5-2013

Development of a Cellular Model for Morquio A Syndrome

Virgen Guadalupe Sierra
Dominican University of California

https://doi.org/10.33015/dominican.edu/2013.bio.04

Development of a Cellular Model for Morquio A Syndrome

A thesis submitted to the faculty of
Dominican University of California
&
BioMarin Pharmaceutical Inc.
in partial fulfillment of the requirements
for the degree

Master of Science
in
Biology

By
V. Guadalupe Sierra
San Rafael, California
May, 2013

CERTIFICATION OF APPROVAL

I certify that I have read *Development of a Cellular Model for Morquio A Syndrome* by V. Guadalupe Sierra, and I approved this thesis to be submitted in partial fulfillment of the requirements for the degree: Master of Sciences in Biology at Dominican University of California and BioMarin Pharmaceutical Inc.

Dr. Michael Vellard, Graduate Research Advisor Date 05/23/2013

Dr. Warren Hoeffler, Adjunct Professor Date 05/23/2013

Dr. Kiowa Bower, Graduate Program Director Date 05/23/2013

Table of Contents

Abstract

Mucopolysaccharidosis IVA (MPS IVA; Morquio A), is a lysosomal storage disorder characterized by the deficiency of N-acetylgalactosamine-6-sulfatase (GALNS), resulting in the accumulation of glycosaminoglycans (GAGs) such as keratan sulfate (KS) (Northover et al., 1996) and chondroitin-6-sulfate. There is no applicable animal model or appropriate cell lines currently available for the study of MPS IVA. Furthermore, patient-specific material is limited and difficult to obtain, rendering the study of MPSIVA pathogenesis challenging. Thus, the generation of an induced pluripotent stem cell (iPSC) line from patient-specific somatic cells shows potential for further examining the pathogenesis of MPS IVA. With this technology, differentiation protocols could be used to acquire desired cell types.

In this study, an iPSC model was established for MPSIVA as well as an unaffected phenotype expressing pluripotency markers, rendering it capable for differentiations. A direct and a progressive approach were taken for the differentiation of iPSCs into a chondrogenic lineage. The direct differentiation of iPSCs into chondrocytes was unsuccessful due to inconsistent results. An alternate indirect route through the establishment of an intermediate mesenchymal stem cell (MSC) lineage before chondrogenesis was then investigated and reached. With the MSCs, it was shown that the severe disease clone not only has a decreased cell growth capacity, but also has larger lysosomes and altered cytoskeleton structure, suggesting a clear phenotype that could be altered upon treatment with rhGALNS. Furthermore, the MSCs can be loaded with KS to recapitulate accumulation within the cell and levels are corrected to the unaffected phenotype with rhGALNS. Thus a bioassay for measuring the intracellular

activity of rhGALNS is introduced. Several attempts were made to further differentiate the MSCs into chondrocytes, a difficult endeavor with varying results. The established iPSC and MSC lines are a promising tool for the further development and understanding of MPSIVA as well of a functional bioassay for rhGALNS using the natural substrate, lumican, of the enzyme.

Acknowledgements

I would like to thank my mentor, Michael Vellard, for his guidance, support, and direction. Thank you for allowing me to take a part in this project and for entrusting me throughout it. It was an incredible learning experience and your challenges helped me tremendously. I am fortunate to have worked for such an inspiring and successful teacher/scientist that truly devotes his time and patience to his students. I owe my deepest gratitude to Andrzej Swistowski, who is an incredible teacher and established the foundations to my work. Thank you for your patience and encouragements, and for helping smooth bumps along the way. It was an absolute pleasure being your student, and I am fortunate to have had the opportunity to work beside you as I broadened my knowledge and experience. A special thanks to the Cell and Molecular Biology research group for their gracious acceptance into the team and help throughout my time spent here. A sincere thank you to everyone else who helped stitch my work together outside of my group, especially Erno Pungor and Danielle Harmon for their expert advice and handling of countless samples. I will remain forever grateful to BioMarin Pharmaceutical and Gordon Vehar for the amazing opportunity to join the research department and complete my thesis. I thank Sibdas Ghosh for guiding me through these collegiate years as well as the faculty at Dominican University who have greatly influenced my success and interests. A heartfelt thank you to Heather Drew for being a great friend and colleague. I could not have been able to do this program without you. Most importantly, I owe my deepest gratitude to my loving and supportive family, who have given me the necessary tools for my accomplishments and continue to motivate me daily.

List of Figures

List of Tables

List of Abbreviations

BMP-2- bone morphogenic protein 2

cDNA- complementary DNA

CS- chondroitin 6-sulfate

EBs- embryoid bodies

ERT- enzyme replacement therapy

ESCs- embryonic stem cells

FACS- fluorescence activated cell sorting

FBS- fetal bovine serum

GAGs- glucosaminoglycans

GALNS - N-acetylgalactosamine-6-sulfatase

ICC- immunicytochemistry

iPSCs- induced pluripotent stem cells

ITS- insulin transferrin selenium

KS- keratan sulfate

MEFs- mouse embryonic fibroblasts

MEM- minimum essential medium

mRNA- messenger RNA

MSCs- mesenchymal stem cells

MPS – mucoplysaccharidoses

MPSIVA- mucoploysaccharidoses type IVA, or Morquio A

PBS- phosphate buffered saline

PE- R-phycoerythrin, a fluorescent probe

qRT-PCR- quantitative real time polymerase chain reaction

rhGALNS- recombinant human GALNS

TGFβ1/3- transforming growth factor beta 1/3

I. Introduction

A. Goal of the Project

Due to the lack of patient material, appropriate cell model, or cell lines, further work remains to be completed for the understanding of Morquio A. The goal of this project is to: 1. Establish cellular disease models to study the pathophysiology of MPS IVA using patient specific induced pluripotent stem cells; 2. Develop a functional bioassay with established cellular model to measure intracellular rhGALNS activity. Differentiation protocols will be explored and instituted for the derivation of patient-specific cell types, specifically chondrocytes, for the comparison of terminally differentiated MPSIVA cells versus unaffected cells. Once the cell types are established, we can investigate how different cell types are affected by MPS IVA and by treatment with rhGALNS. The development of a bioassay will allow for the characterization of biological drugs to demonstrate its ability to enter target cells and act on natural substrates, an extremely useful tool for the biotechnology industry.

B. Background

B1. The mucopolysaccharidoses and Morquio A

The mucopolysaccharidoses (MPS) are a subset of inherited metabolic disorders known as lysosomal storage diseases that are caused by a deficiency of specific lysosomal enzymes, which act as exohydrolases in the degradation of glucosaminoglycans (Northover et al., 1996; Bielicki et al., 1995). In the case of MPS IVA, the deficient GALNS enzyme hydrolyses the sulfate ester bonds of GalNAc6S and Gal6S at the non-reducing termini of chondroitin 6-sulfate (CS) and keratan sulfate (KS)

respectively (Bielicki et al., 1995). The partially degraded CS and KS accumulate within the cells, causing interference with cellular function and excessive urinary excretion of these glucosaminoglycans (Northover et al., 1996; Bielicki et al., 1995). The subsequent cellular pathology is most prominent in connective tissues rich in KS, including cartilage, cornea and heart valve. (Northover et al., 1996; Grande-Allen et. al., 2004; Dvorak-Ewell et al., 2010). Other cell types, such as macrophages and coronary intimal smooth muscle cells also contain excessive accumulation of GAGs, suggesting that the pathophysiology may encompass non-KS-rich tissues (Dvorak-Ewell et al., 2010).

Morquio A, or MPS IVA, has a relatively high incidence rate compared to other MPS disorders and does not involve the central nervous system, preserving normal intellect (Bielicki et al., 1995; Northover et al., 1996; Montaño et al., 2007). Luis Morquio in Montevido, Uruguay first described MPS IVA in 1929 when he observed four Swedish siblings with skeletal dystrophy. James Frederick Brailsford also recognized similar symptoms that same year in Birmingham, England. Since then, the knowledge of this disorder has expanded greatly with a known occurrence rate of approximately 1 in 300,000. Patients are asymptomatic at birth and are usually diagnosed at around three years of age after the evaluation for unusual skeletal features (Tomatsu et al., 2008). Patients will typically undergo major surgical operations in the neck, hip, and leg regions between the ages of 5-10 to improve the quality of life and be wheelchair-bound by the time they are teenagers (Montaño et al., 2007). Patients with severe forms of the disorder do not typically live longer than the second or third decade of life due to complications primarily related with cervical instability and pulmonary compromise (Tomatsu et al., 2008).

The lack of GALNS activity that causes this disorder affects the sequential degradation and results in the progressive accumulation of GAGs in the lysosomes of multiple organs, most apparent in the ligaments, connective tissues, and cartilage. MPS IVA is characterized by: dwarfism, corneal clouding, progressive deafness, dental abnormalities, joint laxity, muscular weakness, systemic skeletal dysplasia, heart valvular disease, and mild hepatosplenomegaly (Bielicki et al., 1995; Tomatsu et al., 2007). Additional compromised systems include the digestive, cardiovascular, and respiratory systems (Northover et al., 1996). The broad spectrum of clinical phenotypes implies the occurrence of several mutant alleles at the G6S locus (Bielicki et al., 1995). Currently, patient care and treatment for MPS IVA include symptomatic management of skeletal manifestations and the associated neurological and orthopedic complications associated with the disease (Tomatsu et al., 2008; Hendriksz et al., 2012). Because of its relatively high incidence rate and the lack of involvement of the central nervous system, MPS IVA is ideal for the clinical evaluation of enzyme replacement therapy, which would move the treatment of the disorder from symptomatic management to therapeutic intervention.

B2. Morquio Research- What's Missing?

The above aforementioned facts infer that further research is needed on the pathophysiology of MPS IVA. Certain limitations exist to conduct this research and alternative methods should be considered. Firstly, an appropriate animal model has yet to be proven for the study of MPS IVA. Using mice, it is possible to create a model with no GALNS activity, but it has been shown that growth plate KS does not accumulate (Montaño et al., 2008). Furthermore, visceral storage of GAGs other than KS was

discovered in a model that unfortunately lacked the skeletal features (Tomatsu et al., 2003). Therefore the human skeletal deformities typical of this disease are not recapitulated in the murine models. These mice had undetectable KS levels in the urine and serum and also failed to show GAG storage in cartilage cells, indicating clear differences to the human phenotypes. Interestingly, the murine model does suffer from substrate accumulation in the brain (Tomatsu et al., 2003; Tomatsu et al., 2008). Indeed there are clear differences in the synthesis and distribution of KS in human and murine tissues (Tomatsu et al., 2003). For example, two forms of KS, KS I and KS II, are found in the cornea and skeleton of humans, respectively. However, the synthesis of KS II of most tissues is much lower in rodents compared with humans and most other animal species (Venn et al., 1985). Moreover, rodents do not have KS chains in the proteoglycan aggrecan while other mammals do. This absence of KS II could explain why MPS IVA mice have no skeletal clinical phenotype (Tomatsu et al., 2008).

Secondly, appropriate cell lines and available patient-specific material are very limited and difficult to obtain for classical in vitro models. MPS IVA patient dermal fibroblasts are accessible, but do not accumulate KS or display any pathology (Hollister et al., 1975; Dvorak-Ewell et al., 2010). Other cell types such as chondrocytes isolated from patients accumulate endogenously produced KS and can display variability (Dvorak-Ewell et al., 2010). And when grown in monolayers, chondrocytes dedifferentiate and no longer express chondrocyte markers such as *Collagen II* and *Aggrecan* (Gosset et al., 2008), and express an increased level of *Collagen I* (De Franceschi et al., 2007). It can be concluded that the reduced ankle and joint movement observed in MPSIVA patients may not only be due to proteoglycan accumulation, but

also the high amount of collagen type I and the low levels of collagen type II fibers in chondrocytes (De Franceschi et al., 2007). In MPS IVA, growth plate chondrocyte pathology is characterized by vacuolar distention and poorly calcified matrix amongst other manifestations. Furthermore, chondrocytes in the articular cartilage are vacuolated, disorganized and show altered expression of extracellular matric component (Anderson et al., 1962; McClure et al., 1986). In contrast, osteoblasts and osteoclasts appear unaffected and histological studies confirm that bone tissue in MPS IVA is qualitatively comparable to unaffected bone (McClure et al., 1986).

The use of human MPS IVA chondrocytes as a model was shown to be adequate in testing enzyme replacement therapy with recombinant human GALNS (rhGALNS) (Dvorak-Ewell, 2010). In this study, it was concluded that the MPS IVA chondrocytes uptake rhGALNS into the lysosomes and that rhGALNS reduces KS accumulation and can restore function to MPS IVA cells. However, this model has many disadvantages that make further studies challenging- such as difficulty to maintain the chondrogenic phenotype and a limited number of cells. Also, the MPS IVA primary human chondrocytes were from iliac crest biopsies whereas the control unaffected chondrocytes were isolated from healthy articular knee cartilage, making comparisons difficult due to different origin. Further studies need to be conducted using an abundant source of MPS IVA chondrocytes.

Moreover, there is a need for an in vitro functional bioassay for rhGALNS to measure its intracellular activity on a natural substrate, lumican. Lumican is one of the major KS-containing proteoglycans expressed in a variety of tissues and belongs to the family of small leucine-rich repeat proteoglycans, which constitute an important fraction

of non-collagenous extracellular matric proteins (Nikitovic et al., 2008). Natural substrate bioassays are used in product comparability and characterization studies to demonstrate its functionality. For the characterization of biological drugs, the regulatory expectation is to have a functional bioassay available that ‘‘describes the specific ability or capacity of a product to achieve a defined biological effect (Pungor et al, 2009; ICH Q6B Specifications, 1999).” Such bioassay would be a great asset to biotech industries launching a new product, as BioMarin Pharmaceutical is doing.

B3. Enzyme Replacement Therapy for Morquio A

BioMarin Pharmaceutical’s proposed treatment for MPS IVA is enzyme replacement of GALNS through the production of recombinant human GALNS (rhGALNS), intended to clear keratan sulfate from the lysosome. As reported by Drovak-Ewell *et al.,* rhGALNS was produced from conditioned media from Chinese Hamster Ovary cells stably overexpressing GALNS and sulfatase modifying factor 1 (SUMF1), which is required for activation of all sulfatases. Previous reports were confirmed of the association of rhGALNS as a non-covalent dimer in solution and as a purified enzyme consisting of a major species of ~55 kDa, a minor species of ~40 kDa, and a species of ~19 kDa under reducing conditions (Masue et al., 1991). It was also shown that the enzyme deficiency of MPS IVA fibroblasts was corrected upon addition of rhGALNS in a dose-dependent manner and that rhGALNS uptake was mannose-6-phosphate (M6P) receptor-dependent. Thus it was concluded that abnormal gene expression could be improved upon treatment with rhGALNS, suggesting enzyme replacement therapy will

not only reduce lysosomal storage, but also possibly restore normal cellular physiology (Drovak-Ewell et al., 2010).

Currently, BioMarin Pharmaceutical's GALNS for MPS IVA, or Vimizim, achieved its primary endpoint in the phase 3 trial, confirming the efficacy of the enzyme replacement therapy. Phase III clinical trials showed endurance improvements, clinically meaningful improvements in two measures of endurance (6-minute walk distance and 3-minute stair climb), and decreased urinary levels of keratan sulfate (BioMarin Pharmaceutical Inc., 2012). These results are very promising, and the company is beginning market applications to make the product available for patients. However, there are still many questions to answer regarding the pathophysiology of MPS IVA, and a cellular model would allow for further studies and serve to test therapeutic efficiency. Using an *in vitro* iPSC-based model, differentiated cells will help understand MPS IVA pathogenesis through the study of KS accumulation on chondrocyte differentiation and gene expression amongst others, as well as the effects on cells upon treatment with rhGALNS. If a cell model is accomplished, the development of a natural substrate bioassay would benefit additional comparability and characterization studies to come.

B4. An Induced Pluripotent Stem Cell Model

We proposed to create an induced pluripotent stem cell-based model to continue studies on the pathophysiology of MPS IVA. Through molecular reprogramming of patient fibroblasts, the generation of diseased and unaffected cell lines has potential in deciphering what remains unknown of this disease. The genetic reprogramming of adult

cells creates an embryonic stem cell-like state where cells are forced to express genes and factors to maintain pluripotent properties. Pluripotent stem cells can differentiate into all three germ layers: ectoderm, mesoderm and endoderm, which can give rise to all cell types of the body (Lanza, 2009). In 2007, it was shown that iPSCs were obtainable from human somatic cells (Takahasi et al., 2007; Yu et al., 2007). The generation of iPSCs has shown to be a breakthrough technology that has great therapeutic potential and could be used for clinical applications.

Since iPSCs can be generated from patients using various tissues, they can be used to obtain cells and progenitors carrying a disease-specific trait and to investigate the impacts of this trait on the disease's phenotypic development (Choi et al., 2011). They can also possibly provide an unlimited supply of cells for drug testing. iPSCs are morphologically similar to embryonic stem cells (ESCs), express pluripotency markers, and also contribute to germ line transmission (Yamanaka, 2009). Because they have pluripotent differentiation potential into committed functional lineages, iPSCs are a valuable asset for the modeling of human diseases (Vitale et al., 2011). This is especially relevant to creating an *in vitro* model to study MPS IVA pathogenesis, since there is no applicable cell lines or available patient-specific material, rendering such studies difficult.

Using this technology, it would be possible to create *in vitro* MPS IVA models through the differentiation of iPSCs to relevant cell types such as chondrocytes, macrophages, myocytes, osteoblasts and osteoclasts for the comparison of normal versus diseased lines. This would allow for the comparison of gene and protein expression profiles as well as the effect on cells upon treatment with rhGALNS. However, the differentiation into such cell types is time-consuming and has yet to be

proven with iPSCs. It has been shown successfully with ES and human pluripotent stem cells using various methods.

C. The Direct and Progressive Differentiation of a Chondrogenic Lineage

C1. Direct Chondrogenic Differentiation

Clinically relevant tissues or cell types are of utmost importance in the elucidation of the extensive pathophysiology in MPS IVA. As Drovak-Ewell et al. demonstrated, the therapeutic enzyme GALNS penetrates through even poorly vascularized tissues, such as growth plate cartilage. Therefore, of particular interest to MPS IVA are chondrocytes, cells that primarily make up cartilage and that produce/maintain the cartilaginous matrix, which consists mainly of collagen and proteoglycans. As described in Section I-B3, growth plate chondrocyte pathology in MPSIVA has many complications and setbacks that demonstrate the importance of these cells in the disease. A steady, reliable chondrocyte cell line would be of interest to scientists for further studies.

Direct chondrogenic differentiation has reportedly been achieved with human embryonic stem cells through techniques such as high-density micromass culture (Figure 1). Ferrari *et al*. (2011) reports culture systems and conditions based on the progressive and uniform differentiation of embryonic limb bud mesenchymal cells into chondrocytes. Through such culture systems the close juxtaposition and subsequent cell-to-cell interactions required for chondrogenic differentiation are recapitulated. Differentiation is promoted with exogenous supplementation of pro-chondrogenic factors such as BMP-2 and TGFβ1, which are typical *in vivo* (Ferrari et al., 2011). Similarly, Gong et al. report the same technique in which hESCs undergo direct, rapid, progressive, and substantially

uniform chondrogenic differentiation in the presence of BMP2 alone or a combination with TGFβ1. A second technique commonly used is a pellet culture (Figure 1), where cells are cultured as a 3-dimensional environment allowing for cell-to-cell interactions. This technique will be later explained in detail.

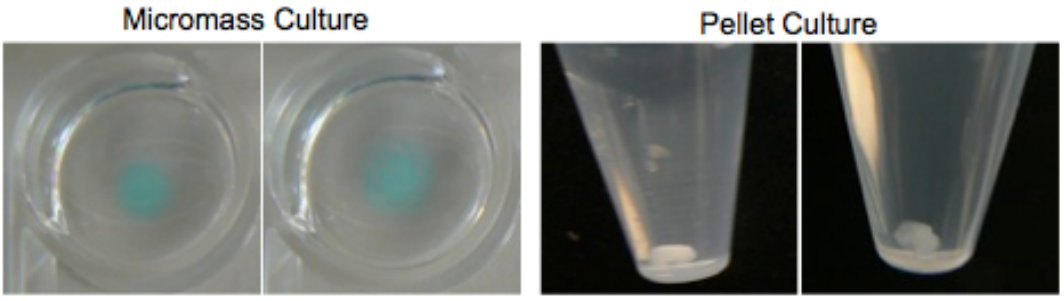


Figure 1. Two culture techniques for the differentiation of chondrocytes. In the micromass culture, cells are attached to a plate and form spheres initially, but flatten out progressively. The pellet culture allows for a suspension culture of the cell sphere, recapitulating interactions in the growth plate. Cells can be exposed to Alcian blue for the detection of matrix accumulation at the end of differentiation.

C2. Progressive Chondrogenic Differentiation

C2.1. Intermediate Mesenchyme Lineage

An alternative technique to consider in the differentiation of chondrocytes is an indirect, intermediate mesenchyme progenitor lineage, which could circumvent the challenges observed with direct differentiation of iPSC protocols. Mesenchymal stem cells (MSCs) are not only tissue-specific adult stem cells that can be used for clinical applications, but can also be further differentiated to other cell types. These multipotent stem cells have a high capacity for self-renewal and can potentially differentiate into osteoblasts, adipocytes, chondrocytes, or myocytes *in vivo* and *in vitro* (Asakura et al., 2001; Mahmood et al., 2010). It was shown that these cells recruit from the perivascular niche representing a tight network throughout the vasculature of the body (Crisan 2008).

The characterization of MSCs is ambiguous and the defining markers are inconsistent among investigators. A thorough literature research conducted by Mafi et al. (2011) as well as additional published articles reveals the most commonly reported positive markers are CD105, CD90 (THY-1), CD44 (HCAM), CD73, CD29, and STRO-1 (See Table 1). The most commonly reported negative markers are CD34 and CD14, both cell surface markers absent on mesenchymal stem cells. Furthermore, the Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy proposes minimal criteria to define human MSC populations. MSCs must be plastic-adherent under standard culture conditions, express CD73, CD90, and CD105, and lack expression of CD11b, CD14, CD19, CD34, CD45, CD79α, and HLA-DR surface molecules (Dominici et al., 2006). There is variable expression of the reported MSC surface markers, which could be accounted for by different stages during cell proliferation, the isolation and derivation of cells, and culture where the markers have been accessed (Mafi et al., 2011).

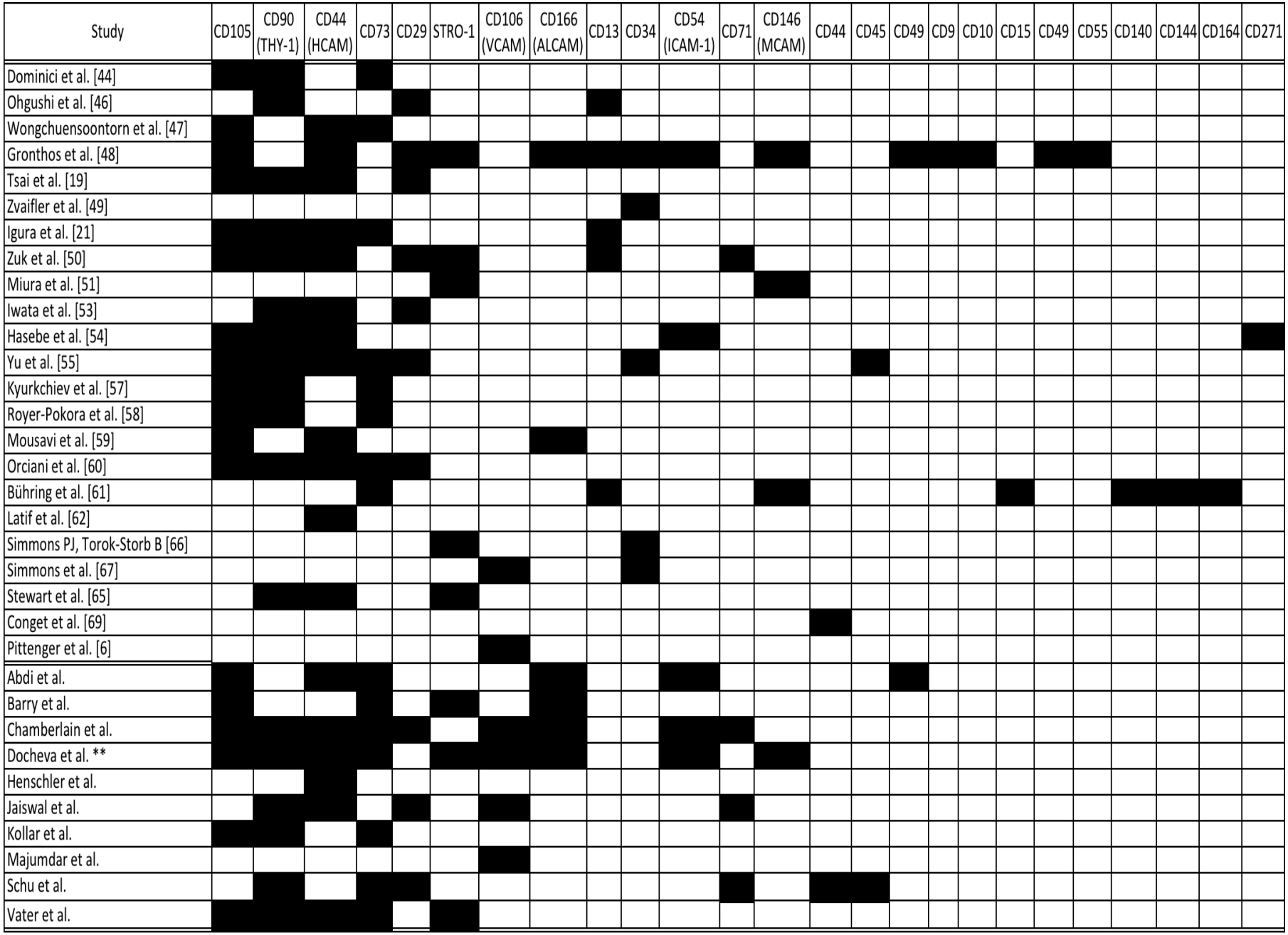

Study
CD105
CD90 (THY-1)
CD44 (HCAM)
CD73
CD29
STRO-1
CD106 (VCAM)
CD166 (ALCAM)
CD13
CD34
CD54 (ICAM-1)
CD71
CD146 (MCAM)
CD44
CD45
CD49
CD9
CD10
CD15
CD49
CD55
CD140
CD144
CD164
CD271
Dominici et al. [44]
Ohgushi et al. [46]
Wongchuensoontorn et al. [47]
Gronthos et al. [48]
Tsai et al. [19]
Zvaifler et al. [49]
Igura et al. [21]
Zuk et al. [50]
Miura et al. [51]
Iwata et al. [53]
Hasebe et al. [54]
Yu et al. [55]
Kyurkchiev et al. [57]
Royer-Pokora et al. [58]
Mousavi et al. [59]
Orciani et al. [60]
Bühring et al. [61]
Latif et al. [62]
Simmons PJ, Torok-Storb B [66]
Simmons et al. [67]
Stewart et al. [65]
Conget et al. [69]
Pittenger et al. [6]
Abdi et al.
Barry et al.
Chamberlain et al.
Docheva et al. **
Henschler et al.
Jaiswal et al.
Kollar et al.
Majumdar et al.
Schu et al.
Vater et al.
Total Number of Studies Reported
18
18
18
15
10
8
6
6
5
5
5
4
4
2
2
2
1
1
1
1
1
1
1
1
1

Table 1. Positive cell surface markers of MSCs. A total of thirty-three studies were investigated in published literature for information on cell surface characterization. Since the identification of MSC populations is not clearly established, thorough research into previous studies revealed the most commonly reported markers: CD105, CD90 (THY-1), CD44 (HCAM), CD73, CD29, and STRO-1. The most common negative markers are CD34, CD14, and CD45. However, the available information in such studies contains inconsistencies with many markers as is apparent in table. Adapted from Mafi, et al.. (2011). The Open Orthopedics Journal. 5, 253-260.

Adult stem cells have certain factors that limit their clinical use however, such as insufficient number of cells from tissue biopsies, impaired growth, and poorly defined phenotype (Mahmood et al., 2010). Therefore using iPSCs to obtain adult stem cells would potentially create a source of unlimited, pluripotent lineage-specific cells. Many protocols have been reported for the derivation of MSCs, and this study will highlight the three protocols that were attempted.

Mahmood *et al* used the embryoid body formation technique efficaciously to directly differentiate human ES cells into mesenchymal progenitor cells (Figure 2). This team demonstrated that paraxial mesodermal markers and myogenic development markers were markedly upregulated with the inhibition of transforming growth factor β (TGF-β) signaling using a small molecule inhibitor SB-431542 (SB) in serum-free medium. This inhibition enhances expression of mesodermal lineage marker genes and can promote cardiomyogenesis. The continuous inhibition of TGF-β enriched myocyte progenitor cells in EB outgrowth cultures. This myogenic lineage further differentiated into mesenchymal progenitors by the inhibition of the TGF-β signaling pathway (Mahmood et al., 2010).

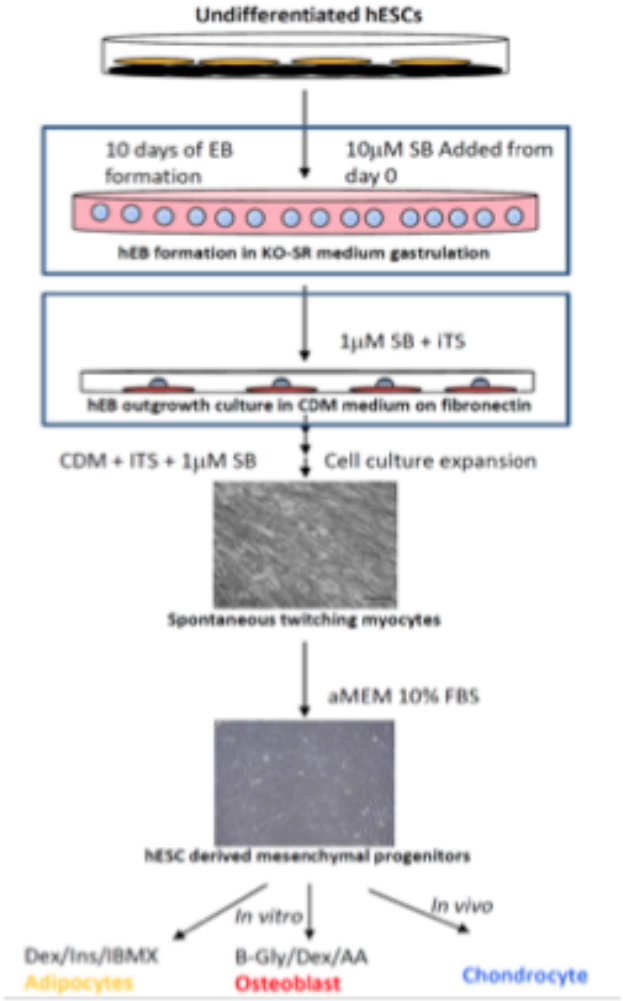


Figure 2. Schematic representation of the directed differentiation of hESCs to mesodermal stem cells. Same protocol was followed with 3 clones of iPSCs. Adapted from Mahmood, Kassem et al., Journal of Bone and Mineral Research 2010

Another technique is described by Stavropulos *et al* for the derivation of multipotent mesenchymal precursors from human ES cells through the culture of the cells at low density with chemically defined serum-free medium. The hESC colonies were seeded on murine embryonic fibroblasts (MEFs) in the serum-free media, which induced the cells to adopt an endomesodermal fate and a subsequent mesenchymal phenotype. This technique is fairly simple and consists of a 3-week differentiation period

followed by a 1-week expansion period. As can be seen below, the results are multipotent mesenchymal precursors with osteogenic, chondrogenic, adipogenic, and myogenic potential (Stavropoulos et al., 2009).

In addition, Oldershaw et al.'s protocol for the direct differentiation of hESCs into chondrocytes reports that mesodermal cells appear at the second stage, day 9 of differentiation. Cells are directed through intermediate developmental stages using substrates and chemically defined media supplemented with exogenous growth factors, progressing through primitive streak or mesenderm to mesoderm before the chondrocytic stage. The full protocol has previously been attempted in the laboratory unsuccessfully for chondrocytes. It would be of interest to attempt this mid-way differentiation to obtain MSCs. Upon accomplishment of attaining CD73+ progenitor cells, further culture of cells is conducted followed by demonstration of the characteristic phenotypes of MSCs, especially the expression of specific genes (See Table 2).

Gene	Gene Name	Notes
CD105	Endoglin	A type I membrane glycoprotein located on cell surfaces and is part of the TGF beta receptor complex. Potentially plays a role in TGF-beta signaling during MSC chodrogenic differentiation
CD90 (THY-1)	Thy-1 cell surface antigen	A cell surface protein that can be used as a marker for a variety of stem cells. This cell adhesion molecule is the smallest member of the immunoglobulin superfamily
CD44 (HCAM)	CD44 molecule (Indian blood group)	A cell-surface glycoprotein involved in cell–cell interactions, cell adhesion and migration; A single transmembrane receptor for hyaluronan
CD73	NT5E	An antigen expressed in a subset of cells; a cell surface enzyme that catalyzes the conversion of AMP to adenosine
CD29	ITGB1, Integrin Beta-1	An integrin unit associated with very late antigen receptors; transmembrane protein that mediates interactions between adhesion molecules on adjacent cells and/or the extracellular matrix (ECM)
STRO-1	STRO-1	A cell surface protein expressed by bone marrow stromal cells and erythroid precursors; Cells expressing antigen are capable of differentiating into multiple mesenchymal lineages
VIM	Vimentin	A type III intermediate filament (IF) protein found in various non-epithelial cells, especially mesenchymal cells; the major cytoskeletal component of cells, supporting and anchoring the position of organelles in the cytosol
CD34	CD34	Common negative marker for MSCs; marks primitive hematopoietic progenitors and endothelial cells
CD14	CD14 molecule	Common negative marker for MSCs; prominently expressed on monocytes and macrophages

Table 2. Genes reportedly expressed by Mesenchymal Stem Cells.

C2.2. The Chondrogenesis of MSCs

The differentiation of MSCs into chondrocytes is not only a promising alternative for the regeneration of cartilage but also for a stable supply of chondrocytes that can be used for disease modeling. Many culture methods have been reported, especially high-density cell cultures such as the micromass and pellet techniques. This study will focus on the pellet culture, which provides a three-dimensional environment that allows cell-to-cell interactions similar to those observed *in vivo* during embryonic developments (Zhang et al, 2010). It has been reported that MSC's are capable of chondrogenic differentiation in pellet culture using serum-free medium and transforming growth factor β family (Johnstone et al. 1998). Other protocols involve media containing FBS or commercial

kits with media containing reagents for inducing the commitment of MSCs into the chondrogenesis pathway. For example, Gibco's STEMPRO® medium provides a standardized culture workflow solution for the culture of MSCs and ultimate derivation of chondrocytes. A modified protocol containing serum-free, serum media, or commercial media will be conducted for the establishment of a differentiation protocol to use with MSCs. After all experiments, characterization of pellets will also be conducted for chondrogenic markers. Gene expression will be carefully examined with the natural niche of chondrocytes in mind where different layers of the growth place express genes that are specific to the cell type (Figure 3)

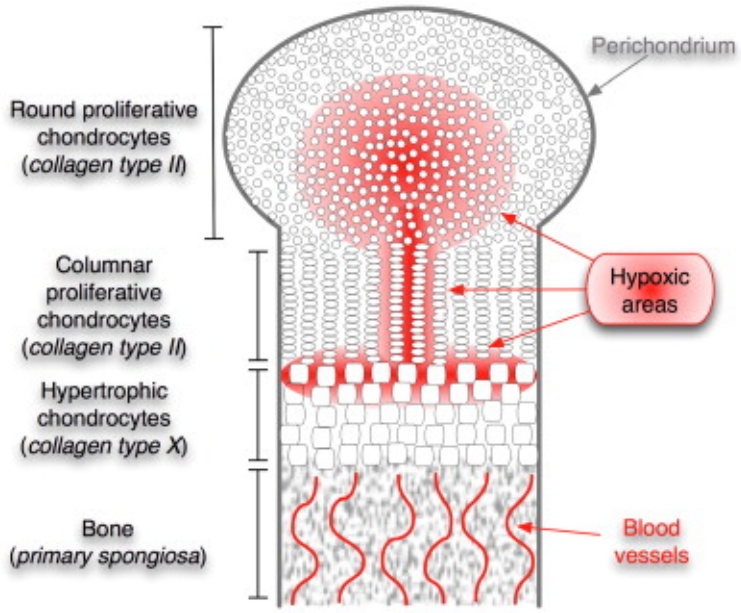


Figure 3. Schematic representation of the growth plate. Chondrocytes are piled in a columnar layer of proliferative chondrocytes, in which collagen type II is produced. Hypertrophic chondrocytes are later-stage ones expressing collagen type X and eventually mineralize their matrix to produce collagen type I. Chondrocytes in deeper layers of the growth plate are exposed to decreased levels of oxygen and nutrients (red). Adapted from Araldi, E. and Schipani, E., *Bone* 47 (2010) 190-196.

D. Summary

Morquio A Syndrome, or MPS IVA, is a lysosomal storage disorder involving an extensive pathophysiology with multiple organs and manifestations. The broad range of clinical characterizations in patients and the lack of an animal model implicate a need for further understanding of the pathophysiology of MPS IVA. Currently, there is no applicable animal model or appropriate cell lines and available patient-specific material is very limited and difficult to obtain for classical in vitro models, rendering the study of the systemic pathophysiology of MPS IVA challenging and limited. Thus, the generation of an induced pluripotent stem cell (iPSCs) line from patient-specific somatic cells is a promising alternative for studying the pathogenesis of MPS IVA, which is what we do in this study.

Using iPSC technology, differentiation protocols can be used to acquire certain cell types, such as a chondrogenic lineage. Challenges to this goal include un-established protocols for iPSC differentiation to follow and the amount of time it takes to differentiate each line. However, once the cell types are acquired, many disease-related factors can be compared, as well as the effects of recombinant human GALNS (rhGALNS) treatment. With this *in vitro* model, an in-depth study of the pathogenesis of MPS IVA is possible as well as the development of a functional bioassay for rhGALNS.

II. Materials and Methods

A. Direct Differentiation of iPSCs into Chondrocytes

A1. iPSC Culture

Normal fibroblasts and two MPSIVA (moderate and severe disease) patient fibroblasts were obtained and reprogrammed using retroviruses encoding human transcription factors Oct4, Sox2, Klf-4, and c-Myc by Andrzej Swistowski in the laboratory (Figure 4). The lines were maintained undifferentiated using either a feeder culture or a feeder free culture. For the feeder culture, iPSC colonies were maintained on primary mouse embryonic fibroblast feeder cells (PMEF-CF, Millipore) seeded at appropriate cell density in plates precoated with gelatin (0.1%, Millipore). Cells were cultured with ES medium containing DMEM/F12 (Invitrogen), 20% Knockout Serum Replacement (KSR, Life Tech.), 200mM Glutamax (100X, Life Tech.), Non-essential Amino Acids(NEAA, 100X, Life Tech.), 2-mercaptoethanol (1000X, Life Tech.), and 25µg/ml bFGF2 (10µg/mL, Life Tech). Media was changed daily after the first 48 hours in culture and splits were conducted enzymatically with collagenase IV (Life Tech., 1mg/mL) every 4-5 days. Feeder-free cultures were used when single iPSC colonies were needed without interaction of feeder cells. iPSC colonies were plated on alternate growth substrates such as Matrigel of Geltrex (Life Tech.)- coated plates, cultured with pre-conditioned ES medium and medium changed every alternate day.

Characterization of derived iPSCs was done initially through immunocytochemistry for pluripotency markers and karyotyping for verification of any chromosomal abnormalities. Pluripotency markers Oct4, Tra 1-60 and Tra 1-81 were

also periodically checked to verify that cells remained pluripotent through passages (See ICC Technique).

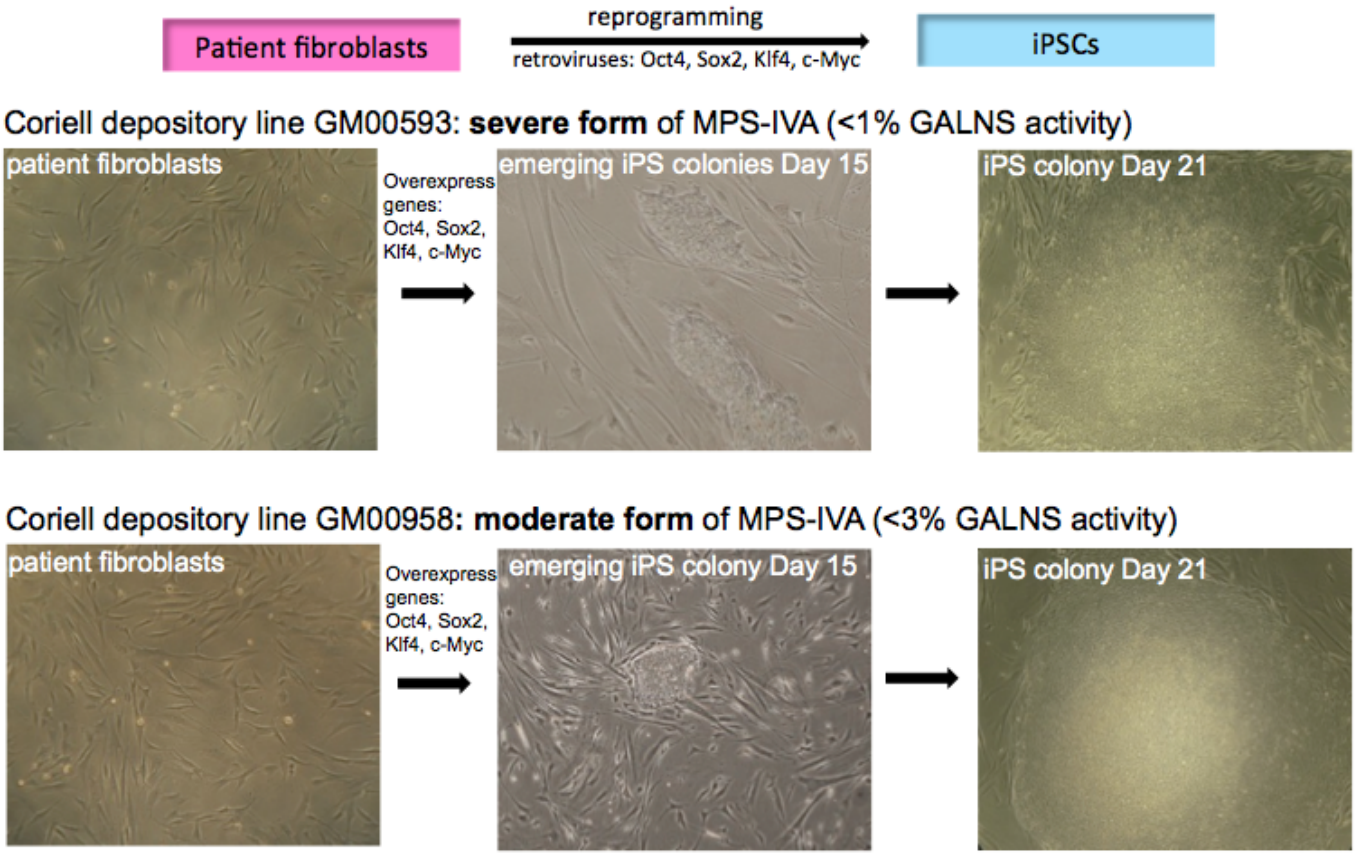


Figure 4. Derivation of MPS-IVA patient specific iPS cells. Normal fibroblasts and two MPSIVA patient fibroblasts were obtained and successfully reprogrammed using retroviruses.

A2. Differentiations

Pellet Culture

Using a protocol that was modified accordingly by Andrzej Swistowski, iPSCs were exposed to a pellet technique for the differentiation of chondrocytes. Colonies of cells were harvested and directed towards a single-cell suspension using accutase. Cells were resuspended in chondrogenic medium (DMEM Hi Glucose, 1% ITS, 40ug/ml L-proline, 1% Sodium Pyruvate, 1% NEAA, 1X PenStrep, 50ug/ml ascorbic acid, 10^{-7}M Dexamethasone, 10ng/mL TGF-β1, and 100ng/mL BMP-2) as a 1 million cell/pellet or

0.5 million cell/pellet mixture and spiked with ROCK inhibitor to avoid cell death. Cell mixture was placed in a 15-mL canonical tube with the tube cap slightly unscrewed to allow oxygen exchange and incubated overnight. Pellets form overnight and media was changed every alternate day for 21 days.

Micromass Culture

A modified micromass culture system was used as previously described (Mello et al. 1999; Toh et al. 2010). iPSCs of each clone were harvested and resuspended in chondrogenic medium (DMEM Hi Glucose, 1% ITS, 40ug/ml L-proline, 1% Sodium Pyruvate, 1% NEAA, 1X PenStrep, 50ug/ml ascorbic acid, 10^{-7}M Dexamethasone, and 10ng/mL TGF-β3). $5X10^5$ cells were carefully placed, or "spotted" in the middle of 24-well plates as droplets and allowed to adhere for 2 hours in incubator. An additional 500ul medium was carefully added after this incubation period and allowed to form spheres overnight. Medium was changed every 3 days with fresh factors for the 14-day differentiation period.

B. Indirect Chondrocyte Differentiations

B1. Establishment of Intermediate Mesenchymal Lineage

B1.1. Embryoid Body Technique

For the derivation of mesenchymal progenitor cells, the first technique used is an EB outgrowth culture treated with a small molecular inhibitor as previously described by Mahmood et al. Normal and severe clone iPSCs were harvested using collagenase IV and fragmented into small clumps for the formation of embryoid bodies. Clumps were placed in a low-adhesion petri dish on a rocker to avoid settling or attachment to plate in

ES medium supplemented with SB-431543 (10μM). Media was changed on days 3, 6, and 8 by gravity sedimentation. On day 10, the EB's were transferred to fibronectin-coated (10mg/mL, Millipore) plates for EB-explant outgrowth culture. Chemically defined medium (CDM) composed of DMEM/F12, 0.5% BSA (Fraction V), 1% Penicillin/Streptomycin, 10% Insulin-Transferrin-Selenium (ITS), and SB-431543 (1mM) was used to allow for cellular outgrowth and the formation of a cell monolayer. The outgrowth culture was allowed to grow to confluency, at which time subsequent passaging was performed using accutase to reach a single-cell mixture. Cells were plated on uncoated cell culture dishes or flasks in CDM medium. After 2-4 passages, cells were transferred into MSC medium for further growth. When cells expanded to 2-3 T175 flasks, magnetic bead (FACS) sorting was performed.

B1.2. Low Density Culture

A modified protocol as reported by Stavropoulos et al. was followed for the derivation of multipotent mesenchymal precursors. A normal split of the iPSCs was first done using collagenase IV and cultured as normal with ES media until colonies reached appropriate size of 30-50cells. Medium was then switched to ITS medium containing DMEM/F12, ITS supplement (Invitrogen), and Pen/Strep for Day 0 time-point. The medium was replaced every 2-3 days with fresh ITS medium. At day 14, cells were harvested using accutase and re-plated on 60mm fibronectin-coated dishes at a split ratio of 1:3 with ITS medium. At day 21, cells were changed to MEM medium containing alpha-MEM, 10% (v/v) heat-inactivated FBS (Invitrogen), 2mM L-glutamine, and 1X Pen/Strep. Cells were cultured in this medium for an additional 7 days, changing media

every 2-3 days. CD73+ cells were isolated through magnetic sorting and further characterization followed after further culturing.

B1.3. Intermediate Development Stage

Oldershaw et al. (2009) reports a protocol for the differentiation of iPSCs into chondrocytes with a mesodermal stage in between. This protocol what followed up to this second stage for the derivation of MSCs using the severe and moderate MPSIVA clones. A feeder-free culture of iPSCs was achieved with mTESR1 medium (Stem Cell Tech.) and harvested with accutase to obtain a single cell suspension. A cell mixture containing $2.1X10^6$ cells was plated on 60mm cell culture dishes coated with fibronectin in DMEM/F12 medium composed of 2mM Glutamax, 1% ITS, 1% NEAA, 2%B27, 90uM B-mercaptoethanol, and appropriate factors according to paper. Passages were carried out as cells became confluent. At day 9, cells were switched to MSC medium (alpha MEM medium containing 10% FBS (Hy Clone) and 100X Pen/Strep) and subsequently passaged on gelatin-coated culture dishes followed onto non-coated tissue culture flasks. After four passages in MSC medium, cells were subjected to magnetic bead sorting using CD73-PE antibody according to protocol (See Techniques Section II-D1). After sorting the CD73+ cells were plated onto 100mm gelatinized culture dishes and cultured with MesenProRS serum-free media (Invitrogen). Further characterization of cells was done using flow cytometry and ICC.

B1.4. MSC Culture

The three lines of derived mesenchymal stem cells were banked and stored in nitrogen until necessary use. When cultured, MSC's were plated on cell culture flasks and grown in MSC media or commercially available MesenProRS media. Cells were

passaged every 3-4 days upon reaching 80-90% confluence with TrypLE using normal cell culture techniques and media was changed every 2-3 days.

B2. Chondrogenic Differentiation of MSCs.

The differentiation of MSCs into chondrocytes is achieved using the pellet culture system and exposure to 5ng/mL transforming growth factor β3 (TGFβ3; R&D Systems) and 200M ascorbic acid (AA; Sigma) in MEM medium. The pellets are cultured for 3 to four weeks with fresh media change every 2-3 days. Pellets were harvested at several time points, washed with PBS, snap frozen, subjected to sonication using the Covaris CryoPrep Cryogenic pulverizer for mRNA extraction (see Gene Expression Analysis). Some pellets were also harvested for immunohistochemistry.

C. Treatment with rhGALNS

The MSCs were used to assess the therapeutic efficiency of rhGALNS and to see effect of treatment on cells, specifically the growth rates. Treatment of cells was done with 10 nM rhGALNS diluted in PBS + 0.1% BSA, typically for seven days. For the negative controls or non-treated groups, cells were exposed to the formulation buffer of GALNS at the same concentration to ensure that no other factors influences results.

D. Techniques

D1. Automated Cell Separation

Magnetic bead sorting of MSCs was done using the autoMACS® Pro separator with CD73-PE antibody and Anti-PE MicroBeads (Miltenyl Biotech) according to manufacturer's instructions. After sorting, cell count and viability of cells was checked

and cells were plated on con-coated culture dishes. Plates were carefully observed for the attachment of cells, focusing on a fibroblast-like morphology typical of MSCs.

D2. Phenotypes Analysis by Flow Cytometry

The BD Accuri C6 flow cytometer was used for the verification of CD73+ cells with the CD73-PE antibody used for cell sorting. Respective fibroblasts were used for each clone as a negative control that does not express CD73+. MSC's were first harvested with accutase for single cell suspension and $2X10^6$ cells re-suspended in FACS buffer (PBS + 1% BSA). The mixture was split in half, one set use with the CD73-PE (MACS) antibody and the second for the PE mouse IgG Control (BioLegend), both at a 1:20 ratio. Tubes incubated with antibodies on ice for 30 minutes and an additional volume of FACS buffer was added to each tube. After spin, supernatant was discarded and pellet was re-suspended in FACS buffer for run on flow cytometer. Instructions according to manufacturer were followed.

D3. Gene Expression Analysis (qRT-PCR)

Total RNA was extracted using the RNeasy Plus Mini Kit (Qiagen) with the tissue sample protocol for iPSCs and MSCs. For chondrogenic pellets, the RNA extraction was performed through the Trizol method according to the PureLink RNA Mini Kit (Ambion) with PureLink DNase (Invitrogen) treatment for DNA-free RNA. A high-capacity cDNA SuperScript® VILO™ (Invitrogen) kit was used for reverse transcription of 1µg RNA according to instructions. cDNA was used for real-time PCR with the LightCycler 480 Real-Time PCR System (Roche), TaqMan Gene Expression Master mix, and TaqMan Gene Expression Assays for SOX9, aggrecan, type II collagen (COL2), type X collagen (COL10), and type 1 collagen (COL1) (see Table 1). A crossing point (CP) determined

for each gene of interest, using a Second Derivative Maximum Method, was normalized to the mean CP for Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) in the same sample.

Gene	Gene Name	Notes
COL2A1	Collagen type II, alpha 1	Basis for articular and hyaline cartilage
COL1A1	Collagen type I, alpha 1	Major Component of type 1 collagen, typically found in skin, tendon, and primarily bone amongs other tissues
COL10	Collagen type X, alpha 1	Hypertrophic chondrocytes typical during endochondral ossification
SOX9	SRY (sex determining region Y)-box 9	A transcription factor that regulates the expression of genes in chondrogenesis
ACAN	Aggrecan	A protein in the extracellular matrix of cartilagenous tissue that provides support to cartilage structure. It is the major proteoglycan found in articular cartilage

Table 3. Genes typically expressed during chondrogenesis.

D4. xCELLigence

Cell viability, size, and growth was monitored using the xCELLigence system (Roche Applied Science), which uses 96-well plates containing inter-digitized microelectrodes, for the noninvasive real-time monitoring of cells. Cells were first plated at increasing densities to observe the optimal density and time point for experiments. Cells were plated at appropriate densities and observed daily. Cell index was used as an indicator for cell size and growth rate, increasing as time progressed. Experiments were stopped once a plateau was reached on graph, indicating reduced proliferation.

D5. Immunocytochemistry

Cells were plated onto multi-channel glass slides or 24-well plates to proper confluency and fixed with 4% PFA for 20 min followed by 3 washes with PBS. Cells were then exposed to a second fixation with ice cold 90% methanol followed by 3 washes with

PBS. Primary antibodies were diluted in a blocking solution containing 0.3% Triton X-100, 10% goat serum, and 1% BSA and left on cells overnight at 4°C. Cells were then washed 3-5 times with PBS and secondary antibodies then applied with blocking buffer for 1 hour at room temperature in dark. Cells were then stained with Alexa-Fluor 488- or Alexa-Fluor 594-conjugated Goat secondary antibodies (Life Tech). After incubation, cells were washed again and slides mounted with ProLong Gold (Invitrogen) containing DAPI for a counterstain of the nuclei.

For confirmation of pluripotency of iPSCs, the antibodies used were: TRA-1-60 (Millipore), a cell surface antigen of human embryonal carcinoma cells; TRA-1-81 (Millipore), a glycoprotein expressed on the surface of stem cells; and Oct4 (AbCam), a transcription factor required for the sustainability of self-renewal and pluripotency of stem cells.

For verifying mesenchymal lineage of MSCs, the markers used were: H-CAM (CD44), a glycoprotein involved in cell-to-cell interaction and hematopoiesis; THY-1 (CD90), a glycoprotein cell surface antigen; STRO-1, a cell surface protein expressed when cells are capable of differentiating into mesenchymal lineages; and MCAM (CD146), a cell adhesion molecule used as a marker for endothelial cell lineage, all from a characterization kit (Millipore). Additional proteins tested included: CD19 and CD14 (Millipore), hematopoietic markers not expressed by MSCs and thus used as negative markers; CD73 (AbCam), a clear marker for MSCs; Vimentin (Millipore), a marker belonging to the intermediate filament family and highly expressed in MSCs; and VCAM-1 (Invitrogen), a member of the immunoglobulin family.

D6. High Content Screening

Using the ImageXpress Micro XL system (Molecular Devices) and the Operetta system (PerkinElmer), high throughput imaging was possible for automated widefield and confocal imaging of the MSCs. Using a 96-well plate, MSCs were plated at increasing cell densities to account for different growth rates and size of cells. Cells were stained with Phalloidin for actin filaments as a whole cell body stain, LAMP-1 for lysosomes, and nuclear DAPI. Fluorescent and brightfield imaging according to manufacturer's instructions was done to measure lysosomal content, cell size, and cell shape/morphology of three clones.

III. Results

A. The iPSC Line

Fibroblast cell lines obtained from Coriell Cell Repositories were cultured for use: a MPSIVA moderately attenuated line expressing 3% GALNS, a MPSIVA severely attenuated line expressing less than 1% GALNS, and an unaffected healthy line. Mutations were verified by activity assays and sequencing (data not shown). The fibroblast lines were successfully reprogrammed into induced pluripotent stem cells using retroviruses previously described in Section II-A1. The three clones were subjected to long-term culture using mitomycin-C treated MEFs as stable colonies in the feeder based system for over 40 passages and were able to generate high quality embryoid bodies (Figure 5). Furthermore, both lines of MPSIVA iPSCs show normal karyotype.

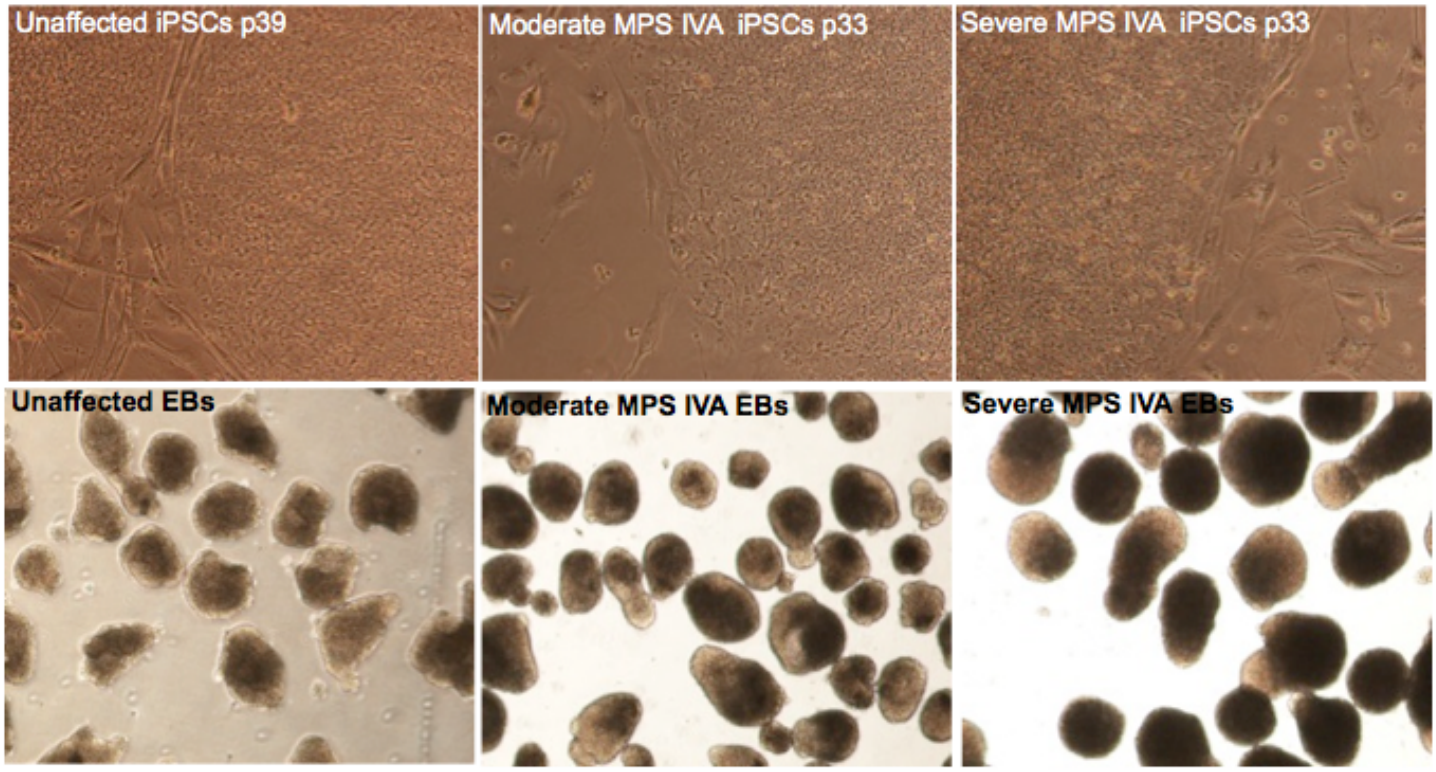


Figure 5. Three iPSC clones can be cultured long-term, for up to 40 passages, with careful observations to maintain stability. All are capable of forming embryoid bodies as well, further demonstrating their pluripotency.

A1. Confirmation of pluripotency

Pluripotency was verified through immunostaining and the ability to form embryoid bodies before beginning any experiment and at intermediate time points in culture maintenance. This was done in order to confirm the retention of an undifferentiated state as well as the potential of cells to differentiate into cells from all three germ layers: endoderm, mesoderm, and ectoderm. All clones showed positive expression of iPSC pluripotency markers OCT4, Tra-1-60, and Tra-1-81 (Figure 6). These expression profiles verify the pluripotent cell populations and their ability to proliferate or differentiate. Furthermore, pluripotency is also confirmed with formation of embryoid bodies *in vitro.* Thus the iPSCs can be used to differentiate any cell type as long as pluripotency is maintained.

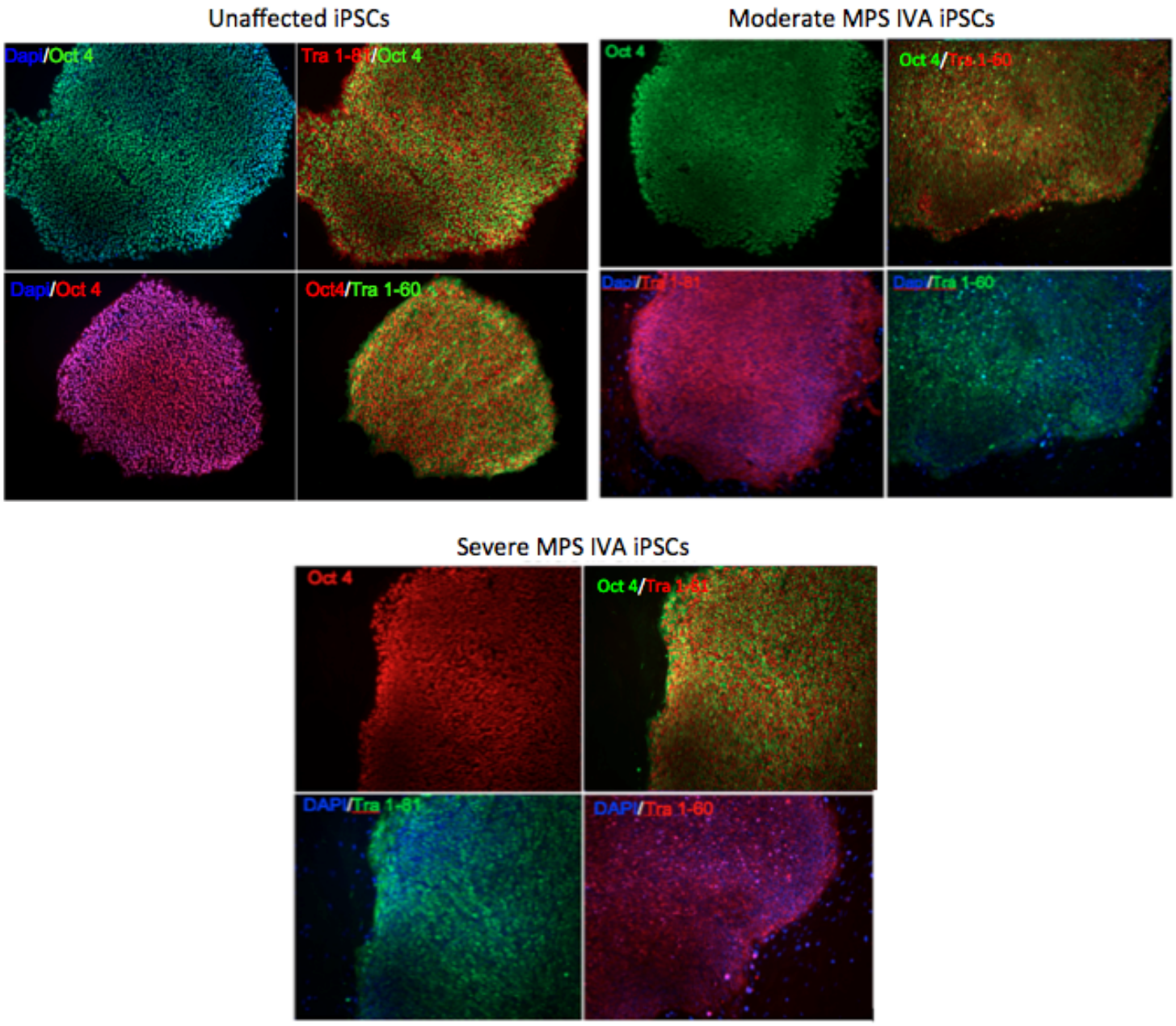


Figure 6. Characterization of derived iPSCs for pluripotency markers, OCT4, Tra 1-60, and Tra 1-81. All three clones showed positive staining of markers.

A2. Initial differences observed between clones

During maintenance of the iPSC culture, several observations were made that suggest a clear difference in phenotype of the severe clone (Table 2). Overall, the severe clone behaves better in the feeder-based culture as opposed to in feeder-free

culture, though random spontaneous differentiation is at times observed. The unaffected and moderate clones remained stable in this culture on the other hand. Manual dissections of differentiating clones were done when necessary, which ensured the maintenance of an undifferentiated state. The feeder-free culture of the severe clone was difficult, with significant random spontaneous differentiation observed. Moreover, the severe clone grows at a slower rate, requiring later passaging and harvests for experiments. The unaffected and moderate clones behaved relatively the same.

Culture system	Unaffected iPSCs	Moderate MPS IVA	Severe MPS IVA
Feeder based	stable	stable	visible spontaneous differentiation (approx. 20%)
Feeder free	stable	stable	significant spontaneous differentiation
Embryoid bodies EBs	stable	stable	stable

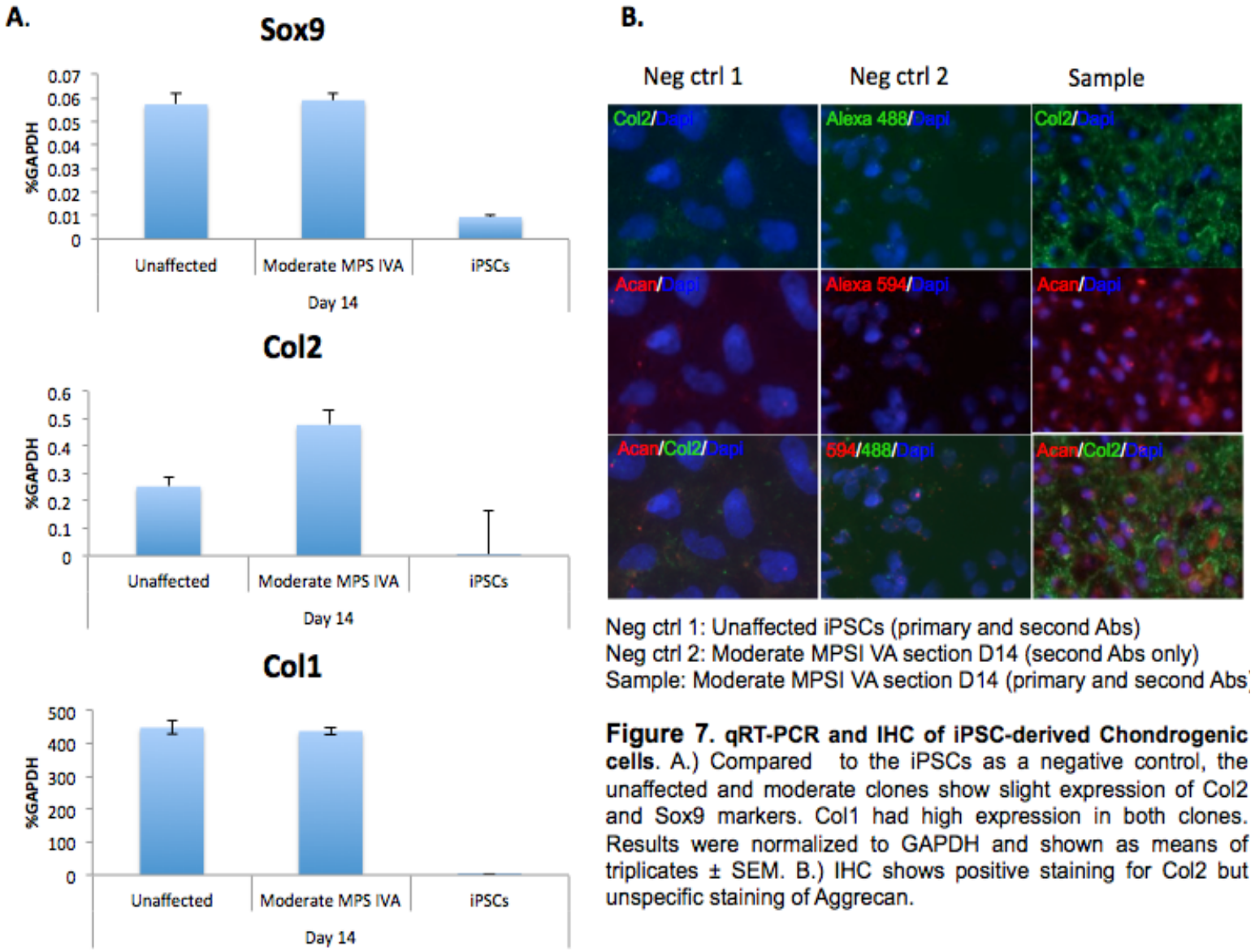
Table 4. Observations of iPSC culture maintenance in long-term feeder based culture and feeder free culture, as well as their ability to form EBs.

B. Direct Chondrocytic Lineage

B1. Multiple attempts made

Using the high-density micromass culture, iPSCs were subjected to a direct and uniform chondrogenic differentiation as reported by Mello and Toh et al. However, cell spheres spotted on tissue plates only remained spheres for approximately 5 days. Around this time, spheres flattened out and there was significant cell loss. Cultures were still maintained until the allotted 14-day differentiation. As shown in figure 7A, there is a relatively low expression of SOX9, a chondrogenic transcription factor, and of COL2, a typical cartilage characteristic collagen at the day 14 time-point. COL2 and SOX9 are

visible in the two samples and not in the negative control, suggesting chondrogenesis was initiated. Similar observations were made with the pellet culture (not shown), in which pellets lasted approximately 8 days before flattening out or dissociating. Expression of COL2, however, was lower in pellets whereas the expression of SOX9 remained similar. COL1 was seen at high levels in both techniques, suggesting late-stage chondrocytes gearing towards a bone-like step. No expression of aggrecan was seen in any samples. The ICC of pellet samples (Figure 7B) shows positive staining of COL2 and negative staining of Aggrecan, since stainings are weak and non-specific, rendering results inconclusive. Several rounds of each differentiation were conducted in which results remained similar, even when supplemented with recombinant human Acid Ceramidase (rhAC), which was thought to facilitate the generation of chondrocytes with enhanced quality and expression of markers. Thus a new approach was necessary for the derivation of chondrocytes.



Neg ctrl 1: Unaffected iPSCs (primary and second Abs)
Neg ctrl 2: Moderate MPSI VA section D14 (second Abs only)
Sample: Moderate MPSI VA section D14 (primary and second Abs)

Figure 7. qRT-PCR and IHC of iPSC-derived Chondrogenic cells. A.) Compared to the iPSCs as a negative control, the unaffected and moderate clones show slight expression of Col2 and Sox9 markers. Col1 had high expression in both clones. Results were normalized to GAPDH and shown as means of triplicates ± SEM. B.) IHC shows positive staining for Col2 but unspecific staining of Aggrecan.

C. Indirect Chondrocytic Lineage

C1. MSC Line Established

To circumvent the challenges observed with the direct differentiation of chondrocytes from iPSCs, creating an intermediate mesenchyme progenitor lineage was investigated through several protocols. The differentiation of MSCs from iPSCs has been

reported in many techniques, three protocols were used: 1) EB-outgrowth, 2) Low density, and 3) Oldershaw. The EB-outgrowth protocol in which cells are cultured with the SB-431542 inhibitor proved the most efficacious. EB's of 3 clones were cultured for 10 days with no issues, at which time an outgrowth culture was set up on fibronectin- or gelatin-coated plates. EB's attached and formed a monolayer better on the fibronectin-coated plates. Interestingly, the moderate MPSIVA clone EBs did not attach as well overall, and dissociation of EB's was observed the following day, suggesting extensive cell loss. On approximately day 17 of experiment, beating colonies were observed for all three clones- most likely an intermediate myocyte stage- that lasted 2 days. Following several splits when confluency was reached, the moderate clone saw an increased cell loss. At the time for sorting, there were not a sufficient number of cells for this clone and was thus not used. The CD73+ sorting of normal and severe MPSIVA clones was successful and resulted in good cell yield, rendering expansion possible for banking and characterization.

The second technique used was the low-density culture of small iPSC colonies seeded on MEFs and treated with ITS in chemically defined media. At day 7 of experiment, differentiation was observed first in the moderate and severe clones, with expansion of colonies and a morphological change of outer cells. 3 days later, MEFs begin lifting off and many dead cells are observed, an observation which progressively worsens as days pass. To avoid further cell loss, plates were switched to MEM media at day 12, which appeared to alleviate the issue of cell loss. At the allotted 14-day period, cells were split according to protocol and re-plated on fibronectin-coated dishes. After second split, the normal and moderate clones had few colonies/ cells that attached

whereas the severe clone had many. At the end of the experiment, only the severe clone remained with sufficient cells for the CD73+ cell sorting. The FACS sorting for CD73+ cells was successful and the clone was expanded and banked.

The third technique used was the Oldershaw protocol that reports a mesodermal stage during differentiation of iPSCs into chondrocytes. Protocol was followed up to this second stage for the moderate clone, since others had already been acquired and due to time constraints. Differentiation occurred during a 9-day period in which time various factors were added according to protocol. A high proliferative rate was observed and there was a sufficient amount of cells at the end for sorting. Sorting resulted in a decent CD73+ yield, which was expanded for banking and characterization. Thus, the mesenchyme progenitor lineage was reached for the three clones using different protocols: the unaffected line was established using the EB-outgrowth protocol, the moderate line was established using the Oldershaw protocol, and the severe clone was established using the EB-outgrowth as well as the low density protocols- but the EB-outgrowth cells will be used for experiments.

C1.1. Characterization and differences in clones

To verify the mesenchyme lineage, sorted cells were subjected to various characterization protocols. The first characterization was a flow cytometric analysis with the CD73 antibody used in cell sorting conjugated to a fluorescent probe. As shown in figure 8, each clone shows a clear shift from the IgG isotype control, verifying the expression of CD73 cell surface marker typical of MSCs. This shift is not observed in the negative control since iPSCs do not express CD73, rendering iPSCs an adequate negative control.

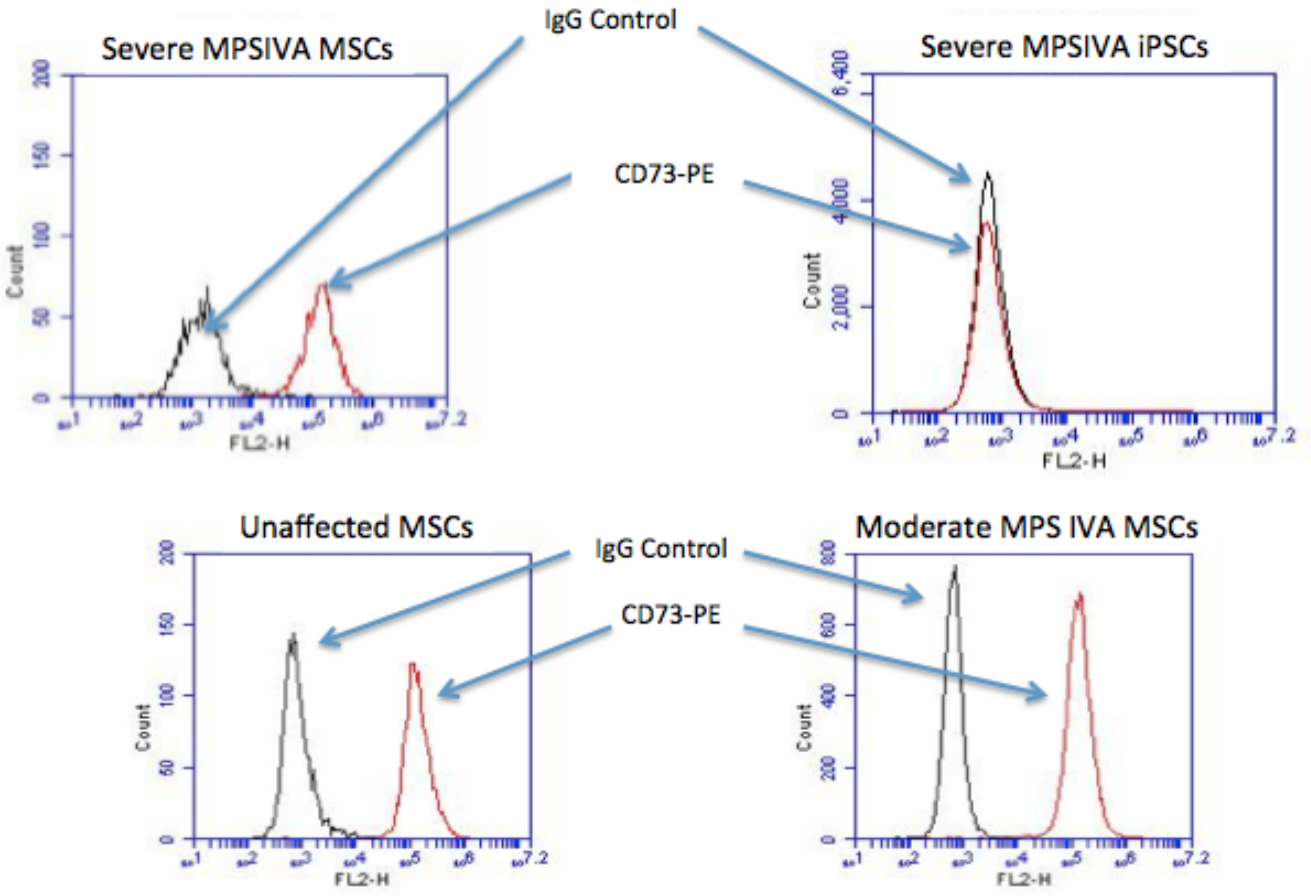


Figure 8. Flow cytometric analysis of iPSC-derived mesenchymal progenitors with MSC-specific cell surface marker, CD73. Same CD73-PE antibody was used with cell sorting. Isotype control IgG used for each run and iPSCs were used as a negative control. All clones show clear shift from IgG control, indicating correct lineage of differentiated cells.

Next, a qualitative real-time analysis was performed on the unaffected and severe MSC clones with 5 genes reportedly expressed by MSCs (recall Tables 1 and 2): CD105, CD44, CD73, CD29, and THY-1 (CD90). Undifferentiated unaffected iPSCs were used as a negative control for all markers. However, positive expression of THY-1 is seen for iPSCs, and research shows it is a common marker for various stem cells. Thus iPSCs are not an adequate negative control for this marker. CD29, an integrin family glycoprotein, and CD44 (HCAM), a cell surface glycoprotein, are both expressed at relatively high levels (Figure 9A) in both unaffected and severe MPSIVA clones

compared to the other genes. Interestingly, 4 out of the 5 markers have higher expression in the unaffected clone except for THY-1. When calculating percent control with the unaffected clone set at 100% (Figures 9B, C), we see that the moderate clone expresses almost double the level of THY-1 and almost the same level of CD105. Interestingly, these two markers are the lowest expressed in the severe clone.

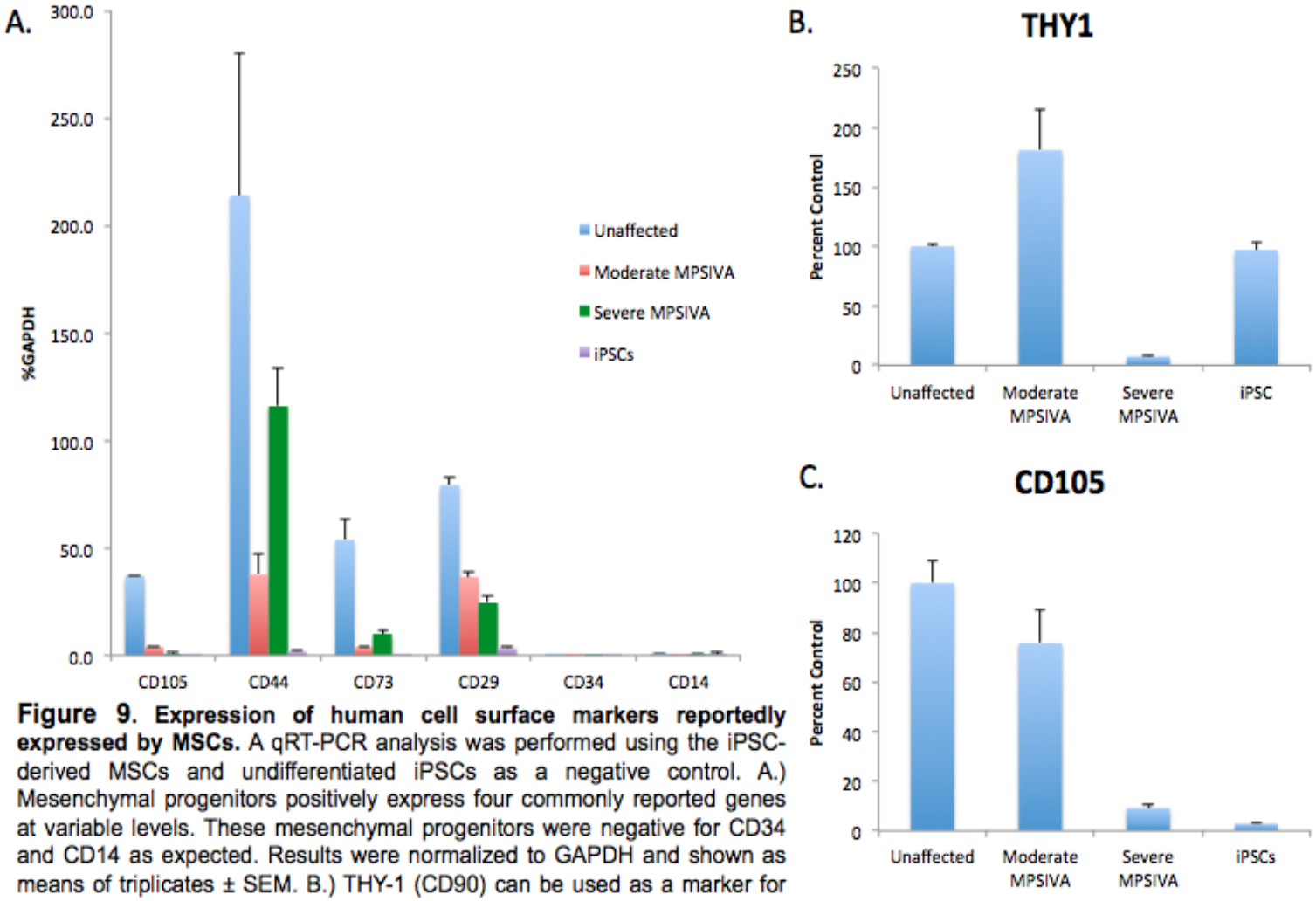


Figure 9. Expression of human cell surface markers reportedly expressed by MSCs. A qRT-PCR analysis was performed using the iPSC-derived MSCs and undifferentiated iPSCs as a negative control. A.) Mesenchymal progenitors positively express four commonly reported genes at variable levels. These mesenchymal progenitors were negative for CD34 and CD14 as expected. Results were normalized to GAPDH and shown as means of triplicates ± SEM. B.) THY-1 (CD90) can be used as a marker for various stem cells, and thus is observed in our cell populations, with the highest expression observed in moderate MPSIVA MSCs. C.) CD105, the most commonly reported gene, shows the highest expression in the unaffected clone. B and C are shown as a percent control, with unaffected clone set to 100% expression.

Lastly, immunofluorescent stainings revealed positively stained cells for MSC markers CD73, Vimentin, STRO-1, and HCAM (Figure 10). The negative control markers CD14

and CD19, expressed in macrophages and lymphocytes respectively, were not expressed in any cells. Stainings for VCAM (not shown), a cell surface protein, repeatedly showed nuclear staining, consistent with the gene expression data which showed no expression of VCAM, suggesting unspecificity of marker or its absence in our MSCs. As a negative control for makers, undifferentiated iPSCs were also stained and demonstrated negative expression of all markers. These immunofluorescence data demonstrate the differentiation of iPSCs to MSCs and expression of cellular markers known to be associated with MSCs.

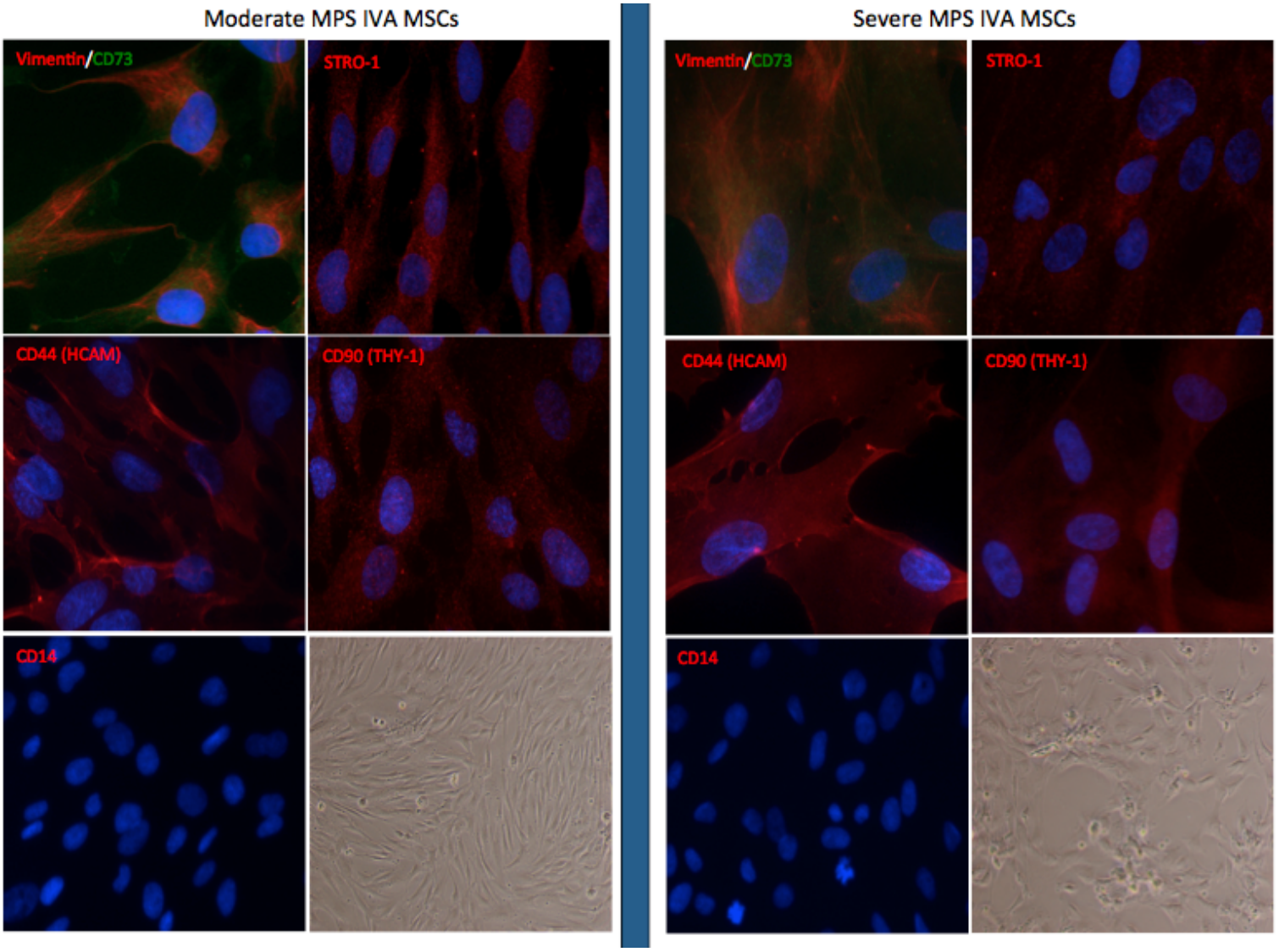


Figure 10. Immunostaining of iPSC-derived MSCs for the detection of human MSC markers. Stainings show positive marked expression of Vimentin, CD73, STRO-1 and HCAM in three clones (Unaffected not shown). CD14 was not expressed in the cell populations tested as expected, and thus serves as a negative control.

Upon culturing the MSCs for banking and characterizations, some initial differences were observed between the three clones. It was apparent early on that the severe MPSIVA clone grows slower than the other two clones- which was also observed in the iPSC culture. As can be seen in the images from figure 10, there are some morphological differences observable between the moderate and severe MPSIVA clones (Figure 10). The moderate and normal clones behave similarly, and both cell populations have a fibroblast-like morphology whereas the severe clone is a bit different. These observed differences initiated new questions and experiments to better understand the cell types and whether these observations could be disease-specific.

C1.2. Growth Rates Investigated

Upon noticing these changes in the MSC culture, several experiments were conducted to check growth rate and confluency. Cell diameter and cell circularity were regularly checked during each passage before beginning any experiment, and it appeared that the severe MPSIVA clone has a larger diameter for the most part (Table 3). At times, the diameter decreases, which could be due to cellular stress or confluency. A quick experiment using the CloneSelect Imager, which allows us to monitor cells and identify densities as well as cell number estimates, demonstrated that normal and moderate MPSIVA cells indeed grow faster and mostly uniformly, as shown by increasing confluency percentages with time (Table 3). The severe MPSIVA clone shows

a decreased growth rate as confluency is reached at later time points and can remain at the same density for several days.

Clone	Diameter at start (µm)	% Confluence at Day 1	% Confluence at Day 3	% Confluence at Day 6
Unaffected	15.97	26	45	71
Moderate	16.32	22	46	86
Severe	17.67	23	30	42

Table 5. Growth rates and confluency checked using a CloneSelect Imager. The three MSC clones were cultured beginning with the same number of cells for various days and confluency was checked daily. As can be seen, the severe clone has a larger diameter and a decreased growth rate as apparent by confluence.

C1.3. Treatment with rhGALNS

After initial confirmation of these preceding observations, it became of interest to see if the growth rate would be altered or rescued upon treatment with rhGALNS, especially for the disease clones. Using the xCELLigence system, a real-time monitoring of cells was possible where cells are measured for confluency (and thus growth rate), viability, and morphology. Several experiments were set up with treated and non-treated groups of cells for comparison to test the effect of treatments on cells in terms of growth rate and cell size. As previously observed, the severe MPSIVA clone did not proliferate until day 6, at which time the treated group saw a dramatic increase. However, the curves in the graph suggest a positive effect of GALNS in all clones, even in the unaffected clone though not as high (Figure 11A). The slope of the curves is highest in the moderate and severe clones after about 4 days, at which time there is a clear increase between the two treatments (Figure 11B).

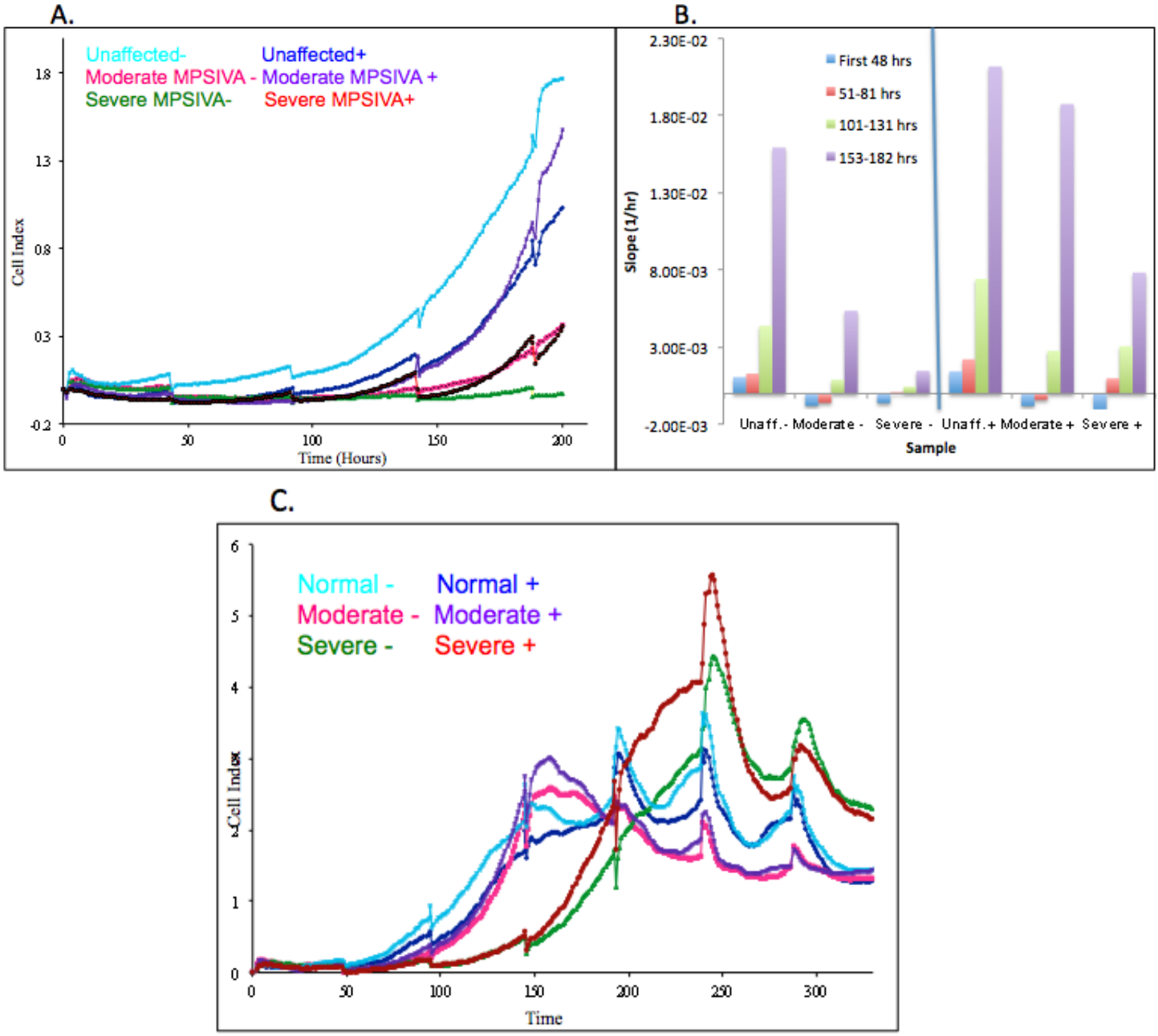


Figure 11. Growth rates of MSCs investigated upon treatment with rhGALNS using xCELLigence. Cell index used as a measure of growth rate and cell size for two treatments of each clone. A.) As is apparent in curves, the severe clone is slowest to proliferate. B.) With treatment, a significant increase in slope was observed in the three clones, specifically moderate and severe MPSIVA clones during the last 24 hours of experiment. C.) A longer experiment showed similar results for the severe clone. However, little to no differences in slopes was observed between normal and moderate clones. Note that the index of the severe clone continues to increase whereas the two others reach a plateau earlier.

Further attempts were made using the same set-up with a longer period of time to confirm these results. Figure 11C shows the previously stated increase in slope for the

severe MPSIVA clone only. The unaffected clone had a slight shift in curve but the slope remains the same between the two treatment groups. The moderate clone remains stable with no clear shifts. At approximately day 10, all clones see a dramatic decrease in slope, most likely due to confluency, stress, or culture conditions. Interestingly, as can be seen in figure 11A, the severe clone has the lowest cell index at the end of the experiment at 200 hours. However, in the second experiment (Fig 11C), we see that at 300 hours, the severe clone is now at a higher cell index than the other two clones. Additionally, the severe clone's slope continues to increase after the other two have reached a plateau, an indication that cells have reached full confluency and begin to die off. These results initiated further questions regarding the size and specificity of our cell line, especially in terms of the cell index. It could be that we are seeing the higher index in the severe clone because of the difference in cell size, or perhaps accumulation within the cell. Since cell index is only a relative measurement, it is of interest to further investigate differences between three clones, especially cell size.

Cells were also plated for Immunostaining and demonstrated that rhGALNS trafficked to lysosomes, as evident by its colocalization with LAMP-1 (Figure 12). High content screening demonstrated 98% colocalization of rhGALNS and LAMP-1 granules. Previous work by Drovak-Ewell et al. (Figure 13A) showed that addition of rhGALNS to MPSIVA fibroblasts corrected their enzyme deficiency dose-dependently. rhGALNS uptake was M6P receptor-dependent (Figure 13B), since it was significantly inhibited by M6P. These findings were further supported and re-capitulated by these results using MSCs.

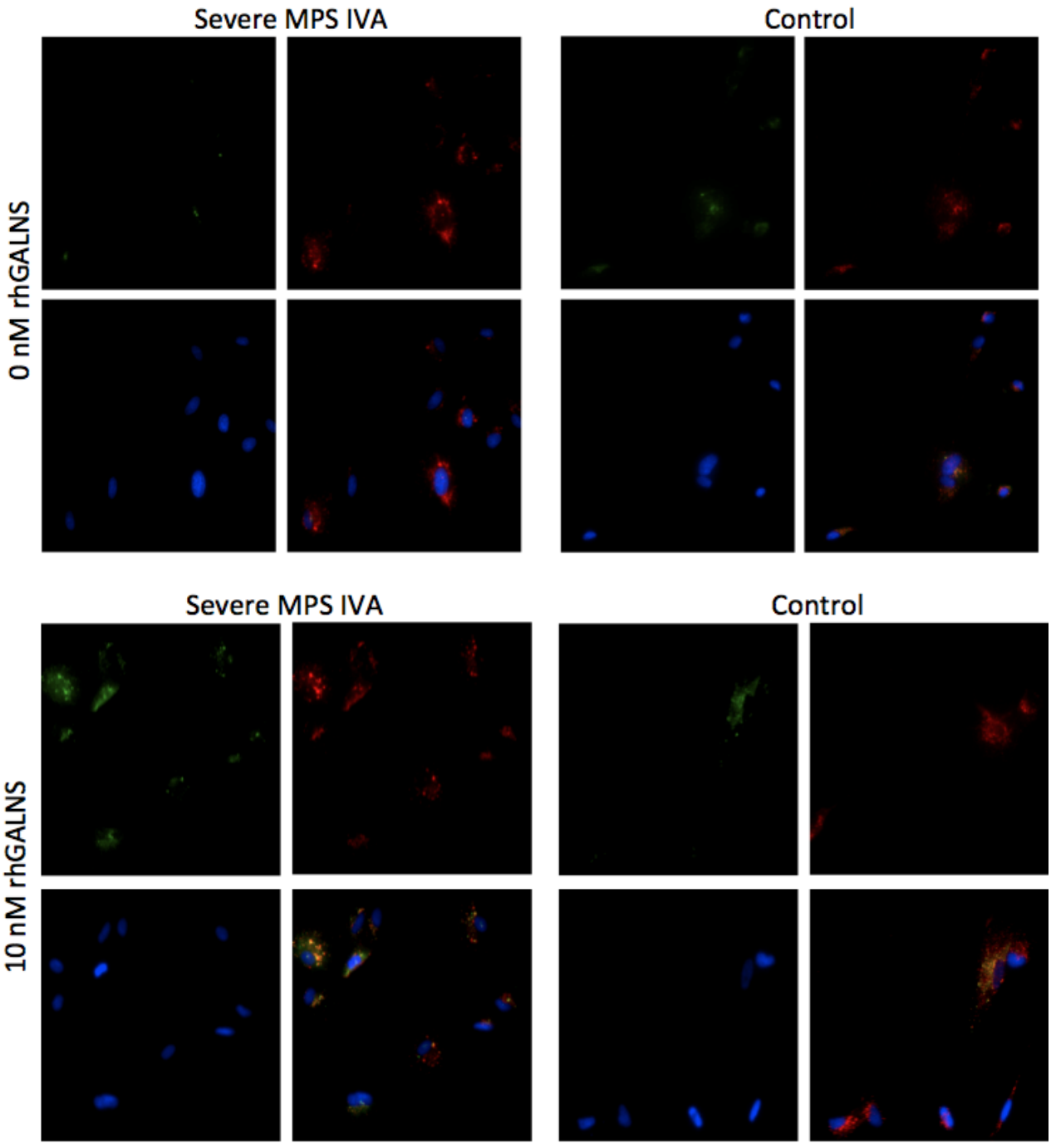


Figure 12. rhGALNS internalizes into lysosomes of MSCs. Cells were cultured for 7 days with 10nM rhGALNS. Using Immunostaining, it was demonstrated that the MSCs uptake rhGALNS (green) and co-localize with lysosomal marker, LAMP1 (red). Control images are unaffected MSCs. Images were acquired with identical parameters.

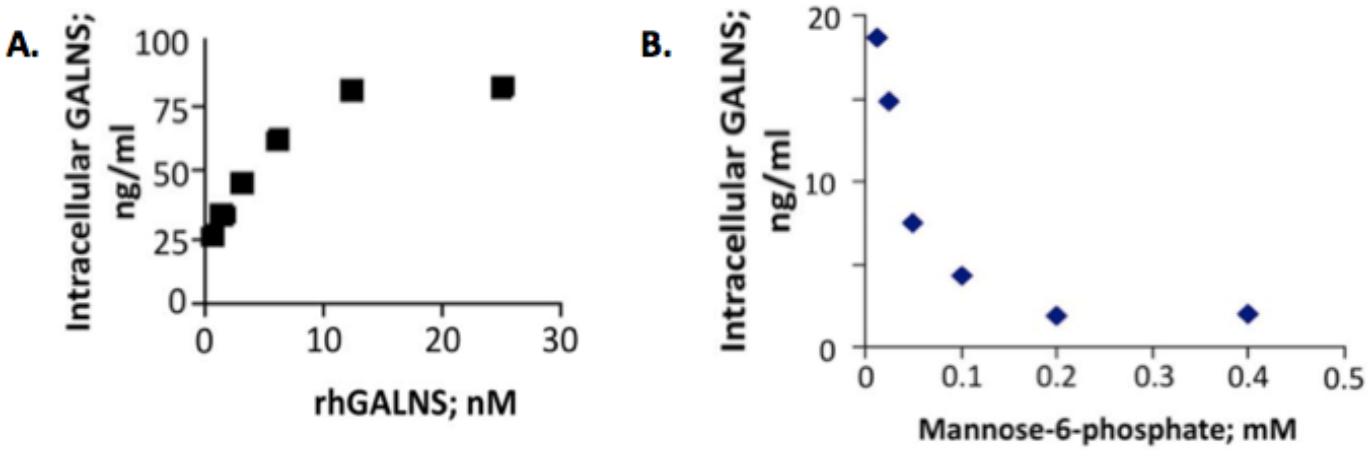
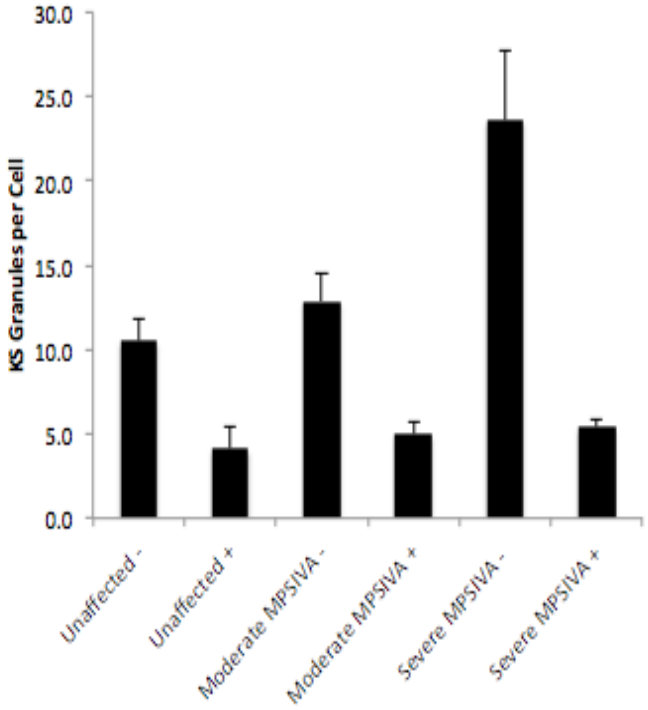


Figure 13. rhGALNS is up taken dose-dependently and M6P receptor-dependent. Previous work by Drovak-Ewell et al. demonstrated that MPSIVA fibroblasts: A) show a dose-dependent uptake of rhGALNS and B) M6P inhibited uptake of 2.5 nM GALNS. Means of 3 independent experiments. Adapted from Dvorak-Ewell et al. (2010). PLoS ONE 5(8): e12194. doi:10.1371/journal.pone.0012194

Additionally, the three MSC clones were cultured for 7 days with and without rhGALNS treatment to test for keratan sulfate accumulation and reduction. Using capillary electrophoresis analysis, a technique used to analyze GAGs, no detectable KS or CS levels were found in any sample (data not shown). The experiment was repeated with the addition of aggrecan, a proteoglycan with CS and KS chains attached to the protein core, to see whether cells could accumulate KS. Cells were immunostained with LAMP-1 and KS for analysis. KS did accumulate and was 99% co-localized to the lysosome. As can be seen in figure 14, there was an increase in KS granules in the moderate and severe clones which was reduced to the unaffected levels upon rhGALNS treatment.

A.



B.

	Unaffected -	Moderate -	Severe -	Unaffected +	Moderate +	Severe +
Unaffected -						
Moderate -	0.285					
Severe -	0.011	0.034				
Unaffected +	0.007					
Moderate +		0.001		0.593		
Severe +			0.001	0.397	0.615	

Figure 14. KS accumulation upon addition of Aggrecan and treatment with rhGALNS. Cells were cultured for 7 days with the addition of aggrecan and with or without rhGALNS. A) Using immunostaining, it was demonstrated that the MSCs can accumulate KS, and levels can be reduced upon treatment. The severe clone shows the highest accumulation and a 4-fold reduction upon treatment. Results shown as means ± SEM (N=6, $p<0.04$). B) All possible p-values were calculated for significance using a student's T-test. Green represents significant.

C1.4. The search for phenotypic differences

Using the technology offered by high content screening, an in-depth analysis of cells is possible. To confirm the growth rate hypothesis, the three clones were cultured for 3 days beginning with the same number of cells. After this time, brightfield imaging was done and cells counted. As previously shown, the severe clone has the least number of cells with normal and moderate clones close together. Next, clones were stained with phalloidin for a whole cell body stain and LAMP-1 for investigating lysosomal content (Figure 15A). These initial stainings demonstrate a possible difference in cell size, with the unaffected clone appearing smaller and thinner. Moderate clone appears slightly larger than the unaffected clone, and the severe clone is the largest. Furthermore, the moderate and severe clones show an increased filamentous phenotype as well as an increase in lysosomal content/size. Analysis of the LAMP-1 staining was

done by calculating the total area of granules per site (mean granule area) and confirmed the observation of increased lysosomal content in the severe clone, with the highest area compared to normal and moderate clones (Figure 15B). The analysis of LAMP-1 spot intensity also showed the highest mean in the severe clone, with moderate clone close behind. (Figure15C). These results indicate possible phenotypic differences amongst the three MSC clones, and it is of interest to investigate the filamentous composition of the cells as well as the lysosomal content. These observations could possibly change upon treatment with rhGALNS, especially the increased lysosomal content of the severe clone. Repeat and further experiments are necessary to further examine these phenotypes.

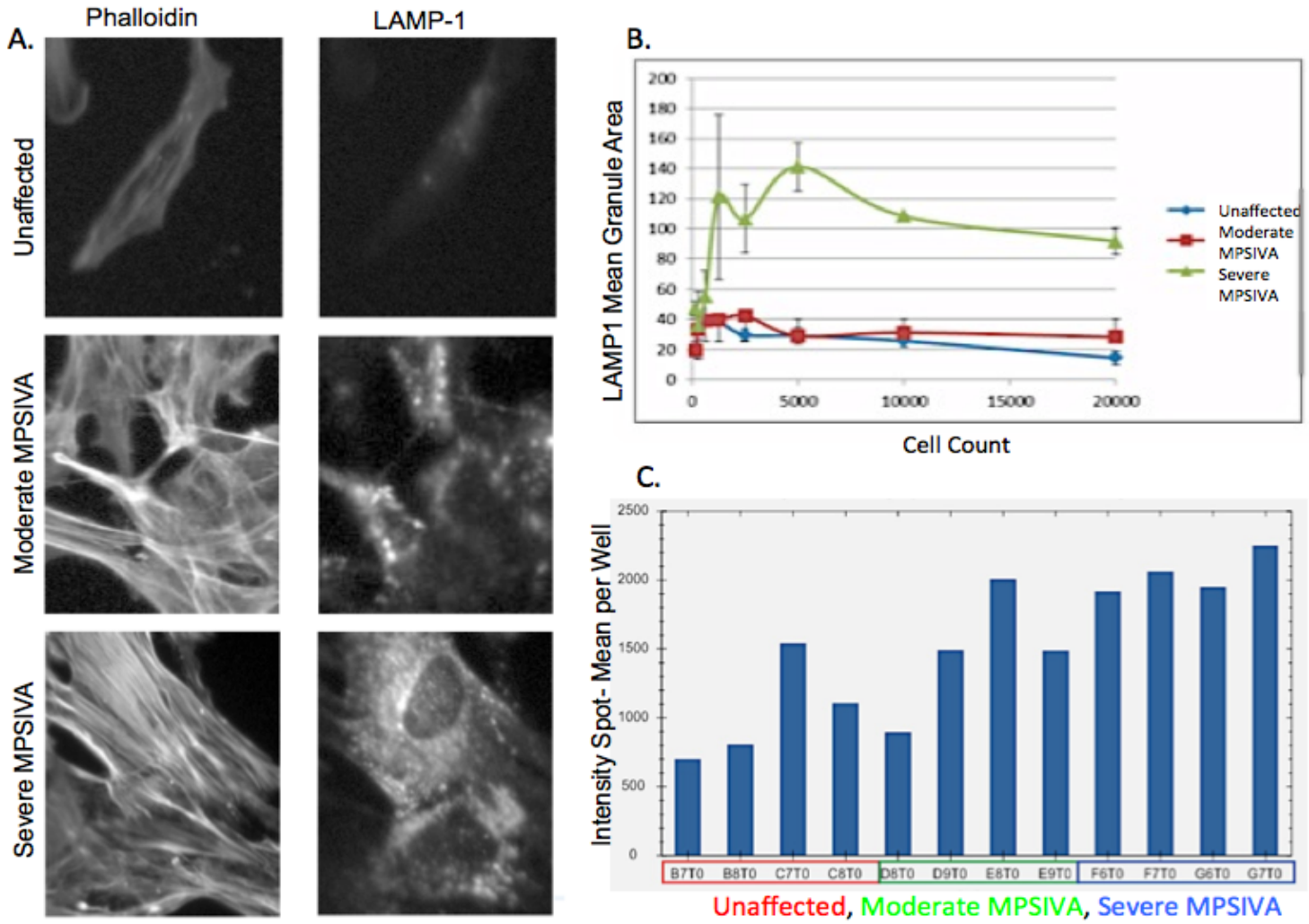


Figure 15. Phenotypic differences between the three MSC clones as analyzed by high content screening. A.) Whole cell body staining with Phalloidin for actin filaments and lysosomal content with LAMP-1 were investigated. Stainings show a possible difference in size, actin filaments, and lysosomal content between three clones. B.) Mean granule area analysis of LAMP-1 shows increased mean of the severe clone, suggesting increased lysosomal content/ size. C.) Spot intensity analysis using LAMP-1 antibody shows increased mean LAMP-1 in the severe clone as well.

C2. The chondrogenesis of MSC's

C2.1. Characterization and differences in clones

After the successful derivation of MSCs, a chondrogenic lineage can be explored through differentiation protocols followed by characterization. Using the pellet technique,

several rounds of differentiations were done under various conditions. A consistent protocol according to Section II-B2 was followed for all the differentiations to ensure accurate comparisons. The first differentiation was done with the unaffected clone for 25 days with harvest points every 5 days. No specific problems were encountered during this experiment, with well-formed pellets that remained consistent for the whole period. As shown in figure 16A, Col2 and aggrecan show a similar expression pattern where the levels peak at day 20 before decreasing once again. In comparison, Sox9 and Col10 expression is relatively low (Figure 16B, C). However, these two markers also show a similar expression pattern where the highest expression is seen on day 15, 5 days before Col2 and aggrecan. It is of interest to observe expression levels of markers at later time points of the differentiations, especially for Col2 and aggrecan, whose expression is still relatively high at 25 days. The expression of chondrogenic markers might change at day 30 in a positive of negative manner. Due to these initial results, this protocol seems promising compared to the direct chondrogenic differentiations previously attempted and described in Section II-A.

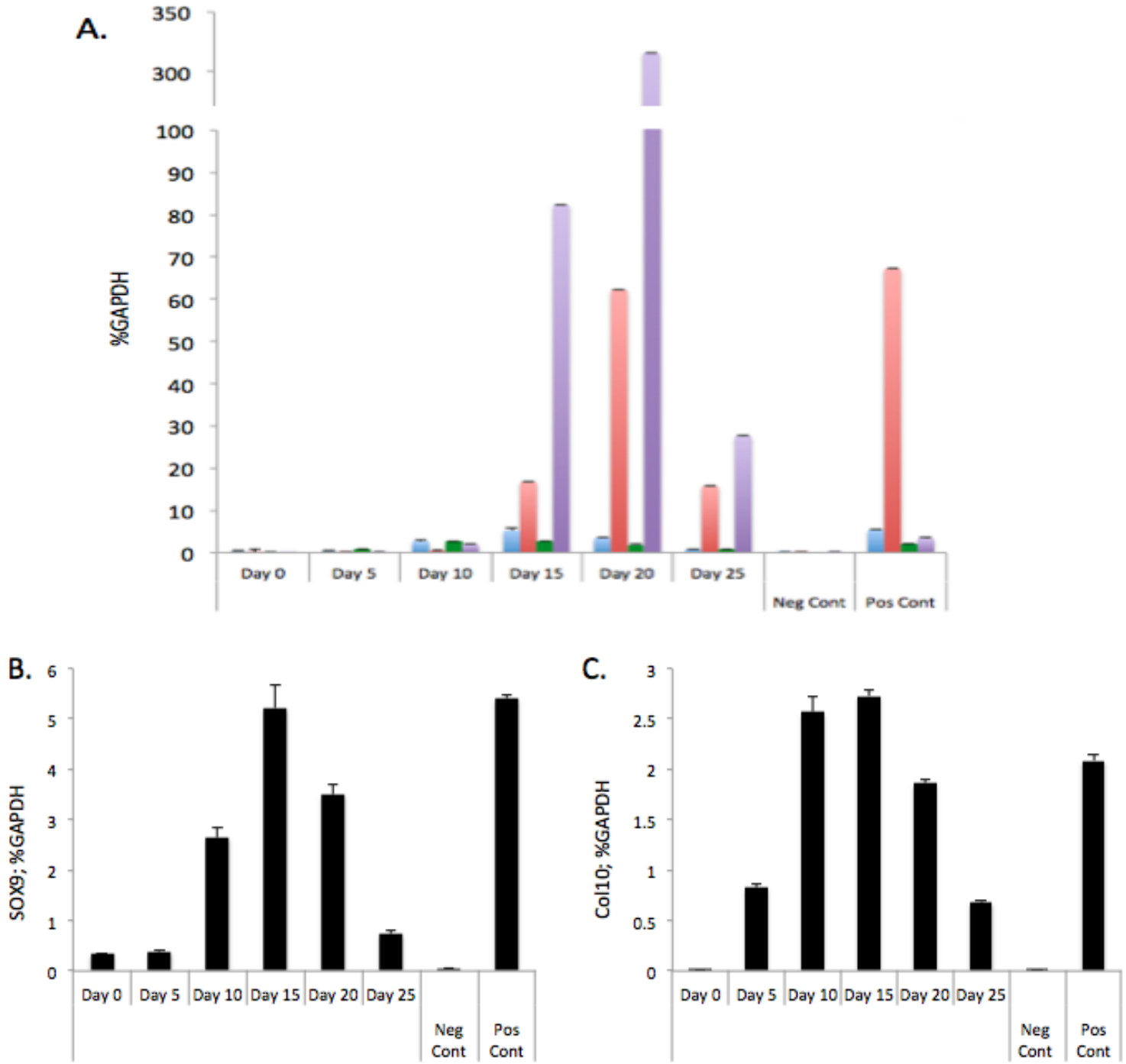


Figure 16. Gene expression analysis for chondrogenic markers of MSC-derived unaffected chondrocytes. 5 pellets were harvested at allotted time points and subjected to RNA extraction, cDNA synthesis and subsequent qRT-PCR. A.) Relative expression of chondrogenic markers at several time points of differentiation. B.) Sox9 expression of Normal pellets shows a bell-shaped curve peaking at day 15. C.) COL10 displays a similar bell-shape distribution than SOX9. Negative control are undifferentiated iPSCs and positive control are 3-week unaffected chondrocytes grown in alginate. Results were normalized to GAPDH and shown as means of triplicates ± SEM.

Ensuing these results, the same protocol was done with the moderate and severe clones to see if the expression of markers changes or if these clones will withstand the differentiation period. Initially, it was observed that the severe clone pellets were bigger after several days in culture, even though pellets begin with the same number of cells. Furthermore, the severe pellets were more flat whereas the moderate clone pellets remained round, although smaller in size. As the harvest time points progressed, the nucleic acid concentration of the moderate clone decreased each time, suggesting possible cell loss with smaller pellets. There was an overall high expression of Col10 in both clones, and the expression pattern was similar to Sox9 (Figure 17A). However, Col2 expression was low compared to positive control and there was little to no aggrecan expression observed, especially in the severe clone. For the moderate clone, the expression pattern of aggrecan and Col2 was equivalent. There was no aggrecan in severe clone, though it had higher overall expression of Col2 (Figures 17B, C).

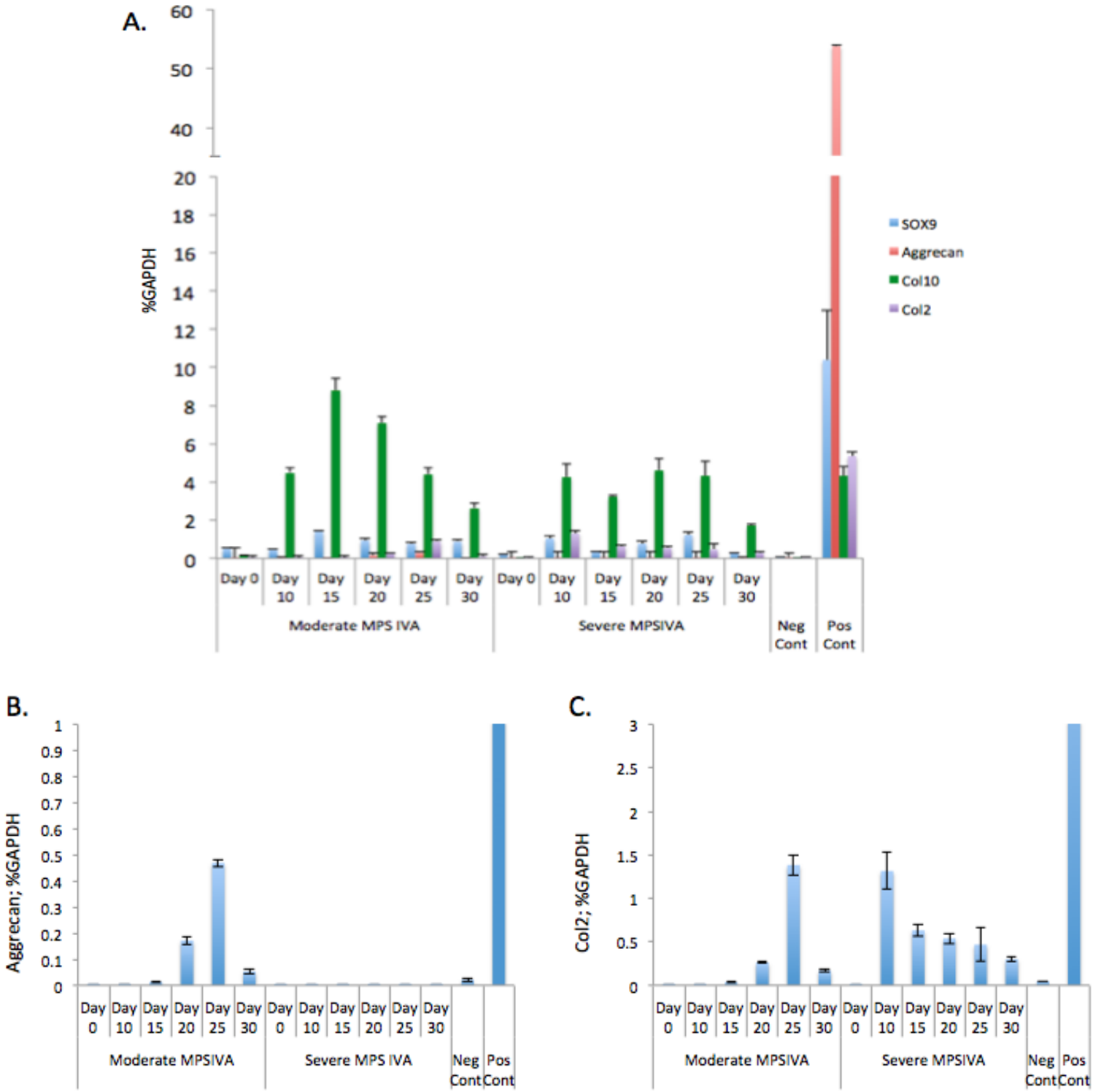


Figure 17. Gene expression analysis for chondrogenic markers of moderate and severe MPS IVA clones. A.) Relative expression of chondrogenic markers at several time points of differentiation. B.) Aggrecan expression is low in both clones compared to the positive control, especially in the severe clone. C.) Expression of Col2 is also low for both compared to positive control, with a slightly higher expression of severe clone peaking at day 10 and progressively decreasing. Negative control are undifferentiated iPSCs and positive control are 3-week unaffected chondrocytes grown in alginate. Results were normalized to GAPDH and shown as means of triplicates ± SEM.

After these rounds of differentiations, some morphologic observations were made in the pellets that portrayed a similar pattern in each case. There were several apparent differences between the three clones in the pellet form. As shown in figure 18, the size of the pellets vary, with severe clone being the biggest and moderate clone the smallest. Furthermore, the severe pellets seem to flatten out more as time progressed. There was also some dissociation observed at times, especially in the moderate and severe clones when compared to unaffected. Some experiments were done to test the effect of hypoxic conditions on the clones' differentiation. Hypoxic conditions are described as increasing the expression of chondrogenic markers by mimicking the poorly vascularized growth plate (Recall Figure 4). A serum-free media and a special chondrocyte commercially available media were also tested to try to ameliorate the chondrocyte differentiation. These 3 new conditions showed similar results to the first presented differentiation and no specific trend, rendering our first protocol adequate for further attempts at differentiations.

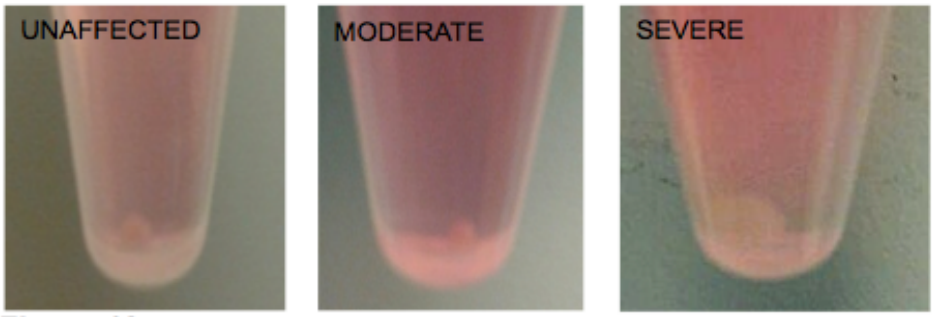


Figure 18. Pellet culture technique for the differentiation of chondrocytes. $1X10^6$ cells are cultured in a 15-mL canonical tube overnight, allowing pellet formation. Several differences observed in clones, primarily size of pellet. Severe clone formed bigger pellets that flattened out with the progression of differentiation period.

Treatment with rhGALNS

The next step taken was to see if these results would be altered upon treatment with rhGALNS, especially for the expression of genes characteristic of chondrocyte differentiations. Harvests and subsequent follow-up experiments were done separately for each clone to avoid confusion. Cells were treated or not with rhGALNS as in the preceding experiments. The unaffected clone behaved well through differentiation, but the cell pellets did decrease in size and there was some slight dissociation observed. As shown in figure 19A, these pellets had an overall positive expression of Col2, which was slightly lower in treated group. Col2 levels maintained a bell-shaped curve in the non-treated group whereas the treated group had variable Col2 expression (Figure 19B). There appeared to be a correlation in expression of Col2 and aggrecan as they increase on the same days as noticed already with the other two clones. Furthermore, aggrecan levels steadily increased, and no major difference is obvious between the two treatment groups (Figure 19C). Expression of Sox9 and Col10 was very low in both groups. In comparison with the preceding experience whereas COL10 was giving the highest signal.

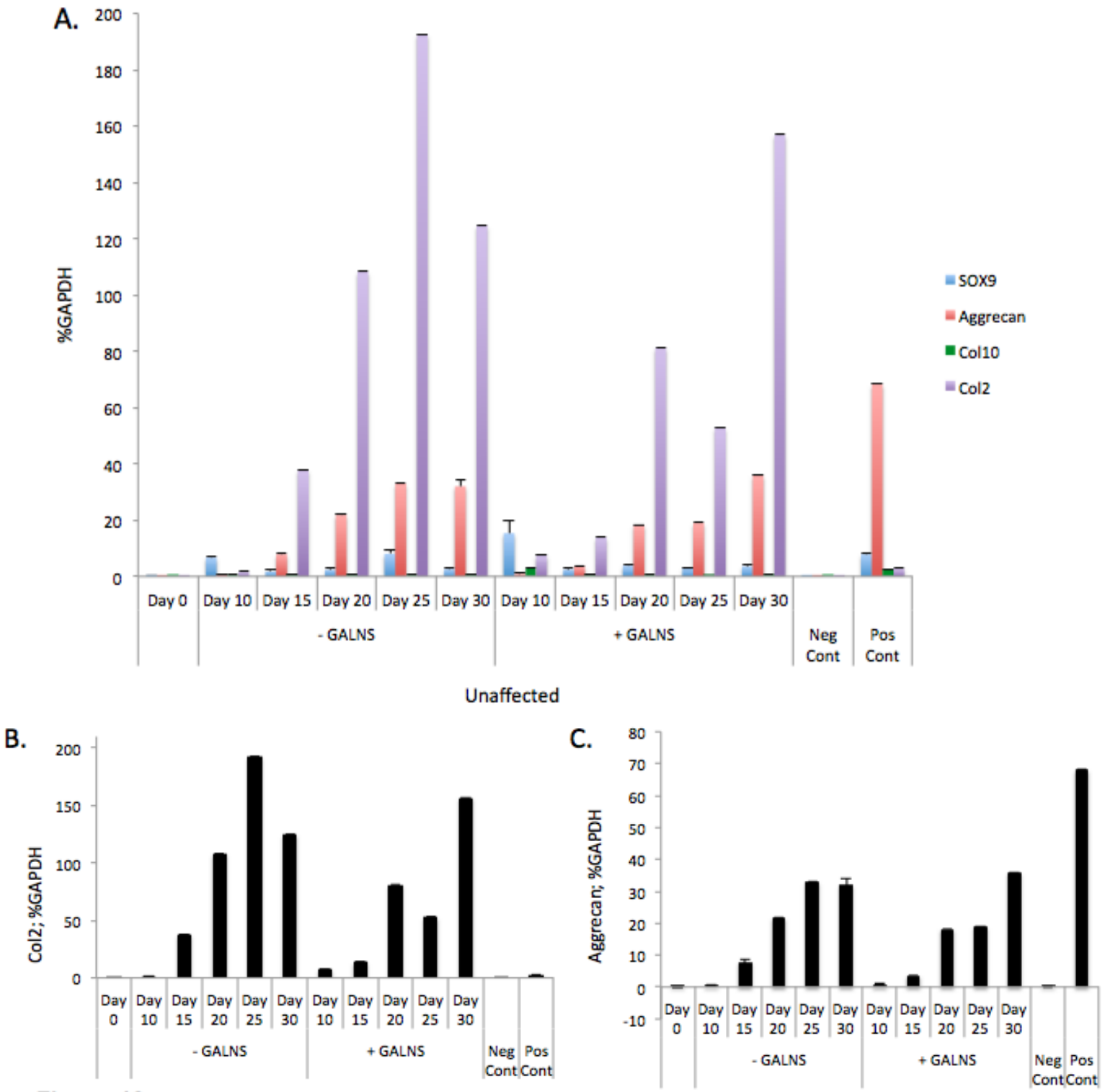


Figure 19. Gene expression analysis for chondrogenic markers of unaffected clone upon treatment with rhGALNS at 10nM. A.) Relative expression of chondrogenic markers at several time points of differentiation in two treatment groups. Overall high expression of Col2 is observed and little to no Sox9 and Col10. B.) Col2 expression steadily increases and then decreases in non-treated group. C.) Aggrecan levels steadily increased in both groups. Negative control are undifferentiated iPSCs and positive control are 3-week unaffected chondrocytes grown in alginate. Results were normalized to GAPDH and shown as means of triplicates ± SEM.

The moderate clone pellets saw a challenging differentiation period in which dissociation occurred often and pellets greatly decreased in size. Harvests had to be done carefully and RNA extraction was not that difficult since remaining pellet pieces were not very firm. The qRT-PCR results were especially interesting for this clone. As can be seen in figure 20A, Col10 expression was relatively high in both groups compared to the other markers, but the levels of Col10 were markedly reduced upon treatment with rhGALNS. Also, Sox9 expression was low in both groups. Interestingly, Col2 and Aggrecan saw a dramatic increase in expression upon treatment at day 30 (Figures 20B, C). This was especially the case for Col2, which saw a 14-fold increase upon treatment. Aggrecan saw a 8-fold increase in expression as well. These observations were not apparent before day 30.

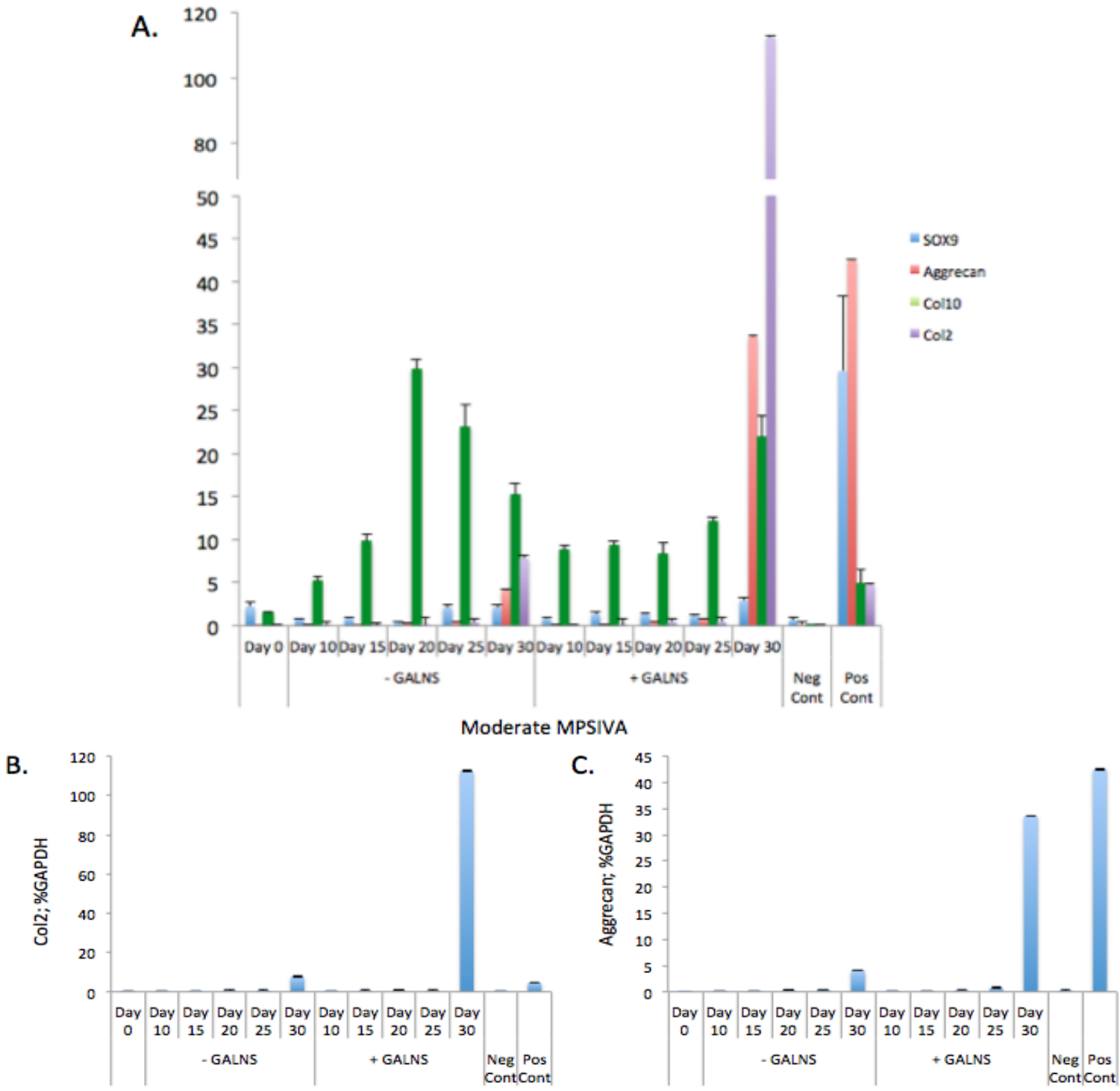


Figure 20. Gene expression analysis for chondrogenic markers of moderate MPSIVA clone upon treatment with rhGALNS at 10nM. A.) Relative expression of chondrogenic markers at several time points of differentiation in two treatment groups. Col10 expression is relatively high in both groups, but decreased upon treatment. Little to no Sox9 expression s observed and no differences in expression detected between two groups. B.) Col2 expression very low up until day 30, where expression is drastically rescued with treatment. C.) Aggrecan levels very low as well until day 30, where same rescue is observed upon treatment. Repeated qRT-PCR for this clone showed same results (not shown). Negative control are undifferentiated iPSCs and positive control are 3-week unaffected chondrocytes grown in alginate. Results were normalized to GAPDH and shown as means of triplicates ± SEM.

Lastly, the severe clone saw an uneventful differentiation period in which pellets remained relatively big and only a slight dissociation was observed. Once again, Col10 expression was relatively high with a decrease upon treatment, a similar observation was made with the moderate clone (Figure 21A). Sox9 expression was higher in this clone, closely matching the pattern of Col10 expression. Interestingly, there was also a slight reduction in Sox9 expression with treatment of rhGALNS as well. In comparison, aggrecan and Col2 expression was very low. As shown in Figure 21B, Col2 expression has no specific pattern but still quite similar to aggrecan. What is different with this marker is that there is slight expression even at day 0, and expression level is decreased at day 25 upon treatment. Aggrecan is also very low, though it increases a bit as experiment progresses (Figure 21C). However, the levels are not significant and there is no difference between treatment groups.

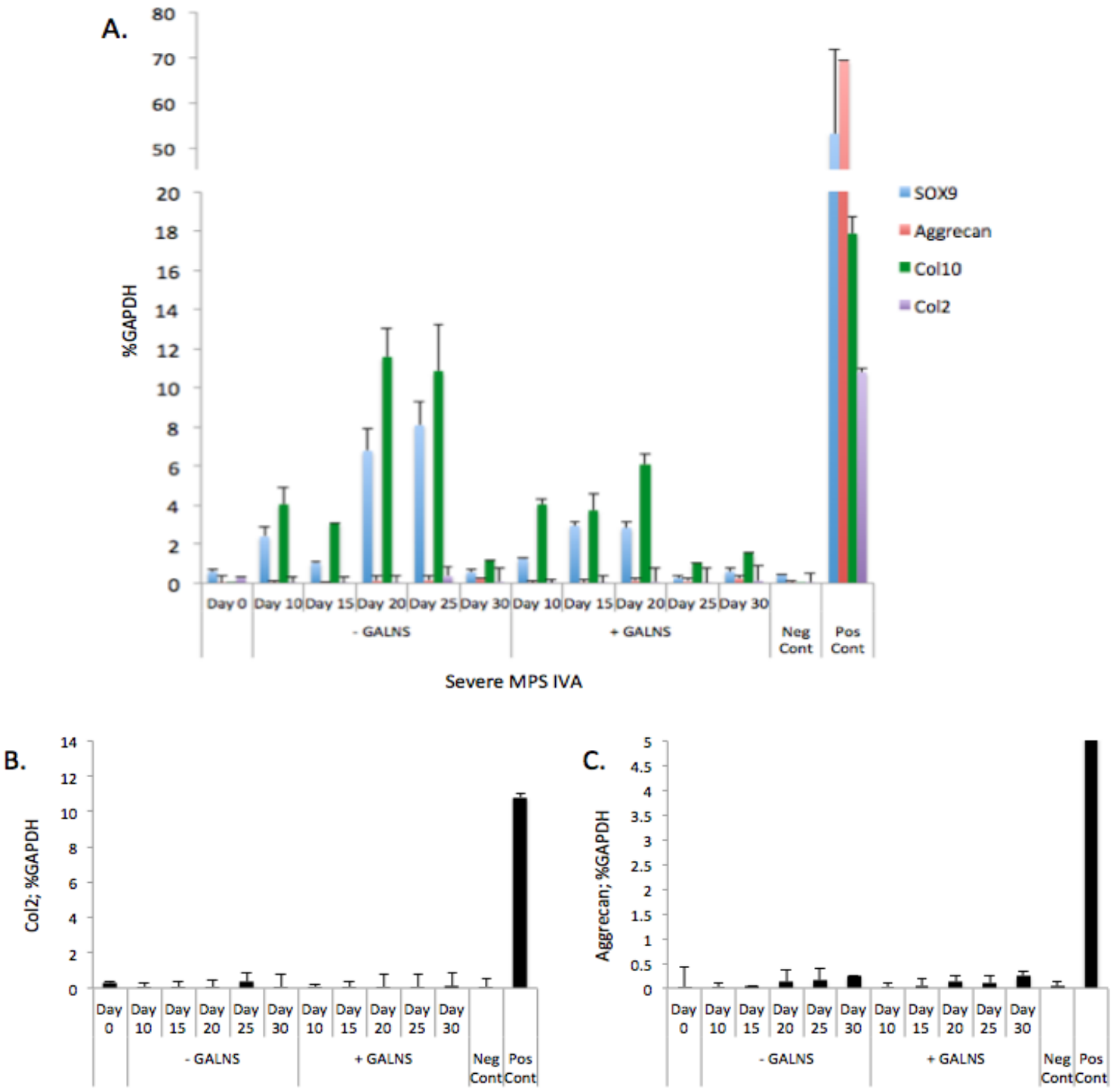


Figure 21. Gene expression analysis for chondrogenic markers of severe MPSIVA clone upon treatment with rhGALNS at 10nM. A.) Relative expression of chondrogenic markers at several time points of differentiation in two treatment groups. Col10 expression is also relatively high in both groups, but decreased slightly upon treatment. Higher Sox9 expression is observed in clone, with levels mimicking the pattern of Col10. A reduction in Sox9 is also observed upon treatment. B.) Col2 expression is very low and shows no specific pattern. C.) Aggrecan levels are also very low. Repeated qRT-PCR for this clone showed same results. Negative control are undifferentiated iPSCs and positive control are 3-week unaffected chondrocytes grown in alginate. Results were normalized to GAPDH and shown as means of triplicates ± SEM.

An additional five pellets were cultured for the duration of the differentiation with the addition of aggrecan, to see if cells could accumulate aggrecan since we have not been able to see a steady expression of it. These five pellets were prepared for capillary electrophoresis analysis, a technique used to analyze GAGs, for keratan sulfate and chondroitin sulfate. As shown in figure 22, there is no detectable KS in any sample. However, there are detectable peaks with the Chondroitinase digestion, especially in the severe clone. In the severe clone, it appears that the peaks are reduced upon treatment with GALNS. Furthermore, calculations of peaks show there is a 20% reduction for the moderate clone and a 60% reduction of the severe clone upon treatment. These results are promising but may be due to treatment with Aggrecan. This experiment was not possible for non-aggrecan-treated pellets since there were no remaining pellets after harvests.

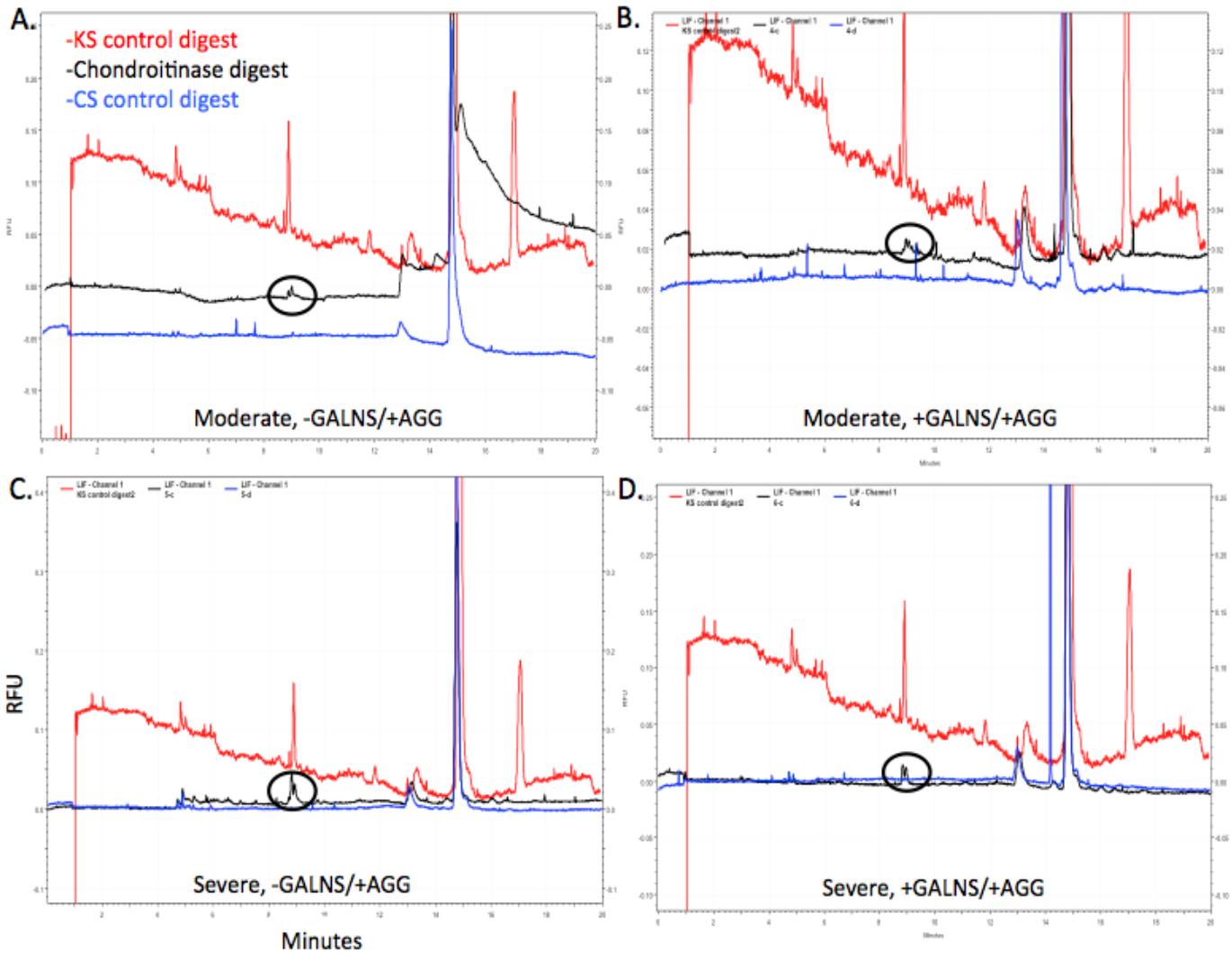


Figure 22. Capillary electrophoresis of keratanase II-digested and chondroitinase digested cell pellet lysates. Pellets were cultured for 30 days with or without rhGALNS and spiked with Aggrecan (10ug/mL). The internal standard spiked into all samples migrates at about 15 minutes. The two keratan digest peaks should migrate at about 8.6 and 17 minutes. There is no detectable KS in any samples. The chondroitinase digestion is a doublet that is migrating at about 8.5 minutes, containing two disaccharides. There are detectable peaks of CS (circled) that appear to shrink in GALNS treated groups. A.) Moderate pellets peaks at the expected time for CS, also observed in treated group B.) The treated moderate pellets see a 20% reduction in CS. C.) In the severe clone, the CS peak is higher. D.) The treated severe pellets see a 60% reduction.

IV. Discussion & Conclusion

With the derived iPSCs, an in-depth study of the pathogenesis of MPS IVA is possible. The reprogramming of patient-specific fibroblasts was successful and shows

positive expression of pluripotent markers. Thus these iPSCs were used for the further differentiation of specific cell types, such as chondrocytes, which are extremely important for studies on Morquio syndrome and which we attempt to differentiate in this study. The iPSC lines displayed observed differences between them, such as slower growth and bigger cell size for the clone representing the severe phenotype. However, maintenance of iPSCs is difficult and requires extensive time commitment.

The direct chondrocytic differentiation of iPSCs was not successful as attested by the absence of significative chondrogenic marker expression. The cell pellets obtained using the micromass or pellet techniques dissociated or flattened out during culture, an issue likely due to iPSC properties or culture conditions. Gene expression analysis showed that Sox9, the main transcription factor regulating chondrogenesis, and Col2, a gene characteristic of chondrogenic differentiation as the basis for articular cartilage, were poorly expressed, suggesting failure to initiate the correct conditions for chondrogenesis. Furthermore, aggrecan, an important proteoglycan of the chondrocyte extracellular matrix, was not detected. Aggrecan contains many KS chains, rendering it as a main source of KS accumulation in MPSIVA. It was apparent through the various attempts of differentiation, however, that chondrogenic induction in improved upon supplementation of TGFβ3 instead of TGFβ1. This information was critical for chondregenic differentiations using MSCs.

As a second option for deriving chondrocytes, we used an indirect approach requiring the establishment of a mesenchymal stem cell line first. Various protocols were used, the 1) EB-outgrowth, 2) Low density, and 3) Oldershaw that resulted in different outcomes. The selected iPSC-derived MSC clones express MSC-specific markers as

seen through flow cytometric analysis, gene expression analysis, and immunostaining, confirming its lineage and ability of further differentiation. The culture maintenance of MSC's also showed some differences amongst clones, primarily in the growth rate and in the cell size. These observations lead to a phenotypic investigation of the three clones with live cell assays and high content screening. It was confirmed that the severe clone does grow the slowest, which could be a phenotype directly related to disease. Other experiments showed the severe clone has bigger cell size and increased lysosomal content or size as well. These results are promising as they show possible phenotypic differences of the MPSIVA clones. Cells were also subjected to experiments testing the therapeutic efficiency of rhGALNS to see if the treatment could restore levels to the unaffected phenotype. Furthermore, it was suggested that the growth rate could be rescued upon treatment with rhGALNS, but additional experiments would be necessary to confirm. Immunostaining of treated cells showed colocalization of rhGALNS to the lysosomes, and levels were decreased to that of the unaffected clone upon treatment. This suggests a functional in vitro bioassay that can be used for rhGALNS with the MSCs.

Once the mesenchymal lineage of each clone was confirmed, they were expanded and banked. Several protocols for chondrocyte differentiations were tried. Initial differentiations done using the pellet technique with the normal clone showed a significant and similar expression pattern of Col2 and aggrecan, suggesting chondrogenesis was reached. The same experiment with the two disease clones, however, showed opposite results. Expression of Col10 was the highest in this case, suggesting hypertrophic chondrocytes that may be leaning towards endochondral

ossification. Upon treatment with rhGALNS, expression of aggrecan and Col2 was greatly recovered in the moderate clone only. Furthermore, expression of Col10 was reduced upon treatment. These results indicate possible therapeutic benefits to the directed differentiation of chondrocytes. It can be hypothesized that perhaps the severe clone would need a long-term treatment with rhGALNS to see similar results observed of the moderate clone. Capillary electrophoresis analysis for KS and CS shows only an accumulation of CS, which appears to be reduced with rhGALNS, especially in the severe clone. This experiment should be repeated for confirmation of these interesting results.

Further work includes follow-up, in-depth experiments to detect phenotypic differences amongst clones, with focus on filamentous composition and lysosomal accumulation. Such experiments should also be attempted upon treatment with rhGALNS. Also, cell-based assays can be developed with the MSCs, such as the effects of KS accumulation in lysosomes, gene expression, and therapeutic efficiency. Because the chondrogenic markers were not expressed equally in each round of differentiation, this protocol needs to be further optimized or other techniques should be investigated. Lastly, the established MPSIVA iPSCs can be used to differentiate other relevant cell types to continue research. With acquired or established cell types, identification and exploration of cellular pathways involved in MPSIVA is possible.

Our results indicate that MPSIVA cell models by using iPSCs and MSCs are attainable and could help us expand our knowledge of this disease. This study has opened a plethora of possibilities and also a lot of questions. A disease model was

established and a bioassay for rhGALNS using these cells introduced. Using the iPSC and MSC lines, a promising tool for *in vitro* studies of MPSIVA is available.

V. References

Anderson C.E., Crane J.T., Harper H.A., Hunter T.W. (1962). Morquio's disease and dysplasia epiphysalis multiplex. A study of epiphyseal cartilage in seven cases. J Bone Joint Surg Am 44-A, 295–306.

Asakura A, Komaki M, Rudnicki M. (2001). Muscle satellite cells are multi- potential stem cells that exhibit myogenic, osteogenic, and adipo- genic differentiation. Differentiation. 68:245–253.

Bailey, L., et al. (2008). An Overview of Enzyme Replacement Therapy for Lysosomal Storage Diseases. *OJIN: The Online Journal of Issues in Nursing* Vol. 13, No. 1, Manuscript 3.

Beck, M. (2001). Variable clinical presentation in lysosomal storage disorders. J Inherit Metab Dis 24 Suppl 2: 47–51; discussion 45–46.

Bielecki, J., Fuller, M., Xiao-Hui, G., et al. (1995). Expression, purification characterization of recombinant human N-acetylgalactosamine-6-sulphatase. Journal of Biochemistry*. 311, 333-339.*

BioMarin Pharmaceutical Inc. (2012). BioMarin Phase 3 Study of GALNS for the Treatment of MPS IVA Meets Primary Endpoint [Press Release]. Retrieved from http://investors.bmrn.com/releasedetail.cfm?ReleaseID=718462

Choi, K., Vodyanik, M., Slukvin, I. (2011). Hematopoietic differentiation and production of mature myeloid cells from human pluripotent stem cells. Nature America 6(3), 296-313.

Crisan M, Yap S, Casteilla L, et al. (2003). A perivascular origin for mesenchymal stem cells in multiple human organs. Cell Stem Cell 2008; 3(3):301-13.

De Franceschi L, Roseti L, Desando G, Facchini A, Grigolo B (2007). A molecular and histological characterization of cartilage from patients with Morquio syndrome. Osteoarthritis Cartilage 15: 1311–1317.

Dvorak-Ewell, M., Richard, M., Vellard, M. (2008). Immortalization of human MPS IVA chondrocytes: Development of a new tool to study pathophysiology and assess therapeutic strategies for MPS IVA [Abstract]. Molecular Genetics and Metabolism. 99(2), S16-S17.

Factor, S.M., Biempica, L,. Goldfischer, S. (1978). Coronary intimal sclerosis in Morquio's syndrome. Virchows Arch A Pathol Anat Histol 379, 1–10.

Gosset M, Berenbaum F, Thirion S, Jacques C (2008) Primary culture and phenotyping of murine chondrocytes. Nat Protoc 3: 1253–1260

Hendriksz, C., Al-Jawad, M., Berger, K., et al. (2012). Clinical overview and treatment options for non-skeletal manifestations of mucopolysaccharidosis type IVA. J Inherit Metab Dis. DOI 10.1007/s10545-012-9459-0.

Hollister DW, Cohen AH, Rimoin DL, Silberberg R (1975) The Morquio syndrome (mucopolysaccharidosis IV): Morphologic and biochemical studies. Johns Hopkins Med J 137: 176–183.

Lanza, R. (2009). *Esssentials of Stem Cell Biology*. Location: Elsevier

Mahmood, A., Harkness, L., et al. (2010). Enhanced differentiation of human embryonic stem cells to mesenchymal progenitors by inhibition of TGF-β/Activin/Nodal signaling using SB-431542. Journal of Bone and Mineral Research. 25(6),1216-1233.

Masue M, Sukegawa K, Orii T, Hashimoto T (1991) N-acetylgalactosamine-6- sulfate sulfatase in human placenta: purification and characteristics. J Biochem 110: 965–970.

McClure, J., Smith, P.S., Sorby-Adams, G., Hopwood, J. (1986). The histological and ultrastructural features of the epiphyseal plate in Morquio type A syndrome (mucopolysaccharidosis type IVA). Pathology. 18, 217–221.

Montaño, A.M., Tomatsu, S., Gottesman, G.S., Smith, M. and Orii, T. (2007). International Morquio A Registry: clinical manifestation and natural course of Morquio A disease. J. Inherit. Metab. Dis., 30, 165 – 174.

Montaño A.M., Goldim, M., Satta, Y., Takahata, N., Tomatsu, S. (2008). Implications of absence of clinical phenotype on Morquio A mice: Why rodents do not require skeletal keratan sulfate? Philadelphia, Pennsylvania.

Nikitovic, D., Katonis, P., Tsatsakis, A., et al. (2008). Lumican, a Small Leucine-rich Proteoglycan. IUBMB Life, 60(12): 818–823

Northover, H., Cowie, R.A., Wraith, J.E. (1996). Mucopolysaccharidosis type IVA (Morquio syndrome): a clinical review. J Inherit Metab Dis. 19, 357–365.

Oldershaw et al. (2010). Directed differentiation of human embryonic stem cells toward chondrocytes. Nat Biotech

Peters, C., Steward, C.G. (2003). Hematopoietic cell transplantation for inherited metabolic diseases: an overview of outcomes and practice guidelines. Bone Marrow Transplant. 31, 229–239.

Pungor, E., Hague, C.M., Chen, G., et al. (2009). Development of a functional bioassay for arylsulfatase B using the natural substrates of the enzyme. Analytical Biochemistry. 395, 144-250.

Stavroupoulos, M.E., Mengarelli, I., Barberi, T. (2009). Differentiation of multipotent mesenchymal precursors and skeletal myoblasts feom human embryonic stem cells. Curr Protoc Stem Cell Biol. 9:1F.8.1-1F.8.10

Takahashi, K. et al. (2007). Induction of pluripotent stem cells from adult human fibroblasts by defined factors. Cell. 131, 861–872.

Tomatsu, S., Orii, K.O., Vogler, C., Nakayama, J., Levy, B., et al. (2003). Mouse model of N-acetylgalactosamine-6-sulfate sulfatase deficiency (Galns-/-) produced by targeted disruption of the gene defective in Morquio A disease. Hum Mol Genet. 12, 3349–3358.

Tomatsu, S., Montaño, A.M., Ohashi, A., et al. (2008). Enzyme replacement in a murine model of Morquio A Syndrome. Human Molecular Genetics. 17(6), 815-824.

U.S. Department of Health and Human Services, ICH Q6B Specifications: Test Procedures and Acceptance Criteria for Biotechnological/Biological Products, U.S. Department of Health and Human Services, Food and Drug Administration, Center for Drug Evaluation and Research, Center for Biologics Evaluation and Research, 1999.

Venn, G. and Mason, R.M. (1985). Absence of keratan sulphate from skeletal tissues of mouse and rat. Biochem. J. 228, 443–450.

Vitale, A.M., Wolvetang, E., Mackay-Sim, A. (2011). Induced pluripotent stem cells: a new technology to study human diseases. The International Journal of Biochemistry & Cell Biology. 43: r5444843-846

Vodyanik, M., Bork, J., Thomson, J, Slukvin, I. (2005). Human embryonic stem cell-derived CD34+ cells: efficient production in the coculture with OP9 stromal cells and analysis of lymphohematopoietic potential. Blood Journal. 105(2),617-626.

Yamanaka, S. (2009).Elite and stochastic models for induced pluripotent stem cell genera- tion. Nature. 460,49–52.

Yu, J. et al. (2007). Induced pluripotent stem cell lines derived from human somatic cells. Science. 318, 1917–1920.