis Cartilage 9: 499-507.

Cleynen, I. and W. J. Van de Ven (2008). The HMGA proteins: a myriad of functions (Review). Int J Oncol 32: 289-305.

Coletti, J. M., Jr., W. H. Akeson and S. L. Woo (1972). A comparison of the physical behavior of normal articular cartilage and the arthroplasty surface. J Bone Joint Surg Am 54: 147-160.

Cook, G. R., M. Minch, G. P. Schroth and E. M. Bradbury (1989). Analysis of the binding of high mobility group protein 17 to the nucleosome core particle by 1H NMR spectroscopy. J Biol Chem 264: 1799-1803.

Cornell, K. K., D. G. Bostwick, D. M. Cooley, G. Hall, H. J. Harvey, M. J. Hendrick, B. U. Pauli, J. A. Render, G. Stoica, D. C. Sweet and D. J. Waters (2000). Clinical and pathologic aspects of spontaneous canine prostate carcinoma: a retrospective analysis of 76 cases. Prostate 45: 173-183.

Crippa, M. P., P. J. Alfonso and M. Bustin (1992). Nucleosome core binding region of chromosomal protein HMG-17 acts as an independent functional domain. J Mol Biol 228: 442-449.

Cummings, B. J., E. Head, W. Ruehl, N. W. Milgram and C. W. Cotman (1996). The canine as an animal model of human aging and dementia. Neurobiol Aging 17: 259-268.

Cummings, B. J., J. H. Su, C. W. Cotman, R. White and M. J. Russell (1993). Beta-amyloid accumulation in aged canine brain: a model of early plaque formation in Alzheimer's disease. Neurobiol Aging 14: 547-560.

Diana, F., J. Di Bernardo, R. Sgarra, M. A. Tessari, A. Rustighi, A. Fusco, V. Giancotti and G. Manfioletti (2005). Differential HMGA expression and post-translational modifications in prostatic tumor cells. Int J Oncol 26: 515-520.

Ding, C., F. Cicuttini, F. Scott, H. Cooley, C. Boon and G. Jones (2006). Natural history of knee cartilage defects and factors affecting change. Arch Intern Med 166: 651-658.

Dowthwaite, G. P., J. C. Bishop, S. N. Redman, I. M. Khan, P. Rooney, D. J. Evans, L. Haughton, Z. Bayram, S. Boyer, B. Thomson, M. S. Wolfe and C. W. Archer (2004). The surface of articular cartilage contains a progenitor cell population. J Cell Sci 117: 889-897.

Esposito, F., G. M. Pierantoni, S. Battista, R. M. Melillo, S. Scala, P. Chieffi, M. Fedele and A. Fusco (2009). Interaction between HMGA1 and retinoblastoma protein is required for adipocyte differentiation. J Biol Chem 284: 25993-26004.

Evans, A., T. W. Lennard and B. R. Davies (2004). High-mobility group protein 1(Y): metastasis-associated or metastasis-inducing? J Surg Oncol 88: 86-99.

Fedele, M., S. Battista, L. Kenyon, G. Baldassarre, V. Fidanza, A. J. Klein-Szanto, A. F. Parlow, R. Visone, G. M. Pierantoni, E. Outwater, M. Santoro, C. M. Croce and A. Fusco (2002). Overexpression of the HMGA2 gene in transgenic mice leads to the onset of pituitary adenomas. Oncogene 21: 3190-3198.

Fedele, M. and A. Fusco (2010). HMGA and cancer. Biochim Biophys Acta 1799: 48-54.

Felson, D. T., R. C. Lawrence, P. A. Dieppe, R. Hirsch, C. G. Helmick, J. M. Jordan, R. S. Kington, N. E. Lane, M. C. Nevitt, Y. Zhang, M. Sowers, T. McAlindon, T. D. Spector, A. R. Poole, S. Z. Yanovski, G. Ateshian, L. Sharma, J. A. Buckwalter, K. D. Brandt and J. F. Fries (2000). Osteoarthritis: new insights. Part 1: the disease and its risk factors. Ann Intern Med 133: 635-646.

Fletcher, J. A., J. Longtine, K. Wallace, S. J. Mentzer and D. J. Sugarbaker (1995). Cytogenetic and histologic findings in 17 pulmonary chondroid hamartomas: evidence for a pathogenetic relationship with lipomas and leiomyomas. Genes Chromosomes Cancer 12: 220-223.

Frenkel, S. R. and P. E. Di Cesare (1999). Degradation and repair of articular cartilage. Front Biosci 4: D671-685.

Frenkel, S. R. and P. E. Di Cesare (2004). Scaffolds for articular cartilage repair. Ann Biomed Eng 32: 26-34.

Frota, R., V. A. Da Silva-Junior, M. Teixeira, A. P. Sobral, S. Emanuel-Dias-de Oliveira e, M. M. Da Silveira and A. C. Aragao-Neto (2011). Histological evaluation of bone repair using beta-tricalcium phosphate. Med Oral Patol Oral Cir Bucal 16: e190-194.

Fusco, A. and M. Fedele (2007). Roles of HMGA proteins in cancer. Nat Rev Cancer 7: 899-910.

Gattas, G. J., B. J. Quade, R. A. Nowak and C. C. Morton (1999). HMGIC expression in human adult and fetal tissues and in uterine leiomyomata. Genes Chromosomes Cancer 25: 316-322.

Geierstanger, B. H., B. F. Volkman, W. Kremer and D. E. Wemmer (1994). Short peptide fragments derived from HMG-I/Y proteins bind specifically to the minor groove of DNA. Biochemistry 33: 5347-5355.

Giancotti, V., M. T. Berlingieri, P. P. DiFiore, A. Fusco, G. Vecchio and C. Crane-Robinson (1985). Changes in nuclear proteins on transformation of rat epithelial thyroid cells by a murine sarcoma retrovirus. Cancer Res 45: 6051-6057.

Giancotti, V., B. Pani, P. D'Andrea, M. T. Berlingieri, P. P. Di Fiore, A. Fusco, G. Vecchio, R. Philp, C. Crane-Robinson, R. H. Nicolas and et al. (1987). Elevated levels of a specific class of nuclear phosphoproteins in cells transformed with v-ras and v-mos oncogenes and by cotransfection with c-myc and polyoma middle T genes. Embo J 6: 1981-1987.

Goodell, M. A., K. Brose, G. Paradis, A. S. Conner and R. C. Mulligan (1996). Isolation and functional properties of murine hematopoietic stem cells that are replicating in vivo. J Exp Med 183: 1797-1806.

Goodwin, G. H., P. N. Cockerill, S. Kellam and C. A. Wright (1985). Fractionation by high-performance liquid chromatography of the low-molecular-mass high-mobility-group (HMG) chromosomal proteins present in proliferating rat cells and an investigation of the HMG proteins present in virus transformed cells. Eur J Biochem 149: 47-51.

Goodwin, G. H. and E. W. Johns (1973). Isolation and characterisation of two calf-thymus chromatin non-histone proteins with high contents of acidic and basic amino acids. Eur J Biochem 40: 215-219.

Goodwin, G. H., R. H. Nicolas and E. W. Johns (1975). An improved large scale fractionation of high mobility group non-histone chromatin proteins. Biochim Biophys Acta 405: 280-291.

Goodwin, G. H., C. Sanders and E. W. Johns (1973). A new group of chromatin-associated proteins with a high content of acidic and basic amino acids. Eur J Biochem 38: 14-19.

Goodwin, G. H., L. Woodhead and E. W. Johns (1977). The presence of high mobility group non-histone chromatin proteins in isolated nucleosomes. FEBS Lett 73: 85-88.

Gotterbarm, T., W. Richter, M. Jung, S. Berardi Vilei, P. Mainil-Varlet, T. Yamashita and S. J. Breusch (2006). An in vivo study of a growth-factor enhanced, cell free, two-layered collagen-tricalcium phosphate in deep osteochondral defects. Biomaterials 27: 3387-3395.

Grogan, S. P., S. Miyaki, H. Asahara, D. D. D'Lima and M. K. Lotz (2009). Mesenchymal progenitor cell markers in human articular cartilage: normal distribution and changes in osteoarthritis. Arthritis Res Ther 11: R85.

Grosschedl, R., K. Giese and J. Pagel (1994). HMG domain proteins: architectural elements in the assembly of nucleoprotein structures. Trends Genet 10: 94-100.

Guo, J. F., G. W. Jourdian and D. K. MacCallum (1989). Culture and growth characteristics of chondrocytes encapsulated in alginate beads. Connect Tissue Res 19: 277-297.

Guo, X., C. Wang, C. Duan, M. Descamps, Q. Zhao, L. Dong, S. Lu, K. Anselme, J. Lu and Y. Q. Song (2004). Repair of osteochondral defects with autologous chondrocytes seeded onto bioceramic scaffold in sheep. Tissue Eng 10: 1830-1840.

Hacein-Bey-Abina, S., F. Le Deist, F. Carlier, C. Bouneaud, C. Hue, J. P. De Villartay, A. J. Thrasher, N. Wulffraat, R. Sorensen, S. Dupuis-Girod, A. Fischer, E. G. Davies, W. Kuis, L. Leiva and M. Cavazzana-Calvo (2002). Sustained correction of X-linked severe combined immunodeficiency by ex vivo gene therapy. N Engl J Med 346: 1185-1193.

Hacein-Bey-Abina, S., C. Von Kalle, M. Schmidt, M. P. McCormack, N. Wulffraat, P. Leboulch, A. Lim, C. S. Osborne, R. Pawliuk, E. Morillon, R. Sorensen, A. Forster, P. Fraser, J. I. Cohen, G. de Saint Basile, I. Alexander, U. Wintergerst, T. Frebourg, A. Aurias, D. Stoppa-Lyonnet, S. Romana, I. Radford-Weiss, F. Gross, F. Valensi, E. Delabesse, E. Macintyre, F. Sigaux, J. Soulier, L. E. Leiva, M. Wissler, C. Prinz, T. H. Rabbitts, F. Le Deist, A. Fischer and M. Cavazzana-Calvo (2003). LMO2-associated clonal T cell proliferation in two patients after gene therapy for SCID-X1. Science 302: 415-419.

Hahn, K. A., L. Bravo, W. H. Adams and D. L. Frazier (1994). Naturally occurring tumors in dogs as comparative models for cancer therapy research. In Vivo 8: 133-143.

Hangody, L., G. Kish, Z. Kárpáti and R. Eberhart (1997). Osteochondral plugs: Autogenousosteochondral mosaicplasty for the treatment of focal chondral and osteochondral articular defects. Operative Techniques in Orthopaedics 7: 312-322.

Hangody, L., G. Vasarhelyi, L. R. Hangody, Z. Sukosd, G. Tibay, L. Bartha and G. Bodo (2008). Autologous osteochondral grafting--technique and long-term results. Injury 39 Suppl 1: S32-39.

Hardingham, T., S. Tew and A. Murdoch (2002). Tissue engineering: chondrocytes and cartilage. Arthritis Res 4 Suppl 3: S63-68.

Harris, J. D., R. A. Siston, R. H. Brophy, C. Lattermann, J. L. Carey and D. C. Flanigan (2011). Failures, re-operations, and complications after autologous chondrocyte implantation--a systematic review. Osteoarthritis Cartilage 19: 779-791.

Harrison, P. E., I. K. Ashton, W. E. Johnson, S. L. Turner, J. B. Richardson and B. A. Ashton (2000). The in vitro growth of human chondrocytes. Cell Tissue Bank 1: 255-260.

Hattori, S., C. Oxford and A. H. Reddi (2007). Identification of superficial zone articular chondrocyte stem/progenitor cells. Biochem Biophys Res Commun 358: 99-103.

Hayes, A. J., S. MacPherson, H. Morrison, G. Dowthwaite and C. W. Archer (2001). The development of articular cartilage: evidence for an appositional growth mechanism. Anat Embryol (Berl) 203: 469-479.

Henriksen, J., M. Stabell, L. A. Meza-Zepeda, S. A. Lauvrak, M. Kassem and O. Myklebost (2010). Identification of target genes for wild type and truncated HMGA2 in mesenchymal stem-like cells. BMC Cancer 10: 329.

Herzog, W. and D. Longino (2007). The role of muscles in joint degeneration and osteoarthritis. J Biomech 40 Suppl 1: S54-63.

Herzog, W., D. Longino and A. Clark (2003). The role of muscles in joint adaptation and degeneration. Langenbecks Arch Surg 388: 305-315.

Hess, J. L. (1998). Chromosomal translocations in benign tumors: the HMGI proteins. Am J Clin Pathol 109: 251-261.

Hirata, M., H. Murata, H. Takeshita, T. Sakabe, Y. Tsuji and T. Kubo (2006). Use of purified beta-tricalcium phosphate for filling defects after curettage of benign bone tumours. Int Orthop 30: 510-513.

Hulth, A., L. Lindberg and H. Telhag (1972). Mitosis in human osteoarthritic cartilage. Clin Orthop Relat Res 84: 197-199.

Hunter, W. (1742). Of the Structure and Diseases of Articulating Cartilages, by William Hunter, Surgeon. Philosophical Transactions (1683-1775) 42: 514-521.

Hunziker, E. B. (2002). Articular cartilage repair: basic science and clinical progress. A review of the current status and prospects. Osteoarthritis Cartilage 10: 432-463.

Inoue, H., H. Nojima and H. Okayama (1990). High efficiency transformation of Escherichia coli with plasmids. Gene 96: 23-28.

Ishiguro, H., N. Nakaigawa, Y. Miyoshi, K. Fujinami, Y. Kubota and H. Uemura (2005). Receptor for advanced glycation end products (RAGE) and its ligand, amphoterin are overexpressed and associated with prostate cancer development. Prostate 64: 92-100.

Iwasaki, N., S. T. Yamane, T. Majima, Y. Kasahara, A. Minami, K. Harada, S. Nonaka, N. Maekawa, H. Tamura, S. Tokura, M. Shiono, K. Monde and S. Nishimura (2004). Feasibility of polysaccharide hybrid materials for scaffolds in cartilage tissue engineering: evaluation of chondrocyte adhesion to polyion complex fibers prepared from alginate and chitosan. Biomacromolecules 5: 828-833.

Jiang, C. C., H. Chiang, C. J. Liao, Y. J. Lin, T. F. Kuo, C. S. Shieh, Y. Y. Huang and R. S. Tuan (2007). Repair of porcine articular cartilage defect with a biphasic osteochondral composite. J Orthop Res 25: 1277-1290.

Johnson, K. R., D. A. Lehn and R. Reeves (1989). Alternative processing of mRNAs encoding mammalian chromosomal high-mobility-group proteins HMG-I and HMG-Y. Mol Cell Biol 9: 2114-2123.

Johnson, S. M., H. Grosshans, J. Shingara, M. Byrom, R. Jarvis, A. Cheng, E. Labourier, K. L. Reinert, D. Brown and F. J. Slack (2005). RAS is regulated by the let-7 microRNA family. Cell 120: 635-647.

Kaddar, T., J. P. Rouault, W. W. Chien, A. Chebel, M. Gadoux, G. Salles, M. Ffrench and J. P. Magaud (2009). Two new miR-16 targets: caprin-1 and HMGA1, proteins implicated in cell proliferation. Biol Cell 101: 511-524.

Kazmierczak, B., J. Bullerdiek, K. H. Pham, S. Bartnitzke and H. Wiesner (1998). Intron 3 of HMGIC is the most frequent target of chromosomal aberrations in human tumors and has been conserved basically for at least 30 million years. Cancer Genet Cytogenet 103: 175-177.

Kazmierczak, B., K. Meyer-Bolte, K. H. Tran, W. Wockel, I. Breightman, J. Rosigkeit, S. Bartnitzke and J. Bullerdiek (1999). A high frequency of tumors with rearrangements of genes of the HMGI(Y) family in a series of 191 pulmonary chondroid hamartomas. Genes Chromosomes Cancer 26: 125-133.

Kazmierczak, B., J. Rosigkeit, S. Wanschura, K. Meyer-Bolte, W. J. Van de Ven, K. Kayser, B. Krieghoff, H. Kastendiek, S. Bartnitzke and J. Bullerdiek (1996). HMGI-C rearrangements as the molecular basis for the majority of pulmonary chondroid hamartomas: a survey of 30 tumors. Oncogene 12: 515-521.

Kazmierczak, B., S. Wanschura, B. Rommel, S. Bartnitzke and J. Bullerdiek (1996). Ten pulmonary chondroid hamartomas with chromosome 6p21 breakpoints within the HMG-I(Y) gene or its immediate surroundings. J Natl Cancer Inst 88: 1234-1236.

Kazmierczak, B., S. Wanschura, J. Rosigkeit, K. Meyer-Bolte, K. Uschinsky, R. Haupt, E. F. Schoenmakers, S. Bartnitzke, W. J. Van de Ven and J. Bullerdiek (1995). Molecular characterization of 12q14-15 rearrangements in three pulmonary chondroid hamartomas. Cancer Res 55: 2497-2499.

Khanna, K. K. and S. P. Jackson (2001). DNA double-strand breaks: signaling, repair and the cancer connection. Nat Genet 27: 247-254.

Kock, N., J. van Susante, A. Wymenga and P. Buma (2004). Histological evaluation of a mosaicplasty of the femoral condyle-retrieval specimens obtained after total knee arthroplasty--a case report. Acta Orthop Scand 75: 505-508.

Koss, M. (1990). Surgical pathology of lung neoplasms. New York, Dekker.

Kubo, T., Y. Matsui, T. Goto, K. Yukata and N. Yasui (2006). Overexpression of HMGA2-LPP fusion transcripts promotes expression of the alpha 2 type XI collagen gene. Biochem Biophys Res Commun 340: 476-481.

Kuniyasu, H., Y. Chihara, H. Kondo, H. Ohmori and R. Ukai (2003). Amphoterin induction in prostatic stromal cells by androgen deprivation is associated with metastatic prostate cancer. Oncol Rep 10: 1863-1868.

Kuska, B. (1999). Sit, DNA, sit: cancer genetics going to the dogs. J Natl Cancer Inst 91: 204-206.

Lane, J. G., W. L. Tontz, Jr., S. T. Ball, J. B. Massie, A. C. Chen, W. C. Bae, M. E. Amiel, R. L. Sah and D. Amiel (2001). A morphologic, biochemical, and biomechanical assessment of short-term effects of osteochondral autograft plug transfer in an animal model. Arthroscopy 17: 856-863.

Lee, Y. S. and A. Dutta (2007). The tumor suppressor microRNA let-7 represses the HMGA2 oncogene. Genes Dev 21: 1025-1030.

Lemke, I., P. Rogalla, F. Grundmann, W. P. Kunze, R. Haupt and J. Bullerdiek (2002). Expression of the HMGA2-LPP fusion transcript in only 1 of 61 karyotypically normal pulmonary chondroid hamartomas. Cancer Genet Cytogenet 138: 160-164.

Li, A. Y., L. M. Boo, S. Y. Wang, H. H. Lin, C. C. Wang, Y. Yen, B. P. Chen, D. J. Chen and D. K. Ann (2009). Suppression of nonhomologous end joining repair by overexpression of HMGA2. Cancer Res 69: 5699-5706.

Li, O., J. Li and P. Droge (2007). DNA architectural factor and proto-oncogene HMGA2 regulates key developmental genes in pluripotent human embryonic stem cells. FEBS Lett 581: 3533-3537.

Li, O., D. Vasudevan, C. A. Davey and P. Droge (2006). High-level expression of DNA architectural factor HMGA2 and its association with nucleosomes in human embryonic stem cells. Genesis 44: 523-529.

Lindblad-Toh, K., C. M. Wade, T. S. Mikkelsen, E. K. Karlsson, D. B. Jaffe, M. Kamal, M. Clamp, J. L. Chang, E. J. Kulbokas, 3rd, M. C. Zody, E. Mauceli, X. Xie, M. Breen, R. K. Wayne, E. A. Ostrander, C. P. Ponting, F. Galibert, D. R. Smith, P. J. DeJong, E. Kirkness, P. Alvarez, T. Biagi, W. Brockman, J. Butler, C. W. Chin, A. Cook, J. Cuff, M. J. Daly, D. DeCaprio, S. Gnerre, M. Grabherr, M. Kellis, M. Kleber, C.

Bardeleben, L. Goodstadt, A. Heger, C. Hitte, L. Kim, K. P. Koepfli, H. G. Parker, J. P. Pollinger, S. M. Searle, N. B. Sutter, R. Thomas, C. Webber, J. Baldwin, A. Abebe, A. Abouelleil, L. Aftuck, M. Ait-Zahra, T. Aldredge, N. Allen, P. An, S. Anderson, C. Antoine, H. Arachchi, A. Aslam, L. Ayotte, P. Bachantsang, A. Barry, T. Bayul, M. Benamara, A. Berlin, D. Bessette, B. Blitshteyn, T. Bloom, J. Blye, L. Boguslavskiy, C. Bonnet, B. Boukhgalter, A. Brown, P. Cahill, N. Calixte, J. Camarata, Y. Cheshatsang, J. Chu, M. Citroen, A. Collymore, P. Cooke, T. Dawoe, R. Daza, K. Decktor, S. DeGray, N. Dhargay, K. Dooley, P. Dorje, K. Dorjee, L. Dorris, N. Duffey, A. Dupes, O. Egbiremolen, R. Elong, J. Falk, A. Farina, S. Faro, D. Ferguson, P. Ferreira, S. Fisher, M. FitzGerald, K. Foley, C. Foley, A. Franke, D. Friedrich, D. Gage, M. Garber, G. Gearin, G. Giannoukos, T. Goode, A. Goyette, J. Graham, E. Grandbois, K. Gyaltsen, N. Hafez, D. Hagopian, B. Hagos, J. Hall, C. Healy, R. Hegarty, T. Honan, A. Horn, N. Houde, L. Hughes, L. Hunnicutt, M. Husby, B. Jester, C. Jones, A. Kamat, B. Kanga, C. Kells, D. Khazanovich, A. C. Kieu, P. Kisner, M. Kumar, K. Lance, T. Landers, M. Lara, W. Lee, J. P. Leger, N. Lennon, L. Leuper, S. LeVine, J. Liu, X. Liu, Y. Lokyitsang, T. Lokyitsang, A. Lui, J. Macdonald, J. Major, R. Marabella, K. Maru, C. Matthews, S. McDonough, T. Mehta, J. Meldrim, A. Melnikov, L. Meneus, A. Mihalev, T. Mihova, K. Miller, R. Mittelman, V. Mlenga, L. Mulrain, G. Munson, A. Navidi, J. Naylor, T. Nguyen, N. Nguyen, C. Nguyen, R. Nicol, N. Norbu, C. Norbu, N. Novod, T. Nyima, P. Olandt, B. O'Neill, K. O'Neill, S. Osman, L. Oyono, C. Patti, D. Perrin, P. Phunkhang, F. Pierre, M. Priest, A. Rachupka, S. Raghuraman, R. Rameau, V. Ray, C. Raymond, F. Rege, C. Rise, J. Rogers, P. Rogov, J. Sahalie, S. Settipalli, T. Sharpe, T. Shea, M. Sheehan, N. Sherpa, J. Shi, D. Shih, J. Sloan, C. Smith, T. Sparrow, J. Stalker, N. Stange-Thomann, S. Stavropoulos, C. Stone, S. Stone, S. Sykes, P. Tchuinga, P. Tenzing, S. Tesfaye, D. Thoulutsang, Y. Thoulutsang, K. Topham, I. Topping, T. Tsamla, H. Vassiliev, V. Venkataraman, A. Vo, T. Wangchuk, T. Wangdi, M. Weiand, J. Wilkinson, A. Wilson, S. Yadav, S. Yang, X. Yang, G. Young, Q. Yu, J. Zainoun, L. Zembek, A. Zimmer and E. S. Lander (2005). Genome sequence, comparative analysis and haplotype structure of the domestic dog. Nature 438: 803-819.

Loeser, R. F. (2009). Aging and osteoarthritis: the role of chondrocyte senescence and aging changes in the cartilage matrix. Osteoarthritis Cartilage 17: 971-979.

Lund, T., J. Holtlund, M. Fredriksen and S. G. Laland (1983). On the presence of two new high mobility group-like proteins in HeLa S3 cells. FEBS Lett 152: 163-167.

Malpeli, M., N. Randazzo, R. Cancedda and B. Dozin (2004). Serum-free growth medium sustains commitment of human articular chondrocyte through maintenance of Sox9 expression. Tissue Eng 10: 145-155.

Mankin, H. J. (1982). The response of articular cartilage to mechanical injury. J Bone Joint Surg Am 64: 460-466.

Mantovani, F., S. Covaceuszach, A. Rustighi, R. Sgarra, C. Heath, G. H. Goodwin and G. Manfioletti (1998). NF-kappaB mediated transcriptional activation is enhanced by the architectural factor HMGI-C. Nucleic Acids Res 26: 1433-1439.

Markowski, D. N., I. von Ahsen, M. H. Nezhad, W. Wosniok, B. M. Helmke and J. Bullerdiek (2010). HMGA2 and the p19Arf-TP53-CDKN1A axis: a delicate balance in the growth of uterine leiomyomas. Genes Chromosomes Cancer 49: 661-668.

Markowski, D. N., N. Winter, F. Meyer, I. von Ahsen, H. Wenk, I. Nolte and J. Bullerdiek (2011). p14Arf acts as an antagonist of HMGA2 in senescence of mesenchymal stem cells-implications for benign tumorigenesis. Genes Chromosomes Cancer 50: 489-498.

Marlovits, S., P. Zeller, P. Singer, C. Resinger and V. Vecsei (2006). Cartilage repair: generations of autologous chondrocyte transplantation. Eur J Radiol 57: 24-31.

Martinez Hoyos, J., M. Fedele, S. Battista, F. Pentimalli, M. Kruhoffer, C. Arra, T. F. Orntoft, C. M. Croce and A. Fusco (2004). Identification of the genes up- and down-regulated by the high mobility group A1 (HMGA1) proteins: tissue specificity of the HMGA1-dependent gene regulation. Cancer Res 64: 5728-5735.

Mayr, C., M. T. Hemann and D. P. Bartel (2007). Disrupting the pairing between let-7 and Hmga2 enhances oncogenic transformation. Science 315: 1576-1579.

McGregor, A. J., B. G. Amsden and S. D. Waldman (2011). Chondrocyte repopulation of the zone of death induced by osteochondral harvest. Osteoarthritis Cartilage 19: 242-248.

Merenmies, J., R. Pihlaskari, J. Laitinen, J. Wartiovaara and H. Rauvala (1991). 30-kDa heparin-binding protein of brain (amphoterin) involved in neurite outgrowth. Amino acid sequence and localization in the filopodia of the advancing plasma membrane. J Biol Chem 266: 16722-16729.

Morgan, J. P., A. Wind and A. P. Davidson (2000). Hereditary bone and joint diseases in the dog : osteochondroses, hip dysplasia, elbow dysplasia. Hannover, Schlüter.

Morishita, K., D. S. Parker, M. L. Mucenski, N. A. Jenkins, N. G. Copeland and J. N. Ihle (1988). Retroviral activation of a novel gene encoding a zinc finger protein in IL-3-dependent myeloid leukemia cell lines. Cell 54: 831-840.

Morrison, S. J., A. M. Wandycz, K. Akashi, A. Globerson and I. L. Weissman (1996). The aging of hematopoietic stem cells. Nat Med 2: 1011-1016.

Murua Escobar, H., B. Meyer, A. Richter, K. Becker, A. M. Flohr, J. Bullerdiek and I. Nolte (2003). Molecular characterization of the canine HMGB1. Cytogenet Genome Res 101: 33-38.

Murua Escobar, H., J. T. Soller, A. Richter, B. Meyer, S. Winkler, J. Bullerdiek and I. Nolte (2005). "Best friends" sharing the HMGA1 gene: comparison of the human and canine HMGA1 to orthologous other species. J Hered 96: 777-781.

Newton, C. D. and D. M. Nunamaker (1985). Textbook of small animal orthopaedics. Philadelphia, Lippincott.

Nishino, J., I. Kim, K. Chada and S. J. Morrison (2008). Hmga2 promotes neural stem cell self-renewal in young but not old mice by reducing p16Ink4a and p19Arf Expression. Cell 135: 227-239.

Ostrander, E. A., F. Galibert and D. F. Patterson (2000). Canine genetics comes of age. Trends Genet 16: 117-124.

Ostrander, E. A. and E. Giniger (1997). Semper fidelis: what man's best friend can teach us about human biology and disease. Am J Hum Genet 61: 475-480.

Ostrander, E. A. and L. Kruglyak (2000). Unleashing the canine genome. Genome Res 10: 1271-1274.

Palmieri, D., D. D'Angelo, T. Valentino, I. De Martino, A. Ferraro, A. Wierinckx, M. Fedele, J. Trouillas and A. Fusco (2011). Downregulation of HMGA-targeting microRNAs has a critical role in human pituitary tumorigenesis. Oncogene.

Palmieri, D., T. Valentino, D. D'Angelo, I. De Martino, I. Postiglione, R. Pacelli, C. M. Croce, M. Fedele and A. Fusco (2011). HMGA proteins promote ATM expression and enhance cancer cell resistance to genotoxic agents. Oncogene 30: 3024-3035.

Park, S. M., S. Shell, A. R. Radjabi, R. Schickel, C. Feig, B. Boyerinas, D. M. Dinulescu, E. Lengyel and M. E. Peter (2007). Let-7 prevents early cancer progression by suppressing expression of the embryonic gene HMGA2. Cell Cycle 6: 2585-2590.

Pentimalli, F., D. Palmieri, R. Pacelli, C. Garbi, R. Cesari, E. Martin, G. M. Pierantoni, P. Chieffi, C. M. Croce, V. Costanzo, M. Fedele and A. Fusco (2008). HMGA1 protein is a novel target of the ATM kinase. Eur J Cancer 44: 2668-2679.

Peter, M. E. (2009). Let-7 and miR-200 microRNAs: guardians against pluripotency and cancer progression. Cell Cycle 8: 843-852.

Pfannkuche, K., H. Summer, O. Li, J. Hescheler and P. Droge (2009). The high mobility group protein HMGA2: a co-regulator of chromatin structure and pluripotency in stem cells? Stem Cell Rev 5: 224-230.

Pomerantz, J., N. Schreiber-Agus, N. J. Liegeois, A. Silverman, L. Alland, L. Chin, J. Potes, K. Chen, I. Orlow, H. W. Lee, C. Cordon-Cardo and R. A. DePinho (1998). The Ink4a tumor suppressor gene product, p19Arf, interacts with MDM2 and neutralizes MDM2's inhibition of p53. Cell 92: 713-723.

Reeves, R. and L. Beckerbauer (2001). HMGI/Y proteins: flexible regulators of transcription and chromatin structure. Biochim Biophys Acta 1519: 13-29.

Reeves, R. and M. S. Nissen (1990). The A.T-DNA-binding domain of mammalian high mobility group I chromosomal proteins. A novel peptide motif for recognizing DNA structure. J Biol Chem 265: 8573-8582.

Richter, A., G. Hauschild, H. Murua Escobar, I. Nolte and J. Bullerdiek (2009). Application of high-mobility-group-A proteins increases the proliferative activity of chondrocytes in vitro. Tissue Eng Part A 15: 473-477.

Richter, A., H. Murua Escobar, K. Gunther, B. Meyer, S. Winkler, G. Dolf, C. Schelling, I. Nolte and J. Bullerdiek (2004). The canine NRAS gene maps to CFA 17. Anim Genet 35: 355-356.

Richter, A., H. Murua Escobar, K. Gunther, J. T. Soller, S. Winkler, I. Nolte and J. Bullerdiek (2005). RAS gene hot-spot mutations in canine neoplasias. J Hered 96: 764-765.

Riley, C. B., W. M. Scott, J. P. Caron, P. B. Fretz, J. V. Bailey and S. M. Barber (1998). Osteochondritis dessicans and subchondral cystic lesions in draft horses: a retrospective study. Can Vet J 39: 627-633.

Rivenson, A. and J. Silverman (1979). The prostatic carcinoma in laboratory animals: a bibliographic survey from 1900 to 1977. Invest Urol 16: 468-472.

Rogalla, P., K. Drechsler, G. Frey, Y. Hennig, B. Helmke, U. Bonk and J. Bullerdiek (1996). HMGI-C expression patterns in human tissues. Implications for the genesis of frequent mesenchymal tumors. Am J Pathol 149: 775-779.

Rogalla, P., I. Lemke, B. Kazmierczak and J. Bullerdiek (2000). An identical HMGIC-LPP fusion transcript is consistently expressed in pulmonary chondroid hamartomas with t(3;12)(q27-28;q14-15). Genes Chromosomes Cancer 29: 363-366.

Scaffidi, P., T. Misteli and M. E. Bianchi (2002). Release of chromatin protein HMGB1 by necrotic cells triggers inflammation. Nature 418: 191-195.

Schlueter, C., H. Weber, B. Meyer, P. Rogalla, K. Roser, S. Hauke and J. Bullerdiek (2005). Angiogenetic Signaling through Hypoxia: HMGB1: An Angiogenetic Switch Molecule. Am J Pathol 166: 1259-1263.

Schwanbeck, R. (2000). Die Phosphorylierung der architektonischen HMGI(Y)-Chromatinproteine : Modulation der Struktur und DNA-Bindung. Göttingen, Cuvillier.

Sekiya, I., J. T. Vuoristo, B. L. Larson and D. J. Prockop (2002). In vitro cartilage formation by human adult stem cells from bone marrow stroma defines the sequence of cellular and molecular events during chondrogenesis. Proc Natl Acad Sci U S A 99: 4397-4402.

Sgarra, R., A. Rustighi, M. A. Tessari, J. Di Bernardo, S. Altamura, A. Fusco, G. Manfioletti and V. Giancotti (2004). Nuclear phosphoproteins HMGA and their relationship with chromatin structure and cancer. FEBS Lett 574: 1-8.

Shearin, A. L. and E. A. Ostrander (2010). Leading the way: canine models of genomics and disease. Dis Model Mech 3: 27-34.

Solomon, M. J., F. Strauss and A. Varshavsky (1986). A mammalian high mobility group protein recognizes any stretch of six A.T base pairs in duplex DNA. Proc Natl Acad Sci U S A 83: 1276-1280.

Starkey, M. P., T. J. Scase, C. S. Mellersh and S. Murphy (2005). Dogs really are man's best friend--canine genomics has applications in veterinary and human medicine! Brief Funct Genomic Proteomic 4: 112-128.

Suba, Z., D. Takacs, D. Matusovits, J. Barabas, A. Fazekas and G. Szabo (2006). Maxillary sinus floor grafting with beta-tricalcium phosphate in humans: density and microarchitecture of the newly formed bone. Clin Oral Implants Res 17: 102-108.

Takaha, N., A. L. Hawkins, C. A. Griffin, W. B. Isaacs and D. S. Coffey (2002). High mobility group protein I(Y): a candidate architectural protein for chromosomal rearrangements in prostate cancer cells. Cancer Res 62: 647-651.

Tallini, G. and P. Dal Cin (1999). HMGI(Y) and HMGI-C dysregulation: a common occurrence in human tumors. Adv Anat Pathol 6: 237-246.

Tallini, G., R. Vanni, G. Manfioletti, B. Kazmierczak, G. Faa, P. Pauwels, J. Bullerdiek, V. Giancotti, H. Van Den Berghe and P. Dal Cin (2000). HMGI-C and HMGI(Y) immunoreactivity correlates with cytogenetic abnormalities in lipomas, pulmonary chondroid hamartomas, endometrial polyps, and uterine leiomyomas and is compatible with rearrangement of the HMGI-C and HMGI(Y) genes. Lab Invest 80: 359-369.

Tamimi, Y., H. G. van der Poel, M. M. Denyn, R. Umbas, H. F. Karthaus, F. M. Debruyne and J. A. Schalken (1993). Increased expression of high mobility group protein I(Y) in high grade prostatic cancer determined by in situ hybridization. Cancer Res 53: 5512-5516.

Tamimi, Y., H. G. van der Poel, H. F. Karthaus, F. M. Debruyne and J. A. Schalken (1996). A retrospective study of high mobility group protein I(Y) as progression marker for prostate cancer determined by in situ hybridization. Br J Cancer 74: 573-578.

Tang, D., R. Kang, H. J. Zeh, 3rd and M. T. Lotze (2010). High-mobility group box 1 and cancer. Biochim Biophys Acta 1799: 131-140.

Vasiliadis, H. S., A. Lindahl, A. D. Georgoulis and L. Peterson (2011). Malalignment and cartilage lesions in the patellofemoral joint treated with autologous chondrocyte implantation. Knee Surg Sports Traumatol Arthrosc 19: 452-457.

Veilleux, N. H., I. V. Yannas and M. Spector (2004). Effect of passage number and collagen type on the proliferative, biosynthetic, and contractile activity of adult canine articular chondrocytes in type I and II collagen-glycosaminoglycan matrices in vitro. Tissue Eng 10: 119-127.

von Ahsen, I., P. Rogalla and J. Bullerdiek (2005). Expression patterns of the LPP-HMGA2 fusion transcript in pulmonary chondroid hamartomas with t(3;12)(q27 approximately 28;q14 approximately 15). Cancer Genet Cytogenet 163: 68-70.

Wang, H., O. Bloom, M. Zhang, J. M. Vishnubhakat, M. Ombrellino, J. Che, A. Frazier, H. Yang, S. Ivanova, L. Borovikova, K. R. Manogue, E. Faist, E. Abraham, J. Andersson, U. Andersson, P. E. Molina, N. N. Abumrad, A. Sama and K. J. Tracey (1999). HMG-1 as a late mediator of endotoxin lethality in mice. Science 285: 248-251.

Wanschura, S., B. Kazmierczak, Y. Pohnke, K. Meyer-Bolte, S. Bartnitzke, W. J. Van de Ven and J. Bullerdiek (1996). Transcriptional activation of HMGI-C in three pulmonary hamartomas each with a der(14)t(12;14) as the sole cytogenetic abnormality. Cancer Lett 102: 17-21.

Waters, D. J., W. A. Sakr, D. W. Hayden, C. M. Lang, L. McKinney, G. P. Murphy, R. Radinsky, R. Ramoner, R. C. Richardson and D. J. Tindall (1998). Workgroup 4: spontaneous prostate carcinoma in dogs and nonhuman primates. Prostate 36: 64-67.

Wei, J. J., X. Wu, Y. Peng, G. Shi, O. Basturk, X. Yang, G. Daniels, I. Osman, J. Ouyang, E. Hernando, A. Pellicer, J. S. Rhim, J. Melamed and P. Lee (2011). Regulation of HMGA1 expression by microRNA-296 affects prostate cancer growth and invasion. Clin Cancer Res 17: 1297-1305.

Whitley, M. Z., D. Thanos, M. A. Read, T. Maniatis and T. Collins (1994). A striking similarity in the organization of the E-selectin and beta interferon gene promoters. Mol Cell Biol 14: 6464-6475.

Winkler, S., H. Murua Escobar, N. Eberle, N. Reimann-Berg, I. Nolte and J. Bullerdiek (2005). Establishment of a cell line derived from a canine prostate carcinoma with a highly rearranged karyotype. J Hered 96: 782-785.

Winkler, S., H. Murua Escobar, B. Meyer, D. Simon, N. Eberle, W. Baumgartner, S. Loeschke, I. Nolte and J. Bullerdiek (2007). HMGA2 expression in a canine model of prostate cancer. Cancer Genet Cytogenet 177: 98-102.

Wisniewski, J. R. and R. Schwanbeck (2000). High mobility group I/Y: multifunctional chromosomal proteins causally involved in tumor progression and malignant transformation (review). Int J Mol Med 6: 409-419.

Yang, C. S., C. G. Lopez and T. M. Rana (2011). Discovery of nonsteroidal anti-inflammatory drug and anticancer drug enhancing reprogramming and induced pluripotent stem cell generation. Stem Cells 29: 1528-1536.

Yang, F., P. C. O'Brien, B. S. Milne, A. S. Graphodatsky, N. Solanky, V. Trifonov, W. Rens, D. Sargan and M. A. Ferguson-Smith (1999). A complete comparative chromosome map for the dog, red fox, and human and its integration with canine genetic maps. Genomics 62: 189-202.

Young, A. R. and M. Narita (2007). Oncogenic HMGA2: short or small? Genes Dev 21: 1005-1009.

Zhang, Y., Y. Xiong and W. G. Yarbrough (1998). ARF promotes MDM2 degradation and stabilizes p53: ARF-INK4a locus deletion impairs both the Rb and p53 tumor suppression pathways. Cell 92: 725-734.

Zhou, X. and K. Chada (1998). HMGI family proteins: architectural transcription factors in mammalian development and cancer. Keio J Med 47: 73-77.

Zuk, P. A., M. Zhu, P. Ashjian, D. A. De Ugarte, J. I. Huang, H. Mizuno, Z. C. Alfonso, J. K. Fraser, P. Benhaim and M. H. Hedrick (2002). Human adipose tissue is a source of multipotent stem cells. Mol Biol Cell 13: 4279-4295.

Zuk, P. A., M. Zhu, H. Mizuno, J. Huang, J. W. Futrell, A. J. Katz, P. Benhaim, H. P. Lorenz and M. H. Hedrick (2001). Multilineage cells from human adipose tissue: implications for cell-based therapies. Tissue Eng 7: 211-228.

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11. Declaration / Erklärung

I herewith declare that

1. the dissertation submitted was completed by me without any unauthorised aid
2. only those sources and aids were used as referenced
3. any uses of the works of other authors in any form are properly acknowledged and referenced

Hiermit erkläre ich, dass ich

1. die Arbeit ohne unerlaubte fremde Hilfe angefertigt habe
2. keine anderen als die von mir angegebenen Quellen und Hilfsmittel benutzt habe
3. die den benutzten Werken wörtlich oder inhaltlich entnommenen Stellen als solche kenntlich gemacht habe

Bremen, 25.01.2012

Andreas Richter