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Differential Gene Expression of Rat Neonatal Heart Analyzed by Suppression Subtractive Hybridization and Expressed Sequence Tag Sequencing

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Abstract Heart diseases have been one of the major killers among the human population worldwide. Because the vast majority of cardiomyocytes cannot regenerate once they cease to proliferate shortly after birth, functionally significant myocardial regeneration is not observed clinically. Whether these cells are terminally differentiated and permanently withdrawn from the cell cycle is controversial, but broadening our understanding of the rapid switch from hyperplastic to hypertrophic growth of cardiomyocytes during neonatal myocardial development may shed light on novel cardiovascular therapies. By suppression subtractive hybridization (SSH) and expressed sequence tag (EST) sequencing, we analyzed the differential gene expression of rat neonatal heart. SSH yielded subtracted and normalized cDNA libraries and enhanced the probability of detecting ESTs, which represent genes pertinent to signal transduction/cell regulation and replication/transcription/translation machinery, as compared to the traditional EST sequencing of heart cDNA libraries. J. Cell. Biochem. 80:24–36, 2000.† © 2000 Wiley-Liss, Inc.

Key words: terminal differentiation; heart development; suppression subtractive hybridization; expressed sequence tags

Heart diseases have been one of the major killers among the human population worldwide. Functionally significant myocardial regeneration has not been documented in cardiovascular diseases and injuries that result in cardiomyocyte loss. Such clinical observation seems to support the notion that the vast majority of cardiomyocytes gradually cease proliferation on differentiation in neonatal myocardial development. However, the debate continues about whether or not cardiomyocytes are genuinely terminally differentiated and permanently withdrawn from the cell cycle shortly after birth in the mammalian heart [Anversa and Kajstura, 1998]. There is evidence demonstrating that cardiomyocytes maintain a low but measurable level of DNA replication beyond the perinatal period in humans [Quaini et al., 1994] and rats [Overy and Priest, 1966; Cheng et al., 1995]. Nevertheless, previous studies on cell number and cell volume documented that, during neonatal development in the rat, cardiomyocytes undergo a transition from hyperplastic to hypertrophic growth within the first 2 weeks after birth [Li et al., 1996]. Further increases in myocardial mass typically are not accompanied by cardiomyocyte proliferation [Soonpaa and Field, 1998]. Therefore, cardiomyocytes respond to growth, increased workload, and injury mainly by hypertrophy.

Currently, the exact mechanism that underlies this rapid transition from hyperplastic to hypertrophic growth of cardiac myocytes in the mammalian heart remains largely unknown [Olson and Srivastava, 1996; Anversa and Kajstura, 1998]. Regardless of the controversy about whether cardiomyocytes undergo terminal differentiation, the elucidation on how proliferation is gradually lost in these cells may shed light on novel therapies for cardiovascular injury or diseases, based on the regeneration of the adult myocardium. Here we present an analysis of the differential gene expression in

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day 1 and day 7 postnatal Sprague-Dawley rat ventricular myocardium, using the techniques of suppression subtractive hybridization (SSH) [Diatchenko et al., 1996, 1999] and expressed sequence tag (EST) sequencing.

Previously, we characterized gene expression in various developmental states of the cardiovascular system by high-throughput sequencing of randomly selected clones from human heart cDNA libraries to generate ESTs [Liew et al., 1994; Hwang et al., 1995; Hwang et al., 1997]. Many developmentally regulated genes in the human heart have been isolated and characterized [Tsui et al., 1994; Chan et al., 1998; Luk et al., 1998; Kotaka et al., 1999]. With SSH, we have recently reported the down-regulated mRNA expression of the 40-kDa subunit of replication factor C, an essential processive factor for DNA replication, as the myocardium differentiates [Chim et al., 2000]. To analyze the differential gene expression more efficiently, we have coupled high-throughput EST sequencing with SSH. The advantages are multifold.

SSH is a highly effective method for generating subtracted cDNA libraries [Diatchenko et al., 1996, 1999]. It dramatically increases the probability of obtaining low-abundance differentially expressed cDNAs and simplifies the analysis of the subtracted cDNA libraries. Moreover, SSH is a more rapid and reliable method than differential display for the detection of differentially expressed mRNAs [Bertram et al., 1998]. However, it is difficult to deal with low-abundance transcripts in the subtracted libraries, because this type of transcript usually does not give a hybridization signal that is strong enough for detection in differential screening or Northern blot analysis. With the advance in DNA sequencing technology, we found that large-scale EST sequencing could be coupled with SSH as a tool for analyzing the gene profiles in subtracted libraries. In this report, we have compared the composition of our subtracted cDNA libraries with an unsubtracted heart cDNA library in terms of the most abundant ESTs and the EST distribution by functional categories. Also, we have validated the differential expression pattern of the neonatal heart compiled by our research method against the current literature on myocardial development and differentiation.

MATERIALS AND METHODS

Experimental Animals and Sampling

Day 1 and day 7 postnatal Sprague-Dawley rats were obtained from the animal facilities of our university. Hearts were carefully dissected from decapitated animals and were rinsed in phosphate-buffered saline. Only the lower two thirds of the myocardium, which contains mostly ventricular cells, was used for RNA isolation.

Total RNA and Polyadenylated RNA Isolation

Total RNA was extracted from rat ventricular myocardium of different ages using TRIZOL reagents (Life Technologies, Gaithersburg, MD), and then polyadenylated RNA was isolated using PolyAttract mRNA Isolation System (Promega, Madison, WI), according to the manufacturer's specifications. The quantity and quality of each sample were determined spectrophotometrically by A_{260} and $A_{260/280}$ ratio, and checked by electrophoresis on a 1.2% agarose/formaldehyde gel.

SSH (Forward- and Reverse-Subtraction)

SSH was performed using PCR-Select cDNA Subtraction Kit (Clontech, Palo Alto, CA) according to manufacturer's protocol. We used day 1 rat ventricular myocardium cDNA as tester and day 7 cDNA as driver for forward-subtraction to identify day 1-dominating cDNA, and vice versa for reverse-subtraction to identify day 7-dominating cDNA. Unsubtracted control for each subtraction was prepared by the same procedures but without the subtractive hybridization step.

Evaluation of Subtraction Efficiency

After two rounds of suppression polymerase chain reaction (PCR) in the subtraction procedures, the subtracted cDNA products were then subjected to 15–30 cycles of PCR amplification of glyceraldehyde-3-phosphate dehydrogenase (G3PDH) and β-actin cDNAs with specific primers. Unsubtracted controls were amplified in parallel.

Construction of Subtracted cDNA Libraries and High-Throughput EST Sequencing

Forward- and reverse-subtracted cDNAs were TA-cloned into pT-Adv vectors (Clontech) and transformed into the *Escherichia coli*

strain DH5α. Subtracted day 1 cDNA library was constructed from forward-subtracted products, in which day 1 rat ventricular myocardium cDNA was used as the tester and day 7 cDNA as the driver for identification of the day 1-dominating genes, and vice versa for subtracted day 7 cDNA library. Batches of random single colonies were inoculated in 100 μl of LB-ampicillin medium on 96-well microtiter plates and grown overnight at 37°C, 250 rpm. Two microliters of the bacterial culture was subjected to PCR amplification of the cDNA inserts using flanking primers (forward: 5′-TCGAGCGGCCGCCCGGGCAGGT-3′; reverse: 5′-AGCGGGTGGTCGCGGCCGAGGT-3′) and cDNA Polymerase Mix (Clontech) built-in with automatic hot start. Two microliters of the PCR product was then used as a DNA template for high-throughput sequencing by DYEnamic ET dye terminator cycle sequencing kit (Amersham Pharmacia Biotech, Buckinghamshire, England). Partial sequences of cDNA clones were acquired by MegaBACE 1000 automated DNA sequencer (Molecular Dynamics, Sunnyvale, CA).

Data Handling

Sequence comparison against all sequences in the nonredundant database (February 21, 2000) at the National Center for Biotechnology Information (NCBI) was performed using the BLAST server [Altschul et al., 1997]. Assignment of putative identities for ESTs matching to known genes required a minimum *P* value of 10^{-10}.

Differential Screening by Subtracted cDNA Probe

To quickly examine the composition of the cDNA libraries, differential screening was performed by colony hybridization using subtracted day 1 (forward-subtracted) and subtracted day 7 (reverse-subtracted) probes, which were *Rsa*I-restricted subtractive-hybridized cDNA. Briefly, a total of 751 randomly selected colonies (389 subtracted day 1 clones, 362 subtracted day 7 clones) were double-spotted identically on two 8-cm × 12-cm Immobilon-N PVDF membranes (Millipore, Bedford, MA), and hybridized at 72°C overnight with either the [α-^{32}P] dCTP random-primed labeled subtracted day 1 or day 7 probe in ExpressHyb Hybridization Solution (Clontech), then washed several times until a final stringency of 68°C in 0.2× SSC, 0.5% sodium dodecyl sulfate was achieved. These membranes were then exposed to autoradiography film at −80°C with two intensifying screens for different exposure time, ranging from 4 days to 2 weeks, to accommodate for both abundant and rare gene transcripts.

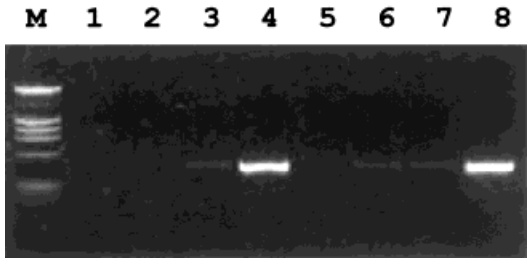

Fig. 1. Evaluation of subtraction efficiency of subtracted cDNAs. After two rounds of suppression polymerase chain reaction (PCR), the subtracted products were subjected to PCR amplification of glyceraldehyde-3-phosphate dehydrogenase (G3PDH). Only barely detectable G3PDH levels were found in the subtracted day 1 (**lanes 1, 2**) and subtracted day 7 (**lanes 5, 6**) products. Significantly higher G3PDH levels were detected in the unsubtracted day 1 (**lanes 3, 4**) and unsubtracted day 7 (**lanes 7, 8**) controls. Odd lanes: 15 PCR cycles. Even lanes: 30 PCR cycles. M: λ DNA-*Hind* III/ϕX174 DNA-*Hae* III DNA marker.

RESULTS

Only a trace amount of G3PDH (Fig. 1) and β-actin (data not shown) could be amplified from the subtracted products. This provides essential, although not sufficient, evidence that the SSH procedure successfully suppressed the cDNAs common to both day 1 and day 7 in the rat ventricular myocardium.

The differential screening of 389 subtracted day 1 clones and 362 subtracted day 7 clones by colony hybridization split the composition of SSH-produced clones into three groups (Fig. 2). The first group of 83 (21.3%) subtracted day 1 clones and 90 (24.9%) subtracted day 7 clones hybridized exclusively with the subtracted day 1 probe or the subtracted day 7 probe, respectively. Besides these "all-or-none" clones, another group of 44 (11.3%) subtracted day 1 clones and 54 (14.9%) subtracted day 7 clones showed substantially stronger signal with their respective subtracted probes, yet approximately 34.5% of the 751 clones showed no detectable hybridization signal for up to 2 weeks of prolonged exposure.

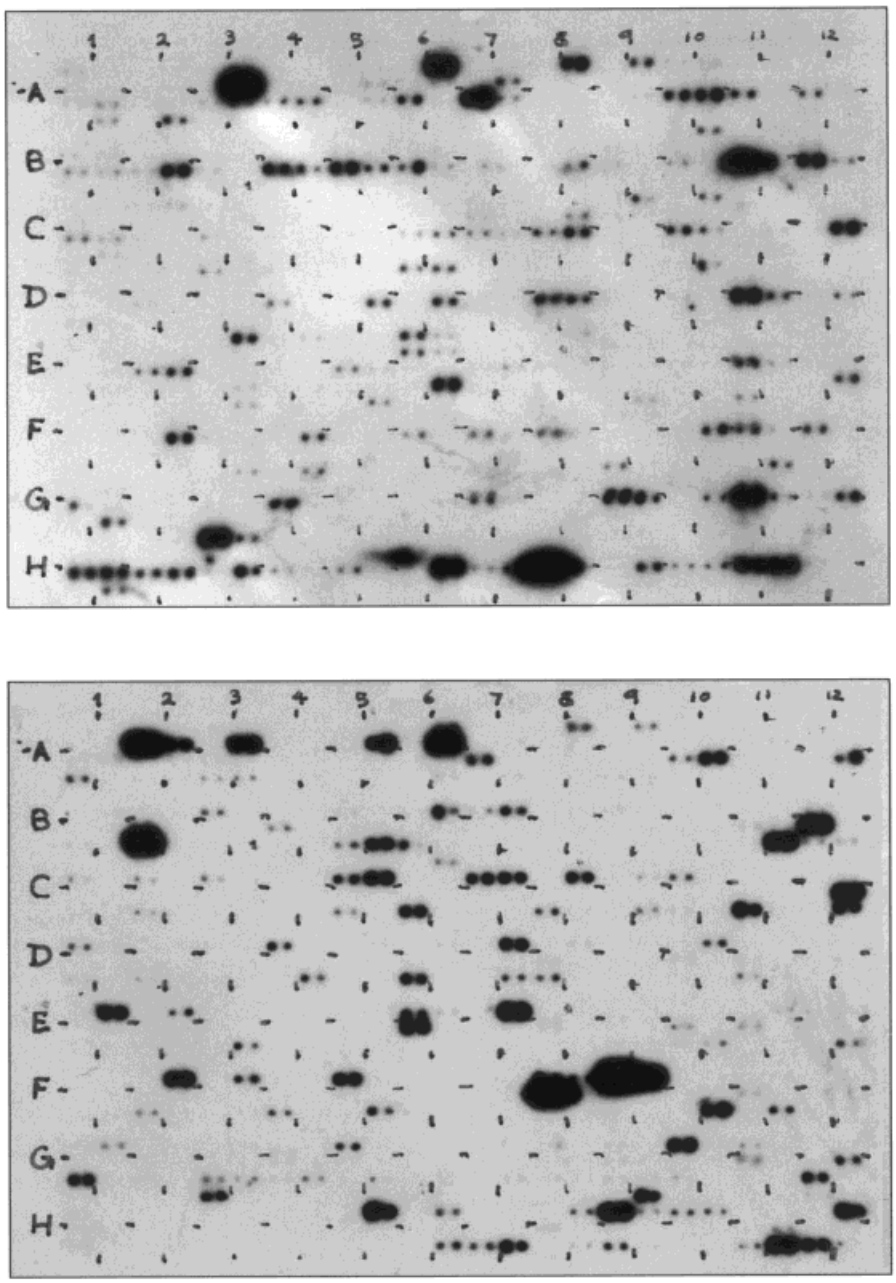

Fig. 2. Colony hybridization of subtracted clones with subtracted day 1 (upper panel) or subtracted day 7 (lower panel) probes. Each clone was double-spotted on two identical membranes. In each 4 × 4 matrix (e.g. A10, D7, etc.), rows 1 and 3 were double-spotted with four subtracted day 1 clones. Rows 2 and 4 mostly contained subtracted day 7 clones, but were embedded with subtracted day 1 clones on dots 3 and 4 of row 4 in each of the 4×4 matrices A1, B1, C1, D1, E1, F1, G1, H1, A2, B2 and E6.

To date, 454 and 493 ESTs have been obtained from the subtracted day 1 and day 7 cDNA libraries, respectively. Of the 947 ESTs presented here, 146 (15.4%) were unmatched to any entries in the nonredundant database at NCBI by BLAST search and hence represented novel, previously uncharacterized genes (Table I). Two hundred ninety-four (31.0%) ESTs matched to other ESTs in the database of ESTs, although not with any genes of known sequence or function. The remaining 507 (53.5%) ESTs matched to and represented 178 different nuclear genes, as well as mitochondrial transcripts (4.0%). Clones corresponding to known genes (excluding mitochondrial transcripts) were classified into nine broad categories according to function (Table II), representing contractile proteins, cytoskeletal or structural proteins, extracellular matrix proteins, proteins involved in energy metabolism, proteins involved in signal transduction or cell regulation, heat shock proteins, proteins involved in basic

TABLE I. Summary of Expressed Sequence Tags (ESTs) from Subtracted Day 1 and Subtracted Day 7 Rat Heart cDNA Libraries (Data Are Numbers of ESTs, with Percentages in Parentheses)

	Subtracted day 1 library	Subtracted day 7 library	Total
No database match	60 (13.2%)	86 (17.4%)	146 (15.4%)
Match only with ESTs	142 (31.3%)	152 (30.8%)	294 (31.0%)
Match with known genes	252 (55.5%)	255 (51.7%)	507 (53.5%)
Total	454 (100.0%)	493 (100.0%)	947 (100.0%)

replication, transcription or translation machinery, membrane-associated proteins, and proteins involved in other aspects of cellular metabolism. Gene profiles of the unsubtracted normal human adult heart [Hwang et al., 1997] and the subtracted day 1 and day 7 rat heart cDNA libraries were compared with each other by comparing the proportion of transcripts in each of the nine functional categories (Table III). Most notably, the percentage of transcripts representing contractile proteins and extracellular matrix proteins were much lower in the subtracted libraries than in the normal adult heart library, whereas the proportions of transcripts representing signal transduction or cell regulatory proteins and basic replication, transcription, or translation machinery were relatively higher in the subtracted libraries than in the normal adult heart library. To assess the efficiency of subtraction, the numbers of ESTs representing abundant genes in normal heart [Hwang et al., 1997] were analyzed (Table IV). Notably, 6 of 12 abundant transcripts in unsubtracted normal heart have been completely eliminated from the subtracted libraries, whereas the other six transcripts may represent genuine change in expression level.

Among the 502 unique ESTs that we have identified, 470 (93.63%) ESTs appeared exclusively in subtracted day 1 library but not in the day 7 library or vice versa, whereas a subset of 139 (27.69%) within them appear more than once.

DISCUSSION

Based on suppression PCR, SSH dramatically increases the probability of obtaining low-abundance differentially expressed cDNAs [Diatchenko et al., 1996, 1999]. However, the normalization feature of SSH results in many low-abundance transcripts, which pose difficulties for detection by differential screening or Northern blot analysis. As in our case, 34.5% of the 751 SSH-generated clones showed no detectable hybridization signals in differential screening up to 2 weeks of prolonged exposure. To increase the sensitivity of differential screening, we had already used subtracted cDNA probes of reduced probe complexity compared to typical cDNA probes. Had we used typical cDNA probes, more cDNA clones would become undetectable. Unlike hybridization-based methods, high-throughput EST sequencing enabled us to analyze the SSH-generated cDNAs regardless of their expression level. Not only did sequencing assign putative identities to the cDNAs, but their relative abundance in the subtractive mixture subjected to library construction could be estimated if enough clones were sampled.

As in other normalized libraries, the frequency of appearance of a certain EST in SSH-generated libraries does not correlate with its expression level, because the cDNA populations have been distorted. Nevertheless, in cases where a certain EST is found exclusively only in the subtracted day 1 library but not in the subtracted day 7 library or vice versa, such distribution pattern implies the prevalent expression of that EST in the tester tissue relative to the driver tissue in the subtraction. Of course, the above contention requires the corresponding effective elimination of the transcripts that are common to the tester and the driver cDNA populations in both the forward- and reverse-subtraction. This presumption on efficient subtraction is validated by the undetectable amplification of housekeeping genes (Fig. 1) and the complete elimination of 6 of the 12 most abundant transcripts in unsubtracted normal heart from our subtracted heart cDNA libraries (Table IV). Because 470 (93.63%) of the 502 unique ESTs that we have identified appeared exclusively in either subtracted library,

TABLE II. Expressed Sequence Tags (ESTs) from Subtracted Libraries Matching to Known Genes (Number in Parentheses Indicates the Frequency of Appearance of the EST. ESTs that Appeared in Both Libraries Are Italicized)

Day 1-dominating genes	Day 7-dominating genes
Contractile elements [33 ESTs, 7 genes]	
Troponin T (10)	*Troponin T (10)*
Actins (3)	Myosin light chain 2, ventricular (3)
Sarcoplasmic reticulum Ca^{2+}-ATPase (3)	*Sarcoplasmic reticulum Ca^{2+}-ATPase (1)*
Myosin heavy chain, alpha (1)	
Myosin binding protein C (1)	
Troponin I (1)	
Cytoskeletal related [59 ESTs, 18 genes]	
Crystallin, alpha B (7)	Actinin, alpha (4)
Titin (7)	*Crystallin, alpha B (3)*
Microtubule-associated protein 4 (5)	Arp2/3 protein complex subunit p16 (3)
Tubulin, alpha (5)	*Cis-Golgi p28 (2)*
Clathrin heavy chain (4)	Kinesin heavy chain (2)
Cis-Golgi p28 (1)	*Tubulin, alpha (2)*
b-Nexilin (2)	*Clathrin heavy chain (1)*
s-Nexilin (2)	M-protein (1)
TEB4 protein (1)	*b-Nexilin (1)*
Tubulin, beta (1)	Sid23p (1)
Tubulin-folding cofactor E (1)	Smoothelin large isoform L2 (1)
Tubulin-specific chaperone e (2)	
Extracellular matrix [7 ESTs, 5 genes]	
Collagen, type V alpha 2 (1)	Collagen, type III alpha 1 (2)
Collagen, type XV (1)	Collagen, type III pro alpha 1 (2)
Protocadherin 2 (1)	
Energy metabolism [60 ESTs, 28 genes]	
Very-long-chain acyl-CoA dehydrogenase (4)	*Triosephosphate isomerase (8)*
ATP synthase subunit c (3)	Beta beta enolase (6)
Cytochrome C oxidase subunit VII homologue (3)	L-3-hydroxyacyl-CoA dehydrogenase (4)
Triosephosphate isomerase (3)	2,4-Dienoyl-CoA reductase (3)
Adenine nucleotide translocator (2)	Electron-transferring-flavoprotein dehydrogenase (3)
Oxoglutarate dehydrogenase (2)	
Aconitase (1)	F1-ATPase alpha subunit (2)
Acyl-CoA synthetase 5 (1)	Acetoacetyl-CoA thiolase (1)
Cytochrome bc-1 complex core P (1)	Aldolase A (1)
Cytosolic malate dehydrogenase (1)	ATP synthase beta subunit (1)
Long-chain acyl-CoA dehydrogenase (1)	ATPase inhibitor gene (1)
NADH dehydrogenase Fe-S protein 2 (1)	Lactate dehydrogenase (1)
NADH dehydrogenase flavoprotein 1 (1)	
Pyruvate dehydrogenase kinase isoenzyme 4 (1)	
Succinyl-CoA synthetase beta subunit (1)	
Succinate dehydrogenase Ip subunit (1)	
Proteolipid 68MP homologue (1)	
Proton/phosphate symporter (1)	
Signal transduction/cell regulation [109 ESTs, 43 genes]	
Protein phosphatase type 1 alpha, catalytic subunit (5)	Calcyclin binding protein (27)
G protein beta 5 subunit (3)	Protein kinase MUK (7)
Peptidylglycine alpha-amidating monooxygenase (3)	cAMP-dependent protein kinase type I, regulatory subunit (5)
Dual-specificity phosphatase (2)	
G protein-coupled receptor LGR4 (2)	Cyclin H (4)
Golgin-245 (2)	BCL2-associated athanogene 5 (3)

"Table II continues on next page"

TABLE II. Expressed Sequence Tags (ESTs) from Subtracted Libraries Matching to Known Genes (Number in Parentheses Indicates the Frequency of Appearance of the EST. ESTs that Appeared in Both Libraries Are Italicized) (continued)

Day 1-dominating genes	Day 7-dominating genes
Herc2 (2)	*Heparin-binding growth-associated molecule (3)*
Protein phosphatase 1, glycogen-binding regulatory subunit (2)	Guanine nucleotide-releasing protein
Protein-kinase inhibitor PRKRI (2)	Mss4 (2)
Receptor for activated protein kinase C (2)	Retinoblastoma-associated protein RAP140 (2)
Serum and glucocorticoid-regulated kinase sgk (2)	Sec7B (2)
ADP-ribosylation factor 1 (1)	Cell immortalization-related clone CIR2 (1)
ADP-ribosylation factor 2 (1)	Leucine-rich repeat interacting protein 1 (1)
Caspase 7 (1)	Phosphatidylinositol transfer protein (1)
CDP-diacylglycerol synthase (1)	Protein kinase B kinase (1)
c-HA-ras protooncogene (1)	*Protein phosphatase 1, glycogen-binding regulatory subunit (1)*
DEK oncogene (1)	RAB7 (1)
Fibroblast growth factor inducible 13 (1)	Reduced expression 3 Rex3 (1)
Fibroblast growth factor inducible 14 (1)	
Glypican 4 (1)	
Heparin-binding growth-associated molecule (1)	
Inhibitor of growth 1-like (1)	
Leucine-rich repeat interacting protein 1 (1)	
Lissencephaly-1 protein (1)	
Protein tyrosine phosphatase (1)	
Phospholipase C-L2 (1)	
Protein kinase C delta subspecies (1)	
Sec61 homologue (1)	
TIS 11 (1)	
Tousled-like kinase (1)	
Replication/transcription/translation [113 ESTs, 37 genes]	
U5 snRNP-specific protein (8)	Elongation factor 1 gamma (23)
Ribosomal protein L9 (6)	H3 histone, family 3B (8)
GATA-6 (5)	Ribosomal protein L21 (6)
Elongation factor 1 alpha (5)	DEAD box polypeptide 5 (3)
Spliceosomal protein SAP 155 (5)	Translational regulatory factor alpha (3)
Polypeptide chain release factor CI1 (3)	Zinc finger protein (3)
MCM6 (2)	Bone morphogenetic protein-6 (2)
Replication factor C subunit 2 (2)	General transcription factor IIE 34 kDa subunit (2)
Calnexin (1)	*Elongation factor 1 alpha (1)*
Ser/Arg-related nuclear matrix protein (2)	hnRNP protein (1)
SP120 (2)	*Nucleosome assembly protein 1 like 4 (1)*
Chromosomal protein HMG2 (1)	RAD23B (1)
dHAND (1)	*Ribosomal protein L9 (1)*
Elongation factor 1 beta (1)	Ribosomal protein S5 (1)
Histone acetyltransferase querkopf (1)	
LFB1/HNF1 promoter (1)	
Methyl-CpG-binding protein 2 (1)	
Nucleosome assembly protein 1 like 4 (1)	
Poly(A) polymerase V (1)	
Poly(A) polymerase VI (1)	
Ribosomal protein S21 (1)	
RNA helicase (1)	
RNA polymerase II transcriptional coactivator (1)	
Scaffold attachment factor A (1)	
Splicing factor SFRS11 (1)	
U6 snRNA-associated Sm-like protein LSm3 (1)	

"Table II continues on next page"

TABLE II. Expressed Sequence Tags (ESTs) from Subtracted Libraries Matching to Known Genes (Number in Parentheses Indicates the Frequency of Appearance of the EST. ESTs that Appeared in Both Libraries Are Italicized) (continued)

Day 1-dominating genes	Day 7-dominating genes
Membrane associated [57 ESTs, 18 genes]	
Mitofilin (4)	ART-4 (18)
Na^+, K^+-ATPase alpha-subunit (2)	Integral membrane protein CII-3 (7)
CD36 (1)	Tim17 (7)
Channel integral membrane protein 28 (1)	Voltage-dependent anion channel 2 (4)
MRC OX-45 surface antigen (1)	CD34 (2)
Outer membrane protein OMP25 (1)	Nicotinamide nucleotide transhydrogenase (2)
Sodium-dependent multivitamin transporter (1)	SCO homolog 1 (2)
	Chemokine receptor LCR1 (1)
	Membrane protein TMS-2 (1)
	Solute carrier family 12, member 7 (1)
	Surfactant protein-A (1)
Heat shock protein [5 ESTs, 3 genes]	
Heat shock protein, HSP86 (2)	Heat shock factor 2 (2)
Heat shock protein, 70 kDa (1)	
Other Metabolism [29 ESTs, 17 genes]	
Galactosyltransferase-associated kinase (5)	Cathepsin L (3)
Cystatin, beta (2)	Porphobilinogen deaminase (2)
Lysyl tRNA synthetase (2)	*3 Beta-hydroxysteroid dehydrogenase isomerase (1)*
Proteasomal ATPase (2)	Calpain small subunit (1)
3 Beta-hydroxysteroid dehydrogenase isomerase (1)	*Lysyl tRNA synthetase (1)*
Glycyl tRNA synthetase (1)	
Proteinase-3 (1)	
Ribonuclease P protein subunit p29 (1)	
Ribonucleotide reductase subunit M1 (1)	
RNase L inhibitor (1)	
Ubiquitin C-terminal hydrolase UCH37 (1)	
Ubiquitin C-terminal hydrolase UCH-L5 (1)	
Ubiquitin protein ligase Nedd-4 (1)	
Uracil-DNA glycosylase (1)	
Miscellaneous [35 ESTs]	

they warranted further investigation for differential expression pattern by literature search.

SSH saved us substantial sequencing efforts with the more abundant transcripts commonly expressed in both day 1 and day 7 rat ventricular myocardium. Compared with the unsubtracted normal heart cDNA library, we found that in our subtracted libraries, the ESTs representing genes involved in contractile elements showed 58.4% and 71.7% reduction in the subtracted day 1 and day 7 libraries, respectively (Table III). Similarly, those proteins involved in extracellular matrix also decreased by 86.0% and 82.8%, respectively. Contractile elements and extracellular matrix are involved mostly with the pumping action but not so much with differentiation and development of the heart. Because many of these genes are commonly expressed in both developmental stages, SSH effectively reduced the appearance of ESTs that fall into these two functional categories. This further adds to the body of evidence that SSH was successfully performed in our hands.

Moreover, the probability of identifying rare but important transcripts related to myocardial development was enhanced after the elimination of these abundant and commonly expressed transcripts. Most strikingly, the category of ESTs representing genes pertinent to signal transduction or cell regulation increased by 79.6% and 127% in the subtracted day 1 and day 7 libraries, respectively (Table III). Also, by a quick perusal of the gene profiles of the ESTs in our subtracted libraries (Table II), we found that the category of genes related to basic replication, transcription, or translation machinery showed a very rich va-

TABLE III. Frequency Distribution of Expressed Sequence Tags (ESTs) from Unsubtracted Normal Human Adult Heart, Subtracted Day 1 and Subtracted Day 7 Rat Heart cDNA Libraries by Functional Categories (Data Are Percentages of ESTs in Each Category, with the Actual Number of ESTs Presented in Parentheses)*

Category	Normal heart library	Subtracted day 1 library	Subtracted day 7 library
Contractile elements	20.2% (232)	8.4% (19)	5.7% (14)
Cytoskeleton related	11.0% (126)	16.7% (38)	8.6% (21)
Extracellular matrix	9.3% (107)	1.3% (3)	1.6% (4)
Energy metabolism	13.3% (153)	12.8% (29)	12.7% (31)
Signal transduction/cell regulation	11.3% (130)	20.3% (46)	25.7% (63)
Replication/transcription/translation	19.9% (229)	25.1% (57)	22.9% (56)
Membrane associated	7.4% (85)	4.8% (11)	18.8% (46)
Heat shock proteins	2.3% (26)	1.3% (3)	0.8% (2)
Other metabolism	5.2% (60)	9.3% (21)	3.3% (8)
Total	100.0% (1,148)	100.0% (227)	100.0% (245)

*Normal human adult heart data from Hwang et al. [1997].

TABLE IV. Most Abundant ESTs in Unsubtracted Normal Human Heart and Their Distribution in Subtracted Rat Heart cDNA Libraries. (Data are Percentages of ESTs Representing Each Gene, with the Actual Number of ESTs Presented in Parentheses)*

Gene	Normal heart library (total 5,164 clones)	Subtracted day 1 library (total 454 clones)	Subtracted day 7 library (total 493 clones)
Myosin heavy chain, cardia beta	1.491% (77)	0.000% (0)	0.000% (0)
Actin, cardia alpha	0.639% (33)	0.220% (1)	0.000% (0)
Elongation factor 1, alpha	0.426% (22)	1.101% (5)	0.203% (1)
Collagen, type I alpha 1	0.407% (21)	0.000% (0)	0.000% (0)
Tropomyosin, skeletal alpha	0.349% (18)	0.000% (0)	0.000% (0)
Cardiodilatation atrial natriuretic factor	0.349% (18)	0.000% (0)	0.000% (0)
Glyceraldehyde-3-phosphate dehydrogenase	0.329% (17)	0.000% (0)	0.000% (0)
Tubulin, beta	0.271% (14)	0.220% (1)	0.000% (0)
Troponin T, cardiac	0.252% (13)	2.203% (10)	2.028% (10)
Tubulin, alpha	0.213% (11)	1.101% (5)	0.406% (2)
Pyruvate kinase, M-2	0.194% (10)	0.000% (0)	0.000% (0)
Myosin light chain 2	0.194% (10)	0.000% (0)	0.609% (3)

*Normal human adult heart data from Hwang et al. [1997].

riety. This is in line with the thought that critical factors for myocardial differentiation are probably signal transduction proteins or proteins related to gene transcription and DNA replication, as implied by the seemingly intact growth factor receptors in differentiated cardiomyocytes with up-regulation of immediate early response genes, like c-myc, in pressure overload-induced cardiac hypertrophy (Izumo et al., 1988).

While Northern blot analyses and reverse transcription-polymerase chain reaction were under way to confirm the differential expression of the genes that we have obtained, we have searched the literature to support the differential expression of at least some of the known genes and to set up our priorities of investigation among the considerable number of candidates. In our following discussion, the frequencies of appearance of the gene are listed as [p, q], where p is the frequency of appearance in the subtracted day 1 library (total 454 ESTs), and q is that for the subtracted day 7 library (total 493 ESTs).

ESTABLISHED MARKERS OF HEART DEVELOPMENT & DIFFERENTIATION

The exclusive appearance of GATA-6 [5, 0] in the subtracted day 1 library implies its preva-

lent expression in day 1 (tester) compared to day 7 (driver) rat myocardium. This implication is supported by previous studies, which showed that GATA-6 expression declined before terminal differentiation, as defined by the expression of cardiac actin and heart-specific myosin light chain, and that overexpression of GATA-6 blocks differentiation of the heart in *Xenopus* [Gove et al., 1997]. The reduction of GATA-6 was demonstrated to be important for the progression of cardiomyogenic differentiation program, and that it may act to maintain heart cells in the precursor state.

Similarly, the helix-loop-helix protein, dHAND, was exclusively found in the subtracted day 1 library [1, 0]. During mouse embryogenesis, dHAND was first detected in the lateral mesoderm of day E7.75 embryos, expressed throughout the developing heart by E8.5, barely detectable in heart by E13.5, and became undetectable within the embryo on E16 by the method of in situ hybridization [Srivastava et al., 1995]. In rat, by SSH, we were still able to detect dHAND on the day 1 myocardium as a rare transcript.

Ventricular myosin light chain 2 (MLC2V) is the earliest known marker of vertebrate ventricular muscle cell lineages [O'Brien et al., 1993]. MLC2V displays segmental expression only in ventricular myocytes. Although it is more of a marker for lineage diversification than differentiation, it supports the implication that MLC2V [0, 3] becomes more prevalent as the ventricular myocardium differentiates.

PROTEINS RELATED TO THE CELL CYCLE

By virtue of mitotic figures, thymidine incorporation, bromodeoxyuridine and proliferating cell nuclear antigen (PCNA) immunochemistry, adult mammalian cardiac myocytes were thought to be incapable of proliferation, because the vast majority of them gradually cease to undergo DNA replication, which is essential for proliferation, during neonatal development. In the rat, this occurs within the first 2 weeks after birth [Clubb and Bishop, 1984; Li et al., 1996].

Recently, by SSH, we reported that the mRNA level of the 40-kDa subunit of replication factor C (RFC2), which is an essential processive factor for PCNA-dependent DNA replication, is downregulated during this time in the myocardium [Chim et al., 2000]. Consistent with the Northern blot analysis, RFC2 appeared exclusively in the subtracted day 1 library [3, 0]. Similarly, the exclusive appearance of MCM6, the subunit of a DNA replication licensing factor, in the subtracted day 1 library [2, 0], was also in line with this cessation of DNA replication during myocardial differentiation. Given the downregulated expression of these essential factors for DNA replication, it is not surprising that the promotion of G1 CDK activities by serum stimulation in rat neonatal cardiac myocytes resulted in no induction of DNA synthesis [Tamamori et al., 1998].

In addition, our current study also suggests the downregulated expression of other cell cycle-related proteins, because most cardiomyocytes stop proliferation and exit the cell cycle. In neonatal rat cardiomyocytes, the activity of cdc2 and cyclin-dependent kinase 2 (CDK2) were dramatically downregulated [Kang and Koh, 1997], whereas the CDK inhibitor p21 was induced [Flink et al., 1998] 2–3 days after birth. A spliceosomal-associated protein, SAP155, which was exclusively found in the subtracted day 1 library [5, 0], was the substrate for cyclin E-cdk2 phosphorylation, which is inhibited by p21 [Seghezzi et al., 1998].

Protein phosphatase type 1 (PP-1) mainly localizes in the cytoplasm of G1- and S-phase cells, accumulates in the nucleus during G2 phase, and intensely colocalizes with individual chromosomes at mitoses [Fernandez et al., 1992]. It was also shown to bind with the tumor-suppressor retinoblastoma (Rb) protein from mitosis to early G1 phase, and that raises the possibility that it may serve the role to dephosphorylate Rb [Durfee et al., 1993]. PP-1's exclusive appearance in the subtracted day 1 library [5, 0] and the implied cessation during myocardial development are consistent with its roles as revealed by these previous studies.

CHROMOSOME-RELATED PROTEINS

Histones are dynamically modified during chromatin assembly, as specific transcriptional patterns are established, and during mitosis and development. Replacement histones were recently found to accumulate in terminally differentiated cells [Bramlage et al., 1997]. By implication of its exclusive appearance in the subtracted day 7 library [0, 8], replacement histone H3.3B predominates in the day 7 rat myocardium relative to day 1.

Also, the high mobility group (HMG) chromosomal proteins may modulate the structure of distinct region in chromatin, affecting development and differentiation. During erythropoiesis in the chicken, mRNAs for HMG decrease as the cells differentiate [Crippa et al., 1991]. By implication, HMG2 [1, 0] mRNA also drops as the myocardium differentiates. Regardless of the different tissues, their differentiation may share similar mechanism to silence certain parts of the chromatin.

Moreover, our SSH-generated libraries also trapped methyl-CpG-binding protein 2 [1, 0], which is a repressor protein that binds to matrix attachment regions, interacts with mSin3A, and recruits a corepressor complex containing histone deacetylases. This in turn is thought to generate a localized silencing chromatin structure [Stratling and Yu, 1999]. Furthermore, a MYST family histone acetyltransferase, Querkopf, was trapped [1, 0]. At least some of the MYST family proteins were known to be involved in transcriptional silencing [Iizuka and Stillman, 1999; Takechi and Nakayama, 1999].

GROWTH FACTORS

Originally identified by the ability of a demineralized bone extract to induce endochondral osteogenesis in vivo, bone morphogenetic protein 6 (BMP6), is a member of the transforming growth factor beta (TGF-beta) superfamily of regulatory molecules, and is expressed in the heart. While the roles of BMP6 in the developing heart awaits further investigation, the elaborate regulation of the TGF-beta family members during embryonic development of the heart, the upregulation of TGF-beta after hemodynamic stress, and the impact of TGF-beta on cardiac gene expression together imply a prominent functional role for this family of growth factors in cardiac organogenesis and hypertrophy [reviewed by McLellan et al., 1993]. Our SSH-generated library also trapped the expression of BMP6, which appeared exclusively in the day 7-subtracted library [0, 2], during myocardial development.

ALPHA- AND BETA-ADRENERGIC SIGNALLING PATHWAYS

Most in vitro studies with neonatal myocytes indicate that the hypertrophic response can be attributed to stimulation of the alpha-adrenergic receptor, which in turn activates the phosphatidylinositol-protein kinase C (PKC) pathway. Formation of inositol phosphatase and/or PKC activation is mediated by various other hypertrophic stimuli (mechanical stretch, endothelin, angiotensin II). To name just a few examples, the mRNA levels of PKC receptor [2, 0], phospholipase C-L2 [1, 0], and phosphatidylinositol transfer protein [0, 1] were suggested by our study to be perturbed when the rat myocardium switches from hyperplastic to hypertrophic growth. In contrast, beta-adrenergic receptor is coupled to adenosine 3′, 5′-cyclic monophosphate (cAMP) formation through adenylate cyclase, activation of protein kinase A (PKA) and enhanced Ca^{2+} influx through the L-type calcium channel [reviewed by van Bilsen and Chien, 1993].

$Alpha_1$-adrenergic stimulation activates the expression and release of peptidylglycine alpha-amidating monooxygenase (PAM) from myocytes [Girard et al., 1999]. PAM catalyzes the two-step formation of bioactive alpha-amidated hormones or neuropeptides from their glycine-extended precursors. Earlier studies revealed that, in rat, ventricular PAM mRNA and activity were highest from embryonic days 14–18, declined at the time of birth, rose slightly during the first postnatal week, and declined toward adult levels [Ouafik et al., 1989]. Our study also revealed this subtle fluctuation of PAM, with declined expression on day 7 [3, 0].

In skeletal myoblasts, elevated levels of cAMP and overexpression of PKA inhibit myogenic differentiation [Winter et al., 1993]. PKA represses the transcriptional activation of muscle-specific genes by the myogenic regulators Myf-5 and MyoD. Activation of PKA occurs when cAMP is bound to the two regulatory subunits of the tetrameric PKA holoenzyme complex, thereby releasing its catalytic subunits [Clegg et al., 1987]. The regulatory subunit of cAMP-dependent kinase type I (PKA) was exclusively found in the subtracted day 7 library [0, 5], but extrapolation of PKA down-regulation during differentiation from skeletal myoblasts to cardiomyocytes requires further experimental evidence.

ELONGATION FACTORS

Elongation factor-1 alpha (EF-1α) is a highly conserved, abundantly expressed protein that

functions in peptide elongation during mRNA translation. EF-1α mRNA level decreases sharply in skeletal muscle and heart, and less sharply in brain during postnatal development [Lee et al., 1993]. The downregulation of EF-1α coincides well with the upregulation of its sister gene, S1, which shares 92% amino acid similarity and whose expression is limited to brain, heart, and skeletal muscle. This alternation from EF-1α to S1 was confirmed to be terminal differentiation dependent in neuron and may also be true for skeletal myocytes and cardiomyocytes [Lee et al., 1995]. Again, our current study also trapped this change, as implied by the predominant appearance of EF-1α [5, 1] in the subtracted day 1 library.

Surprisingly, elongation factor-1 gamma (EF-1γ), a substrate of cdc2 kinase [Belle et al., 1989], was exclusively found at a high frequency in the subtracted day 7 library [0, 23]. Being a universal regulator of the cell cycle, cdc2 kinase regulates the G2 to M transition. During cell division, a burst of protein phosphorylation of EF-1γ occurs [Maller et al., 1977]. Its predominant expression in the day 7 relative to day 1 myocardium is contrary to what we expect, because the cardiomyocytes cease to undergo the cell cycle. Therefore, EF-1γ may serve other functions beyond a cell cycle substrate during this postnatal period. Notably, EF-1γ was overexpressed in gastric and pancreatic carcinoma, as well as the cell lines of hepatic, ileocecal, duodenal, and colon carcinoma, with respect to the healthy normal cells [Mimori et al., 1995].

Although the data that we reported here do not account for the amount of protein products and were confined to the early postnatal period, our findings enhance the current understanding on the inhibited proliferation of cardiomyocytes during postnatal development. On the grounds that a considerable number of them are consistent with the current literature on cell cycle and differentiation, we believe that this pool of data consists of factors that are important for myocardial development and is worth further investigation.

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