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Fibrosis, Gene Expression, and Orbital Inflammatory Disease

James T. Rosenbaum[1,2,3], **Dongseok Choi**[1,4], **David J. Wilson**[1], **Hans E. Grossniklaus**[5], **Christina A. Harrington**[6], **Roger A. Dailey**[1], **John D. Ng**[1], **Eric A. Steele**[1], **Craig N. Czyz**[7], **Jill A. Foster**[8], **David Tse**[9], **Chris Alabiad**[9], **Sander Dubovy**[9], **Prashant Parekh**[9], **Gerald J Harris**[10], **Michael Kazim**[11], **Payal Patel**[11], **Valerie White**[12], **Peter Dolman**[12], **Deepak P. Edward**[13], **Hind Alkatan**[13], **Hailah al Hussain**[13], **Dinesh Selva**[14], **Patrick Yeatts**[15], **Bobby Korn**[16], **Don Kikkawa**[16], **Patrick Stauffer**[1], and **Stephen R. Planck**[1,2,3]

[1]Casey Eye Institute, Oregon Health & Science University, Portland OR, USA

[2]Department of Medicine, Oregon Health & Science University, Portland OR, USA

[3]Devers Eye Institute, Legacy Health Systems, Portland OR, USA

[4]Department of Public Health and Preventive Medicine, Oregon Health & Science University, Portland OR, USA

[5]Department of Ophthalmology, Emory University, Atlanta, GA, USA

[6]Integrated Genomics Laboratory, Oregon Health & Science University, Portland OR, USA

[7]Division of Ophthalmology, Ohio University, Columbus, OH, USA

[8]Department of Ophthalmology, The Ohio State University, Columbus, OH, USA

[9]Department of Ophthalmology, University of Miami, FL, USA

[10]Department of Ophthalmology, Medical College of Wisconsin, Milwaukee, WI, USA

[11]Department of Ophthalmology, Columbia University, New York, NY, USA

[12]Department of Ophthalmology and Visual Sciences, University of British Columbia, Vancouver, BC, Canada

[13]Research Department, King Khaled Eye Specialist Hospital, Riyadh, Saudi Arabia

[14]Ophthalmology Network, Royal Adelaide Hospital, Adelaide, SA, Australia

[15]Department of Ophthalmology, Wake Forest University, Winston-Salem, NC, USA

[16]Department of Ophthalmology, University of California, San Diego, La Jolla CA, USA

Abstract

Corresponding author James T. Rosenbaum, M.D., Casey Eye Institute, Oregon Health & Science University, 3375 SW Terwilliger Blvd, Portland, OR 97239, rosenbaj@ohsu.edu, Phone: 503-494-5023, Fax: 503-494-6875.

Competing Interest Disclosures: Dr. Rosenbaum has in the past consulted for Genentech and was a co-investigator on a studied funded by Genentech to evaluate the use of rituximab for orbital inflammatory diseases. The other authors report no potential competing interest.

Background/aims—To clarify the pathogenesis of fibrosis in inflammatory orbital diseases, we analyzed the gene expression in orbital biopsies and compared our results to those reported for idiopathic pulmonary fibrosis.

Methods—We collected 140 biopsies from 138 patients (58 lacrimal gland; 82 orbital fat). Diagnoses included healthy controls (n=27), nonspecific orbital inflammation (NSOI) (n=61), thyroid eye disease (TED) (n=29), sarcoidosis (n=14), and granulomatosis with polyangiitis (GPA) (n=7). Fibrosis was scored on a zero to three scale by two expert, ophthalmic pathologists. Gene expression was quantified using Affymetrix U133 plus 2.0 microarray.

Results—Within orbital fat, fibrosis was greatest among subjects with GPA (2.75±0.46) and significantly increased in tissue from subjects with GPA, NSOI, or sarcoidosis ($p<0.01$), but not for TED, compared to healthy controls (1.13±0.69). For lacrimal gland, the average score among controls (1.36±0.48) did not differ statistically from any of the 4 disease groups. Seventy-three probe sets identified transcripts correlating with fibrosis in orbital fat (false discovery rate < 0.05) after accounting for batch effects, disease type, age and sex. Transcripts with increased expression included fibronectin, lumican, thrombospondin, and collagen types I and VIII, each of which has been reported upregulated in pulmonary fibrosis.

Conclusion—A pathologist's recognition of fibrosis in orbital tissue correlates well with increased expression of transcripts considered essential in fibrosis. Many of the transcripts implicated in orbital fibrosis have been previously implicated in pulmonary fibrosis. TED differs from other causes of orbital fat inflammation in that fibrosis is not a major component. Marked fibrosis is less common in the lacrimal gland compared to orbital adipose tissue.

Keywords

Orbital inflammation; fibrosis; gene expression; lacrimal gland

INTRODUCTION

Fibrosis is an important component of the inflammatory response. In many diseases including proliferative vitreoretinopathy, mucous membrane pemphigoid, cirrhosis, scleroderma, idiopathic pulmonary fibrosis, and retroperitoneal fibrosis, the fibrotic component of the disease is a dominant clinical feature. The ability to prevent or reverse fibrosis requires an understanding of its pathogenesis.

Exophthalmos can be due to infections, malignancies, or inflammation. The inflammatory processes include Graves disease (also known as thyroid eye disease or TED), sarcoidosis, granulomatosis with polyangiitis (GPA) (previously known as Wegener's granulomatosis), IgG4 disease, and Erdheim-Chester disease [1,2]. Fibrosis can be a prominent component in orbital inflammation. It is considered to be an ominous prognostic finding [3].

In order to clarify the pathogenesis of orbital inflammatory diseases, we have assembled an international consortium of orbital surgeons and ophthalmic pathologists. We have collected a library of formalin-fixed biopsies of either the lacrimal gland or orbital fat. We have scored these biopsies for fibrosis and then correlated the fibrosis score with gene expression.

Finally we compared genes with increased expression in orbital fibrosis to transcripts reportedly increased in idiopathic pulmonary fibrosis.

METHODS

Tissue and pathology review

The orbital biopsies were performed by surgeons at ten different centers from 3 continents. All biopsies were fixed in formalin. The diagnosis was made on the basis of clinical information and pathological review at the center where the biopsy was obtained. Normal, uninflamed control orbital tissue was obtained from subjects with no history of orbital disease at the time of surgery such as fat typically excised and discarded as part of routine blepharoplasty or retrobulbar fat that is snared with the eye during enucleation. The pathology was further reviewed by two of the authors (DJW and HEG). Most pathological diagnoses were accepted but in a small number of cases, an alternative diagnosis was suggested and the tissue was not included in further analysis. DJW and HEG independently scored the fibrosis as absent (0), mild (1), moderate (2), or severe (3). One pathologist based the grading scheme on the percent of tissue that was fibrotic: no fibrosis was graded as 0; up to 1/3 of the specimen containing fibrosis was graded as mild (1); 1/3 to 2/3 of the specimen containing fibrosis was graded as moderate (2); and greater than 2/3 of the specimen containing fibrosis was graded as severe (3). The other pathologist graded the tissue more qualitatively overall as displaying no fibrosis versus mild, moderate or severe fibrosis.

Microarray

All tissue was sent to Oregon Health & Science University, Portland, Oregon, where RNA was extracted. cDNA was synthesized from the RNA and hybridized in two batches to Affymetrix U 133 plus 2.0 arrays, which include about 54,000 probe sets. The methodology for the RNA extraction and microarray have been previously described [4]. Further, we have reported on the RNA quality (Rosenbaum et al., manuscript submitted) and the correlation between our array data and quantitative PCR [5].

Statistical methods

Each batch of Affymetrix cel files of a tissue type was preprocessed by Robust Multiarray Analysis separately [6]. Then, a linear regression model was fitted to the preprocessed data to estimate trend in intensity with respect fibrosis score while accounting for batch effects, disease type, age and sex to all data of a tissue type independently. To fit the model, we used RUVinv in conjunction with empirical Bayes and false discovery rate adjustment for multiple test corrections [7]. These methods are available in ruv and limma packages of R Statistical Computing Environment (http://www.r-project.org). For negative controls, we used the human housekeeping genes reported in Eisenberg and Levanon (2003) [8].

RESULTS

We analyzed 150 biopsies including 85 from orbital fat and 65 from the lacrimal gland. The 85 orbital biopsies were obtained from 82 subjects with two biopsies read from 3 subjects. The 65 lacrimal biopsies were obtained from 56 subjects. The lacrimal biopsies included

from two subjects who provided two separate tissue blocks and 7 subjects for whom two tissues from the same block were independently scored. Table 1 shows the age, gender, and number of biopsies for each diagnosis for the orbit and lacrimal gland respectively. Each pathologist scored the tissue for fibrosis independently using a 0 to 3 scale. All except nine normal control tissues were scored by both pathologists. The scores were averaged. For 64 lacrimal gland biopsies scored by both pathologists, 20 received an identical score (31.3%). In 35 instances (54.7%) the scores differed by one. In 9 instances, the scores differed by two (14.1%). For the orbital biopsies, 77 biopsies were scored by both pathologists. In 38 instances (49.3%), the scores were identical. Thirty-one times the scores differed by one (40.3%) and 8 times the scores differed by two (10.4%). The average fibrosis scores for diseases affecting orbital fat are shown in Figure 1A. The greatest degree of fibrosis was noted among subjects with GPA ($p<0.0001$ relative to healthy controls). Subjects with NSOI ($p<0.002$) or sarcoidosis ($p=0.005$) also demonstrated significantly more fibrosis than what was detected in control tissue, although each value was significantly less than for GPA ($p<0.01$). In contrast, patients with TED had an average fibrosis score that did not differ from controls.

Fibrosis scores for lacrimal gland diseases are also shown in Figure 1A. In contrast to inflammation affecting orbital fat, none of the disease processes resulted in fibrosis that differed statistically from the healthy controls. However, we only had a single lacrimal gland with the diagnosis of GPA. Figure 1B shows the frequency scores for fibrosis for each tissue site. The values should not be compared directly because the frequency for specific diagnoses differed between the tissues. However, it is apparent that a fibrosis score of 3 was far more likely to be scored in disease affecting orbital adipose tissue.

We then generated a list of transcripts that correlated with the scores of fibrosis in the orbit (FDR <0.05) while accounting for batch effects, disease type, age and sex. This is shown in Tables 2 and 3 with probe sets indicating increased and decreased expression, respectively. It includes 73 probe sets. Several of these code for proteins that are strongly implicated in fibrosis [9] including several types of collagen, thrombospondin, lumican, and fibronectin. With one exception, we were not able to correlate increased transcript expression with fibrosis within the lacrimal gland, a result that we attribute to the relative lack of fibrosis in that tissue. The only exception was an increase in nuclear-pore complex interacting protein-like 2.

Fibrosis in specific tissues might be regulated by unique mediators, or it could be that there is substantial overlap between fibrosis within the orbit and fibrosis in other tissues. In order to test this latter hypothesis, we compared the list of transcripts increased in orbital disease with a list previously reported for idiopathic pulmonary fibrosis [9]. As shown in Table 4, many transcripts up regulated in the orbit affected by fibrosis have also been detected as up regulated in idiopathic pulmonary fibrosis.

DISCUSSION

Fibrosis is a characteristic feature of many inflammatory diseases. We believe that our study is the first to compare the likelihood of fibrosis among various forms of orbital disease. Our

results indicate that GPA is especially likely to be associated with fibrosis. We have recently noted that many patients with NSOI might have a limited form of GPA (Rosenbaum, JT, Choi, D, Wilson, DJ, et al., Orbital Pseudotumor Can Be a Localized Form of Granulomatosis with Polyangiitis Based on Gene Expression Profiling, manuscript submitted), so it should not be surprising that this entity is also associated with fibrosis. On the other hand, fibrosis was minimal among patients with TED. We anticipated that fibrosis would also accompany disease in the lacrimal gland, but our data indicate that fibrosis is generally mild in lacrimal specimens and statistically the fibrosis from diseased, lacrimal tissue did not differ from fibrosis in healthy controls.

IgG4 related disease has recently been defined as a multisystem disease associated with storiform fibrosis [10]. IgG4 related disease is well described within the orbit [11–14]. We recently reported on the prevalence of IgG4 staining in tissue from patients with orbital inflammatory disease [4]. We found only a slight correlation between the degree of fibrosis and IgG4 staining. Further, we found that many patients with GPA, NSOI, or sarcoidosis had IgG4 staining in their orbital tissue [4]. We did not specifically investigate the role of IgG4 in the present study, but the majority of the tissues described in this present report were included in our previous report on IgG4 [4], and none of subjects in the prior report had a multisystem disease that could be classified as IgG4 related disease.

The two pathologists scoring fibrosis in tissue for this project agreed in only a minority of instances, 31% for lacrimal gland and 49% for orbital adipose tissue. In 14% and 10% for lacrimal gland and orbital fat respectively, the pathologists differed by a score of two. In fact, one of the pathologists who scored 9 lacrimal gland biopsies twice (because two separate blocks were evaluated) provided discordant scores 8 of the 9 instances. The intra-observer and inter-observer discrepancies could result from the subjective nature of the scoring system as well as from sampling error. The pathologists accurately recognized fibrosis since tissue with greater fibrosis expressed transcripts associated with fibrosis in another tissue, the lung. We speculate that a fibrosis score based on transcript expression might eventually be standard in biopsied tissue because it can be quantified and would minimize the sampling errors that confront a histopathologist.

We noted more fibrosis in orbital fat than in lacrimal specimens. A possible explanation for this is that the adipocytes themselves contribute to fibrosis. For example, the adipokines, leptin and adiponectin, have been implicated in hepatic fibrosis [15]. In skin affected by scleroderma, adipocytes reportedly demonstrate the potential to transform into myofibroblasts that are a critical part of the fibrosing process [16]. Future studies should compare the levels of adipokines in the lacrimal gland versus the anterior orbit as these cytokines might represent a potential therapeutic target.

The transcripts which we found to be up regulated in association with fibrosis include many that code for proteins generally implicated in fibrosis including collagens, fibronectin, and thrombospondin. This suggests that pathological grading of fibrosis has an expected molecular correlate with gene expression and it validates the histopathological interpretation. The finding of overlapping, up regulated genes in fibrosis affecting the lung and orbit also supports the validity of the technique and indicates that fibrosis in diverse

tissues must share common elements in pathogenesis. An analysis of gene expression in fibrosing diseases holds the promise to discover novel targets for pharmacotherapy.

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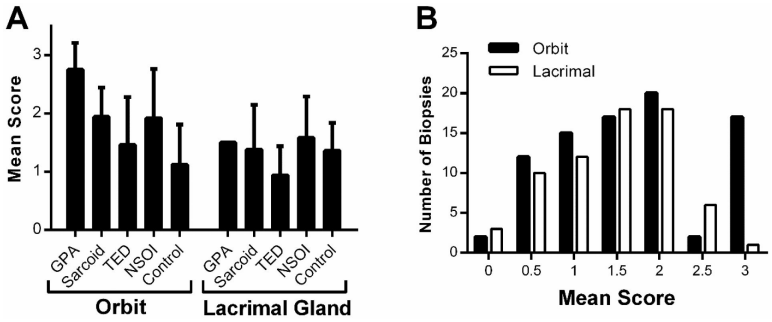

Figure 1.

Orbital adipose tissue from subjects with GPA had the highest fibrosis scores. A: Orbital adipose and lacrimal gland fibrosis scores for each disease group (mean ± standard deviation). B: The distribution of fibrosis scores relative to the site of biopsy.

Table 1

Subject demographics

	Orbital Adipose		Lacrimal Gland	
Disease	**Female:Male**	**Age (mean ± SD)**	**Female:Male**	**Age (mean ± SD)**
GPA	4:2	41.7 ± 10.8	1:0	12.9
NSOI	16:8	50.8 ± 23.9	27:10	46.8 ± 17.4
Sarcoidosis	5:2	47.7 ± 12.3	6:2	35.4 ± 11.5
TED	19:6	51.6 ± 14.0	4:1	54.6 ± 6.2
Normal	14:6	63.6 ± 14.5	6:1	68.4 ± 8.9

Table 2

Probe sets that indicate increased gene expression in orbital adipose with fibrosis.

Probe Set	Gene Title	Fold Change*	FDR p-value
243085_at	---	1.2181	0.0374
224694_at	anthrax toxin receptor 1	1.3928	0.0016
220092_s_at		1.2915	0.0310
202965_s_at	calpain 6	1.4451	0.0406
224619_at	cancer susceptibility candidate 4	1.1945	0.0449
207173_x_at	cadherin 11, type 2, OB-cadherin (osteoblast)	1.4538	0.0014
207172_s_at		1.3217	0.0100
202404_s_at	collagen, type I, alpha 2	1.5119	0.0016
221900_at	collagen, type VIII, alpha 2	1.3230	0.0098
225681_at	collagen triple helix repeat containing 1	1.5247	0.0265
232343_at	dynactin 5 (p25)	1.1912	0.0252
213853_at	DnaJ (Hsp40) homolog, subfamily C, member 24	1.2404	0.0450
201430_s_at	dihydropyrimidinase-like 3	1.2101	0.0406
212231_at	F-box protein 21	1.1767	0.0494
212464_s_at	fibronectin 1	1.5643	0.0015
211719_x_at		1.5108	0.0016
216442_x_at		1.5068	0.0016
210495_x_at		1.4716	0.0036
214701_s_at		1.4095	0.0077
226930_at	fibronectin type III domain containing 1	1.6789	0.0406
209524_at	hepatoma-derived growth factor, related protein 3	1.2324	0.0406
211868_x_at	immunoglobulin (IgG) heavy locus; IgG heavy constant alpha 1; IgG heavy constant alpha 2 (A2m marker); IgG heavy constant delta; IgG heavy constant gamma 1 (G1m marker); IgG heavy constant gamma 2 (G2m marker); IgG heavy constant gamma 3 (G3m marker); IgG heavy constant mu; IgG heavy variable 4–31	1.2907	0.0406
211635_x_at	IgG heavy constant alpha 1; IgG heavy constant alpha 2 (A2m marker); IgG heavy constant delta; IgG heavy constant gamma 1 (G1m marker); IgG heavy constant gamma 3 (G3m marker); IgG heavy constant gamma 4 (G4m marker); IgG heavy constant mu; IgG heavy variable 4–31	1.4915	0.0252
211640_x_at	IgG heavy constant gamma 1 (G1m marker); IgG heavy constant mu	1.5036	0.0415
216541_x_at		1.4027	0.0406
211647_x_at		1.3756	0.0382
216853_x_at	IgG lambda variable 3–19; NULL	1.4989	0.0265

Probe Set	Gene Title	Fold Change*	FDR p-value
201744_s_at	lumican	1.6664	0.0006
218729_at	latexin	1.3505	0.0489
37005_at	MINOS1-NBL1 readthrough; neuroblastoma 1, DAN family BMP antagonist	1.2373	0.0016
201621_at	neuroblastoma 1, DAN family BMP antagonist	1.2149	0.0098
1554591_at	prostate cancer associated transcript 4 (non-protein coding)	1.2300	0.0277
227276_at	plexin domain containing 2	1.2265	0.0406
204517_at	peptidylprolyl isomerase C (cyclophilin C)	1.2210	0.0449
211737_x_at	pleiotrophin	1.4403	0.0098
209466_x_at		1.3701	0.0449
224901_at	stearoyl-CoA desaturase 5	1.2773	0.0277
228844_at	solute carrier family 13 (sodium-dependent citrate transporter), member 5	1.2062	0.0449
212354_at	sulfatase 1	1.3868	0.0449
201107_s_at	thrombospondin 1		0.0252
201108_s_at		1.4172	0.0406
201109_s_at			0.0449
201666_at	TIMP metallopeptidase inhibitor 1	1.2909	0.0406
210986_s_at	tropomyosin 1 (alpha)	1.2643	0.0491
1553718_at	zinc finger protein 548	1.2220	0.0449

*Based on a linear trend

Table 3

Probe sets that indicate decreased gene expression in orbital adipose with fibrosis.

Probe Set	Gene Title	Fold Change*	FDR p-value
206548_at	---	−1.2552	0.0075
49452_at	acetyl-CoA carboxylase beta	−1.2267	0.0449
207275_s_at	acyl-CoA synthetase long-chain family member 1	−1.3043	0.0449
209612_s_at	alcohol dehydrogenase 1B (class I), beta polypeptide	−1.7190	0.0014
209613_s_at		−1.5021	0.0397
207175_at	adiponectin, C1Q and collagen domain containing	−1.8639	0.0101
204151_x_at	aldo-keto reductase family 1, member C1	−1.4219	0.0006
216594_x_at		−1.2731	0.0449
209699_x_at	aldo-keto reductase family 1, member C2; aldo-keto reductase family 1 member C2-like	−1.4008	0.0014
218803_at	checkpoint with forkhead and ring finger domains, E3 ubiquitin protein ligase	−1.2139	0.0305
209683_at	family with sequence similarity 49, member A	−1.2642	0.0305
215602_at	FYVE, RhoGEF and PH domain containing 2	−1.2499	0.0075
201540 at	four and a half LIM domains 1	−1.4247	0.0274
214505_s_at		−1.4073	0.0397
210299_s_at		−1.3667	0.0480
226648_at	hypoxia inducible factor 1, alpha subunit inhibitor	−1.1994	0.0458
214767_s_at	heat shock protein, alpha-crystallin-related, B6	−1.4170	0.0406
226304_at		−1.3860	0.0449
1566472_s_at	all-trans-retinol 13,14-reductase-like; retinol saturase (all-trans-retinol 13,14-reductase)	−1.2564	0.0406
202016_at	mesoderm specific transcript	−1.3110	0.0449
231736_x_at	microsomal glutathione S-transferase 1	−1.3182	0.0077
224918_x_at		−1.2982	0.0139
1555740_a_at	melanocortin 2 receptor accessory protein	−1.3791	0.0305
205913_at	perilipin 1	−1.7415	0.0077
205478_at	protein phosphatase 1, regulatory (inhibitor) subunit 1A	−1.5391	0.0077
1563542_a_at	sex comb on midleg-like 4 (Drosophila)	−1.2117	0.0372
215505_s_at	striatin, calmodulin binding protein 3	−1.1932	0.0449
229477_at	thyroid hormone responsive	−1.4388	0.0449

* Based on a linear trend

Table 4

Genes with increased expression in orbital fibrotic disease (Table 2) in common with genes with increased expression in idiopathic pulmonary fibrosis [9].

	Orbital Adipose		Lung	
Gene Title	**Fold Change***	**FDR p-value**	**Fold Change**	**Q value**
anthrax toxin receptor 1	1.3928	0.0016	0.81	2.10E-04
	1.2915	0.031		
cadherin 11, type 2, OB-cadherin (osteoblast)	1.4538	0.0014	0.64	8.40E-03
	1.3217	0.01		
calpain 6	1.4451	0.0406	1.96	6.70E-04
collagen triple helix repeat containing 1	1.5247	0.0265	8.41	2.20E-07
collagen, type I, alpha 2	1.5119	0.0016	1.21	4.90E-03
collagen, type VIII, alpha 2	1.323	0.0098	1.44	2.60E-07
dihydropyrimidinase-like 3	1.2101	0.0406	1	1.30E-04
fibronectin 1	1.5643	0.0015	2.25	2.10E-04
	1.5108	0.0016		
	1.5068	0.0016		
	1.4716	0.0036		
	1.4095	0.0077		
fibronectin type III domain containing 1	1.6789	0.0406	14.44	2.80E-08
immunoglobulin heavy constant alpha 1; immunoglobulin heavy constant alpha 2 (A2m marker); immunoglobulin heavy constant delta; immunoglobulin heavy constant gamma 1 (G1m marker); immunoglobulin heavy constant gamma 3 (G3m marker); immunoglobulin heavy constant gamma 4 (G4m marker); immunoglobulin heavy constant mu; immunoglobulin heavy variable 4–31	1.4915	0.0252	3.61	9.30E-03
immunoglobulin heavy locus; immunoglobulin heavy constant alpha 1; immunoglobulin heavy constant alpha 2 (A2m marker); immunoglobulin heavy constant delta; immunoglobulin heavy constant gamma 1 (G1m marker); immunoglobulin heavy constant gamma 2 (G2m marker); immunoglobulin heavy constant gamma 3 (G3m marker); immunoglobulin heavy constant mu; immunoglobulin heavy variable 4–31	1.2907	0.0406	10.89	5.40E-08
immunoglobulin heavy constant gamma 1 (G1m marker); immunoglobulin heavy constant mu	1.5036	0.0415	4.84	6.60E-03
	1.4027	0.0406		
	1.3756	0.0382		
latexin	1.3505	0.0489	2.25	4.90E-06
lumican	1.6664	6.00E-04	2.25	6.50E-04
sulfatase 1	1.3868	0.0449	7.29	3.60E-10
thrombospondin 1	1.4609	0.0252	0.81	4.00E-02

Gene Title	Orbital Adipose		Lung	
	Fold Change*	FDR p-value	Fold Change	Q value
	1.4199	0.0406		
	1.4172	0.0449		
TIMP metallopeptidase inhibitor 1	1.2909	0.0406	0.81	7.60E-03

*Based on a linear trend