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Thermally labile components of aqueous humor potently induce osteogenic potential in adipose-derived mesenchymal stem cells

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Abstract

Adipose-derived mesenchymal stem cells (ASCs) hold promise for use in cell-based therapies. Their intrinsic anti-inflammatory properties are potentially useful for treatments of inflammatory conditions such as uveitis, while their ability to differentiate along multiple cell lineages suggests use in regenerating damaged or degenerated tissue. However, how ASCs will respond to the intraocular environment is poorly studied. We have recently reported that aqueous humor (AH), the fluid that nourishes the anterior segment of the eye, potently increases alkaline phosphatase (ALP) activity of ASCs, indicating osteogenic differentiation. Here, we expand on our previous findings to better define the nature of this response. To this end, we cultured ASCs in the presence of 0, 5, 10, and 20% AH and assayed them for ALP activity. We found ALP activity correlates with increasing AH concentrations from 5 to 20%, and that longer treatments result in increased ALP activity. By using serum free media and pretreating AH with dextran-coated charcoal, we found that serum and charcoal-adsorbable AH components augment but are not required for this response. Further, by heat-treating the AH, we established that thermally labile components are required for the osteogenic response. Finally, we showed myocilin, a protein present in AH, could induce ALP activity in ASCs. However, this was to a lesser extent than untreated 5% AH, and myocilin could only partially rescue the effect after heat treatment, documenting there were additional thermally labile constituents of AH involved in the osteogenic response. Our work adds to the understanding of the induction of ALP in ASCs following exposure to AH, providing

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important insight in how ASCs will be influenced by the ocular environment. In conclusion, increased osteogenic potential upon exposure to AH represents a potential challenge to developing ASC cell-based therapies directed at the eye.

Keywords

aqueous humor; mesenchymal stem cells; osteogenic potential; myocilin; alkaline phosphatase

1. Introduction

Mesenchymal Stromal/Stem Cells (MSCs) have been derived from a variety of tissue sources including adipose tissue (Bourin et al., 2013). MSCs are characterized by expression of CD73, CD90, CD105, CD146 surface markers, lack expression of CD31, CD34, or CD45 surface markers, and are capable of tri-lineage differentiation into adipocytes, osteocytes, chondrocytes (Dominici et al., 2006; Horwitz et al., 2005). Additionally, MSCs do not express MHC II, making them ideal for allogenic as well as autologous cell therapies. Low immunogenicity and their differentiation potential positions MSCs for regenerative medicine applications including bone and tendon repair (Caplan, 2013). Furthermore, MSCs have also been shown to be immunomodulatory and have been investigated in clinical trials for the treatment of immune mediated diseases (Nauta and Fibbe, 2007; Voswinkel et al., 2013). Adipose-derived MSCs (ASCs) are an especially promising source for use in regenerative medicine, as they can be acquired in high numbers from minimally invasive lipectomy procedures (Zuk et al., 2001).

The promise of cell therapies in general and ASC therapy specifically is no less apparent in the eye (Joe and Gregory-Evans, 2010; Rajashekhar, 2014). Degenerative ocular diseases such as diabetic retinopathy, age-related macular degeneration (AMD), and glaucoma are candidates for cell replacement therapies. Additionally, these diseases have known immune components in their progression, which could potentially be ameliorated by the immunosuppressive effects of ASCs. Several recent publications have highlighted the potential for cell therapy in the eye. MSCs have been successfully differentiated in retinal progenitor cells (Moviglia et al., 2012), keratocyte-like cells (Arnalich-Montiel et al., 2008; Liu et al., 2012; Park et al., 2012), corneal endothelial-like cells (Joyce et al., 2012), retinal pigmented epithelium cells (Vossmerbaeumer et al., 2009), and photorecptors (Kicic et al., 2003). Further, MSCs have been used to treat models of glaucoma (Johnson et al., 2010; Manuguerra-Gagne et al., 2013), retinopathy (Chung et al., 2011; Inoue et al., 2007; Jiang et al., 2014; Machalinska et al., 2013), autoimmune uveoretinitis (Li et al., 2013; Zhang et al., 2011), and corneal wounds (Arnalich-Montiel et al., 2008; Jia et al., 2012; Oh et al., 2008; Yao et al., 2012). The bulk of these studies focus on how MSCs influence the ocular environment and limited consideration is given to how the ocular environment influences the behavior of MSCs.

We have recently reported that aqueous humor (AH), the fluid that nourishes the tissues adjoining the anterior segment of the eye, potently stimulates osteogenic potential in ASCs (Morgan et al., 2014). AH is a complex mixture of proteins, lipids, salts, and other small

molecules (Cousins et al., 1991; De Berardinis et al., 1965; Duan et al., 2010; Edwards et al., 2014; Greiner et al., 1991; Iyer et al., 2012; Knisely et al., 1994; Lee et al., 1977; Rao et al., 2000; Russell et al., 2001; Tripathi et al., 1989). Several of these solutes have been previously implicated in osteogenesis, including ascorbate and glucocorticoids (Bellows et al., 1987; Bellows et al., 1986; Herbertson and Aubin, 1995; Maniatopoulos et al., 1988; Tenenbaum and Heersche, 1982), growth-factor like lipids such as lysophosphatidic acid (LPA) (Liu et al., 2010), and the protein myocilin (Kwon et al., 2013). Understanding this effect is important to ensuring the safety and efficacy of ASC administration to the eye. Further, illuminating the osteogenic potential of AH may provide insight into the calcification of anterior chamber tissues such as the trabecular meshwork, believed to play a role in the progression of glaucoma (Borras and Comes, 2009; Gonzalez et al., 2004; Gonzalez et al., 2000; Vittitow and Borras, 2004; Xue et al., 2007; Xue et al., 2006). In this study, we expanded on our previous results to define the effects of AH dose and treatment duration. We further identified the importance of thermally labile and charcoal adsorbable components in AH to the differentiation process. Finally, we showed myocilin by itself can increase osteogenic potential, although this induction was not as robust as that of complete AH.

2. Methods

2.1 Preparation of AH and myocilin

AH was extracted using a 25-gauge needle from enucleated bovine eyes shipped overnight on ice (Pel-freez, Rogers, AR). AH was sterile filtered, aliquoted, and stored at −20°C until use. Some AH was heat treated (AH/HT) to denature protein components by heating it to 90°C for 30 min. AH was also treated with dextran-coated charcoal (AH/DCC) to reduce the concentration of hormones and lipids (Herbert et al., 1965; Keane et al., 1968; Lee et al., 1998). Briefly, 20 mg DCC (Sigma-Aldrich, St. Louis, MO) was added per 1 mL AH and incubated with rocking overnight at 4°C. The sample was then centrifuged at 5,000*g* for 15 min and sterile filtered to remove the DCC.

Full length human MYOCILIN cDNA was cloned into the pCS2-FLAG vector as described (Kwon et al., 2009) and used for transient transfection of HEK293 cells. Transfection was performed with Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. Serum-containing medium was replaced by serum-free medium 14-16 h after transfection, and cells were incubated for 48 h. Conditioned medium was collected and myocilin-FLAG protein was purified using anti-FLAG M2 agarose beads according to the manufacturer's instructions (Sigma, St. Louis, MO). Myocilin was further purified by ion-exchange chromatography using HiTrap-SP FF 1-ml columns (GE Healthcare). The purity of the isolated myocilin was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Two closely migrated bands with mobilities corresponding to myocilin were observed after Coomassie blue staining of the gel, similar to shown in Fig. 1 of (Kwon et al., 2009).

2.2 Cell Culture

Primary cultures of ASCs were cultured from human donor adipose tissue as previously described (Chung et al., 2012; Morgan et al., 2014; Toupadakis et al., 2010; Wood et al., 2012). Briefly, 10–13 g of fat was minced and incubated with rocking 2 h at 37°C in 50 mL of PBS (Invitrogen, Carlsbad, CA) with 0.1% collagenase/1% bovine serum albumin (Worthington, Lakewood, NJ). The tissue was then centrifuged to remove the lipid layer and repeatedly washed with PBS. Cell pellets were re-suspended with low glucose DMEM supplemented with 10% FBS and 1% penicillin/streptomycin (Life Technologies, Carlsbad, CA), plated, and incubated at 37°C, 5% CO_2. Cells were passaged at 70% confluence and maintained in the supplemented DMEM, henceforth referred to as full media.

For experiments, cells were plated at 50,000 cells per well in a 24-well plate in full media and allowed to attach overnight. Cells were rinsed with PBS and placed in either full or serum free DMEM with AH or myocilin supplements. To avoid disrupting the cell monolayer, half-volume media exchanges were performed twice weekly. At either 2 or 3 weeks, the cells were briefly fixed in 4% formaldehyde and rinsed in PBS.

2.3 Staining and imaging of cells

Immediately after fixation, cells were stained for ALP activity as previously described (Morgan et al., 2014). Briefly, they were stained for 15 min with 0.1% naphthol AS-MX phosphate (Sigma) and 0.1% fast red violet LB (Sigma) dissolved in 56mM 2-amino-2-methyl-1,3-propanediol (pH 9.9; Sigma). In the initial dose response experiments, cells were costained for the presence of sulphated acid proteoglycans using Alcian blue after ALP staining (Asahina et al., 1993). Briefly, the cells were rinsed in 0.1N HCl (pH 1.0), stained for 15 min with 1% w/v Alcian Blue 8GX (Sigma) in 0.1N HCl, and rinsed with 0.1N HCl to remove non-specific staining. Following staining, the coverslips were rinsed in PBS and mounted on slides for imaging. Coverslips were imaged using a Nikon DS-Fi1 color camera attached to a Nikon Diaphot inverted microscope. Six random 0.89 mm^2 fields were taken of each coverslip. ALP activity was quantified and averaged across the six images using custom analysis programs written in the MATLAB (Mathworks, Natick, MA) software package.

2.4 Statistics

All experiments were performed on ASCs isolated from three donors. Data for each donor were normalized. For each experiment, significance was assessed on the normalized values by one way ANOVA and Fisher's post-hoc test. Levels of significance are denoted throughout the manuscript by *** = $p<0.001$, ** = $p<0.01$, and * = $p<0.05$. All bar graphs are shown as mean ± SEM.

3 Results

3.1 Dose and time dependence of aqueous humor effect on ASCs

We cultured human ASCs in serum supplemented DMEM with AH added at concentrations of 5, 10, and 20%. Control cells were cultured similarly without the addition of AH. At 2 and 3 weeks, the ASCs were fixed and stained for ALP activity and Alcian blue, indicating

osteogenic and chondrogenic potential, respectively (Asahina et al., 1993). Control cells showed weak ALP activity (Figure 1A; left panel; red stain) and similarly exhibited little Alcian blue staining (Figure 1A; left panel; blue stain). However, after treatment with 5% aqueous humor for 3 weeks, ASCs exhibited a dramatic increase in ALP activity (Figure 1A; right panel; red stain) with a lesser effect on Alcian blue staining. We therefore focused on ALP activity for further characterization of the dose and duration response to AH.

Using cells isolated from 3 donors, we quantified the ALP activity of ASCs treated with 5, 10 and 20% AH at both 2 and 3 weeks. To control for variability between donors, we normalized staining intensity for a donor to the 20% addition of AH for 3 weeks. At 2 weeks (Figure 1B), there was a clear dose response, with ALP activity with correlating with AH concentration, resulting in significant increases at both 10 and 20%. At 3 weeks (Figure 1C), all three concentrations resulted in significant increases in ALP activity and little concentration dependence could be discerned at 3 weeks. It is also important to note that 2 weeks of treatment was sufficient for the 20% to induce close to maximal activation (black bars of Figures 1B and 1C).

3.2 Effect of serum free culture aqueous humor effect on ASCs

As serum contains numerous growth factors and other signaling molecules, we considered the possibility that serum was providing an essential component to the osteogenic differentiation process. To test this possibility, we also performed the above experiments in serum free DMEM. Similar to the results with serum, AH resulted in an increase in ALP activity (Figure 2A). However, the increase in ALP activity was substantially reduced compared to cells cultured with AH in the presence of serum. Similar to the serum containing experiments, we found a lesser effect on Alcian blue activity and focused on ALP activity.

We similarly tested the effect of time and dose in the absence of serum for cells. All values for a donor were normalized to the 20%/3 week values for the serum-containing cultures. This allowed direct comparison between the ALP activity in the presence and absence of serum. At 2 weeks (Figure 2B), there was a trend towards increasing ALP activity, although it was less compared to the AH plus serum data (Figure 1B, note the altered Y-axis) and did not reach statistical significance. At 3 weeks (Figure 2C), there was a more apparent dose response, although only exposure to 20% AH reached significance (but was still far reduced compared to the cells treated with equivalent AH in serum containing media-Figure 1C, note the altered Y-axis).

3.3 Deactivation of aqueous humor by charcoal stripping and heat

To gain insight into which components of AH may contribute to the increased ALP activity; we utilized dextran-coated charcoal stripping (AH/DCC) to remove hormones and lipids (Herbert et al., 1965; Keane et al., 1968; Lee et al., 1998) and heating (AH/HT) to denature protein components. As our previous results indicated a stronger overall response with serum-containing media, and little effect of dose at 3 weeks, we tested AH/DCC and AH/HT at 5% AH in full media. As before, there was a dramatic increase in ALP activity after

treatment with AH, but this was reduced with AH/DCC and eliminated with AH/HT (Figure 3A).

We again performed this study on three donors, and normalized the ALP activity to the response to 5% AH (Figure 3B). Treatment with 5% AH/DCC significantly increased ALP activity compared to control cells, but was significantly lower than the unmodified AH. ALP activity after 5% AH/HT was not different than control, but was significantly lower than both unmodified AH and AH/DCC.

3.4 Effect of myocilin on ALP activity of ASCs

The aforementioned results demonstrated a requirement for a thermally labile component of the AH for promotion of osteogenic differentiation. The AH component protein myocilin (Rao et al., 2000; Russell et al., 2001) has recently been shown to accelerate osteogenesis in bone-marrow derived MSCs cultured in osteogenic media (Kwon et al., 2013). However, it was not shown if myocilin would have a similar effect in normal culture media, or if it would rescue the loss of ALP induction in AH/HT. To this end, we treated cells with 3 μg/mL myocilin in full media, or full media supplemented with 5% AH/HT. This dose was selected as it elicited maximal response when used with osteogenic media in a previous study (Kwon et al., 2013).

Exogenous myocilin induced a subtle though significant increase in ALP activity, both in the presence or absence of 5% AH/HT (Figure 4A, second row) when compared to the appropriate control (Figure 4A, first row). However, it was not as dramatic as unmodified 5% AH (Figure 4A, third row). Similar to the previous experiments, we quantified this for three donors, normalizing to the 5% AH response (Figure 4B). Myocilin treatment significantly increased ALP activity compared to control cells, although not to the levels observed in AH treated cells. Similar results were observed in cells treated with AH/HT. Myocilin treatment significantly elevated ALP activity in these cells, but again not to the level of unmodified AH.

4. Discussion

The results of this study expand on our previous finding that AH potently increases the osteogenic potential of ASCs, even in the absence of traditional osteogenic media (Morgan et al., 2014). In this study, we initially considered chondrogenic potential as well, using Alcian blue staining. However, we found the effect of AH on Alcian blue staining was less pronounced than the induction of ALP activity. Therefore, we chose to focus on the ALP promoting activity of AH when investigating the dose and time dependant effects of AH treatment.

When cultured for 2 weeks, there was a clear trend with increasing dose of AH (Figure 1B). However, after 3 weeks, that trend had diminished, with 5% treatment resulting in roughly equivalent ALP activity as supplementation of 10 and 20% AH (Figure 1C). Additionally, there was little difference between 20% treatment at 2 weeks and the 5, 10 and 20% treatments at 3 weeks (compare Figure 1B and 1C). These results suggest that the ultimate osteogenic potential of AH on the ASC population is not directly influenced by AH dose per

se, but that lower doses (e.g. 5 and 10%) just take a longer time to reach this maximal level of activation. This may indicate subpopulations of responsive and non-responsive cells, which would be consistent with previous reports that have shown distinct subpopulations in bone-marrow derived MSC cultures with varying osteogenic potential (Kuznetsov et al., 1997; Liu et al., 2009).

AH has only a fraction (~0.4%) of the protein content of serum (Dernouchamps, 1982). It is possible that the osteogenic effect of AH depends on the presence of serum as a rich source of additional mitogens and other signaling molecules. To study this, we performed serum free experiments in parallel, finding that serum amplified, but was not required for, the increase in ALP activity following AH treatment (Figure 2). The effect of AH was much reduced without serum; however, extended treatments at 20% AH in serum free media resulting in a significant increase in ALP activity.

AH is known to possess chemical factors important in osteogenesis, including lipids such as LPA and steroid hormones (Bellows et al., 1987; Bellows et al., 1986; Herbertson and Aubin, 1995; Iyer et al., 2012; Knisely et al., 1994; Maniatopoulos et al., 1988; Tenenbaum and Heersche, 1982). To test the role of the lipids and steroids known to be present in AH, we incubated AH with dextran-coated charcoal (DCC), known to strip lipids and steroids (Dang and Lowik, 2005; Herbert et al., 1965; Keane et al., 1968; Lee et al., 1998). DCC pretreatment significantly reduced, but did not eliminate, ALP activity following AH treatment. This demonstrates that there is a charcoal adsorbable component of AH involved in the osteogenic response. Since AH is known to contain LPA and steroids, those are likely constituents involved in the osteogenic activation. It further demonstrates that there are additional ALP inducing components that were not adsorbed by charcoal, such as proteins.

To determine the effect of thermally labile components, including proteins, we denatured the AH by heating it to 90°C for 30 min (AH/HT) and found the induction of ALP activity completely abolished (Figure 3). A prominent protein component of normal AH is myocilin, which has previously been shown to increase osteogenic potential of MSCs treated cultured in osteogenic media (Kwon et al., 2013; Rao et al., 2000; Russell et al., 2001). Complete loss of ALP induction by AH/HT would be consistent with myocilin being a key contributor to osteogenic potential of AH to ASCs.

To further support this hypothesis, we treated cells with recombinant myocilin and found that myocilin alone is sufficient to induce ALP activity in ASCs, even in the absence of osteogenic media (Figure 4). This result expands previous findings of the effect of myocilin, tested in the context of osteogenic media (Kwon et al., 2013). However, myocilin alone was not sufficient to fully explain the effects of AH. Indeed, further experiments on the heat treated AH revealed that the addition of myocilin only partially rescued the loss of induction caused by heat treatment. Our results show conclusively that thermally labile components in AH are essential for the induction of osteogenic differentiation of ASCs. Myocilin, known to be present in AH, is one of these components.

These findings may also provide insight into the pathogenesis of glaucoma. The primary outflow pathway of AH is the trabecular meshwork (Gottanka et al., 1997; Johnson, 2006;

Lutjen-Drecoll, 2005; Rohen et al., 1993), calcification of which has been implicated in glaucoma (Borras and Comes, 2009; Gonzalez et al., 2004; Gonzalez et al., 2000; Vittitow and Borras, 2004; Xue et al., 2007; Xue et al., 2006). This is consistent with glaucoma-associated increased AH expression of molecules linked to calcification, such as growth factor like lipids (Iyer et al., 2012; Liu et al., 2010), myocilin (Howell et al., 2010; Jacobson et al., 2001; Menaa et al., 2011; Nguyen et al., 1998; Polansky et al., 1997; Rao et al., 2000; Russell et al., 2001; Tamm, 2002), and connective tissue growth factor (Browne et al., 2011; Ho et al., 2005; Junglas et al., 2012; Junglas et al., 2009). In healthy meshwork, the activity of these stimulators of calcification is likely inhibited by the high expression of proteins such as matrix Gla protein (Gonzalez et al., 2004; Gonzalez et al., 2000; Tomarev et al., 2003; Vittitow and Borras, 2004). Indeed, matrix Gla inhibits the upregulation of ALP activity in cultured trabecular meshwork cells and likely plays a similar role *in vivo* (Xue et al., 2007; Xue et al., 2006). Taken with these studies showing the role of calcification in glaucoma, our findings offer further evidence that AH continuously bathes the anterior chamber with osteogenic stimuli.

While AH alone and myocilin in serum containing media did both increase the osteogenic potential of ASCs, additional serum components greatly augmented this process. Further, maximal osteogenic induction involved thermally labile components of AH, one of which was myocilin. Additional components of AH that adsorb to charcoal and ones present in serum greatly enhanced the osteogenic signals. In aggregate, these data add to the understanding of the induction of ALP in ASCs following AH treatment and provide important insight in how ASCs will be influenced by the ocular environment. The promotion of osteogenic potential for ASC's upon exposure to AH indicates the need for careful evaluation of safety for ASCs grafted into the intraocular environment.

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- Aqueous humor induces alkaline phosphatase activity in mesenchymal stem cells
- There is both a dose and temporal dependence on the response
- This is mediated by both charcoal adsorbable and thermally labile components
- Exogenous myocilin, a component of aqueous humor, has a similar effect
- Myocilin partially reverses the effects of heat treatment

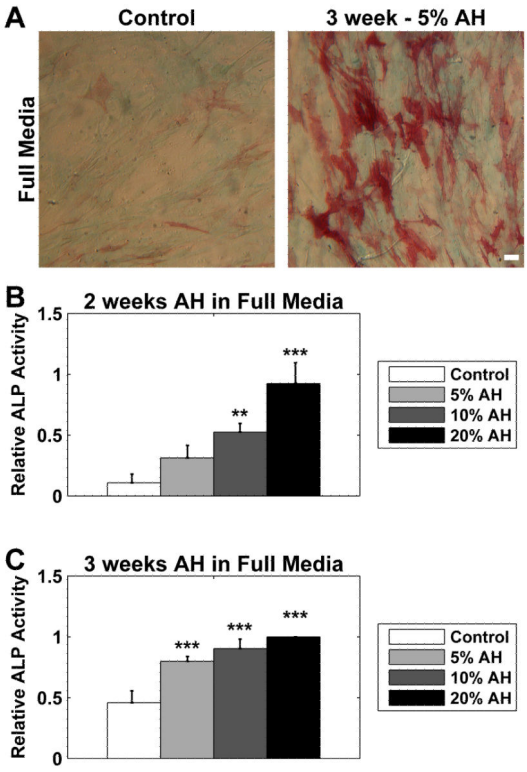

Figure 1. ASCs exhibited a time and dose dependent response to aqueous humor

ASCs were cultured in full media (serum supplemented DMEM) in the presence of 5, 10, or 20% AH. (A) Cells were stained for alkaline phosphatase (ALP) activity (red) and with Alcian blue (blue). Some ALP activity was observed in untreated cells (Control), but cells treated with AH exhibit dramatic increase in ALP activity, exemplified by cells cultured for 3 weeks in the presence of 5% AH (3 week - 5% AH). There was apparently less effect of AH on Alcian blue staining. (B) At 2 weeks, ALP activity was increased in a dose dependent manner, with 10% and 20% eliciting a significant response when compared to control cells. (C) At 3 weeks, all doses resulted in a significant increase in ALP activity compared to control. All data were normalized to the response of 3 week treatment with 20% AH. Data are mean ± SEM (n = 3 donors). * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. Scale bar 50 μm.

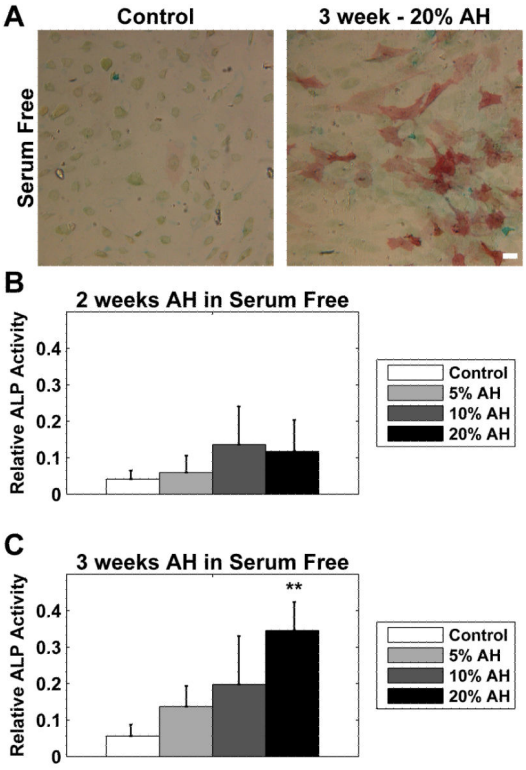

Figure 2. Serum was not required for the osteogenic effect of aqueous humor on ASC

ASCs were cultured serum free (un-supplemented DMEM) in the presence of 5, 10, or 20% AH. (A) Cells were stained for ALP activity (red) and with Alcian blue (blue). ALP activity was virtually absent in untreated cells (Control), but cells treated with AH exhibited noticeable ALP activity, exemplified by cells cultured for 3 weeks in the presence of 20% AH (3 week - 20% AH). There was less effect of AH on Alcian blue staining. (B) At 2 weeks, ALP activity was not significantly increased at any dose, although there was an apparent trend towards increase. (C) At 3 weeks, there was a dose response, and cells treated with 20% exhibited a significant increase over control. All data were normalized to the response of 3 week treatment with 20% AH in serum containing media. Note the smaller Y-axis when compared to Figure 1. Data are mean ± SEM (n = 3 donors). * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. Scale bar 50 μm.

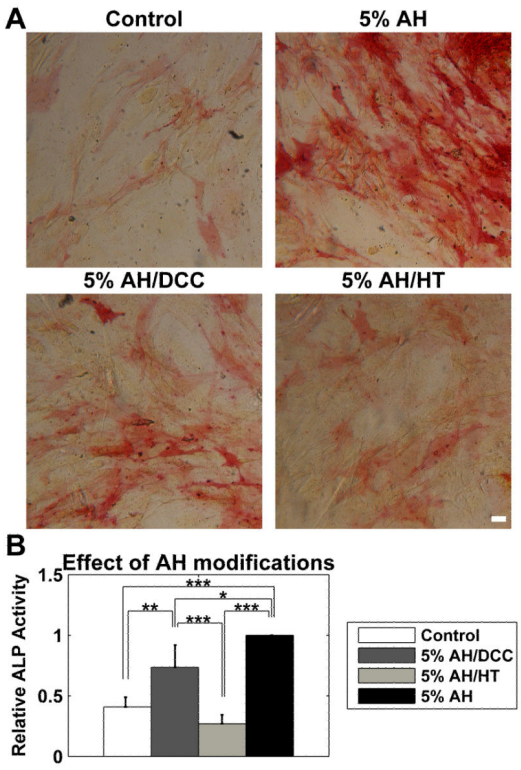

Figure 3. The effect of aqueous humor on the osteogenic potential of ASCs was dependent on thermally labile and charcoal-adsorbable components

ASCs were cultured for 3 weeks in full media (serum supplemented DMEM) in the presence of 5% AH. The AH was either untreated, incubated with dextran-coated charcoal (DCC), or heat treated. (A) Both DCC (5% AH/DCC) and HT (5% AH/HT) treatments reduced ALP activity when compared to untreated AH (5% AH), with HT reducing activity to the level seen in untreated cells (Control). (B) Both DCC and HT significantly reduced the ALP induction caused by AH. The effect of DCC was partial, and was still significantly elevated compared to control cells, while HT resulted in complete loss of AH osteogenic activity. All data normalized to the response of 5% AH case. Data are mean ± SEM (n = 3 donors). * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. Scale bar 50 μm.

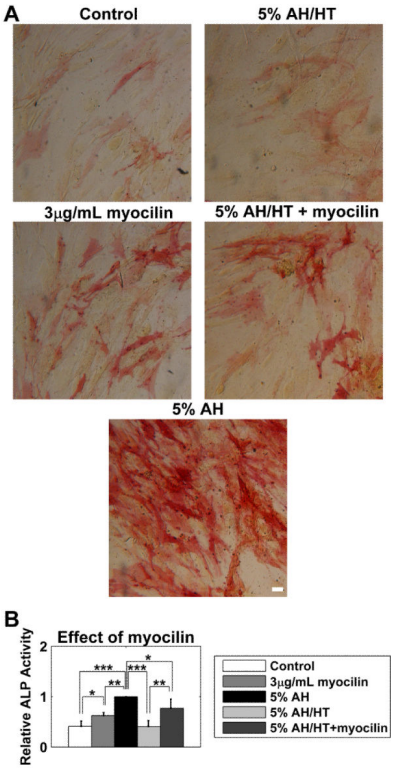

Figure 4. Myocilin partially rescued the loss of activity induced by HT

ASCs were cultured for 3 weeks in full media (serum supplemented DMEM) in the presence of 3 μg/mL myocilin (3 μg/mL myocilin), 5% aqueous humor (5% AH), heat treated 5% AH (5% AH/HT), or myocilin and 5% AH/HT (5% AH/HT + myocilin). (A) Myocilin, both separately and in conjunction with AH/HT, increased ALP activity. (B) Quantification of ALP activity. All data normalized to the response of 5% AH case. Data are mean ± SEM (n = 3 donors). * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. Scale bar 50 μm.