Thorax Online First, published on January 31, 2006 as 10.1136/thx.2005.050005

ENHANCED UPREGULATION OF SMOOTH MUSCLE-RELATED TRANSCRIPTS BY TGFβ_2 IN ASTHMATIC (MYO)FIBROBLASTS.

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Keywords: Fibroblast, myo-fibroblasts, TGFβ, geNorm, Differentiation.

ABSTRACT

TGFβ upregulates a number of smooth-muscle 'specific' genes in (myo)fibroblasts. As asthma is characterised by an increase in airway smooth muscle, we postulated that $TGF\beta_2$ favours differentiation of asthmatic (myo)fibroblasts towards a smooth muscle phenotype. Primary fibroblasts were grown from bronchial biopsies of normal (n=6) and asthmatic (n=7) donors and treated with $TGF\beta_2$ to induce myofibroblast differentiation. We identified the most stable genes for normalisation using RT-qPCR and the geNorm software applied to a panel of 12 'housekeeping' genes. Expression of α-smooth muscle actin (αSMA), heavy chain myosin (HCM), calponin 1 (CPN1), desmin and γ-actin were measured by RT-qPCR. Protein expression was assessed by immunocytochemistry and western blotting. Phospholipase A2 and ubiquitin C were identified as the most stably expressed and practically useful genes for normalisation of gene expression during myofibroblast differentiation. $TGF\beta_2$ induced mRNA expression for all 5 smooth muscle related transcripts; αSMA, HCM and CPN1 protein were also increased, however desmin protein was not detectable. Although there was no difference in basal expression, HCM, CPN1 and desmin were induced to a significantly greater extent in asthmatic fibroblasts compared with normal controls (p=0.041 and 0.011 respectively). Although $TGF\beta_2$ induced the transcription of several smooth muscle-related genes, not all were translated into protein. Thus, while $TGF\beta_2$ is unable to induce a *bona fide* smooth muscle cell phenotype, it may 'prime' (myo)fibroblasts for further differentiation, especially if the cells are derived from asthmatic airways.

KEYWORDS: Normalisation, Fibroblast, Myofibroblast, Differentiation, geNorm, Smooth-Muscle, TGFβ

Abbreviations

αSMA:	Alpha smooth muscle actin.
HCM:	Heavy chain myosin.
CPN1:	Calponin 1
TGFβ:	Transforming growth factor beta.
RT-qPCR:	Reverse transcription quantitative polymerase chain reaction.
FBS :	Foetal bovine serum
DMEM :	Dulbecco's modified Eagle's medium
SFM:	Serum free medium

INTRODUCTION

Bronchial hyperresponsiveness (BHR) is a fundamental abnormality in asthma, which is increased in proportion to disease severity and is functionally antagonised by β2-agonists. The mechanisms underlying BHR are not known for certain, but an increase in airway smooth muscle is considered important [1]. In diseases such as pulmonary hypertension, the increase in smooth muscle bulk has been attributed, in part, to the recruitment of activated fibroblasts, termed myofibroblasts, which align with resident smooth muscle and differentiate further to contribute to the smooth muscle [2]. Although asthma is also characterised by an increase in myofibroblast numbers [3], the relationship between myofibroblasts and smooth muscle cells has not been extensively explored.

The myofibroblast is often described as possessing a phenotype between that of a fibroblast and a smooth muscle cell. Classically, the presence of alpha smooth muscle actin (αSMA) has been considered a marker of myofibroblast differentiation. However, myofibroblasts do not always express αSMA *in vivo* [4, 5]. Cells containing stress fibres containing cytoplasmic actins are termed proto-myofibroblasts, whilst those containing stress fibres including αSMA are termed differentiated myofibroblasts. Although several factors induce the differentiation of fibroblasts into proto-myofibroblasts [6], by far the most potent inducer *in vitro* is $TGF\beta_1$ [7-10]. This profibrogenic growth factor is increased in asthma and has been associated with myofibroblast proliferation and airway remodelling [3]. In particular, the $TGF\beta_2$ isoform has been implicated in the pathogenesis of asthma due to its release from damaged epithelial cells [11].
Microarray analysis of gene expression in foetal lung fibroblasts identified several smooth muscle-associated genes including Heavy Chain Myosin (HCM), Calponin 1 (CPN1) and smoothelin, previously considered a smooth muscle specific gene [12], that were upregulated in response to $TGF\beta_1$. These observations were confirmed at the protein level in adult fibroblasts [7]. Work with animal models has highlighted similar phenotypic switching *in vivo.* For example, in a rabbit model of serosal thickening, smooth muscle-like cells were seen to develop from fibroblasts and expressed a full range of smooth muscle specific markers, including desmin [13]. Such findings suggest the possibility of a complete phenotypic switch from fibroblast to smooth muscle cell.

Recognising the importance of smooth muscle in asthma, we postulated that $TGF\beta_2$ promotes differentiation of asthmatic (myo)fibroblasts into smooth muscle cells at both mRNA and protein levels. To achieve accurate quantitation of expression of smooth muscle-related transcripts, we used the geNorm software to establish the optimum housekeeping genes for use in our differentiation model. Although we found that smooth muscle transcripts were upregulated by $TGF\beta_2$, protein expression was not detectable in all cases. We propose a model whereby $TGF\beta_2$ induces the myofibroblast phenotype whilst priming the cell for further differentiation into a smooth muscle phenotype. This is consistent with a recently published model for smooth muscle differentiation that suggests that *de novo* transcription of mRNA is not an essential prerequisite to the differentiation of smooth muscle to a contractile phenotype [14].

MATERIALS AND METHODS

Primary bronchial fibroblast cultures

Fibroblasts were grown from bronchial biopsies obtained from normal (n=6) or asthmatic (n=7) volunteers by fibreoptic bronchoscopy following standard guidelines [15]. The normal subjects

(4:2 M:F, mean age 21 (range: 20-21) years) had an FEV_1 of 97.25±5.2% predicted and the asthmatic subjects (4:3 M:F, mean age 21 (range: 20-26) had an FEV_1 of 76.4±7.1 % predicted. Asthmatic subjects were using β2-agonists only, as required. Primary fibroblast cultures were established as previously described [16] and were used between passage 2 and 7; typically, all cells were positive for vimentin and <1% of cells were αSMA positive in the absence of $TGF\beta_2$.

To induce myofibroblast differentiation, fibroblasts were seeded on to collagen coated (30µg/ml Vitrogen in H_2O; Nutacon, Leimuiden, NL) dishes and grown to 80-95% confluence before serum starvation (Ultraculture; Biowhittaker, Wokingham, UK) for 24h followed by treatment with $TGF\beta_2$ (Sigma, Poole, UK).

RNA extraction and Reverse Transcription

RNA was extracted using TRIzol reagent (Invitrogen, Paisley, UK) and contaminating DNA removed using DNase (Ambion, Austin, USA) according to the manufacturer's instructions. 1µg total RNA was reversed transcribed using 1mM dNTP (Invitrogen, Paisley, UK), 3ng random hexamer primer (MWG biotech, Milton Keynes, UK) and 100U M-MLV RT enzyme (Promega, Chilworth, UK) according to the manufacturer's instructions.

Real-time qPCR analysis and normalization using genNorm

To establish the most stable genes for normalising, control gene expression was measured in 12 fibroblast cultures before and after myofibroblasts differentiation. 12 normalising genes (Eurogentech, Seraing, Belgium) were selected for analysis; 18S ribosomal RNA (18S), 28S ribosomal RNA (28S), beta actin (ACTβ), glyceraldehyde-3-phosphate (GAPDH), ubiquitin C (UBC), beta-2-microglobulin (B2M), phospholipase A2 (A2), ribosomal protein L13a (RPL13A), succinate dehydrogenase (SDHA), hypoxanthine phosphoribosyl-transferase 1 (HPRT1), TATA box binding protein (TBP), and hydroxymethyl bilane synthase (HMBS). To validate these assays for use with the Δ_{CT} method, melt curve analyses were performed to determine specificity and standard curves plotted as CT value *versus* log dilution from serially diluted cDNA. From these data, the priming efficiency was calculated according the formula $(10^{(-1/gradient)})$-1 and was found to be close to the theoretical maximum for each assay. Target genes were analysed in the same way and validated for ΔC_T based methods of quantification.

geNorm analysis was performed using the geNorm applet (http://medgen31.ugent.be/jvdesomp/genorm/) according to the guidelines and theoretical framework previously described [17]. This approach is based on the principle that, regardless of conditions, the expression ratio of two ideal normalising genes will remain constant in all samples. Hence any variation in expression ratio between the two is indicative of one (or both) genes being variably expressed. In brief, for each normalising gene, Δ_{CT} calculations were performed relative to the strongest signal which was assigned the value 1 and these data used as the input for geNorm. Output files rating gene stability (M) and variation in normalising signal (NF) were collected for fibroblasts, myofibroblasts and the combined data set for both (Fig.1).

For detection of the smooth muscle-related genes, αSMA, HC Myosin, Calponin1, Desmin and γ-actin, primers and fluorogenic probes labelled with the 5' reporter dye, 6-carboxy-fluorescein (FAM) and 3' quencher dye 6-carboxy-*N*,*N*,*N'*,*N'*-tetramethyl-rhodamine (TAMRA) were designed using beacon designer 2.1 (Premier bio-soft). The sequences were as follows:

αSMA: forward primer 5'GACAGCTACGTGGGTGACGAA3',

reverse primer 5'TTTTCCATGTCGTCCCAGTTG3',
probe FAM-5'TGACCCTGAAGTACCCGATAGAACATGGCT3'-TAMRA

HCM: forward primer 5'GCCTCCGTGCTACACAACCT3',
reverse primer 5'CACGCAGAAGAGGCCAGAG 3',
probe FAM-5'ACGTATATATTAGCCCTGAGAAGTACCGCTCCCTT3'-TAMRA

CPN1: forward primer 5'GGTGAAGCCCCACGACATT3',
reverse primer 5'GTTCACCTTGTTTCCTTTCGTCTT3',
probe FAM-5'TGCAGTCCACCCTCCTGGCTTTGT3'-TAMRA

desmin : forward primer 5'GGAGAGGAGAGCCGGATCA3',
reverse primer 5'GGGCTGGTTTCTCGGAAGTT3',
probe FAM-5'TCTCCCCATCCAGACCTACTCTGCCCTT3'-TAMRA

γ-actin : forward primer 5'CAGGTTATCACCATTGGCAATG3',
reverse primer 5'ATGAATTCCAGCGGACTCCAT3',
probe FAM-5'CCCTGAGACCCTCTTCCAGCCTTCCTTT3'-TAMRA

For each sample, the PCR reaction was performed in duplicate and contained 25ng cDNA template, 3pmol fluorogenic probe, 15pmol forward and reverse primers, 12.5µl universal qPCR master mix (Eurogentech, Seraing, Belgium), made up to 25µl with water. RT negative samples confirmed that the signals were not due to genomic contamination. The PCR cycling conditions were: 95°C, 10 min, followed by 42 cycles of denaturation 95°C, 15s, and annealing/extension 60°C, 1 min. Quantitation and real-time detection of the PCR products was followed on an icycler IQ real time detection system (Bio-rad, Hemel Hempstead, UK).

Based on the geNorm analysis (Fig.1), the geometric mean of UBC and A2 was used as the normalising signal. Data were either analysed relative to the geometric mean of UBC and A2 using a standard ΔC_T calculation or normalised to the geometric mean of UBC and A2 and expressed relative to the signal obtained for the average the untreated time-zero controls using a $\Delta\Delta C_T$ calculation.

Immunocytochemistry

Cells were acetone-fixed and air-dried prior to rehydration for immunocytochemical staining using the following primary antibodies: anti-αSMA (1:500), anti-HCM (1:100), anti-Calponin1 (1:200), anti-desmin (1:40) (all antibodies were from Sigma, Poole, UK) with anti-mouse horse-radish peroxidase-conjugated secondary antibody (1:100; Dako, Glostrup, Denmark).

Western Blotting

Cell lysates were prepared in SDS buffer (0.3M Tris-HCl pH6.8, 50% glycerol, 25% 2-mercaptoethanol, 10% SDS, 0.01% bromophenol blue) containing protease inhibitors. Samples were subjected to SDS-PAGE in 12.5% polyacrylamide gels and then transferred onto PVDF membranes (Amersham) for immunoblotting using antibodies against αSMA (1:5000), CPN1 (1:10,000), β-Actin (1:20,000) with ECL detection. Semi-quantitative analysis of protein

expression was performed by densitometry using Genetools software (Syngene, Cambridge, UK); during this analysis, the operator was blinded to the identity of the samples. Since β-Actin was relatively stably expressed during TGFβ$_2$ induced myofibroblast differentiation, it was used to control for protein loading. Data are expressed relative to time-zero control and are presented as box and whisker plots showing the median and interquartile range with the 95% confidence intervals.

Statistical Analyses Data were analyzed using non parametric tests for within (Wilcoxon Signed Rank Test) or between (Mann Whitney U test) group comparisons using SPSS for Windows (version 11.5, Chicago, IL, USA).

RESULTS

In order to determine basal and stimulated mRNA expression of smooth muscle-related transcripts, we used RT-qPCR. However, accurate gene quantification can be problematical, and is especially difficult when quantifying changes in expression occurring during the process of differentiation as "housekeeping" genes used to normalise signals are also likely to be regulated during differentiation. In addition, rates of proliferation and overall transcriptional activity within primary cell cultures tend to be highly variable. For these reasons, we considered it particularly important to establish which housekeeping genes were the most suitable for our purposes.

geNorm analysis of control genes.

The geNorm analysis compares the ratio of the ratio of control genes in different samples to find those that are most stable, as previously described [17]. The 12 normalising genes were rated for stability (M) with the most stable producing the lowest M value (Fig. 1). Analysis of fibroblasts and myofibroblasts produced contrasting orders of gene stability although we consistently found that HPRT1 and RPL13A were the least stable genes, and were therefore unsuitable for use as normalising genes to measure fibroblast/myofibroblast differentiation. Combining the two data sets produced a third sequence of gene stability ratings indicating the genes that are most stable during the differentiation of fibroblasts to myofibroblasts. In all cases, the four best genes gave M values <0.5 indicating that they are reasonably stable. The top two genes cannot be ranked in order because of the requirement for a gene ratio to calculate gene stability. The pair-wise variation analysis in normalising signal (NF_n) was also calculated for the three data sets using geNorm. This measure shows the degree of variation in normalisation signal that is achieved by using n control genes compared to n+1, with genes added step-wise into the analysis in the order of their gene stability rankings (Fig. 2). The stability of the normalisation signal improves up to the addition of the eighth gene and then deteriorates as the four least stable genes are added to the analysis. In all three cases, the top three genes gave values close to the recommended threshold of 0.15 for accurate normalisation. Based on these findings, we chose to use the geometric mean of A2 and UBC for our subsequent analyses.

Smooth muscle-related transcript expression in fibroblasts.

Basal mRNA expression of smooth muscle-related transcripts in undifferentiated fibroblast cultures was quantified relative to UBC and A2. Since these housekeeping genes are highly abundant, expression relative to these controls gives some indication of the overall expression level of target genes (Fig. 3). αSMA was highly expressed at the mRNA level in fibroblasts

cultures having a ratio of approximately 1:1 relative to the geometric mean of UBC and A2. CPN1 and γ-actin were also expressed relatively abundantly whilst both HCM and desmin showed very low basal transcription. No difference in the levels of any smooth muscle-related transcripts was detected between asthmatic or healthy control cells.

Induction of smooth muscle-related transcripts in response to TGFβ$_2$-induced myofibroblast differentiation.

Expression of all five smooth muscle-related transcripts increased significantly from baseline after 24h treatment with 0.04nM or 0.4nM TGFβ$_2$ ($p<0.05$ in all cases, see figure legend) (Fig. 4). Further changes in expression occurred over the next 24h and were dependent on TGFβ$_2$ dose, the origin of the cells and the target gene. A dose of 0.04nM TGFβ$_2$ was sufficient to cause maximal induction of αSMA and γ-actin expression in asthmatic fibroblast cultures (Fig 4), however in control fibroblasts, increasing TGFβ$_2$ to 0.4nM caused a further increase in αSMA and γ-actin expression. Expression of HCM, CPN1 and desmin increased with increasing TGFβ$_2$ doses in all cultures. A lower dose of TGFβ$_2$ (0.004nM) did not cause a significant induction of any of the genes by comparison with the untreated controls although there was a trend for an increase in gene expression by 24h that had diminished by 48h.

A number of disease-related differences in smooth muscle-related transcript expression were detected in the cultures (Fig 3b). αSMA and γ-actin gene expression were maximally induced at 0.04nM TGFβ$_2$ in asthmatic myofibroblasts whilst higher doses of TGFβ$_2$ further induced expression in healthy control fibroblasts (Fig 4). The expression of HCM, CPN1, and desmin mRNA was induced to a significantly greater extent in asthmatic myofibroblasts than in normal controls ($p=0.041$, 0.011 and 0.024 respectively) when treated with either 0.04nM TGFβ$_2$ or 0.4nM TGFβ$_2$ for 48h (Fig 3b). γ-Actin mRNA expression was induced to a significantly greater extent by 0.04nM TGFβ$_2$ treatment for 24h in asthmatic fibroblast cultures than in healthy controls.

Smooth muscle-related transcript protein expression in myofibroblasts.

Protein staining for αSMA was absent from the majority (estimated >99%) of untreated cells (Fig. 5), confirming the fibroblast origin of these cells and the absence of smooth muscle contamination. After treatment with 0.04nM TGFβ$_2$, clearly defined filamentous staining was present in the majority (estimated >90%) of cells. HCM immunostaining was present within the nuclei of untreated cells but was absent in the cytoplasm. Treatment with 0.4nM TGFβ$_2$ induced formation of cytoplasmic myosin filaments in around 80% of cells. Diffuse immunostaining for CPN1 was present within the cytoplasm of the majority (>95%) of untreated cells and, upon TGFβ$_2$ treatment, the intensity of this staining increased and localised to resemble the filamentous staining of αSMA and HCM. Desmin staining was absent from treated and untreated cells (Fig. 6). The activity of this antibody was confirmed by staining of smooth muscle in bronchial biopsy sections (Fig. 6).

Semi-quantitative analysis of αSMA and CPN1 protein expression by western blotting (Fig. 7) showed αSMA protein to be induced to the same extent in asthmatic fibroblasts as healthy control cells, following 48h treatment with 0.4nM TGFβ$_2$. However, consistent with the mRNA

data, CPN1 protein expression was induced to a significantly greater extent (p=0.011) in asthmatic cells than healthy control cells under the same conditions.

DISCUSSION

Asthma is characterised by an increase in myofibroblast numbers and smooth muscle mass. Myofibroblasts have features intermediate between fibroblasts and smooth muscle cells [18] and it has been suggested that they may act as precursors for smooth muscle cells [2, 13, 19]. In this study we provide novel evidence that asthmatic fibroblasts are more responsive than normal fibroblasts for the induction of smooth muscle related genes following differentiation induced by $TGF\beta_2$.

Selection of normalising genes using geNorm analysis

During differentiation, a large number of genes, including 'housekeeping' genes, are up- or down-regulated [7]. This was confirmed by the geNorm analysis where the average expression stability of the control genes was lower in the fibroblast or myofibroblast alone populations than in the combined analysis. For example, B2M is ranked as the most stable gene in undifferentiated fibroblasts, third in myofibroblast cultures, but drops to eighth in the combined analysis indicating that B2M is regulated during differentiation. By contrast SDHA is ranked only seventh, in terms of stability in undifferentiated fibroblasts, third within myofibroblasts but is the best in the combined fibroblast/myofibroblast analysis. SDHA is therefore more stably expressed during differentiation than the other genes that it has "overtaken" in the rankings.

The geNorm analysis indicated that the optimum number of house keeping genes to measure within a combined fibroblast/myofibroblast culture would be eight. However the criteria suggested by the geNorm authors of a pairwise variation analysis between NF_n and NF_{n+1} of less than 0.15 was met by the inclusion of a fifth gene. In fact the inclusion of only the best three genes gave a pairwise variation analysis between NF_n and NF_{n+1} of 0.157. It seems therefore that the measurement of a large number of housekeeping genes is not necessary in this case. Selection of housekeeping genes often reflects a balance between what is scientifically desirable with what is practically achievable. We chose to use the geometric mean of A2 and UBC for our subsequent analyses as they are two of the best three genes in the combined analysis and are available as a multiplex assay allowing simultaneous detection of both genes. This improves accuracy by minimising sampling error and, more importantly, minimises the amount of cDNA required to measure both genes. When working with limited amounts of cDNA, this is of particular importance.

Smooth muscle-related transcript expression in differentiating fibroblasts

Our analysis of smooth muscle-related transcript expression at mRNA and protein level gives us some insight in to the nature and extent of the switch that occurs in differentiating fibroblasts as they move towards a smooth muscle phenotype. Stimulation with $TGF\beta_2$ significantly induced mRNA expression for the five smooth muscle transcripts that were measured, however not all were induced at protein level.

Undifferentiated fibroblasts expressed high levels of αSMA mRNA, almost identical to the highly abundant housekeeping genes used for normalisation. However αSMA protein was present within only a minute proportion of these untreated cells. Upon stimulation with $TGF\beta_2$,

αSMA mRNA expression increased further and protein expression was greatly induced and assembled into fibres. This suggests that fibroblasts are 'primed' for myofibroblast differentiation through the presence of a large pool of αSMA mRNA. Upon stimulation with $TGF\beta_2$, fibroblasts quickly differentiate to a proto-myofibroblast phenotype, presumably by overcoming the block in translation of pre-existing αSMA transcripts coupled with the induction of nascent mRNA.

The responses of HCM and CPN1 to $TGF\beta_2$ stimulation were more conventional. HCM mRNA and protein expression were both very low in fibroblasts and both were significantly induced during differentiation. CPN1 expression at both the mRNA and protein level was relatively high in untreated fibroblasts and increased during differentiation. However, the distribution of protein changed from diffuse cytoplasmic staining to filamentous staining which is due to the recruitment of CPN1 to the rapidly assembling contractile apparatus within the cell. HCM mRNA and protein were both good indicators of phenotype whilst CPN1 was widely expressed prior to $TGF\beta_2$ treatment and was a poor maker of differentiation.

Desmin mRNA expression was low in untreated fibroblasts but increased significantly upon $TGF\beta_2$ stimulation, although there was no induction of desmin protein within the cells. The expression of desmin is known to decrease in proliferating cultures of smooth muscle cells, suggesting that it is an important marker of muscle differentiation. γActin mRNA was relatively abundant in untreated cultures and was further induced upon stimulation with $TGF\beta_2$. However γ-actin is generally considered an excellent marker for fully differentiated smooth muscle cells [20, 21] and is unlikely to be present in these myofibroblast cultures.

Overall, $TGF\beta_2$ treatment did not achieved a genuine phenotypic switch from fibroblast to differentiated smooth muscle cells, proven by the absence of essential protein markers such as desmin. Nonetheless, mRNA pools for desmin existed but appeared not to be translated. The absence of desmin protein, but an abundance of mRNA in $TGF\beta_2$-differentiated fibroblasts is analogous to the large pool of untranslated αSMA mRNA that was present in untreated fibroblasts. We therefore propose a model whereby resting airways fibroblasts are primed for phenotypic differentiation into myofibroblasts and this may be achieved by $TGF\beta_2$ treatment. Likewise, these myofibroblasts appear to be primed for further differentiation but lack the appropriate stimulus to overcome the block in the translation of certain smooth muscle mRNA species. This is consistent with a recently published model for smooth muscle differentiation and suggests that post-transcriptional regulation is a key determinant of cell fate in this context [14].

The dose dependency for $TGF\beta_2$-induced differentiation further emphasises the potential importance of $TGF\beta_2$ in asthma. It is known that $TGF\beta_2$ is increased in asthma [3] and our data suggest that this is likely to lead to an increased number of myofibroblasts each expressing more αSMA, HCM and CPN1 protein that may provide greater contractile potential. Assuming that myofibroblasts are primed for phenotypic transition to smooth muscle through the accumulation of smooth muscle-related transcripts, the disease related differences in the mRNA expression of HCM, CPN1, desmin and γ-actin gene may also provide be important for the pathogenesis of asthma. Thus, it is possible that asthmatic fibroblasts are "primed" to a greater extent by $TGF\beta_2$

through the transcription of larger mRNA pools of these genes. If appropriate stimulation for a further phenotypic transition towards smooth muscle occurs *in vivo*, we hypothesise that (myo)fibroblasts within asthmatic airways are better primed for this transition than in a healthy subject. The sensitivity of asthmatic fibroblasts to TGFβ_2 "priming" is emphasised further by the fact that αSMA and γ-actin mRNA were maximally induced by only 0.04nM TGFβ_2, whereas healthy control fibroblasts required a greater dose to achieve maximal transcription of mRNA for these genes. Therefore, within asthmatic airways several processes may be acting in concert to promote myofibroblast and smooth muscle differentiation leading to altered airway structure.

Acknowledgements

We would like to thank the Medical Research Council (UK), the Wellcome Trust (London, UK) and the AAIR Charity (Southampton General Hospital, Southampton UK) for supporting this work.

Competing Interests

None of the authors contributing to this manuscript have any competing interests.

Ethical approval

Ethical approval was obtained from the Southampton and South West Hampshire Joint Ethics Committee.

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Figure Legends

Figure 1.
The stability of constitutively expressed housekeeping genes was measured in fibroblasts, myofibroblasts and in a combined data set using geNorm software. The M value is calculated based on the changing ratios of genes in the analysis as previously described [17] such that lower M values indicate more stably expressed genes and an M value <0.5 indicates a relatively stable control gene.

Figure 2.
Pairwise variations in the normalising signal (NF) derived using different numbers of control genes were calculated using the geNorm software. This measure shows the degree of variation in normalisation signal that is achieved by using n control genes compared to n+1. Control genes are added to the analysis in the order of decreasing stability as determined above (Fig 1.) and a change in NF with n genes compared to NF using n+1 genes. Changes in NF value less than 0.15 are considered ideal for accurate normalisation.

Figure 3.
(a.) The steady state levels of mRNA expression of smooth muscle cells markers were measured in both asthmatic (light grey boxes) and healthy control (dark grey boxes) untreated fibroblasts and expressed relative to the geometric mean of the highly abundant and constitutively expressed genes A2 and UBC. αSMA was expressed at an equivalent level (ratio of 1:1) whilst HC myosin and desmin mRNA were comparatively rare transcripts. Calponin and γ-actin were moderately highly expressed being approximately 10 fold less that A2/UBC. Data were analysed using a Mann Whitney U test. (b.) The $TGF\beta_2$-induced mRNA expression of smooth muscle-related transcripts was measured in both asthmatic and healthy control fibroblasts and expressed relative to the geometric mean of the highly abundant and constitute expressed genes A2 and UBC. Data were analysed using a Mann Whitney U test.

Figure 4.
The levels of mRNA expression for αSMA (a,b) and CPN1 (c,d) were measured in asthmatic (n=6) and healthy control fibroblast (n=7) cultures either untreated or treated with 0.004, 0.04 and 0.4nM $TGF\beta_2$ at 0, 24 and 48h post treatment. Expression levels were normalised to the geometric mean of A2 and UBC and expressed relative to the median ΔC_t value for the untreated time zero controls. * indicates earliest time point and lowest $TGF\beta_2$ concentration at which a significant increase (a. p=0.044, b. p=0.049, c. p=0.042, d=0.044) in mRNA expression was achieved.

Figure 5.
Protein expression and localisation for αSMA (**a**), HCM (**b**) and CPN1 (**c)** was measured by immunohistochemistry in untreated fibroblast (**a1-c1**) cultures and those treated with 0.4nM $TGF\beta_2$ for 48h (**a2-c2**). Both untreated (**d1**) and treated (**d2**) control cultures were immunostained with an isotype matched IgG preparation
Data are representative of immunostaining of asthma and healthy control cells (n=6).

Figure 6.

Desmin protein expression and localisation was measured by immunohistochemistry in untreated fibroblast (**a1**) cultures and those treated with 0.4nM TGFβ_2 for 48h (**a2**). Both untreated (**b1**) and treated (**b2**) control cultures were immunostained with an isotype matched IgG preparation (n=6).. Affinity of anti-desmin antibody was demonstrated by staining bronchial biopsy section (**c2**). Location of muscle in a sequential section as demonstrated with anti-αSMA staining. Data are representative of immunostaining of asthma and healthy control cells.

Figure 7.

Semi-quantitative analysis of calponin protein as detected by western blot. Healthy control (dark shading) and asthma (light shading) fibroblasts were incubated in serum free medium ± TGFβ_2 for 48h and then lysed for SDS PAGE and western blot analysis. Representative western blots of αSMA and calponin as well are shown. βActin was used a as a loading control.
Densitometry data was obtained by using Genetools software by Syngene and expressed relative to time-zero control.

Fig 1

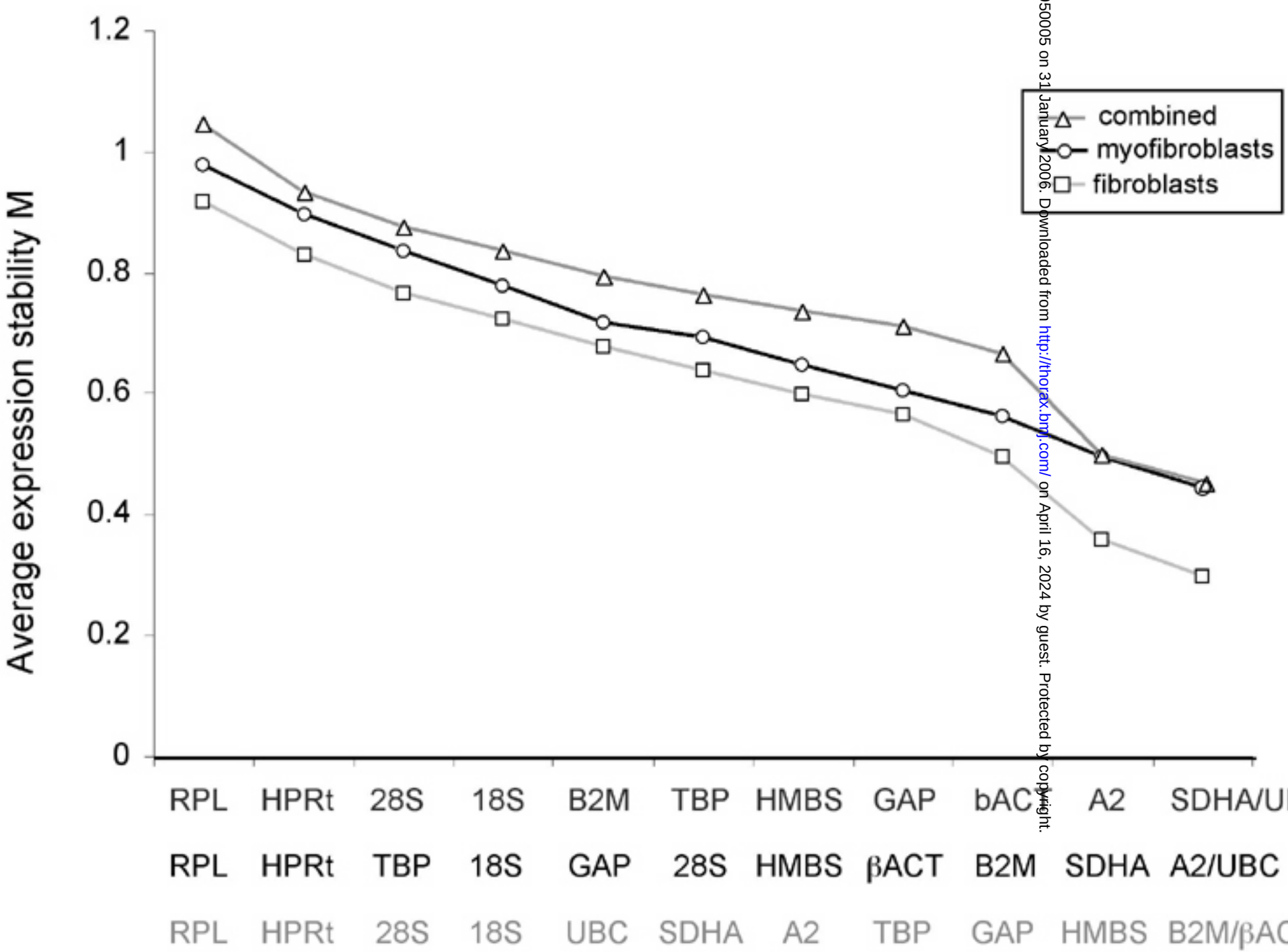
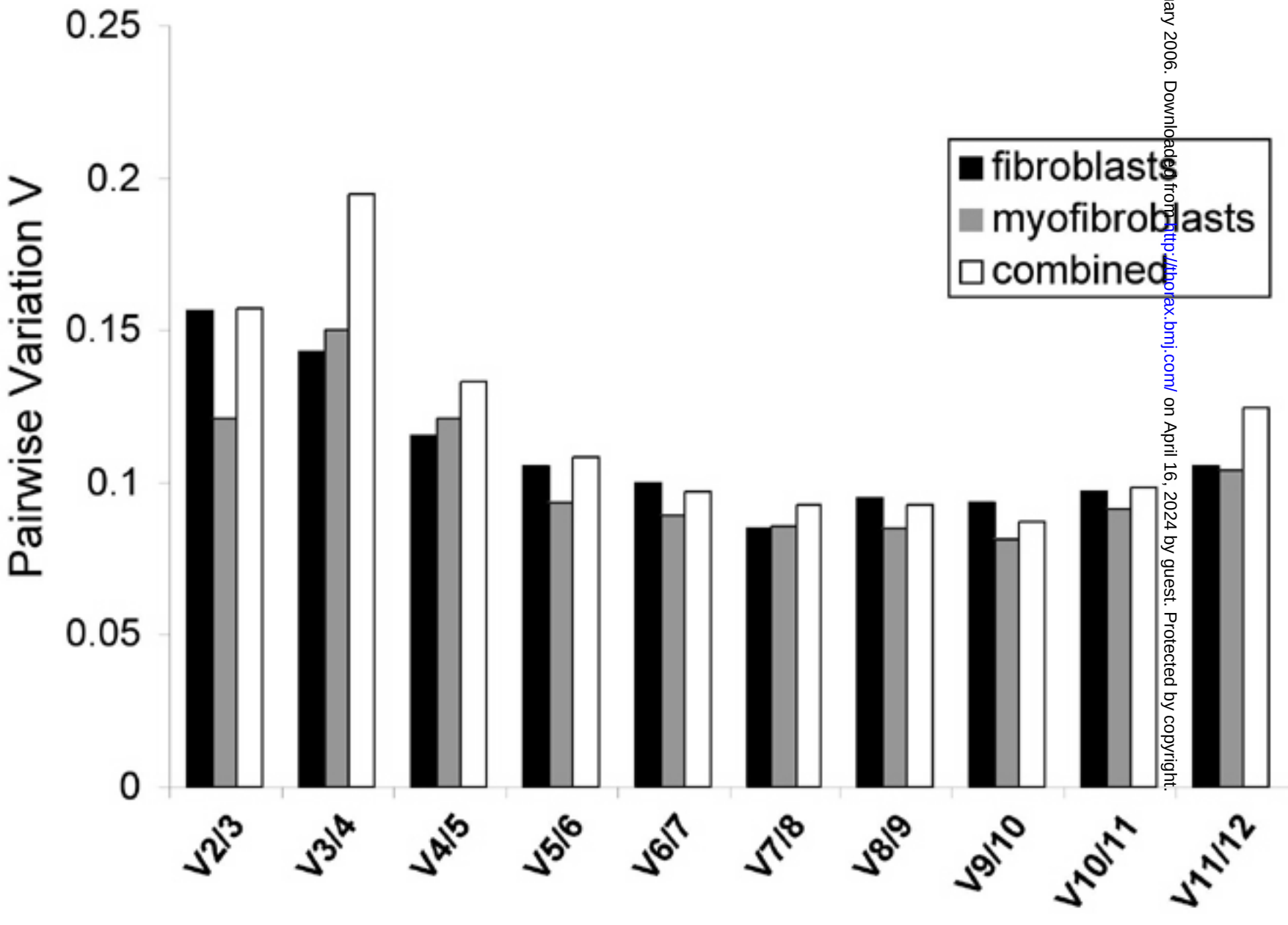


Fig 2.



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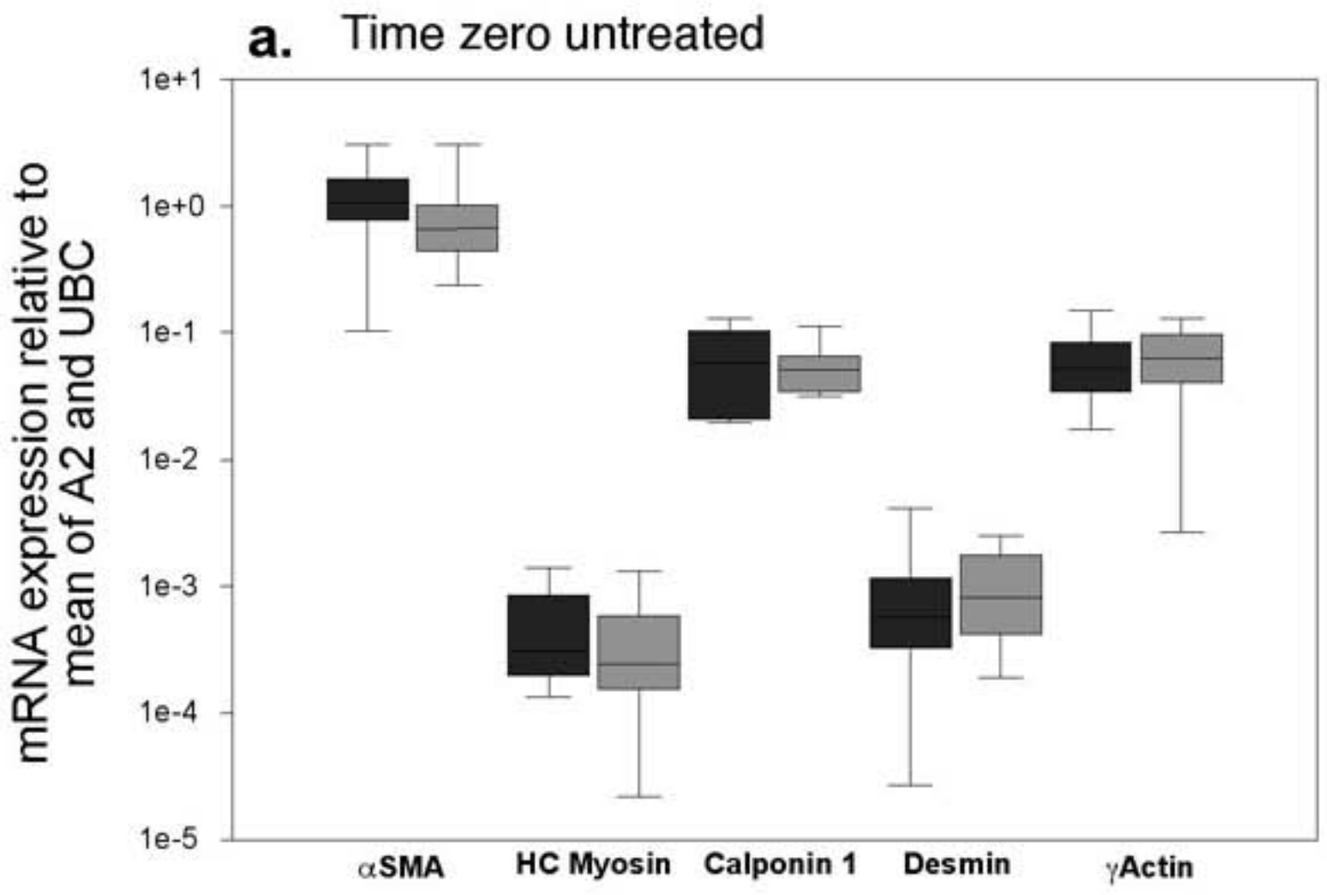


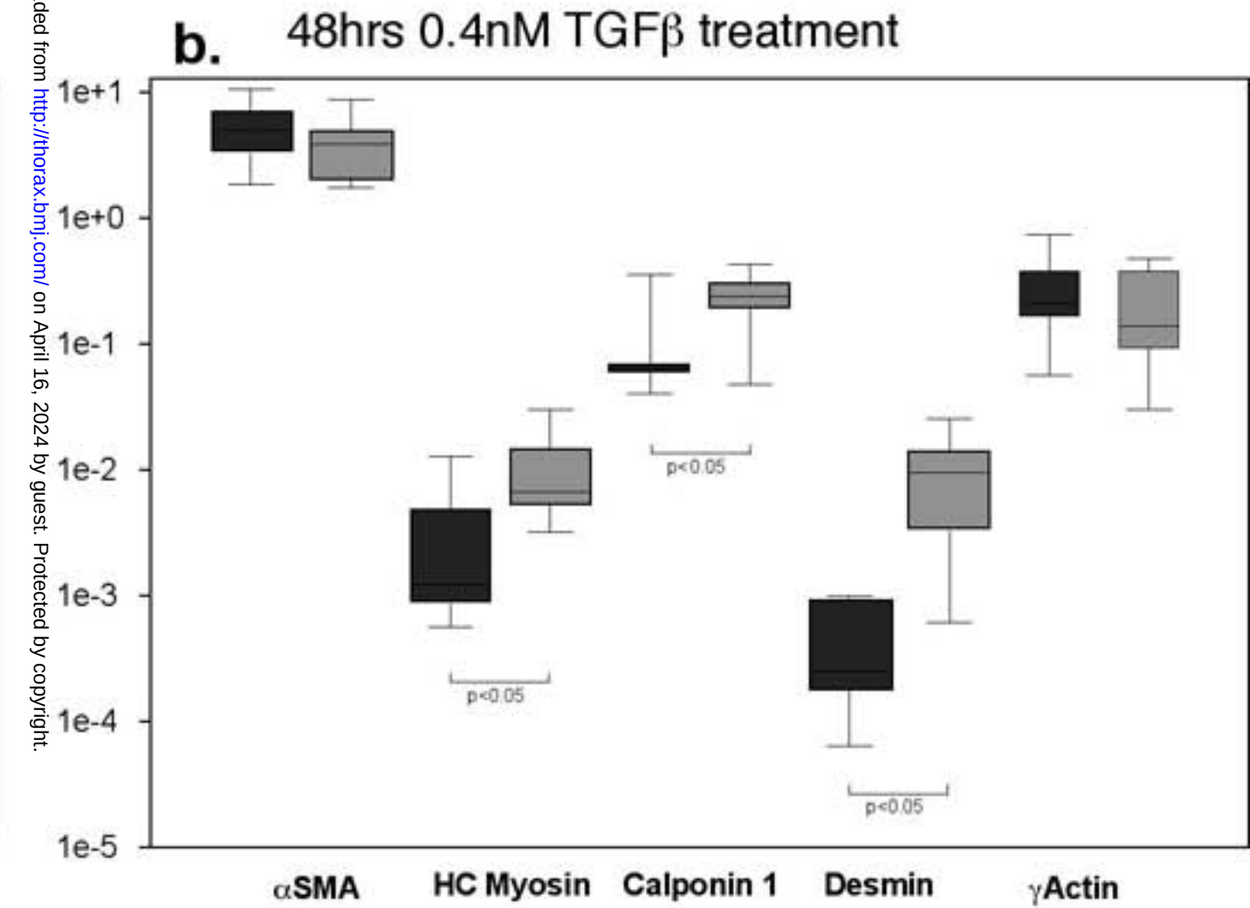
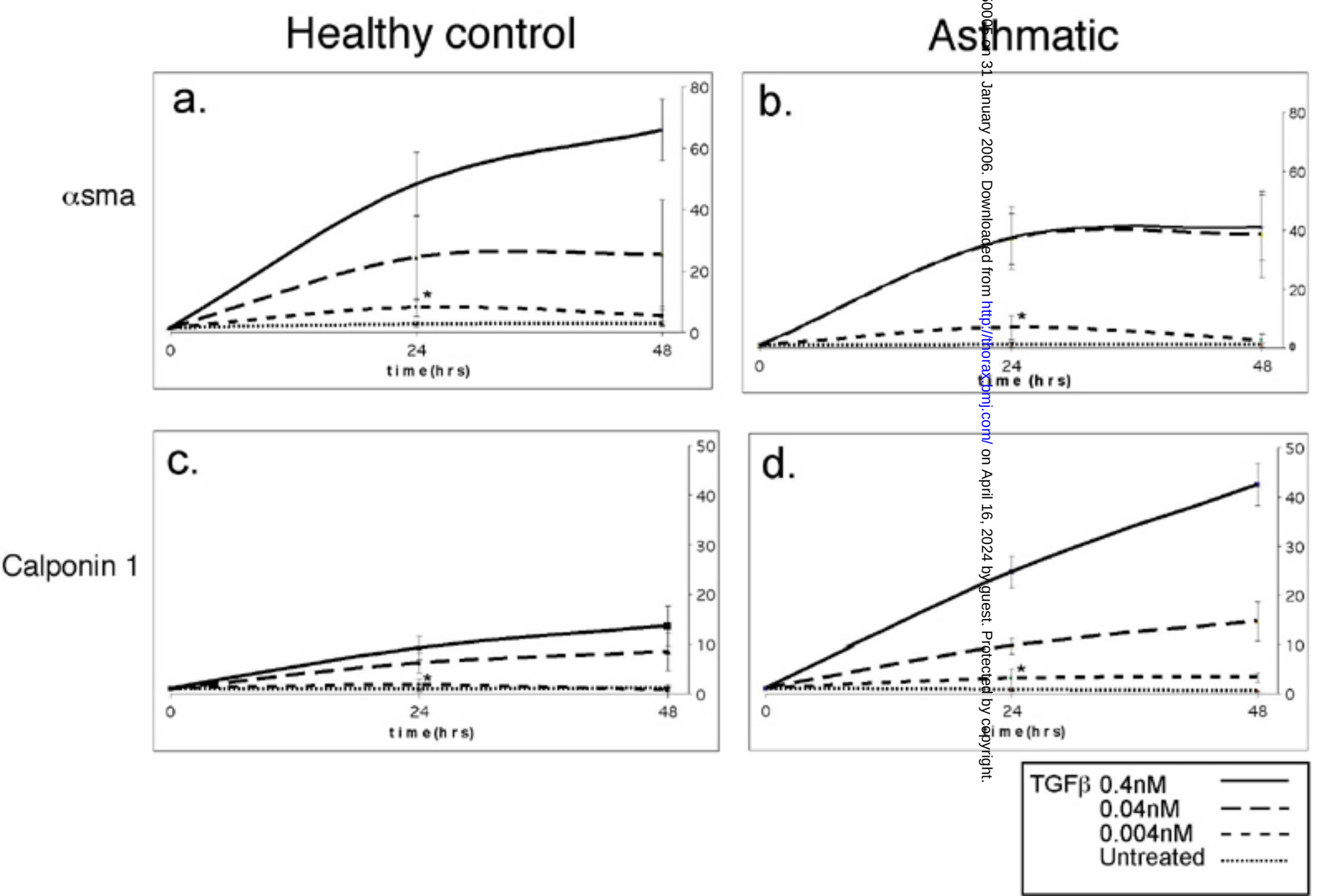
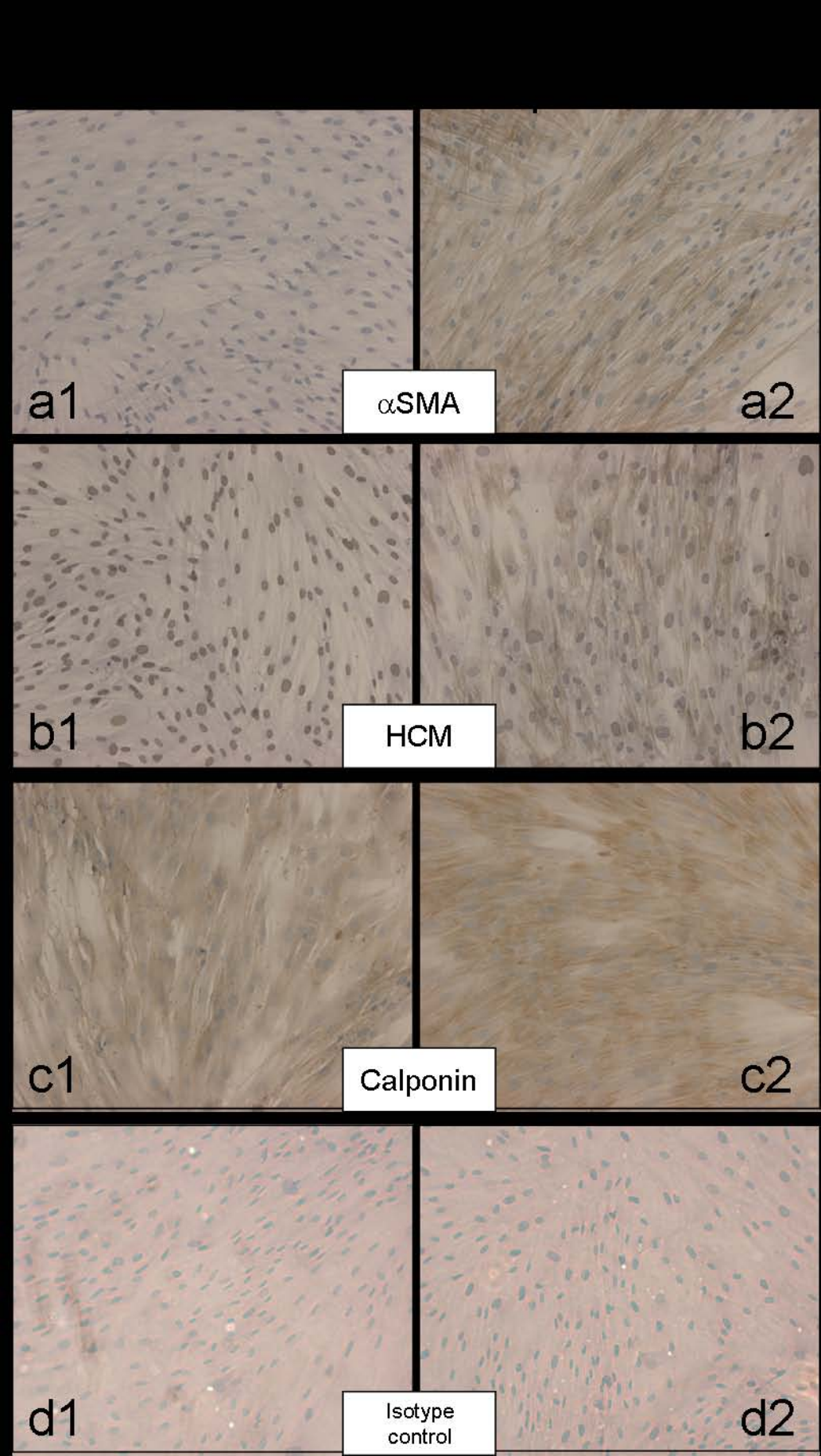


Fig 4.




a1
αSMA
a2
b1
HCM
b2
c1
Calponin
c2
d1
Isotype control
d2

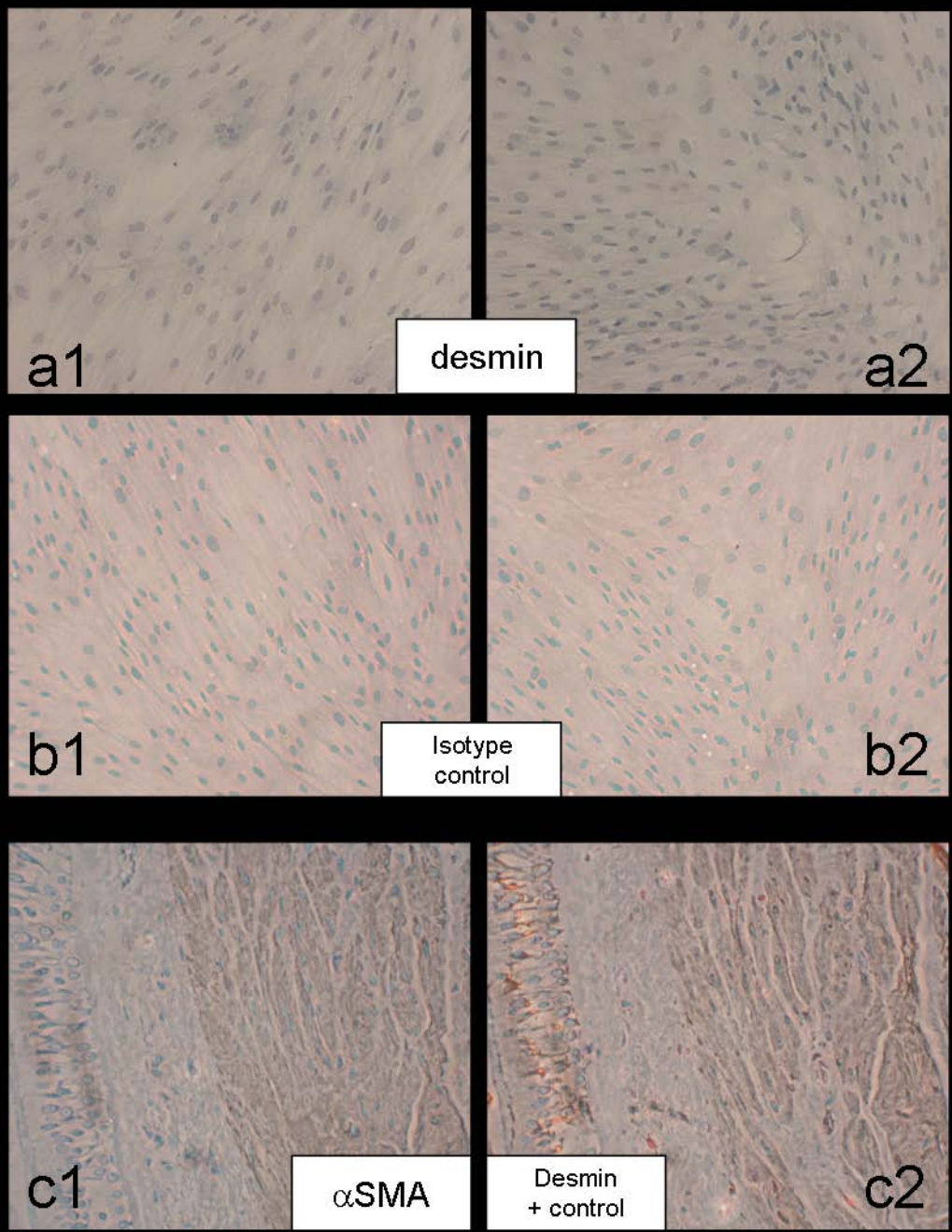
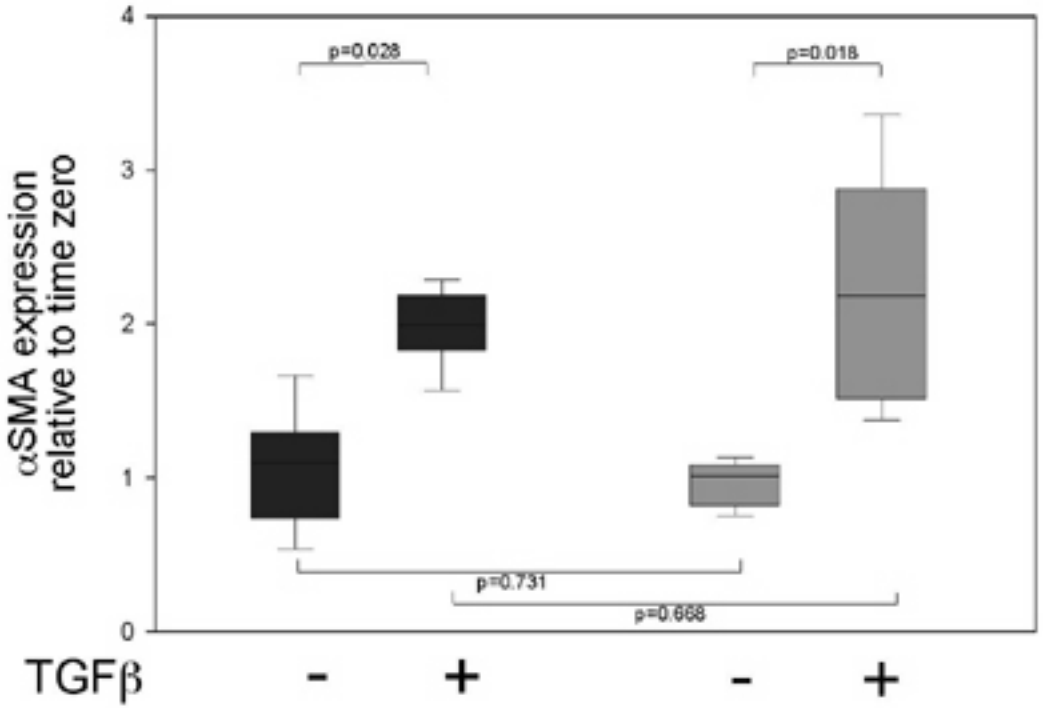
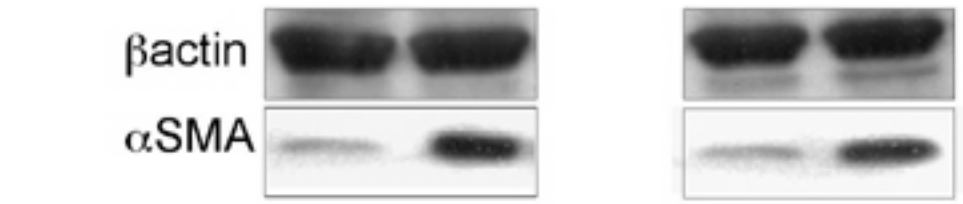
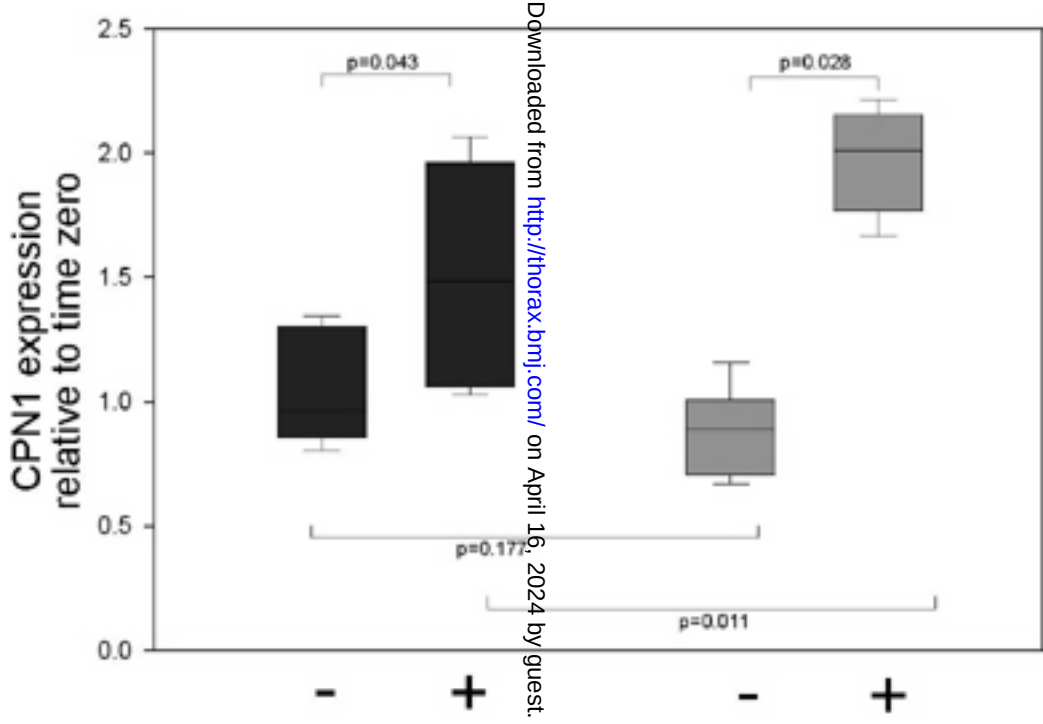

a1
desmin
a2
b1
Isotype
control
b2
c1
αSMA
Desmin
+ control
c2

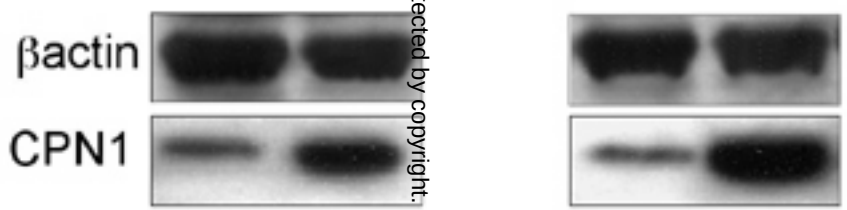
Fig 7.





representative western blots





representative western blots