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Quantitative analysis of the cardiac fibroblast transcriptome - implications for NO/cGMP signalling

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Summary

Cardiac fibroblasts regulate tissue repair and remodeling in the heart. To quantify transcript levels in these cells we performed a comprehensive gene expression study using serial analysis of gene expression (SAGE). Among 110,169 sequenced tags we could identify 30,507 unique transcripts. A comparison of SAGE data from cardiac fibroblasts with data derived from total mouse heart revealed a number of fibroblast-specific genes. Cardiac fibroblasts expressed a specific collection of collagens, matrix proteins and metalloproteinases, growth factors, and components of signalling pathways. The NO/cGMP signalling pathway was represented by the mRNAs for α_1 and β_1 subunits of guanylyl cyclase, cGMP-dependent protein kinase type I (cGK I) and interestingly the G-kinase anchoring protein GKAP42. The expression of cGK I was verified by RT-PCR and Western Blot. To establish a functional role for cGK I in cardiac fibroblasts we studied its effect on cell proliferation. Selective activation of cGK I with a cGMP-analog inhibited the proliferation of serum-stimulated cardiac fibroblasts which express cGK I, but not higher passage fibroblasts which contain no detectable cGK I. Currently, our data suggest that cGK I mediates the inhibitory effects of the NO/cGMP pathway on cardiac fibroblast growth. Furthermore the SAGE library of transcripts expressed in cardiac fibroblasts provides a basis for future investigations into the pathological regulatory mechanisms underlying cardiac fibrosis.

Keywords: cardiac fibroblast / SAGE / gene expression / cGMP / protein kinase / proliferation

Introduction

Cardiac fibrosis is a disease process leading to structural reorganization of the ventricular wall, with increased myocardial stiffness, ventricular dysfunction, and ultimately heart failure [1]. The major cell type involved in this process is the cardiac fibroblast. Injury to the myocardium, e.g. during myocardial infarction, as well as hypertensive heart disease lead to an activation of cardiac fibroblast proliferation and extracellular matrix production [2]. In general the response of the heart to injury shares many features in common with wound healing and fibrosis observed in other tissues, however significant differences have also been observed which suggest mechanisms unique to heart. For example, opposite effects of certain cytokines have been observed to be dependent on the type of fibroblast studied. Interleukin-I increased proliferation and collagen synthesis in skin fibroblasts, whereas it inhibited these in cardiac fibroblasts [3]. Important molecular factors shown to mediate phenotypic changes in cardiac fibroblasts in response to injury are angiotensin II [4-7], transforming growth factor β [8,9], norepinephrine [10] and endothelin-1 [11]. Cardiac myocytes also secrete atrial and brain natriuretic peptides (ANP, BNP) which have potent systemic activities such as natriuresis, diuresis and vasodilation. In addition BNP has been attributed a local antifibrotic function; BNP knockout mice exhibited signs of cardiac fibrosis which were increased in response to ventricular pressure overload [12].

Although the importance of cardiac fibroblasts in the development of heart failure is widely accepted, the understanding of the molecular regulation of these cells is still quite limited. To date only individual activation and signal transduction pathways have been analyzed. A new approach to studying cell function is to gain a comprehensive view of all expressed gene products and their interactions in a given cell [13]. The first method used to perform global expression analyses in mammals was SAGE (serial analysis of gene expression, [14,15]). Microarray analysis [16] has since become the most widely used tool for comprehensive gene expression studies. However in comparison to microarrays, SAGE has important advantages making it a method of choice for certain applications. SAGE is based on the sequencing of short 14-bp tags at the 3'-end of a gene. Frequencies of gene-specific tags found by sequencing correspond to levels of gene expression. The major advantages of SAGE in comparison to microarray analyses are that absolute values (tag counts) are obtained which can be easily compared with other SAGE libraries, and that SAGE yields very extensive transcriptome information whereas microarray studies are restricted by the number of DNAs spotted onto the arrays. The SAGE method has been used extensively to characterize

differences in gene expression profiles of cancer versus normal cells [14,17], and has proven to be an appropriate tool for the analysis of complete transcriptomes [15,18,19]. Currently, no global gene expression data for cardiac fibroblasts is available.

Using SAGE, 30,507 unique transcripts were identified within cardiac fibroblasts. We obtained quantitative information on individual transcript expression levels, and identified fibroblast-specific transcripts in comparison to a total heart SAGE library. Importantly, we detected transcripts of the cGMP signal transduction pathway in cardiac fibroblasts, which we verified at the protein level. Finally we present data demonstrating this pathway's involvement in inhibition of mouse cardiac fibroblast proliferation.

Results

Comprehensive analysis of gene expression in mouse cardiac fibroblasts

To establish a SAGE library from cardiac fibroblasts we used cells in passage 15. After sequencing 118,654 tags and excluding duplicate ditags and linker-derived sequences, we obtained a total tag number of 110,169 representing 30,507 unique tags/transcripts (Table 1). Assuming a sequencing error of 1% (translates to 10% error in tag analysis), the total number of unique tags would be reduced to 27,456. Some transcripts were found at a frequency of only one tag (68% of the total unique tags), whereas other transcripts were found at least 10 times or more (3.6% of unique tags), and very few transcripts were present as more than 100 copies (only 0.4% of unique tags). However tags present at >100 copies accounted for 31% of the total number of tags. The ten most abundant transcripts in cardiac fibroblasts are shown in Table 2. To facilitate comparisons, tag counts were normalized to tags per million (tpm), i.e., 100 copies correspond to 907 tpm. Most of the highly expressed transcripts are involved in protein synthesis and energy metabolism. The highest expression level was observed for translation elongation factor 1α1 (12,844 tpm, corresponding to 1.3 % of total transcripts).

Comparison of SAGE and Northern blot data

We next compared expression levels of transcripts obtained by SAGE with Northern Blot signal intensities. For this we chose 32 transcripts ranging in expression level from 64 to 2050 tpm and performed Northern blots for these transcripts. All chosen transcripts could be demonstrated by Northern blot analysis. High tag counts tended to correlate with strong Northern blot signals (Fig. 1), although no linear relationship could be observed.

Comparison of cardiac fibroblast and total heart gene expression

We compared our data with a recently published SAGE library derived from total adult mouse heart [20] to identify cardiac fibroblast specific transcripts. Table 3 summarizes 50 transcripts with the highest fibroblast/heart expression ratio ranging from 20- to 188-fold. To verify that the identified transcripts were equally expressed in early versus later passage cells we randomly chose 11 transcripts from Table 3 and analyzed their expression by RT-PCR. In this study we also included the cGK II transcript as a low level expressed gene (see below). All transcripts studied were expressed both in passage 1 as well as passage 20 cells (Fig. 2). In addition no major changes in transcript level could be observed between these two cell preparations. The list of cardiac fibroblast specific transcripts contains many extracellular

matrix proteins, e.g., collagens and other regulatory extracellular proteins such as annexin 1, metalloproteinase-3 and the inhibitor of metalloproteinase-3. Our data also agree with reports of the presence of amyloid A3 and prion protein in fibroblasts [21,22]. However, unlike previous data, SAGE gives quantitative information about the expression level of these different gene products. Among the top 50 fibroblast-specific tags, 17 matched to uncharacterized cDNA or EST sequences. The corresponding transcripts could play important new roles in cardiac fibroblast physiology.

Expression of extracellular matrix proteins and growth factors

Next we searched our expression library for the presence of extracellular matrix proteins (Table 4). The pattern of expressed collagens comprised the ubiquitous type III and type I collagens, as well as collagen IV which, together with laminins, is a building block of basal laminas. In addition, other unconventional, non-helix forming collagens, such as type VI and XVI were highly expressed in cardiac fibroblasts. Among the most prominent extracellular matrix proteins were SPARC (secreted acidic cysteine rich glycoprotein, also called osteonectin) and matrix metalloproteinase 3, two proteins involved in the regulation of extracellular matrix composition and function [23,24]. Interestingly superoxide dismutase was expressed in cardiac fibroblasts. Superoxide has been shown previously to induce proliferation of cardiac fibroblasts [25]. The most abundant growth factor detected was connective tissue growth factor which has been ascribed a role in the pathogenesis of fibrosis [26]. At the time of resubmission of our manuscript, a paper was published showing that cGK I inhibits the fibrogenic potential of high glucose by repressing thrombospondin 1 – dependent transforming growth factor-beta activation; both of these proteins are also expressed in cardiac fibroblasts (Table 4) [27]. The complete SAGE expression library of mouse cardiac fibroblasts is available at the gene expression omnibus, a publicly accessible database of gene expression data at http://www.ncbi.nlm.nih.gov/geo/ under the accession number GSM7764.

Expression of intracellular signalling molecules in cardiac fibroblasts

Table 5 summarizes the ten most highly expressed protein kinases and protein phospatases found. We found high expression of cyclin-dependent kinase 4 involved in cell cycle progression. Furthermore two kinases from the mitogen activated protein kinase pathway were abundantly expressed (MAPKK 1 and Erk-1). Protein phosphatases were found to be expressed at high levels, a fact stressing their important role in signal transduction pathways.

We were also especially interested in components of the cGMP signalling pathway since nitric oxide (NO), natriuretic peptides (ANP, BNP), and cGMP have been suggested to inhibit fibrotic processes in the heart [12]. Both subunits of soluble guanylyl cyclase were expressed in mouse cardiac fibroblasts suggesting that at least some of the described NO-effects on cardiac fibroblasts might be mediated by cGMP. In this SAGE analysis we did not identify tags derived from the GC-A natriuretic peptide receptor nor the recently described cGMP-stimulated phosphodiesterase (PDE2), a new effector of cGMP in rat cardiac fibroblasts [28]. This might be due to a loss of gene expression after cell isolation and passaging. A second possible explanation is the incompleteness of current tag-to-gene mappings. For exact tag-to-gene mapping, information on the 3'-end of an mRNA molecule is necessary. Although a draft seqeuence of the complete mouse genome has been presented recently [29], the information necessary for comprehensive tag-to-gene mapping will only be provided by a completion of mouse cDNA and EST sequencing projects in the future. For example only a partial cDNA sequence of cGMP-dependent protein kinase type I (cGK I) is available in the databases. After performing BLAST searches a longer cDNA could be constructed through alignments of the RefSeq. NM_011160 of cGK I and the sequences AK036955 and finally AK052877 which contains a polyadenylation signal. About 5-fold less cGMP-dependent protein kinase type I (cGK I) was expressed compared to one of the most abundant serine/threonine kinases, the Peutz-Jeghers syndrome kinase. The catalytic subunit of cAMP-dependent protein kinase was detected at a similar expression level (1.5-fold higher than cGK I). Surprisingly we detected expression of GKAP42, a cGK Iα specific G-kinase anchoring protein (GKAP) initially described in male germ cells [30]. GKAP42 expression was confirmed by RT-PCR (data not shown).

Verification of cGK I expression and demonstration of functional activity

To verify the presence of cGK I transcripts in cardiac fibroblasts, we performed RT-PCR analysis. Using isoform-specific primers that covered exon/intron boundaries to exclude priming from genomic DNA, we identified both cGK Iα as well as Iβ, two known splice isoforms [31] (Fig. 3A). Interestingly, also type II cGK was detected (Fig. 3A). The example of cGK I illustrates an important limitation of the SAGE method: SAGE cannot distinguish between different splice isoforms of a certain transcript if splicing occurs upstream of the SAGE anchoring enzyme's restriction site. Only by RT-PCR could we prove the expression of both cGK Iα and β subtypes in cardiac fibroblasts.

To examine a possible role of cGKs in cGMP-mediated processes in mouse cardiac fibroblasts, we first tested for protein expression and activity. In Western blots we demonstrated cGK I expression in freshly isolated cardiac fibroblasts as well as in passaged cells (Fig. 3B). However cGK II protein expression was not detected (data not shown). To measure the activity of cGK I in intact cells, we treated cardiac fibroblasts with the cGK-selective cGMP-analog 8-pCPT-cGMP and studied phosphorylation of the vasodilator-stimulated phosphoprotein (VASP), an established substrate of cGK I, using phospho-specific antibodies. Activation of cGK resulted in phosphorylation of VASP in cardiac fibroblasts (Fig. 3B). However during passaging the amount of VASP phosphorylation was reduced reflecting a reduction in cGK I activity. In higher passage cells cGK I could no longer be detected.

Inhibition of mouse cardiac fibroblast proliferation by cGK I

Cells in passages 15-20 which expressed endogenous cGK I were used to investigate cGK effects on cell proliferation. Quiescent cardiac fibroblasts switched from medium with 0.1 % serum to 5 % serum for two days underwent a 10-fold increase in cell number (Fig. 4). Activation of cGK with 8-pCPT-cGMP (100 μM) maximally inhibited proliferation by 40 % after 2 days (Fig.4, first 2 bars). Higher passage cells (P27-39), which express only very low levels of cGK I that can no longer be detected by Western blot, showed no significant inhibition of proliferation by 8-pCPT-cGMP could be detected, thus confirming the role of the kinase for the observed effects.

Discussion

The NO/natriuretic peptide/cGMP signalling pathways have been shown by numerous groups to inhibit the activation of cardiac fibroblasts which leads to pathology culminating in cardiac fibrosis. Natriuretic peptides, NO, and cGMP-analogs have been shown to inhibit a number of important functions such as DNA synthesis and proliferation in cultured neonatal rat cardiac fibroblasts [32-35]. However the signalling pathway downstream of cGMP has not been clarified. In this manuscript we provide evidence that cGK I mediates cGMP-induced inhibition of cardiac fibroblast proliferation. Both isoforms of cGK I α and β were demonstrated to be present in cardiac fibroblasts and could potentially participate in antiproliferative action. Antiproliferative effects of cGK I have been described for the β isoform of cGK I in smooth muscle [36], BHK cells [37], mesangial cells [38] and T cells [39]. Recently cGMP and cAMP effects on PDGF-BB-induced proliferation were studied in rat cardiac fibroblasts [40]. However in contrast to our data in mouse, cGMP exhibited no significant effects in the rat study. This discrepancy might be explained by species differences as well as by the different growth stimuli used (whole serum in our case, versus PDGF alone in the other). GK phosphorylation sites in a given protein are often not conserved across all species [41], which could result in a GK function also not being conserved. In our study we detected high expression of the cGK Iα specific G-kinase anchor protein GKAP42. The function of GKAP42 is presently unknown [30] but could have a role in targeting cGK Iα to cell sites important for antiproliferative effects. RT-PCR also detected expression of type II cGK, however a function of cGK II in cardiac fibroblasts remains questionable since we could not detect any cGK II protein. Interestingly, cell cycle dependent expression of cGK II in human fibroblast cell lines has been reported [42].

A potential limitation of the present study is the passage number of the cells used. After isolation cardiac fibroblasts undergo a process of senescence and crisis at around passages 5 to 10 before they convert into a stable proliferating state. Since we analyzed the transcriptome from isolated cells at passage 15, certain transcripts could be expressed differently under cell culture conditions than in the intact heart. However we also provide evidence that gene expression is very similar between freshly isolated cells passaged for the first time and older cells at passage 20. In addition our SAGE library contains a broad spectrum of genuine fibroblast markers such as many different collagens and other matrix proteins. We conclude that our library is an initial approximation of the *in situ* expression pattern of fibroblasts in the intact mouse heart.

The generated cardiac fibroblast SAGE library comprises the major part of the transcriptome of these cells. The relatively high number of unique transcripts per cell obtained with the SAGE technology (30,507 unique transcripts in the present study) in comparison with estimates of 10,000 – 15,000 different transcripts per average cell has been a matter of debate. Chen and coworkers [43] recently could show that, in general, the majority of identified SAGE transcripts can be verified with independent methods and thus are not due to sequencing errors. Perhaps not all of these transcripts are translated into protein but may serve regulatory purposes instead.

Quantitation of global gene expression data, as well as comparison of such data from different laboratories, is highly desirable. However in general, quantitative data obtained by SAGE cannot be easily correlated with data from other methods such as Northern Blots, microarrays or RT-PCRs [20,44]. Such comparisons seem to be basically hindered by the intrinsically different underlying principles of detection, e.g., tag isolation and sequencing in SAGE versus hybridization in Northern and array technology. Microarray data are hampered by numerous inter-experimental variabilities such as labelling and hybridization conditions, although efforts are underway to establish standards for recording and reporting microarray-based gene expression data [45]. SAGE is presently the only method providing an easy way to compare data from different tissues and laboratories. Our comparison of the cardiac fibroblast library with a total heart library revealed a number of cardiac fibroblast-specific genes. Further characterization of these transcripts may provide new information on cardiac fibroblast function. Finally, knowledge of the specific collection of transcripts expressed by cardiac fibroblasts will facilitate focussed investigations into the differences between fibroblast processes directing beneficial wound healing versus pathological fibrosis.

Materials and Methods

Cell culture

Primary adult mouse cardiac fibroblasts were isolated as described previously [46] and cultured at 37°C with 5 % CO_2 in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10 % fetal calf serum (FCS) in the presence of penicillin and streptomycin.

Serial analysis of gene expression (SAGE)

RNA was prepared from quiescent, serum-starved mouse cardiac fibroblasts (passage 15) using TRIzol reagent (Invitrogen), and mRNA was isolated by binding to oligo dT cellulose (Ambion, Austin, TX). Then SAGE analysis was performed as described in the 1.0c version of the SAGE protocol [14] with the following modifications. For all PCR reactions, a PCR master mix (Qiagen) was used; after NlaIII digestion of amplified ditags, samples were directly loaded onto PAA-gels without prior phenol extraction and ethanol precipitation (step 10b of the SAGE protocol version 1.0c); concatamers were heated to 65°C for 10 min before loading onto gels (step 11 of the SAGE protocol). After cloning of concatamers into the pZERO cloning vector (Invitrogen), inserts were PCR-amplified and sequenced with an ABI377 automatic sequencer using BigDye-Terminator chemistry (Applied Biosystems). For tag extraction, and elimination of duplicate ditags and contaminating linker sequences, we used the SAGE 2000 software kindly provided by Dr. K. Kinzler (Johns Hopkins University). A database of tag-to-gene mappings was generated from a GenBank download of 83,490 complete mouse mRNAs using SAGE 2000 software. Tags derived from mitochondrial transcripts were identified by sequence comparisons with the complete mouse mitochondrial genome (GenBank accession no. J01420). Tag counts were normalized to tags per million (tpm) to facilitate comparisons with other SAGE libraries. For all tags presented in Tables 2-5 we checked tag-to-gene assignments using the tag-to-gene mapper on the SAGEmap homepage (http://www.ncbi.nlm.nih.gov/sage/). Furthermore we verified if the obtained GenBank reference sequences contained polyadenylation signals. In the Tables and Figures the HUGO nomenclature for gene names and symbols was used.

Northern blotting

Mouse cardiac fibroblasts (passage 18) were seeded onto T75 cell culture flasks (Greiner). Cells were harvested in TRIzol (Invitrogen) and total RNA was isolated. Northern blotting

was carried out using positively charged Nylon membranes (Schleicher and Schuell, Dassel, Germany). After UV crosslinking, membranes were prehybridized with hybridization buffer (DIG EasyHyb, Roche) at 50°C for 30 min, and hybridized overnight at 50°C with probe in hybridization buffer. Hybridization probes were obtained by RT-PCR using specific primers. Each RT-PCR product was used as a template for PCR with digoxigenin (DIG)-labeled dUTP (PCR DIG probe synthesis kit, Roche). After hybridization, membranes were washed twice with 2x SSC (300 mM NaCl, 30 mM Tris-Na-Citrate, pH 7) at room temperature for 5 min, and twice with 0.1 x SSC, 0.1 % SDS for 15 min at 68°C. Bound probe was detected with alkaline phosphatase-coupled anti-DIG antibody and CSPD^{TM} as chemiluminescent phosphatase substrate (Roche).

RT-PCR analysis

Total RNA was isolated from cultured mouse cardiac fibroblasts (passages 1 and 20) using TRIzol (Invitrogen), contaminating DNA was digested with DNase I (Roche) and afterwards RNA was purified using RNeasy columns (Qiagen). Reverse transcription as well as PCR reactions were performed using the GeneAmp PCR-System 9700 (Applied Biosystems). The first strand antisense primers used for reverse transcription were oligo(dT) for all mRNAs. In some reactions, the following cGK subtype-specific antisense primers were used in place of oligo(dt) for reverse transcription: 5′-TGTCTTTAAATGTCCGGTATA-3′ common to both cGK Iα and cGK Iβ, and 5′-ATTGTCCTTGAAAGTACGATAC-3′ for cGK II. Each first strand reaction was carried out using Superscript II Reverse Transcriptase (Invitrogen) and 5 µg total RNA from mouse cardiac fibroblasts.

The PCR reaction primers for cGK Iα and cGK Iβ were as follows: sense, 5′-ATTGTATGTACCCCGTGGAAT-3′ (common to both cGK Iα and Iβ), 5′-GGAAGACTTTGCGAAGATT-3′ (cGK Iα-specific), and 5′-ACTGGACAAGTATCGCTCG-3′ (cGK Iβ-specific); and antisense, 5′-TTGGTGAGTCTTCTCGGGTAA-3′ (common to both cGK Iα and Iβ). PCR primers for cGK II were as follows: sense, 5′-AACTTCAGGAGCGCGAGTA-3′ and antisense 5′-ATCATTTTCTTCCGCGATT-3′. Additional PCR reactions were performed from the oligo-(dT) primed first strand reactions using the described sense primers together with the cGK subtype-specific antisense primers used for reverse transcription. Sequences were aligned to published cGK sequences from mouse.

The PCR primers (sense and antisense) for the transcripts shown in figure 2 were as follows: 5'-TCAGTTCAGCTATGGCCCTCC-3', 5'-GTTGCTCTGCAGATGGGCTAG-3' (Col3a1);

5'-CAGACTTGTCCCGTTTCCATC-3', 5'-CCACCCTTGAGTCAACACCT-3' (Mmp3);
5'-AAGGTGTGGATGAAGCAACCA-3', 5'-GATGAGGAAAGCTCCTGCTG-3' (Anxa1);
5'-GGTGGCAAGAGCAAGAAAAGC-3', 5'-TGATCTTGGGCAAGGAAAACTT-3' (AA589382);
5'-ATTTGTGTCTGCCTCGCTCT-3', 5'-CATTGGTGTGCACCTGTTTC-3' (Efemp2);
5'-TTCCTGCTCATCCTCTGCTTG-3', 5'-GAGCCTCTCCAGCATCATTC-3' (Ctla2a);
5'-GGTCCTTCCGAGGAAGCTAA-3', 5'-GACCAAAGCCCATGTCATCT-3' (Rplp1);
5'-TCATTCCCTTGCATCTTTCCC-3', 5'-TCTATGAGGAGGAGGCTGGA-3' (Timp3);
5'-GCTTGTTCCTTCGCATTCTC-3', 5'-CCACTTTGGAATGGAGCCTA-3' (Prnp);
5'-CCACACTGGGATGAGAGGTT-3', 5'-TCTGGGCTCCATGTCCTAAC-3' (Serpinh1);
5'-GCAGAGTCTCTGTGGCGTACC-3', 5'-CTACTCTAGCTCATTCGCAAGAA-3' (Sdc4).

Western blotting

Cardiac fibroblasts (passages 1-19) grown on 6-well plates were lysed in hot SDS sample buffer and proteins were separated by SDS-PAGE using 9 % gels. Western blotting was performed using nitrocellulose membranes, polyclonal anti-cGKI antiserum [47], and a monoclonal anti-phospho-Ser239 VASP antibody raised against a phosphorylated VASP peptide [48].

Cell proliferation assay

For proliferation assays, mouse cardiac fibroblasts (passage 15-20 as well as passage 27-39) were seeded onto 12 well plates (Greiner, Frickenhausen, Germany) at a density of 500 cells/cm^2. Subconfluent cells were starved in medium containing 0.1 % FCS for 2 days, then cell proliferation was stimulated for 48 h by the addition of fresh medium containing 5 % FCS in the presence or absence of 8-pCPT-cGMP (Biolog, Bremen, Germany). Afterwards cells were detached using EDTA and trypsin, and cell counts were determined with a CASY®1 cell counter (Schärfe System, Reutlingen, Germany).

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Figure legends

Figure 1: Comparison of SAGE tag and Northern blot data obtained from cardiac fibroblasts. Northern blots of 32 transcripts ranging in SAGE tag expression level from 64 to 2050 tags per million (tpm) were performed. Representative blots obtained for 5 transcripts are shown. High SAGE tag counts tended to correlate with strong signals obtained in Northern blots.

Figure 2: Comparison of gene expression in isolated cardiac fibroblasts at different passage numbers. RNA was isolated from cardiac fibroblasts in passage 1 as well as passage 20. Subsequently RT-PCR analysis was performed using oligo-dT for priming of the RT-reaction and specific primers for 12 different transcripts. A control reaction using random hexamers for priming of the RT-reaction yielded identical results (not shown). Cell passaging did not induce major gene expression changes.

Figure 3: Expression and activity of cGK I in cultured adult mouse cardiac fibroblasts. RNA from cardiac fibroblasts (passage 20) was isolated and RT-PCRs for cGKs were performed as described in Methods. Panel A: Lane 1 contains a DNA molecular weight marker. cGK I was detected with a common primer not distinguishing between cGK isoforms (lane 2), and cGK Iα and β were individually detected with isoform-specific primers (lanes 3 and 4). In addition expression of cGK II (II) was observed using two different primer pairs (lanes 5 and 6). All bands were isolated and sequenced to confirm identities. Panel B: Cardiac fibroblasts were cultured at different passage numbers as shown, incubated for 30 min with or without 100 μM 8-pCPT-cGMP, and harvested. Proteins from cell lysates were subjected to SDS-PAGE and Western blotting. cGK I expression was detected using a polyclonal antibody, and cGK I activity was demonstrated as VASP phosphorylation at Ser 239 using a monoclonal phospho-site specific antibody.

Figure 4: cGK I inhibition of cardiac fibroblast proliferation. Quiescent, serum-starved cardiac fibroblasts (passages 15-20 as well as passage 27-39) were stimulated to proliferate with 5 % serum in the absence or presence of 100 μM 8-pCPT-cGMP for 2 days as described in Methods. The number of cells present in 5 % serum in the absence of 8-pCPT-cGMP was defined as 100 % (control), and other data was expressed relative to this. Shown are data ±

SEM of three separate experiments each performed in triplicate. cGK activation led to a maximal 40 % decrease of cell number in cells expressing cGK I but not in later passage cells in which cGK I expression was undetectable.

Table 1: Frequency distribution of identified transcripts in cardiac fibroblasts

Frequency distribution	Unique tags	%	Total tag number	%
>100 tags	130	0.4 %	34009	31 %
11-99 tags	1071	3.6 %	24833	22 %
2-10 tags	8609	28 %	30630	28 %
1 tag	20697	68 %	20697	19 %
Total	30507		110169	

Table 2: Most abundant transcripts in cardiac fibroblasts

Tag sequence	Tag count (tpm)[1]	Transcript	GenBank Acc. no.
AGGCAGACAG	12844	Eef1a1, eukaryotic translation elongation factor 1 alpha 1	BC018485
TGGGTTGTCT	7815	Tpt1, tumor protein, translationally-controlled 1	X06407
TTGCTGCCTT	6889	various ESTs	BE691506
ATACTGACAT	6345	Mtco3, Mitochondrial cytochrome oxidase subunit 3	J01420
TCAGGCTGCC	6000	Fth, Ferritin heavy chain	BC011096
AGCAGTCCCC	5510	Mtco2, Mitochondrial cytochrome oxidase subunit 2	J01420
GCGGCGGATG	5192	Lgals1, Lectin, galactose binding, soluble 1	BC002063
GTGGCTCACA	5128	various mRNA sequences	NM_021282
AGGTCGGGTG	4938	Rp13a, 60S ribosomal protein L13A	NM_009438
ATAATACATA	4602	Mtatp6, Mitochondrial ATP synthase 6	J01420

[1] tpm, tags per million

Table 3: Top 50 cardiac fibroblast specific transcripts

Tag sequence	**Fibroblast** (tpm)	**Heart** (tpm)	**Ratio**	**Transcript**	**GenBank Acc. no.**
TGTTCATCTT	2260	12	188	Col3a1, Procollagen, type III, alpha 1	AK019448
TGCTATGGCA	3068	24	127	Mmp3, Matrix metalloproteinase 3	BC006725
GGCTTAAGTA	944	12	78	Anxa1, Annexin A1	NM_010730
CTCACAGTGG	808	12	67	RIKEN cDNA 1810009M01	BC019539
CTGCCCCCCG	1607	24	67	Saa3, Serum amyloid A 3	AK010675
GACCTGGAGC	4457	71	63	various ESTs	AK019465
TTGCTGCCTT	6889	131	52	various ESTs	AA172933
TTCTGGTTTG	617	12	51	various ESTs	R75163
AGAAGACAGA	1071	24	44	various ESTs	BY704822
TAACAGAAGC	499	12	41	Col5a2, Collagen, type V, alpha 2	AK084285
AATGTACAAG	499	12	41	Expressed sequence AA589382	BC021494
TGGGGGCCCC	481	12	40	Efemp2, EGF-containing fibulin-like extracellular matrix protein 2	BC012269
CAATAAAACT	2805	71	39	Ctla2a, Cytotoxic T lymphocyte-associated protein 2 alpha	AY034577
TAACAACCAA	445	12	37	Cfh, Complement protein H	AK087707
TGTAGGCTGT	853	24	35	various ESTs	AI428774
GGCTTTGGTC	4076	119	34	Rplp1, ribosomal protein, large, P1	AK010656
TGCACAGTGC	1643	48	34	S100a4, S100 calcium binding protein A4	X05835
TCAGGGTAGA	408	12	34	Tpm1, Tropomyosin 1 (alpha)	NM_024427
TTATTTATGA	408	12	34	Timp3, Tissue inhibitor of metalloproteinases 3	Z30970
TGTATAAAAA	390	12	32	Tra1, Tumor rejection antigen (gp96) 1	AK031918
TATTTTGTTT	390	12	32	Prnp, Prion protein	BC006703
GATACTTGGA	1552	48	32	various ESTs	BY386032
AACTTTTGTT	772	24	32	Serpinh1, serine proteinase inhibitor, clade H1	X60676
TTGGCTGGAT	381	12	31	Sdc4, Syndecan 4	NM_011521
CCAACGCTTT	1752	59	30	Fn1, Fibronectin 1	BE310012
TAACTGACAA	345	12	29	Mt2, Metallothionein-II	AK002567
CTGTAAAAAA	1017	36	28	Cxcl12, chemokine (C-X-C motif) ligand 12	BC029335
CACCTTGGTG	336	12	28	Riken clone C130020A02	AK081478
TTGTTGCTAC	2623	95	28	EST	C77421
ACAGTGTCGT	327	12	27	Tm4sf7, transmembrane 4 superfamily member 7	NM_053082
GCGCCGAAAG	327	12	27	RIKEN cDNA 3110001N18	BC026533
TCTGACTTCC	644	24	27	Bgn, Biglycan	NM_007542
AGGCTGACAA	1561	59	26	various ESTs	AA242461
AGGCAGACAG	12844	487	26	Eefa1, Eukaryotic translation elongation factor 1 alpha 1	X13661
AAAACATCTC	309	12	26	Nedd4, Neural precursor cell expressed, developmentally down-regulated gene 4	U96635
GCCACTTTGA	309	12	26	Hmox1, Heme oxygenase (decycling) 1	BC010757
TTTTTGATGA	300	12	25	various ESTs	BB157389
GACTGTGCCA	281	12	23	Dnclc1, Dynein, cytoplasmic, light chain 1	AK002522
ACACCAAAAA	263	12	22	Aebp1, AE binding protein 1	X80478
ATTTTCGAGG	263	12	22	Atp1b1, ATPase, Na+/K+ transporting, beta 1 polypeptide	X16646
GGACGCCCAA	263	12	22	Mmp14, Matrix metalloproteinase 14	X83536
AACATTCGCA	517	24	22	various ESTs	CB587655
TTGTGCTTCT	517	24	22	various ESTs	AA104526
ATCCTGGTAA	508	24	21	Cpe, Carboxypeptidase E	BC010197
AACATTCAAA	254	12	21	various ESTs	BB155332
TTCATTATAA	254	12	21	Ptma, Prothymosin alpha	X56135
GATTGTCAGA	245	12	20	Ier3, Immediate early response 3	BC006950
CATTCTTCCT	245	12	20	Raly, hnRNP-associated with lethal yellow	BC016587
TCTCTAGCTG	245	12	20	RIKEN cDNA 2810026P18	AK012825
GGGTGGCCCA	1933	95	20	various ESTs	CB589063

Table 4: Extracellular matrix components in cardiac fibroblasts

Tag sequence	Tag count (tpm)	Transcript	GenBank Acc. no.
		Collagens	
TGTTCATCTT	2260	Col3a1, Collagen, type III, alpha 1	AK019448
CGCCTGCTAG	1079	Col1a2, Collagen, type I, alpha 2	BC007158
CCCAATGGCC	635	Col6a2, Collagen, type VI, alpha 2	X65582
TAACAGAAGC	499	Col5a2, Collagen, type V, alpha 2	AK084285
GGGTTCCCCT	236	Col16a4, Collagen, type XVI, alpha 1	BC027766
TGAGAAAATG	118	Col4a5, Collagen, type IV, alpha 5	BC043317
GCTCTAGCCA	91	Col4a2, Collagen, type IV, alpha 2	X04647
GTGTCTGATA	64	Col4a1, Collagen, type IV, alpha 1	J04694
ACCTAGCCAC	45	Col5a1, Collagen, type V, alpha 1	AB009993
		Other matrix proteins	
CAAACTCTCA	2632	Sparc, Secreted acidic cysteine rich glycoprotein	X04017
CCAACGCTTT	1752	Fn1, Fibronectin 1	BE310012
TGGGGGCCCC	481	Efemp2, EGF-containing fibulin-like extracellular matrix protein 2	BC012269
GATTTTTCCA	363	Thbs1, Thrombospondin 1	BC042422
GGCCAGCTCT	109	Ecm1, Extracellular matrix protein 1	NM_007899
TTCCCGATCA	100	Sod3, Superoxide dismutase 3	BC010975
GGCGACGTGC	82	Lamb2, Laminin, beta 2	BC026051
TCAATGCTGT	73	Smoc2, Secreted modular calcium binding protein 2	NM_022315
GTGAAGGAAG	64	Lamb1-1, Laminin B1 subunit 1	M15525
TAGTTGGGCC	27	Lama4, Laminin, alpha 4	U59865
CAAACACCGT	27	Spp1, Secreted phosphoprotein 1 (osteopontin)	NM_009263
GTCATTCTCC	18	Tnc, Tenascin C	X56304
		Matrix metalloproteinases	
TGCTATGGCA	3068	Mmp3, Matrix metalloproteinase 3 (stromelysin)	BC006725
GGACGCCCAA	263	Mmp14, Matrix metalloproteinase 14	X83536
GGAAATGGCA	54	Mmp2, Matrix metalloproteinase 2 (gelatinase)	NM_008610
TGTGGTACGC	18	Mmp23, Matrix metalloproteinase 23	NM_011985
TGCACAAATA	9	Mmp7, Matrix metalloproteinase 7 (matrilysin)	NM_010810
		Tissue inhibitors of matrix metalloproteinases	
TTATTTATGA	408	Timp3, Tissue inhibitor of matrix metalloproteinase 3	Z30970
GAAAGCCTCT	145	Timp1, Tissue inhibitor of matrix metalloproteinase 1	BC008107
		Growth factors	
TTTGCACCTT	136	Ctgf, Connective tissue growth factor	BC006783
TCTCACCTCA	82	Vegfb, Vascular endothelial growth factor B	BC046303
TGCGACCTCC	73	Pdgfa, Platelet derived growth factor, alpha	BC003817
ATTGTGCGCT	45	Tgfb1, Transforming growth factor, beta 1	BC013738
CAAAACTATG	18	Vegfc, Vascular endothelial growth factor C	U73620
GATGACTTCA	18	Tgfb3, Transforming growth factor, beta 3	NM_009368
AGGATTTCCA	9	Hgf, Hepatocyte growth factor	X84046
GGGTGTATTA	9	Tgfb2, Transforming growth factor, beta 2	NM_009367

Table 5: Intracellular signalling components in cardiac fibroblasts

Tag sequence	Tag count (tpm)	Transcript	GenBank Acc. no.
		Protein kinases	
TGGAGCGTTG	378	Cdk4, Cyclin-dependent kinase 4	BC046336
TAACGCCCTT	189	Mapk3, Mitogen activated protein kinase 3 (erk-1)	NM_011952
GGAGGGATCA	162	Ilk, Integrin linked kinase	NM_010562
ATAGCTGGGC	135	Map2k1, Mitogen activated protein kinase kinase 1	NM_008927
GACTATGCGA	126	Nek6, NIMA-related serine/threonine kinase 6	AK004925
GCCAAACCAA	117	Clk, CDC-like kinase	NM_009905
CCGCCCTGCG	90	Stk11, Serine/threonine kinase 11 (Peutz-Jeghers syndrome)	AF129870
GTGCATCCAG	81	Csnk2b, Casein kinase 2, beta polypeptide	BC003775
CTTCACTCAC	81	Prkar1a, Protein kinase, cAMP-dependent, regulatory, type I, alpha	BC005697
CCAATTGTCC	27	Prkaca, Protein kinase, cAMP-dependent, catalytic, alpha	BC003238
		Protein phosphatases	
GAGGACCTGG	198	Ptprs, Protein tyrosine phosphatase receptor type S	BC052462
TGCTGAGAAT	180	Ppp1cc, Protein phosphatase 1	NM_013636
ACTGTAGATG	81	Ppp2ca, Protein phosphatase 2a	BC003856
ATGGCTTAAT	72	Ptp4a1, Protein tyrosine phosphatase 4a1	NM_011200
GCTCCTCCCC	63	Ppp5c, Protein phosphatase 5	BC003744
AAGTTTTCAG	36	Ppp4c, Protein phosphatase 4	NM_019674
GACCAAAAAA	36	Ppm1d, Protein phosphatase 1D magnesium-dependent	NM_016910
GAGATTCAGT	27	Ptpn12, Protein tyrosine phosphatase, non-receptor type 12	X63440
CGCGTATGAG	27	Dusp1, Dual specificity phosphatase 1	BC006967
GGTTAAAATA	27	Ppp2r5c, Protein phosphatase 2, regulatory subunit B	BC003979
		cGMP signalling	
CCGCACTGGA	54	Gkap42, 42 kD cGMP-dependent protein kinase anchoring protein	NM_019832
AACGAGTCTT	18	Prkg1, Protein kinase, cGMP-dependent, type I	AK052877
GTCACACCCA	18	Vasp, Vasodilator-stimulated phosphoprotein	BC015289
TTTTGTCATT	9	Gucy1a1, Guanylyl cyclase 1, soluble, $\alpha 1$	AK004815
TTAATTGTGT	9	Gucy1b1, Guanylyl cyclase 1, soluble, $\beta 1$	AF297083
CTCTTGGTCT	9	Pde1b, Phosphodiesterase 1B, calmodulin-dependent	L01695

Figure 1

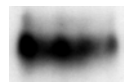
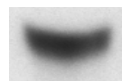
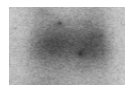
	tag count (tpm)	Northern Blot
Actin, gamma	2050	
Collagen Iα2	1079	
Sec61	245	
Interleukin 25	136	
Fibrillarin	108	

Figure 2

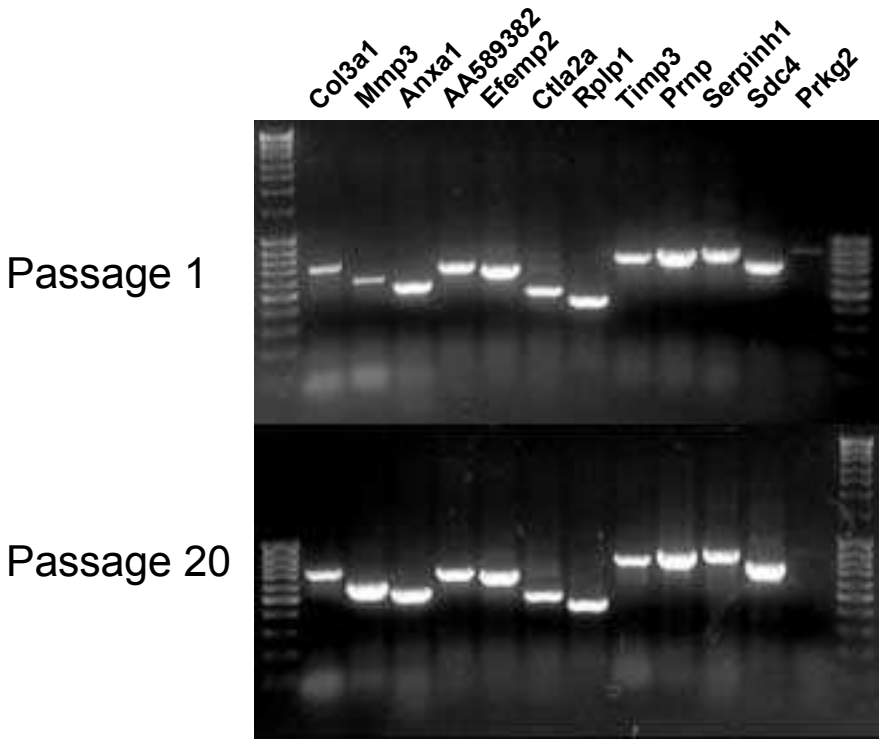
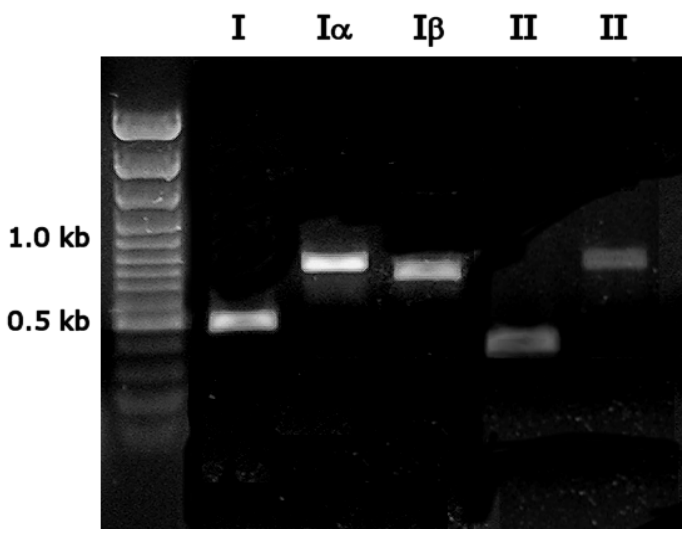

Col3a1
Mmp3
Anxa1
AA589382
Efemp2
Ctla2a
Rplp1
Timp3
Prnp
Serpinh1
Sdc4
Prkg2
Passage 1
Passage 20

Figure 3

A


I
Iα
Iβ
II
II
1.0 kb
0.5 kb

B

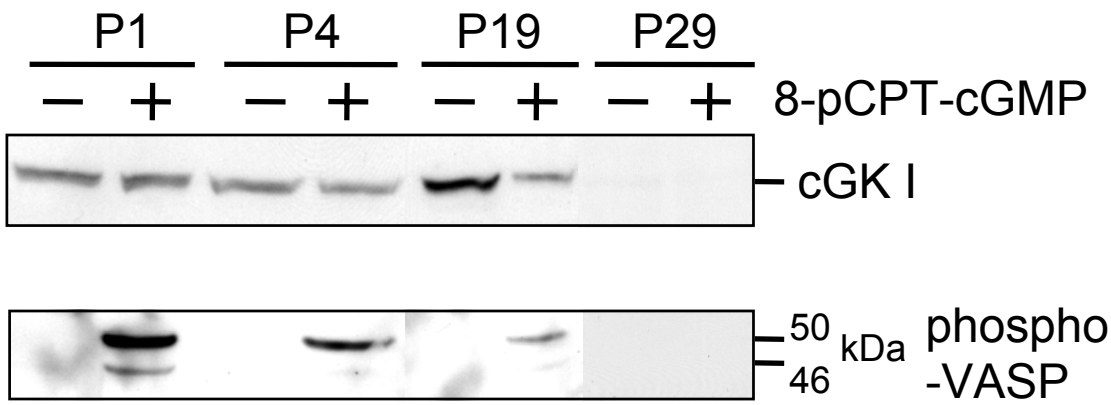
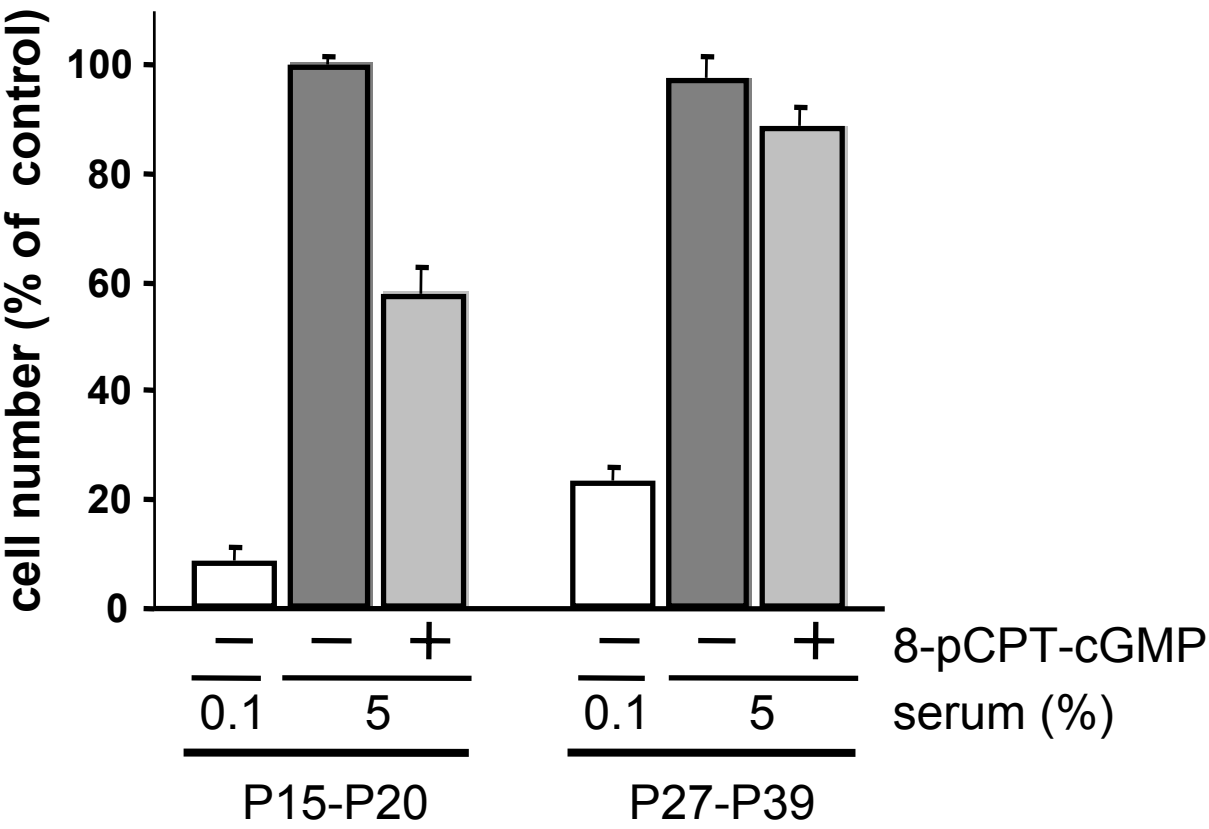

P1
P4
P19
P29
– + – + – + – +
8-pCPT-cGMP
cGK I
50
46
kDa
phospho
-VASP

Figure 4


cell number (% of control)
100
80
60
40
20
0
– – + – – +
8-pCPT-cGMP
0.1 5 0.1 5
serum (%)
P15-P20
P27-P39