

ARTICLE

Co-expression of *FBN1* with mesenchyme-specific genes in mouse cell lines: implications for phenotypic variability in Marfan syndrome

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Mutations in the human *FBN1* gene cause Marfan syndrome, a complex disease affecting connective tissues but with a highly variable phenotype. To identify genes that might participate in epistatic interactions with *FBN1*, and could therefore explain the observed phenotypic variability, we have looked for genes that are co-expressed with *Fbn1* in the mouse. Microarray expression data derived from a range of primary mouse cells and cell lines were analysed using the network analysis tool BioLayout Express^{3D}. A cluster of 205 genes, including *Fbn1*, were selectively expressed by mouse cell lines of different mesenchymal lineages and by mouse primary mesenchymal cells (preadipocytes, myoblasts, fibroblasts, osteoblasts). Promoter analysis of this gene set identified several candidate transcriptional regulators. Genes within this co-expressed cluster are candidate genetic modifiers for Marfan syndrome and for other connective tissue diseases.

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INTRODUCTION

Marfan syndrome (OMIM 154700) is an autosomal dominant condition that affects connective tissue.^{1–4} Individuals show overgrowth of the long bones, lack of adipose and muscle tissue, and abnormalities of the eyes and skin. The major cause of morbidity and mortality is dilatation and dissection of the ascending aorta. Marfan syndrome is usually associated with mutations in the *FBN1* gene (OMIM 134797), encoding the microfibrillar protein, fibrillin-1.^{2,5,6} A bovine Marfan-like syndrome (OMIA 1204) is also due to a mutation in *FBN1*. Homozygous mice that lack a functional *Fbn1* gene have some manifestations similar to Marfan syndrome in humans, although the heterozygous phenotype is mild.⁵ In addition, a transgenic mouse line carrying a mutation known to cause severe disease in humans has a dose-dependent phenotype showing aspects of Marfan syndrome.⁷ A homozygous lethal natural mutation involving duplication of exons 17–40 of the mouse *Fbn1* gene⁸ causes the tight skin (*Tsk*) phenotype in which heterozygotes have abnormalities of skin, viscera, lungs, cartilage, bone, heart and tendons,⁹ with some characteristics of Marfan syndrome such as overgrowth of long bones.

The tissues primarily affected by *FBN1* mutation (including bone, aorta and pulmonary artery, mitral valve, zonular fibres of the eye, dura mater, skin and adipose) contain cells of mesenchymal origin, which synthesize connective tissue extracellular matrix (ECM), composed of fibrous proteins and glycosaminoglycans. The ECM provides strength and elasticity for these tissues. Fibrillin-1 is the major structural component of the extracellular microfibrils of the ECM¹⁰ and also seems to be involved in sequestering the growth factor TGF β in inactive form.^{5,11,12} In adults, mesenchymal cells derive from stem cells residing in the bone marrow and mesenchymal tissues.^{13,14} These stem cells retain the ability to differentiate into cells of connective

tissue lineages, including adipocytes, osteoblasts, chondrocytes, smooth and skeletal muscle, endothelial cells of blood vessels and fibroblasts (reviewed in Barry and Murphy¹⁴). Differentiation of mesenchymal cells into specific cell types requires induction of a range of transcription factors¹⁴ and may also involve interaction with cells of monocyte origin.¹⁵ During organogenesis, mesenchymal cells can also undergo transition to epithelial phenotype (mesenchymal–epithelial transition), with concomitant inhibition of mesenchyme-specific genes and activation of genes required to form intercellular adhesions characteristic of epithelium. The transition between the two states is regulated by a number of cellular factors, especially TGF β family members.^{16,17} The actions of TGF β on mesenchymal cells are mediated through transcription factors such as SNAIL and SLUG (encoded by *SNAIL1* and *SNAIL2* genes),¹⁸ and result in expression of mesenchymal genes and suppression of the epithelial marker E-cadherin.

The phenotype of Marfan syndrome is extremely variable, even among family members carrying the same mutation (see refs.^{4,19,20}). Potential modifier genes for Marfan syndrome are likely to be found in the network of genes that are co-expressed in tissues affected by *FBN1* mutation. Such genes would also be strong candidates for a role in diseases with related phenotypes. In this article, we identify and analyse genes that are stringently co-regulated with *FBN1*.

MATERIALS AND METHODS

Identification and annotation of an *FBN1*-associated cluster of genes

The analysis was performed on publicly available gene expression data (to which we contributed) generated from 44 mouse cell types and 2 mouse organs^{15,21} (Supplementary Table S1) using the Affymetrix MOE430_2

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GeneChip and normalized using MAS5 (Affymetrix, Santa Clara, CA, USA). The data were accessed through GEO DataSets (accession number GSE10246). Correlation networks were constructed from the data on the basis of pairwise Pearson's correlation relationships. A network graph comprising 8578 nodes (probes) and 153 418 edges was generated using BioLayout *Express*^{3D,22}. The resulting graph was then clustered using the Markov Clustering algorithm at an MCL inflation value of 1.7.²³ Clustering was also performed on expression data from mouse tissues.²⁴ Because the initial data did not include chondrocytes, which are likely to be involved in the skeletal phenotype of Marfan syndrome, the expression of cluster genes was also examined in data from a published study of chondrocyte differentiation (cultured limb bud mesenchymal cells; GEO Profiles accession no. GDS1865).²⁵ In addition, we considered data sets from developing mouse kidney (E12.5; GEO Profiles accession no. GDS1583)²⁶ and from developing mouse gastrointestinal tract (E18.5; GEO Profiles accession no. GDS2699).²⁷

Location and function of cluster genes

Genes in the cluster of interest were assessed for recognized homologies, cellular localization and function using publicly available databases (Ensembl, NCBI). Possible or verified involvement in disease was determined by searching the Online Mendelian Inheritance in Man (OMIM) and Online Mendelian Inheritance in Animals (OMIA) databases on the NCBI website.

Determination of functional transcription factor binding sites in promoter regions of *Fbn1*-associated cluster genes

The Affymetrix MOE430_2 probe set was mapped to mouse RefSeq genes and the beginning of RefSeq was taken as a predicted transcription start site. Bioinformatic analysis of motif activity and motif target predictions were performed as described previously.²⁸ All genes represented on the microarray, which had been allocated a RefSeq (12752 in total), were classified as being either among the 205 genes of the *Fbn1*-associated cluster or not within the set. The proportion of genes with a $z(p, m)$ score of greater than 1 for each transcription factor binding motif m was calculated for the two groups and a z -value for this difference was determined. This provides a measure of overrepresentation of predicted targets of the transcription factor in the mesenchymal cluster relative to other genes.

RESULTS

Identification and annotation of *Fbn1*-associated genes in proliferating cells

To identify genes that were strictly co-regulated with mouse *Fbn1* in a cell-autonomous manner, we focused on a large data set derived from primary mouse cells, including primary calvarial osteoblasts undergoing differentiation and a range of haemopoietic cell types (see Supplementary Table S1), produced as described previously.¹⁵ BioLayout *Express*^{3D} analysis of the cell line data generated 480 clusters containing at least five nodes on the basis of their connectivity within the co-expression network graph. The third largest cluster contained 304 transcripts, including two probes for *Fbn1* (1425896_a_at and 1460208_at) (Figure 1a and b). In total, 205 different genes were represented by the 304 probe sets. The full list of genes represented in this cluster is available in Supplementary Material (Supplementary Table S2). This cluster was enriched for genes associated with the ECM. *Fbn1* was a central gene in the cluster (Figure 1b), which was termed the *Fbn1*-associated cluster. The two *Fbn1* probes were correlated (at $r \geq 0.90$) with 241 and 229 probes. Figure 1c shows the averaged expression in 23 cell types of the 304 probes of the cluster. Cells with a high expression of genes in this cluster included mesenchymal cell types such as preadipocytes, myoblasts, fibroblasts and osteoblasts. *Fbn1* had a high expression in mesenchymal cells and minimal expression in other cell types (Figure 1d). Two other probes for *Fbn1* (1438870_at and 1458593_at) did not cluster with this set of genes. This is probably because the latter two probes detected sequences with a very low

expression and high variability (see expression profiles on BioGPS). Both mapped to intronic sequences (Affymetrix website) that have a low frequency of transcript initiation, indicating that these probes may detect rare variant *Fbn1* transcripts that do not show clustering with the major *Fbn1* probe sets. Probes for the other mouse fibrillin gene, *Fbn2*, which has overlapping functions with *Fbn1*,²⁹ did not cluster with *Fbn1* in this data set. *Fbn2* showed expression only in osteoblasts and C3H 10T1/2 cells, and is therefore likely to function more specifically in bone.

Fbn1-associated cluster genes in other data sets

The cell lines assessed in the initial analysis did not include all mesenchymal cell types that would be found within tissues, nor all states of mesenchymal differentiation. To identify a subset of genes that were robustly expressed in mesenchymal tissues rather than cell lines, we clustered expression patterns across tissues in the publicly available GNF1M data set of gene expression in mouse tissues.²⁴ Gene expression showed more diversity across these tissues and there was substantially greater noise in this data set, as evidenced by smaller clusters and overall lower correlation coefficients. Hence, a lower correlation level (at $r \geq 0.75$) was required to detect associations. A total of 119 genes clustered with *Fbn1* in this analysis (Supplementary Table S3). Of these, 24 overlapped with the cluster derived from proliferating cells (indicated in Supplementary Table S3). Classic ECM genes such as *Eln*, *Fbln2*, *Mfap4*, *Mfap5* and *Fbn2* also clustered with *Fbn1* in this analysis of expression in tissues.

One major mesenchyme-derived cell type excluded from the cellular data was the chondrocyte. We therefore examined a published study of the differentiation of primary chondrocytes derived from embryonic footpads.²⁵ Results for 235 of the 304 probes were available, representing 160 different genes. In all, 81% of these genes, including *Fbn1*, were in the highest 25% of expression at most or all time points, extending the view that these genes are co-expressed by proliferating mesenchyme, regardless of lineage.

As noted above, mesenchyme-epithelial transition is a key event in organogenesis. The transition has been analysed separately in developing mouse kidney²⁶ and gastrointestinal tract.²⁷ *Fbn1* expression was strongly associated with mesenchyme in these data sets. *Fbn1*-associated cluster genes such as *Bgn* (biglycan), *Cald1* (caldesmon 1), *Coll1a2* (collagen type 1- α 2 subunit), *Il6st* (interleukin-6 signal transducer), *Ror1* (receptor tyrosine kinase-like orphan receptor 1), *Sparc* (osteonectin; secreted protein, acidic, cysteine rich) and *Timp 2* (tissue inhibitor of metalloproteinases 2) showed a similar pattern of expression to *Fbn1* in both the data sets, whereas others were profile neighbours of *Fbn1* in one or the other data set (not shown).

Cellular location and function of *Fbn1*-associated cluster genes

As summarized in Table 1, 171 members of the *Fbn1*-associated cluster could be assigned a cellular location on the basis of experimental evidence or electronic annotation. The majority were extracellular but a substantial number were involved in secretion. For example, 10% of the annotated genes encoded proteins of the endoplasmic reticulum (including trafficking proteins and molecular chaperones), indicating a surprising level of target specificity for these processing proteins. Table 1 also shows that 181 genes could be assigned a function (Table 1). The largest group (25%) was a broad category of proteins involved in regulating cell size and number. A total of 10% were involved in ECM structure. There were 17 (9.4%) genes encoding known or putative transcription factors, including some families (SLUG/SNAIL, TWIST, PRRX, NFAT, ID, SOX) known to regulate mesenchyme differentiation or function. There were nine genes for

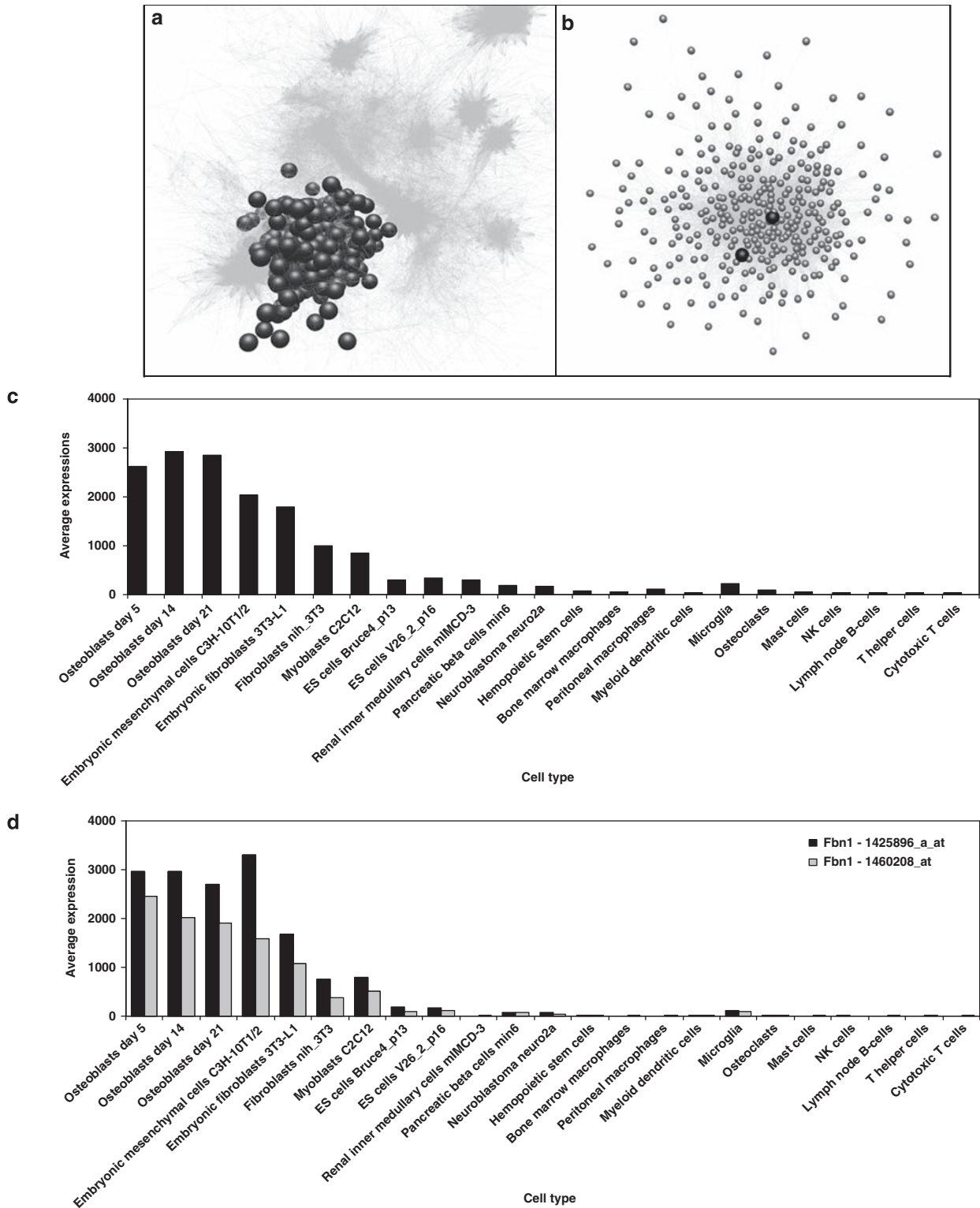


Figure 1 Characteristics of the *Fbn1*-associated expression cluster. (a) A three-dimensional image of the *Fbn1*-associated cluster (nodes shown by black spheres, edges by grey lines) within the network. Other clusters are shown by edges only. (b) A two-dimensional image of the *Fbn1*-associated cluster with the two *Fbn1* probe sets shown as black spheres. (c) Normalized expression of genes in representative cell types, averaged across all probes in the cluster. The means of two experiments performed in triplicate are shown. (d) Expression of two *Fbn1* probes, 1460208_at (black) and 1425896_a_at (grey). The means of two experiments performed in triplicate are shown for each probe. Data are available at GEO DataSets (accession number GSE10246).

Table 1 Cellular location and function of genes in the *Fbn1*-associated gene cluster

Localization	Number	Percent	Function	Number	Percent
Extracellular	60	33.9	Control of cell proliferation	45	25.0
Transmembrane	46	26.0	Enzymes	29	16.1
Endoplasmic reticulum	17	9.6	ECM structure	18	10.0
Nuclear	22	12.4	Transcription factors	17	9.4
Cytoskeleton	20	11.3	Cell adherence	14	7.8
Mitochondrial	1	0.6	Cytoskeleton structure	14	7.8
Unspecified intracellular	11	6.2	G-protein receptors	9	5.0
			Receptor tyrosine kinases	5	2.8
			Other receptors	3	1.7
			Other assigned functions	26	14.4
Total	177		Total	180	

G-protein-coupled receptors and five for receptor tyrosine kinases. Most of the receptors had unknown ligands. Four genes had no informative annotation, with no similarity to known genes or assignable function or location.

The role of genes of the *Fbn1*-associated cluster in disease was examined by assessing entries in OMIM. Of 168 genes with an entry, 60 were associated with a phenotype in mouse (41) or human (29) (Supplementary Table S4). Of these, bones, skin, eyes and blood vessels were most frequently affected in humans, and bones, blood vessels and lung were most frequently noted for mouse. Eight mouse knockout models resulted in embryonic lethality. The results are consistent with a critical role for these genes in the development of the ECM.

Determination of common functional transcription factor binding sites in promoter regions of *Fbn1*-associated cluster genes

To assess the basis for their apparent co-regulation, we subjected the 205 genes of the *Fbn1*-associated cluster to an analysis of transcription factor binding sites.²⁸ Table 2 lists the 15 transcription factors that had the highest positive correlations with the expression pattern of cluster genes. Comparison was also carried out between genes within the cluster and the remaining genes of the data set. Supplementary Table S5 shows the 65 transcription factor binding motifs that showed significant overrepresentation in the cluster genes. Seven transcription factor binding motifs showed a high correlation between activity and expression of the cluster genes and were consistently overrepresented in cluster gene promoters. The motifs were consensus sequences for binding proteins of the TEAD, RP58, MAZ, KLF4, IK1/IK2, BLIMP1 and CIZ families (Table 2). No *Fbn1*-associated cluster gene was significantly ($Z > 2$) associated with activity of all seven of these motifs, and *Fbn1* alone was associated with six. Five of the genes were significantly associated with activity of five of these motifs and fifteen were associated with four.

Identification of genes highly correlated with *Fbn1*

When the initial clustering analysis using cell line data was repeated at a higher stringency of $r \geq 0.95$, 46 probes (31 genes) were found to be in the same cluster as *Fbn1* (Supplementary Table S6). Twelve of these genes were annotated as being located in the ECM, extracellular region or extracellular space. There were five recognized transcription factors and seven receptors. Eight of these genes had no or limited annotation, including a TGF β -induced transcript (*Tgfb1i1*), a steroid-sensitive coiled-coil domain protein (*Ccdc80*) and a transmembrane protein (*Tmem45a*).

As noted above, in this study *Fbn1* expression showed strong association ($Z > 6.5$) with the activity of six of the seven transcription factor motifs identified as having high activity in the cluster (for TEAD, RP58, MAZ, KLF4, BLIMP1 and CIZ family members; Table 2). Three genes (*Loxl3*, *Nfatc4* and *Atoh8*) were associated with activity of five of the six motifs in common with *Fbn1* and 11 were associated with four of the six motifs in common with *Fbn1* (*Nuak1*, *Col1a2*, *Col3a1*, *Gas1*, *Serpinh1*, *Cdh11*, *Thbs2*, *Tpm1*, *Pcdh18*, *Boc*, *Grp23*). In addition, *Fbn1* expression was significantly associated ($Z > 4$) with a number of other motifs found to be overrepresented in the cluster. These included binding motifs for AP-4, MAZR, Broad Complex and SP1-gershenson (Table 2). Five genes (*Gas1*, *Capn6*, *Atoh8*, *Col1a2* and *Snai2*) were associated ($Z > 2$) with 10–15 of the same factors as *Fbn1*.

DISCUSSION

This analysis of gene expression data revealed that the mouse *Fbn1* gene was in a cluster of 205 genes representing a lineage-independent expression signature for mesenchymal cells. Transcription factors binding TEAD, CIZ, RP58, KLF4, MAZ, BLIMP1 and IK1/IK2 sites are candidate regulators of this *Fbn1*-associated cluster. Several of these have known roles in mesenchymal cell types. MAZ (myc-associated zinc-finger protein) has been shown to regulate muscle-specific gene expression.³⁰ RP58 has an essential role in skeletal myogenesis,³¹ and BLIMP1 is involved in myocyte differentiation.³² CIZ is implicated in regulation of bone mass biology.³³ TEAD2 and TEAD4, although not previously implicated in mesenchyme biology, had a similar expression pattern to the genes of the *Fbn1* cluster. KLF family members and IK1/IK2 are associated with transcriptional repression in haemopoietic cells, and their function may be to prevent ectopic expression of the *Fbn1*-associated cluster genes in non-mesenchymal cells. *Fbn1* was associated with six of these seven transcription factor motifs, the only gene with this level of association. The *Fbn1*-associated cluster itself includes genes for a number of transcriptional regulators that are known to be involved in epithelial–mesenchyme transition, including the *Snai1*, *Snai2*, *Prrx 1*, *Prrx2* and *Twist1* genes. Our recent analysis³⁴ detected motifs for PRRX family members in the *Fbn1* proximal promoter region.

The study is limited by a number of factors. The published data were from cells of a single mouse strain, and it would be interesting to use a different strain, especially as there is considerable between-strain variability in gene expression (see Wells *et al*³⁵ and mouse e-QTL data on BioGPS); we would predict that the same genes would continue to cluster on the basis of expression pattern, even though those patterns might vary with different strains. Many members of this *Fbn1* cluster

Table 2 Transcription factor motifs showing the highest correlations of activity with expression of *Fbn1*-associated cluster genes and with *Fbn1*

Transcription factor motif ID	Transcription factor family ^a	Function ^b	Correlation between motif activity and expression of cluster genes ^c	Correlation between motif activity and <i>Fbn1</i> expression ^d
MA0090	TEA domain (TEAD)/transcription enhancer factor (TEF)	Transcriptional activators, interact with vestigial homologues including Vgll3 ^{42,43}	20.01	10.53
M00532	58 kDa repressor protein (RP58)/zinc-finger protein 238 (ZNF238)	Neuronal transcription repressor ⁴⁴	18.95	14.18
M00649	MYC-associated zinc-finger protein (MAZ)	Regulation of inflammation-responsive and muscle genes; regulates initiation and termination of transcription ^{30,45}	16.96	11.57
M00483	ATF6		13.54	NS
MA0039	KLF4	Cell-cycle check-point regulator; transcriptional repressor ⁴⁶	12.31	9.22
MA0083	SRF		11.24	NS
M00086	IK-1/IK-2/ZNFN1A	Chromatin remodelling ^{47,48}	10.08	NS
M00087				
MA0005	Agamous		9.50	NS
M00243	EGR-1/NGFI-C/EGR-3/EGR-2		9.21	NS
M00244				
M00245				
M00246				
M00734	CIZ/ZNF384	Nucleocytoplasmic shuttling; suppresses bone formation ^{33,49}	8.99	6.66
M00133	TST-1/POU3F		8.98	NS
MA0116	ROAZ/ZNF423		8.81	NS
M00639	HNF-6/ONECUT2		8.27	NS
M01066	BLIMP1/PRDM1	Regulation of B and T cell and myocyte differentiation, transcriptional repressor ^{32,50}	8.16	6.77
SP1_gershenson	SP1/Gershenson		7.92	5.37
M00033	p300		7.45	5.61
M00005	AP-4		7.01	6.40
MA0013	Broad Complex 4		6.50	4.99
M00451	NKX3A		6.12	5.80
MA0029	EVI1		6.06	4.90
Homeobox_class: Homeo MF0010			5.77	4.30
MA0089	TCF11_MAFc/NFE211		5.55	3.87
M00706	TFII-1		5.50	4.41
M00432	TTF1		4.64	4.29
M00491	MAZR		4.57	4.38
nanog_mm8	NANOG		4.45	4.26
octsox_dimer_mm8			3.65	2.59
sox2_human	SOX2		3.12	3.32
M00935	NF-AT		2.31	3.08
M00302				
M00456	FAC1		2.20	2.27
M00418	TGIF		NS	2.20

NS, not significant ($z < 2$).^aTranscription factors or families known to bind to the consensus motif used in the search.^bFunction given for the seven closely associated transcription factor families.^cz-value for the correlation between the activity profile for the motif and the average expression profile for the cluster.^dz-value for the correlation between the activity profile for the motif and the expression profile of *Fbn1*.

(including *BGN*, key collagen genes, *SERPINH1* and the transcription factor genes *SNAI2*, *PRRX1* and *TWIST1*) were also co-expressed with *FBN1* in human tumours and tissues (TC Freeman and TN Doig, unpublished results), as were several minimally annotated genes such as *CD248*, *FKBP9*, *LRRIC17* and *TGFBIII*. We did not assess all cell types that are abnormal in Marfan syndrome. For example, the main

morbidity comes from dissection of the aorta, and there were no aortic cells in the study, nor were there cells from the anterior segment of the eye or from dura mater. If these cell types were included, some of the genes would drop out of the cluster and those that remained would represent tightly co-regulated genes that are powerful candidates for a role in modulating the Marfan syndrome phenotype.

The rationale behind our analysis is that genes that are co-expressed with *Fbn1* are candidate modifiers of the effects of *FBN1* mutation in humans and may contribute to other diseases of connective tissue with similar phenotypes. In spite of the limitations, several examples validate this rationale. For instance, a strong association of *Fbn1* with the *Lox* and *Bgn* genes was noted. Biglycan protein (encoded by *Bgn*) has been reported to stimulate synthesis of fibrillin-1 in pressure-induced renal injury and may have a more general role in assembly of connective tissue.³⁶ No disease has been associated with *BGN* mutation in humans but *Bgn*-deficient mice have a skeletal phenotype.³⁷ Lysyl oxidase (*Lox* gene) may be important in overall assembly of elastic microfibrils (reviewed in Wagenseil and Mecham³⁸) and may be involved in preparing tumour cells for metastasis.³⁹ The human homologue of another of the cluster genes, *SERPINH1*, was recently implicated in a recessive form of osteogenesis imperfecta, a bone disease.⁴⁰ Others within the cluster are not well characterized. They include novel transcription factors, G-protein-coupled receptors, nuclear receptors and receptor tyrosine kinases that are clearly potential drug targets and may be important in cell signalling during differentiation of mesenchymal cell types. A group of novel genes encoding hypothetical proteins was also present. The functions of these genes can now be inferred from their co-expression in the cluster,⁴¹ and they clearly warrant a detailed characterization in cells of mesenchymal lineage, and also consideration as modifiers of the Marfan phenotype or as candidate genes in human connective tissue diseases.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGEMENTS

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WEB RESOURCES

The URLs for data presented herein are as follows:

Affymetrix, <http://www.affymetrix.com>.

BioGPS, <http://biogps.gnf.org>.

BioLayout *Express*^{3D}, <http://www.biayout.org>.

Ensembl Human Genome Server, <http://www.ensembl.org>.

National Center for Biotechnology Information, <http://www.ncbi.nlm.nih.gov/>.

NCBI Gene Expression Omnibus, <http://www.ncbi.nlm.nih.gov/geo/>.

Online Mendelian Inheritance in Animals (OMIA), <http://omia.angis.org.au/>.

Online Mendelian Inheritance in Man (OMIM), <http://www.ncbi.nlm.nih.gov/OMIM>.

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