TEF-1 and C/EBP β are major p38 α MAPK-regulated transcription factors in proliferating cardiomyocytes

Concetta AMBROSINO*^{†1}, Tomoko IWATA^{‡1,2}, Claudio SCAFOGLIO[†], Massimo MALLARDO[§], Rüdiger KLEIN[‡] and Angel R. NEBRED*^{||3}

*European Molecular Biology Laboratory, 69117 Heidelberg, Germany, [†]Dipartimento di Patologia Generale, Seconda Università degli Studi di Napoli, 80138 Napoli, Italy, [‡]Department of Molecular Neurobiology, Max-Planck Institute of Neurobiology, 82152 Martinsried, Germany, [§]Dipartimento di Biochimica e Biotecnologie Mediche, Università di Napoli 'Federico II', Italy, and ^{||}CNIO (Spanish National Cancer Center), Melchor Fernández Almagro 3, E-28029 Madrid, Spain

p38 MAPKs (mitogen-activated protein kinases) play important roles in the regulation of cellular responses to environmental stress. Recently, this signalling pathway has also been implicated in the regulation of processes unrelated to stress, for example, in T lymphocytes and cardiomyocytes. In order to identify molecular targets responsible for the housekeeping functions of p38 MAPKs, we have analysed the differences in the transcriptomes of normally proliferating wild-type and p38 α knockout immortalized embryonic cardiomyocytes. Interestingly, many potential components of the myocardium extracellular matrix were found to be upregulated in the absence of p38 α . Further analysis of the microarray data identified TEF-1 (transcriptional enhancer factor-1), a known regulator of heart-specific gene expression, and C/EBP β (CCAAT/enhancer-binding protein β), as the two

transcription factors the binding sites of which were most enriched in the promoters of p38 α -regulated genes. We have focused on the study of the extracellular matrix component COL1A1 (α 1 chain of type I collagen) and found evidence for the involvement of both TEF-1 and C/EBP β in the p38 α -dependent inhibition of COL1A1 transcription. Our data therefore show that p38 MAPKs regulate TEF-1 and C/EBP β transcriptional activity in the absence of environmental stress and suggests a role for p38 α in the expression of extracellular matrix components that maintain organ architecture.

Key words: cardiomyocyte, collagen, extracellular matrix (ECM), gene expression, p38 MAPK, transcription factor.

INTRODUCTION

The p38 MAPK (mitogen-activated protein kinase) pathway was originally described as a signalling cascade activated in response to stress conditions and pro-inflammatory cytokines. However, it has become clear that p38 MAPKs can also regulate cellular processes that are unrelated to inflammatory and environmental stress responses, such as proliferation, differentiation and the survival of several cell types (reviewed in [1]). Four p38 MAPK family members have been identified and termed p38 α , β , γ and δ . p38 α is ubiquitously expressed and is thought to be the most abundant p38 MAPK in most tissues. Many types of proteins can be phosphorylated by p38 MAPKs, but transcription factors probably constitute the largest group of substrates [2]. The activity of several transcription factors, such as ATF (activating transcription factor)-1/2, CHOP [C/EBP α (CCAAT enhancer-binding protein)-homologous protein], p53, MEF (myocyte enhancer factor) 2A/C and C/EBP α , can be regulated by p38 MAPKs either via direct phosphorylation [3] or through downstream kinases such as MSK1 (mitogen- and stress-activated protein kinase 1) [4]. In addition, p38 MAPKs can also regulate gene expression at the post-transcriptional level [5].

The existence of shared substrates among p38 MAPK family members suggests that they may have some overlapping functions.

However, the degree of redundancy between the different p38 MAPKs in the regulation of cellular processes, both under normal and stress conditions, is not clear yet. The generation of knockout mice has revealed that p38 α plays an essential role in placental morphogenesis [6–8]. Furthermore, a role for the p38 MAPK pathway has been shown in the regulation of T-cell function [9], as well as in the development of or pathophysiology in diverse organs, including the heart [10].

Stimuli that promote hypertrophic growth of cardiomyocytes, such as pressure overload and endothelin-1 stimulation, induce the activation of the ERK (extracellular-signal-regulated kinase) and p38 MAPK pathways [11–13]. Osmotic, oxidative and ischaemia/reperfusion stressors also induce p38 MAPK activation in the heart [14]. Heart injury provokes a complex response that initiates the process of myocardium remodelling, which involves cardiomyocyte hypertrophy, fibrosis and cell death. This fibrosis is caused by the accumulation of the main components of the cardiac ECM (extracellular matrix), such as COL1 (collagen type 1) and COL3 (type 3) molecules, which play essential roles in the maintenance of myocardium integrity under normal conditions [15].

In order to better understand the role of p38 MAPKs in the control of heart pathophysiology and the regulation of cardiomyocyte function, we have investigated genes regulated by p38 α

Abbreviations used: AP-1, activator protein-1; C/EBP β , CCAAT/enhancer-binding protein β ; ChIP, chromatin immunoprecipitation; COL1, collagen type 1; COL1A1, α 1 chain of COL1; DTT, dithiothreitol; ECM, extracellular matrix; EMSA, electrophoretic mobility shift assays; ERK, extracellular-signal-regulated kinase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; MAPK, mitogen-activated protein kinase; M-CAT, muscle C, A and T sites; MKK, MAPK kinase; MSCV, murine stem cell virus; PRIMA, promoter integration in microarray analysis; ROM, ratio of medians; STAT, signal transducer and activator of transcription; TEF-1, transcriptional enhancer factor-1; TNF, tumour necrosis factor; wt, wild-type.

¹ These authors contributed equally to this work.

² Present address: Division of Cancer Sciences and Molecular Pathology, Faculty of Medicine, University of Glasgow, Beatson Laboratories, Bearsden, Glasgow G61 1BD, U.K.

³ To whom correspondence should be addressed (email anebreda@cnio.es).

in cardiomyocyte cell lines derived from wt (wild-type) and $p38\alpha^{-/-}$ ($p38\alpha$ null mutant) embryo hearts. In the present study, we provide evidence for the stress-independent control of gene expression by $p38\alpha$ in non-stimulated cells. We show that there is an increased expression of several components of the myocardium ECM in $p38\alpha^{-/-}$ cardiomyocytes, suggesting that $p38\alpha$ plays a role in the maintenance of myocardium structural integrity. Moreover, we have identified two $p38\alpha$ -regulated transcription factors, C/EBP β and TEF-1 (transcriptional enhancer factor-1), which are involved in the control of ECM gene expression in cardiomyocytes. The involvement of TEF-1 in $p38$ MAPK-regulated gene expression has not been described to date.

MATERIALS AND METHODS

Cell lines

The generation of immortalized $p38\alpha^{-/-}$ and wt cardiomyocyte cell lines and culture conditions have been previously described [6,16]. The construction of MSCV (murine stem cell virus)- $p38\alpha$ and MSCV- $p38\alpha$ -D/A (with the mutation D168A) and the procedure for retroviral infections have been already reported [17]. Stably infected cell lines were selected and grown in culture medium supplemented with 100 μ g/ml hygromycin (Sigma).

Microarray analysis

Total RNA was extracted with RNA Clean (Hybaid) and further purified with an RNeasy mini kit (Qiagen). Direct labelling of 20 μ g of total RNA primed with oligo(dT) was performed in the presence of Cy-5- and Cy-3-dUTP (Amersham Pharmacia Biotech) using SuperScript II (Invitrogen). The Cy5- and Cy3-labelled cDNA samples were then mixed together and purified using a GFX DNA purification kit (Amersham Pharmacia Biotech). Before hybridization, samples were supplemented with 1.2 μ g of salmon sperm DNA and 5 μ g of poly(dA) and dried. The high-density glass microarray chips were prepared at the EMBL (European Molecular Biology Laboratory) core facility by spotting of the PCR products prepared from the NIA (National Institute on Ageing) 15K mouse cDNA set [18]. The microarray slides were pre-hybridized in $6 \times$ SSC, 0.5 % SDS and 1 % BSA at 42°C for 1 h and incubated in boiled water for 2 min prior to hybridization. The cDNA samples were resuspended in 15 μ l of hybridization buffer (50 % formamide, $6 \times$ SSC, 0.5 % SDS and 5 % Denhardt's solution) and denatured by incubating at 95°C for 2 min. Hybridization was carried out at 42°C for 16 h. The slides were washed for 10 min each with $2 \times$ SSC, then with $0.5 \times$ SSC/0.1 % SDS followed by $0.1 \times$ SSC/0.1 % SDS at room temperature. Scanning was performed with GenePix 4000B (Axon). The data acquisition and initial data analysis was performed with GenePix Pro 3.0 software and the data tables were analysed further with the program Excel in order to obtain the gene list.

Quality control was performed to confirm scanner alignment and the absence of significant bubbles and scratches. Scatter plots were further used to eliminate unacceptable hybridization data. Hybridizations were performed in triplicate, using total RNA samples extracted from three independent batches of proliferating cells. In two hybridizations, the RNAs from $p38\alpha^{-/-}$ and wt cells were labelled with Cy-5- and Cy-3-dUTP respectively, whereas in the third hybridization, the dyes were swapped. The multiple spike-in RNA control templates were added to the sample upon direct labelling, and successful labelling and hybridization was confirmed in each hybridization [19]. Twelve housekeeping genes in the array gave an average ROM (ratio of medians) value of 1.30 ± 0.26 , which was confirmed to be acceptable. The GenePix Pro program calculates the normalization factor for each hybrid-

ization, based on the premise that the arithmetic mean of the ratios from every feature on the given array should be equal to 1. Normalization was therefore performed by multiplying this factor with the ROM value for each gene. The program also identifies elements that do not produce good alignment to the expected spotted area as 'flagged' spots, indicative of a low-quality hybridization of the specific genes. All flagged genes were removed from the list. Genes fulfilling all these criteria were sorted by ROM and those that showed a greater than ± 1.5 -fold change were selected from each hybridization. Finally, data from 3 independent hybridizations were compared and only those genes that were identified in all 3 experiments were selected for the final list. The microarray data have been deposited in the GEO database (under accession number GSE1568).

Northern blotting and nuclear run-on

Total RNA was prepared from proliferating cells with TRIzol reagent (Life Technology) and a sample of 20 μ g was used for Northern blotting analysis as previously described [17]. The COL1A1 ($\alpha 1$ chain of COL1) and the COL3A1 ($\alpha 3$ chain of COL3) probes were prepared from the corresponding clones of the NIA mouse cDNA set used for the microarray. A fragment of the GAPDH (glyceraldehyde-3-phosphate dehydrogenase) open reading frame (nt 134–600) was used for normalization. After autoradiography, the bands were excised and DNA quantified using a Fuji FLA 2000 PhosphorImager instrument. The experiments were performed at least twice.

The run-on analysis was performed as previously described [17]. The membranes were analysed and quantified using a PhosphorImager instrument.

Cell extracts and immunoblotting

Whole-cell extracts were prepared and analysed by immunoblotting as described previously [17,20]. The membranes were probed with the following antibodies according to the manufacturer's instructions: anti- $p38\alpha$ and anti-C/EBP β (Santa Cruz Biotechnology), anti-TEF-1 (Transduction Laboratories), anti-COL1 (Calbiochem), anti-tubulin (Sigma), anti-phospho-ERK (Cell Signaling) and anti-MKK (MAPK kinase) 6 [20].

PRIMA (promoter integration in microarray analysis)

The PRIMA program identifies transcription factors the binding sites of which are significantly over-represented in particular promoters [21]. The fingerprint contains the number of hits (putative binding-sites) of the various transcription factors identified in the promoter. EXPANDER then executes the PRIMA program on a fixed set of pre-computed fingerprints, which are constructed as a set of about 15 000 mouse promoter sequences, spanning from 1000 bp upstream to 200 bp downstream of the transcriptional start site [22]. In the present study, the scan was performed for each transcription factor motif in the TRANSFAC (version 5.4) database that corresponds to a human transcription factor. The set of fingerprints was prepared on mouse promoters (15 000 promoters, Ensemble release 13.30) using a threshold that corresponds to a hit frequency of 10 % in random sequences. We have used the locus ID of genes that were mostly retrieved from the NIA site (<http://lgsun.grc.nia.nih.gov/cDNA/15k.html>) and the rest were from Source (<http://source.stanford.edu>). In total, we used locus IDs for 9113 genes on the array and for 114 $p38\alpha$ -regulated genes.

Nuclear lysates and EMSA (electrophoretic mobility-shift assay)

Nuclear and cytosolic extracts to be used in assaying DNA binding activity and the amount of C/EBP protein were prepared

as described previously [23]. For C/EBP EMSA, the following oligonucleotide probes were used: 5'-GATCGGACGTCACATTGCACAATCTTAATAAT-3' (interleukin 6 C/EBP binding site), 5'-GGACGTCACACTACAACTCTTAATAA-3' (mutant interleukin 6 C/EBP binding site). Nuclear extracts (3 μ g) were incubated for 5 min on ice in 20 μ l of 10% glycerol, 60 mM KCl, 1 mM EDTA, 1 mM DTT (dithiothreitol), 2 μ g of poly(dI-dC) (Boehringer Mannheim). The γ -³²P-labelled double-stranded probe [0.2 ng, (4–6) $\times$ 10⁴ c.p.m.] was then added with or without a 100-fold molar excess of the competitor wt or mutant oligonucleotide. Where indicated, 1 μ g of the anti-C/EBP antibody (Santa Cruz Biotechnology) or purified rabbit IgG (Sigma) was added before the probe and the mixture was incubated on ice for 1 h. The reactions were incubated at room temperature for 20 min and analysed on a 5% acrylamide/bisacrylamide (30:1) gel in 22.5 mM Tris/borate, 0.5 mM EDTA and 3.7 mM mercaptoethanol. Gels were dried and subjected to autoradiography.

For the TEF-1 EMSA, the M-CAT [muscle C, A and T sites, sequence related to CATTC(A/T)] oligonucleotide probe 5'-GATCGGACGTCACATTGCACAATCTTAATAAT-3' was used. Nuclear extracts (5 μ g) were prepared as described previously [24] and incubated for 5 min on ice in 20 μ l of 25 mM Tris (pH 7.5), 10% glycerol, 100 mM KCl, 2 mM EDTA, 1 mM DTT and 2 μ g of poly(dI-dC) (Boehringer Mannheim). The γ -³²P-labelled double-stranded probe (0.2 ng, 10 $\times$ 10⁴ c.p.m.) was then added with or without a 100-fold molar excess of competitor wt oligonucleotide. For the supershift assay, 0.5 μ g of anti-TEF-1 antibody (Transduction) or purified rabbit IgG was added before the probe and the mixture was incubated on ice for 30 min. The reactions were processed and analysed as described above for C/EBP.

ChIP (chromatin immunoprecipitation)

ChIP assays were performed using the protocol for the acetyl-histone H4 ChIP Assay kit (Upstate Biotechnology) using 4 $\times$ 10⁷ wt or p38 α ^{-/-} exponentially growing cells for each immunoprecipitation. Cells were cross-linked by the addition of formaldehyde (1% final concentration) to the medium for 30 min at 37°C and the reaction was stopped by the addition of glycine. Cells were washed and scraped in ice-cold PBS supplemented with protease inhibitors, collected by centrifugation and resuspended at 1 $\times$ 10⁸/ml in ChIP lysis buffer [1% SDS, 10 mM EDTA, 50 mM Tris/HCl (pH 8.0) and protease inhibitors]. After 10 min of incubation on ice, aliquots of 0.4 ml were sonicated. The lysates were then diluted $\times$ 10 with ChIP dilution buffer [0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris/HCl (pH 8.0) and 167 mM NaCl] and pre-cleared by incubation for 1 h at 4°C with Protein A beads previously washed and resuspended in ChIP dilution buffer containing 0.4 mg/ml salmon sperm DNA. After pre-clearing, 4 μ g of the anti-C/EBP β or anti-TEF-1 antibodies was added. For wt cells, a control using purified rabbit IgG was included. After overnight incubation at 4°C, the immunocomplexes were collected with Protein A beads and washed twice with both low-salt and high-salt buffers, LiCl wash buffer and finally TE [10 mM Tris/HCl (pH 7.5)/1 mM EDTA] 1 $\times$. The complexes were then eluted by 3 sequential incubations with 170 μ l of freshly prepared elution buffer (1% SDS, 0.1 M NaHCO₃) for 15 min at room temperature. The cross-linking was reverted by the addition of 20 μ l of 5 M NaCl and by heating at 65°C for 5 h. The control DNAs were also incubated at 65°C after the addition of 5 M NaCl and processed as described for the immunoprecipitated DNA. After Proteinase K digestion, the DNA was purified by phenol/chloroform extraction and precipitated. The pellets were resuspended in 20 μ l and 1 μ l

was used for PCR using AmpliTaq (Perkin Elmer). The PCR primers (forward, 5'-GCCAGAGGTGCTGTAC-3'; reverse, 5'-GGTGTGTCTGGCATGGCAG-3') were designed based on the published mouse COL1A1 promoter sequence [25], to amplify a region conserved in the rat and human COL1A1 promoters.

RESULTS

The inactivation of p38 α in cardiomyocytes changes the levels of mRNAs encoding ECM components

We have previously proposed that the low level of p38 α basal activity present in normally proliferating cells could modulate the expression of specific genes, which may be related to house-keeping functions of the p38 MAPK pathway [17]. This idea prompted us to look for genes regulated by p38 α under normal growing conditions. We have examined the gene expression profiles of wt and p38 α ^{-/-} cell lines derived from mouse embryo cardiomyocytes, which express heart-specific markers (results not shown and [6]).

Total RNA was prepared from three independent batches of proliferating p38 α ^{-/-} and wt cardiomyocyte cultures and hybridized on to high-density glass microarray chips containing the NIA 15K mouse cDNA clone set [18]. Strict measures were applied for the purpose of quality control (as described in the Materials and methods section) and the data were obtained from the three experiments that had the best possible conditions. The genes that showed a greater than $\pm$ 1.5-fold change in all three hybridization experiments were included in the final list. The expression level of 54 genes was found to be upregulated, whereas 60 genes were downregulated in p38 α ^{-/-} cardiomyocytes under normal growing conditions (Figure 1; see Supplementary Tables 1 and 2 at <http://www.BiochemJ.org/bj/396/bj3960163add.htm>). Approx. 40% of the genes in the list were Riken or IMAGE clones that did not show homology with any known genes based on BLAST searches against DNA sequence databases. Analysis by Northern blotting of several genes that showed either large or small changes in their expression level confirmed the validity of the microarray results (results not shown).

A statistical validation of the results was performed using the Onto-Express program [26], which associates Unigene clusters with GeneOntology terms relative to biochemical and molecular functions, biological processes, cellular components and roles, and then computes for each function the probability that the regulated genes could have been picked randomly from the population (i.e. the total number of genes in the microarray). This analysis revealed that the expression of ECM components was the cellular function potentially regulated by p38 α with the best *P* value (7.0e⁻⁵), which is also in agreement with the functional categorization based on the NIA mouse 15K cDNA gene ID list (Figure 1).

p38 α downregulates collagen transcripts in cardiomyocytes

Several collagen transcripts were upregulated in the p38 α ^{-/-} cells (see Supplementary Table 1 at <http://www.BiochemJ.org/bj/396/bj3960163add.htm>). COL1 is a major component of bone, skin, tendons, blood vessel walls and connective tissues and represents approx. 85% of myocardium ECM proteins. The COL1A1 and COL1A2 genes, which encode the α 1 and α 2 chains of COL1 respectively, were upregulated to approx. equivalent levels in our microarray analysis, supporting the specificity of the result. Previous studies have shown that the p38 MAPK pathway is involved in the control of COL1A1 and COL1A2 gene expression upon stress treatment [27,28]. By Northern blotting we confirmed a 3–4-fold increase in the COL1A1 transcript content of p38 α

Figure 1 Summary of the genes for which expression levels significantly changed in proliferating p38 α ^{-/-} versus wt cardiomyocytes

Three independent p38 α ^{-/-} and wt cardiomyocyte cultures were analysed and only those genes that showed a greater than ± 1.5 -fold changes in all three experiments were selected. Gene functions were initially categorized based on the NIA mouse 15K cDNA gene ID list and modified when necessary. Note that many genes that are differentially regulated in the p38 α ^{-/-} cardiomyocytes encode matrix and structural proteins.

knockout cardiomyocytes (Figures 2A and 2B). COL3A1 mRNA expression was also upregulated in p38 α ^{-/-} cardiomyocytes (Figures 2B and 2C), which is in agreement with previous reports showing that the COL1A1 and COL3A1 genes frequently have the same expression pattern [29]. We focused on the COL1A1 gene for further experiments.

COL1A1 mRNA levels are regulated by p38 α under stress and normal growth conditions

To confirm that the upregulation of the COL1A1 transcript level was directly related to the absence of p38 α , we re-introduced either wt p38 α or the kinase-dead mutant p38 α -D/A into p38 α ^{-/-} cardiomyocytes [17]. As shown in Figure 3(A), the presence of p38 α decreased the COL1A1 mRNA content in p38 α ^{-/-} cardiomyocytes to approx. the same levels as in wt cells. However, the expression of p38 α -D/A further increased the cellular level of COL1A1 mRNA. At the protein level, the amount of COL1A1 was also increased in the p38 α ^{-/-} cardiomyocytes and was decreased by expression of wt p38 α , but not by p38 α -D/A (Figure 3B, upper panel). As a control, MKK6 upregulation [17] was confirmed in the absence of p38 α activity, either in p38 α ^{-/-} cardiomyocytes or in those transfected with p38 α -D/A (Figure 3B, middle panel).

Figure 2 Up-regulation of COL1A1 and COL3A1 in p38 α ^{-/-} cardiomyocytes

(A) and (C) Northern blotting analysis of RNAs prepared from wt and p38 α ^{-/-} cardiomyocytes. The membranes were hybridized with COL1A1, COL3A1 and GAPDH probes as indicated. (B) Phosphorimager quantification of COL1A1 and COL3A1 transcript levels (normalized to GAPDH values) in the indicated cell lines. The results were compiled from six or two independent experiments for COL1A1 and COL3A1 respectively.

The expression of the COL1A1 gene is tightly regulated and several transcription factors are known to bind to its promoter [30]. To analyse the mechanism of COL1A1 upregulation, we investigated the activation status of the ERK MAPK pathway in normally proliferating p38 α ^{-/-} cells, as this pathway can also regulate COL1A1 expression [31] and sometimes is negatively regulated by p38 MAPK [32]. In agreement with previous results [16], we found that the deletion of p38 α increased the basal levels of phosphorylated and active ERK MAPKs (Figure 4A, upper panel). The re-introduction of wt p38 α , but not the inactive form p38 α -D/A, in p38 α ^{-/-} cardiomyocytes decreased ERK phosphorylation to the same level as in wt cells (Figure 4A, upper panel). These results suggested that ERK activation could potentially contribute to the upregulation of COL1A1 expression observed in p38 α ^{-/-} cardiomyocytes. To address this possibility, we used the ERK inhibitors PD98059 and U0126. Incubation of p38 α ^{-/-} cardiomyocytes with either of these inhibitors for up to 36 h did not affect the level of COL1A1 mRNA, as determined by Northern blotting (results not shown). It therefore seems that the main cause of an increased COL1A1 mRNA level in the p38 α ^{-/-} cardiomyocytes is p38 α inactivation rather than the upregulation of ERK.

The pro-inflammatory cytokine TNF (tumour necrosis factor) α has a negative effect on collagen expression in cardiac fibroblasts [33]. Therefore we analysed the role of p38 α in decreasing the level of COL1A1 mRNA in the cardiomyocyte lines stimulated with TNF α . For this purpose, cells were stimulated with a low concentration of TNF α (20 ng/ml) for 6 h. This mild treatment was necessary to avoid the induction of apoptosis [16]. We found that TNF α stimulation resulted in the inhibition of COL1A1 expression only in wt cardiomyocytes, consistent with the TNF α -induced activation of p38 α , whereas COL1A1 transcription was

Figure 3 COL1A1 up-regulation in p38α^{-/-} cardiomyocytes is reversed by p38α expression

(A) Cardiomyocytes were infected with a retrovirus expressing wt p38α or the kinase-dead mutant p38α-D/A. RNA was prepared from the infected cells and the COL1A1 and GAPDH transcript levels were determined by Northern blotting. PhosphorImager quantification (normalized to GAPDH) from four independent experiments is shown. (B) COL1 protein expression was analysed in the same cell lines by immunoblotting. The filter was re-probed with antibodies to MKK6, the expression of which is known to be negatively regulated by p38α activity, and tubulin as a loading control. The results are representative of three independent experiments.

further induced by TNFα stimulation in the p38α^{-/-} cardiomyocytes (Figure 4B).

Taken together these results show that, in cardiomyocytes, COL1A1 mRNA levels are negatively regulated by p38α in a kinase-dependent manner under both normal proliferative and stress conditions.

The p38 MAPK pathway negatively regulates COL1A1 promoter transcriptional activity

To characterize the molecular mechanism by which p38α regulated the COL1A1 mRNA expression level, we performed nuclear run-on experiments. Using nuclei purified from proliferating cardiomyocytes, transcription from the COL1A1 promoter was found to be about 2-fold higher in p38α^{-/-} than in wt cardiomyocytes (Figure 5A). This observation suggested a direct negative role for p38α in the regulation of the COL1A1 promoter's transcriptional activity. As COL1A1 has been reported to be post-

Figure 4 Increased basal ERK activity in the p38α^{-/-} cardiomyocytes and super-induction of COL1A1 upon TNFα treatment

(A) Cell lysates were prepared from the indicated cardiomyocyte lines and analysed by immunoblotting. Antibodies that specifically recognize the phosphorylated and active forms of ERK1/2 were used. The total amount of p38α or ERK1/2 was also determined and the filter was re-probed with a tubulin antibody as the loading control. (B) Cardiomyocytes were starved overnight (0.5% foetal calf serum) and then treated with TNFα (20 ng/ml) for 6 h. Total RNA was prepared and analysed by Northern blotting. The PhosphorImager quantification data (normalized to GAPDH values) are shown at the bottom of the blots. The data are compiled from two independent experiments.

transcriptionally regulated in some cell lines (reviewed in [34]), we also investigated this possibility. Cardiomyocytes were treated with actinomycin D for 3 and 6 h (longer actinomycin D treatment is toxic in these cells; C. Ambrosino unpublished work) and the COL1A1 mRNA level was determined by Northern blotting. We observed only minor differences, that were reproducible and are unlikely to be significant, in the stability of the COL1A1 transcript between wt and p38α^{-/-} cardiomyocytes (Figure 5B), therefore we concentrated on the transcriptional regulation of COL1A1 promoter.

Identification of the transcriptional network regulated by p38α in cardiomyocytes

To characterize the transcriptional network involved in gene expression regulated by p38α, we performed a PRIMA analysis on the microarray data set. PRIMA is a program that identifies

Figure 5 COL1A1 gene expression is regulated by p38 α at the transcriptional level

(A) Proliferating cardiomyocytes were used for the run-on analysis with 5 μ g of the indicated plasmids. The signals were visualized by autoradiography (upper panel) and quantified by PhosphorImager (arbitrary units). The data were normalized to GAPDH values and plotted at the bottom of the blots. (B) Cardiomyocytes were treated with actinomycin D (Act; 2.5 μ g/ml) and RNA was prepared, at the indicated times, and analysed by Northern blotting (upper panel). The amount of COL1A1 mRNA remaining after the treatment (normalized to GAPDH values) is shown at the bottom of the blots.

transcription factors for which the binding sites are enriched in a defined set of promoters [21]. The analysis was performed by comparing the promoter regions of 9113 genes on the array (considered as a background set) either with those of the entire set of p38 α -regulated genes, or with the sets of genes up-regulated or down-regulated in two different clusters. In the first case, we found that C/EBP had the best P value (2.46×10^{-4}) among the transcription factors that were present in the promoter of differentially regulated genes, followed by TEF-1 and Nkx6-2 that had P values of 8.32×10^{-3} and 2.7×10^{-2} respectively. In the cluster of up-regulated genes, TEF-1 had the largest value ($P = 1.36 \times 10^{-4}$), followed by the transcription factors C/EBP ($P = 6.72 \times 10^{-3}$), Octamer ($P = 9.71 \times 10^{-3}$) and NF-AT (nuclear factor of activated T-cells) ($P = 1.89 \times 10^{-2}$). In the cluster of down-regulated genes, only AP-1 (activator protein-1) was found with a P value of less than 0.005. Taken together, there was a clear enrichment of binding sites for TEF-1 and C/EBP in the promoters of p38 α -regulated genes, suggesting an important role for these transcription factors in the regulation of gene expression by p38 α in cardiomyocytes. Furthermore, the genes harbouring a TEF-1 binding site in their promoter were all found to be up-regulated in the p38 $\alpha^{-/-}$ cells, whereas those with C/EBP binding sites included both up-regulated and down-regulated genes (Table 1). Interestingly, the only genes that had binding sites for both TEF-1 and C/EBP in their promoters were COL1A1, COL1A2, Tra1 and cathepsin D.

p38 α regulates TEF-1 and C/EBP β binding to the COL1A1 promoter

The regulation of COL1A1 gene expression is known to involve several transcription factors, including C/EBP [30]. We have also identified 3 potential TEF-1 binding sites in the COL1A1

promoter [25] at positions -603 bp to -598 bp, -540 bp to -535 bp and -359 bp to -354 bp (as described below; Figure 7A), but the possible regulation of COL1 expression by TEF-1 has never been investigated before.

We analysed C/EBP and TEF-1 activity in wt and p38 $\alpha^{-/-}$ cardiomyocytes by EMSA. We found that C/EBP DNA binding activity was decreased in cells lacking p38 α (Figure 6A, lanes 1 and 7) and re-introduction of wt p38 α , but not of catalytically inactive p38 α -D/A, was able to restore C/EBP DNA binding activity to the same level as in wt cells (Figure 6A). By supershift assay, we found that C/EBP β was the most abundant component of the C/EBP protein complex and was specifically regulated in the cardiomyocyte lines (Figure 6A, *). The increase in C/EBP binding was probably due to direct regulation of its DNA binding activity, as the amount of the protein was very similar in wt and p38 $\alpha^{-/-}$ cardiomyocytes (Figure 6B). No changes in the nuclear/cytoplasmic distribution of the two isoforms of C/EBP β , p35 and p20, were observed (results not shown).

We also investigated TEF-1 activity and found more pronounced binding to the M-CAT probe in the p38 $\alpha^{-/-}$ cardiomyocytes (Figures 6C and 6D). Moreover, upon re-expression of p38 α in p38 $\alpha^{-/-}$ cells, TEF-1 DNA binding activity reverted to the same level as in wt cells. By supershift assay, TEF-1 appears to be the most relevant component of such complexes among the TEF-1 family members. We confirmed that the level of TEF-1 expression was not very different between wt and p38 $\alpha^{-/-}$ cell lines, supporting the idea that the p38 MAPK pathway regulates TEF-1 DNA binding activity, rather than its expression (Figure 6D).

Finally, we also investigated differences in the loading of C/EBP β and TEF-1 on to the COL1A1 promoter between wt and p38 $\alpha^{-/-}$ cardiomyocytes. ChIP assays, which provide a real-time estimation of DNA binding proteins loaded on to a particular promoter, were performed using exponentially growing wt and p38 $\alpha^{-/-}$ cardiomyocytes. The primers were chosen in order to amplify the region of the COL1A1 promoter (-620 to -225) containing the different C/EBP and TEF-1 binding sites (Figure 7A). Consistent with the EMSAs, we detected more C/EBP β loaded on to the COL1A1 promoter in the wt cells (Figure 7B, upper panel). The re-introduction of p38 α into the p38 $\alpha^{-/-}$ cardiomyocytes partially reverted C/EBP β binding to the same level as that observed in wt cells (Figure 7B, lane 3). TEF-1 was also detected on the COL1A1 promoter but only in the p38 α -lacking cells (Figure 7B, lower panel).

These results strongly suggest that the up-regulation of COL1A1 gene transcription in p38 $\alpha^{-/-}$ cells is related to the ability of p38 α to regulate the basal DNA binding activities of C/EBP β and TEF-1.

DISCUSSION

The involvement of the p38 MAPK pathway in inflammatory and environmental stress responses has been extensively investigated in many different cellular contexts. However, the molecular targets responsible for the housekeeping functions of p38 MAPK are poorly characterized. By analysing global differences in the transcriptome between wt and p38 $\alpha^{-/-}$ cardiomyocytes, we found changes in the expression of genes that are associated with several cellular processes (Figure 1), implying possible functions for the low-level activity of p38 MAPKs in non-stressed proliferating cells. A similar question has recently been addressed in lymphocyte cell lines treated with the p38 MAPK inhibitor SB203580 [35]. However, there was little overlap between the SB203580-regulated genes in lymphocytes and our data set of p38

Table 1 Results of the PRIMA analysis showing genes for which promoters are enriched in binding sites for TEF-1 and C/EBP transcription factorsThe cut-off *P* values for TEF-1 and C/EBP were 0.000136 and 0.000431 respectively. GB, Gen Bank.

Gene name	Gene symbol	GB accession number	UniGene ID	Average fold-change	Binding site	
					TEF-1	C/EBP
Cathepsin D	<i>Ctsd</i>	BG087382	Mm.231395	2.66	*	*
Procollagen, type I, alpha 1	<i>Col1a1</i>	BG073196	Mm.277735	5.10	*	*
Procollagen, type I, alpha 2	<i>Col1a2</i>	BG073735	Mm.277792	4.39	*	*
Tumour rejection antigen gp96	<i>Tra1</i>	BG078337	Mm.87773	2.94	*	*
High mobility group AT-hook 2	<i>Hmga2</i>	BG086396	Mm.157190	-1.92		*
Ladinin	<i>Lad1</i>	BG076566	Mm.36726	-2.62		*
Myeloid ecotropic viral integration site-related gene 1	<i>Mrg1</i>	BG067314	Mm.247566	-1.69		*
Plexin A4	<i>Plxn4</i>	BG086250	Mm.242174	-2.30		*
RIKEN cDNA 1110032D12 gene	<i>1110032D12Rik</i>	BG076581	Mm.325307	1.98		*
RIKEN cDNA 1110033009 gene	<i>1110033009Rik</i>	BG067483	Mm.21171	2.08		*
ROD1 regulator of differentiation 1 (<i>Schizosaccharomyces pombe</i>)	<i>Rod1</i>	BG063016	Mm.331640	-3.87		*
TBC1 domain family, member 13	<i>Tbc1d13</i>	BG072158	Mm.19172	1.70		*
Activated leucocyte cell adhesion molecule	<i>Alcam</i>	BG086024	Mm.288282	1.66	*	
ADP-ribosylation factor 1	<i>Arf1</i>	BG082675	Mm.371546	1.66	*	
ATPase, Na ⁺ /K ⁺ transporting, beta 1 polypeptide	<i>Atp1b1</i>	AW544616	Mm.4550	2.19	*	
Barren homolog (<i>Drosophila</i>)	<i>Brrn1</i>	BG085939	Mm.29786	3.01	*	
Centaurin, delta 2	<i>Centd2</i>	BG087361	Mm.277687	2.00	*	
Cullin 3	<i>Cul3</i>	BG085494	Mm.12665	1.58	*	
Cyclin D3	<i>Ccnd3</i>	BG066310	Mm.246520	1.85	*	
Decorin Dcn	<i>D16Bwg1547e</i>	BG088078	Mm.56769	2.73	*	
DNA segment, Chr 16, Brigham & Women's Genetics 1547 expressed	<i>D16Bwg1547e</i>	BG063060	Mm.567	4.12	*	
Immunity-related GTPase family, Q1	<i>AF322649</i>	BG069854	Mm.41108	2.19	*	
Integral membrane protein 2B	<i>Itm2b</i>	BG088523	Mm.4266	1.89	*	
Mannose-P-dolichol utilization defect 1	<i>Mpdu1</i>	BG083639	Mm.89579	2.81	*	
Microsomal glutathione S-transferase 1	<i>Mgst1</i>	BG086330	Mm.14796	2.23	*	
Pleiomorphic adenoma gene-like 1	<i>Plagl1</i>	BG085853	Mm.287857	3.60	*	
Potassium intermediate/small conductance calcium-activated channel, subfamily N, member 4	<i>Mpdu1</i>	BG080543	Mm.9911	2.52	*	
Procollagen, type III, alpha 1	<i>Col3a1</i>	BG085576	Mm.249555	4.25	*	
RIKEN cDNA 1110059P08 gene	<i>1110059P08Rik</i>	C85340	Mm.227983	2.15	*	
RIKEN cDNA 1190007F08 gene	<i>1190007F08Rik</i>	BG076322	Mm.260690	2.32	*	
Secreted acidic cysteine rich glycoprotein	<i>Sparc</i>	BG078305	Mm.291442	3.25	*	
Sema domain, immunoglobulin domain (Ig), short basic domain, secreted, (semaphorin) 3 F	<i>Sema3f</i>	BG074382	Mm.12903	2.15	*	
Vinculin	<i>Vcl</i>	BG063694	Mm.279361	2.29	*	

MAPK-regulated genes in cardiomyocytes, with the exception of genes involved in cell cycle regulation, such as cyclin D3 and cullin 3, which are present in both cases. We also found that many of the main components of the myocardium ECM were over-expressed in p38 α ^{-/-} cardiomyocytes. As ECM components play a key role in heart development and function [15], our results indicate a potential role for p38 α in the regulation of postnatal heart physiology. Therefore we focused our studies on the mechanism of COL1 regulation, which represents approx. 85 % of the protein content of the myocardium ECM.

We found that a significant number of the genes differentially expressed in p38 α ^{-/-} cells were potentially regulated by the transcription factors C/EBP and TEF-1. Although C/EBP was known to be regulated by the p38 and ERK MAPK pathways, heart-specific transcriptional activity of TEF-1 has not been shown to be regulated by MAPK pathways to date. The TEF-1 gene family consists of 4 transcriptional activators, which regulate promoters containing the M-CAT sites in several tissues [36]. In cardiac muscle, M-CAT sites are required for the full activity of promoters induced by hypertrophic signals [37]. C/EBPs include 6 transcription factors in the leucine zipper family, which play key regulatory roles in cell proliferation and differentiation and can be regulated by MAPK phosphorylation (reviewed in [38]). C/EBP β proteins have been proposed to be involved in the down-

regulation of COL1A1 gene expression [39], the promoter of which contains three C/EBP binding sites (Figure 7A) [40].

In some cell types, down-regulation of p38 MAPKs has been shown to correlate with up-regulation of the ERK pathway [16,32]. While this manuscript was in preparation, a study has been published reporting the regulation of collagen expression by p38 α in primary rat cardiac fibroblasts subjected to mechanical load. Using the inhibitor SB203580, these authors concluded that p38 MAPK negatively regulates collagen transcription by acting through ERK [41]. We also found a higher level of ERK basal activity in p38 α ^{-/-} cardiomyocytes, however, incubation with ERK inhibitors did not change the level of COL1A1 mRNA expression in p38 α ^{-/-} cells. Moreover, TNF α treatment promotes the inhibition of COL1A1 expression in wt cardiomyocytes, but increased COL1A1 expression in p38 α ^{-/-} cells; similar results were observed upon expression of the p38 α -D/A mutant in the p38 α ^{-/-} background. Taken together, these results suggest a negative role for p38 MAPK, independent of ERK activity, in the regulation of COL1A1 in normally growing and cytokine-stimulated cardiomyocytes.

Our studies indicate that gene transcription is the primary mechanism of COL1A1 expression regulated by p38 α in cardiomyocytes. The expression of COL1 genes is controlled by multiple regulatory modules, which are differentially required in

induced during myocardium injury and can bind to the COL1A1 promoter [43]. Depending on the cell type and experimental conditions, the transcriptional activity of C/EBP may be regulated in a positive or negative manner through phosphorylation by different signal transduction pathways including ERK [44] and p38 MAPKs [45]. We observed that less C/EBP β was loaded on to the COL1A1 promoter in p38 $\alpha^{-/-}$ cells, indicating that it could mediate the inhibition of COL1A1 transcription induced by p38 α in normally proliferating cardiomyocytes. Conversely, both TEF-1 DNA binding activity and its loading on to the COL1A1 promoter were increased in the p38 $\alpha^{-/-}$ cells. Therefore the up-regulation of COL1A1 gene expression in p38 $\alpha^{-/-}$ cells may be related to the binding of the transcriptional activator TEF-1 and the simultaneous release of the transcriptional repressor C/EBP β . The COL1A1 promoter also contains Sp1 sites that have been suggested to mediate TEF-1 binding to promoters that lack an M-CAT binding site [46]. Moreover, we have noticed that the C/EBP binding sites on the murine COL1A1 promoter overlap with sites recognized by STAT (signal transducer and activator of transcription) transcription factors, and p38 $\alpha^{-/-}$ cardiomyocytes are known to contain increased amounts of nuclear phosphorylated STAT3 [16]. Further experiments will be necessary to investigate the potential contribution of all of these proteins to the upregulation of COL1A1 gene expression in p38 $\alpha^{-/-}$ cells.

The generation of knockout mice lacking p38 α or the upstream activators MKK6 and MKK3 has allowed us to investigate the role of this pathway in mammalian embryogenesis. The absence of p38 α expression or activation resulted in mouse embryonic lethality due to a defect in placental morphogenesis, probably due to defective vascularization [6,7,47,48]. Collagens are the main components of blood vessel ECM, as well as of the chorion-allantoic ECM that provides a scaffold for placental vascular growth. Therefore our results regarding the regulation of COL1 and COL3 gene expression by p38 MAPKs suggest that the placental vascularization defect in p38 $\alpha^{-/-}$ mice could be related to impaired collagen accumulation. This is consistent with the proposed role of p38 α in the vascular remodelling associated with angiogenesis, rather than with the processes involved in the formation of the primary capillary plexus [7]. The study of COL1 and COL3 expression in the placentas of p38 $\alpha^{-/-}$ embryos will be necessary to confirm this possibility. Previous work has suggested a role for p38 MAPKs in cardiac morphogenesis and pathological conditions such as hypertrophy and fibrosis (reviewed by [49]). However, defects in cardiac development were not detected when the placental phenotype of p38 $\alpha^{-/-}$ embryos was rescued by fusion with tetraploid wt embryos [6]. In the present study, we have shown that p38 MAPK controls the expression of several myocardium ECM components in normally proliferating cells. Our results are in agreement with previous work showing that the expression of transgenes for dominant negative forms of p38 α or their activators MKK6 and MKK3 in mouse heart results in interstitial fibrosis, which leads to the modification of myocardium integrity and function [13]. Interestingly, cardiac-specific p38 α knockout mice have recently been reported to display normal global cardiac structure and function, but these mice develop an abnormal response to pressure overload, which is accompanied by massive cardiac fibrosis [50]. In agreement with our results, the expression levels of COL1A1 and COL3A1 mRNAs after pressure overload were also significantly increased in the hearts of cardiac-specific p38 α knockout mice compared with the levels in wt control mice [50].

In summary, we propose that the basal level of p38 MAPK activity in non-stimulated cardiomyocytes may be implicated in the inhibition of pro-fibrotic and pro-hypertrophic pathways, which are activated upon heart injury and play a key role in the

repair response (reviewed in [49]). Our results also indicate that C/EBP β and TEF-1 transcription factors are important mediators of p38 MAPK function in cardiomyocytes.

We thank Ran Elkon for support with bioinformatics studies, Ana Cuadrado and Ignacio Dolado for critically reading the manuscript before submission and Emma Black for excellent technical assistance. C.A. thanks Professor Alessandro Weisz for his helpful discussions, and the Associazione Italiana per la Ricerca sul Cancro (Investigator and Regional Research Grants) and Ministero dell'Istruzione, Università e Ricerca (Grants FIRB RBNE0157EH and PRIN 2004067020.002) for the financial support required to complete the work reported in the present study. C. S. is a Dottorato di Ricerca in Oncologia medica e chirurgica ed Immunologia clinica (XVIII° ciclo) student at the Seconda Università degli Studi di Napoli. A.R.N. is supported by a grant from the Fundacion Cientifica de la Asociacion Espanola Contra el Cancer.

REFERENCES

- 1 Nebreda, A. R. and Porras, A. (2000) p38 MAP kinases: beyond the stress response. *Trends Biochem. Sci.* **25**, 257–260
- 2 Cohen, P. (1997) The search for physiological substrates of MAP and SAP kinases in mammalian cells. *Trends Cell Biol.* **7**, 353–361
- 3 Ono, K. and Han, J. (2000) The p38 signal transduction pathway: activation and function. *Cell. Signalling* **12**, 1–13
- 4 Darragh, J., Soloaga, A., Beardmore, V. A., Wingate, A. D., Wiggin, G. R., Pegg, M. and Arthur, J. S. (2005) MSKs are required for the transcription of the nuclear orphan receptors Nur77, Nurr1 and Nor1 downstream of MAPK signalling. *Biochem. J.* **390**, 749–759
- 5 Clark, A. R., Dean, J. L. and Saklatvala, J. (2003) Post-transcriptional regulation of gene expression by mitogen-activated protein kinase p38. *FEBS Lett.* **546**, 37–44
- 6 Adams, R. H., Porras, A., Alonso, G., Jones, M., Vintersten, K., Panelli, S., Valladares, A., Perez, L., Klein, R. and Nebreda, A. R. (2000) Essential role of p38 α MAP kinase in placental but not embryonic cardiovascular development. *Mol. Cell* **6**, 109–116
- 7 Mudgett, J. S., Ding, J., Guh-Siesel, L., Chartrain, N. A., Yang, L., Gopal, S. and Shen, M. M. (2000) Essential role for p38 α mitogen-activated protein kinase in placental angiogenesis. *Proc. Natl. Acad. Sci. U.S.A.* **97**, 10454–10459
- 8 Tamura, K., Sudo, T., Sentfleben, U., Dadak, A. M., Johnson, R. and Karin, M. (2000) Requirement for p38 α in erythropoietin expression: a role for stress kinases in erythropoiesis. *Cell* **102**, 221–231
- 9 Tanaka, N., Kamanaka, M., Enslin, H., Dong, C., Wysk, M., Davis, R. J. and Flavell, R. A. (2002) Differential involvement of p38 mitogen-activated protein kinase kinases MKK3 and MKK6 in T-cell apoptosis. *EMBO Rep.* **3**, 785–791
- 10 Sugden, P. H. (2001) Signalling pathways in cardiac myocyte hypertrophy. *Ann. Med.* **33**, 611–622
- 11 Yue, T. L., Gu, J. L., Wang, C., Reith, A. D., Lee, J. C., Mirabile, R. C., Kreutz, R., Wang, Y., Maleeff, B., Parsons, A. A. and Ohlstein, E. H. (2000) Extracellular signal-regulated kinase plays an essential role in hypertrophic agonists, endothelin-1 and phenylephrine-induced cardiomyocyte hypertrophy. *J. Biol. Chem.* **275**, 37895–37901
- 12 Bueno, O. F., De Windt, L. J., Tymitz, K. M., Witt, S. A., Kimball, T. R., Klevitsky, R., Hewett, T. E., Jones, S. P., Lefer, D. J., Peng, C. F. et al. (2000) The MEK1-ERK1/2 signaling pathway promotes compensated cardiac hypertrophy in transgenic mice. *EMBO J.* **19**, 6341–6350
- 13 Braz, J. C., Bueno, O. F., Liang, Q., Wilkins, B. J., Dai, Y. S., Parsons, S., Braunwart, J., Glascock, B. J., Klevitsky, R., Kimball, T. F. et al. (2003) Targeted inhibition of p38 MAPK promotes hypertrophic cardiomyopathy through upregulation of calcineurin-NFAT signaling. *J. Clin. Invest.* **111**, 1475–1486
- 14 Bogoyevitch, M. A., Gillespie-Brown, J., Ketterman, A. J., Fuller, S. J., Ben-Levy, R., Ashworth, A., Marshall, C. J. and Sugden, P. H. (1996) Stimulation of the stress-activated mitogen-activated protein kinase subfamilies in perfused heart. p38/RK mitogen-activated protein kinases and c-Jun N-terminal kinases are activated by ischemia/reperfusion. *Circ. Res.* **79**, 162–173
- 15 Weber, K. (1997) Cardiac Interstitium. In *Heart Failure* (Poole-Wilson, P., Colucci, W., Massie, P., Chatterjee, K. and Coats, A., eds.), pp. 13–31, Churchill Livingstone, New York
- 16 Porras, A., Zuluaga, S., Black, E., Valladares, A., Alvarez, A. M., Ambrosio, C., Benito, M. and Nebreda, A. R. (2004) p38 α mitogen-activated protein kinase sensitizes cells to apoptosis induced by different stimuli. *Mol. Biol. Cell* **15**, 922–933
- 17 Ambrosio, C., Mace, G., Galban, S., Fritsch, C., Vintersten, K., Black, E., Gorospe, M. and Nebreda, A. R. (2003) Negative feedback regulation of MKK6 mRNA stability by p38 α mitogen-activated protein kinase. *Mol. Cell. Biol.* **23**, 370–381
- 18 Tanaka, T. S., Jaradat, S. A., Lim, M. K., Kargul, G. J., Wang, X., Grahovac, M. J., Pantano, S., Sano, Y., Piao, Y., Nagaraja, R. et al. (2000) Genome-wide expression profiling of mid-gestation placenta and embryo using a 15,000 mouse developmental cDNA microarray. *Proc. Natl. Acad. Sci. U.S.A.* **97**, 9127–9132

- 19 Richter, A., Schwager, C., Hentze, S., Ansorge, W., Hentze, M. W. and Muckenthaler, M. (2002) Comparison of fluorescent tag DNA labeling methods used for expression analysis by DNA microarrays. *BioTechniques* **33**, 620–630.
- 20 Alonso, G., Ambrosino, C., Jones, M. and Nebreda, A. R. (2000) Differential activation of p38 mitogen-activated protein kinase isoforms depending on signal strength. *J. Biol. Chem.* **275**, 40641–40648.
- 21 Elkon, R., Linhart, C., Sharan, R., Shamir, R. and Shiloh, Y. (2003) Genome-wide *in silico* identification of transcriptional regulators controlling the cell cycle in human cells. *Genome Res.* **13**, 773–780.
- 22 Sharan, R., Maron-Katz, A. and Shamir, R. (2003) CLICK and EXPANDER: a system for clustering and visualizing gene expression data. *Bioinformatics* **19**, 1787–1799.
- 23 Baldassarre, F., Mallardo, M., Mezza, E., Scala, G. and Quinto, I. (1995) Regulation of NF- κ B through the nuclear processing of p105 (NF- κ B1) in Epstein-Barr virus-immortalized B cell lines. *J. Biol. Chem.* **270**, 31244–31248.
- 24 Dignam, J. D., Lebovitz, R. M. and Roeder, R. G. (1983) Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei. *Nucleic Acids Res.* **11**, 1475–1489.
- 25 Ravazzolo, R., Karsenty, G. and de Crombrughe, B. (1991) A fibroblast-specific factor binds to an upstream negative control element in the promoter of the mouse α 1(I) collagen gene. *J. Biol. Chem.* **266**, 7382–7387.
- 26 Khatri, P., Draghici, S., Ostermeier, G. C. and Krawetz, S. A. (2002) Profiling gene expression using onto-express. *Genomics* **79**, 266–270.
- 27 Rodriguez-Barbero, A., Obreo, J., Yuste, L., Montero, J. C., Rodriguez-Pena, A., Pandiella, A., Bernabeu, C. and Lopez-Novoa, J. M. (2002) Transforming growth factor- β 1 induces collagen synthesis and accumulation via p38 mitogen-activated protein kinase (MAPK) pathway in cultured L(6)E(9) myoblasts. *FEBS Lett.* **513**, 282–288.
- 28 Varela-Rey, M., Montiel-Duarte, C., Osés-Prieto, J. A., Lopez-Zabalza, M. J., Jaffrezou, J. P., Rojkind, M. and Iriburu, M. J. (2002) p38 MAPK mediates the regulation of α 1(I) procollagen mRNA levels by TNF- α and TGF- β in a cell line of rat hepatic stellate cells. *FEBS Lett.* **528**, 133–138.
- 29 Jimenez, S. A., Gaidarova, S., Saitta, B., Sandorfi, N., Herrich, D. J., Rosenbloom, J. C., Kucich, U., Abrams, W. R. and Rosenbloom, J. (2001) Role of protein kinase C- δ in the regulation of collagen gene expression in scleroderma fibroblasts. *J. Clin. Invest.* **108**, 1395–1403.
- 30 Ghosh, A. K. (2002) Factors involved in the regulation of type I collagen gene expression: implication in fibrosis. *Exp. Biol. Med.* **227**, 301–314.
- 31 Tharaux, P. L., Chatziantoniou, C., Fakhouri, F. and Dussault, J. C. (2000) Angiotensin II activates collagen I gene through a mechanism involving the MAP/ER kinase pathway. *Hypertension* **36**, 330–336.
- 32 Westermarck, J., Li, S. P., Kallunki, T., Han, J. and Kahari, V. M. (2001) p38 mitogen-activated protein kinase-dependent activation of protein phosphatases 1 and 2A inhibits MEK1 and MEK2 activity and collagenase 1 (MMP-1) gene expression. *Mol. Cell. Biol.* **21**, 2373–2383.
- 33 Siwik, D. A., Chang, D. L. and Colucci, W. S. (2000) Interleukin-1 β and tumor necrosis factor- α decrease collagen synthesis and increase matrix metalloproteinase activity in cardiac fibroblasts *in vitro*. *Circ. Res.* **86**, 1259–1265.
- 34 Lindquist, J. N., Marzluft, W. F. and Stefanovic, B. (2000) Fibrogenesis. III. Posttranscriptional regulation of type I collagen. *Am. J. Physiol. Gastrointest. Liver Physiol.* **279**, G471–G476.
- 35 Lin, Z., Crockett, D. K., Jensen, S. D., Lim, M. S. and Elenitoba-Johnson, K. S. (2004) Quantitative proteomic and transcriptional analysis of the response to the p38 mitogen-activated protein kinase inhibitor SB203580 in transformed follicular lymphoma cells. *Mol. Cell. Proteomics* **3**, 820–833.
- 36 Larkin, S. B., Farrance, I. K. and Ordahl, C. P. (1996) Flanking sequences modulate the cell specificity of M-CAT elements. *Mol. Cell. Biol.* **16**, 3742–3755.
- 37 McLean, B. G., Lee, K. S., Simpson, P. C. and Farrance, I. K. (2003) Basal and β 1-adrenergic-induced activity of minimal rat MHC promoters in cardiac myocytes requires multiple TEF-1 but not NFAT binding sites. *J. Mol. Cell. Cardiol.* **35**, 461–471.
- 38 McKnight, S. L. (2001) McBindall—a better name for CCAAT/enhancer binding proteins? *Cell* **107**, 259–261.
- 39 Greenwel, P., Tanaka, S., Penkov, D., Zhang, W., Olive, M., Moll, J., Vinson, C., Di Liberto, M. and Ramirez, F. (2000) Tumor necrosis factor α inhibits type I collagen synthesis through repressive CCAAT/enhancer-binding proteins. *Mol. Cell. Biol.* **20**, 912–918.
- 40 Attard, F. A., Wang, L., Potter, J. J., Rennie-Tankersley, L. and Mezey, E. (2001) Identification of new sites of binding and activation of the murine α 1(I) collagen promoter by CCAAT/enhancer binding protein beta. *DNA Cell Biol.* **20**, 455–463.
- 41 Papakrivopoulou, J., Lindahl, G. E., Bishop, J. E. and Laurent, G. J. (2004) Differential roles of extracellular signal-regulated kinase 1/2 and p38MAPK in mechanical load-induced procollagen α 1(I) gene expression in cardiac fibroblasts. *Cardiovasc. Res.* **61**, 736–744.
- 42 Goldberg, H., Helaakoski, T., Garrett, L. A., Karsenty, G., Pellegrino, A., Lozano, G., Maity, S. and de Crombrughe, B. (1992) Tissue-specific expression of the mouse α 2(I) collagen promoter. Studies in transgenic mice and in tissue culture cells. *J. Biol. Chem.* **267**, 19622–19630.
- 43 Mori, K., Hatamochi, A., Ueki, H., Olsen, A. and Jimenez, S. A. (1996) The transcription of human α 1(I) procollagen gene (COL1A1) is suppressed by tumour necrosis factor- α through proximal short promoter elements: evidence for suppression mechanisms mediated by two nuclear-factor-binding sites. *Biochem. J.* **319**, 811–816.
- 44 Nakajima, T., Kinoshita, S., Sasagawa, T., Sasaki, K., Naruto, M., Kishimoto, T. and Akira, S. (1993) Phosphorylation at Threonine-235 by a ras-Dependent Mitogen-Activated Protein Kinase Cascade is Essential for Transcription Factor NF-IL6. *Proc. Natl. Acad. Sci. U.S.A.* **90**, 2207–2211.
- 45 Caivano, M., Gorgoni, B., Cohen, P. and Poli, V. (2001) The induction of cyclooxygenase-2 mRNA in macrophages is biphasic and requires both CCAAT enhancer-binding protein beta (C/EBP beta) and C/EBP delta transcription factors. *J. Biol. Chem.* **276**, 48693–48701.
- 46 Shie, J. L., Wu, G. X., Wu, J., Liu, F. F., Laham, R. J., Oettgen, P. and Li, J. (2004) RTEF-1, a novel transcriptional stimulator of vascular endothelial growth factor in hypoxic endothelial cells. *J. Biol. Chem.* **279**, 25010–25016.
- 47 Allen, M., Svensson, L., Roach, M., Hambor, J., McNeish, J. and Gabel, C. A. (2000) Deficiency of the stress kinase p38 α results in embryonic lethality: characterization of the kinase dependence of stress responses of enzyme-deficient embryonic stem cells. *J. Exp. Med.* **191**, 859–870.
- 48 Brancho, D., Tanaka, N., Jaeschke, A., Ventura, J. J., Kelkar, N., Tanaka, Y., Kyuuma, M., Takeshita, T., Flavell, R. A. and Davis, R. J. (2003) Mechanism of p38 MAP kinase activation *in vivo*. *Genes Dev.* **17**, 1969–1978.
- 49 McKinsey, T. A. and Olson, E. N. (1999) Cardiac hypertrophy: sorting out the circuitry. *Curr. Opin. Genet. Dev.* **9**, 267–274.
- 50 Nishida, K., Yamaguchi, O., Hirotsu, S., Hikoso, S., Higuchi, Y., Watanabe, T., Takeda, T., Osuka, S., Morita, T., Kondoh, G., Uno, Y., Kashiwase, K., Taniike, M., Nakai, A., Matsumura, Y., Miyazaki, J., Sudo, T., Hongo, K., Kusakari, Y., Kurihara, S., Chien, K. R., Takeda, J., Hori, M. and Otsu, K. (2004) p38 α mitogen-activated protein kinase plays a critical role in cardiomyocyte survival but not in cardiac hypertrophic growth in response to pressure overload. *Mol. Cell. Biol.* **24**, 10611–10620.

Received 12 September 2005/1 February 2006; accepted 21 February 2006

Published as BJ Immediate Publication 21 February 2006, doi:10.1042/BJ20051502