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# Osmolarity regulates chondrogenic differentiation potential of synovial fluid derived mesenchymal progenitor cells

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ABSTRACT

Cartilage is one of few tissues where adult stem/progenitor cells have not been putatively identified. Recent studies have provided strong evidence that a sub-population of mesenchymal progenitor cells (MPCs) derived from the synovial fluid may be able to affect some degree of cartilage repair both *in vivo* and *in vitro/ex vivo*, however this does not appear to be the case in patients with arthritis. Previously, it has been found that synovial fluid osmolarity is decreased in patients with osteoarthritis (OA) or Rheumatoid arthritis (RA) and these changes in osmolarity have been linked to changes in chondrocyte gene regulation. However, it is yet unknown if changes in osmolarity regulate the gene expression in synovial fluid MPCs (sfMPCs), and by extension, chondrogenesis of this cell population. In the present study we have collected synovial fluid samples from normal, OA and RA knee joints, quantified the osmolarity of the fluid and modified the culture/differentiation media to span a range of osmolarities (264–375 mOsm). Chondrogenesis was measured with Alcian blue staining of cultures in addition to quantitative PCR (qPCR) using probes to Sox9, ACAN and Col2A1. Overall, sfMPCs from arthritic joints demonstrated decreased chondrogenic potential compared to sfMPCs isolated from normal synovial fluid. Furthermore, the sfMPCs retained increased chondrogenic potential if differentiated under the same osmolarity conditions for which they were initially derived within. In conclusion, it does appear the synovial fluid osmolarity regulates the chondrogenic potential of sfMPCs, however, further study is required to elucidate the mechanism by which the changes in osmolarity are sensed by the cells and regulate chondrogenic gene expression.



## 1. Introduction

Articular cartilage is a remarkably resilient tissue that ordinarily lasts a lifetime. Although it was long treated as an inert material, it is clear that articular cartilage is a living tissue that undergoes constant regeneration [1,2]. However, the exact mechanism whereby articular cartilage regenerates a functional extracellular matrix remains obscure. Mesenchymal progenitor cells (MPCs) found within the synovial membrane and synovial fluid may affect some level of cartilage repair *in vivo*, however, this does not appear to be the case in diseases such as OA and RA. Previous studies have demonstrated changes in MPC characteristics in diseased joints such as those afflicted with OA and/or RA [3–6]. Importantly though, in these studies the MPCs are derived, cultured and differentiated under near identical conditions (media components, growth factors, etc.), which is normally considered as "best practice" for cell culturing and experimental design. In this case, however, it is important to realize the *in vivo* synovial environment within a diseased joint is quite different in normal joints, not only in regards to cytokines, chemokines and other biologic factors [7,8], but also in regards to the physiological properties. For example, the synovial fluid osmolarity can dramatically change with the onset of joint disease [9], with synovial fluid ranging from 404 ± 57 mOsm in normal joints, to 297 ± 16.9 mOsm and 280 ± 7.7 mOsm in OA and RA joints respectively [9]. The effect of changes in osmolarity has been previously studied in-depth on chondrocyte cell populations present in articular cartilage, and based on these results, it appears that osmolarity can indeed trigger regulate gene expression (Sox9 in particular) in these cells [10–12]. The effects of osmolarity on chondrocyte ECM synthesis have been investigated with varied results [13,14]. It has been shown that chondrocyte ECM synthesis can be decreased either with the application of hyper- or hypo-osmotic conditions, or in a recent study hypo-osmotic stresses resulted in increased expression of cartilage ECM genes [15]. However, to date, no studies have been published examining the effects of osmolarity on sfMPCs. Based on recent

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*in vivo* evidence suggesting that these cells may play an active role in cartilage repair/regeneration [16–18], it is of paramount importance to understand how the joint environment, in this case osmolarity, regulates the chondro-potential of this stem/progenitor cell population. Therefore, we have undertaken a study to examine the effects of physiological ranges (normal and arthritic) of osmolarity on the derivation, cell morphology, behavior and chondrogenic differentiation potential of synovial fluid derived progenitor cells.

## 2. Methods and materials

### 2.1. Patient criteria

Informed consent to participate was obtained by written agreement. The study protocol was approved by the University of Calgary Research Ethics Board. *Normal Group*: Inclusion criteria for control cadaveric donations were an age of 30 years or older, no history of arthritis, joint injury or surgery (including visual inspection of the cartilage surfaces during recovery), no prescription anti-inflammatory medications, no co-morbidities (such as diabetes/cancer), and availability within 4 h of death. The Southern Alberta Organ and Tissue Donation Program (SAOTDP) screens the medical history of every donor including current medication, eliminating individuals with a previous history of joint disease and other co-morbidities (e.g. cancer, diabetes, inflammatory diseases). *OA and RA group*: Inclusion criteria were an age of 30 years or older, OA diagnosed based on the American College of Rheumatology [19] criteria with X-ray documentation, and no evidence of autoimmune disease or RA, RA diagnosed based on the American College of Rheumatology criteria [20].

### 2.2. Outline of experimental methods

A flow chart has been provided that described the methods used in this study Fig. 1.

### 2.3. Cell derivation

The fresh synovial fluid was plated in untreated culture dishes and after 1–2 h at 370 °C/5%$CO_2$ culture media was added. Culture media consisted of DMEM (Invitrogen # 11965), 10% FBS, 1% Pen/Strep, 1% Non-essential amino acids (NEAA), 0.2% Beta-mercaptoethanol (BME) (all Invitrogen, Carlsbad, CA). Once cells had adhered to the plastic and reached 30–40% confluence, the media was changed and the cells were allowed to reach 60–70% confluence. At this point the cells were dissociated and resuspended in Dulbecco's PBS (DPBS) at 1 million cells/ml. Progenitors were isolated using magnetic separation. First the total cell population was depleted for cells expressing CD3, CD14, CD16, CD19, CD41a, CD56 and Glycophorin A (All Becton, Dickinson and Company (BD), Franklin Lakes, NJ). The resultant cell population was then purified for cells expressing CD90 (BD), and induced to differentiate.

### 2.4. Chondrogenic differentiation

sfMPCs were plated in triplicate (100,000 cells/well/24 well dish) and exposed to chondrogenic media for 21 days with prior micro-mass aggregation. Differentiation media consisted of culture media with 500 ng/mL BMP-2 (Peprotech, Rocky Hill, NJ), 10 ng/mL TGF-β3 (Peprotech), $10^{-8}$ M dexamethasone (Sigma, St. Louis, MO), 50 μg/mL ascorbic acid (Sigma), 40 μg/mL proline (Invitrogen), 100 μg/mL pyruvate (Sigma) and supplemented with insulin, transferrin and selenium (Sigma). Media was changed every three days during the 21 day differentiation period.

### 2.5. Osmolarity quantification and media adjustment

Two hundred micro-liters of native synovial fluid was placed mixed with 10ul (1 mg/ml) of hyaluronidase (Bovine derived, Sigma, St. Louis, MO.) to disrupt the viscous nature of the joint fluid [21]. The sample was then assayed with an Osmette A (freezing point depression) osmometer (Precision Scientific) that had been calibrated with known standards (100 mOsm and 500 mOsm). In the experiments performed, the osmolarity of the base DMEM was adjusted with NaCl or water so that when all the supplements were added the final osmolarity was either: 264, 273, 322, 337 or 375 mOsm. The osmolarity of the standard media used (described above) was consistently 300 mOsm.

### 2.6. Cell sizing

Sizing of the sfMPCs was performed using the Multi-sizer 3 (Beckman Coulter).

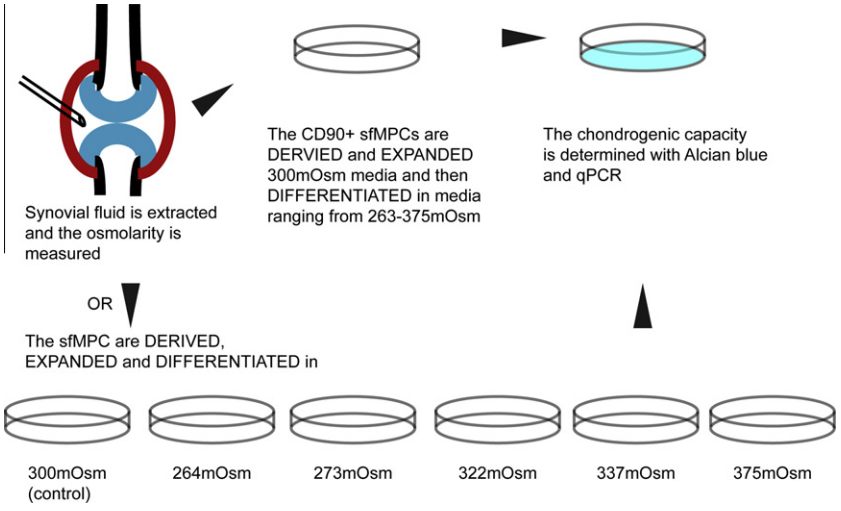



**Fig. 1.** Experimental outline flow chart. sfMPCs were derived from normal and arthritic synovial fluid and either derived and expanded in 300 mOsm media, or media ranging from 264–375 mOsm. The sfMPCs were then differentiated in media ranging from 264–375 mOsm and chondrogenic output was analyzed with Alcian blue staining and qPCR using col2A1, Sox9 and ACAN as chondrocytic specific markers.

### 2.7. Statistical analysis

Each treatment (cell differentiation) and qPCR was performed in triplicate. Statistical analysis (ANOVA) was performed on qPCR data using GraphPad Prism4 (GraphPad Software) and significance was set at $p < 0.05$.

## 3. Results

### 3.1. Synovial fluid osmolarity

Synovial fluid harvested from normal joints ranged from 295–340 mOsm, while values from OA joints ranged from 249–277 mOsm and RA joints ranged from 273–283 mOsm Fig. 2. While OA and RA values were not significantly different from each other, both OA and RA osmolarity values were significantly different were compared to normal joints Fig. 2.

### 3.2. sfMPC morphology under different osmolarities

sfMPCs derived from all patients were initially derived and expanded in control media (300 mOsm). After the sfMPCs were purified and expanded, they were seeded in dishes containing media with specific osmolarities ranging from 264–375 mOsm Fig. 2. While all cell lines derived acted in a similar fashion, the data from two representative cell lines (one derived from normal synovial fluid and one derived from arthritic synovial fluid: OA) is presented. There was no recognizable difference in cell morphology between normal and arthritic sfMPCs Fig. 2, and furthermore, no differences in morphology were observed when sfMPC were cultured under varying osmolarity conditions Fig. 2. No difference was observed between the sizes of sfMPCs cultured under low (264 mOsm) or high (375 mOsm) conditions Fig. 2, with the vast majority of the cells maintaining a cell diameter of 20 μm.

### 3.3. Chondrogenic potential of sfMPCs derived at 300 mOsm

sfMPCs from normal and arthritic joints were derived, purified and expanded at 300 mOsm then induced to differentiate in chondrogenic media ranging from 264–375 mOsm. After 21 days of differentiation, the sfMPC cultures were examined with Alcian blue staining and qPCR Fig. 3. Based on the Alcian blue staining we observed a decrease in chondrogenic potential in arthritic sfMPCs when compared to normal sfMPCs Fig. 3. Arthritic cells differentiated in 300 mOsm media displayed the most prominent Alcian blue staining, while Alcian blue staining in the normal sfMPCs was strongest at lower osmolarities (264, 273) in addition to the control 300 mOsm media Fig. 3.

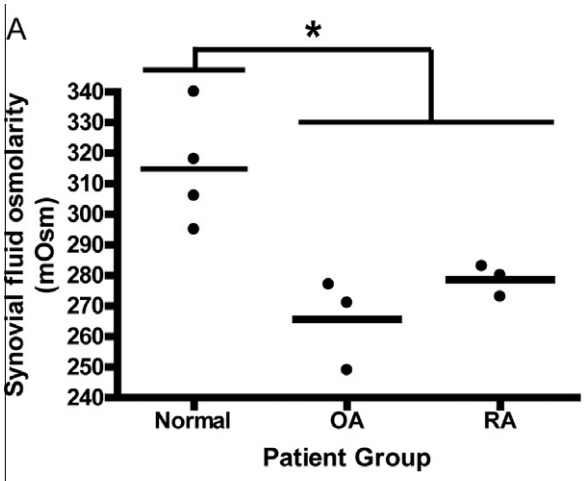
The qPCR data demonstrated similar results to what was observed with the Alcian blue staining. We normalized all qPCR data to 18s (housekeeping gene) and the changes in fold expression presented are relative to cells differentiated in 300 mOsm medium. In both cases (normal and arthritic), Sox9, Col2A1 and ACAN were uniformly down-regulated in all osmolarities tested when compared to cells differentiated at 300 mOsm (with some exceptions as observed in Fig. 3.

### 3.4. Chondrogenic potential of sfMPCs derived at multiple osmolarities

sfMPCs were derived, expanded and differentiated in one of seven specific osmolarities (264, 273, 300, 322, 337 or 375 mOsm) then assayed for chondrogenic potential with Alcian Blue or qPCR Fig. 4. The normal sfMPCs presented in Fig. 4 were derived from synovial fluid with an *in vivo* osmolarity of 318 mOsm. When the Alcian blue staining was examined, the cells appeared to lay down Alcian blue positive ECM in all conditions tested Fig. 4. qPCR data verified these results demonstrating increased expression of col2A1, Sox9 and ACAN under nearly all conditions tested, with the maximal col2A1 expression observed at 322 mOsm. When arthritic sfMPCs (derived from 249 mOsm synovial fluid) were examined, as before it was observed that less Alcian blue positive ECM was present (compared to normal sfMPCs) Fig. 4, however, the qPCR data demonstrated increased expression of col2A1, Sox9 and ACAN when the sfMPCs were derived, expanded and differentiated at 264 mOsm Fig. 4.

## 4. Discussion

It has been suggested in the literature that cartilage has little to no intrinsic repair capacity [22–24], however, a growing body of research is beginning to provide evidence that progenitor cells within the synovial joint environment maybe indeed contribute to endogenous cartilage repair [16–18]. These synovial mesenchymal progenitor cells can be isolated from either the synovial membrane (sm) or synovial fluid (sf). Although smMPCs and sfMPCs act very similarly in culture, no study to date has described phenotypic differences between the progenitor populations, and the origin of the sfMPCs is still unclear. In this study we have chosen to use sfMPCs for a number of reasons, first of all they are less invasive to procure that smMPCs as only a small joint fluid sample needs to be acquired, and secondly, previous research describing the chondrogenic capacity of these cells derived from normal and arthritic joints has been variable [3,25–27]. To potentially shed some light on the variability of this data, we have chosen to investigate the effect of differences between the *in vivo* environment and the *in vitro* culture environment. Past research has identified growth factors and cytokines can differentially expressed between normal, OA and RA synovial fluid [7,8], however, even in multiple studies focusing on progenitors derived from one group of patients (e.g. RA) have been inconsistent [3,27]. Therefore, we decided to examine if physical changes in the synovial fluid environment may regulate the chondrogenic potential of these sfMPCs. It is well known that oxygen tension [28,29], lubricating ability [30] and viscosity [31] of synovial fluid changes between normal and arthritic joints, however, few studies have examined changes in osmolarity [9,32]. In these limited studies it has been demonstrated that the osmolarity in normal joints is higher when compared to OA and RA joint fluid [9]. The effect of changes in osmolarity has been widely studied in regards to chondrocyte biology [10–15], but has not been examined in the context of synovial progenitors. A number of recent studies have demonstrated that chondrocytes respond to changes in osmolarity by regulating gene expression of chondrogenic transcription factors (Sox9) and/or constituents of the ECM (col2A1) [10–15]. Most of these studies suggest that increasing or decreasing the osmolarity of the culture fluid (from isotonic) results in a decrease of chondrogenic gene expression, however, a recent dissenting report suggests that chondrocytes may in fact increase gene expression of ECM related genes in response to hypo-osmotic solutions [15]. Based on these inconsistencies, additional research is required to identify the response of chondrocytes to osmolarity, however, it is clear that this cell population is in part regulated by changes in joint fluid osmolarity. Therefore, in this study we have examined if osmolarity can regulate the chondrogenic capacity of synovial fluid derived progenitors. To undertake this study it was first important to identify the physiological range of osmolarity present in normal and arthritic synovial fluid, this was essential since culturing sfMPCs in conditions they would never see *in vivo* is not relevant. When we compared our results Fig. 2 to those already published [9], we found that our values for OA and RA synovial fluid were comparable, however, our values




| Synovial fluid osmolarity (mOsm) | Patient Description |
|---|---|
| Normal (N=4) | |
| 340 | Male, 75 years of age |
| 318 | Male, 56 years of age |
| 306 | Female, 49 years of age |
| 295 | Male, 75 years of age |
| OA (N=3) | |
| 271 | Female, 33 years of age, right knee |
| 277 | Male, 64 years of age, right knee |
| 249 | Male, 81 years of age, both knees, left knee sampled |
| RA (N=3) | |
| 280 | Female, 48 years of age, left knee sampled, on Methotrexate |
| 273 | Female, 41 years of age, left knee sampled, on Methotrexate |
| 283 | Female, 32 years of age, right knee sampled, Abatacept |

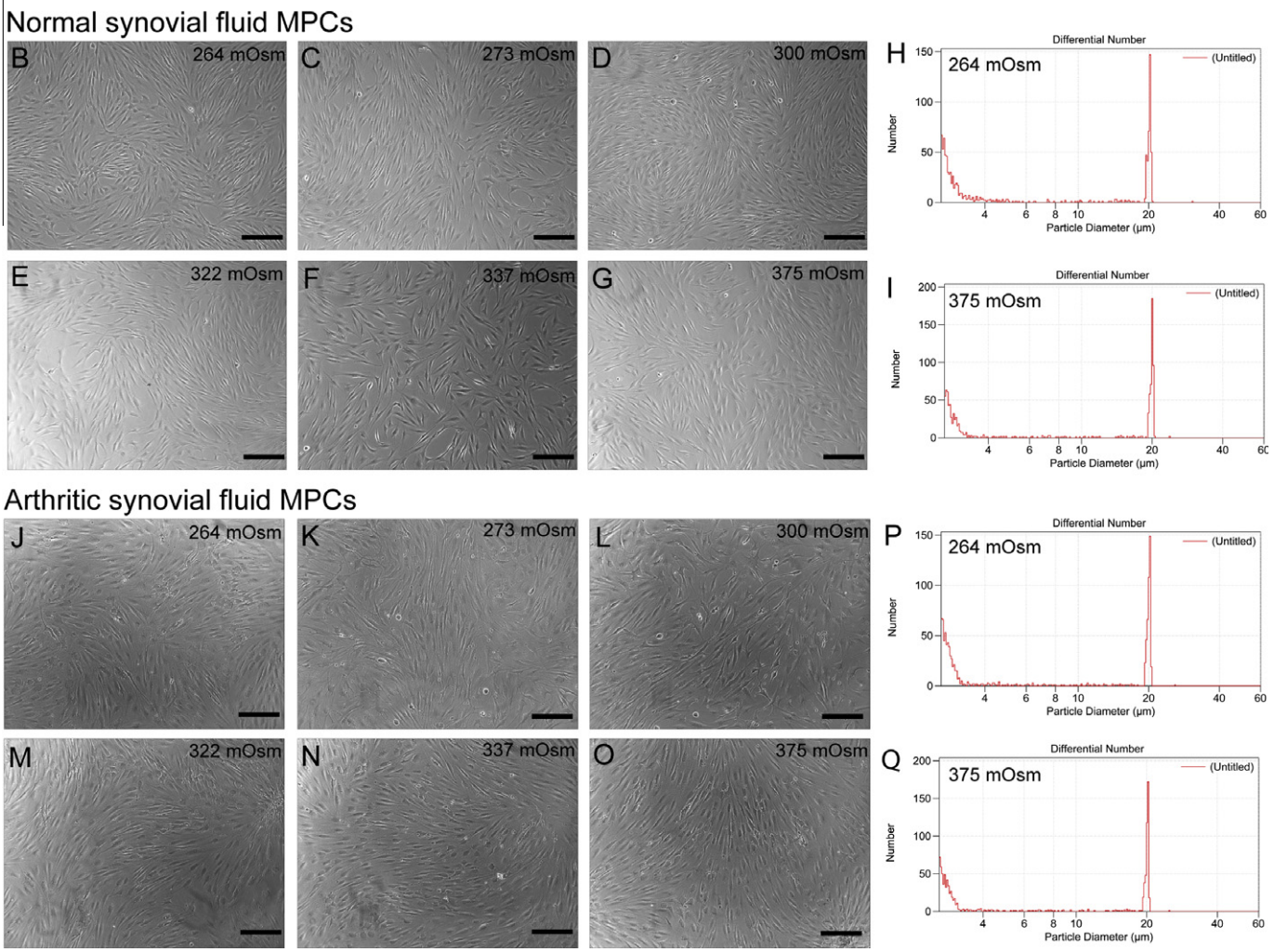



**Fig. 2.** Morphology of sfMPCs cultured under a range of osmolarity. Synovial fluid was derived from normal (4), OA (3) and RA (3) patients and the osmolarity was quantified (A). Normal (B–G) and arthritic (J–O) sfMPCs were cultured under a range of osmolarities and the morphology was analyzed with bright-field microscopy. The size of normal (H,I) and arthritic (P,Q) sfMPCs under all osmolarity conditions was measured and no changes were observed (264 and 375 mOsm shown).

for normal synovial fluid were noticeably lower that previously published. This could be the result of how the osmolarity was measured as we used a freezing point osmometer and most other studies published on synovial fluid use vapor–pressure osmometry [9,32]. While vapor–pressure methods are normally used for viscous solutions, freezing point depression is considered to be more accurate. Regardless, both our study and previous ones have agreed that a range of 250–375 would be considered physiological for synovial fluid. Therefore we examined the response of sfMPCs to a range of osmolarities that included: 264, 273, 300, 322, 337

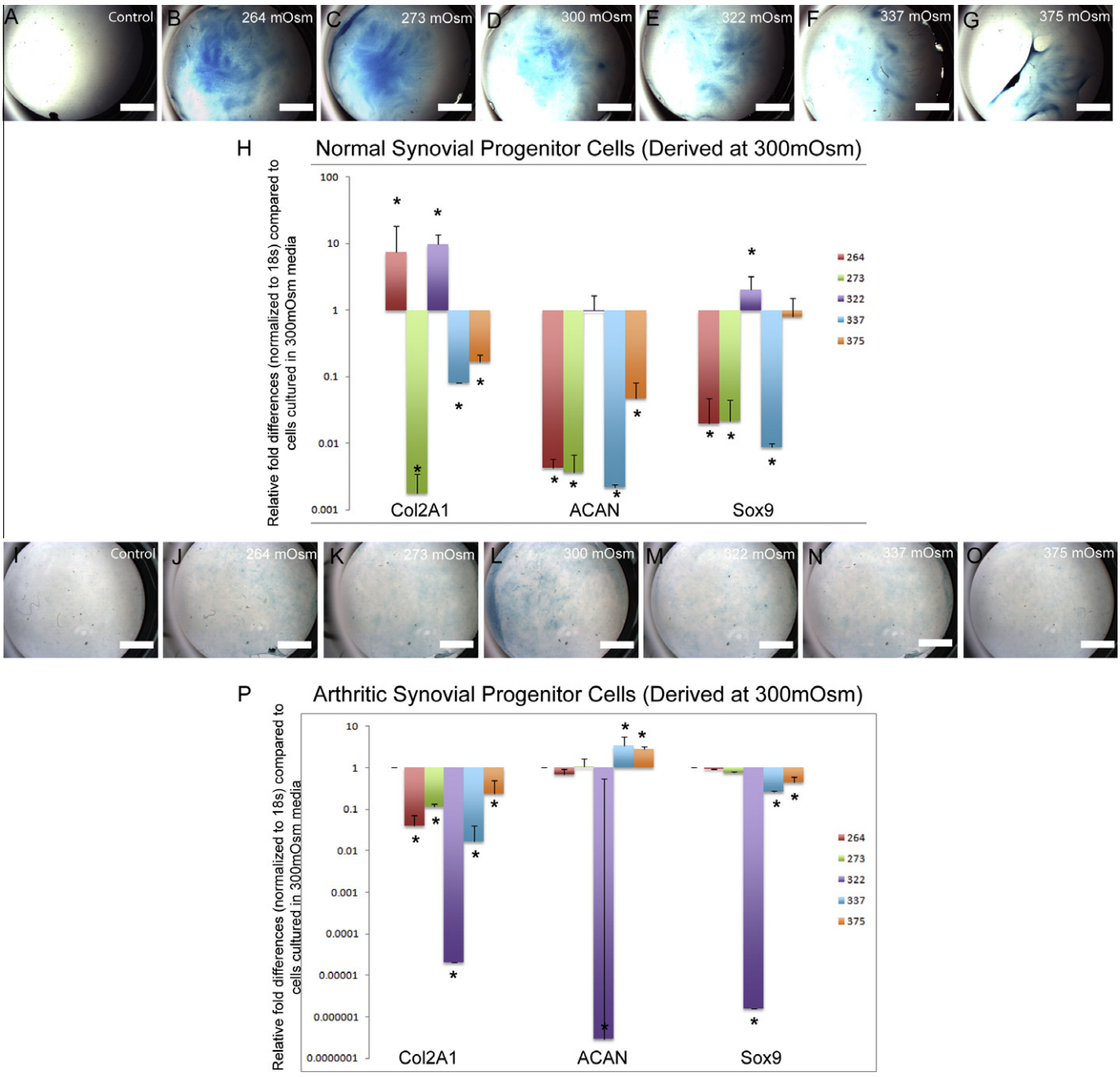



**Fig. 3.** Chondrogenic potential of sfMPCs derived and expanded at 300 mOsm. Normal (A–H) and arthritic (I–P) sfMPCs were derived and expanded in standard culture media (300 mOsm). The sfMPCs were then differentiated in chondrogenic media ranging from 264–375 mOsm. Alcian blue staining of normal (A–G) and arthritic (I–O) demonstrates the deposition of proteoglycans, except in control media without chondrogenic factors present (A,I). qPCR of sfMPCs derived at 300 mOsm with col2A1, Sox9 and ACAN as chondrogenic markers (H,P). Alcian blue and qPCR data suggests that normal (D,H) and arthritic (L,P) sfMPC most efficiently differentiated at 300 mOsm. $^{*}p < 0.05$.

and 375 mOsm. Although we attempted to generate media with an osmolarity of less than 264 mOsm, this was not possible once all the media supplements were added, for example, when the media supplements (FBS, amino acids, etc.) were added to water, the osmolarity was found be just over 250 mOsm. When we cultured sfMPCs derived from the same individual across this range of osmolarity, no changes in morphology or cell diameter were observed Fig. 2. This result seems to suggest that sfMPCs have tight control over their intercellular ion stores and can stringently balance the flow of water and ions across the plasma membrane over a wide range of environmental osmolarity. Little is actually known about the ion channels present on synoviocytes in general, and no studies have been published describing the ion channel present on the progenitor populations in the synovial member of synovial fluid, however, based on this result, further studies should be undertaken to determine which channels are responsible for this observed homeostasis under a wide range of osmolarity.

When we examined the chondrogenic potential of normal and arthritic sfMPCs derived at 300 mOsm and then differentiated over a range of osmolarity, we observed that normal sfMPCs exhibited increased chondrogenic capacity when compared to arthritic sfMPCs (including OA and RA derived cells). Interestingly, all sfMPCs tested demonstrated maximal chondrogenic potential at 300 mOsm and this did not appear to differ among patient populations. This

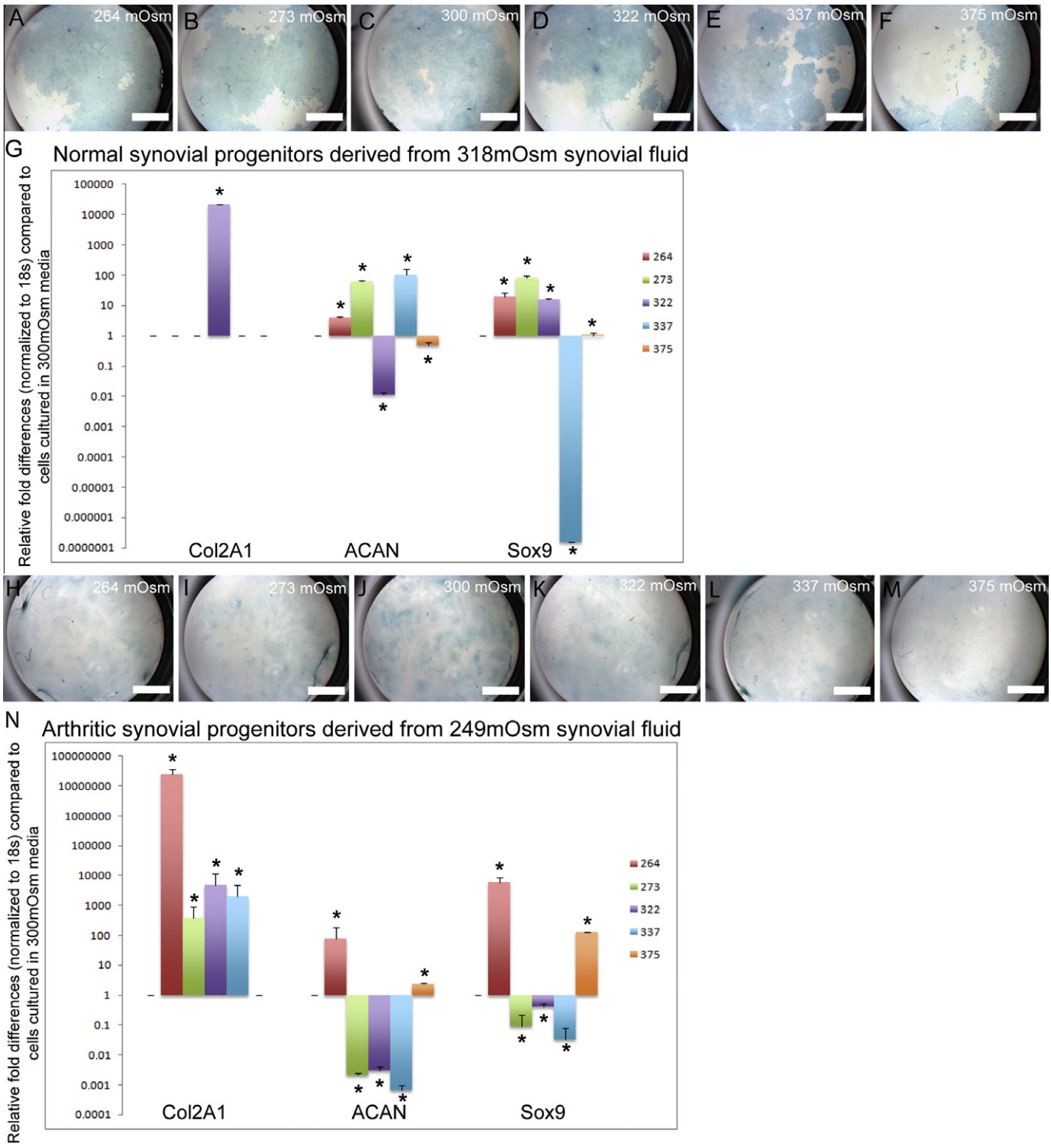



**Fig. 4.** Chondrogenic potential of sfMPCs derived, expanded and differentiated at various osmolarities. The sfMPCs were derived, expanded and differentiated in media ranging from 264–375 mOsm. Alcian blue staining of normal (A–F) and arthritic (H–M) demonstrates the deposition of proteoglycans. qPCR of sfMPCs derived and differentiated at 264–375 mOsm with col2A1, Sox9 and ACAN as chondrogenic markers (G,N). Alcian blue and qPCR data suggests that normal (G) and arthritic (N) sfMPC most efficiently differentiated at the osmolarity that replicated the osmolarity of the synovial fluid *in vivo*. $^{*}p < 0.05$.

strongly suggests that we have selected for a sub-population of progenitors that thrive at 300 mOsm, and that these selected sfMPCs were not as potent at differing osmolarities. This result is particularly interesting when we examine it in the context of the data obtained from deriving sfMPCs in varying osmolarities (including the physiological level of the original synovial fluid: which was variable between patients), where we have found that sfMPCs retain maximal chondrogenic differentiation capacity when derived, expanded and differentiated in an *in vitro* environment that mimics the endogenous *in vivo* osmolarity. These results, taken together,

suggest that sfMPCs retain maximal *in vitro* chondrogenic potential, when cultured in an environment that best mimic the osmolarity of the synovial fluid *in vivo*.

Even potentially more important is the consequences of lower synovial fluid osmolarities *in vivo*. When comparing all sfMPC populations, it was clear that normal sfMPCs had increased chondrogenic capacity vs. arthritic sfMPCs (OA & RA), we propose that *in vivo* changes in osmolarity may act as a selection pressure on sfMPCs, possibly selecting for a sfMPC sub-population that exhibits decreased chondrogenic potential. Although, this may explain the lack of endogenous cartilage repair in arthritic joints (in part), this hypothesis will require further study before any conclusions can be drawn.

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