ACADEMIC PRESS

Biochemical and Biophysical Research Communications 294 (2002) 161–166

BBRC

www.academicpress.com

A novel gene IC53 stimulates ECV304 cell proliferation and is upregulated in failing heart

Jingzhou Chen,[1] Baohua Liu, Yuqing Liu, Yu Han, Hui Yu, Yinhui Zhang, Lihe Lu, Yisong Zhen, and Rutai Hui*

Sino-German Laboratory for Molecular Medicine and Center for Molecular Cardiology, Fuwai Hospital, Peking Union Medical College and Chinese Academy of Medical Sciences, 167 Beilishilu, Beijing 100037, China

Received 29 April 2002

Abstract

C53, cloned from rat brain cDNA library, can bind to p35, the precursor of activator of Cdk5. A novel gene with 84% homolog to C53, named IC53, was cloned from our 5300 EST database of human aorta cDNA library (GenBank Accession No. AF110322). Computational analysis showed that IC53 cDNA is 2538 bp long, encoding 419 amino acids, mapped to chromosome 17q21.31 with 12 exons, ubiquitously expressed in 12 tested normal tissues and 8 tumor cell lines from MTN membranes and vascular endothelial cells by Northern blot and in situ hybridization, and upregulated in the rat models of subacute heart failure and chronic ischemic heart failure by left coronary ligation. Stable transfection of IC53 stimulates ECV304 cell proliferation by 2.1-fold compared to cells with empty vector ($P < 0.05$). The results support that IC53 is a novel gene, mainly expressed in vascular endothelial cells and mediates cell proliferation.

Keywords: IC53; Endothelium; Proliferation

Angiogenesis is a process that forms new blood vessels responsible for physiological and pathological demanding in our bodies [1]. A very important step in initiation of the angiogenesis process is the activation of endothelial cells that line the inside of the vessels upon specific signals, degradation of vessel basement membrane, migration and proliferation of endothelial cells in the surrounding stroma to form capillary ''sprouts'', and subsequently forming a jacket around the outside by smooth muscle cells. Excessive or insufficient endothelial cell proliferation contributes to numerous neovascularization-related diseases, including cancer, blindness in diabetes, arthritis, atherosclerotic plaque rupture, and thrombosis [2]. Recently vascular endothelial growth factor (VEGF) has been shown to enhance atherosclerotic plaque progression in animal model [3].

The activation of endothelial cells is strictly regulated under many endothelial specific and non-specific mediators and transcriptors which are negative or positive of cell proliferation. Endothelial cell proliferation is very slow, compared with many other cell types. Even though many regulators of endothelial cell growth such as VEGF [4] have been cloned but not many are specific mitogen in mediation of endothelial cell proliferation. Identification of novel molecules that regulate endothelial cell proliferation could improve our understanding of these complex processes and lead to novel therapeutic approaches.

Cell proliferation is under the tight control of cell cycle mediators at different cell cycle phases. The association of cyclin-dependent protein kinase (Cdk) leads to the transition of phases during progression of the cell cycle. Cdk5 has been cloned and shown to be involved in neuronal cell differentiation in the form of an active kinase [5,6]. The highly purified Cdk5 is a heterodimer consisting of a 33 kDa catalytic subunit (as Cdk5) and a

* Corresponding author. Fax: +8610-6833-1730, 8610-6831-3012.
E-mail address: huirutai@sglab.org (R. Hui).
[1] Jingzhou Chen is a PhD candidate in Sino-German Laboratory, Cardiovascular Institute, Peking Union Medical College, Beijing, China.

0006-291X/02/$ - see front matter
PII: S0006-291X(02)00446-1

25-kDa activator protein (p25). The later has been shown to be the proteolytic product of a 35-kDa protein. All p25 and p35 in the cells appear to have existed in complex with Cdk5. Ching et al. [7] isolated a number of cDNAs whose products bound to p35 with unknown biological functions. They were designed as C42, C48, and C53.

We have cloned a novel gene from human aorta cDNA library (GenBank Accession No. AF110322) encoding 419 amino acids with 84% of sequence homology to C53, named it as isoform of C53 (IC53). In the present study, we characterized the structural features and the biological functions of IC53.

Materials and methods

Computational characterization of IC53. Computational characterization of IC53 was performed as follows: homologous comparisons between species and chromosome localization of IC53 through BLAST, the database "nr" and "Human Genome", at http://www.ncbi.nlm.nih.gov/BLAST/, prediction of theoretical molecular weight and isoelectric point at http://www.expasy.ch/tools/protparam.html, searching membrane-spanning motif at http://www.cbs.dtu.dk/services/TMHMM-1.0/, characterizing pattern and profiles through ScanProsite at http://www.expasy.ch/tools/scnpsit1.html.

Molecular cloning and constructing mammalian expression plasmids. The open reading frame (ORF) of IC53 was amplified by polymerase chain reaction (PCR) using the EST clone (Accession No. AF110322) as template with pfu polymerase (Promega, Nepean, Canada), sense primer (5′-CTA GTC TAG AGG ATG TGT GTG CAT CCT GGG GCA-3′) and antisense primer (5′-CCC GGT ACC TCA CAG AGA GGT TCC CAT CAG-3′). To produce pcDNA3.1/Myc-His(-)A-IC53, the resultant PCR product was digested and ligated into the *Xba*I and *Kpn*I (New England Biolabs, Maryland, USA) sites of pcDNA3.1/Myc-His(-)A vector (Invitrogen, California, USA). It was sequenced fully and found the ORF to be intact and no PCR errors occurred in the amplified fragment before use in experiment described here.

Cell culture. ECV304 cell line was a gift from Professor Dalong Ma in Peking University. The cells were incubated in a 5% CO_2 and 95% air atmosphere at 37 °C and cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS, Hyclone, Utah, USA), 2 mM glutamate, 25 mM HEPES, 100 μg/ml penicillin, and 100 μg/ml streptomycin sulfate. Cell viability was determined by trypan blue exclusion. All cultures were checked routinely and determined to be mycoplasma free.

Stable transfection. ECV304 cells were plated in 6-well plates at a density 10^6 cells/ml (0.5 ml cells/well). After 20 h, the cells were transfected with 2 μg of pcDNA3.1/Myc-His(-)A (empty vector) or 2 μg of pcDNA3.1/Myc-His(-)A-IC53 using Clonfectin kit according to supplier's instruction (CLONTECH, California, USA). Stable clones were selected by G418 (200 μg/ml, GIBCO BRL, Maryland, USA) resistance 48 h later.

Northern blot analysis. Tissue distribution of human IC53 mRNA was analyzed by Northern blot on normal and tumor MTN blots purchased from CLONTECH (California, USA). ^{32}P-labeled probes were synthesized according to the ORF of human IC53 using a Prime-a-Gene Labeling System (Promega, Nepean, Canada) based on supplier's protocol. After hybridization with ^{32}P-labeled IC53 probe and a sequential washing, the membranes were subjected to autoradiography.

Cell counting. The effect of stable transfection of IC53 on cell proliferation was evaluated in ECV304 cells, 1×10^4 of cells, transfected with empty vector (pcDNA3.1/Myc-His(-)A) or IC53 containing pcDNA3.1/Myc-His(-)A, respectively, were plated in a 24-well plate in triplicate and fresh media were replaced every 48 h and the cells were counted every 24 h with hemocytometer.

In situ hybridization. In situ hybridization was performed according to the method described by Nicolas Solban et al. [8]. Briefly, the fresh tissues from Sprague–Dawley rats were cut at 8 μm thick and mounted on microscope slides, pretreated with aminopropylthioethoxysilane, dried at 37 °C, and then at 60 °C for 10 min prior to use. The probe applied was a unique 260-bp fragment amplified with primers (5′-GGCGTCTGTGGGGTTTGT-3′) and (5′-TGCCCCAGTCGATTCCAG-3′), corresponding to IC53 ORF. The DNA was transcribed using T7 or SP6 polymerases to create sense and antisense riboprobes, which were labeled with digoxigenin-UTP and in situ hybridization was performed according to DIG Luminescent Detection Kit (Roche Molecular Biochemicals, Mannheim, Germany).

Heart failure animal models. All rats were conformed to the guiding principal of China National Law for Animal Use in Medical Research and proved by Fuwai Hospital Committee for Animal Care and Use.

Animal model of subacute heart failure: Male Sprague–Dawley rats, body weight 250 ± 10 g, were purchased from Medical Animal Center at Beijing University and kept for at least one week in our hospital animal room with normal rat chaw and water ad libitum. Subacute heart failure model was created according to Muders' methods [9] modified as the following: 20 rats were treated with subcutaneous injection of adrenaline (20 mg/kg body weight) dissolved in 0.9% NaCl freshly prepared everyday and 20 control rats with 0.9% normal saline as vehicle. After 4–5 days of treatment, the animals were sacrificed with guillotine and the heart, aorta, kidney, liver, and brain were removed immediately for pathological examination in determining existence of heart failure and the expression of IC53.

Chronic ischemic heart failure model: Myocardial infarction was induced in male Sprague–Dawley rats by left coronary arterial ligation as described previously [10], totally 70 rats were operated on under anesthesia with 50 mg/kg ketamine hydrochloride intraperitoneally. Sham operation was performed on 20 control rats. After 6–8 weeks of operation, 20 rats developed chronic ischemic heart failure according to echocardiography and postmortem pathological examination and were selected for the study.

Statistical analysis. Data are expressed as means ± SD. The number of samples in each experiment was indicated in the figure legends. Statistical analysis was performed with Student's t test. $P < 0.05$ was considered significant.

Results

Cloning and molecular features of IC53

We isolated a full-length IC53 cDNA (2538 bp) from human aorta cDNA library, encoding a putative protein of 419 amino acids (Fig. 1A), mapped to chromosome 17q21.31 with 12 exons and a theoretical isoelectric point of 4.56, and calculated molecular mass of 46.3 kDa. Database searches indicated that the predicted protein is 84% identity throughout human C53 (Figs. 1B and C). No known membrane-spanning motif and putative signal peptide sequence were detected, suggesting an intracellular protein. The deduced amino acid sequence has several putative protein kinase C phosphorylation sites (residues 41–43, 57–59, 77–79, 339–341, 404–406, 408–410), casein kinase II phosphorylation sites (residues 15–18, 57–60, 113–116, 131–134, 150–153, 156–159, 161–164, 235–

238, 255–258, 316–319), N-myristorylation sites (residues 13–18, 157–162, 193–198, 204–209, 222–227), cAMP- and cGMP-dependent protein kinase phosphorylation sites (resides 405–408), N-glycosylation site (residues 145–148), and leucine zipper motifs (residues 270–291, 277–298). We concluded that the novel gene that we have cloned is likely to be a homolog of C53 (Fig. 1B).

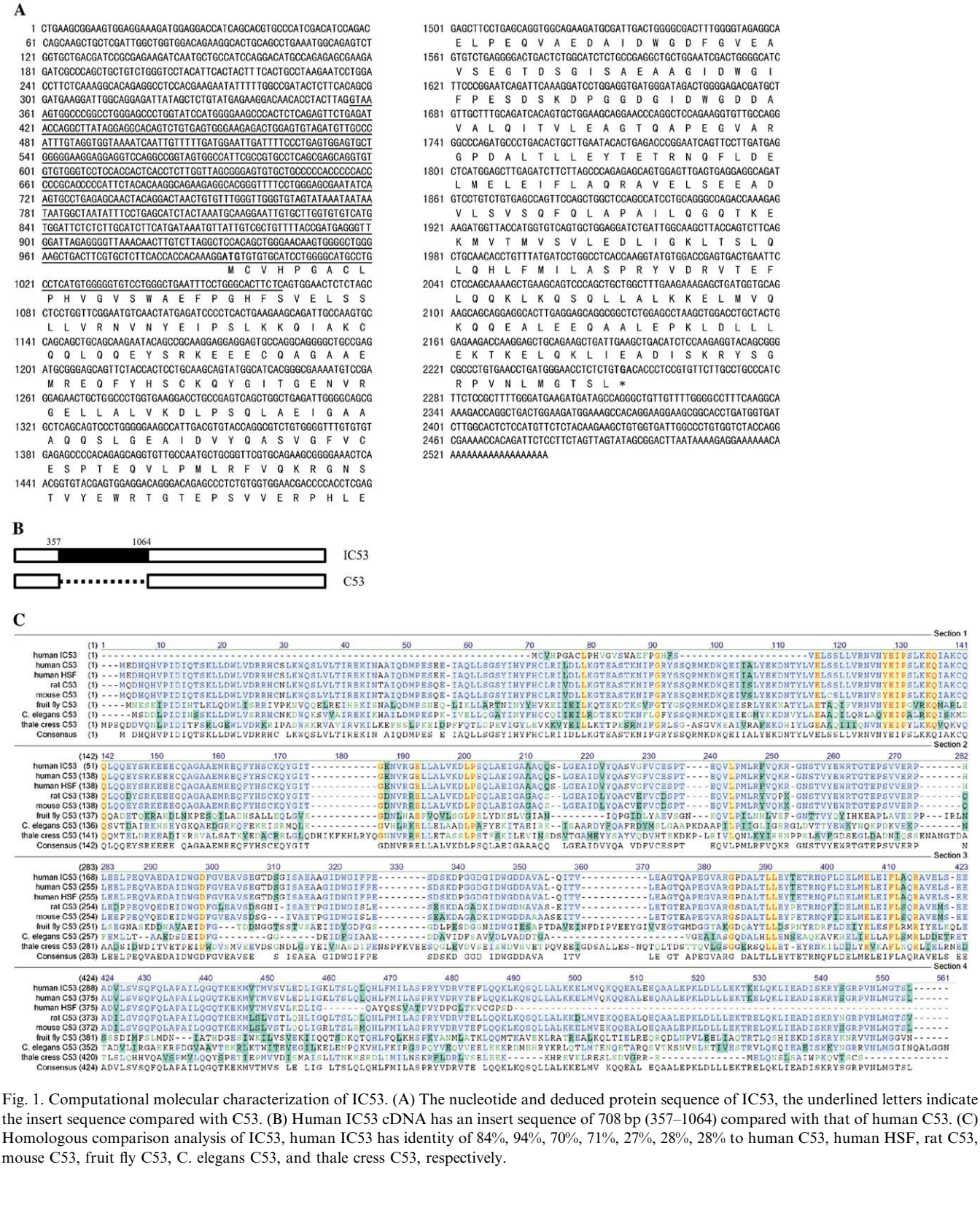

Fig. 1. Computational molecular characterization of IC53. (A) The nucleotide and deduced protein sequence of IC53, the underlined letters indicate the insert sequence compared with C53. (B) Human IC53 cDNA has an insert sequence of 708 bp (357–1064) compared with that of human C53. (C) Homologous comparison analysis of IC53, human IC53 has identity of 84%, 94%, 70%, 71%, 27%, 28%, 28% to human C53, human HSF, rat C53, mouse C53, fruit fly C53, C. elegans C53, and thale cress C53, respectively.

Ubiquitous tissue distribution of IC53

The distribution of IC53 RNA transcript in 12 tissues and 8 cell lines was detected (Fig. 2), two transcripts of 2.0 and 3.2 kilobase pairs (kb) were identified in all the normal tissues examined with selectively high expression of 3.2-kb transcript in the peripheral blood leukocytes, liver, and kidney and high expression of 2.0-kb transcript in heart. These two transcripts were also present in all tested tumor cell lines with the highest expression in promyelocytic leukemia HL-60, lymphoblastic leukemia MOLT-4, and Burkitt's lymphoma Raji.

The presence of two hybridized bands suggests either existence of two genes or differential splicing. To confirm which transcript is the full-length cDNA of IC53, we used IC53 insert of 708 bp (from 357 to 1064) as probe to perform Northern blot hybridizing on tumor cell line membrane (CLONTECH, California, USA). Only 3.2-kb transcript was detected in all tumor cell lines, corresponding to the size and expression pattern of the transcript previously observed by Northern blot analysis. Calculated transcript ratio of IC53/2.0-kb transcript is about 2:1 in most tumor cell lines (data not shown).

High expression in failing heart

IC53 structure feature analysis shows that IC53 has 84% identity to C53. To search possible engagement of IC53 in heart failure, we performed in situ hybridization

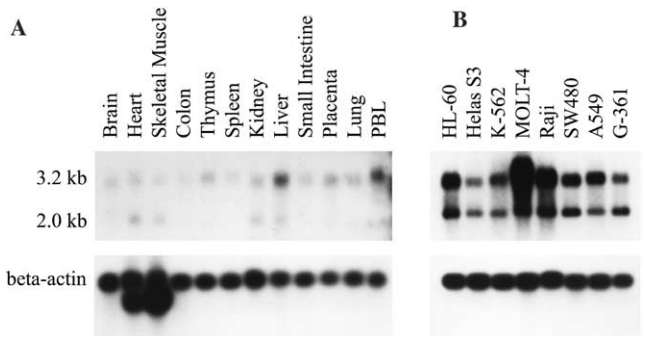

Fig. 2. Tissue distribution of IC53 mRNA. (A) Comparison of IC53 expression in 12 normal tissues. 1: brain, 2: heart, 3: skeletal muscle, 4: colon, 5: thymus, 6: spleen, 7: kidney, 8: liver, 9: small intestine, 10: placenta, 11: lung, 12: peripheral blood leukocytes (PBL). Two transcripts of 3.2 and 2.0 kb were detected in all tissues examined with particular high expression in PBL and liver for 3.2-kb transcript and relatively high expression of 2.0-kb transcript in heart. (B) IC53 expression level in 8 tumor cell lines which indicate HL-60: promyelocytic leukemia HL-60, K-562: chronic myelogenous leukemia K-562, MOLT-4: lymphoblastic leukemia MOLT-4, Raji: Burkitt's lymphoma Raji, SW480: colorectal adenocarcinoma SW480, A549: lung carcinoma A549, G-361: melanoma G-361. Two transcripts of 3.2 and 2.0 kb were detected in all tumor cell lines with particularly high expression in HL-60, MOLT-4, Burkitt's lymphoma Raji. β-Actin was used to evaluate the loading of mRNA in each lane and RNA markers were indicated on the left.

in heart ventricular sections of normal rats and rats with adrenaline-induced subacute heart failure and ischemic heart failure in which angiogenesis was activated due to blood supply demanding. Our results show that IC53 mRNA was expressed weakly in normal rat heart tissues and over-expressed in the ventricular sections in the model of subacute heart failure and chronic ischemic heart failure (Fig. 3).

Over-expression of IC53 promotes ECV304 cell proliferation

From our in situ hybridization studies, we found that IC53 was mainly expressed in vascular endothelium in failing hearts. Since endothelial cells play very important roles in angiogenesis, we characterized the effect of over-expression of IC53 on cell growth. The human umbilical vein endothelial cell-derived cell line ECV 304 cells were stably transfected with either plasmid alone or with plasmid carrying human IC53. The expression level of IC53 mRNA was determined by Northern blot. The clones expressing the highest levels of IC53 were selected for cell proliferation studies under normal growth condition (10% FBS and 200 μg/ml of G418) by counting cell numbers everyday for a period of 6 days. Cells that were transfected with the vector alone served as controls. Our results show that the cells transfected with vector alone have a similar growth rate as non-transfected cells (data not shown), the cells transfected with IC53 cDNA revealed a much higher proliferating rate compared with empty vector controls (Fig. 4). Consistent with a higher proliferation rate, stable transfection cells showed significantly ($P < 0.05$) increased numbers of viable cells 4 days after plating by MTT assay (data not shown).

Discussion

In this study, we have identified and characterized a previously uncharacterized gene termed IC53 that possesses structural similarity to C53, a Cdk5 binding protein precursor with unknown function, but IC53 has ability to stimulate endothelial cell to grow. IC53 mRNA is ubiquitously expressed in all tested tissues and tumor cell lines with different levels and over-expressed in failing heart in animal models. During heart failure, especially ischemic heart failure, cardiac myocytes need more oxygen; they release molecules that encourage blood vessels to grow, known as angiogenesis. Endothelial cell activation plays very important roles in this process. IC53 is expressed in vascular endothelium in the tested cardiac tissues. We speculated that over-expression of IC53 stimulates endothelial cell proliferation and contributes to the new vessel formation in failing heart.

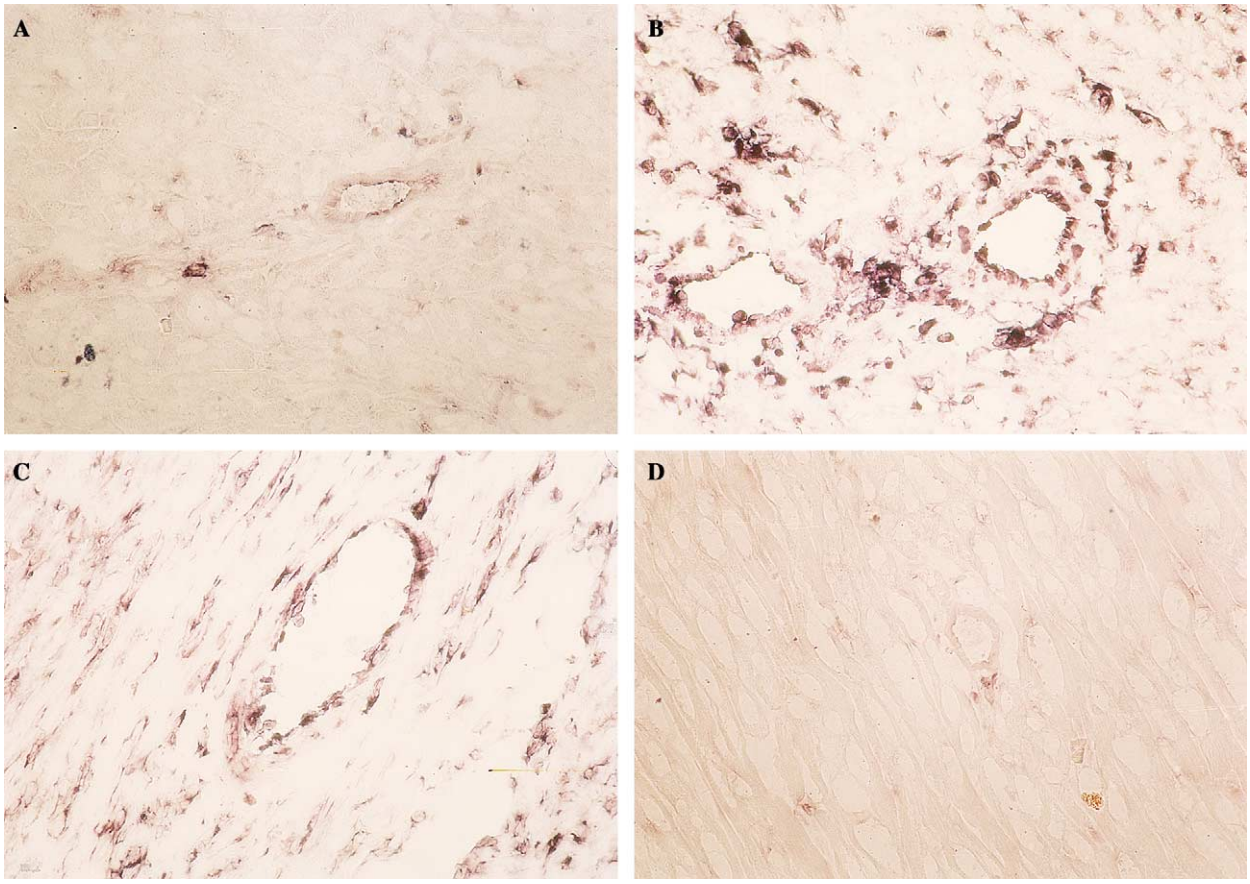

Fig. 3. Comparison of expression of IC53 in rat ventricular sections from normal rats and rats with subacute heart failure and with chronic ischemic heart failure. Cellular localization of IC53 gene expression in rat ventricular tissue as revealed by in situ hybridization used digoxigenin-labeled antisense IC53 riboprobe. Panel A: IC53 expressed weakly in vascular endothelium in normal rat ventricular section. Panels B and C indicate that expression of IC53 is upregulated in the ventricular sections after 5 days of treatment with adrenaline and in ischemic heart failure. Panel D: No signal is seen with the corresponding sense probe (400× folds).

To test the hypothesis, we transfected IC53 cDNA into the human umbilical vein endothelial cell-derived cell line ECV304 cells and found that over-expression IC53 promotes ECV304 cell proliferation. The similar results were observed in MTT assay as well. It is conceivable that losing cardiac myocytes caused by infarctions or other causes may significantly alter myocardiac paracrine signaling and have effects beyond those attributable purely to the losing contractile elements. It has been indicated that there is acute induction of VEGF expression in the peri-infarction zone [11]. In this study, we have found high expression of IC53 in the ventricular sections in subacute and chronic ischemic heart failure models. IC53 is able to promote ECV304 cell proliferation in vitro. So, in our experiments, the high expression of IC53 is speculated to be a compensation effect on hypoxia stimulation during heart failure. Chronic regional reduction of IC53 (or other growth factors) might negatively influence post-infarction ventricular remodeling or contribute to the pathophysiology of progressive ischemic myocardial dysfunctions. This is important for us to

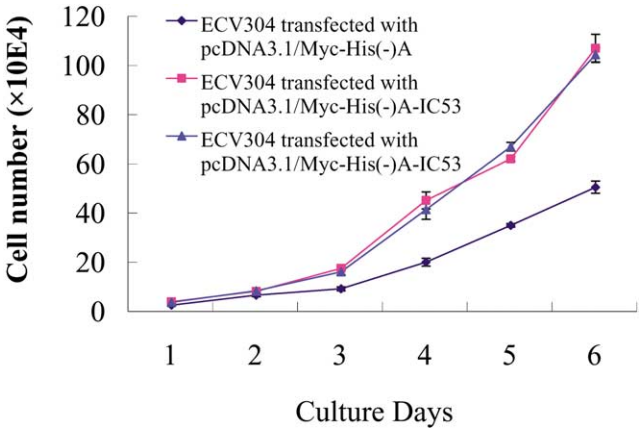

Fig. 4. IC53 induces ECV304 cell proliferation. ECV304 cells, transfected with pcDNA3.1/Myc-His(-)A or pcDNA3.1/Myc-His(-)A-IC53, were examined for cell proliferation by counting cell numbers everyday for a period of 6 days after plating. The cells were maintained in RPMI 1640 medium containing 10% fetal bovine serum and G418 200 μg/ml, cultured in 5% CO_2 and 95% air at 37 °C; medium was replaced every 48 h. The cells were trypsinized and counted every 24 h. The figure represents one of the three independent experiments, the results are means ± SD in triplicate.

consider use of growth factors therapeutically in ischemic heart diseases.

Atherosclerotic lesions begin as fatty streaks underlying the endothelium of large arteries. With progression of the lesions, the complications such as formation of plaque, hemorrhage, rupture, and thrombosis of the plaque result in acute coronary syndrome (unstable angina and acute myocardial infarction) or stroke [12]. Angiogenesis occurs in association with remodeling and with protease activation in plaque surrounding tissues, suggesting that neovascularization could contribute to plaque instability and rupture [12].

Our results showed that IC53 is a cell proliferator localized at vascular endothelium and upregulated in failure heart. Based on our observation, the over-expression of IC53 may contribute to compensatory formation of vessels, dysregulation of IC53 may lead to insufficient compensation in some pathological conditions, and over-expression of IC53 may contribute to atherosclerotic progression. If this were the case, manipulation of IC53 would have some clinical relevance to the treatment of neovascularization-related diseases.

In conclusion, these results indicate that IC53 is a novel angiogenic molecule and may have implications in inducing the growth of new blood vessels in blood-deprived (ischemic) heart tissues. In future, the IC53 ability to stimulate angiogenesis in ischemic heart tissue needs to be clarified.

Acknowledgments

This work was supported by China National 863 High-tech Grants Z19-02-02-01 and 102100405 and National P73 Basic Research Grant (to Dr. Rutai Hui).

References

[1] P. Carmeliet, R.K. Jain, Angiogenesis in cancer and other diseases, Nature 407 (2000) 249–257.
[2] J. Folkman, Angiogenesis in cancer, vascular, rheumatoid, and other disease, Nat. Med. 1 (1995) 27–31.
[3] F.L. Celletti, J.M. Waugh, P.G. Amabile, A. Brendolan, P.R. Hilfiker, M.D. Dake, Vascular endothelial growth factor enhances atherosclerotic plaque progression, Nat. Med. 7 (2001) 425–429.
[4] G.D. Yancopoulos, S. Davis, N.W. Gale, J.S. Rudge, S.J. Wiegand, J. Holash, Vascular-specific growth factors and blood vessel formation, Nature 407 (2000) 242–248.
[5] J. Lew, J.H. Wang, Neuronal cdc2-like kinase, Trends Biochem. Sci. 20 (1995) 33–37.
[6] R. Dhavan, L.H. Tsai, A decade of CDK5, Nat. Rev. Mol. Cell. Biol. 2 (2001) 749–759.
[7] Y.P. Ching, Z. Qi, J.H. Wang, Cloning of three novel neuronal Cdk5 activator binding proteins, Gene 242 (2000) 285–294.
[8] N. Solban, H.P. Jia, S. Richard, S. Tremblay, A.M. Devlin, J. Peng, F. Gossard, D.F. Guo, G. Morel, P. Hamet, R. Lewanczuk, J. Tremblay, HCaRG, a novel calcium-regulated gene coding for a nuclear protein, is potentially involved in the regulation of cell proliferation, J. Biol. Chem. 275 (2000) 32234–32243.
[9] F. Muders, E. Friedrich, A. Luchner, M. Pfeifer, G. Ickenstein, B. Hamelbeck, G.A. Riegger, D. Elsner, Hemodynamic changes and neurohumoral regulation during development of congestive heart failure in a model of epinephrine-induced cardiomyopathy in conscious rabbits, J. Card. Fail. 5 (2) (1999) 109–116.
[10] J.M. Pfeffer, M.A. Pfeffer, P.J. Fletcher, E. Braunwald, Progressive ventricular remodeling in rat with myocardial infarction, Am. J. Physiol. 260 (1991) H1406–H1414.
[11] K.S. Moulton, E. Heller, M.A. Konerding, E. Flynn, W. Palinski, J. Folkman, Angiogenesis inhibitors endostatin or TNP-470 reduce intimal neovascularization and plaque growth in apolipoprotein E-deficient mice, Circulation 99 (1999) 1726–1732.
[12] C.K. Glass, J.L. Witztum, Atherosclerosis, the road ahead, Cell 104 (2001) 503–516.