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Original paper

Semi-automatic grading system in histologic and immunohistochemistry analysis to evaluate *in vitro* chondrogenesis

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Abstract

During embryological limb formation mesenchymal cells condense and differentiate into chondrocytes, in a process known as chondrogenesis. These chondrocytes synthesize glycosaminoglycans (GAGs), thus playing an important role in this process. A simplified system *in vitro* chondrogenesis, using adult mesenchymal stromal cells (MSCs) has been demonstrated. This differentiation potential is usually assessed by histological staining. **Objective.** Establishment of a semi-automatic grading system for histochemistry stains and immunohistochemistry assays. **Materials and methods.** For chondrogenesis cells were cultured for three weeks in aggregates with inducing media. Total GAGs were measured using dimethylmethylene blue (DMB) method. For histological analyses aggregates were stained with Alcian blue for total GAGs detection and immunohistochemistry (IHC) for aggrecan was performed. Semi-automatic grading for all slides was obtained after ImageJ analysis. **Results.** MSCs cultured as aggregates in chondrogenic differentiation media had similar protein concentrations for all time points, suggesting cellularity remained homogenous during culture. Total GAGs was higher for aggregates cultured in chondrogenic compared to complete media. The same trend was observed for Alcian blue stain grades by blinded observer and analysis using ImageJ software. Aggrecan's IHC analysis had a decreasing tendency with time for aggregates in chondrogenic media for blinded observer and ImageJ evaluation. **Conclusion.** We developed a functional system for semi-automatic slide grading. We corroborated these results by biochemical analysis with comparable results. To our knowledge, for *in vitro* chondrogenesis, this is the first report to evaluate stains using this methodology. This procedure might be useful for other applications in the field of Biology and Medical Sciences.

Key words: mesenchymal stromal cells, *in vitro* chondrogenesis, glycosaminoglycans, ImageJ

Resumen

Establecimiento de un sistema de puntaje semi-automático para ensayos histoquímicos e inmunohistoquímicos con el fin de evaluar diferenciación condrogénica *in vitro*. Durante el desarrollo embriológico las extremidades surgen de la condensación de células mesenquimales y su diferenciación a condrocitos en un proceso llamado condrogénesis. Estos condrocitos sintetizan glicosaminoglicanos, jugando un papel importante durante este proceso. Existe un sistema de condrogénesis *in vitro* utilizando células mesenquimales generalmente evaluado mediante histoquímica. **Objetivo**. Establecer un sistema de puntaje semi-automático para ensayos histoquímicos e inmunohistoquímicos. **Materiales y métodos.** Para condrogénesis las células fueron cultivadas con medio inductor en agregados por tres semanas. Los glicosaminoglicanos totales fueron determinados mediante azul de dimetileno. Para el análisis histológico los agregados fueron teñidos con azul de alcian e inmunohistoquímica para detección de agrecán. La puntuación semi-automática fue obtenida utilizando el programa ImageJ. **Resultados**. Las células mesenquimales cultivadas en medio de diferenciación condrogénica tuvieron una concentración de proteína comparable durante las tres semanas de cultivo, sugiriendo una celularidad similar. La concentración de glicosaminoglicanos fue superior para los agregados cultivados en medio condrogénico. La misma tendencia fue observada para la tinción de azul de alcian mediante puntajes del observador ciego y análisis con ImageJ. Finalmente, los resultados de inmunohistoquímica de puntajes asignados por el observador y los del análisis por ImageJ revelaron una tendencia decreciente con el tiempo para agregados en medio condrogénico. **Conclusión**. Desarrollamos un

sistema funcional para generación de puntaje semi-automático para diferenciación condrogénica. Corroboramos estos resultados mediante análisis bioquímico con resultados comparables. En nuestro saber este es el primer reporte en evaluar esta metodología, la cual puede ser útil para otras aplicaciones en el campo biológico o médico.

Palabras clave: células mesenquimales, condrogénesis *in vitro*, glicosaminoglicanos, ImageJ.

Resumo

Estabelecimento de um sistema de pontuação semi-automático para ensaios histoquímicos e imuno-histoquímicos para avaliar diferenciação condrogênica *in vitro*. Durante o desenvolvimento embrionário os membros emergem a partir da condensação de células mesenquimais e sua diferenciação em condrócitos em um processo chamado condrogênese. Estes condrócitos sintetizam glicosaminoglicanos, desempenhando um papel importante neste processo. Existe um sistema de condrogénese *in vitro* utilizando células mesenquimatosas, geralmente avaliado por histoquímica. **Objetivo.** Estabelecer um sistema de pontuação semi-automático para ensaios histoquímicos e imuno-histoquímicos. **Materiais e métodos.** Na condrogênese as células foram cultivadas com meio indutor em agregados, durante três semanas. Os glicosaminoglicanos totais foram determinados pelo azul de dimetileno. Para a análise histológica os agregados foram corados com Azul Alciano e imuno-histoquímica para detecção de agrecan. A pontuação semi-automática foi obtida utilizando o programa ImageJ. **Resultados.** As células mesenquimais cultivadas em meio de diferenciação condrogênica tiveram uma concentração de proteína comparável durante as três semanas de cultura, o que sugere uma celularidade similar. A concentração de glicosaminoglicanos foi maior para os agregados cultivados em meio condrogênico. A mesma tendência foi observada para a coloração com Azul Alciano segundo as pontuações do observador cego e a análise com ImageJ. Finalmente, os resultados de imuno-histoquímica de pontuações dados pelo observador e aqueles dados pela análise ImageJ revelaram uma tendência decrescente ao longo do tempo para os agregados em meio condrogênico. **Conclusão.** Nós realizamos um sistema funcional para gerar pontuação semi-automática para diferenciação condrogênica. Nós corroboramos esses resultados por análise bioquímica com resultados comparáveis. Segundo nosso conhecimento, este é o primeiro estudo a avaliar esta metodologia, que pode ser útil para outras aplicações no campo biológico ou médico.

Palavras-chave: células mesenquimais, condrogênese *in vitro*, glicosaminoglicanos, ImageJ.

Introduction

Chondrogenesis, the first step in endochondral bone formation, is a process where the skeleton is ensued from a cartilage mould or anlagen (1, 2). It begins with the recruitment of mesenchymal cells aggregating at the site where future skeletal elements will be formed. Following migration, epithelial-mesenchymal interactions result in condensation (3). Most bones are formed by this process where mesenchymal cells condense and differentiate into chondrocytes to form a cartilage mould (2). During differentiation mesenchymal cells become chondrocytes and secrete an extracellular matrix (ECM) rich in aggrecan, a type of proteoglycan composed of a core protein and glycosaminoglycans (GAGs), and collagen type 2 (4). After the cartilage mould has been formed, chondrocytes in the center stop proliferating, enlarge and hypertrophy. These modifications are regulated by changes in gene expression, with collagen 10 becoming the principal ECM protein. These hypertrophic chondrocytes direct mineralization of their enveloping matrix and attract blood vessels. Last, hypertrophic chondrocytes undergo apoptosis to provide the necessary scaffold for true bone matrix deposition with osteoblast and blood vessel invasion (4). These series of highly orchestrated events are characterized by different cellular processes and separate genetic controls (3).

Since chondrogenesis in the embryo is such a complex process for over a decade a simplified system, *in vitro* chondrogenesis, has been used for studying cellular differentiation using mesenchymal stromal cells (MSCs) (5). Mesenchymal stromal cells are multipotent progenitors of connective tissue (6). These cells have the potential to differentiate *in vivo* or *in vitro* into mesodermic lineages: adipo-, osteo-, and chondrogenic (7). In addition to their differentiation potential in order to define a MSC two other criteria must be met: MSCs should adhere to plastic and have specific surface antigen expression (8).

Isolated MSCs from human bone marrow cultured in aggregates with defined media containing TGF-β appear to promote initial cell density and signaling pathways needed to induce chondrogenic differentiation (9). Furthermore, *in vitro* chondrogenesis has been demonstrated with MSCs isolated from adipose tissue (10-12). Studies in MSCs isolated from adipose tissue have evidenced lower chondrogenic efficiency. However, addition of BMP-6 has been demonstrated to improve chondrogenic potential (13). After the previously mentioned conditions MSCs should undergo chondrogenic differentiation within the following weeks with a characteristic extracellular matrix composed of aggrecan and collagen type 2 (14). Expression of these markers has been used to evidence differentiation.

In vitro chondrogenic properties have been assessed by biochemical analysis and histological staining (15). Most reports have evaluated total sulfated GAGs accumulated in the ECM of aggregates. To this end some have modified a Safranin-O dye binding assay or dimethylmethylene blue method (16). Chondrogenesis induction of adipose derived MSCs has been confirmed by the presence of sulfated proteoglycans within the matrix by Alcian blue stain, and specific determination of GAGs by immunohistochemistry (IHC) marker assessment (17).

Using staining to demonstrate *in vitro* differentiation into chondrogenic lineage has been an accepted methodology, since it provides evidence for the presence of GAGs during the chondrogenic process (8). Furthermore, IHC for chondrogenic markers specifically determines the molecule synthesized. Many reports mention that histological assessment was associated with a positive stain in the ECM (10). Others, report a grade based on values given by a blinded observer. It is known that a visual grading system might present an overestimation by the observer. Considering histological analysis is a critical tool to determine *in vitro* chondrogenesis an objective and quantifiable technique is needed to assess a histological result.

Techniques for quantifying histological stains or IHC markers have been attempted by a number of authors using chromophores and color deconvolution (18, 19). We were able to perform an objective and accurate assessment of histochemical stains using color segmentation and IHC marker determination by evaluating hue, saturation and brightness. Hence, in this report we describe how a public domain, Java-based image processing program developed by the National Institutes of Health ImageJ (20), can be used as a semi-automatic grading system for both histochemical stains and IHC marker determination obtained from sections in aggregate cultures during *in vitro* chondrogenesis.

Materials and methods

Mesenchymal stromal cell isolation and culture expansion

Adipose tissue was collected after informed signed consent from females (n = 3 ages: 24 to 38) undergoing cosmetic surgery with the approval of the Bioethics Committee at the Pontificia Universidad Javeriana. Adipose tissue MSCs were isolated as previously described with slight modifications (10). Briefly, samples were washed, digested with collagenase (Invitrogen Carlsbad, CA USA) and the stromal vascular fraction (SVF) containing MSCs was resuspended in α-MEM (Gibco, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS, Eurobio, CEDEX B France), and 100 U/ml penicillin, 100 µg/ml streptomycin and 0.25 µg/ml B: amphothericin B (Lonza, Walkerville, MD), hereafter complete media or CM. Cells were seeded and tissue culture plates were incubated at 37 °C with 5% CO_2 and humidified atmosphere. Media was changed 24 h after plating.

Mesenchymal stromal cell characterization

Plastic adherence and Immunophenotypification

Adipose derived cells below passage seven were cultured in complete media to evaluate adherence to plastic. Immunophenotypification was determined by MSCs incubation with allophycocyanin (APC), fluorescein isothiocyanate (FITC) and phycoerythrin (PE) conjugated monoclonal antibodies against human CD34 (Dako, Eching Germany), human CD73 (eBioscience, San Diego, CA, USA), human CD90 (FITC Dako, Eching Germany) and human CD105 (PE, eBiosciences). Background fluorescence was established by MSCs incubation with antibodies against IgG1 (APC and FITC, Dako) and IgG1K for PE (eBioscience). Flow cytometry was performed on a FACSAria (Becton Dickson, San Jose, CA) and results were analyzed using FlowJo software (Tree Star, Ashland OR).

Multilineage differentiation

Cells at passage one were trypsinized and 96 x 10^3 cells were seeded in six well plates and incubated with complete media for 24 h. Adipogenesis and osteogenesis was evaluated culturing cells with media supplemented with inducers according to Zuk *et al*. (17) with modifications. For adipogenesis we used half the concentration of indomethacin and a 5.8 fold decrease in insulin concentration, and for osteogenesis a fourfold increase in ascorbic acid.

Chondrogenic differentiation

For chondrogenesis an aggregate culture system was used. Briefly, approximately 250 x 10^3 cells passage two in CM were placed in a polypropylene tube and centrifuged at 300 x *g* for five minutes to form a pellet. Twenty-four hours after centrifugation induction was initiated. The cells were cultured in either complete media or chondrogenic differentiation media (Lonza, Walkerville, MD USA) supplemented with BMP-6 (Sigma-Aldrich, St. Louis MO USA) and TGF-β3

(Invitrogen) at 10 ng/ml respectively. Media was changed twice a week for 21 days.

Biochemical analyses

Total protein was quantified using BCA assay (Pierce,Thermo Scientific Rockford, IL USA) according to the manufacturer's instructions from aggregate lysate in 0.9% NaCl solution. Total sulfated GAGs (sGAGs) were assayed by spectrophotometric procedure with 1,9-dimethylmethylene blue (Sigma-Aldrich). Total GAG determination by DMB (mg/ml) was normalized to total protein (mg/ml).

Histologic and immunohistochemical analysis

For microscopy analysis, spheres were fixed in 4% formaldehyde and embedded in paraffin. For total GAG detection 4 μm sections were stained with Alcian blue. Aggrecan expression in aggregates was examined by IHC using Leica Biosystem automated BOND (Leica Biosystems, Buffalo Grove, IL). Briefly, epitope was retrieved for 20 minutes at pH 8.8, followed by primary antibody incubation against aggrecan (Santa Cruz Biotechnology, Santa Cruz, CA) for 15 minutes. Immunolabeling was detected using a Bond Polymer Detection kit (Leica Biosystems) with horseradish peroxidase conjugate and diaminobenzidine (DAB) as substrate.

ImageJ analysis

Tagged image file format (TIFF) Images were captured by Nikon E600 microscope at 20X magnification (Nikon Corporation, Melville NY, USA), and composite images (magnification) by the robotics microscope at the BioIngenium facility at Universidad Nacional de Colombia at 40X. For robotics microscopy image sections were captured and assembled using an algorithm developed by this facility. Histological assessment of Alcian blue stain and IHC marker for aggrecan were performed. For Alcian blue stain the TIFF image was opened with ImageJ (**Figure 1 A**). Then the image was *Adjusted* to *Color Threshold* from hue, saturation and brightness (*HSB*) to red, green, and blue (*RGB*) color space (**Figure 1 B black arrow 1**). In addition, *Threshold color* was selected on *Red* (**Figure 1 B black arrow 2**). The color threshold was adjusted for each image to depict the areas that display the positive areas of Alcian blue by defining minimum and maximum values for each color (**Figure 1 B purple arrows**). The mask created by the threshold color was then changed from *Red* to black and white (*B&W*) (**Figure 1 C**). The *Image* was changed to grayscale by setting *Image Type* to *8-bit*. After converting the image to grayscale the *Image* was *Adjusted* to *Threshold* into *B&W* (**Figure 1 D**). To define entire aggregate section area *Freehand selections* delineated the total area (**Figure 1 E**). To eliminate pixels outside of selected area *Edit Clear Outside* was chosen (**Figure 1 F**). To calculate total aggregate section area *Analyze* was utilized and *Measure* was chosen (**Figure 1 F**). The value for total section area was recorded. To determine the area of positive Alcian blue stain *Analyze* followed by *Analyze Particles* was defined. The value obtained was recorded (**Figure 1 G**). To calculate the area of positive Alcian blue stain the Analyze particle value was divided by the value recorded for total aggregate section area. The value obtained was defined in percentage by multiplying this last result by 100 (**Figure 1 H**). For IHC percentage determination the same procedure aforementioned was utilized with the exception that instead of color segmentation by threshold (RGB) *Image* was *Adjust*ed to *Color Threshold* set in hue, saturation, and brightness (*HSB*). Since ImageJ assessment for both histochemical Alcian blue stain and aggrecan's IHC is a semi-automatic grading system, false positives are not included in the analysis because the person performing the evaluation can omit these areas. To calculate Percentage improvement the following equations were calculated.

$$\frac{sem}{mean} \times 100 = sem\ percentage \qquad (1)$$

Where sem is standard error mean for the parameter measured, and mean is the average of the parameter measured. Sem percentage was calculated for each complete or chondrogenic differentiation media for both blinded observer (BO) or ImageJ (IJ) analysis.

$$\frac{IJ\ sem\ percentage}{BO\ sem\ percentage} = IJ\ vs.\ BO\ difference \qquad (2)$$

Where IJ refers to ImageJ standard error mean (sem) percentage as determined in equation (1) and BO refers to blinded observer sem percentage as determined in equation (1).

$$1 - IJ\ vs.\ BO\ difference \times 100\ percentage\ improvement \qquad (3)$$

Where IJ vs. BO difference were determined in equation (2).

Statistical analysis

Results are presented as mean ± standard error mean (n = 3 with replicas for each sample). To determine levels of significance results were analyzed using One Way ANOVA followed by Tukey *post hoc* test. Differences were considered significant at $p < 0.05$. Graph Pad Prism Version 3.1 (Graph Pad Software, San Diego Ca, USA) was used for statistical analysis.

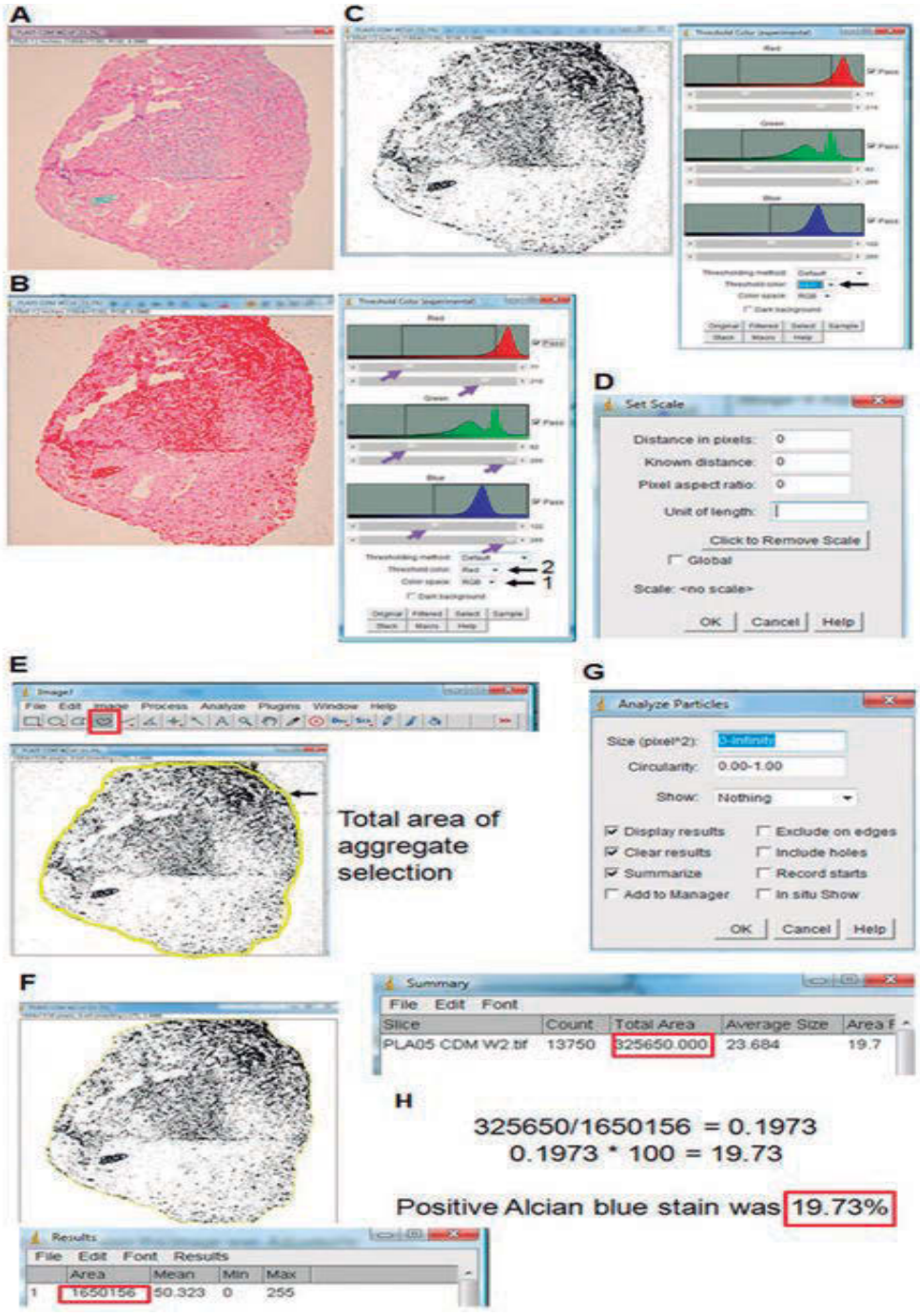

Figure 1. Schematic representation of methodology used for ImageJ analysis. (**A**) Open Alcian blue image by selecting ***File*** → *Open Samples* → PLA05 CDM W2. (**B**) Adjustment to Red Green and Blue: ***Image*** → ***Adjust*** → ***Color Threshold***. In window *Color space* to RGB (arrow 1) and *Color Threshold* to Red (arrow 2) were adjusted. Threshold values for each color (purple arrows) were defined. (**C**) *Color Threshold* from *Red* to *B&W* (arrow) was changed. Threshold Color window was closed. (**D**) Color image was converted to grayscale: ***Image*** → ***Type*** → ***8-bit. Image*** → ***Adjust*** → ***Threshold*** → ***BW*** → ***apply. Analyze*** → ***Set scale.*** Values were selected as depicted. ***OK*** was selected. (**F**) To eliminate background noise ***Edit*** → ***Clear outside*** was elected. To measure pixel number in selected area ***Analyze*** → ***Measure*** was chosen. (**G**) Positive Alcian blue area was determined by ***Analyze*** → ***Analyze particle***. Value was recorded. (**H**) Percentage positive area was calculated by dividing: 325650/1650156 = 0.1973. This obtained value was multiplied by 100 to obtain percentage 0.1973 x 100 = 19.73. Positive Alcian blue stain was 19.73%.

Results

Adipose tissue derived cells were adherent to plastic and met all the characteristics as previously described in the literature for MSCs (8). They were positive for CD73, CD90, and CD105 and less than 2% of the population assayed was positive for CD34 (**Figure 2 A and B**). Cells differentiated into adipogenic, osteogenic and chondrogenic lineages as determined by positive stain for each lineage (**Figure 2 D, F and 8 B, D**).

Protein concentration for aggregate cultures in chondrogenic differentiation media was comparable (0.05 ± 0.01 mg/ml) for all three weeks (**Figure 3**), with a non-significant protein concentration decrease for the third week of culture (ANOVA $p > 0.05$). Protein within aggregates cultured in

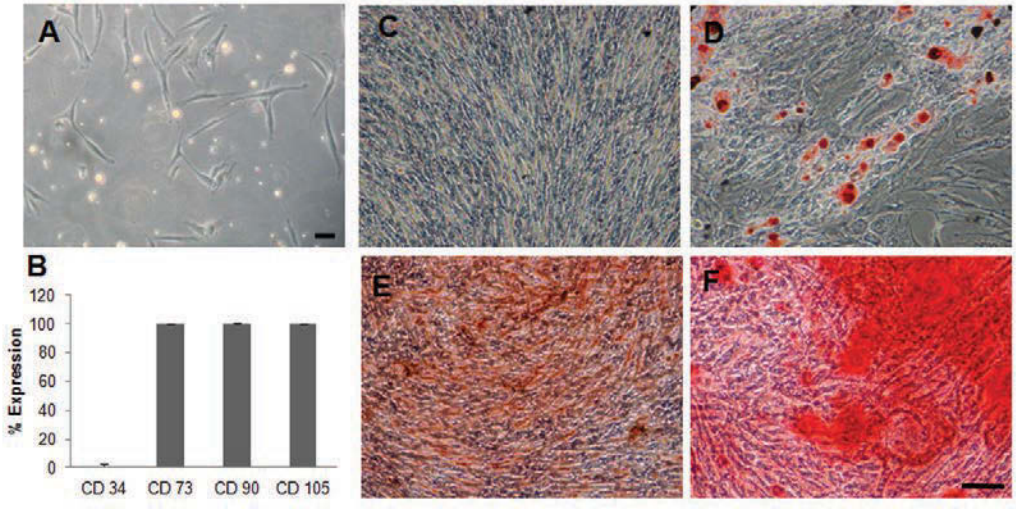

Figure 2. MSC identification criteria. (**A**) MSC morphology isolated from processed lipoaspirate. Fibroblast-like cells adherent to plastic at passage one. Scale bar 200 µm, 10X magnification. (**B**) Immunophenotypic profile of adipose derived MSCs. Cells were incubated with antibodies against human CD34, CD73, CD90 and CD105 with respective isotype control (n = 3). **Adipogenic differentiation.** (**C**) Representative sample of cells cultured in complete media. (**D**) Staining of neutral fat lipid droplets to confirm adipogenesis was detected by Oil Red O stain for cells cultured in media with inducers. **Osteogenic differentiation.** (**E**) Cells cultured in complete media. (**F**) Extracellular calcium deposits were determined by Alizarin Red stain in cells cultured in complete media with inducers. Scale bar 100 µm. C – F photographs at 20X magnification.

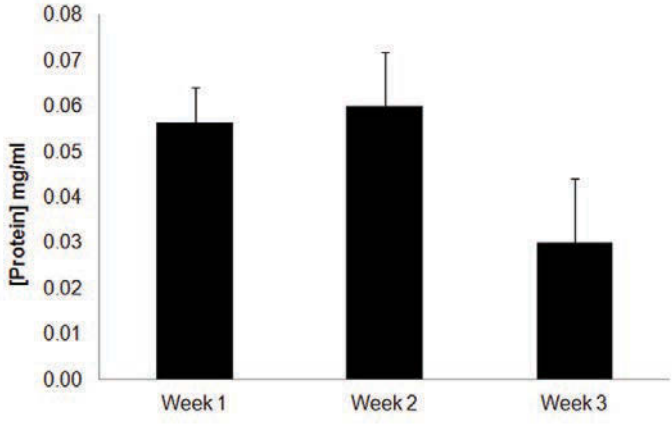

Figure 3. Aggregate total protein quantity. Bicinchoninic acid protein (BCA) quantity of cell lysate reported in mg/ml for 250 x 10^3 cell aggregate formed at day zero by centrifugation and cultured in chondrogenic differentiation media during one, two or three weeks. Values are the mean ± sem. ANOVA $p > 0.05$

CM was not detected for all three weeks of culture. For all three time points (week 1, week 2, and week 3) total GAGs were higher for aggregates cultured in chondrogenic differentiation media compared to MSCs in CM (**Figure 4 A**). Furthermore, DMB results normalized to total protein had the same trend as total GAG concentration for aggregates cultured in chondrogenic differentiation media (**Figure 4 B**). Normalization of DMB results to total protein could not be performed for aggregates cultured in CM, given that total protein was not detected.

Since total protein values for aggregates cultured in CM were undetected we proceeded to analyze total GAGs to compare aggregates cultured in CM vs. chondrogenic differentiation media by microscopy histological analysis of Alcian blue based on two blinded observers with a grading system from zero (no stain) to three. Positive Alcian blue evidenced a significant higher grade for all aggregates cultured in chondrogenic differentiation media compared to aggregates cultured in CM for the same time points (ANOVA $p < 0.05$), suggesting chondrogenic differentiation (**Figure 5**). In addition these results had a comparable trend to that observed for total GAGs determined by DMB, and DMB normalized to protein. Furthermore, Alcian blue stain based on percentage of positive stain determined by ImageJ analysis also evidenced a significant higher percentage of positive area for aggregates in chondrogenic differentiation media compared to sections from aggregates cultured in CM during all three time points (ANOVA $p < 0.05$), again confirming chondrogenic differentiation. The trend for total GAGs determined by Alcian blue percentage analyzed by ImageJ for aggregates cultured in chondrogenic differentiation media was different from that obtained by DMB and DMB normalized to total protein. Alcian blue percentage decreased with time with a gradual diminishment from 19.50 ± 1.15 to 8.77 ±1.13. Additionally, highly significant differences were also observed among aggregates in chondrogenic differentiation media between the first and second week compared to the third week of culture (**Figure 6**).

Last, IHC evaluations using either blinded grading or ImageJ percentage analysis were similar for aggregates under chondrogenic differentiation media. For both types of analysis i.e blinded observer and ImageJ, a non significant decreasing tendency with time for aggrecan's IHC marker assessment was observed. Furthermore, ImageJ IHC analysis had an analogous tendency compared to that of Alcian blue percentage also determined by ImageJ. The opposite effect was observed for aggregates in CM with an increase in IHC marker determination for both methodologies (**Figure 7**).

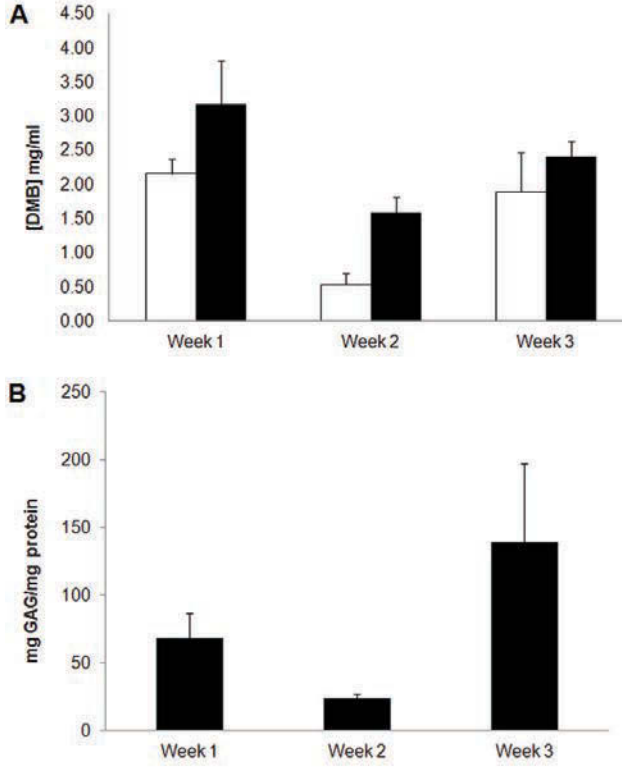

Figure 4. Total sGAG. Biochemical composition of MSC aggregates on days 7, 14, and 21 of culture. Total GAG content was measured with dimethylmethylene blue method (DMB). (**A**) White bars represent aggregates cultured in complete media (CM). Black bars represent aggregates cultured in chondrogenic differentiation media. No significant differences were observed between media during three weeks of culture (**B**) DMB values (mg/ml) normalized to total protein (mg/ml) for aggregates cultured in chondrogenic differentiation media. One way ANOVA $p > 0.05$.

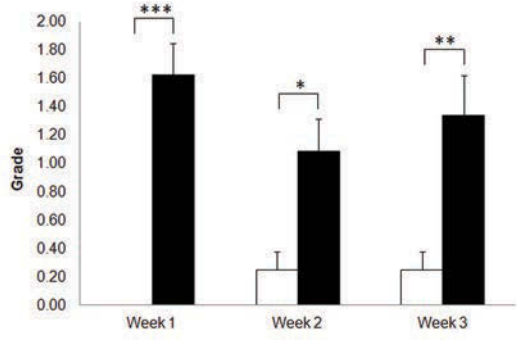

Figure 5. Alcian blue blinded observer score. Cells cultured in aggregates in CM (white bars) or chondrogenic differentiation media (black bars) during first, second and third week of culture. Stain was qualified based on a grading system from zero to three (0 = no stain). One way ANOVA * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

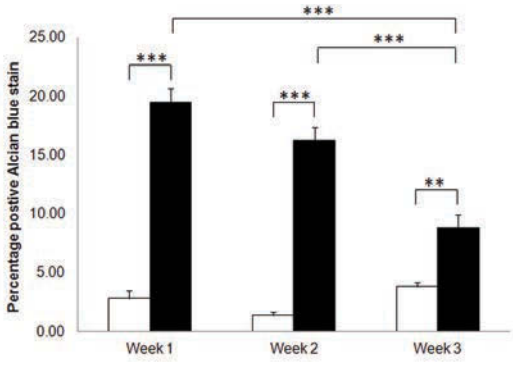

Figure 6. Alcian blue positive stain percentage using ImageJ. Cells cultured in aggregates in CM (white bars) or chondrogenic differentiation media (black bars) during first, second and third week of culture. Percentage was determined as described in detail in Materials and Methods section. One way ANOVA ** $p < 0.01$, *** $p < 0.001$.

When comparing aggrecan's IHC results between blinded observer grade and ImageJ percentage a starker contrast between CM and chondrogenic differentiation media was observed for the ImageJ analysis. No significant differences were observed for blinded observer or ImageJ analysis.

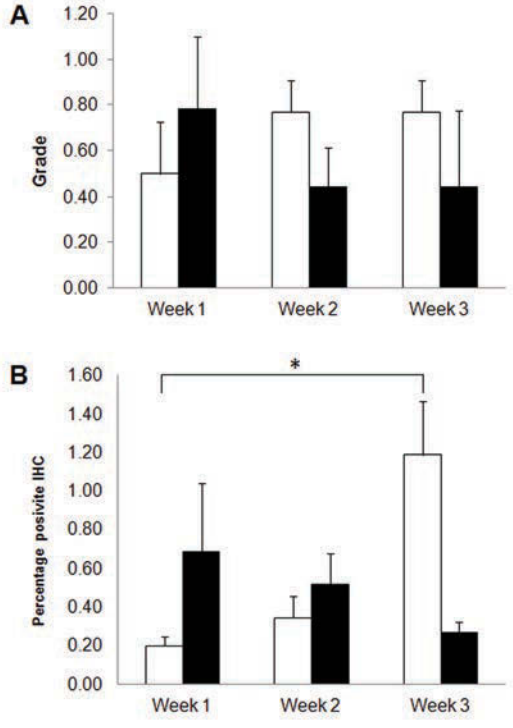

Figure 7. Aggrecan IHC score. IHC aggrecan marker in MSCs cultured in complete (white bars) or chondrogenic differentiation media (black bars) during three week aggregate culture. (**A**) **Blinded observer grade.** One way ANOVA $p > 0.05$. (**B**) **ImageJ percentage.** One way ANOVA * $p < 0.05$.

Discussion

We developed a functional system for semi-automatic slide grading for MSCs *in vitro* chondrogenic differentiation using ImageJ, a public domain, Java-based image processing program developed by the National Institutes of Health in 1997 (20). This open-source software permits cutting-edge image processing and can be used as an indispensible support for researchers in different fields. To our knowledge this methodology innovates in the way histological slides are evaluated for *in vitro* chondrogenic determination. We corroborated image analysis results by biochemical analysis with comparable results. For histochemical procedures color segmentation was applied, with color filter values selected by the evaluator. Furthermore, we developed a novel IHC quantification technique not relying on color segmentation based on RGB intensities, but rather on hue saturation and brightness (HSB). Consequently, this procedure could be used for other applications in the biological and medical fields depending on the user's need.

Chondrogenesis was activated by a commercial available media supplemented with IGF-1, insulin, transferrin, TGF-β1 TGF-β3 and BMP-6, all are known chondrogenic inducers (21-23). Chondrogenic differentiation was confirmed by Alcian blue stain by blinded observer evaluation and ImageJ percentage determination. Spheroids cultured for three weeks in CM had a lower grade (blinded observer) or lower percentage (ImageJ) compared to aggregates in chondrogenic differentiation media. Thus, confirming that inducers had an effect on total GAG synthesis during aggregate culture, and suggest cells in chondrogenic differentiation media were undergoing chondrogenic differentiation process.

For Alcian blue stains in aggregates cultured in complete or chondrogenic differentiation media we calculated the percentage difference in standard error mean (sem) between ImageJ and conventional blinded observer methodology (**Table 1**). Results obtained using ImageJ software demonstrated a greater than 50% improvement in relation to conventional blinded observer methodology. These results suggest that error can be substantially reduced when using a more objective methodology for image analysis and scoring.

Our results obtained by histological analysis were supported by biochemical assays that proved aggregates cultured in chondrogenic differentiation media had a higher GAG concentration in their ECM compared to those cultured in CM. These results agree with those previously reported in the literature (17). Furthermore, DMB results for CM could not be normalized to total protein, because of undetectable protein values. These results evidence the

Table 1. Alcian blue Percentage Improvement.

Aggregates cultured in complete or chondrogenic differentiation media were assayed at days 7, 14 or 21 for either media. Improvement analysis referred to as percentage improvement was calculated as described in materials and methods section. IJ: ImageJ. BO: Blinded observer. N.D. Not determined.

Alcian blue		
Media	**Time of culture (days)**	**IJ vs. BO percentage improvement**
Complete media	7	N.D
	14	53.38%
	21	79.34%
Chondrogenic Differentiation media	7	56.87%
	14	68.25%
	21	39.52%

limitations of biochemical assays for very small samples. Since chondrogenic differentiation characteristics based on biochemical criteria presented an obstacle (undetectable protein for aggregates in CM) we developed this semi-automatic functional system using ImageJ to perform a more objective and accurate assessment of histochemical stains and IHC marker to determine *in vitro* chondrogenesis.

For biochemical assays we used DMB method as described by Farndale with modifications (24). This highly sensitive technique permitted to assay total GAGs concentration from 5 µg/ml to 50 µg/ml from a sample with low protein quantities, as those obtained in this work from a lysed aggregate with a range of protein content from 0.03 ± 0.01 mg/ml to 0.060 ± 0.01 mg/ml. We observed total protein did not have a significant change throughout the chondrogenic process. This suggests cellularity remained similar during the three weeks when aggregates were cultured in chondrogenic differentiation media.

Moreover, we normalized total GAGs to total protein. Using total protein to normalize total GAGs provides advantages such as cost reduction and a less cumbersome methodology compared to other parameters such as total DNA (9, 25). On the other hand, this technique is not sensitive enough to quantify very low protein values. This could explain why we were unable to detect protein for CM, and could not normalize DMB results for aggregates cultured in CM.

For aggrecan's IHC we observed ImageJ methodology had a better performance for aggregates cultured in chondrogenic media compared to MSCs cultured in CM (**Table 2**). Again, as for the Alcian blue stain this technique presents greater advantages as compared to conventional grading methodologies.

Table 2. Aggrecan IHC Percentage Improvement.

Aggregates cultured in complete or chondrogenic differentiation media were assayed at days 7, 14 or 21 for either media. Improvement analysis referred to as percentage improvement was calculated as described in the materials and methods section. IJ: ImageJ. BO: Blinded observer.

Aggrecan IHC		
Media	**Time of culture (days)**	**IJ vs. BO percentage improvement**
Complete media	7	43.39 %
	14	No improvement
	21	No improvement
Chondrogenic Differentiation media	7	No improvement
	14	18.31 %
	21	70.50 %

Even though the objective of this study was to establish a semi-automatic grading system; contrasting results between total GAGs and aggrecan detection by IHC are important for their biological implications. Aggrecan is composed of two types of GAGs, a small keratan sulfate domain and a larger chondroitin sulfate domain (26). We observed a gradual aggrecan decrease with time for aggregates cultured in chondrogenic differentiation media in contrast to GAG/protein biochemical analysis with an increase for the last week of culture. Aggrecan is not the only GAG synthesized during chondrogenesis (27). Other GAGs, such as heparan sulfate, dermatan sulfate and syndecan, could be involved in the process, probably explaining these contrasting results.

During the earlier stages of chondrogenesis mesenchymal cells differentiate into chondrocytes and begin to synthesize collagen type 2 and aggrecan. This event sets the stage for mesenchymal cell differentiation into chondrogenesis (28). In a report of human adipose MSCs undergoing chondrogenic differentiation aggrecan expression was restricted to early events, days seven and ten, and was specific to aggregates under chondrogenic induction (17). Other studies with human adipose derived MSCs also have demonstrated aggrecan's expression after seven days of culture in chodrogenic differentiation media (11, 22). Aggrecan synthesis initiates during chondroprogenitor proliferation and differentiation and its synthesis stops with terminal differentiation (1). Thus,

our blinded observer and ImageJ percentage analysis results for aggregates cultured in chondrogenic media agree with reports in the literature regarding aggrecan's expression. These results demonstrate the biological importance of computer assisted quantification of histological results during *in vitro* chondrogenesis that corroborate with biochemical and immunological attributes to determine differentiation.

For histological analysis images were captured by conventional microscopy. This technique permitted to obtain for most slides the entire section at 20X magnification with low resolution. At higher magnification (40X) images have higher resolution, but the section cannot be captured completely. In order to provide a solution we used robotic microscopy. We compared at random captured images from conventional microscopy and robotic microscopy and observed an improvement with the latter. A comparison between both microscopy capture methodologies for Alcian blue stain is depicted in Figure 8. On panel A, B, and C we observe images captured by robotics microscopy. As can be seen, robotics captured images appear as if part of the background were missing. This is the result of the assembling process, since each section is captured, according to the algorithm designed, and the assembled image does not guarantee an image with a homogenous background. The robotics image capture provides the researcher several advantages over images captured with a conventional microscope. For one, it allowed greater detail, since the complete section can be integrated as one image. In contrast, conventional microscopy, as that observed by panel D does not permit this type of detail. In order to capture the entire aggregate section the objective must be at 20X. For larger slide sections using conventional microscopy capture techniques, for example those a pathologist might encounter, integration of images would have to be carried out manually. Collectively, robotics microscopy improves detail, because it integrates the complete section at 40X magnification with aid of an algorithm developed by BioIngenium engineers. This complete image can then be analyzed by ImageJ.

Panels C and D in figure 8 demonstrate ImageJ analysis percentage determination. The area that was positive for Alcian blue stain is highlighted in red. This is more easily observed for panel D, where the aggregate was cultured in chondrogenic differentiation media. This methodology permits more objectivity compared to blinded observer

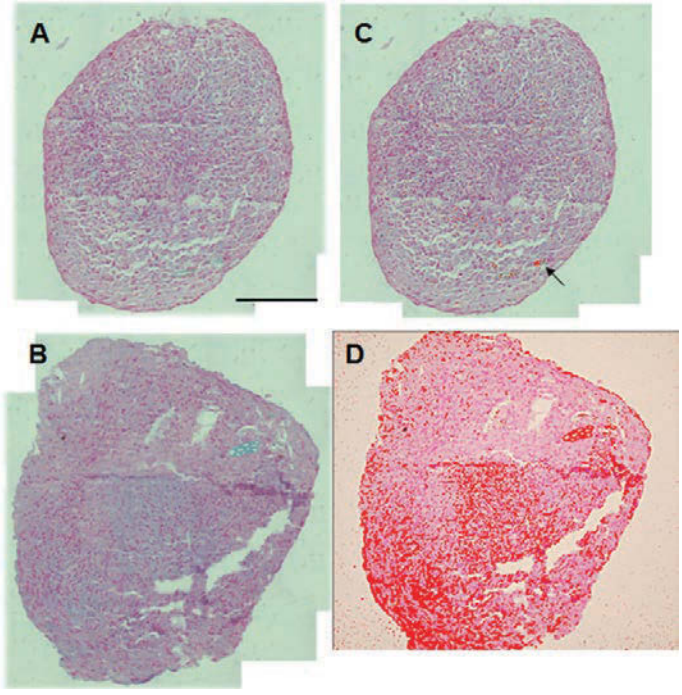

Figure 8. Alcian blue stain analysis using ImageJ. Aggregates cultured in complete **(A and C)** or chondrogenic differentiation media **(B and D)** for 14 days. **(A)** Aggregate section of representative sample during two weeks of culture in complete media. Digitally photographed at high resolution (40X) by automated system. Captures were assembled into one image by algorithm designed by BioIngenium. Scale bar 100 µm. **(B)**. Representative sample of aggregate cultured in chondrogenic differentiation media. **(C)** Color segmentation to determine threshold values for capture in A. **(D)** Color segmentation to determine threshold values for capture in B.

evaluation, because the observer by using ImageJ can quantify in a semi-automatic manner the slide section and give a percentage of positive stain or IHC chondrogenic marker to determine differentiation.

Although techniques for quantifying histological stains have been reported (18, 19, 29), recent development in automated image analysis describe the use of sophisticated digital capture of histological slides. This type of equipment cost can range from $ 100,000 to $ 300,000 US dollars. Furthermore, IHC analysis requires use of DAB deconvolution to digitally separate different stains, and some researchers describe using methods implementing ImageJ macro (18). Other image processing programs offer an image processing toolbox that can be used for color space conversion and image filtering, such as MATLAB, yet this software must be purchased. In this report we present an option for capturing images at high resolution and analyzing them using free software.

Conclusion

We developed a functional system for semi-automatic slide grading, and corroborated these results by biochemical analysis with comparable results for *in vitro* chondrogenesis. This computer assisted quantification provided a percentage improvement compared to blinded observer methodology. To our knowledge this is the first report to evaluate stains using this methodology providing a quantifiable and impartial technique to assess *in vitro* differentiation. In addition, automated image analysis enables improved detail and accuracy for slide assessment. This methodology represents an advantage for the researcher analyzing and scoring slide sections. Overall, these findings suggest that semi-automatic grading system using ImageJ is a more objective way to analyze microscopy images and can be applied to other fields.

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Conflicts of Interest

The authors indicate no potential conflicts of interest.

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