

Articular Mesenchymal Stem Cell (MSC) and Effects of Growth Differentiation Factor-5 (GDF-5)

An Internship Report Presented to
the Faculty of the Master of Science in Biotechnology and Bioinformatics
and Master of Business Administration Programs
California State University Channel Islands

By

YueJia Wu

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Dr. Ching-Hua Wang

10/25/2010

Date

APPROVED BY THE MVS SCHOOL OF BUSINESS AND ECONOMICS



Dr. William P. Cordeiro

10-25-10

Date

APPROVED BY THE UNIVERSITY



Dr. Gary A. Berg

10-25-10

Date

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Abstract

Objectives.

1. To determine the presence of mesenchymal stem cells (MSC) in rat embryo limb buds and adult rat knee joints.
2. To determine the pharmacological effects of recombinant human GDF-5 (rhGDF-5) on rat MSCs.
3. To determine the effect of rhGDF-5 on load bearing in a rodent model of osteoarthritis.

Methods. Fluorescent-Activated Cell Sorting (FACS) was used to identify and purify MSCs from E18 rat embryo and adult rat knee cartilage. Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR) was performed to assess the RNA profile of MSCs. qRT-PCR was specifically used to quantify the expression of chondrogenesis markers including Aggrecan (Agc), Type II collagen (Col2), and Sox9 and osteogenesis markers including Runx2 and Type I collagen. An Alamar Blue (AB) assay was utilized to assess effects of rhGDF-5 on MSC proliferation. An Alkaline Phosphatase (ALP) assay was used to study the effects of rhGDF-5 on MSC differentiation. Neuroprobe migration chambers were used to assess rhGDF-5 effects on chondrocyte chemotaxis. In situ hybridization (ISH) was conducted to identify MSC (expression of chondrogenic RNA biomarkers) in rat embryos and in adult knee joints from normal and osteoarthritic rats . Differential weight bearing analysis was assessed using Bioseb's DWB system.

Results. MSCs were obtained from E18 rat embryo limb buds and adult rat knee joint cartilage. qRT-PCR showed that MSC comprised pre-osteochondrocytes expressing *Agc*, *Col2a1*, *Sox9*, and *GDF-5* RNAs. rhGDF-5 treated MSC showed a dose-dependent increase in AB intensity from Day 1 to Day 14, followed by an increase in rhGDF-5 mediated ALP activity on Day 14 and Day 21. The increased ALP activity coincided with increased *Agc*, *Col2a1*, *Sox9*, and *GDF-5* RNA expression indicating that rhGDF-5 selectively promoted chondrogenesis. Greater chemotaxis effect of rhGDF-5 was observed with MSC that presumably were more differentiated after 23 days in culture with rhGDF-5. Following 23 days of treatment with rhGDF-5, MSC appeared as condensations and precartilaginous structures. ISH confirmed the presence of *GDF-5*, *Sox9*, and *Runx2* in rat embryo hind limbs and in the experimentally-induced osteoarthritic joint of adult animals. DWB analysis revealed significant differential joint loading in the Medial Meniscus Tear (MMT) injured knee, but no reverse differential weight bearing was detected in rhGDF-5 treated rats after a 9-week treatment period.

Conclusion. Mesenchymal stem cells are present in rat embryonic limb buds and adult rat knee joint. Our studies show that rhGDF-5 may promote an increase in metabolic activity of MSCs prior to commitment to the chondrocyte lineage. The positive chemotactic effect of *GDF-5* suggests a role in MSC cell homing, cell condensation, and formation of pre-cartilaginous structures. Taken together, *GDF-5* is a selective chondrocyte differentiation factor. The presence of MSC in the damaged joint suggests that rhGDF-5 may act to differentiate endogenous MSC to chondrocytes.

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Introduction

Osteoarthritis (OA) is one of the most common forms of arthritic disease, affecting 27 million people in the United States. OA patients suffer from impaired movement and chronic pain. OA associates with progressive death of chondrocytes and destruction of smooth articular surface. Articular cartilage is a unique connective tissue that provides weight bearing capacity to allow smooth joint movement. However, due to the lack of blood supplies and scattered chondrocytes in normal joint tissue, articular cartilage has poor regenerative mechanisms once injured.

Since clinical application of chondrocyte-based therapy may be limited due to insufficient numbers of autologous chondrocytes, mesenchymal stem cells (MSC) may represent a promising cell population for cartilage regeneration. Previous research has indicated that MSC can self-renew despite an individual's age and maintain the ability to differentiate into chondrocytes, osteocytes, and adipocytes (De Bari, 2001). Therefore, understanding the molecular mechanisms involved in MSC differentiation and proliferation may provide clues for developing potential OA treatment.

Several molecules in the transforming growth factor beta (TGFP) super family are key regulators in skeletal development including bone morphogenic proteins (BMP) and growth differentiation factors (GDF) (Ducy and Karsenty, 2000; Coleman and Tuan, 2003; Bai et al., 2004). Growth differentiation factor-5 (GDF-5, also known as cartilage-derived morphogenic protein-1 (CDMP-1), may be of importance for understanding cartilage maintenance and repair. Together with other polypeptide growth factors, GDF-5 actively participates in early embryonic skeletal development (Storm, 1999). GDF-5 is

predominantly expressed at the joint interzone where condensation is observed (Tsumaki, 1999). The autosomal recessive syndromes, brachypod (bp) in mice and Hunter-Thompson (CHTT) in human are caused by the missense mutation in GDF-5 which leads to the loss of the GDF-5 function (Miyamoto, 2007). These syndromes are characterized by the shortening of skeletal elements and loss of joints such as missing finger phalanges in severe cases (Francis-West, et al., 1999; Thomas et al., 1997; Seemann et al., 2005). Francis-West (1999) and Buxton (2001) also showed that overexpressing GDF-5 would result in increased skeletal elements but not another joint formation. These findings implicate GDF-5's critical role in limb development and cartilage formation.

In the present study, we investigated the presence and distribution of MSCs in rat embryo limb buds and adult rat knee joints using Flow Cytometry (FC) and In Situ Hybridization (ISH). We studied the pharmacological effect of recombinant human GDF-5 (rhGDF-5) in E18 rat embryo MSC using Alkaline Phosphatase (ALP), Alamar Blue (AB), Quantitative Real-Time Polymerase Chain Reaction (qPCR), and cell migration assay in response to the rhGDF-5 gradient. Our findings suggested that rhGDF-5 induces MSC proliferation and chondrocytic differentiation *in vitro*. It also showed cell homing effects to attract differentiating MSC to migrate toward its gradient *in vitro*. With the *in vitro* results, we planned and conducted a 17-week animal functional experiment to study the effects of rhGDF-5 in an adult rat MMT model.

Materials and Methods

Cell Culture

Mesenchymal stem cells (MSC) were harvested from E18 rat embryo limb buds. Both forelimbs and hindlimbs were severed from the body with scissors and forceps. The collected limb buds were temporarily stored in a 50 ml conical tube (with 50 ml Dulbecco's Phosphate Buffered Saline, DPBS) on ice then treated with 0.025% Trypsin for enzyme digest cell isolation. The isolated cells were cultured in a T175 flask with growth media (Ham's F12 + 10% Fetal Bovine Serum) in an incubator (37°C with 5% CO₂ and humidified air). Cultured cells have a doubling time of 18 hours.

Flow Cytometry (Single staining)

Cells were lifted from the T175 flask with the non-enzymatic dissociation buffer and counted. These cells were diluted to 100,000 cells/ml and transferred to FACS tubes in a staining buffer (PBS + 0.5% BSA). The CD29, CD44, CD71, CD90, CD34, and CD45 conjugated antibodies or their specific isotype controls were added to separate FACS tubes. The cells were immunostained for 30 minutes on ice followed by two washes with a staining buffer. Cells were analyzed to determine the antibody expression for MSC identification (BD FACSCalibur).

Fluorescence-Activated Cell Sorting (FACS) (Double staining)

FACS analysis was used to purify the CD44⁺/CD90⁺ cell population from others (BD FACSAria III). Similar to the FC single staining, cells were lifted and diluted to 100,000 cells/ml and transferred to 4 specific FACS tubes to prepare for machine calibration: 100,000 cells were stained with the CD44 antibody, 100,000 cells with the

CD90 antibody, 100,000 with the CD44/CD90 isotype control, and 100,000 cells with no staining. In addition, 500,000 cells were stained with CD44 and CD90 for cell sorting.

The purified cell populations were cultured for further investigation.

Antibody Selection

CD29 antibody and isotype (BD: 555005; 553960)

CD44 antibody and isotype (BD: 550974; 550056)

CD71 antibody and isotype (BD: 554891; 553930)

CD90 antibody and isotype (BD: 554898; 550617)

CD106 antibody and isotype (BD: 559229; 553972)

Negative control:

CD34 antibody and isotype (Invitrogen: CD34-581-04; MG104)

CD45 antibody and isotype (BD: 554877; 553971)

Quantitative Real-Time PCR

RNA samples were collected and purified following the RNeasy protocol by solubilizing the cells with a QiaShredder and preparing total RNA with an RNeasy mini spin kit (Qiagen). The concentration and quality of the RNA samples were determined by the Nanodrop spectrophotometer (Thermo Scientific). All test RNA samples were reconstituted in 1µg/50µl and treated with DNase (New England Biolabs) on a hotplate at 37°C for 30 minutes. of 250mM EDTA was added to stop the DNase treatment.

Specific primers and oligonucleotides were designed to detect the expression of aggrecan (Agc), type II collagen (Col2), growth differentiation factor-5 (GDF-5), Sox9, Runx2, type I collagen (Col1), and hprt. The Applied Biosystems 7900HT FAST Real-Time

PCR system was used for the qPCR experiment, and the data were analyzed with ABI Prism SDS software.

Table 1: Primers and Oligonucleotides

	forward primer	reverse primer	oligonucleotide
AGC	5-GGG TCA GGA GAA ACA GAG TCA-3'	5-AAG TGT CCA AGG CAT CCA C-3'	<6FAM>AA GCC CTT GTC TGA ATG GAG CCA C<TAM>
COL2	5-AGA GCA AGG AGA AGA AGC ACA-3'	5-GGA CAG TAG ACG GAG GAA AGT C-3'	<6FAM>TC TGG TTT GGA GAG ACC ATG AAC GG<TAM>
GDF5	5-TCC TTC TCC AAG GCA CTG AT-3'	5-CCA ACA CCA TCA CCA GCT TT-3'	<6FAM>CT GTG GTC AGG AAG CAG AGG TAC GTG <TAM>
SOX9	5-CTC GGG CAT GAG TGA GGT-3'	5-GAT CAA CTT TCC CAG CTT GC-3'	<6FAM>CC ACC CAC CAC TCC CAA AAC AGA C<TAM>
RUNX2	5-GAC AGC CCC AAC TTC CTG T-3'	5-GTA ACC ACG GTC CCA TCG-3'	<6FAM>AC TGG CGG TGC AAC AAG ACC CT<TAM>
COL1	5-GAA CAA GGT GAC AGA GGC ATA A-3'	5-GGA CCA GAA GCT CCA GAG G-3'	<6FAM>AG GGT CAT CGT GGC TTC TCT GGT CT<TAM>
HFRT	5-CAG ACT TTG CTT TCC TTG GTC-3'	5-GCC TGT ATC CAA CAC TTC GAG-3'	<6FAM>AG TAC AGC CCC AAA ATG GTT AAG GTT GC<TAM>

Alkaline Phosphatase Cell Differentiation Analysis

Cells were treated with recombinant human GDF-5 (30[^]g/ml, 10[^]g/ml, 3[^]g/ml, and 1[^]g/ml, respectively) every other day for 21 days. Bone morphogenic protein 2/7 (BMP2/7) was used as a positive control. Alkaline Phosphatase analysis (ALP) was performed on Days 1, 7, 14, and 21. Cells were lysed with a 100[^]l lysis buffer (0.1% Triton X-100). 10[^]l of the cell lysate was transferred to a 96-well ELISA plate. Each well was mixed with an 80[^]l ALP buffer and a 10[^]l ALP substrate (prepared immediately prior to use): 5ml ALP buffer + 1 tablet of pNPP. The ELISA plate was gently rocked for 30 minutes then read with the spectrophotometer (SpectraMax 340) at 405 nm.

Alamar Blue Cell Metabolic Activity Analysis

Metabolic activity was measured concurrently with ALP analysis. 10% Alamar Blue dye was added to each well in the original 96-well plate with the MSC culture. The plates were incubated for 3 hours at 37°C then read at 530 nm with the SAFIRE spectrophotometer (Tecan).

Cell Migration Analysis

The chemotaxic effect of rhGDF-5 was assessed using a Neuroprobe migration chamber (Neuroprobe). The upper chamber was loaded with 100,000 rhGDF-5 pre-treated MSC in 30 μ l growth media. The lower chamber was loaded with the rhGDF-5 titration (30[^] μ g/ml highest with 3 fold dilution) in 30 μ l growth media. A filter with 8 μ m pore size was chosen (MSC size 18 μ m) to prevent nonspecific migration. The chamber was placed at 37°C incubator (5% CO₂, humidified air) for one hour followed by the removal of the upper chamber and the filter membrane. 10% of Alamar Blue was added

to the lower chamber. The lower chamber was incubated at 37°C for an additional 3 hours followed by the spectrophotometer reading with the SAFIRE machine (Tecan) at 530 nm.

Animals

Male Sprague Dawley rats (initial body weight = 200-215g) were used in this study. They were housed two animals per cage in climate and humidity controlled room with 12-hour dark/light reverse cycle. They had free access to food and water and no daily activity restriction within the cage. All live animal experiments were conducted following the Guide for the Care and Use of Laboratory Animals and were approved by Amgen's Institutional Animal Care and Use Committee (IACUC).

Techniques for in vivo experiments

1. Monosodium Iodoacetate (MIA) model

Monosodium Iodoacetate was dissolved in physiological saline at 1 mg/50 µl. This dose was chosen based on previous studies to induce sufficient cartilage destruction mimicking OA-like injury (Bove, 2003).

Rats were anesthetized with 1.5%-4% isoflurane + O₂ and maintained under a nose cone for the duration of the procedure. A single intra-articular injection of 1 mg/50 µl MIA was delivered unilaterally to the hind limb with a 28 gauge needle under aseptic conditions. The contralateral joint was injected with 50 µl physiological saline as a control. The animals were returned to cages and monitored during the recovery from anesthesia. This method was performed by Amgen staff.

2. Medial Meniscus Tear model

Rats were anesthetized with 1.5%-4% isoflurane + O₂ and maintained under a nose cone for the duration of the procedure. The right knee was prepared for surgery. A skin incision was made over the medial aspect of the knee, and the medial collateral ligament was exposed by blunt dissection and transected. The medial meniscus was reflected medially, and a cut was made through the full thickness to simulate a complete tear. The skin was then closed with suture. This method was performed by Amgen staff.

3. SHAM-Operated model

Rats were anesthetized with 1.5%-4% isoflurane + O₂ and maintained under a nose cone for the duration of the procedure. The unilateral knee was prepared for surgery. A skin incision was made over the medial aspect of the knee, and the skin was then closed with suture. This method was performed by Amgen staff.

In situ hybridization: Table 2: In Situ Hybridization Probes

Gene (region)	Genbank?	Corresponding Nucleotides (nts)	Cloning Vector	RNA Polymerase	Restriction Enzyme
Rat GDF5 (CDS region)	XM_001066344	MS- ⁸⁰³ 1156	pCR4-TOP0	T7 (antisense) T3 (sense)	SpeI Not I
Rat Sox9 (CDS region)	XM_001081628	nts 705-961	pCR4-TOPO	T3 (antisense) T7 (sense)	Not I SpeI
Rat Runx2 (CDS region)	NM_053470	nts. 1952-2302	pCR4-TOP0	T3 (antisense) T7 (sense)	Not I SpeI

Isotopic In Situ Hybridization (ISH) was performed using cDNA templates for Rat GDF5, Rat Sox9, and Rat Runx2. The templates, consisting of fragments from the coding sequence (see Table 1), were generated by PCR and cloned into the pCR4-TOPO vector (Invitrogen, Carlsbad, CA). The identity of each template was verified by sequencing. Antisense and sense³³ P-labeled riboprobes were generated from the cDNA vector construct after linearization with the appropriate restriction enzyme and then transcribed using either T3 or T7 RNA polymerases (Promega, Madison, WI).

In Situ Hybridization was performed on 5 μ m sections of formalin-fixed and paraffin-embedded sections of knee joints from vehicle-control (n=5), monosodium iodoacetate (MIA) (n=5) and medial meniscus tear (MMT) (n=5) rats. In brief, sections were deparaffinized, deproteinized (0.2M HCl; 10 minutes), treated with Proteinase K (10 μ g/ml; 10 minutes), acetylated (0.25% acetic anhydride in 0.1M triethanolamine; 10 minutes), and prehybridized with a hybridization buffer (300 mM NaCl, 20 mM Tris-HCl pH 8.0, 5 mM EDTA pH 8.0, 1x Denhardt's solution, 0.2% sodium dodecyl sulfate, 10 mM dithiothreitol, 0.25 mg/ml yeast tRNA, 25 μ g/ml polyadenylic acid, and 50% deionized formamide) for 1 hour in a humidified chamber at 56°C. After prehybridization, 1.5 x 10⁶ cpm of³³ P-labeled probe (1x hybridization buffer/10% dextran; 50 μ l/slide) was added to each section, covered with parafilm, and the slides incubated in a humidified chamber at 56°C for 18 hours. The slides were then washed in 4x SSC (3 M NaCl, 300 mM trisodium citrate pH 7.0) at 55°C to remove the coverslips, followed by 2 successive washes in 4x SSC at 55°C for 10 minutes each, then 2 washes at 2x SSC at 55C for 10 minutes each. The sections were treated with 20 μ g/ml RNase A in

a RNase buffer (0.5 M NaCl, 10 mM Tris-HCl pH 8.0, 5 mM EDTA) at 37°C for 30 minutes. Following digestion, the slides were again washed through decreasing SSC concentrations (2x SSC, 1x SSC, 0.5x SSC; all 5 minutes each at room temperature) until reaching the final stringency of 0.1x SSC at 55°C for 30 minutes. Sections were then dehydrated through graded ethanols containing 300 mM ammonium acetate, air-dried and coated with Kodak NTB emulsion (Kodak, Rochester, NY; diluted 1:1 with 600 mM ammonium acetate), stored in light tight boxes at 4°C with desiccant, and exposed for 3 weeks. Finally, the slides were developed in Kodak D19 developer (diluted 1:1 with water), fixed in Kodak Professional Fixer, and counterstained with hematoxylin and eosin. This method was performed in collaboration with Amgen staff.

Dynamic Weight Bearing system

DWB is a new type of incapacitance test developed by Bioseb (France). A freely moving rat was placed in a biometric floor instrumented cage (W30 x L30 x H25) for 5 minutes to measure the weight bearing event for each limb. The raw data were synchronized with a video image and transferred to a PC with a 10Hz sampling rate. The raw data were analyzed using the DWB software.

In the 14 week DWB analysis, DWB data were collected from three different cohorts:

Cohort 1: MMT + IA vehicle

Cohort 2: MMT + IA rhGDF-5

Cohort 3: SHAM operated

The DWB data were collected every other week starting from Week 7 post MMT surgeries. The weight born on the hind left and right limbs were recorded. A ratio of the difference in weight born on the injured/uninjured leg was plotted to assess the establishment of the MMT injury and the potential reversal of the injury by rhGDF-5.

Results

Cell Culture

The MSC population was isolated and expanded in culture from E18 rat embryos. These cells were passaged no more than four times prior to use in these experiments. Freshly harvested MSC and cryopreserved MSC yielded similar ALP and AB results.

Flow Cytometry (Single Staining)

Similar to human bone marrow mesenchymal stem cells (Pittenger, 1999), rat embryo MSC were identified to be CD29+, CD44+ (Fig.1), CD71+, CD90+ (Fig.2), CD34 -, and CD45-.

Figure 1. Rat Embryo MSC CD44 Single Staining

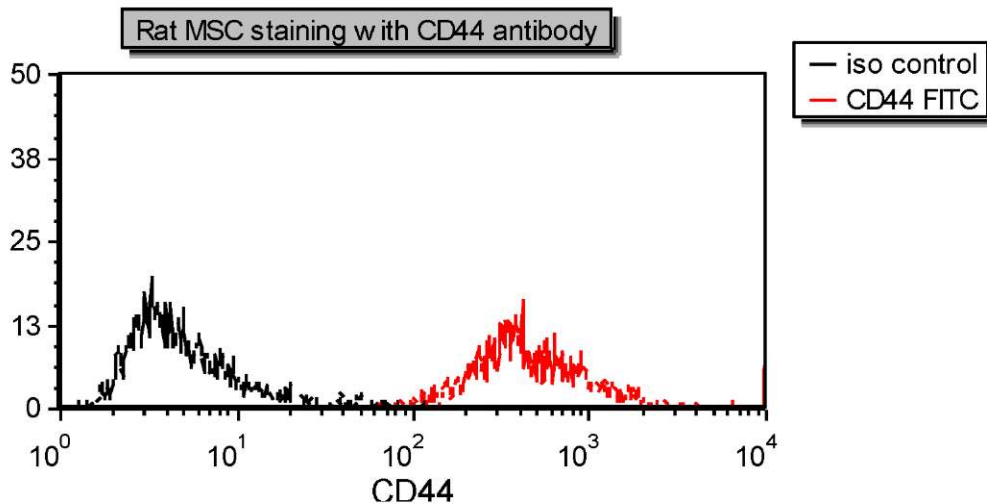
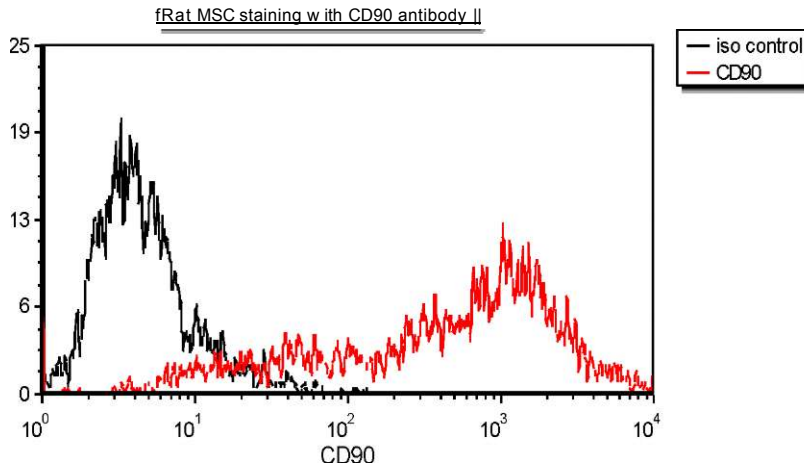


Figure 2. Rat Embryo MSC CD90 Single Staining



Fluorescence-Activated Cell Sorting (FACS) (Double staining)

To investigate the phenotypic differences between the MSC and non-MSC cell population, CD44/CD90 double staining was adapted to identify and purify two populations in the rat embryo cell culture: CD44+/CD90+ and CD44+/CD90- MSC (Fig.3). Applying similar FACS parameter, we further identified and purified both CD44+/CD90+ and CD44+/CD90- populations from the adult rat knee joint cell culture (Fig.4). The total RNA of each cell culture sub-population was prepared for phenotyping analysis.

Figure 3. Rat Embryo MSC CD44+/CD90+ Double Staining

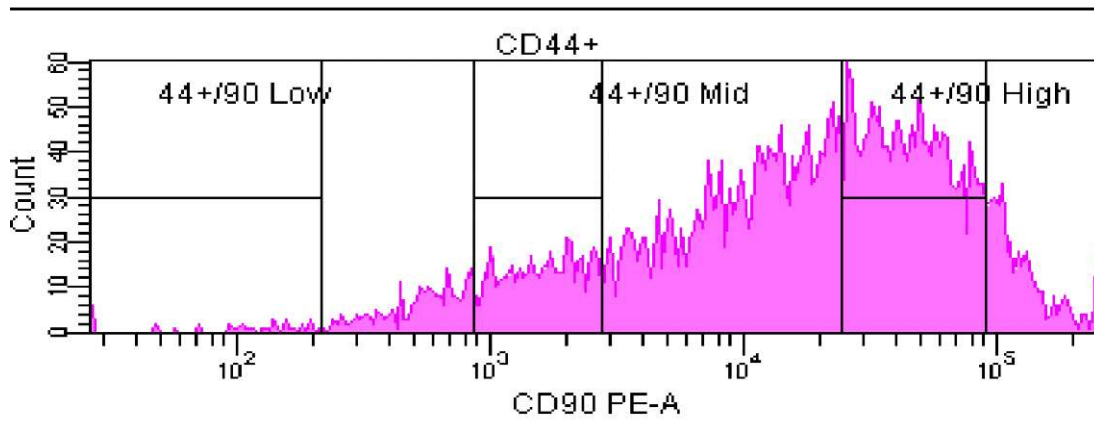
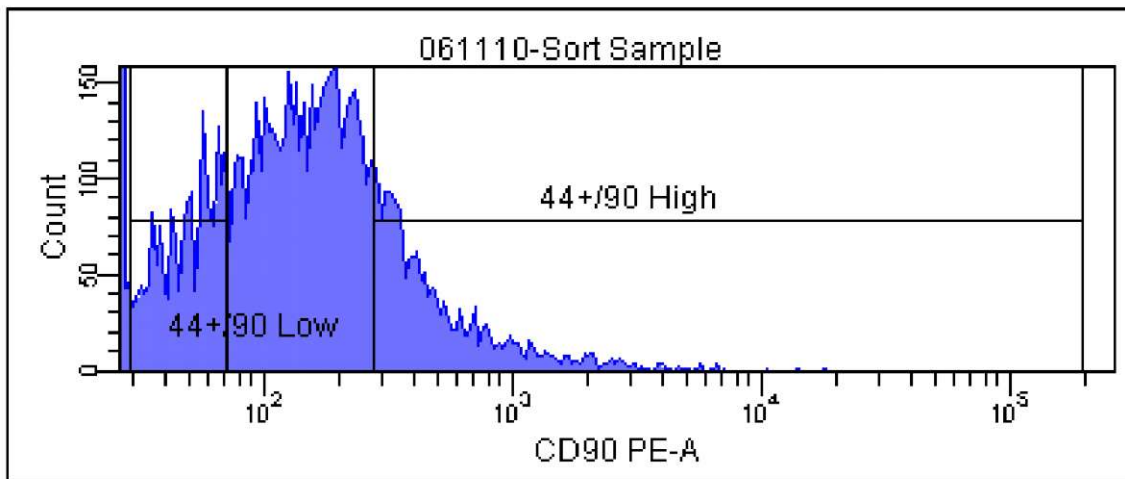


Figure 4. Rat Adult Articular Cartilage MSC CD44+/CD90+ Double Staining



In Situ Hybridization

In Situ Hybridization provides visualization of the presence and localization of GDF-5, Sox9, and Runx2 expression in both rat embryo and adult rat knee joint. In a normal E17 rat embryo, GDF-5 was expressed exclusively in the joint interzone, underlying the future knee joint location. SOX9 and RUNX2 were primarily expressed in the cartilage primordium (Fig.5).

Both normal control and MIA diseased adult rat model knee joints were examined with in situ hybridization. We found different endogenous GDF-5 expression patterns in the two model comparison. The expression of GDF-5 was under the detection level in the normal adult rat knee joint tissue. However, clear GDF-5 expression was detected around the clusters of chondrocytes adjacent to areas of cartilage necrosis and degeneration in the MIA model knee joint. Such expression patterns were consistent in both normal control and MIA diseased models throughout the experiment. The Sox9 was expressed prominently in the articular cartilage in both normal and MIA-model joints. On

the contrary, strong expression of RUNX2 was detected in the subchondral area but not the articular cartilage from neither normal nor MIA diseased model knee joints samples (Fig.6).

Figure 5. In Situ Hybridization: GDF-5, Sox9, and Runx2 expression in Rat Embryo

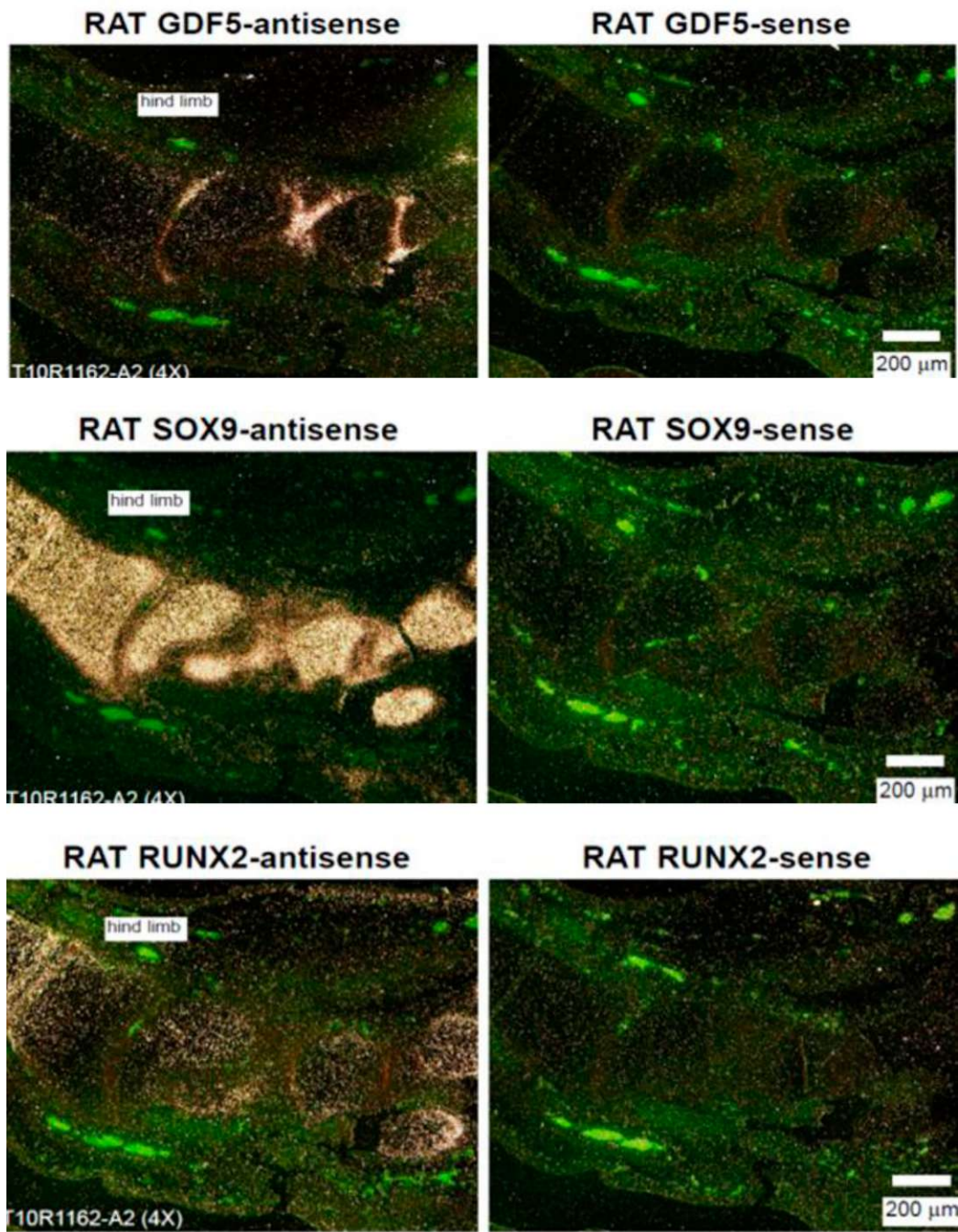
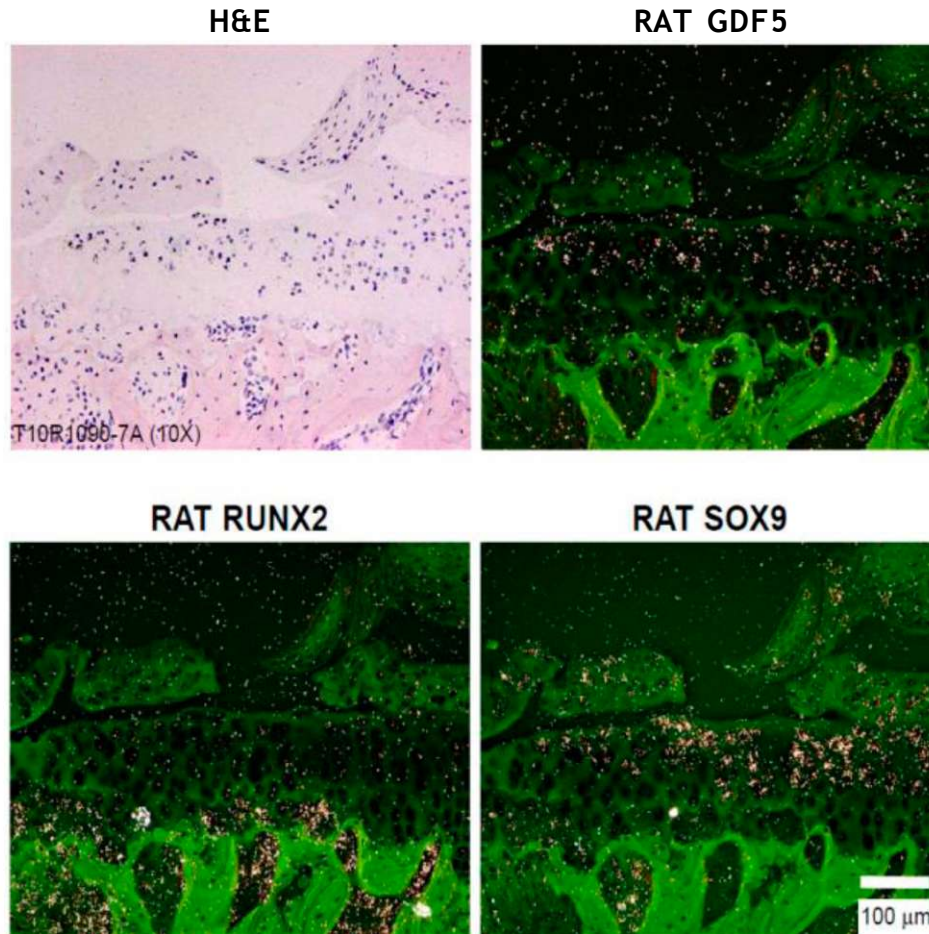


Figure 6. Situ Hybridization: GDF-5, Sox9, and Runx2 Expression in Adult Rat

Damaged Knee Joint



Quantitative Real-Time PCR (qPCR)

qPCR was utilized for MSC identification and rhGDF-5 responsive MSC phenotyping. All RNA primers and oligonucleotides were designed and synthesized in house. As the progenitor cells for both chondrocytes and osteocytes, MSCs were identified with the co-expression of chondrocyte transcription factor Sox9 and osteocyte transcription factor Runx2. The selective pharmacological effect on MSC by rhGDF-5 was evaluated by the expression of chondrocyte lineage specific markers (aggrecan, type

II collagen, Sox9), osteocyte lineage specific markers (type I collagen and Runx2) and endogenous expression of GDF-5.

1. Embryo MSC verification

To further verify the FACS purified MSC identification, total RNA samples were prepared from the CD44+/CD90- and CD44+/CD90+ E18 rat embryo cell populations.

The results indicated that the CD44+/CD90+ cell population expressed Sox9 and Runx2 but little GDF-5 RNA. On the contrary, the CD44+/CD90- cell population showed little Sox9, Runx2, or GDF-5 expression.

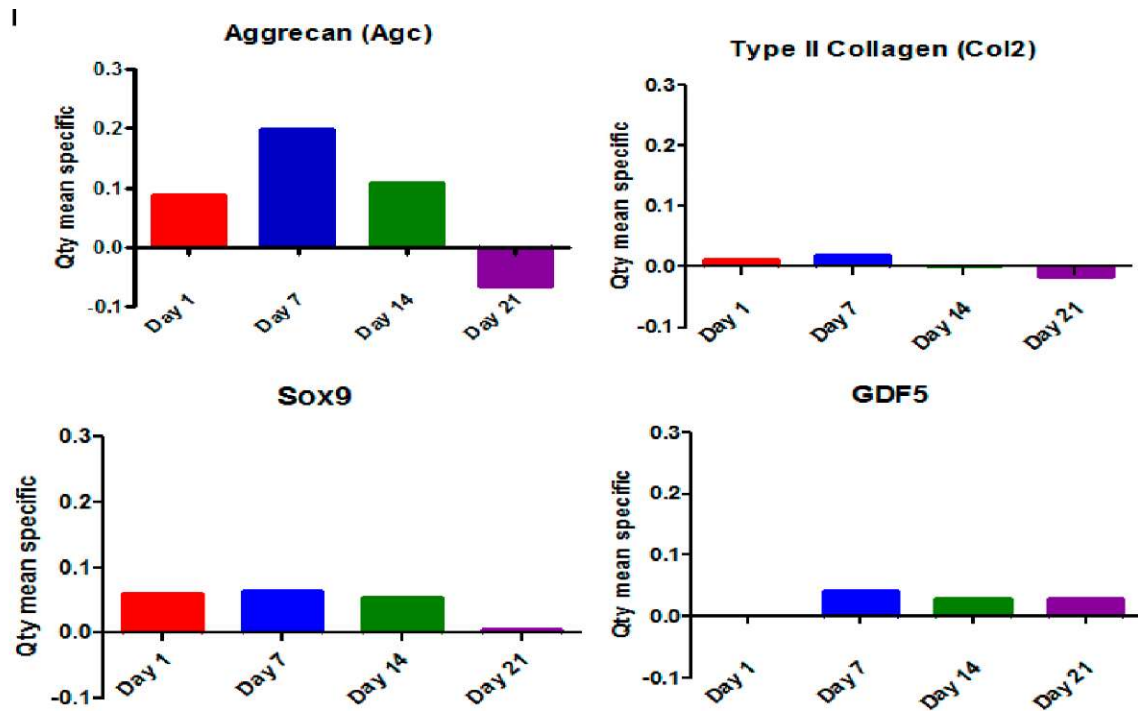
2. Adult rat knee joint MSC verification

Total RNA samples were prepared with cells isolated from normal adult rat knee joints. Two cell populations were also identified with FACS analysis: CD44+/CD90- and CD44+/CD90+. qPCR results showed that the CD44+/CD90+ population had significantly higher Sox9 (p value = 0.0037) and Runx2 (p value = 0.0162) expressions than the CD44+/CD90- population. Moreover, endogenous GDF-5 expression was detected in the CD44+/CD90+ population.

3. Embryo MSC phenotyping

qPCR was used to analyze the embryo MSC phenotyping concurrently with ALP, AB, and cell migration assay. Total RNA prepared with rhGDF-5 treated MSC showed generally increased expression of chondrocyte markers including Agc, Col2, Sox9, and GDF-5 in the 21 day experiment (Fig.7). In contrast, osteocyte markers Runx2 and Coll were suppressed during the same 21 day time frame (data not shown).

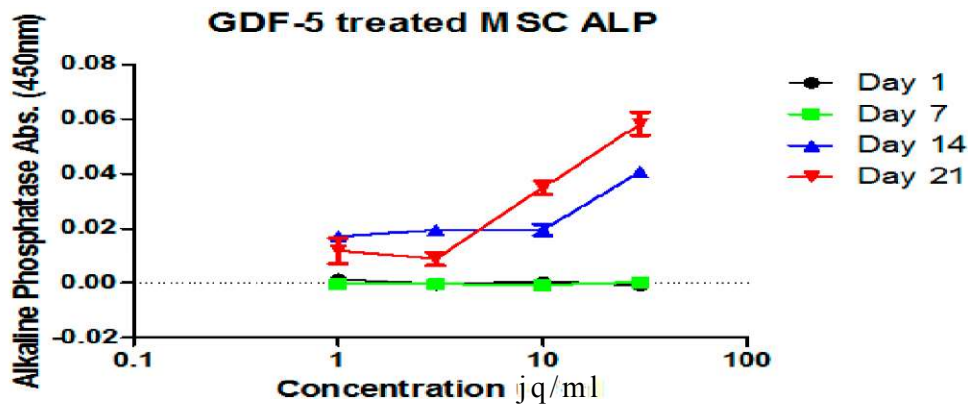
Figure 7. rhGDF-5 treated rat embryo MSC qPCR (specific reading)



Alkaline Phosphatase Cell Differentiation Analysis

ALP is an important indicator for chondrogenesis and osteogenesis. Higher ALP content is synthesized during differentiation. In the ALP assay, we measured the ALP reading from the rhGDF-5 treated MSC and compared the results from the vehicle treated MSC. Continuous treatment of rhGDF-5 to MSC gradually stimulated ALP synthesis by Day 14. A maximum effect of ALP synthesis was observed on Day 21. It was also observed that the higher rhGDF-5 concentration treatment (up to 30 μ g/ml) stimulated higher ALP synthesis at any specific given time point (Fig.8).

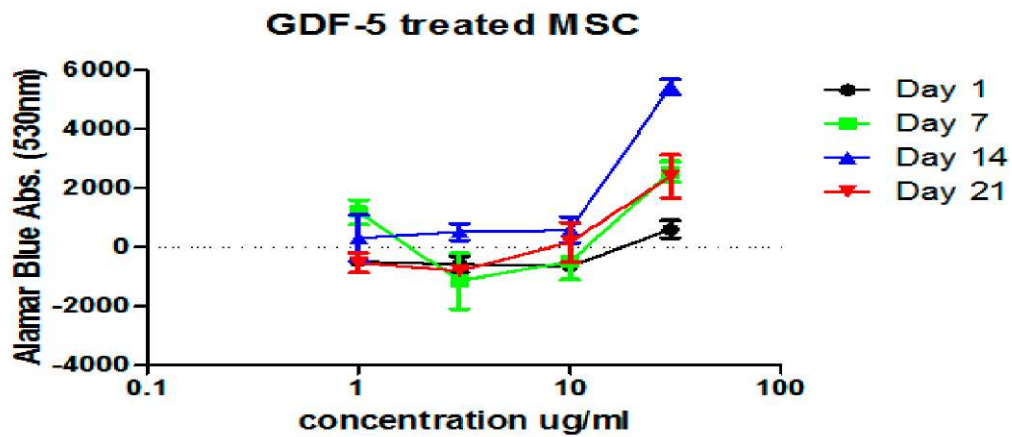
Figure 8. rhGDF-5 Treated MSC ALP



Alamar Blue Cell Metabolic Activity Analysis

Alamar Blue is a redox indicator that yields a colorimetric change and a fluorescent signal in response to cell metabolic activities (Ahmed, 1994). It provides an alternative method to measure cell health and metabolic activity for assessing cell proliferation activity. It was observed that the increasing dose of rhGDF-5 treatment to the MSC stimulated positive proportional AB reads at any given time point. It was also observed that the AB activity gradually increased from Day 1 to Day 7 and peaked by Day 14 with an AB reading decline on Day 21 (Fig.9).

Figure 9. rhGDF-5 Treated MSC AB



Cell Migration Analysis

The Cell Migration Analysis showed that the rhGDF-5 concentration attracted the rhGDF-5 differentiated MSC towards its gradient. The cell migration rate was directly proportionate to the increasing rhGDF-5 concentration gradient. It was noted that such effect was not confirmed in the 14-day rhGDF-5 pre-treated MSC (Fig.10). On the contrary, the highest chemotactic effect was observed with the highest concentration gradient (30 μ g/ml) with the 21-day rhGDF-5 pre-treated MSC. The same trend was shown in 1 hour and confirmed by a 2-hour incubation period. In parallel, we noted that cartilaginous condensation formation was observed in 23 day rhGDF-5 pre-treated MSC but not the 14 day ones (Fig.11).

Figure 10. rhGDF-5 Treated MSC and rhGDF-5 Chemotaxis Effect

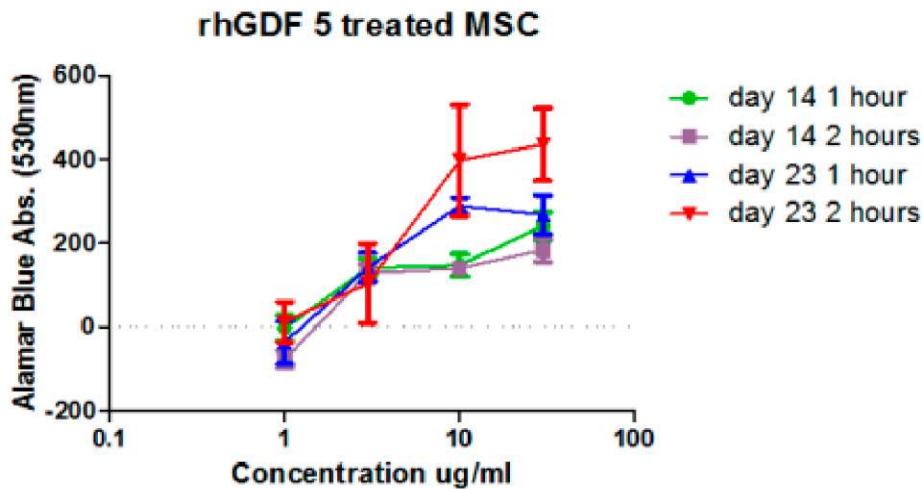
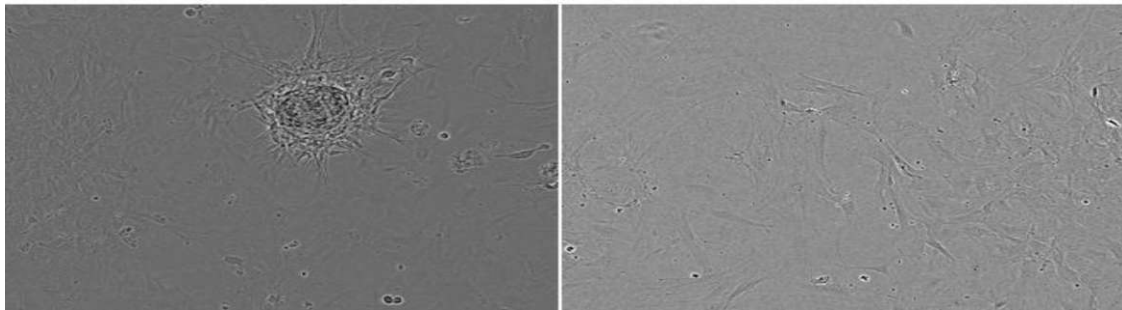


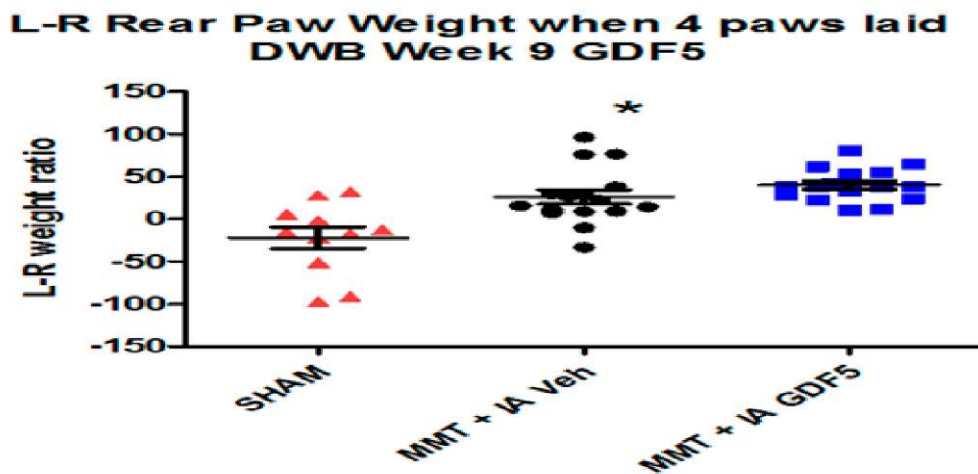
Figure 11. rhGDF-5 Treated MSC and Cartilaginous Condensation Formation



Animal Functional Study

We used the DWB system to calculate each rat's weight bearing differential between the left and right hind limb. Osteoarthritic rats would be expected to show differential weight bearing. No difference was detected between the left and right limb in the SHAM operated cohort. The weight bearing differential was detected in the MMT operated cohort. Moreover, the weight bearing differential was significantly higher in the MMT cohort than the SHAM operated cohort. (Week 7: p value = 0.0072; Week 9: p value = 0.0027). IA-rhGDF-5 treatment did not reverse the differential weight bearing in the MMT operated rats in either Week 7 or Week 9 (Fig.11).

Figure 12. rhGDF-5 Treated MMT OA-like Model Week 9 DWB Analysis



Discussion

In the present study, we studied rat embryo and adult knee joint MSCs as they had the capacity for multilineage differentiation similar to human bone marrow and synovial membrane MSC following various external stimuli (De Bari et al., 2001; Yoshimura et al., 2007; and Pittenger et al. 1999). Our goal was to study the pharmacological effects of rhGDF-5 on rat embryo MSC and to evaluate whether rhGDF-5 was efficacious in the joint repair mechanism in a rat model of osteoarthritis.

We first investigated the methods for MSC identification. Pittenger and co-workers had defined a set of cell surface antibodies to identify human mesenchymal stem cells. Our Flow Cytometry analysis with rat embryo cells agreed with most of the human MSC defining antibodies, in which CD44 and CD90 were selected for our FACS analysis. Double positive CD44+/CD90+ cells were identified and purified from both embryo and adult knee joint MSC populations in the FACS analysis. These findings suggested the presence of MSC in the embryonic stage and in the rat knee joint post-natal development. The identification of MSC was critical in the experiment design since MSC was our treatment target. Therefore, experiments were further designed to identify MSC at RNA expression level.

qPCR analysis was implemented to study the RNA expression difference between MSC and non-MSC populations. As the progenitor cell for chondrocytes and osteocytes, MSCs express two specific transcription factors, Sox9 and Runx2 (Zhou et al. 2006). Sox9 and Runx2 were expressed and regulate the development of chondrocytes and osteocytes respectively (Komori, 2008; Bi et al. 1999; Tsuchiya et al. 2003). We not

only found Sox9 and Runx2 RNA co-expression but also detected higher Sox9 and Runx2 expression in CD44+/CD90+ MSC population than other non-MSC cell population. Similar results were obtained from both rat embryos MSCs and adult rat knee joint MSCs in the qPCR analysis. The findings from FACS and qPCR suggested the presence of MSC in both embryo and adult rat knee joint. However, neither FACS nor qPCR was able to provide the visualization of MSC deposition within the knee joints.

Since the biomarker that could specifically pin point MSC was not available, In Situ Hybridization was conducted to visualize the region where GDF-5, Sox9, and Runx2 were expressed since the co-expression of Sox9 and Runx2 would suggest the presence of MSC. Such co-expression was detected in the E18 rat embryo hind limb, indicating the abundance of MSC in the region. Endogenous GDF-5 was primarily expressed in the embryo joint interzone, which matched the findings from Terrig Thomas and co-workers (2006) who investigated the role of GDF-5 in early stage joint formation. On the contrary, strong co-expression of Sox9 and Runx2 was under the detectable level from the adult rat knee joint image. The Sox9 was primarily expressed in the articular cartilage region whereas Runx2 was prominently expressed in the subchondrol bone area in both normal and MIA diseased adult knee joints. This finding suggested that the MSC may be of scarce as expected. However, our ISH findings also indicated that endogenous GDF-5 was expressed adjacent to the degenerating chondrocyte clusters in the damaged articular cartilage. Therefore, we hypothesized that endogenous GDF-5 might not only play a role in embryo joint development but also protect and maintain knee joint homeostasis when cartilage is damaged. Consequently, we wanted to understand if

rhGDF-5 would have any effect on the adult knee joint MSC. Due to the cell availability and similar characteristic between adult knee joint and embryo MSC, we designed experiments to study the pharmacological effect of rhGDF-5 on embryo MSC.

It was reported that rhGDF-5 had the ability to induce human bone marrow MSC to differentiate into chondrocytes and to stimulate cartilage matrix synthesis. (Bai et al., 2004; Pittenger et al., 1999; and Bobacz et al., 2001) Therefore, we hypothesized that the rhGDF-5 treatment has chondrocytic differentiation, proliferation, and chemotaxis effects on the E18 rat embryo MSC. Therefore, MSC samples were taken at 4 different time points in the 21 day experiment period and analyzed with ALP, AB, qPCR, and cell migration assay.

The rhGDF-5 induced ALP expression observed by Day 14 and 21 matched Hotten and Bai's findings (Hotten et al., 1996; Bai et al., 2004), which suggested the differentiation effect of rhGDF-5 on the embryo MSCs. Moreover, the total RNA prepared from the rhGDF-5 treated MSCs showed increasing chondrogenic markers in the qPCR analysis, which suggested the rhGDF-5 chondrogenic selectivity on embryo MSC population.

Alamar Blue analysis was used to measure metabolic activity and could also be an indication for cell proliferation. Our study showed that rhGDF-5 could induce metabolic activity in embryo MSC population. Moreover, in combined with the results from the concurrent ALP study, the results suggested that the rhGDF-5 stimulated the MSC to proliferate prior to the commitment to chondrocytic differentiation.

In combined with the cartilaginous condensation formation observed with 23 day rhGDF-5 pre-treated MSC, the findings from the cell migration assay suggested that rhGDF-5 elicits cell homing effects in rhGDF-5's responsive and differentiated MSC. This cell homing effect was consistent with Lee et al. in the regenerating articular surface in synovial joint using Transforming Growth Factor beta 3 (TGF-beta 3) in the rabbit model (Lee et al., 2010).

Results from the *in vitro* experiments confirmed the pharmacological role of rhGDF-5 to embryo MSC population: it stimulates the embryo MSC to proliferate, chondrogenic differentiate, and can attract MSC towards its gradient. These properties might suggest a critical role of rhGDF-5 in adult animal cartilage regeneration.

Therefore, we further investigated the effect of rhGDF-5 in rodent a joint-instability (MMT) model of osteoarthritis after studying the pharmacological effects of rhGDF-5 on rat embryo MSC. We hypothesized that rats would preferentially place less weigh on the injured limb than the normal limb due to the associated cartilage degeneration and pain resulting from joint-instability. DWB analysis was used to assess the weight bearing differential of the left and right limbs. A significant weight bearing differential was observed in the MMT rats the right limb by Week 7 and Week 9. However, with the weekly rhGDF-5 IA injection (30 μ g/50 μ l), such pain reading was not reversed by Week 9. Histology and pathology analysis are required for further effect of rhGDF-5 assessment.

Summary

Our findings demonstrate that the MSC are present in both embryo and adult knee joints. Increased endogenous GDF-5 expression in the damaged articular cartilage further speculated its role in cartilage maintenance. We also demonstrate that exogenous rhGDF-5 treatment induced MSC chondrocytic differentiation, proliferation, and chemotaxis. The understanding of the pharmacological effects of rhGDF-5 may provide insights into new possible treatments for OA.

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Vita

YueJia Wu graduated from California State University, Stanislaus with a bachelor degree in Biological Science in 2007. He then entered the Master of Biotechnology and Master of Business Administration dual master program at California State University, Channel Islands in the same fall. In summer 2009, he took one step further in his career development and applied for the Stem Cell Technology and Laboratory Management Emphasis at CSUCI. He was accepted and started the one-year internship at Amgen Inc. (Thousand Oaks) where he found his passion in the biotechnology industry. He plans on staying at Amgen after graduation and keeping an open mind for further education when the opportunity comes along.