Perimysial Fibroblasts of Extraocular Muscle, as Unique as the Muscle Fibers

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PURPOSE. Extraocular muscle (EOM) has a distinct skeletal muscle phenotype. The hypothesis for the study was that fibroblasts support the unique EOM phenotype and that perimysial fibroblasts derived from EOM have properties that distinguish them from fibroblasts derived from other skeletal muscle.

METHODS. Perimysial fibroblasts from leg muscle (LM-Fibro) and EOM (EOM-Fibro) of mice were derived and maintained in culture. EOM- and LM-Fibro were assessed morphologically and for vimentin, smooth muscle actin, and Thy-1 immunoreactivity. DNA microarray analysis was performed on LM- and EOM-Fibro grown in conditions that support myoblast differentiation. To assess trophic interactions, co-cultures of myoblasts from established cell lines, CL-EOM and CL-LM with, EOM- or LM-Fibro were performed in direct contact and in a permeable filter support culture. The degree of myotube maturation was assessed by the percentage of myotubes with more than three myonuclei per myotube.

RESULTS. EOM- and LM-Fibro cells exhibited distinct morphologies. Both cell types proliferated as a monolayer and expressed vimentin. Fifty-five percent (SD 4.4%) of EOM-Fibro were Thy-1 positive compared with only 24% (SD 4.4%) of LM-Fibro. DNA microarray analysis demonstrated differential expression of structural, immune response, and metabolism-related genes between EOM- and LM-Fibro. Co-cultures demonstrated that mature myotube formation in EOM-derived cell lines was supported to a greater extent by EOM-Fibro than by LM-Fibro, compared with CL-EOM grown with LM-Fibro.

CONCLUSIONS. Fibroblasts from EOM demonstrate distinct properties that distinguish them from leg muscle-derived fibroblasts. The distinct properties of EOM-Fibro may support the unique EOM phenotype and contribute to their differential involvement in disease. (*Invest Ophthalmol Vis Sci.* 2010;51:192–200) DOI:10.1167/iovs.08.2857

The unique nature of extraocular muscle (EOM) and its differential disease susceptibility have been extensively established by anatomic, histologic, physiological, and

genomic characterizations.^{1,2} However, studies to date have focused on the most prominent cell component of EOM—the muscle fibers—and have ignored the contribution of other cells, such as fibroblasts or endothelial cells, to the properties of the muscle as a whole. Dissection of the functions of each cell type in the context of the tissue is not trivial. Ultimately, investigations will necessitate application of multiple methods to gain understanding of the integration of each cell type in a cellular and chemical milieu and their individual contributions to a distinct tissue phenotype.

There is a growing appreciation that fibroblasts do not simply function in formation of extracellular matrix, but also provide critical signaling molecules for the cell migration, proliferation, and adherence involved in a broad array of physiological functions.^{3,4} Fibroblasts possess extensive phenotypic heterogeneity and plasticity across tissues⁵ with respect to extracellular matrix production, immune response, metabolism, and proliferative capabilities, but even within a given tissue there is heterogeneity based on expression of cell surface markers^{6,7} and response to growth factors and cytokines.^{5,8,9}

Human orbital fibroblasts and the fibroblasts that reside within the EOM have been a focus of intense study in the context of the pathology of Graves' ophthalmopathy.^{10,11} The thyrotropin receptor and the insulin-like growth factor receptor, which have been proposed to be autoantigens, are expressed by both the orbital fibroblasts and the perimysial fibroblasts of the EOMs.^{12,13} Evaluation of synovial, dermal, and orbital fibroblasts has demonstrated their differential response to inflammatory mediators in a disease-specific manner for rheumatoid arthritis, Graves' dermopathy, and Graves' orbitopathy (see Smith¹⁰ for a comprehensive review). Therefore, based on investigations of Graves' disease, it is clear that fibroblasts possess tissue-specific properties.

Because of the phenotypic differences between EOM and limb skeletal muscles, we hypothesized that fibroblasts derived from EOM (designated, EOM-Fibro) possess properties that differentiate them from fibroblasts of limb muscle (designated LM-Fibro). In vitro investigations indicate that fibroblasts support the differentiation and survival of skeletal myoblasts, providing a substrate for contraction and trophic support.¹⁴ Therefore, we further hypothesized that EOM-Fibro would preferentially support the in vitro maturation of myotubes from EOM cell lines (CL-EOM) compared with LM-Fibro. To evaluate these hypotheses, we first performed comprehensive assessment of EOM-Fibro morphology, expression of Thy-1, a standard marker of fibroblast subpopulations, and broad-based characterization of gene expression. The results supported our hypothesis that EOM-Fibro possess distinct properties compared with LM-Fibro and encouraged further functional experiments, which demonstrated that EOM-Fibro sustain greater in vitro maturation of EOM myotubes than LM-Fibro. The data suggest that the perimysial fibroblasts of EOMs contribute to the unique phenotype of EOMs and therefore, may serve as a

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target for manipulation to modify the EOM phenotype, especially for therapeutic purposes.

METHODS

Fibroblast Cell Line Derivation

EOM and hindlimb muscles of five 5-week-old C57BL/6J mice were dissected from their tendon ends. The muscles were cleaned of surrounding tissue, including the epimysium, and finely minced and pooled. The samples were digested by 0.25% trypsin and 0.1% collagenase (Sigma-Aldrich, St. Louis, MO) at 37°C. Cells were collected and resuspended in DMEM (HyQ; HyClone, Logan, UT) containing 4 mM L-glutamine, 2500 mg/L glucose, sodium pyruvate, 10% fetal bovine serum (FBS), and 1% penicillin/streptomycin (referred to as growth medium) and transferred to 100-mm tissue culture plates. Fibroblasts were expanded and passaged until a purified population of cells was obtained at passage four. The pure fibroblast populations were designated EOM- and LM-Fibro. Cells were frozen in growth medium containing 10% DMSO and stored in liquid nitrogen. Unless otherwise stated, EOM- and LM-Fibro were used only to passage 15.

Immunocytochemistry of EOM- and LM-Fibro

EOM- and LM-Fibro were plated and allowed to grow for 24 hours in growth medium. The cells were fixed in 4% paraformaldehyde and 0.1 M phosphate buffer (pH 7.4) for 10 minutes at room temperature. Rat anti-mouse CD90.2 (Thy1.2) antibody (BD Biosciences Pharmingen, San Diego, CA), chicken anti-vimentin polyclonal antibody (Chemicon, Temecula, CA), or α -smooth muscle actin (SMA) monoclonal antibody (Sigma, St. Louis, MO) in 5% BSA/PBS was applied for 1 hour at room temperature. The secondary antibodies for detection of mouse Thy-1 and vimentin were Alexa-Fluor 488 donkey anti-rat IgG (Invitrogen, Eugene, OR) and Alexa-Fluor 488 goat anti-chicken IgG (Invitrogen). The secondary antibody against α -SMA was Oregon Green 488 goat anti-mouse IgG (Invitrogen). The cells were viewed by microscope (BX50; Olympus, Center Valley, PA), and digital images were captured (Spot RT digital camera; Diagnostic Instruments, Sterling Heights, MI) and analyzed (Image Pro image-analysis software; Media Cybernetics, Silver Springs, MD). For light microscopy, the cells were visualized (Eclipse TS100 microscope; Nikon, Tokyo, Japan) and images were captured on a 3 CCD camera.

Flow Cytometric Analysis

EOM- and LM-Fibro were cultured for 5 to 10 passages before Thy-1 expression analysis. They were then grown in growth medium to 70% confluence, which was attained within 24 hours of culture initiation. The cells were briefly treated with trypsin and resuspended in 10% FBS/PBS followed by incubation with rat anti-mouse CD90.2 (Thy1.2) antibody and Alexa-Fluor 488 donkey anti-rat IgG. They were resuspended in buffer (FACSFlow; BD Biosciences) before sorting. Flow cytometric analysis was then performed (LSR system; BD Biosciences, in conjunction with BD Cellquest Pro software).

Sample Preparation for DNA Microarray Analysis

To ensure consistency among the samples, three individual vials of LM- and EOM-Fibro derived from passage 5 were thawed and grown on individual plates. Each culture was passaged once to increase the number of cells for RNA isolation. LM- and EOM-Fibro were incubated in myoblast differentiation media (F10C and 2% horse serum) for 24 hours before harvesting for RNA purification. DNA microarray analysis was performed on three independent samples. Total RNA was extracted (TRIzol reagent; Life Technologies, Rockville, MD), purified (RNeasy Kit; Qiagen Inc., Valencia, CA), and resuspended at 1 μ g/ μ L in DEPC-treated water. Eight micrograms of RNA was used for reverse transcription (SuperScript II; Life Technologies) to generate first-strand cDNA. Double-stranded cDNA was synthesized and used in an *in vitro* transcription (IVT) reaction to generate biotinylated cRNA. Frag-

mented cRNA (15 μ g) was used in a 300- μ L hybridization cocktail containing herring sperm DNA and BSA as carrier molecules, spiked IVT controls, and buffering agents. A 200- μ L aliquot of the cocktail was used for hybridization to a mouse microarray (MOE430; Affymetrix, Santa Clara, CA) for 16 hours at 45°C. The manufacturer's standard posthybridization wash, double-staining, and scanning protocols were used (GeneChip Fluidics Station 400; Affymetrix; and a Gene Array scanner; Hewlett Packard, Palo Alto, CA).

DNA Microarray Data Analysis

Raw data from microarray scans were analyzed with microarray analysis software (GCOS 2.0; Affymetrix). The software evaluated sets of perfect-match (PM) and mismatch (MM) probe sequences to obtain hybridization signal values and present/absent calls for each transcript. Microarrays were scaled to the same target intensity, and pair-wise comparisons were made between experimental and control samples. Transcripts defined as differentially regulated met the following criteria: (1) consistent increase/decrease call across nine of nine replicate comparisons, based on Wilcoxon's signed rank test (algorithm assesses probe pair saturation, calculates a probability and determines increase, decrease, or no-change calls) and (2) absolute value of the mean difference (α -fold) between EOM- and LM-Fibro $\geq$ 8.0.

Quantitative Real-Time PCR (qPCR)

Select transcripts were analyzed by qPCR for verification of genomic profiling or myosin heavy chain gene expression. Transcript-specific primers (see Supplementary Table S1, <http://www.iovs.org/cgi/content/full/51/1/192/DC1>) were designed on computer (Primer Express 2.0 software; Applied Biosystems, Inc. [ABI], Foster City, CA) and their specificity confirmed by BLAST (NCBI, Bethesda, MD). RNA was isolated from three independent cultures (passages 7–12) of LM- and EOM-Fibro. For myosin expression analysis, RNA was isolated from co-cultures of EOM-Fibro with CL-EOM or -LM. Reverse transcription was performed using 1 μ g total RNA with a reverse transcription reagent (*TaqMan*; ABI). qPCR reactions used the SYBR green PCR core reagent in a 25 μ L total reaction volume with a sequence-detection system (Prism 7000; ABI). GAPDH was used as an internal positive loading control. Data were expressed as the mean change (α -fold) of triplicate measurements, using the $2^{-\Delta\Delta CT}$ method.¹⁵

Cell Lines and Co-cultures

Stock cell lines of extraocular muscle (CL-EOM) and limb muscle (CL-LM), which have previously been described and extensively verified as pure populations of myoblasts,¹⁶ were used for all co-culture studies. For proliferation, myoblasts were seeded on 0.5% gelatin-coated plates with myoblast growth medium (F10C: F10 plus 1% penicillin-streptomycin, 1 mM L-glutamine, 1.2 mM CaCl₂, 15% horse serum, and 6 ng/mL fibroblast growth factor [FGF]). Co-culture of EOM- or LM-Fibro with CL-EOM or -LM myoblasts was performed as follows: EOM- and LM-Fibro were grown to 80% confluence in DMEM growth media over 2 days. Myoblasts ($5.7 \times 10^3/\text{cm}^2$) were then added in F10C growth medium (minus FGF) and cultured for 24 hours. The co-cultures were switched to myoblast differentiation medium (F10C with 2% horse serum minus FGF) to induce myotube formation and were monitored using light microscopy for 12 days. For all experiments, the medium was changed every 48 hours.

Images were captured with a digital camera (MTI 3CCD; DAGE-MTI, Michigan City, IN, Image J acquisition software [developed by Wayne Rasband, National Institutes of Health, Bethesda, MD; available at <http://rsb.info.nih.gov/ij/index.html>], and Adobe Photoshop 7.0 imaging software, Adobe, San Jose, CA). To assess the percentage of mature myotubes, all individual myotubes were counted, and mature myotubes were defined as those with more than three nuclei.^{14,17} Cultures were established in triplicate, and the experiment was repeated to verify results.

Permeable Filter Support Cultures

EOM- or LM-Fibro were plated on polycarbonate permeable filter supports (0.4- μ m pore size; Transwell; Costar, Cambridge, MA) at a den-

FIGURE 1. Light microscopy demonstrated differences in morphology of EOM- and LM-Fibro (A, B). EOM-Fibro were stellate and appeared to have larger cytoplasmic areas and large central nuclei, whereas LM-Fibro were more heterogeneous in size and shape. Immunoreactivity for vimentin was identified in all cells in both cultures (C, D), whereas immunoreactivity to smooth muscle actin was identified in a limited number of cells (E, F). Thy-1 expression was characterized by immunostaining (G, H). Flow cytometry was used to assess the percentage of Thy-1-positive cells (I, J; one representative experiment). The average of results in three independent experiments identified $54.8\% \pm 4.4\%$ of Thy-1 positivity in EOM-Fibro compared with $24.4\% \pm 4.4\%$ in LM-Fibro cultures ($P < 0.001$).

sity of $9.4 \times 10^3/\text{cm}^2$ in DMEM growth medium and grown to 80% confluence, which was attained within 24 hours. CL-EOM or -LM myoblasts ($5.7 \times 10^3/\text{cm}^2$) were added to the bottom chambers of the plates, which were coated with 0.5% gelatin. As controls, CL-EOM and -LM were also established on the permeable supports without fibroblasts, to serve as the control. The cultures were switched to differentiation medium (F10C and 2% horse serum) to induce myotube formation. The nuclei were identified by staining with DAPI (Sigma-Aldrich). In each culture, 75 to 100 myotubes were counted for each time point, and the percentage of myotubes with more than three myonuclei. The myonuclei count is the standard measurement for assessing myotube maturity in culture.^{14,17}

Immunocytochemistry of Co-cultures

Cultures of CL-EOM and -LM were grown on EOM-Fibro and incubated in differentiation medium for 6 days before fixation in 4% paraformaldehyde. The slides were incubated with antibodies directed against actin (anti- α -sarcomeric actin, clone 5C5; Sigma-Aldrich), slow myosin (anti-slow myosin heavy chain, MAB1628; Chemicon Inc.), or fast myosin (anti-skeletal myosin; clone MY-32; Sigma-Aldrich) followed by the appropriate secondary fluorescein conjugated antibody. The cells were viewed by microscope (BX50; Olympus) and digital images were captured with a digital camera (Spot RT; Diagnostic Instruments) and analyzed on computer (Image Pro software; Media Cybernetics).

Statistical Analysis

All quantitative data are expressed as the mean $\pm$ SD. Comparisons between groups were analyzed with the Student's one-tailed paired *t*-test (Excel; Microsoft, Redmond, WA).

RESULTS

Morphology

EOM- and LM-Fibro demonstrated distinct morphologic characteristics. The EOM-Fibro cells were uniform in size and stellate in shape, with large amounts of cytoplasm and large central nuclei (Fig. 1A). In contrast, the LM-Fibro appeared more

heterogeneous, containing both small, angularly shaped cells with long dendritic processes and larger stellate cells (Fig. 1B). Growth patterns of each culture were consistent with the behavior of fibroblasts: no requirement for extracellular matrix for attachment, strong attachment to stratum, and rapid growth.

We assessed specific markers of fibroblasts that distinguish them from skeletal muscle, endothelial, and neuronal cells in culture. Immunostaining for vimentin, an intermediate filament protein that is generally found in cells of mesenchymal origin, was positive in all cells of EOM- and LM-Fibro (Figs. 1C, 1D). As is common for fibroblasts in culture,¹⁸ at 24 hours some of the cells (<5%) in each culture were immunoreactive for α -SMA, a marker of myofibroblasts (Figs. 1E, 1F).

Thy-1 (CD90) Expression

The expression of Thy-1 has been extensively used to characterize fibroblast populations because of its association with specific phenotypic characteristics, including cell morphology and response to disease.^{7,19,20} Cytometric analysis (Figs. 1G-J) revealed that $54.8\% \pm 4.4\%$ of EOM-Fibro expressed Thy-1, compared with $24.4\% \pm 4.4\%$ of LM-Fibro ($P < 0.001$). Figures 1I and 1J are representative of the flow cytometry studies. Consistent with the cytometry data showing higher levels of Thy-1 expression in EOM-Fibro, qPCR showed that *Thy-1* gene expression was 1.49 ± 0.24 -fold higher in EOM- than in LM-Fibro ($P < 0.05$).

DNA Microarray Analysis

To further characterize EOM-Fibro, we performed a broad-based assessment of transcriptional variation by using DNA microarray technology. The microarray (MOE430 Affymetrix), which includes 45,101 probe sets representing more than 39,000 unique transcripts was used to identify genes that are differentially expressed between EOM- and LM-Fibro. The percentage of transcripts detected as expressed ranged from 51.1 to 56.3, with a mean of 54.4 ± 1.7 and the GAPDH 3'/5' ratio

FIGURE 2. Hierarchical cluster and functional characterization of genomic profiles of EOM- and LM-Fibro. Hierarchical cluster hybridization signal intensity is shown by a color scale (blue, low; red, high). Three independent replicates per group are shown. Functional distribution showed differentially regulated gene transcripts. A cutoff of ≥ 8.0 for the average difference (x -fold) between EOM- and LM-Fibro resulted in identification of 156 gene transcripts that were increased and 129 that were decreased in EOM- compared with LM-Fibro.

ranged from 0.78 to 0.88 with a mean of 0.82 ± 0.04 . These data showed little variability among samples in the same group, as demonstrated by the hierarchical cluster (Fig. 2A). We used a stringent significance filter with consistent calls across all replicates (nine of nine replicate comparisons). We focused on transcripts that showed a greater than eightfold difference in expression between EOM- and LM-Fibro. In total, 156 transcripts were expressed at higher levels in EOM-Fibro compared with LM-Fibro and 129 transcripts were expressed at a lower level.

All transcripts that showed differential expression were classified into basic categories of function: immune response, metabolism, cell signaling, transcriptional regulation, membrane protein, structural protein, extracellular matrix, apoptosis, EST, or other (Fig. 2B; Supplementary Tables S2, S3, <http://www.iovs.org/cgi/content/full/51/1/192/DC1>). The functional classification demonstrated that highly expressed transcripts in EOM-Fibro were predominantly in the areas of immune response, metabolism, and structurally related genes, whereas LM-Fibro demonstrated a greater number of transcripts involved in transcriptional regulation.

Validation of DNA Microarray Analysis by qPCR

We performed qPCR on selected transcripts to verify the DNA microarray results (Table 1). Specific transcripts were chosen for further evaluation based on functional categories (transcrip-

tional regulation, immune response, and extracellular matrix production) as well as potential relevance to trophic support of skeletal muscle. Table 1 shows that the microarray and qPCR gave consistent results for the direction of increase or decrease for each of the gene transcripts evaluated. The differences between EOM- and LM-Fibro were all significant at $P < 0.01$. Within the transcriptional regulation category, the homeobox genes have been shown to be critical in development through provision of signals that target tissue formation to a specific anatomic area.⁵ This may be of particular relevance for the differentiation of EOM and hind limb muscles.

Functional Characterization of EOM-Fibro by Myoblast Co-culture

When plated on gelatin-coated tissue culture dishes, both CL-EOM and CL-LM myoblasts developed into myotubes in a manner consistent with the original study, with CL-EOM myotubes appearing qualitatively smaller in diameter than CL-LM myotubes at 48 hours.¹⁶ We co-cultured EOM- and LM-Fibro with the CL-EOM and CL-LM to assess the impact of fibroblasts on mature myotube formation, as determined by the percentage of myotubes with greater than three nuclei. Co-culture of CL-EOM or -LM with EOM- or LM-Fibro led to a greater percentage of myotubes with more than three nuclei for longer times in culture than CL-EOM or -LM plated on gelatin (data not shown).

TABLE 1. Validation of DNA Microarray Results by qPCR

Gene Name	Gene Symbol	Category	Microarray	qPCR
Paired-like homeodomain transcription factor 2	<i>Pitx2</i>	Transcriptional regulation	9.33	7.15 ± 1.37
Homeo box A10	<i>Hoxa10</i>	Transcriptional regulation	-29.18	-23.28 ± 3.55
Homeo box C10	<i>Hoxc10</i>	Transcriptional regulation	-69.66	-11.13 ± 1.70
Homeo box B3	<i>Hoxb3</i>	Transcriptional regulation	-12.60	-90.74 ± 14.83
CD59a antigen	<i>Cd59a</i>	Immune response	10.89	79.62 ± 4.72
Interleukin 6	<i>Il6</i>	Immune response	36.20	11.30 ± 2.65
Chemokine (C-X-C motif) ligand 14	<i>Cxcl14</i>	Immune response	40.01	13.60 ± 1.46
CD109 antigen	<i>Cd109</i>	Immune response	8.64	2.76 ± 0.76
Laminin, alpha 2	<i>Lama2</i>	Extracellular	17.96	25.23 ± 3.71
Pleiotrophin	<i>Ptn</i>	Extracellular	-55.29	-786.89 ± 85.16
Fibulin 1	<i>Fbln1</i>	Extracellular	-13.40	-9.72 ± 5.62
Netrin 4	<i>Ntn4</i>	Extracellular	20.95	17.92 ± 1.66
Connective tissue growth factor	<i>Ctgf</i>	Extracellular	9.93	25.76 ± 1.66

Positive values indicate that levels are elevated in the EOM-Fibro compared with the LM-Fibro.

All qPCR values are significant at $P < 0.01$.

FIGURE 3. Light microscopy of co-cultures. CL-EOM or -LM were co-cultured with EOM-Fibro and allowed to differentiate over 12 days. The number of nuclei per myotube was determined and the percentage of mature myotubes was assessed based on the number of myotubes with more than three myonuclei compared with the total number of myotubes in culture. Data presented (Q) are the mean of three independent experiments. *Significantly different, $P < 0.03$ comparing CL-EOM/EOM-Fibro with CL-LM/EOM-Fibro. Arrows denote examples of myotubes in co-cultures.

The addition of EOM-Fibro to either CL-EOM or -LM cultures produced greater percentages of mature myotubes than cultures with LM-Fibro (Fig. 3). The percentage of mature myotubes in co-cultures of CL-EOM/EOM-Fibro was 40.6 ± 1.4 on day 4 and the CL-EOM myotubes appeared larger in diameter with spontaneous contractions (Fig. 3), but the percentage of mature myotubes in a CL-LM/EOM-Fibro culture was similar $39.2 \pm 1.6\%$ ($P < 0.5$). By day 10 and 12, CL-LM/EOM-Fibro had a lower percentage of mature myotubes compared to CL-EOM/EOM-Fibro, $8.5 \pm 0.6\%$ and $24.8 \pm 6.3\%$ ($P < 0.03$), respectively.

Culture of LM-Fibro with either CL-EOM or -LM led to significantly lower percentages of mature myotubes (Fig. 3). Myotube maturity peaked at day 4. The percentage of mature myotubes in the CL-LM/LM-Fibro and CL-EOM/LM-Fibro was $15.3 \pm 4.5\%$ and $10.7 \pm 4.5\%$ (NS). Qualitatively, myotubes appeared thinner and shorter compared with those grown with EOM-Fibro. Over the course of the experiment, the percentage decreased.

Assessment of Trophic Support

The myotube maturation that was observed in the co-culture experiments could have been influenced by physical connections

with the fibroblasts, secreted factor(s), or both. To determine whether EOM- or LM-Fibro secrete factors, that support myotube maturation in culture, we used a permeable filter support culture dish. Culture in this system permits physical separation of the fibroblast and myoblast cultures, but allows for secreted factors to diffuse through the culture medium. We analyzed the percentage of myotubes with greater than three myonuclei after 4 days in culture, which was the point of maximum mature myotube formation. The cultures in which the EOM-Fibro were grown on the top filter chamber (Fig. 4) had the greatest percentage of mature myotubes in both the CL-LM and -EOM cultures ($P < 0.001$). The CL-LM and -EOM cultures with LM-Fibro demonstrated no significant difference in mature myotube formation compared with the control without the addition of fibroblasts.

Myosin Heavy Chain Gene Expression in Co-cultures

To further evaluate the ability of EOM-Fibro to support myotube differentiation, we assessed the expression of myosin heavy chain isoforms in CL-EOM and -LM when co-cultured directly on EOM-Fibro. In cultures of C2C12 cells, expression of developmental isoforms occurs initially, followed by slow

FIGURE 4. Light microscopy of CL-EOM or -LM grown in permeable filter support cultures. CL-EOM or -LM were cultured on the bottom chamber of a dual-level culture dish that contained EOM- or LM-Fibro or media only in the top chamber and were allowed to differentiate over 4 days. (A) Representative CL-EOM myotubes grown with EOM-Fibro are shown. The cells were visualized by microscope with a 10×/0.40 Ph1 objective. Images were captured with a digital camera and analyzed by computer. The number of nuclei per myotube was determined and the percentage of mature myotubes determined based on the number of myotubes with more than three myonuclei divided by the total number of myotubes in culture. Data presented (B) are the mean of results in three independent experiments (* $P < 0.001$).

myosin, and then rarely, fast myosin isoforms.^{14,21} We performed qPCR to assess expression of *Myh3* (embryonic), *Myh7* (slow), and *Myh4* (fast) expression in CL-EOM/EOM-Fibro and CL-LM/EOM-Fibro. There was no difference in gene expression through day 12 for *Myh3* or *Myh4*. *Myh7* expression in CL-EOM/EOM-Fibro was significantly higher at 6.3 ± 0.96 , 9.4 ± 0.14 , and 55.6 ± 4.6 at days 4, 8, and 12, respectively, compared with CL-LM/EOM-Fibro ($P < 0.01$). The results indicate that the EOM-Fibro differentially support maturation of CL-EOM.

Myotube Structure in Co-cultures

Myotubes in culture develop evidence of sarcomeric structure that reaches various degrees of refinement.¹⁴ On day 6 of culture, sarcomeric periodicity was identified by the myosin heavy chain immunostaining in both the CL-EOM/EOM-Fibro and CL-LM/EOM-Fibro myotubes (Fig. 5); however, the pattern was more easily identified in CL-EOM myotubes and evident to a greater longitudinal extent (Fig. 5). There was no obvious difference in the number of slow or fast myosin heavy chain immunoreactive myotubes in the co-cultures, but this observation was not quantitated. The CL-LM myotubes were shorter with less branching (Fig. 5).

DISCUSSION

Our investigations demonstrated that EOM-Fibro are distinct from LM-Fibro in morphology, Thy-1 expression pattern,

genomic signature, and support of myotube maturation. The genomic profile of EOM-Fibro identified differentially expressed genes involved in metabolism, production of extracellular matrix genes, and immune responses. The results supported our hypothesis that fibroblast populations are heterogeneous across tissues and are consistent with findings of other groups.^{5,9} Overall, our results indicated that fibroblasts of the EOM have unique basic features compared with limb skeletal muscle²² and that EOM-Fibro provide specific trophic support of the muscle fibers.

General Morphology and Cell Surface Markers

Although both EOM- and LM-Fibro demonstrated uniform expression of vimentin, the cells in culture displayed clear morphologic differences. Both EOM- and LM-Fibro cultures contained a small percentage of myofibroblasts identified by the expression of smooth muscle actin. Fibroblasts in situ and in vitro may become activated, taking on the properties of smooth muscle cells, as in our study, a small percentage of myofibroblasts may be identified.²³ The myofibroblasts may play a functional role in the facilitation of myoblasts fusion and assist new muscle formation during injury.²⁴ The EOM- and LM-Fibro were grown in a high serum medium that induced a proliferative response, but has also been shown to induce gene expression patterns that mimic those observed in wound repair and inflammation.²⁵ Therefore, the serum conditions used in our study could explain the observation of myofibroblasts in the EOM- and LM-Fibro cultures.

FIGURE 5. Immunocytochemistry of CL-EOM and -LM co-cultures with EOM-Fibro demonstrating sarcomeric arrangement. CL-EOM myoblasts were allowed to differentiate in EOM-Fibro co-cultures for 6 days followed by fixation and staining with antibody to sarcomeric α -actin (A), adult fast myosin heavy chain (B), and adult slow myosin heavy chain (C). CL-LM myoblasts were allowed to differentiate in an EOM-Fibro co-culture for 6 days followed by fixation and staining with antibody to sarcomeric α -actin (D), adult fast myosin heavy chain (E) and adult slow myosin heavy chain (F). UPlanFI objectives. Magnification: (A, D) $\times 20$; (B, C, E, F) $\times 40$.

Thy-1 is a member of the immunoglobulin superfamily and is expressed in several cell types—in particular, lymphocytes and neurons. It also serves as a common marker for categorization of fibroblast populations. Despite its extensive use as a cell surface marker, its function is poorly understood, although cell–cell and cell–extracellular matrix interactions appear to be involved.¹⁹ In humans, Thy-1-positive fibroblasts tend to have a spindle shape and produce greater amounts of collagen I and III than the larger Thy-1-negative fibroblasts.²⁶ Although half of the EOM-Fibro were Thy-1 positive compared with 24% of the LM-Fibro, the EOM-Fibro exhibited a uniform morphology. In contrast, human perimysial fibroblasts of the EOM uniformly express Thy-1²⁷ (Kusner LL, et al., unpublished results, 2007). Thy-1 expression has been correlated with fibroblast response to cytokines and growth factors,²⁷ and our observation of a lower percentage of Thy-1 expressing EOM-Fibro in mouse compared with human orbital fibroblasts could offer an insight into the inability to develop a robust mouse model of Graves' ophthalmopathy. An important caveat to this statement is that the evaluation of Thy-1 expression of human orbital fibroblasts was performed in freshly isolated fibroblasts,²⁷ rather than cultured fibroblasts, as in the present investigation. However, we have performed studies on human fibroblasts derived from EOMs in a fashion identical to that described in this report and have observed a Thy-1 expression pattern by cytometric analysis (Kusner LL, et al., unpublished observations, 2007), similar to that described by Smith.²⁷

Genomic Profile

Based on previous investigations of human skin fibroblasts,⁵ it was not surprising that genomic profiling identified a broad range of genes that were differentially expressed between EOM- and LM-Fibro. In particular, genes responsible for synthesis of the extracellular matrix, cell signaling, and transcriptional factors have been found to be differentially expressed among human fibroblasts from different anatomic sites,⁵ similar to the present investigation of muscle-derived fibroblasts.

Several transcription factors were specifically expressed in EOM-Fibro (*Myog*, *Eya2*, and *Ankrd1*) or LM-Fibro (*Hoxa9*, *Hoxa5*, and *Mab211l*). The differential expression of transcription factors guides the formation of a particular tissue and can provide localization cues that control spatial patterning during embryogenesis.⁵ Fibroblasts of the orbit are derived from neural ectoderm, in contrast to fibroblasts of other regions, including the leg musculature, which are of mesenchymal origin.^{3,28} Therefore, basic differences in transcriptional factor expression would be expected.^{28,29}

Specific *Hox* genes were expressed at a higher level in LM- than in EOM-Fibro. Fibroblast localization may depend on a unique code of *HOX* gene expression that determines positional memory. Although the localization cue for EOM has not been determined, *Pitx2* expression in the EOM is necessary for development of EOM.³⁰ EOM-Fibro expressed higher levels of *Pitx2* than did LM-Fibro, suggesting that *Pitx2* plays a role in fibroblasts to support EOM development; however, at present no other data exist to support this proposal.

Expression of laminin (*Lama2*) was increased 17-fold in EOM-Fibro compared with LM-Fibro; this is consistent with EOM having a much denser connective tissue matrix compared with skeletal muscle in general, including leg muscle. In humans, the extrasynaptic basement membrane of the EOM contains laminin- α 2, - β 1, - β 2, and - γ 1, and in contrast to limb muscle, also contains laminin- α 4 and - α 5.²² In addition, EOM expresses Lutheran protein, which is an α 5-chain-specific receptor not found in limb muscle.²² Laminin isoform expression differs at the neuromuscular junctions of EOM compared with other skeletal muscle junctions, consistent with the differential

expression of other synaptic proteins at the neuromuscular junctions of EOM.³¹ Vitrin (*Vit*), a glycosaminoglycan binding protein, involved in cell-substrate adhesion, was differentially expressed in EOM-Fibro³² as was collagen type VIIIa (*Col8a1*), a critical component of the anterior segment of the eye.³³ These results suggest that EOM-Fibro possess a genomic signature that leads to production of an extracellular matrix distinct from that of LM-Fibro.

The heterogeneity of fibroblasts is manifest in certain disorders and with particular stimuli—for example, it can be observed in variations in the extent of fibrosis. In Duchenne muscular dystrophy, there is a wide variation in fibrosis between different muscle groups³⁴ raising the possibility that the specific response of EOM fibroblasts could contribute to sparing of the EOM in muscular dystrophy.³⁵ Therefore, a greater understanding of the biology of EOM perimysial fibroblasts could be relevant to therapeutic intervention for muscular dystrophy.

The connective tissue matrix contributes to tension, which in turn influences the forces involved in eye movements.³⁶ This influence may be appreciated by the return of the globe to a central position after physical displacement when a patient is under anesthesia.³⁷ Investigations of fibroblasts in three-dimensional matrices have demonstrated a range of collagen formation and fibroblast morphology that falls along a spectrum from compliant to stiff.³⁸ Similar to dermal fibroblasts,³⁸ we have observed that EOM-Fibro in three-dimensional cultures (Kusner LL, unpublished data, 2008) align themselves along the lines of force, suggesting that the fibroblasts themselves might contribute to tension (Kusner LL, unpublished data, 2008). Therefore, connective tissue properties could be modified as a means of manipulating eye movements, especially in pathologic states.^{39,40}

The response of EOM to immune mediators differs from that of other skeletal muscle^{1,41} and fibroblasts may play a role in this response. Several immune system-related genes were differentially expressed between EOM- and LM-Fibro. In particular, expression of *CD59a*, a complement regulator that influences the severity of experimental myasthenia gravis,^{42,43} and *IL-6*, which is involved in Graves' ophthalmopathy,^{44,45} were both increased in EOM-Fibro. Fibroblasts act as immunomodulators by secreting factors such as cytokines, lipid mediators, and growth factors.⁷ Fibroblasts react to early signals of injury and coordinate recruitment of immunocompetent cells from bone marrow,⁴⁶ thereby, performing a critical role in wound healing and tissue remodeling. Accumulation of extracellular matrix may be detrimental in diseases such as Graves' ophthalmopathy.⁴⁷ Evaluation of the properties of fibroblasts in Graves' ophthalmopathy has brought focus on the orbital fibroblast, as well as the perimysial fibroblasts of EOMs²⁷ and EOM fibers.^{48–50} Whole EOM genomic profiles demonstrated increased expression of calsequestrin, which may be an autoantigen in Graves ophthalmopathy.⁵⁰ It should be noted that EOM-Fibro did not differentially express the calsequestrin gene, and its expression appears isolated to EOM fibers. Our genomic profile and the work of others supports the concept of a unique immunologic environment for the EOM and the orbit.

The genomic profiles of EOM reflect distinct metabolic requirements, which are also indicated by enzymatic histologic characterization, mitochondrial contents, fatigue-resistance, and vascularity.^{41,51} The EOM-Fibro genomic profile identified certain differentially expressed transcripts that suggest that EOM-Fibro may influence EOM fiber metabolism. For example, the increase in secretion of factors, such as IL-6, from EOM-Fibro could enhance glucose absorbance by the highly active EOM fibers, as observed in other skeletal muscle.^{52–54}

Functional Analysis

Fusion of myoblasts is a necessary step in myogenesis that allows for formation and growth of myotubes.^{55,56} We designed a co-culture system of EOM- or LM-Fibro with CL-EOM or -LM and used the standard measure of myotube percentage as a functional measure of myotube maturity. Our experiments identified a clear propensity for CL-EOM to form myotubes with a greater number of myonuclei when cultured with EOM-Fibro, compared with LM-Fibro, whereas the addition of LM- or EOM-Fibro to CL-LM had no influence on the number of myotubes. Incubation of murine myoblasts from the C2C12 cell line with primary human fibroblast cultures enhances myoblast maturity, as defined by identification of a mature sarcomeric structure and contractile myotubes.¹⁴ We observed the same effect when CL-EOM were cultured on the EOM-Fibro. In the same study,¹⁴ the investigators suggested that the adherence of myotubes to underlying fibroblasts provided an elastic support, that allowed myotube contraction while protecting from contraction-related injury. Moreover, the contractions themselves could serve as a trigger for enhanced maturation. The same may be true in our studies; however, the results of the permeable filter support culture experiments strongly indicate that a soluble factor produced by the EOM-Fibro provided trophic support of CL-EOM and, surprisingly, also enhanced CL-LM myotube formation. If EOM fibers are in a continuous state of remodeling, as has been hypothesized,⁵⁷ soluble factors from perimysial fibroblasts of the EOM could provide signals for myoblast fusion.

CONCLUSIONS

This study demonstrates that component cell types can be important determinants of the final EOM phenotype. Fibroblasts of the EOM may support the metabolic demands of the muscle fibers, contribute to tensile force, and be an important part of the immunologic environment. Although the differential response of EOMs to disease is well appreciated, to date the focus has primarily been on the muscle fibers. Further characterization of fibroblasts of the orbit may identify a significant role in differential disease response, and if so, manipulation of fibroblast properties could lead to novel therapeutic approaches.

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