FUNCTIONAL ROLE OF HEPARANASE IN CHONDROGENESIS

by

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LIST OF ABBREVIATIONS

Bone Morphogenic Protein (BMP)

Dulbeco's Modified Eagle Medium (DMEM)

Extracellular Matrix (ECM)

Fetal Bovine Serum (FBS)

Fibroblastic Growth Factor (FGF)

Glycosoaminoglycans (GAGs)

Geneticin (G418)

Heparan Sulfate (HS)

Heparin Binding Growth Factor (HBGF)

Indian hedgehog (Ihh)

Minimum Essential Medium (MEM)

Non-Essential Amino Acids (NEAA)

Parathyroid Hormone Related Peptide (PTHrP)

Proteoglycans (PG)

Quantitative Polymerase Chain Reaction (Q-PCR)

ABSTRACT

Heparanase, an endo-β-D-glucuronidase that cleaves heparan sulfate (HS) chains from various heparan sulfate proteoglycans, has been identified in a wide variety of tissues. Despite the ability of heparanase to release active HS binding growth factors in various tissues, the role that heparanase plays in chondrogenesis has not been determined. Quantitative PCR (Q-PCR) and activity assays studying heparanase expression and activity during *in vitro* chondrogenic differentiation of mouse ATDC5 cells show a 72-fold transient increase in heparanase transcript expression and robust heparanase activity throughout the differentiation period. This thesis is based on the hypothesis that the enzyme heparanase is an important mediator of growth factor delivery during chondrogenic differentiation. This hypothesis was tested by knocking down heparanase expression in ATDC5 cells by stably transducing ATDC5 cells with a lentivirus carrying a ribozyme targeting heparanase mRNA. Clones expressing an 80% or greater knockdown in heparanase expression were selected for *in vitro* chondrogenic assays. Perturbing normal patterns of heparanase expression during chondrogenic differentiation was found to reduce collagen type II mRNA levels and lead to delayed accumulation of glycosoaminoglycans (GAGs) during chondrogenic differentiation. ATDC5 cells treated with the heparanase inhibitor PI-88 showed increased cell proliferation and reduced GAG accumulation. Preliminary studies indicate that heparanase overexpression in

ATDC5 cells leads to decreased GAG accumulation. Taken together the data suggest that heparanase expression is important for efficient chondrogenic differentiation.

Chapter 1

INTRODUCTION

1.1 Chondrogenesis and Endochondral Ossification

Bone development occurs through two different processes: intramenbranous ossification and endochondral ossification. The first leads to the formation of flat bones of the skull, the sternum, and the scapula; and involves the direct differentiation of mesenchymal cells into osteoblasts, the bone forming cells. The second process leads to the formation of long bones of the body and is a two-step process wherein chondrocytes form a matrix template in which osteoblasts can differentiate and begin ossification (Provot et al., 2005). Endochondral ossification is a complex process that requires co-ordination between cell-cell; cell-matrix and growth factor mediated signaling.

Chondrogenesis is an important process in vertebral development leading to the development of cartilaginous tissue providing structural support in the articular joints and the respiratory and auditory tracts. Cartilage malformation diseases account for a large proportion of birth defects in humans and can lead to embryonic or perinatal lethality or life long handicaps (Lefebvre et al., 2005).

Endochondral bone formation begins when mesenchymal cells in the limb bud form cell condensations that aggregate due to the expression of cell adhesion molecules. The cells in the condensation differentiate into round proliferative chondrocytes that

secrete a matrix rich in collagen type II. The cells in the condensation proliferate while the cells at the periphery of the condensation differentiate into a thin layer of flattened cells known as the perichondrium, which then defines the boundary of the condensation and prevent the further recruitment of mesenchymal cells. Chondrocytes in the center of the condensation stop proliferating undergo hypertrophy and secrete a matrix rich in type X collagen. Hypertrophic chondrocytes then direct the mineralization of the surrounding matrix and attract blood vessels through the expression of angiogenic factors such as the vascular endothelial growth factor (VEGF). The cartilage matrix left behind acts as a scaffold for the osteoblasts (bone forming cells) that invade the cartilaginous condensation along with the blood vessels forming the primary center of ossification. Ossification then continues from this center toward the ends of the bones (Kronenberg, 2003).

1.2 Heparan Sulfate Proteoglycans

Heparan Sulfate (HS) Proteoglycans (PGs), (HSPGs), located on the cell-surface or the extracellular matrix (ECM) consist of a core protein covalently attached to HS chain(s). The HS chain is composed of alternating hexuronic acid (D-glucuronic acid or L-iduronic acid) and D-glucosamine residues, which may be modified at various positions by sulfation, epimerization and N-acetylation (Gallagher et al, 1992; Stringer et al, 1997; Nakato at al, 2002). HS biosynthesis requires the activity of numerous genes encoding enzymes required for synthesizing linkage regions, HS co-polymerases and

HS modifying enzymes (Nakato et al, 2002). Numerous studies in invertebrates as well as vertebrates have shown that genetic defects in any of the above classes of genes disrupt growth factor signaling during tissue morphogenesis (Nakato et al, 2001; Grobe et al, 2002).

HSPGs are known to play a role in growth control, signal transduction, cell adhesion, wound healing, homeostasis, lipid metabolism and tumor metastasis (Nakato et al, 2001; Esko et al, 2002). Although majority of the HSPG activities involve electrostatic binding of the anionic glycosoaminoglycan (GAG) to protein sequences enriched in basic amino acids, some interactions may involve hydrogen bonds, van der Waals packing and hydrophobic interactions (Thompson et al, 1994).

Various studies have demonstrated that bioactive factors including Indian hedgehog (Ihh), parathyroid hormone-related peptide (PTHrP), fibroblast growth factors (FGFs), and bone morphogenetic proteins (BMPs) as well as different components of the pericellular matrix in cartilage are potential regulators of chondrocyte differentiation (Wallis, 1996; Ohta et al, 1999; Tapp et al, 1999; Murakami et al, 2000; Pizette and Niswander, 2000; Gritli-Linde et al, 2001). HS chains have the ability to bind various growth factors such as FGF-2 and BMP-2, which enhance growth factor delivery.Evidence suggests that HS chain(s) attached to HSPGs can enhance cell signaling by forming trimolecular complexes between certain growth factors and their cognate receptors (Ornitz, 2000, Powers et al, 2000, Schlessinger et al, 2000, Mikami et al, 2004).

Perlecan, a 470 kDa HSPG, is generally found in the basal lamina of adult and embryonic tissues. Various studies have shown that perlecan plays an important role in chondrogenic differentiation both *in vivo* and *in vitro* (Costell et al., 1999; Arikawa-Hirasawa et al., 1999). The perlecan protein core is composed of five domains, four of which bear similarities to other known proteins. However, the N-terminal domain of perlecan is unique and contains three probable sites for the attachment of HS side chains. Studies in the Farach-Carson and Carson laboratories have shown that perlecan coated surfaces maintain the differentiated chondrogenic phenotype of human chondrocytes and promote the condensation and chondrogenic differentiation of C3H10T1/2 cells (murine fibroblasts) *in vitro* (French et al., 1999). Domain mapping studies employing recombinant fragments to the various domains of perlecan have shown that domain I of perlecan (along with the attached GAG chains) promotes a chondrogenic phenotype in C3H10T1/2 fibroblasts (French et al., 2002). Using a ribozyme mediated knockdown approach, this laboratory has shown that endogenous perlecan expression is required for aggregation, chondrogenic differentiation and maturation of C3H10T1/2 cells *in vitro* (Gomes et al, 2006).

1.3 Heparanase

As the HS chains attached to a HSPGs play an important role in the biological activity of the HSPG, the cleavage of the HS chains is likely to affect its biological activity. Heparanase is an endo-β-D-glucuronidase that selectively recognizes relatively infrequent oligosaccharide sequences within modified regions of the HS chain and can cause internal cleavage of the HS chain. Heparanase activity has been detected in a wide variety of normal and malignant cells such as cytotropoblasts, endothelial cells, keratinocytes, platelets, mast cells, neutrophils, macrophages, T and B-lymphocytes, lymphoma, melanoma and carcinoma cells and tissues such as lung, kidney, skin and placenta (Vlodavsky et al., 2002). Heparanase plays a role in diverse biological processes such as wound repair, tissue generation, immune surveillance and embryo implantation (Dempsey et al, 2000). Various studies have demonstrated increased heparanase activity in malignant and aggressive tumors (Parish et al, 2001). However the role of heparanase as a regulator of growth factor delivery during chondrogenesis has not been studied.

The human heparanase gene was cloned by several research groups and is known to have an open reading frame of 1629 base pairs encoding a protein of 543 amino acids and appears as a ~65 kDa band in SDS-PAGE analysis (Vlodavsky et al, 2002; Kussie et al, 1999; Hulett et al, 1999; Toyoshima et al, 1999). The protein undergoes proteolytic cleavage at Glu^{109}-Ser^{110} and Gln^{157}-Lys^{158} resulting in an 8kDa polypeptide at the N-terminal, a 50kDa peptide at the C-terminus and a 6kDa linker polypeptide. The

active heparanase is a heterodimer consisting of the 50kDa subunit non-covalently linked to the 8 kDa subunit (Fairbanks et al, 1999; Levy-Adam et al, 2003). Miao et al. cloned, expressed and purified the mouse heparanase gene. The gene has an opening reading frame of 1605 bp encoding a protein of 535 amino acids that is 77% identical to the human protein. Mouse heparanase first is processed into a 60kDa pro-heparanase by the cleavage of the signal peptide. The active enzyme exists as a non-covalently bound heterodimer of a 50kDa protein (Lys^{150}-Ile^{535}) and the 8kDa peptide (Asp28-Lys100) resulting from the internal cleavage of a 49-residue peptide (Glu101- Gln149). Maximum mouse heparanase activity is observed under slightly acidic conditions, such as would exist in the hypoxic avascular regions of cartilage, but is less active at neutral pH (Miao et al, 2002). Thus the mouse heparanase is similar to its human homologue.

1.3 Hypothesis

The purpose of this study is to test the hypothesis that the enzyme heparanase is an important mediator of growth factor delivery during chondrogenic differentiation. During chondrogenic differentiation, heparin binding growth factors (HBGFs) bind to the HS chains attached to HSPGs such as perlecan, which are expressed in cartilage. Heparanase then cleaves the HS chain from the HSPG such that the HS chain is still associated with the HBGF. The HS-HBGF complex then is delivered to its receptor on the cell surface where it initiates signaling pathways leading to chondrogenic differentiation. Just the HS chain alone or just the HBGF alone is not sufficient to

initiate signaling. Perturbing the normal pattern of heparanase expression is therefore expected to impact HS-dependent processes during cartilage differentiation.

1.4 Experimental Approach

Studies in our laboratory have shown that heparanase is expressed *in vitro* in the murine chondrogenic cell line, ATDC5. ATDC5 is a clonal cell line derived from the tetracarcinoma AT805 and can display both early and late markers of chondrogenic differentiation when cultured in the presence of insulin (Atsumi et al., 1990). ATDC5 cells can also initiate mineral deposition when cultured under appropriate conditions (Shukunami et al., 1997). Hence the ATDC5 cells provide a good *in vitro* model to study endochondral ossification. Quantitative PCR (Q-PCR) and activity assays studying heparanase expression and activity during *in vitro* chondrogenic differentiation of mouse ATDC5 cells show a 72-fold transient increase in heparanase transcript expression and robust heparanase activity throughout the differentiation period (unpublished data).

The role of heparanase as an important mediator of growth factor delivery during chondrogenic differentiation was tested by stably transducing ATDC5 cells with a ribozyme targeting heparanase mRNA. ATDC5 clones exhibiting a greater than 80% knockdown in heparanase mRNA then were subjected to chondrogenic assays. ATDC5 cells also were treated with the heparanase inhibitor PI-88 and cultured in *vitro* chondrogenic assays. PI-88 is a highly sulfonated oligosaccharide that inhibits

heparanase activity and competes with HS for binding HBGFs (Rosenthal et al., 2002). These experiments studied the effect of reduced heparanase activity on the expression of cartilage specific genes. ATDC5 cells also were stably transfected with a plasmid expressing human heparanase and the resulting ATDC5 heparanase over-expression clones were studied to access the effect of increased heparanase expression on chondrogenic differentiation.

1.5 Practical Implications

The main aim of this thesis is to examine the role of heparanase in HBGF delivery during endochondral ossification. This study is conducted in an *in vitro* system and the results of this study will be used to propose potential heparanase function(s) in an *in vivo* model.

Chapter 2

MATERIALS AND METHODS

2.1 Cell Culture

ATDC5 cells, a murine carcinoma derived chondrogenic cell line, were obtained from Dr. Véronique Lefebvre (Lerner Research Institute, Cleveland, OH) and were maintained as monolayer cultures. Cells were cultured at 37^{o}C in 95% air: 5% (v/v) CO_2 in Dulbecco's modified Eagle's medium-F12 (DMEM-F12) supplemented with 5% (v/v) fetal bovine serum (FBS). All cell culture reagents were purchased from Invitrogen (Carlsbad, CA) unless otherwise stated. Cells were maintained in 75cm² cell culture flasks (Corning, Corning, NY) and passaged at 90% confluency with trypsin-EDTA (Invitrogen) prior to being plated for further experiments. The ATDC5 clones were cultured in DMEM-F12 suplemented with 5% FBS and the appropriate concentration of the antibiotic used for selection.

293FT cells, a cell line derived fron the human embryonal kidney 293 cells and stably expressing the SV40 large T antigen (Naldini et al.,1996) were cultured at 37^{o}C in 95% air: 5% (v/v) CO_2 in Dulbecco's modified Eagle's medium (D-MEM) high glucose, supplemented with 10% (v/v) FBS, 0.1 mM MEM Non-Essential Amino Acids (NEAA) and 500 μg/ml of Geneticin (G418).

2.2 Killing Curve Analysis

ATDC5 cells were plated in six well plates at a density of 100,000 cells/well. After 24 hrs, the cells were treated with different concentrations of blasticidin (Invitrogen) (0, 2.5, 3, 3.5, 4 and 4.5 μg/ml) or with different concentrations of G418 (Invitrogen) (0, 100, 250, 400, 550, 700 μg/ml). Following treatment cells were trypsinized and counted using a hemocytometer at various time points. The media was changed every four days. Cell viability was measured by staining the cells with 0.4% (w/v) Trypan Blue (Sigma, St. Louis, MO, USA).

2.3 Generating a Ribozyme Targeting Heparanase mRNA

A hammerhead ribozyme targeting heparanase mRNA was designed and tested by the Ribozyme Core at the University of Delaware according to previously published procedures (Liu et al., 2002). The ribozyme targeting heparanase mRNA is shown in Figure 1. The ribozyme was cloned into a U6 RNAi cassette. The U6 RNAi cassette then was transferred to a pLenti6/BLOCK-iT™-DEST destination vector. The destination vector contains elements that allow packaging of the construct into virions and the blasticidin resistance gene for the selection of stably transduced cell lines.

2.4 Generating Lentivirus Carrying the Heparanase Ribozyme

To generate lentivirus carrying the heparanase ribozyme, 293FT cells were co-

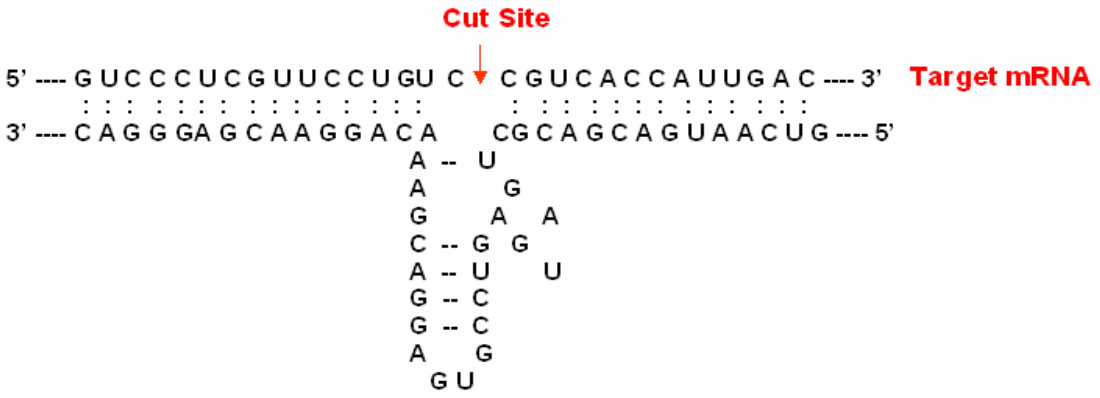

Figure 2.1: Hammerhead Ribozyme Targeting Heparanase mRNA
The heparanase hammerhead ribozyme contains a 24-nt hammerhead domain flanked by a 10-15 nucleotide region complementary to the target site of the heparanase mRNA. The cut site is the bond at which the ribozyme cleaves.

transfected with the ViraPower (Invitrogen) packaging mix and the pLenti6/heparanase ribozyme. DNA liposomes were created as follows: 9 μg of the ViraPower packaging mix and 3 μg of pLenti6/heparanase ribozyme plasmid DNA were diluted in 1.5 ml of OPTI-MEM (Invitrogen) in a 12mm x 75mm polystyrene tube, 36 μl of Lipofectamine2000 (Invitrogen) were diluted in 1.5 ml of OPTI-MEM in a separate tube and incubated at room temperature for five min. The contents of the two tubes were then combined and incubated for 20 min at room temperature. During this incubation, 293FT cells were trypsinized, counted using a hemocytometer and resuspended at a concentration of 1.2 x 10^6 cells/ml in OPTI-MEM supplemented with 10% (v/v) FBS. The DNA liposome complexes were added to five ml of cell suspension and five ml of OPTI-MEM supplemented with 10% (v/v) FBS in a T-75 flask (Corning) and incubated overnight at 37^{o}C in 95% air: 5% (v/v) CO_2. The DNA-liposome containing media then was replaced with DMEM-F12 supplemented with 10% (v/v) FBS, 0.1 mM MEM NEAA and 1mM sodium pyruvate. Forty-eight hours post transfection, the virus containing supernatant was harvested, centrifuged at 3000 rpm for 5 min, syringe filtered using a 0.45 μm filter, and aliquot were stored at -80^{o}C.

2.5 Titering of the Viral Stock

ATDC5 cells were plated in six well plates at a density of 100,000 cells/ml. After 24 hours, the cells were transduced with one ml 10-fold series dilution of viral stock (1:100-1:1,000,000). Twenty-four hours post transduction, the media was

replaced with DMEM-F12 supplemented with 5% (v/v) FBS. Seventy-two hours post transduction; the media was replaced with regular media supplemented with blasticidin (4.5μg/ml). Media was changed every four days and was replaced with media containing blasticidin. After 14 days of treatment with blasticidin, cells were washed twice with Dulbecco's Phosphate Buffer Saline (Invitrogen) (PBS) and were stained with one ml of 1% (w/v) crystal violet for two min. The cells were then washed twice with PBS and the number of colonies formed was recorded. The viral titer was calculated as the average of the number of colonies formed multiplied by the dilution factor of the virus.

2.6 Generation of Cell Lines Stably Transduced with the Heparanase Ribozyme

ATDC5 cell were plated in a 12-well plate at a density of 50,000 cells/well. After 24 hours, 0.5 ml of the heparanase ribozyme viral stock or the scrambled ribozyme viral stock (1:100,000 in DMEM-F12 supplemented with 5% [v/v] FBS) was added to each well. Twenty-four hours post transduction, the media was replaced with DMEM-F12 supplemented with 5% (v/v) FBS. Seventy-two hours post transduction, the media was replaced with regular media supplemented with blasticidin (4.5 μg/ml) and changed every four days. Following 14 days of treatment with blasticidin, colonies were identified and trypsinized separately at room temperature for one minute and were transferred to a 24 well plate containing one ml of DMEM-F12 supplemented with 5% (v/v) FBS supplemented with blasticidin (4.5 μg/ml). After the colonies were 90%

confluent, the clones were trypsinized and split 1:3 in 35mm plates (Corning). At confluency, both protein and RNA were extracted from the cells for further analysis.

2.7 Generating Construct Expressing Human Heparanase

A pBK-CMV plasmid (Stratagene, La Jolla, CA) containing a 1.7 kb fragment containing the entire open reading frame of human heparanase (GenBank Accession Number AF144325, from SV40 transformed human fibroblast cell line Wi38/VA13 cDNA library) was kindly provided by Jian Dong (Tsukuba Research Institute, Novartis Pharma, K. K., Tsukuba, Japan). The 1.7 Kb fragment was cloned into the EcoRI site of the T&A vector (Invitrogen). To assist with future analysis, the 1.7kb fragment was transferred to the pcDNA3.1TM -myc-His (-) vector (Invitrogen) to allow for detection using an anti-myc antibody. High-level constitutive expression of human heparanase-1 (hpa-1) was driven by the CMV promoter.

2.8 Generating of Cell Lines Stably Transfected with Human Heparanase

ATDC5 cells (1 x 10^5 cells/well) were seeded in six well plates 24 hours prior to transfection with Lipofectamine 2000 (Invitrogen). To perform transfection, the heparanase plasmid DNA was diluted in OPTI-MEM to a concentration of 1.5 µg/ml in a in a 12mm x 75mm polystyrene tube. In a second tube, lipofectamine 2000 was diluted with OPT-MEM such that 1 ml of OPTI-MEM contained 7.5 µl of lipofectamine 2000 and was allowed to incubate for five min at room temperature. The contents of the two

tubes were combined and allowed to incubate for 20 min at room temperature. The ATDC5 cells were washed twice with PBS and incubated with 1.5 ml of OPTI-MEM /well for 15 min and 200 µl of the liposomes were added to each well. Four hours post transfection, the media was replaced with DMEM-F12 supplemented with 5% (v/v) FBS, 72 hours post transfection, the media was replaced with DMEM-F12 supplemented with 5% (v/v) FBS and G418 (400µg/ml). Thereafter, the media was changed every three days and replaced with DMEM-F12 supplemented with 5% (v/v) FBS and G418 (400µg/ml). Individual colonies were identified after two weeks of culture. The colonies were trypsinized separately at room temperature for one minute and were transferred to a 24 well plate containing one ml of DMEM-F12 supplemented with 5% (v/v) FBS and G418 (400 µg/ml). After the colonies were 90% confluent, the clones were trypsinized and split 1:3 in 35 mm plates. At confluency, both protein and RNA were extracted from the cells for further analysis.

2.9 Differentiation Culture

For differentiation, ATDC5 cells in DMEM-F12 supplemented with 5% (v/v) FBS were seeded in 6-well tissue culture plates (Corning Inc, Corning, NY) until they reached 70-80% confluency. Culture media then was replaced with regular growth media (DMEM-F12 + 5% [v/v] FBS) containing 10µg/ml bovine insulin (I), 10µg/ml human transferrin (T), and 3 x 10^{-8} mol/L sodium selenite (S), (ITS). After 21 days in differentiation media, cells were transferred to 97% air: 3% (v/v) CO_2 and switched to

α-MEM containing 5% (v/v) FBS, and 1% (v/v) ITS for further differentiation into calcifying chondrocytes as described in Shukunami et al. (1997). At various time points, cells were harvested for RNA and protein analysis.

2.10 Micromass Cultures

To prepare micromass cultures, 1 x 10^5 cells in a total volume of 10µl of DMEM/F-12 supplemented with 5% (v/v) FBS was placed in the center of the wells of a polystyrene 4 well plate (1.9cm^2/well, Nalge, Nunc, Roskilde, Denmark). The plates were incubated for three hours in a tissue culture incubator set at 37^{o}C and 95% air: 5% (v/v) CO_2. Following this incubation, one ml of DMEM/F-12 supplemented with 5% (v/v) FBS 1% (v/v) ITS was added to the cultures. The media was changed every other day and the cells were harvested at various time points and stained with alcian blue or crystal violet.

For PI-88 cultures, the drug PI-88 was generously provided by Progen Industries (Toowong, Queensland, Australia). The PI-88 powder was dissolved in PBS and aliquots were stored at -20 ^{o}C. Micromass cultures were treated with different concentrations of PI-88 and additional PI-88 was added during each media change.

For transient transduction experiments, ATDC5 cells were treated with different dilutions of the Lentivirus carrying a ribozyme targeting heparanase mRNA either 24 hours prior to plating or three hours after in micromass cultures. More virus was added

during each media change and cells were stained with crystal violet and alcian blue at different time points.

2.11 Staining

For staining with alcian blue cells were washed twice with Phosphate Buffer Saline (PBS) containing 0.901 mM calcium and 0.493 mM magnesium (Invitrogen). The cells were fixed for 10 min at room temperature with 10% (w/v) neutral buffered formalin (Sigma), containing 0.5% (w/v) cetylpyridinium chloride (Sigma) and rinsed three times with 3% (v/v) glacial acetic acid (pH 1.0). Cells were incubated overnight at room temperature with 1% (w/v) Alcian Blue 8GX (Sigma) in 3% (v/v) glacial acetic acid (pH 1.0). Post staining, cells were rinsed trice with 3% (v/v) glacial acetic acid (pH 1.0), then trice with 3% (v/v) glacial acetic acid (pH 2.5) to remove the unbound alcian blue, air dried, and finally photographed with a digital camera (Nikon SMZ21500) attached to a microscope.

To perform crystal violet staining, cells were washed twice with PBS containing calcium and magnesium (Invitrogen). The cells were then treated with 1% (w/v) Crystal violet (Sigma) for two min at room temperature. The cells were washed trice with PBS containing calcium and magnesium (Invitrogen), air dried and photographed as above.

2.12 Quantitative RT-PCR

Total RNA from ATDC5 cells was extracted using the RNeasy® Mini Kit and QIAshredder column (Qiagen, Valencia, CA) and digested with DNAseI with the DNA-free kit (Ambion, Austin, TX) according to the manufacturer's instructions. RNA purity and concentration was assessed by measuring the absorbance of each RNA sample at both 260nm and 280nm. Reverse transcription (RT) was performed with Omniscript™ Reverse Transcriptase (Qiagen, Valencia, CA). Q-PCR was performed using SYBR® green PCR master mix (Applied Biosystems, Foster City, CA). Heparanase, Collagen type II, Collagen type X and β-Actin primer sequences were designed using Primer Express (Applied Biosystems, Foster City, CA). PCR samples were cycled for 15 sec at 95^{o}C and 60 sec at 60^{o}C for 45 cycles using the ABI Prism® 7000 Sequence Detection System (Applied Biosystems, Foster City, CA). All data collected from Q-PCR were calculated using the ABI Prism® 7000 software (Applied Biosystems). The relative amounts of the mRNA of interest were identified using the comparative threshold cycle (Ct) method (ABI Prism® 7000 Sequence Bulletin Number 2). To confirm amplification specificity of the fluorescent detection system, Q-PCR products were analyzed in a 1.5% (w/v) agarose gel stained with etidium bromide. All gels revealed a single PCR product at the appropriate size.

2.13 SDS-PAGE and Western Blotting

Total cell extract from ATDC5 cells at different time intervals was obtained by lysing cells with Radio-Immunoprecipitation Assay (RIPA) Buffer. Protein concentration was determined using the bicinchoninic acid (BCA) assay. Cell extracts (40 μg) were incubated for five min at 100^{o} C with Laemmli Sample Buffer (BioRad Laboratories, Hercules, CA) and electrophoresed by SDS-PAGE using a 10% (w/v) Porzio-Pearson polyacrylamide gel (Invitrogen,). After electrophoresis, gels were transferred to Protran® Pure Nitrocellulose and Immobilization Membrane (Scheleicher & Schuell Bioscience Inc, Keene, NH) for western blotting with rabbit anti-mouse heparanase antibody 1453 (obtained from Dr. Israel Vlodavsky, Cancer and Vascular Biology Research Center, The Bruce Rappaport Faculty of Medicine, Technion, Haifa, Israel). Briefly, the blots were blocked overnight at 4^{o}C with 5% (w/v) milk in PBS plus 0.1% (v/v) Tween 20 (PBS-T) and then incubated overnight at 4^{o}C with the primary antibody at a final dilution of 1: 2,500 in 5% (w/v) Milk/PBS-T. Blots were rinsed three times, 10 min each at room temperature with PBS-T to remove unbound antibody. Next, blots were incubated for two hours at 4^{o}C with anti-rabbit IgG peroxidase conjugate (Sigma, St Louis, MO) at a final dilution of 1:200,000 in 5% (w/v) Milk/PBS-T. Finally, the blots were rinsed three times for 10 min each at room temperature and detected using the ECL system (Pierce, Rockford, IL) as described by the manufacturer. Recombinant human heparanase (obtained from Dr Israel Vlodavsky)(0.0049ug) was used as a positive control.

β-Actin expression was used as load control. After blocking, blots were incubated with primary antibody (Abcam, Cambridge, MA) at a final dilution of 1: 10,500 in 3% (w/v) bovine serum albumin (BSA) in PBS-T. Blots were rinsed three times, 10 min each at room temperature with PBS-T to remove unbound antibody. Next, blots were incubated for two hours at 4^{o}C with sheep anti-mouse IgG peroxidase conjugate (Sigma, St Louis, MO) at a final dilution of 1:200,000 in 5% (w/v) Milk/PBS-T. Finally, the blots were rinsed three times for 10 min each at room temperature and detected using the ECL system (Pierce).

For detecting myc tagged human heparanase expression, blots were incubated with primary antibody (Invitrogen) at a final dilution of 1: 2,500 in 5% (w/v) milk in PBS-T. Blots were rinsed three times, 10 min each at room temperature with PBS-T to remove unbound antibody. Next, blots were incubated for two hours at 4^{o}C with sheep anti-mouse IgG peroxidase conjugate (Sigma) at a final dilution of 1:200,000 in 5% (w/v) Milk/PBS-T. Finally, the blots were rinsed three times for 10 min each at room temperature and detected using the ECL system (Pierce).

2.14 Immunocytochemistry

Cells were washed twice with PBS containing calcium and magnesium and fixed with 100% methanol for 10 minutes at room temperature. The cells were blocked with 1% (w/v) BSA in PBS for 30 min at room temperature and washed trice with PBS containing calcium and magnesium. Human heparanase antibody (Insight) was

conjugated with Alexa Fluor 488 using a Zenon mouse IgG labeling kit (Invitrogen). The cells were incubated with the conjugated antibody (1:40) and DRAQ5 (1:2000) in 1% (w/v) BSA in PBS for 0ne hour at room temperature and washed trice with PBS containing calcium and magnesium. The cells then were post fixed with 4% (v/v) paraformaldehyde for 10 min at room temperature and washed trice with PBS containing calcium and magnesium.

Chapter 3

RESULTS

3.1 Killing Curve Analysis

To generate ATDC5 cells stably expressing the ribozyme targeting heparanase mRNA, ATDC5 cells were transduced with the heparanase ribozyme virus and transduced cells were selected by their ability to grow in the presence of the antibiotic, blasticidin. Prior to selection of stable clones, ATDC5 cells were treated with different concentrations of blasticidin and stained with trypan blue to determine cell viability at various time points. The results of this experiment are shown in Figure 3.1. As seen in the figure, essentially all ATDC5 cells grown in the presence of 4.5 μg/ml of blasticidin die within a 10-day period and therefore ATDC5 cells stably expressing the ribozyme targeting heparanase mRNA were selected by culturing the transduced cells in the presence of 4.5 μg/ml of blasticidin over a 14 day time period.

To generate ATDC5 cells stably overexpressing heparanase, ATDC5 cells were transfected with a plasmid encoding human heparanase and resistance to geneticin (G418). Prior to selection of stable clones, killing curve analysis was performed as above. As seen in the Figure 3.2, 400 μg/ml of G418 was sufficient to kill essentially all cells in a 10-day time period. ATDC5 cells stably expressing human heparanase were selected by culturing

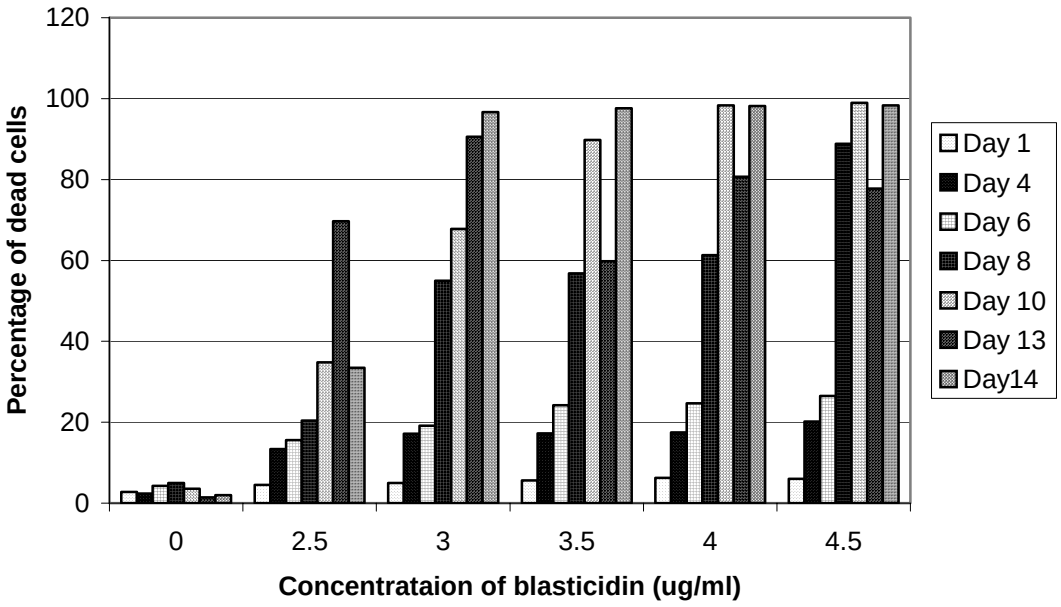

Figure 3.1: Blasticidin Killing Curve Analysis
ATDC5 cells were treated with different concentrations of blasticidin and stained with trypan blue to determine cell viability at various time points. The bars above show the percentage of dead cells. ATDC5 cells grown in the presence of 4.5 μg/ml of blasticidin die within a ten-day period.

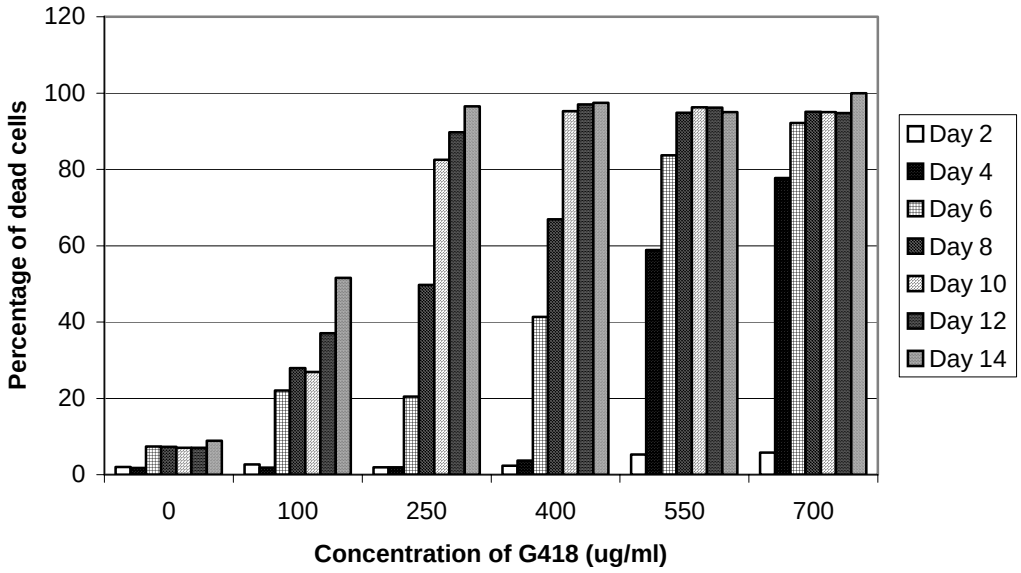

Figure 3.2: Geneticin Killing Curve Analysis
ATDC5 cells were treated with different concentrations of G418 and stained with trypan blue to determine cell viability at different time points. The graph above shows the percentage of dead cells. ATDC5 cells grown in the presence of 400 μg/ml of G418 die within a ten-day period.

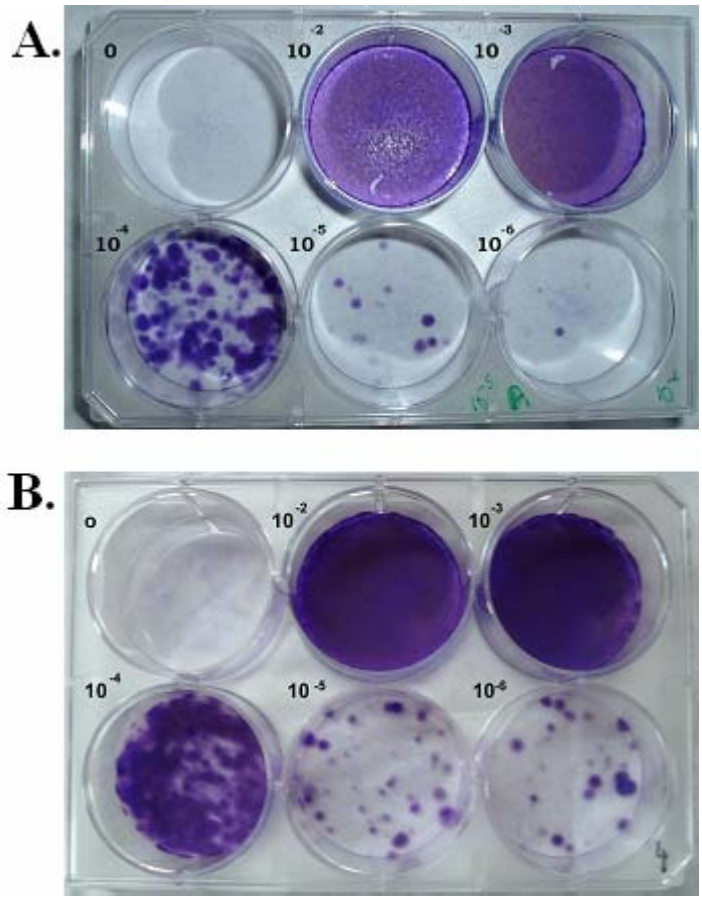

Figure 3.3: Titering of Lentiviral Stock
ATDC5 cells were treated with the indicated ten fold dilutions of the heparanase ribozyme virus (A) or the control ribozyme virus (B). The cells were cultured for two weeks in the presence of blasticidin prior to staining with crystal violet. The titer of the heparanase virus and the control virus were found to 4.25 x 10^6 Transduction units (TU)/ml and 9.65 x 10^6 TU/ml respectively.

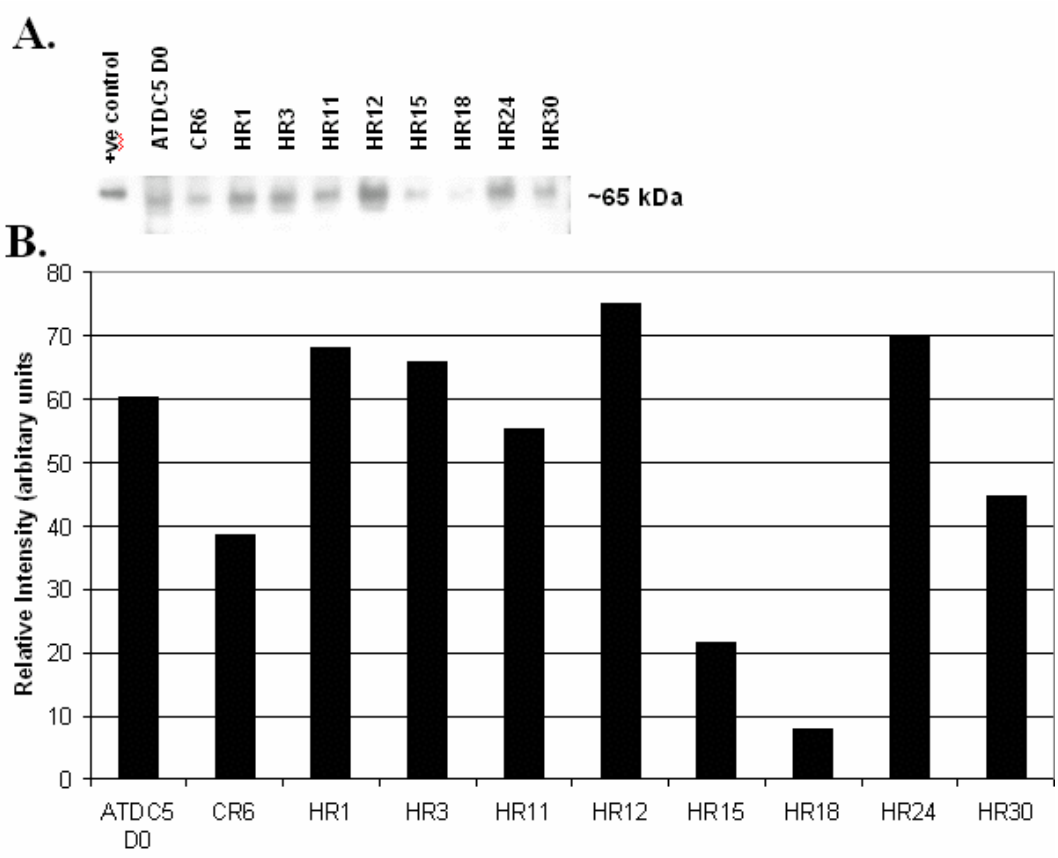

Figure 3.4: Western Blot Analysis of Heparanase Knockdown Clones
Total cell extracts were obtained from the ribozyme clones upon reaching 100% confluency. Protein samples (40µg) were electrophoresed on a 10% Porzio-Pearson gel and probed with rabbit anti-mouse heparanase antibody followed by anti-rabbit IgG peroxidase conjugate detection. The Western Blot (A) was subjected to densitometric analysis (B) using the Scion Image software. Recombinant human heparanase was used as a positive control. As seen in the figure the clone HR18 had an 86% knockdown in heparanase expression.

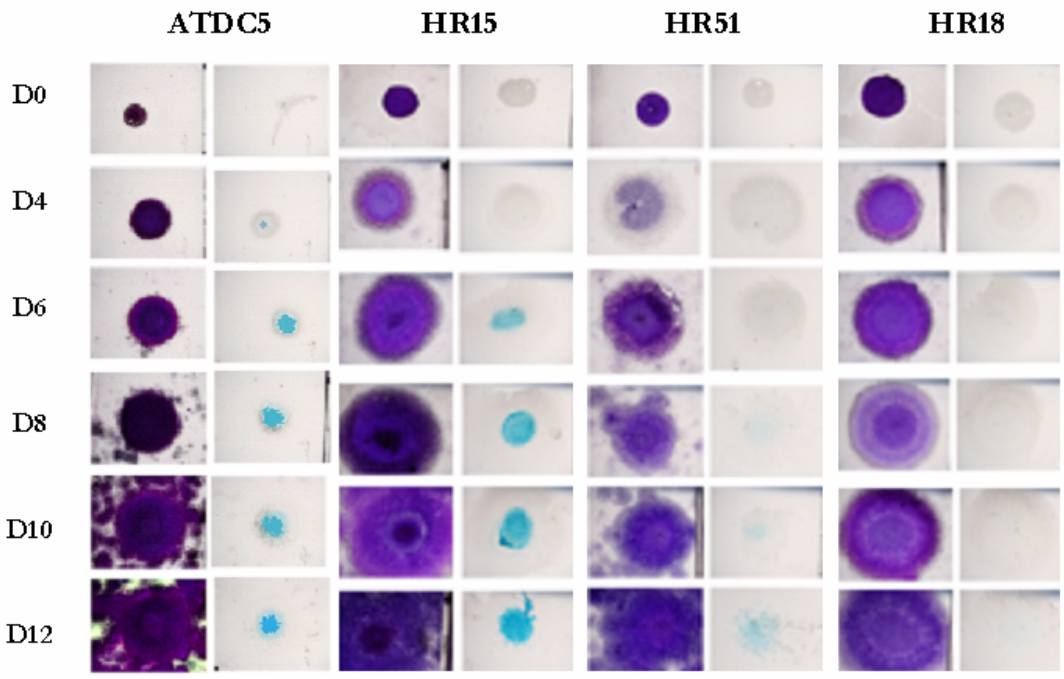

Figure 3.5: Alcian Blue Labeling of Heparanase Knockdown Clones
The parental ATDC5 cells as well as the selected heparanase knockdown clones were cultured in high-density micromass cultures and were stained with alcian blue (right panel) or crystal violet (left panel) at different time points. As seen in the figure, the knockdown clones exhibit delayed accumulation of GAGs, but have a similar cell proliferation pattern as compared to the parental ATDC5 cells.

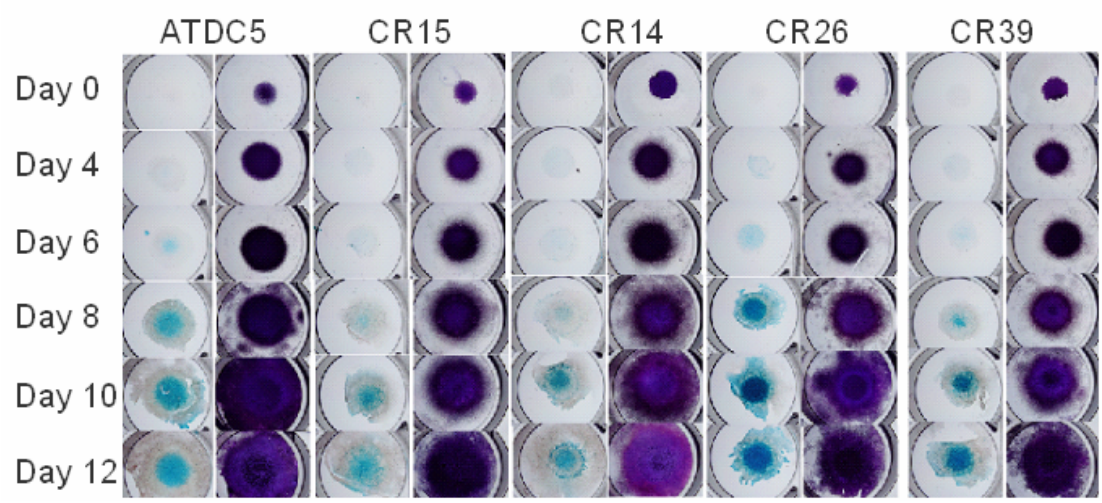

Figure 3.6: Alcian Blue Labeling of Scrambled Ribozyme Clones
The parental ATDC5 cells as well as the selected scrambled ribozyme clones were cultured in high-density micromass cultures and were stained with alcian blue (left panel) or crystal violet (right panel) at different time points. As seen in the figure, control clones exhibit a similar pattern for accumulation of GAGs and a similar cell proliferation pattern as compared to the parental ATDC5 cells.

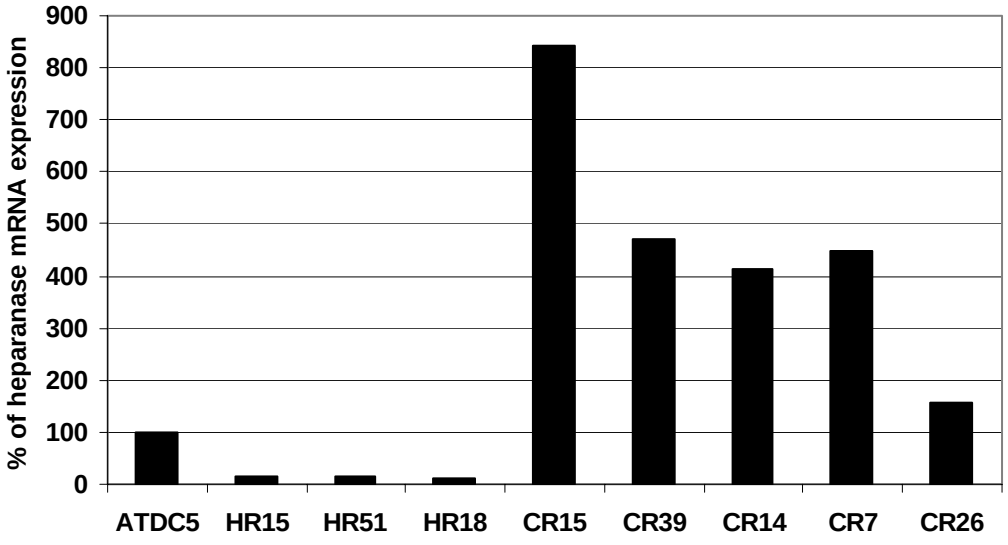

Figure 3.7: Heparanase mRNA Expression in Selected Heparanase Ribozyme and Scrambled Ribozyme Clones
The heparanase mRNA levels in selected clones were analyzed using Q-PCR. As seen in the figure, the clones HR15, HR51 and HR18 have a greater than 80% knockdown in heparanase mRNA expression as compared to the parental ATDC5 cells. The scrambled ribozyme clones CR15, CR39, CR14, CR7, and CR26 express higher levels of heparanase mRNA compared to the parental ATDC5 cells.

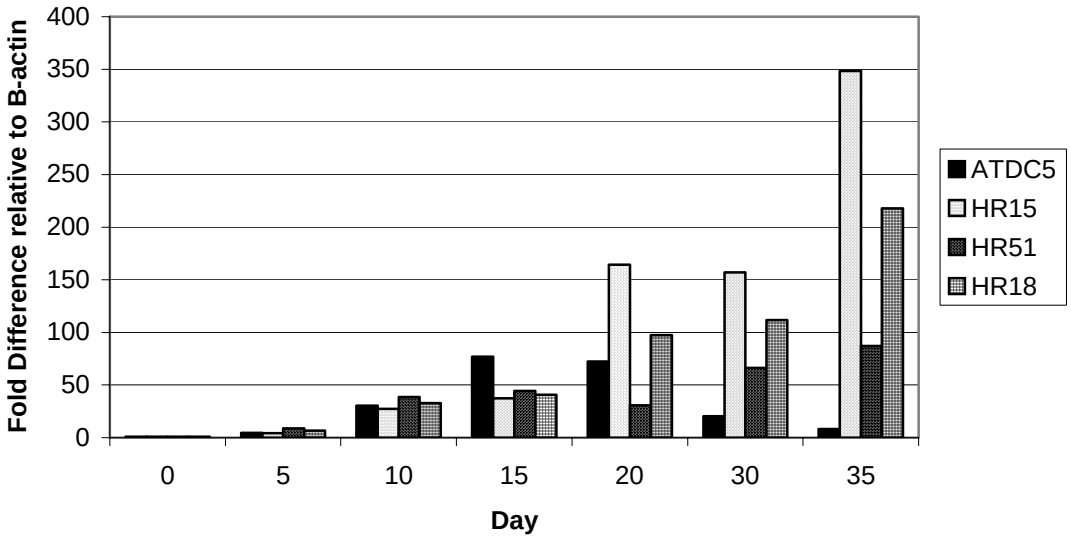

Figure 3.8: Heparanase mRNA Expression in Heparanase Knockdown Clones During Chondrogenic Differentiation
The parental ATDC5 cells and the selected heparanase ribozyme clones were subjected to 35 day differentiation cultures and RNA samples collected at different time points were analyzed for the expression of heparanase mRNA through Q-PCR. As seen in the figure, the knockdown clones exhibit an altered heparanase mRNA pattern as compared to the parental ATDC5 cells.

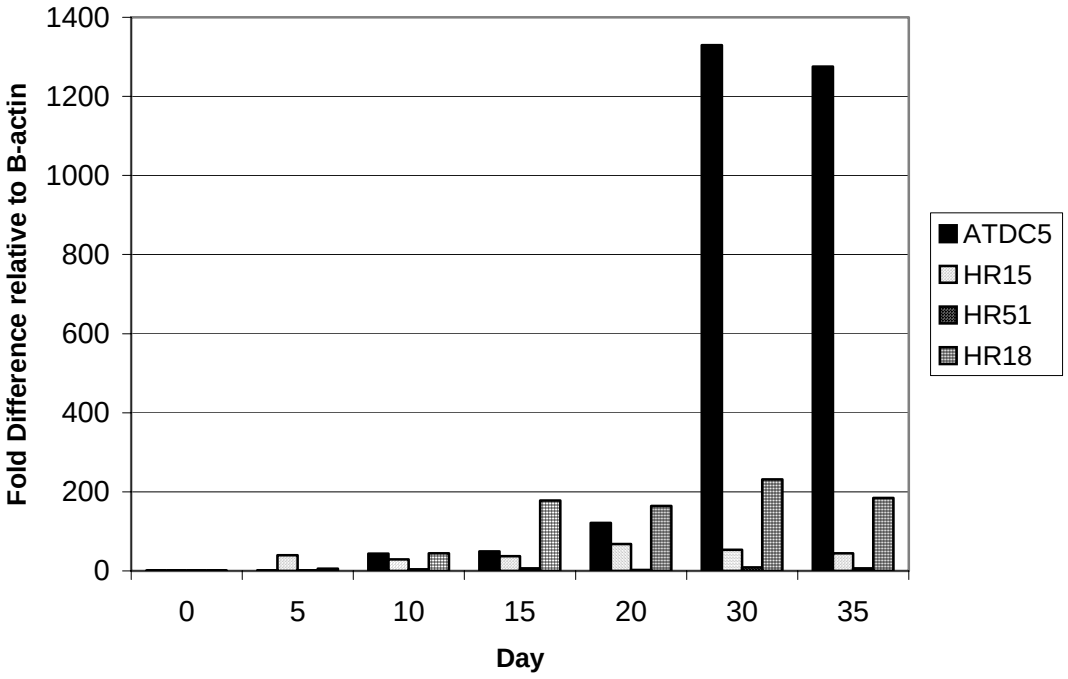

Figure 3.9: Collagen type II mRNA Expression in Heparanase Knockdown Clones During Chondrogenic Differentiation
The parental ATDC5 cells and the selected heparanase ribozyme clones were subjected to 35 day differentiation cultures and RNA samples collected at different time points were analyzed for the expression of collagen type II mRNA through Q-PCR. As seen in the figure, the heparanase knockdown clones exhibit a reduced expression of collagen type II particularly at days 30 and 35.

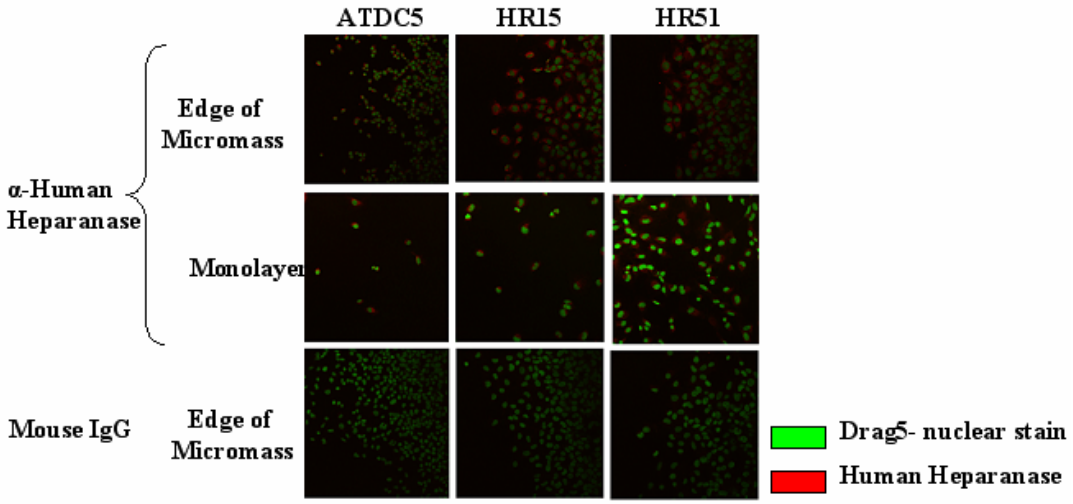

Figure 3.10: Analysis of Heparanase Knockdown in Heparanase Ribozyme Clones
ATDC5 cells and the heparanase ribozyme clones 15 and 51 were plated in micromass and monolayer cultures. Heparanase protein expression was detected using immunocytochemical techniques using an antibody against human heparanase. As seen in the above figure, clones 51 and 15 express heparanase comparable to or greater than the parental ATDC5 cells. The above images were taken at 20X magnification.

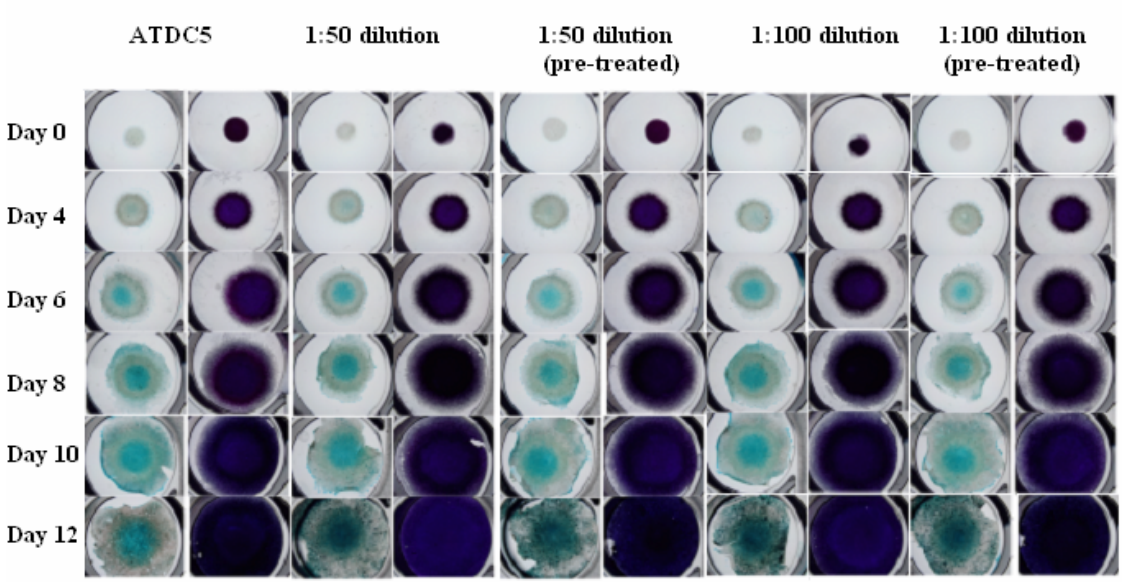

Figure 3.11: Analysis of Chondrogenic Differentiation in ATDC5 Cells Transiently Transduced with the Heparanase Ribozyme
ATDC5 cells were plated in micromass cultures and treated with different dilutions of the lentivirus carrying a ribozyme targeting heparanase mRNA on alternate days for a 12 day time period. ATDC5 cells in two T-75 flasks were treated with different dilutions of the virus 24 hours prior to plating in micromass cultures. As seen the above figure there is no obvious difference in alcian blue staining intensity between the parental ATDC5 cells and cells treated with the lentivirus targeting heparanase mRNA.

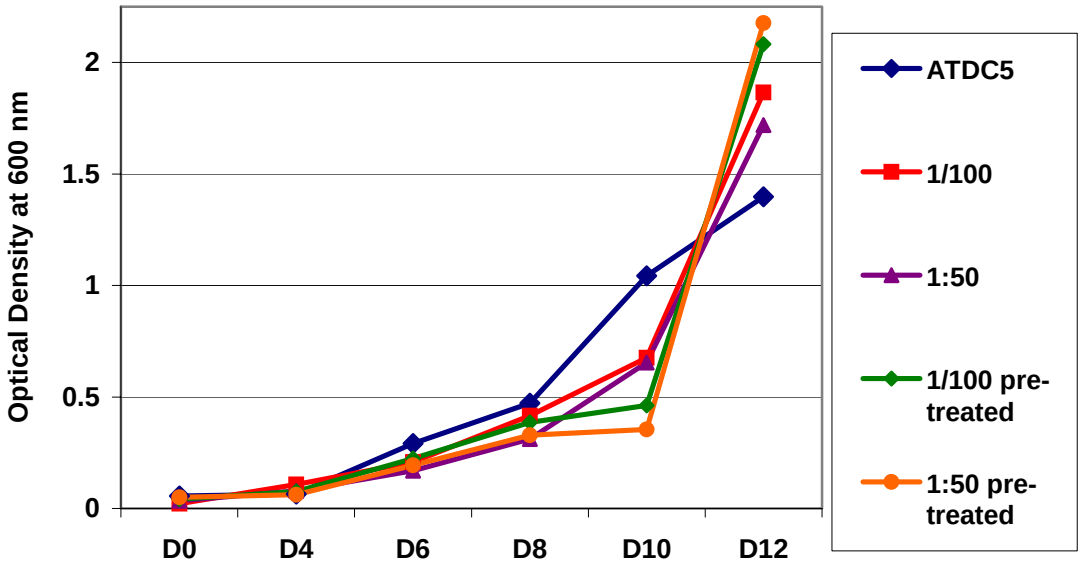

Figure 3.12: Quantification of Alcian Blue Staining Intensity for ATDC5 cells Treated with the Ribozyme Targeting Heparanase mRNA

The alcian blue staining intensity for the micromass cultures shown in Figure 3.11 was measured by extracting alcian blue with 6M guanidine HCl and recording absorbance at 600 nm. As seen in the above figure lentivirus treated micromass cultures show similar alcian blue staining intensity until day 4 and show exhibit a lower staining intensity from day 6-10. Alcian blue intensity for the lentivirus treated micromass cultures is higher on day 12 as compared to untreated micromass cultures.

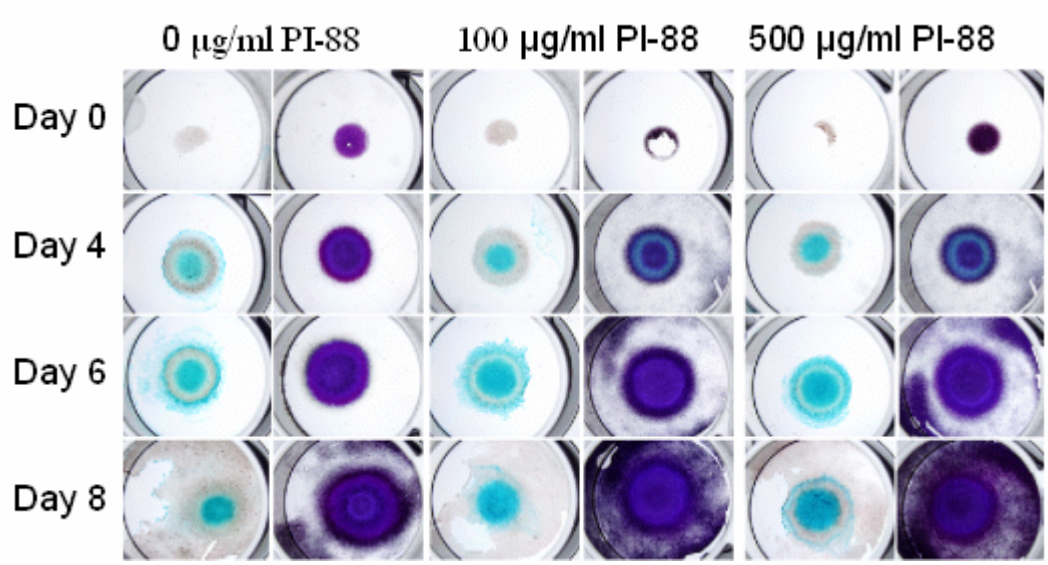

Figure 3.13: Alcian Blue Labeling of ATDC5 Micromass Cultures Treated with the Heparanase Inhibitor, PI-88
ATDC5 cells cultured in micromass cultures were treated with the indicated concentrations of the heparanase inhibitor, PI-88. The micromass cultures were stained with alcian blue (left panel) or crystal violet (right panel) to determine GAG accumulation and cell proliferation respectively. As seen in the above figure there is no obvious difference in the alcian blue staining intensity for the cells treated with PI-88, although the PI-88 treated micromass cultures may exhibit increased cell proliferation.

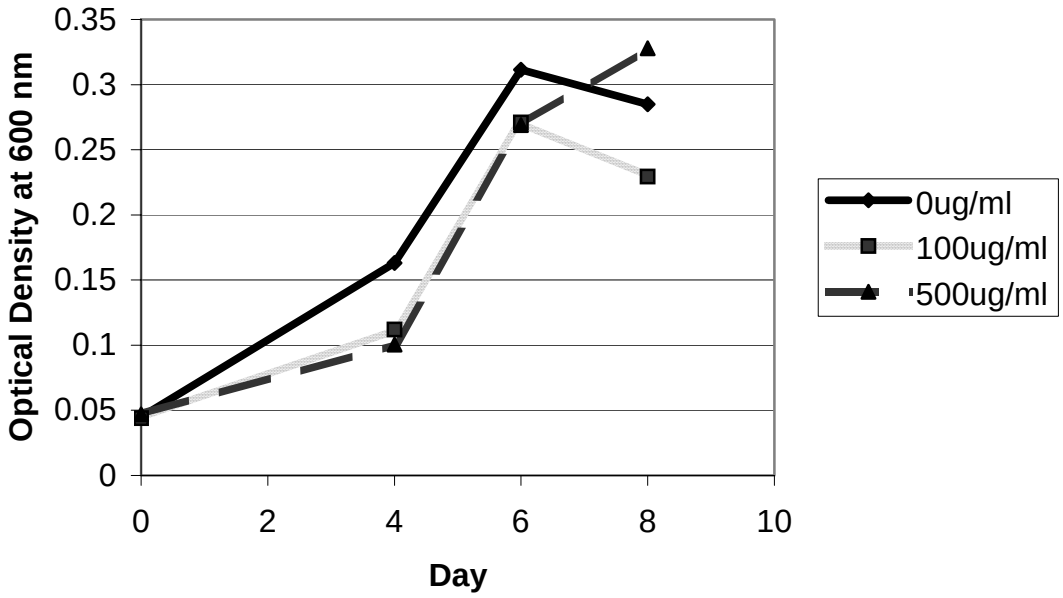

Figure 3.14: Quantification of Alcian Blue Staining for PI-88 Treated Micromass Cultures

The alcian blue staining intensity for ATDC5 micromass cultures treated with PI-88 was quantified by extracting the alcian blue with 6M Guanidine HCl and recording the absorbance at 600 nm. As seen in the above figure the PI-88 treated micromass cultures have a reduced alcian blue staining intensity.

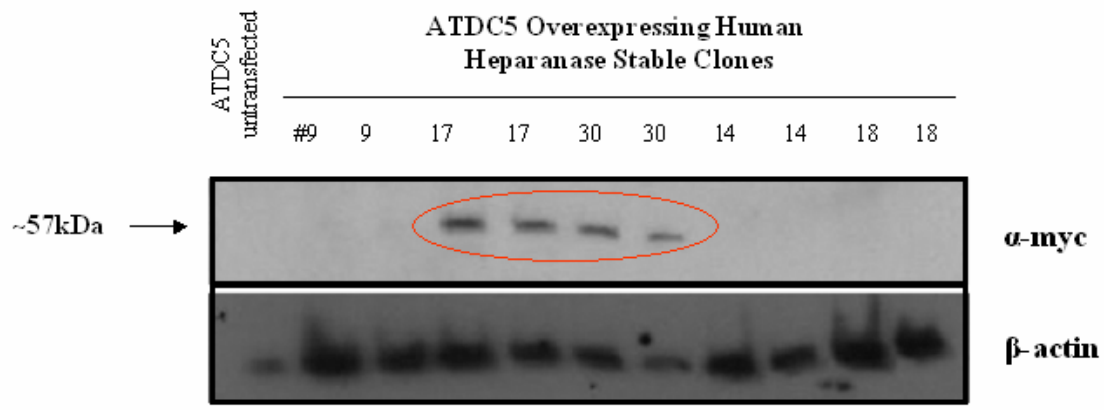

Figure 3.15: Analysis of Heparanase Overexpression Clones
ATDC5 cells stably transduced with a plasmid expressing human heparanase with a myc tag were analyzed by western blotting with a myc antibody. As seen in the above figure clones 17 and 30 exhibit a band of appropriate size.

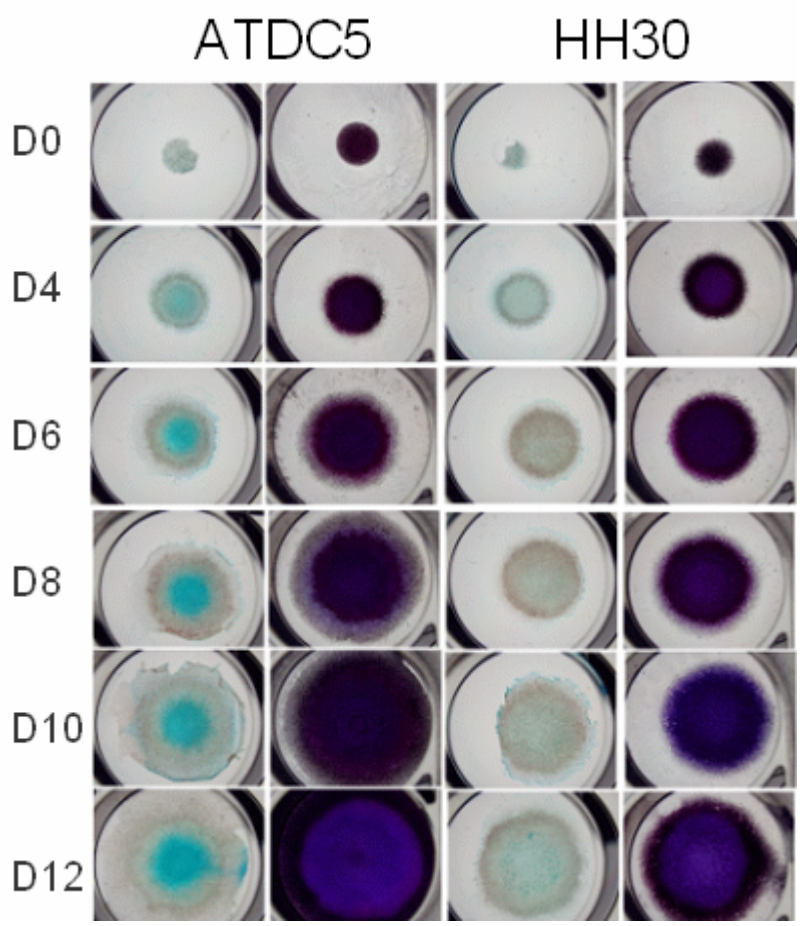

Figure 3.16: Alcian Blue Labeling for Heparanase Overexpression Clone
The parental ATDC5 cells as well as the clone overexpressing heparanase were cultured in high-density micromass cultures and stained with alcian blue (left panel) or crystal violet (right panel) at different time points. As seen in the above figure, the overexpression clone HH30 exhibits reduced accumulation of GAGs and decreased crystal violet stained area.

the transfected cells in the presence of 400 μg/ml of G418 over a 14 day time period.

3.2 Titering of Lentiviral Stock

Lentivirus carrying the heparanase ribozyme or the scrambled ribozyme was generated using the ViraPower lentiviral expression system. The viral stocks were titered to determine the viral concentration. ATDC5 cells were treated with 10 fold dilutions of the viral stock, cultured for 14 days in the presence of blasticidin and stained with crystal violet. The viral titer was calculated as the average of the number of colonies formed multiplied by the dilution factor of the virus. The results of the titering are shown in Figure 3.3. The heparanase ribozyme viral stock was found to have a titer of 4.25×10^6 transduction units (TU)/ml while the scrambled ribozyme virus was found to have a titer of 9.65×10^6 TU/ml. As seen in Figure 3.3, cells infected with $1{:}10^5$ dilution of the viral stock resulted in well-separated colonies and hence ATDC5 cells were infected with $1{:}10^5$ dilution of the viral stock to generate stable clones.

3.3 Analysis of Heparanase Knockdown Clones

Sixty-six clones transduced with the heparanase ribozyme virus and 37 clones transduced with the scrambled ribozyme were screened using Q-PCR. Heparanase ribozyme clones demonstrating a greater than 80% knockdown in heparanase mRNA expression were selected for further study. Some of the clones also were analyzed for heparanase protein expression using western blotting techniques. The results are shown in Figure 3.4. As seen in the figure heparanase ribozyme clones 18 and 15 demonstrate

an 86% and 65% knockdown in heparanase expression respectively. Control ribozyme clones expressing heparanase mRNA levels similar to or greater than the parental ATDC5 cells were subjected to alcian blue staining in micromass cultures to access GAG accumulation.

3.4 Alcian Blue Screening of Heparanase Ribozyme Clones

Selected clones were cultured in high-density micromass cultures to determine the consequences of heparanase knockdown on chondrogenic differentiation as identified by alcian blue staining (Figure 3.5). As seen in the figure, the parental ATDC5 cells demonstrate positive alcian blue staining by day 4. Clones HR15, HR51 did not demonstrate positive alcian blue staining until day 6 and day 10 respectively while clone HR18 is negative for alcian blue staining. The cells also were stained with crystal violet a cationic dye used as a general biological stain in order to monitor the growth of the clones in micromass cultures. As seen in Figure 3.5, the parental ATDC5 cells and the heparanase knockdown clones exhibit similar growth patterns in micromass cultures.

Selected control ribozyme clones were screened for alcian blue staining and clones exhibiting an alcian blue staining pattern similar to the parental ATDC5 cells were selected for further study. The alcian blue staining for the selected clones is shown in Figure 3.6. As seen in the figure, the scrambled ribozyme clones CR15, CR14, CR26, and CR39 demonstrate an alcian blue staining pattern similar to the parental ATDC5 cells. The control clones also were stained with crystal violet and as seen in Figure 3.6,

the control clones and the parental ATDC5 cells have similar growth patterns in micromass cultures.

The heparanase mRNA expression levels for the clones selected for further study are shown in Figure 3.7. The heparanase knockdown clones HR15, HR51 and HR18 express 15%, 17% and 11.5% of heparanase mRNA as compared to the parental ATDC5 cells, respectively. The control clones CR15, CR39, CR14, CR7 and CR26 express 840%, 470%, 412%, 448% and 156% of heparanase mRNA as compared to the parental ATDC5 cells, respectively.

3.5 Chondrogenic Differentiation of Heparanase Knockdown Clones

The parental ATDC5 cells as well as the selected heparanase knockdown clones were subjected to a 35-day differentiation protocol and RNA and protein samples were collected at different time points. Heparanase mRNA expression in the heparanase knockdown clones was analyzed using Q-PCR. The results are shown in Figure 3.8. As seen in the figure, in the parental ATDC5 cells, heparanase mRNA expression increased until day 20 in differentiation cultures where it was about 72 fold higher than day 0 heparanase mRNA expression. Thereafter, heparanase mRNA levels decreased and on day 35 were 8 fold higher compared to day 0 heparanase mRNA expression. For the heparanase knockdown clones HR15, HR51 and HR18, heparanase mRNA levels continue to increase through out the differentiation experiment. On day 20, heparanase mRNA levels for HR15, HR51 and HR18, were 164, 30 and 98 times higher than day 0

mRNA levels, respectively. On day 35 of differentiation heparanase mRNA levels for HR15, HR51 and HR18 are 348, 88 and 218 times higher as compared to day 0 heparanase mRNA expression, respectively. Thus normal pattern of heparanase mRNA expression pattern was disrupted in heparanase knockdown clones.

The heparanase knockdown clones also were analyzed for the expression of type II collagen in differentiation cultures using Q-PCR. The results are shown in Figure 3.8. For the parental ATDC5 cells, collagen type II expression increased throughout the 35-day differentiation period. On day 20, when the heparanase mRNA levels were the highest, the collagen type II mRNA levels are 120 fold higher as compared to day 0 levels and increased significantly by day 30 such the collagen type II mRNA expression was 1300 times higher as compared to day 0 mRNA levels. On day 35, collagen type II mRNA levels were 1276 times higher compared to day 0 mRNA levels. For the heparanase knockdown clone HR15, collagen type II mRNA levels continued to increase until day 20 and were slightly lower by day 30 and 35. On days 20, 30 and 35, collagen type II mRNA levels were 68 times, 52 times and 44 times higher compared to day 0 mRNA levels, respectively. For clone HR51, collagen type II mRNA levels were six fold higher as compared to day 0 levels by day 15 and were two times higher than basal levels on day 20. By day 30 and 35, type II collagen mRNA levels were eight times and six times higher than basal levels, respectively. For the heparanase knockdown clone HR18, type II collagen mRNA expression increased to 178 times the basal levels by day 15. Thereafter, the collagen type II mRNA levels remained relatively stable and

were much lower than collagen type II mRNA levels in the parental ATDC5 cells on days 30 and 35. Thus, perturbing the normal heparanase mRNA expression pattern in differentiating ATDC5 cells results in a decrease in the expression of type II collagen, an early marker of chondrogenic differentiation.

3.6 Analysis of Heparanase Expression in Heparanase Knockdown Clones Following Several Passages

To examine whether heparanase knockdown clones maintained reduced heparanase expression after several passages, the knockdown clones were plated in micromass and monolayer cultures and analyzed for the expression of heparanase using immunocytochemical techniques. As seen in figure 3.10, the heparanase knockdown clones exhibited increased heparanase expression indicating that they overcame the effects of the heparanase ribozyme. The knockdown clones also exhibit an increase in nuclear size.

3.7 Analysis of Chondrogenic Differentiation in ATDC5 cells Transiently Transduced with the Heparanase Ribozyme

ATDC5 cells were plated in micromass cultures and treated with different dilutions of the lentivirus carrying a ribozyme targeting heparanase mRNA every alternate day for a 12 day time period. ATDC5 cells in two T-75 flasks were treated with different dilutions of the virus 24 hours prior to plating in micromass cultures. The cells were stained with alcian blue and crystal violet at various time points to monitor GAG accumulation and cell proliferation respectively. The results of this experiment are as shown in Figure 3.10. As seen in the figure there is no obvious difference in the alcian blue staining intensity for the micromass cultures treated with the lentivirus carrying the heparanase ribozyme virus; these cultures show a cell proliferation pattern similar to the untreated micromass cultures. The alcian blue staining then was quantified by extracting the bound alcian blue with 6M guanidine HCl and absorbance was recorded at 600 nm. The results are shown in Figure 3.11. As seen in the figure, the treated and untreated cultures have a similar alcian blue staining intensity. From day 6-10, the lentivirus treated cultures show decreased alcian blue staining as compared to the untreated cultures. The alcian blue staining intensity is proportional to the concentration of the virus and the micromass cultures treated with the lentivirus 24 hours prior to plating exhibit decreased alcian blue staining as compared to the micromass cultures treated with the lentivirus after being plating in micromass cultures. On day 12, the GAG accumulation for the lentivirus treated micromass cultures is higher than the untreated cultures indicating that the cells have overcome the effects of

the ribozyme. Collectively, these results show that heparanase expression is required for the ATDC5 cells to efficiently produce GAGs when plated in micromass cultures

3.8 Chondrogenic Differentiation in the Presence of a Heparanase Inhibitor

As an alternative to the ribozyme approach, ATDC5 cells were plated in high-density micromass cultures and were treated with 100μg/ml and 500 μg/ml of PI-88, a heparanase inhibitor. The micromass cultures were stained with alcian blue and crystal violet to monitor the accumulation of GAGs and cell proliferation, respectively. The results of this experiment are shown in Figure 3.13. As seen in the figure, there was no obvious difference in alcian blue staining intensity in the micromasses treated with PI-88. The alcian blue staining intensity was quantified by extracting the alcian blue with 6M guanidine HCl and the absorbance was recorded at 600 nm. The results of alcian blue quantification are shown in Figure 3.14. As seen in figure, in the cells not treated with PI-88, the alcian blue levels increased until day 6 and show a slight decrease on day 8. The cells treated with 100 μg/ml of PI-88 showed a similar pattern, but the alcian blue staining intensity was lower on days 4-8 compared to the untreated cells. The cells treated with 500 μg/ml of PI-88 had a similar alcian blue staining intensity on days 0-6 compared to the cells treated with 100μg/ml of PI-88. By day 8, the cells treated with 500μg/ml of PI-88 had a higher staining intensity compared to the untreated cells. These preliminary results indicate that inhibition of heparanase activity reduces the accumulation of GAGs in ATDC5 cells cultured in micromass cultures.

As seen in figure 3.10, the PI-88 treated cells had a greater crystal violet stained area compared to the untreated cells indicating that inhibition of heparanase activity induces proliferation of ATDC5 cells in micromass cultures.

3.9 Analysis of Heparanase Overexpression Clones

ATDC5 cells stably transfected with a plasmid expressing human heparanase tagged with a myc epitope were screened by western blotting with a myc tag antibody. As seen in Figure 3.15, the human heparanase clones 17 and 30 express a myc band similar in size to human heparanase. Clone HH30 was plated in micromass cultures and stained with alcian blue or crystal violet at different time points. As seen in Figure 3.16, the clone did not exhibit alcian blue staining until day 12. Clone HH30 also exhibited decreased crystal violet stained area.

Chapter 4

DISCUSSION

4.1 Screening of Scrambled Ribozyme Clones

ATDC5 cells were transduced with a lentivirus carrying a scrambled ribozyme that should not target any specific mRNA sequence. After the cells were stably transduced and screened for the expression of heparanase mRNA, it was found that only 2 out of the 37 clones had a heparanase mRNA levels comparable to the parental ATDC5 cells. Alcian blue staining of these clones showed reduced GAG accumulation (results not shown). To better understand this observation, the substrate binding arms of the scrambled ribozyme were used to perform a Blast search. The search revealed that the mRNA binding arms do not align with any known mouse sequence. However the control ribozyme may align with a yet unidentified sequence leading to the observed results. To overcome this problem, ATDC5 scrambled ribozyme clones expressing heparanase mRNA levels equal to or greater than parental ATDC5 cells were subjected to chondrogenic differentiation in micromass cultures and stained with alcian blue. Clones exhibiting an alcian blue staining pattern similar to the parental ATDC5 are shown in Figure 3.6 and their heparanase mRNA levels as compared to the parental ATDC5 cells are shown in Figure 3.7. All of the selected clones have a higher level of heparanase mRNA as compared to the parental ATDC5 cells. It is important to note that changes in mRNA levels do not always reflect changes in protein levels. Therefore, the

scrambled ribozyme clones will be subjected to heparanase activity assays. The activity assay data will give a better picture about whether the elevated heparanase mRNA levels reflect increased in heparanase activity. Alternatively heparanase ribozyme clones exhibiting heparanase mRNA levels similar to the parental ATDC5 cells can be used as controls.

4.2 Chondrogenic Differentiation of Heparanase Knockdown Clones

Heparanase knockdown clones exhibiting greater than 80% knockdown in heparanase expression were plated in micromass cultures and stained with alcian blue to index accumulation of GAGs. As seen in Figure 3.5, the knockdown clones exhibited delayed accumulation of GAG providing preliminary evidence that heparanase knockdown has a negative effect on chondrogenic differentiation. The crystal violet staining showed that the defect in chondrogenic differentiation was not due to impaired cell proliferation.

To further study the effect of reduced heparanase expression on chondrogenic differentiation, the knockdown clones were plated in 35 day differentiation cultures and analyzed for the expression of heparanase and collagen type II mRNA at different time points. As seen in Figure 3.8, heparanase mRNA levels in knockdown clones were elevated as compared to the parental ATDC5 cells. When cultured in differentiation cultures, the parental ATDC5 cells demonstrate an increase in heparanase mRNA expression during day 15 and 20 and a decrease in heparanase mRNA levels thereafter.

However in the knockdown clones, heparanase mRNA levels continue to increase throughout the differentiation period exhibiting an alteration in the regular pattern of heparanase mRNA expression. As mentioned earlier an increase in mRNA levels may not correspond with increased heparanase activity, and therefore, the clones will be assayed for heparanase activity. As the knockdown clones are cultured for a 35-day period in differentiation media in the absence of antibiotic selection, the clones may have overcome the effects of the heparanase ribozyme. The loss of selective pressure may have silenced heparanase ribozyme expression by DNA methylation. Heparanase expression may be regulated by feedback from growth factor signaling and the initial reduction in growth factor signaling may upregulate heparanase expression. As heparanase is localized in the lysosomes and endosomes it may play a role in degradation/recycling of cell surface HSPG internalized by endosytosis. Problems with HSPG degradation may upregulate heparanase expression in the knockdown clones. Alternatively, the increased heparanase expression in the knockdown clones could also be a result of increased mRNA stability.

Analysis of collagen type II expression in the knockdown clones (Figure 3.9) shows reduced expression particularly during day 30 and 35. Taken together these results indicate that changes in heparanase mRNA patterns during chondrogenic differentiation have a negative effect on the expression of collagen type II, an early marker of chondrogenic differentiation.

Further analysis of the knockdown clones showed that the knockdown clones could overcome the heparanase ribozyme after several rounds of passaging (Figure 3.10). The knockdown clones also show an increase in nuclear size. In a study conducted by Hsia et al. (2003), studying the factors affecting the nuclear location of FGF showed that FGF nuclear localization is mediated by HSPG and treatment with bacterial heparatinase III inhibits FGF and HSPG nuclear localization (Hsai et al., 2003). Another study has demonstrated that human heparanase can localize to the nucleus where it is enzymatically active (Schubert et al., 2004). The initial knockdown in heparanase activity may lead to HSPG accumulation in the nucleus and an increase in nuclear size.

To gain a better understanding of the role on heparanase in chondrogenesis, ATDC5 micromass cultures were treated with different dilutions of the lentivirus containing the heparanase ribozyme and stained with alcian blue and crystal violet. As seen in Figure 3.11 and 3.12, the lentivirus treated cultures exhibit reduced GAG accumulation between days 6-10. As the ribozyme targets heparanase mRNA and not the protein, it is not surprising that the lentivirus treated micromass cultures exhibit similar alcian blue staining intensity as the untreated micromass cultures on day 4. On day 12, all the lentivirus treated micromass cultures exhibit increased alcian blue staining indicating that they have overcome the heparanase ribozyme. Transduction with lentivirus requires that cells are not greater than 70-80% confluent prior to transduction. As micromass cultures are high-density cultures, the cells in the center of

the micromass may not be transduced in spite of repeated viral treatment. The cells in the center of the micromass may have overcome the effects of the heparanase ribozyme and this may explain the sudden increase in GAG accumulation by day 12.

Taken together these results indicate that heparanase expression is important for efficient chondrogenic differentiation.

4.3 Chondrogenic Differentiation in the Presence of a Heparanase Inhibitor

To further understand the role of heparanase in chondrogenesis, ATDC5 cells were plated in micromass cultures, treated with different concentrations of the heparanase inhibitor PI-88 and stained with alcian blue and crystal violet to monitor GAG accumulation and cell proliferation respectively. The PI-88 treated micromass cultures exhibited reduced accumulation of GAGs and an increase in the crystal violet stained area as compared to the untreated micromass cultures.

Although the reduction in GAG accumulation was not as significant as with the heparanase knockdown clones, these preliminary results show that inhibition of heparanase activity has a negative effect on chondrogenic differentiation. In case of the PI-88 treated micromass cultures, the cells are plated in high density cultures and the PI-88 may not be able to penetrate the center of the micromass and this may account for increased proliferation and relatively little reduction in GAG accumulation. On the other hand, the heparanase knockdown clones have reduced heparanase mRNA expression prior to plating in micromass cultures and this may contribute to the

difference in the reduction of GAG accumulation. PI-88 can also inhibit HBGF interactions with HS chains and this May partly account for decreased GAG accumulation.

The increased crystal violet stained area in PI-88 treated micromass cultures may be due to increased cell proliferation or migration of the cells at the periphery of the micromass cultures.

4.4 Heparanase Overexpression

ATDC5 cells were stably transfected with a plasmid expressing human heparanase under the control of CMV promoter to support constitutive heparanase expression. The plasmid also contains a myc epitope that can be recognized with a myc antibody. Sixty clones stably transfected with the above plasmid were selected and RNA and protein samples extracted. Screening of the clones led to the identification of two clones expressing a myc tagged protein with a molecular weight similar to human heparanase. One of the selected clones plated in micromass cultures and stained with alcian blue and crystal violet demonstrated reduced GAG accumulation and slightly decreased crystal violet staining. Heparanase overexpression may lead to ATDC5 cells differentiating into perichondrial cells. Studies in the Farach-Carson and Carson laboratories have show that mouse bone sections stained with a heparanase antibody exhibit predominant heparanase staining in the perichondrium (unpublished data).

Genetic studies have shown that changes in the rate of chondrocyte proliferation and chondrocyte hypertrophy can lead to defects in endochondral ossification. The rate of chondrocyte proliferation and hypertrophy is regulated by the Indian hedgehog (Ihh) and the parathyroid hormone related peptide (PTHrP) feedback loop. PTHrP is secreted by cells in the perichondrium and suppresses the differentiation of prehypertrophic chondrocytes into hypertrophic chondrocytes. Ihh is secreted by prehypertrophic chondrocytes and upregulated the expression of PTHrP in the cells in the perichondrium. As Ihh can bind HS chains, heparanase may play an important role in regulating this feedback loop (Gao et al., 2004).

As only one of the heparanase overexpression clones was studied in micromass cultures these results are preliminary and the differentiation of other clones should be studied in order to make stronger conclusions.

Chapter 5

CONCLUSION

In this study the role of the enzyme, heparanase, in chondrogenesis was studied. Analysis of clones stably transduced with the heparanase ribozyme (knockdown clones) showed that heparanase expression is required for the efficient accumulation of GAGs. The knockdown clones exhibited an altered heparanase expression pattern when cultured in differentiation cultures. These clones also were found to have decreased collagen type II mRNA levels, particularly during the later time points in the differentiation period. Treating ATDC5 micromass cultures with PI-88, a heparanase inhibitor, showed that inhibition of heparanase activity leads to a decrease in the accumulation of GAGs and an increase in cell proliferation. Preliminary studies indicate that heparanase overexpression in ATDC5 cells leads to decreased GAG accumulation. Collectively these results show that heparanase plays an important role in the regulation of chondrogenic differentiation in ATDC5 cells.

Chapter 6

FUTURE DIRECTIONS

To further understand the effect of altered heparanase expression patterns on chondrogenic differentiation, the knockdown clones should be analyzed for the expression of collagen type X, a late marker of chondrogenic differentiation. The control clones also should be subjected to 35 day differentiation cultures and be analyzed for the expression of heparanase, collagen type II and collagen type X mRNA. The heparanase overexpression clones should be further studied for the GAG accumulation and the expression of cartilage specific genes.

To better understand the role of heparanase in growth factor delivery during chondrogenesis, the differentiation of the knockdown clones as well as the overexpression clones on treatment with HBGFs such as BMP-2 should be studied.

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